DEVELOPMENT AND EVALUATION OF A FOOD FREQUENCY QUESTIONNAIRE TO ASSESS DAILY TOTAL FLAVONOID INTAKE USING A ROOIBOS INTERVENTION STUDY MODEL

by
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DECLARATION

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ABSTRACT

A comprehensive food frequency questionnaire (FFQ) was developed to assess the daily total flavonoid intake over the past fortnight within a 14-week intervention that consisted of four periods to determine the effect of rooibos consumption on oxidative stress in adults (n=40) at intermediate to high coronary heart disease (CHD) risk. Within the intervention the comprehensive FFQ validity (against six estimated dietary records and biomarkers), reproducibility (on administrations in the washout and control periods six weeks apart as these periods had similar flavonoid intake restrictions) and responsiveness (across the four intervention periods of changed dietary conditions) was evaluated. The baseline period dietary record and FFQ dietary sources found to contribute most to the participants' daily total flavonoid intake, considering the percentage contribution, and the between-person variation in intake, considering the stepwise multiple regression analysis, formed the food list of the resultant abbreviated FFQ. The validity, reproducibility and responsiveness of the latter were also evaluated within the intervention and its validity (against dietary records) and reproducibility (on re-administration two weeks apart) in an additional group (n=90) being at low and intermediate CHD risk to evaluate its external strength.

The validity and reproducibility evaluations of the comprehensive and abbreviated FFQs in the intervention and abbreviated FFQ within the additional group comprised paired difference tests (to establish the ability to estimate group intakes), correlation coefficients (to establish the ability to rank individual participants), category agreement and gross misclassification next to the weighted *kappa* statistic (to establish the ability to classify the participants into tertiles and quintiles of intake) and Bland-Altman plots (as representation of the limits of agreement between the two dietary assessment methods). Correlation coefficients were also used for biomarker validity evaluations in the baseline period. The repeated measures analysis of variance (ANOVA) (Bonferroni correction) was used for the responsiveness evaluations of the comprehensive and abbreviated FFQs across the intervention periods alongside that of the biomarkers as evidence for the changed dietary conditions.

The study demonstrated that the comprehensive FFQ could be modified to a format with a brief food list as few items contributed appreciably to the total flavonoid intake and of which most also contributed to the between-person intake variability. The comprehensive and moreover the abbreviated FFQ in the validity evaluations provided sufficiently accurate daily total flavonoid intake estimates. They could determine the intake at group level in correspondence with that of the dietary records. The participant intakes could additionally be categorized and in particular ranked greatly alike to the dietary record intakes. The Bland-Altman plots revealed proportional bias regarding overestimation at the higher intake level. The reproducibility also appeared to be greatly satisfactory although seasonal fruit exclusions from the abbreviated FFQ food list may hamper its repeated administration. Both FFQs also confirmed the changed total flavonoid intakes across the intervention periods in relation to changes in the expected direction concerning the plasma total polyphenol, conjugated diene and thiobarbituric acid reactive substance concentrations.

OPSOMMING

'n Omvattende voedsel frekwensie vraelys (VFV) is ontwikkel om die daaglikse totale flavonoïed inname oor twee agtereenvolgende weke te beraam te midde van 'n 14-week intervensie. Die intervensie het uit vier periodes bestaan wat die effek van rooibosinname op oksidatiewe stres in volwassenes (n=40), met 'n intermediêre tot hoë koronêre hartsiekte (KHS) risiko, bepaal het. Binne die intervensie is die geldigheid (teen ses geskatte dieetrekords en biochemiese merkers), herhaalbaarheid (op aanwending ses weke uitmekaar in die uitwas en kontrole intervensie periodes met dieselfde flavonoïed inname bepalings) en waarneembaarheid (oor vier intervensie periodes van veranderde dieet bepalings) van die omvattende VFV geëvalueer. Die dieetbronne in die basislyn periode dieetrekords en vraelyste wat die meeste tot die deelnemers se daaglikse totale flavonoïed inname (baseer op die persentasie bydrae) en die tussen-persoon variasie in inname (baseer op die stapsgewyse meervuldige regressie analise) bygedra het, het die voedsellys van die voortvloeiende verkorte VFV gevorm. Die geldigheid, herhaalbaarheid en waarneembaarheid van dié VFV is binne die intervensie geëvalueer en die geldigheid (teen dieetrekords) en herhaalbaarheid (heradministrasie twee weke later) daarvan in 'n verdere groep (n=90) met lae en intermediêre KHS risiko as evaluasie van die eksterne vermoë van die VFV.

Die geldigheid en herhaalbaarheid evaluasies van die omvattende en verkorte VFV in die intervensie en die verkorte VFV in die verdere groep het bestaan uit gepaarde verskil toetse (bepaling van die groepinname skattingsvermoë), korrelasie koëffisiënte (bepaling van individuele deelnemer rangorde skattingsvermoë), kategorie ooreenstemming en erge wanklassifikasie naas die aangepaste *kappa* statistiek (bepaling van die vermoë om die deelnemer innames in derdes en vyfdes te klassifiseer) en die Bland-Altman karterings (verteenwoordiging van ooreenstemmingslimiete tussen die twee dieetinname metodes). Korrelasie koëffisiënte is ook gebruik vir biochemiese merker geldigheid evaluasies in die basislyn periode. Die herhaalde metings analise van variansie (ANOVA) (Bonferroni regstelling) is gebruik om die waarneembaarheid evaluasies van die omvattende en verkorte VFV oor die intervensie periodes naas dit van die biochemiese merkers te evalueer as bewys van die veranderde dieet bepalings.

Die studie het aangedui dat die omvattende VFV gewysig kon word tot 'n formaat met 'n verkorte voedsellys omdat slegs 'n aantal items merkbaar tot die totale flavonoïed inname bygedra het en die meeste hiervan ook tot die tussen-persoon variasie in inname. Die omvattende en die verkorte VFV het in die geldigheid evaluasies daarvan voldoende akkurate daaglikse totale flavonoïed inname skattings opgelewer omdat groep innames bepaal kon word in ooreenstemming met dit verkry van die dieetrekords en die deelnemer innames bykomend kategoriseer en in besonder grootliks eenders rangeer kon word as met hul dieetrekord innames. 'n Proporsionele oorskatting by die hoër inname vlakke is wel vir al twee getoon in die Bland-Altman karterings. Die herhaalbaarheid was ook grootliks aanvaarbaar, alhoewel seisoenale vrugte uitsluitings in die verkorte VFV voedsellys die heruitvoering kan bemoeilik. Al twee vraelyste kon ook die veranderinge in die daaglikse totale flavonoïed inname oor die intervensie periodes bevestig in ooreenstemming met veranderinge in die verwagte rigting van die plasma totale polifenool, konjugaat diëne en tiobarbituursuur reaktiewe stof konsentrasies.

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CONTRIBUTIONS BY PRINCIPAL RESEARCHER AND FELLOW RESEARCHERS

LIST OF OPERATIONAL TERMS AND CONCEPTS

Antioxidants: Compounds that inhibit or delay the oxidation of molecules by restraining the initiation or propagation of oxidizing chain reactions.(1)

Antioxidant activity: The antioxidant activity of a compound corresponds to the rate constant of that specific antioxidant compound against a particular free radical. An antioxidant would under different testing conditions represent different activities which depend on the species used to initiate the oxidation reaction.(2)

Antioxidant capacity (AC): The antioxidant capacity is the measure of the scavenging ability of a mixture of a given free radical.(2)

Bioactive compounds: Food components that exert physiological or cellular activities which result in beneficial health effects, but are not nutrients and not essential for life.(3)

Cardiovascular disease (CVD): Diseases of the heart and the blood vessels.(4)

Dataset: Although Joachim (5) refers to the total information or data collected using the structure of the questionnaire as the database this is referred to as the dataset in this research and comprises the computed mean daily total flavonoid intakes of the participant dietary records and the participant food frequency questionnaires.

Estimated dietary records: Daily records of actual food and beverage intake with indication of estimated or reasonably accurate measured portion sizes of the dietary items consumed.(6)

Flavonoids: A class of polyphenolic compounds (7,8) of low molecular weight that are usually bound to sugar molecules (7,9) and occur in plant sources.(8,10) They form several subclasses based on their chemical structure and generally act as antioxidants (3) ensuing positive effects on human health.(11,12)

Food frequency questionnaire (FFQ): A questionnaire used as dietary assessment tool with the framework to collect data on the consumption of a list of foods and in addition on elements like portion size and frequency of consumption.(13) For the purpose of this research, a list of foods and beverages pooled in food/beverage categories of which the participant recalls the food/beverage, its quantified portion size as small, medium or large based on portion descriptions provided, and how often it was consumed over the past two weeks as per day and per week or per two weeks in an open frequency indication and not in pre-defined frequency categories.

Inter-individual (inter-person/inter-subject): Between individuals/persons/subjects (14) with the term used in this research as between-person.

Intra-individual (intra-person/intra-subject): Within one individual/person/subject (14) with the term used in this research as within-person.

Lipid oxidation/peroxidation: The oxidation/peroxidation of polyunsaturated fatty acids in membranes by exposure to reactive oxygen species and/or to transition metal ions in a free radical chain reaction.(15)

Oxidative stress: Non-enzymatic oxidative damage to molecules that impair the normal functioning of the cell or the organism.(16)

Phytochemicals: See bioactive compounds

Reproducibility: The association between the responses obtained from administering the same questionnaire to the same group of subjects over a time interval,(17) in this research the FFQ, which is also considered "repeatability" or "reliability" in dietary methodology evaluation (18) and determines whether or not the data can be reproduced.(5)

Responsiveness: For the purpose of the rooibos intervention trial phase of the study and arising from the description of Kristal et al,(19) the sensitivity of the FFQ to changes in the mean daily total flavonoid intake which is the dietary intervention behaviour under study in the trial.

Validity: The degree to which the questionnaire developed as method measures items, that is foods or nutrients, for which it was designed and intended to measure (17) conferring the truthfulness of the database ("dataset") generated (5) which for this research is the mean daily total flavonoid intake as measured by the FFQ.

LIST OF ABBREVIATIONS AND SYMBOLS

A

AAE	Ascorbic acid equivalents
AAPH	2,2'-azobis(2,4-aminopropane)dihydrochloride
ABTS	2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate)
AC	Antioxidant capacity
$ACAT_2$	Acyl-CoA cholesterol acyltransferase
AHA	American Heart Association
ANOVA	Analysis of variance
ATP	Adenosine triphosphate

B

BH4	Tetrahydropiopterin
BMI	Body mass index
BMR	Basal metabolic rate
BSßG	Broad substrate specificity ß-glycosidase

C

C	Carbon
CAD	Coronary artery disease
CD(s)	Conjugated diene(s)
CHD	Coronary heart disease
CI(s)	Confidence interval(s)
cm	Centimeter
CO_2	Carbon dioxide
COMT	Catechol-*O*-methyltransferase
COX	Cyclooxygenase
CPUT	Cape Peninsula University of Technology
CRP	C-reactive protein
CVD	Cardiovascular disease
CYP	Cytochrome P450

D

DAD	Diode array spectrometry
DHQ	Dietary history questionnaire
di-OH	Dihydroxylated
DNA	Deoxyribonucleic acid

E

EC	Epicatechin
EDRF	Endothelial-dependent relaxing factor
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
EI	Energy intake

F

FFQ	Food frequency questionnaire
fl oz	Fluid ounces
FMD	Flow-mediated dilation
FORT	Free oxygen radical test
FRAP	Ferric reducing ability of plasma

G

g	Gram
GC-MS	Gas chromatography-mass spectroscopy
GIS	Geographic information systems

GP   Glycoprotein
GRAS   Generally recognized as safe
GSH   Glutathione
GSSG   Glutathione disulfide
GST   Glutathione S-transferase
H
H   Hydrogen
OH   Hydroxyl
HDL   High density lipoprotein
HPLC   High performance liquid chromotography
I
ICAM   Intracellular adhesion molecule
ICC(s)   Intraclass correlation coefficient(s)
IHD   Ischemic heart disease
IkB-alpha   Inhibitory kappa B-alpha
ISCO   International Standard Classification of Occupations
K
K Kappa
kcal   Kilocalorie
kg   Kilogram
Kw Weighted Kappa
L
L   Liter
LDL   Low density lipoprotein
LOX   Lipoxygenase
LPH   Lactase phlorizin hydrolase
LPL   Lipoprotein lipase
M
CH_3 Methyl
mg   Milligram
MJ   Megajoule
mL   Milliliter
μM   Micromolar
MAPK   Mitogen activity protein kinase
MCT   Monocarboxylate transporter
MDA   Malondialdehyde
MMP(s)   Metalloproteinase(s)
MRC   Medical Research Council
MRP   Multidrug resistance-associated proteins
MS   Mass spectometry
MTP   Microsomal transfer protein
N
NAD(P)H   Nicotinamide adenine dinucleotide (Phosphate)
NCEP   National Cholesterol Education Program
NCI   National Cancer Institute
NDB   Nutrient data bank
NF-kB   Nuclear transcription factor-kappa B
NHANES   National Health and Nutrition Examination Survey
NHMRC   National Health and Medical Research Council
nm   Nanometer
NO   Nitric oxide

NPV	Negative predictive value
O	
o	Ortho
n-3	Omega-3
n-6	Omega-6
ORAC	Oxygen radical absorbance capacity
P	
PA(s)	Plasminogen activator(s)
PG(s)	Prostaglandin(s)
PPV	Positive predictive value
PUFA(s)	Polyunsaturated fatty acid(s)
Q	
QC	Quality control
QSAR(s)	Quantitative structure-activity relationship(s)
R	
ROC	Receiver operator characteristic
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RP-HPLC	Reverse-phase high-performance liquid chromatography
RR	Relative risk
S	
SA	South Africa
SAR(s)	Structure-activity relationship(s)
SD(s)	Standard deviation(s)
SGLT1	Sodium-dependent glucose transporter
SOD	Superoxide dismutase
SULT	Sulfotransferases
T	
TAC	Total antioxidant capacity
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TEAC	Trolox equivalent antioxidant activity
t-PAs	Tissue-plasminogen activators
TX	Thromboxane
U	
UDPGT, UGT	UDP glucuronosyl transferase
UK	United Kingdom
u-PA(s)	Urinary-type plasminogen activator(s)
US	United States
USDA	United States Department of Agriculture
UV	Ultraviolet
V	
VC	Validity coefficient
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoprotein
VSMC(s)	Vascular smooth muscle cell(s)

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CHAPTER 1

STATEMENT OF THE RESEARCH PROBLEM

1.1 BACKGROUND TO RESEARCH

Cardiovascular disease (CVD) is a leading cause of morbidity and mortality (20) with coronary heart disease (CHD) or ischaemic heart disease (IHD),(4) a primary health problem and cause of death, particularly in developed countries.(4,21,22) CHD is a multi-factorial chronic disease (4) with dietary intake one of the important risk factors that originated in recommendations to change dietary intake, in addition to other lifestyle factors, as approach for cardioprotection.(23) Nutrition-related non-communicable diseases have emerged as a major problem in developing countries.(24) CHD is now not only a major health problem of developed countries, but also of developing countries. This may be due to improved socio-economic status and changes in dietary intake and lifestyle among these nations.(22) CHD is one of the leading causes of overall morbidity and mortality in South Africa (SA).(25) As these chronic diseases, like CHD, account for considerable morbidity and mortality, their prevention is crucial to support public health.(26)

The nutrition field is at present trying to elucidate how the numerous diverse non-nutritive bioactive compounds in food can be used in the prevention and treatment of chronic degenerative diseases like CHD.(27) Plant foods, in addition to the nutrients that they contain, contain numerous of these phytochemicals that through independent or synergistic functioning *in vivo* may benefit vascular health.(28) As dietary intake needs to be considered when exploring the chronic degenerative diseases,(29) the intake of these numerous non-nutritive bioactive compounds need to be assessed,(30) as there is an increasing recognition of their potential health importance in the aetiology of these diseases.(31) Phytochemicals include the plant polyphenolic compounds,(32) a large group of natural antioxidants,(33) for which considerable interest has arisen in the polyphenolic flavonoids (34,35) because of their multiple potential beneficial effects on cardiovascular health.(27)

Food remains the core source of flavonoids for human ingestion.(22) Since CVD is a primary cause of morbidity and mortality, foods that contain traits that can have a beneficial cardioprotective effect, should take a distinctive place in the diet.(28) Locally, emphasis has been placed on the consumption of rooibos (*Aspalathus linearis*) herbal tea. Rooibos is an important dietary source of antioxidants, containing mostly flavonoids,(36) which led to its utilization in a human clinical intervention trial with the aim to append to the particulars on its cardioprotective effects.(37)

This increasing recognition of the potential health importance of phytochemicals conveys the need for methods to assess their intake that can be used satisfactorily in epidemiological studies. This would enable researchers to ascertain whether there is any association between their intake and disease risk and provide the needed quantification of the validity and reproducibility status of such methods.(31) This places the emphasis on the development and evaluation of a food frequency questionnaire (FFQ) to assess the daily total flavonoid intake within, for example as intended with the rooibos intervention trial integrated in this research, along with using dietary records. Dietary records are generally considered to be a more detailed and precise dietary assessment method and are the most indicative of true dietary intake to serve as reference method.(18,38-41)

Dietary records are considered to be a suitable assessment method in small clinical studies, whereas in the case of large scale investigations, there would be a high participant burden and high costs involved to analyze the dietary data due to its open-ended recording format.(42) Thus a FFQ may be a more suitable dietary assessment method due to ease of completion, data reviewing and analysis.(43) FFQs often assess frequency of consumption of specific foods to provide data on a particular research question,(44) which would be perfectly appropriate for this research with total flavonoid intake as the focal point. It was envisaged that fruits, vegetables and beverages would form the bulk of the developed FFQ food list due to the abundant presence of flavonoids in these foods.(8,45-48) The majority of studies assessing fruit and vegetable intake have used some form of a FFQ approach.(42)

It has also been advocated to use more than one dietary assessment method in intervention trials and to look for consistency in the results.(49,50) All dietary assessment methods have measurement errors or biases associated with the method itself.(51,52) The sequential administration of the same assessment method to the same subjects within an intervention trial may be a further source of measurement error resulting from a training or learning effect over time,(43,53) compliance bias (50,54) or response set bias, like those attributable to social desirability.(55-57) The current research, to develop and evaluate a FFQ to assess daily total flavonoid intake, was therefore devised from the intervention trial to investigate the utility of rooibos consumption as dietary source of flavonoids on oxidative stress in adults at risk of CHD. The research on which this dissertation is based is thus nested within the rooibos intervention trial with the trial itself not being the focus of this research.

Validations of dietary assessment measures have been conducted within clinical trials, but they were mostly coupled with larger nutrition or health surveys or investigations of diet-disease associations.(58) However, the dietary intake assessment of bioactive compounds may require biochemical data,(30) because comprehensive databases for calculating the intakes of bioactive

compounds are not available. However, clinical intervention trials may provide biochemical data. In addition, available food composition databases reporting bioactive compound contents, are subject to errors because in its compilation, the variations in food preparation and processing methods are not considered in their entirety.(30)

1.2 RATIONALE FOR AND SIGNIFICANCE OF THE RESEARCH

Dietary and other lifestyle changes are often recommended as a means to support health and prevent disease.(23) The endogenous antioxidant levels cannot easily be manipulated. Since dietary antioxidants are exogenous in nature, their levels can be manipulated by dietary modification or supplement use.(22) The findings that polyphenolic flavonoids are bioactive dietary compounds capable of increasing plasma antioxidant defences (59) and that flavonoid-rich foods may reduce CVD risk,(27) have far-reaching implications for health promotion and disease prevention. Intervention trials using whole foods rich in such antioxidants are more appropriate for assessing the effects of phytochemicals than using dietary antioxidant supplements.(60) This is thus the rationale for conducting intervention trials using rooibos.

The unique South African herbal tea, rooibos, is made from the leaves and stems of the fynbos plant, *Aspalathus linearis*. Rooibos is naturally caffeine free, contains very low levels of tannins (61,62) and is a rich dietary source of flavonoids.(36) The most widespread rooibos on the market is the traditional 'fermented' product. 'Fermentation' involves extensive oxidation of the phenolic compounds which is required for the formation of its characteristic sweetish flavour and red-brown to brown colour. The popularity of rooibos has grown both locally and internationally.(63) Although little clinical data is available on the antioxidant and cardioprotective properties of rooibos consumption, results from animal studies reported rooibos to modulate oxidative stress (64,65) and to wield cardioprotective effects.(66)

The few human studies published on these health effects of rooibos overall seem encouraging. While the consumption of a single oral dose of rooibos by healthy volunteers in the study of Breiter et al. (67) resulted in no change in the antioxidant status of the plasma, Villaño et al. (68) found a significant increase in the serum antioxidant status. In the study of Persson et al.,(69) a significant inhibition of the angiotensin-converting enzyme activity was found suggesting cardioprotective effects. One intervention study reported on the antioxidant status of lead factory workers after chronic rooibos consumption. Modulation of the redox status of these workers was shown by a decreased level of lipid peroxidation.(70) The rooibos intervention trial itself would therefore investigate the modulation of oxidative stress on chronic rooibos consumption in a population at risk of developing CHD.(37)

Studies evaluating associations between dietary intake and health require assessment of dietary intake.(71) Some form of dietary assessment is required to assess the effectiveness of dietary interventions.(52) Such dietary assessments may be interested in monitoring food intake and/or nutrient intake.(52,72) In a rooibos intervention trial, the consumption of rooibos as a measure of the intervention outcome would be confounded by other dietary sources of flavonoids. In addition to the total dietary intake assessment, a rooibos intervention trial would necessitate monitoring the consumption of rooibos and the total dietary flavonoid intake as a measure of the outcome. Dietary records would be a suitable choice for such an assessment. However, dietary records collect far more information than is needed to monitor the consumption of rooibos and in particular to assess daily total flavonoid intake. A very high price is paid to collect extra data that may not be useful for a study.(73) FFQs often assess the frequency of the consumption of specific foods to provide data on a particular research question (44) and it has been indicated that a brief FFQ may be a suitable method for estimating flavonoid intake.(74)

Studies based mostly on retrospective data indicate that a small number of dietary items contribute significantly to flavonoid intake,(9) which includes tea (black and green), red wine, fruits (apples, citrus fruits, purple grapes and grape juice), vegetables (onion), cocoa and chocolate.(9,75) Furthermore, Marshall et al. (76) point out the importance of selecting a questionnaire that accurately collects nutrient data relevant to the research questions in a study. In the setting of the rooibos intervention trial, a FFQ needed to be developed and evaluated to assess daily total flavonoid intake. A purpose of clinical intervention trials is to provide fundamental information for differentiating single components of foods as being important in a disease process.(77) The development and use of a FFQ alongside dietary records in a rooibos intervention trial, would thus provide the opportunity to compare the daily total flavonoid intake estimates of the two dietary assessment methods, thereby allowing for the evaluation of a developed FFQ.

In a pilot study of a calcium intervention trial, the subjects were asked to complete the 98-item Block FFQ twice in two months. However, the second questionnaire was only returned by half of the subjects.(78) The investigators found that completing the comprehensive FFQ a second time was a burden and suggested that a shorter version of the questionnaire might provide greater compliance. Therefore the FFQ to be developed with its comprehensive food list (incorporating in particular fruits, vegetables and beverages) for use in a rooibos intervention trial that provides for a more precise absolute daily total flavonoid intake estimate would have to be modified to produce a shorter food list. This resultant abbreviated FFQ with its shorter food list, would have to be evaluated not only within the rooibos intervention trial itself, but also in a further participant group administration to determine its external strength and likely generalizability.

Time-consuming dietary assessment methods (dietary records and, for the purpose of this research, the comprehensive FFQ to be developed) are considered uneconomical. Shortcut methods (abbreviated FFQs) should still produce accurate results,(79) hence the evaluation of the resultant abbreviated FFQ in this research would be required. Such short yet accurate methods are needed for epidemiological studies.(80) The long list of individual fruit and vegetables, envisaged to form the bulk of the comprehensive FFQ food list, might impact on the precision of the daily total flavonoid intake estimates. It has been stated that over-reporting, and thus an overestimation, of total fruit and vegetable intake occurs in FFQs with a long fruit and vegetable food list.(18,40,81,82) Therefore, short FFQs that assess fruit and vegetable intake, may have an advantage over longer questionnaires as the over-reporting and overestimation possibly may be reduced.(83)

The research should firstly contribute to the global scarcity of dietary assessment measures specifically aimed at the assessment of the daily total flavonoid intake. Nearly all FFQs used to date to determine flavonoid intake, do not have a prior flavonoid-specific hypothesis in the compilation, nor do they have a well-defined brief food list,(84) which would make the FFQ developed entirely unique in purpose and design. A FFQ should have a limited food item list by only including items of which the frequency of intake and nutrient content result in an important nutrient intake contribution and/or differentiate the intake between subjects.(18,85) As the latter is not known for the compilation of the FFQ, the FFQ developed initially with its comprehensive food list will have to be modified to obtain a further simpler FFQ with a well-defined food list considering these food list characteristics. Thus it would be imperative to evaluate the resultant abbreviated FFQ as indicated previously. The use of a FFQ to assess daily total flavonoid intake will depend upon whether it is the only dietary component studied or whether its intake will be assessed as part of the total diet. In this case it can be utilized in conjunction with a more intensive dietary assessment method to assess the total diet. The increasing interest in the possible role of flavonoids in CHD consequently implies that practical and efficient methods of assessing such intakes, which are suitable for large-scale investigations, are required. An abbreviated FFQ can meet this requirement. The envisaged development and evaluation of a FFQ to assess daily total flavonoid intake in this research within a South African context, could provide useful information for other investigators who are considering this kind of activity.

Currently, no information is available on the consumption of flavonoids in SA. No abbreviated FFQ that determines daily total flavonoid intake exists at this time for use in SA. It is anticipated that an abbreviated FFQ could be used to determine a South African daily total flavonoid intake. The intended use of dietary records alongside a FFQ in the rooibos intervention trial and in the further participant group administration should provide an indication of a South African average daily total

flavonoid intake and intake range. Scalbert and Williamson (8) indicated that it is desirable to know the main classes of flavonoids consumed and the main foods that contain them. Such quantification would allow nutritionists/dieticians to make recommendations on the intake of flavonoids as a means of contributing to the body's antioxidant defences and ultimately improving public health, which may be important for a country like SA where CHD is a public health concern.(25)

1.3   PROBLEM STATEMENT

The growing interest in the role of flavonoids in CHD means that efficient, but practical methods of assessing the intake is necessary. Accurate assessment of the daily flavonoid intake is essential to expand health research in this area. While the lack of association between flavonoid intake and health benefits may be real, an explanation may include the lack of an acceptable measure to assess the intake.(47) To shed light on the role of flavonoids in health promotion and disease prevention, valid estimates of its intake longitudinally (as in intervention and cohort research) and retrospectively (as in epidemiological research) in particular, are necessary. The rooibos intervention trial requires longitudinal estimates, which can be provided by means of the employment of dietary records. However, making use of a FFQ in the trial would provide a measure to estimate the intake retrospectively. It is generally advocated that optimally more than one dietary assessment method be employed in research settings,(54,86) including intervention trials.(49,50)

Dietary records and available FFQs, collect far more information than is needed to assess daily total flavonoid intake. For instance, in the earlier absence of food composition data, Peterson and Dwyer (87) applied botanical taxonomic classification to identify flavonoid-containing foods in the food list of the National Cancer Institute (NCI) FFQ. Botanical taxonomic classifications of foods provide clues about their phytochemical composition. In this NCI FFQ, 153 foods were screened after duplications were removed. Among these foods, 54 foods (35%) were judged to contain flavonoids, 19 composite foods (12%) to incorporate flavonoid-containing components or ingredients, and 39 foods (25%) to contain some flavonoids, but most of which had been reduced or removed during milling and other processing.(87)

FFQs are generally designed for assessing total diet which would be unnecessarily lengthy when used for estimating the intake of only one nutrient and might not be as valid for assessing the intake of that single nutrient,(18,88) as is the case of flavonoids. Most FFQs employed in epidemiological studies have not been designed to assess phytochemical intake specifically,(47) including that of flavonoids. Johannot and Somerset (89) point out that dietary records offer more convincing flavonoid intake data than FFQs since the latter often have not been validated for

flavonoid intake. Individual dietary flavonoid sources can be overlooked due to their exclusion from the food list. This necessitates the development of a FFQ to assess daily total flavonoid intake in the context of the rooibos intervention trial. Although dietary assessment requires that no food category be omitted,(56) it is evident that certain food categories would be absent in a FFQ developed with the sole purpose of assessing daily flavonoid intake. It is evident that food categories, which make up a large part of the South African diet,(90) are devoid of flavonoids or the flavonoids have been reduced or removed during processing, and therefore could be excluded from a FFQ food list.

Self-report FFQs or brief measures with a tapered focus on whole foods, or a particular class of nutrient, are typically employed to assess dietary change in intervention studies. Few biomarkers that accurately reflect intake, are available and if they are, they are often prohibitively expensive to use. Some intervention trials do not have the resources to conduct intensive dietary assessment so they, although not the best approach, might rely on short measures to help evaluate the efficacy of the intervention.(50) Flavonoid intake estimates are important, not only for intervention settings, but also for epidemiological studies if health research on these compounds is to increase. In these instances, shorter questionnaires that include foods/food groups that discriminate between high and low intakes would be useful.(18)

In the rooibos intervention trial, the dietary stipulations for each of the trial periods (the washout versus the intervention trial period) would allow for a responsive evaluation of the developed abbreviated FFQ regarding its ability to differentiate between high and low intakes. There is an increasing need for simple assessment measures that can quantify the intake of dietary components as determinants in the aetiology of chronic diseases.(91) An abbreviated FFQ may thus be of further worth as more rooibos intervention trials are necessary to append to the particulars on its beneficial health effects for humans. Adequately assessing dietary change can substantially contribute to the evidence for health benefits resulting from change in dietary intake.(23)

1.4 RESEARCH QUESTIONS

Would the comprehensive and the resultant abbreviated FFQ developed for use in the rooibos intervention trial, to assess the daily total flavonoid intake, be a valid, reproducible and responsive dietary assessment measure?

Would the resultant abbreviated FFQ, developed to assess the daily total flavonoid intake, be a valid and reproducible dietary assessment measure on administration in a further external participant group?

1.5   RESEARCH OBJECTIVES

The research objectives are:

(a)   To develop a self-administered comprehensive quantitative FFQ to assess the daily total flavonoid intake and to evaluate its validity, reproducibility and responsiveness (see list of operational terms and concepts for description) within the context of the rooibos intervention trial;

(b)   To identify those foods and beverages that contributed most to the daily total flavonoid and the between-person variation in intake, according to the estimated dietary record and comprehensive FFQ estimates in order to create an abbreviated FFQ; and

(c)   To evaluate the validity, reproducibility and responsiveness of the resultant abbreviated FFQ within the context of the rooibos intervention trial, and its validity and reproducibility in a further participant group administration as appraisal of its external strength.

1.6   RESEARCH HYPOTHESES

The following null-hypotheses (H_0) were investigated (at a significance level of 5%):

- There is no difference between the test (comprehensive FFQ and resultant abbreviated FFQ) and reference (estimated dietary record) method estimates when evaluating its validity at the group level;

- There is no association between the test and the reference (estimated dietary records/biomarkers of exposure and of effect) method datasets in its validity evaluation at the individual level;

- There is no difference between the estimates of the test method administrations in its reproducibility and responsiveness evaluation at the group level; and

- There is no association between the datasets of the test method administrations in its reproducibility evaluation on the individual level for the mean daily total flavonoid intake(s).

1.7   OUTLINE OF THE DISSERTATION

Chapter 1 includes the background and the rationale for the research with the research significance, along with the statement of the research problem, the research questions, objectives and hypotheses. A literature review is provided in chapters 2 through to 6 in support of the proposed research. These chapters form a major component of the dissertation in view of this supportive literature with the fundamentals being the polyphenolic flavonoids as bioactive compounds (Chapter 2), their cardioprotection utility (Chapter 3) and the FFQ as dietary

assessment methodology pertaining to the aspects to consider in its development (Chapter 4), evaluation (Chapter 5) and utilization as a brief measure (Chapter 6).

Chapter 7 provides a description of the research design and the methodology comprising firstly the development and evaluation of the comprehensive FFQ in the rooibos intervention trial, then the development of the resultant abbreviated FFQ and its evaluation in the rooibos intervention trial, and lastly its evaluation within a further participant group administration as evaluation of its external strength. The results of the research reported in Chapter 8, are followed by a discussion thereof in Chapter 9. The dissertation culminates with the conclusions of the research and finally, the recommendations for further research (Chapter 10).

It should be noted that the literature sources used in support of the research are the original primary sources, which in the present day are still reported in publications on aspects of the research fundamentals. In particular, publications regarding the biological properties of flavonoids, several of their cardioprotective mechanisms and the developmental and evaluation essentials surrounding a FFQ have been consulted and are sited. With regard to the FFQ, the supportive literature for its development and evaluation essentials were obtained from publications related to nutrients and not specifically to flavonoids due to the absence of FFQ literature that relate to the assessment of flavonoid intake.

CHAPTER 2

THEORETICAL BACKGROUND FOR INTERPRETING DIETARY FLAVONOID INTAKE AND STATUS

2.1   PREAMBLE

Numerous non-nutrient but bioactive compounds, which have become known as phytochemicals or phytonutrients, are found in plant foods.(7,47,92)   The polyphenols or polyphenolic compounds, are polyhydroxylated phytochemicals (93) and hence reducing agents, commonly referred to as antioxidants.(8,94)   These polyphenols are one of the largest groups of phytochemicals (7,47,92) and the most abundant antioxidants in the human diet.(8,12,94-96)

Flavonoids and phenolic acids comprise the two main classes of the polyphenols (93) with flavonoids forming the larger class.(7,45,47,92)   Flavonoids are low molecular weight secondary metabolites synthesized by plants (75,97,98) for structural growth and defence against injury and infection.(99-101)   Flavonoids, principally considered plant pigments,(97) occur widely throughout the plant kingdom, and thus are in many foods and beverages, where they are in particular responsible for the sensory characteristics (99) of colour and taste.(34,102,103)   The main flavours associated with flavonoids are bitterness and astringency (102-104) that is connected to their defence function in plants.(104)

Polyphenols, being one of the largest groups of phytochemicals,(7,47,92) are regular food and hence dietary components.(95)   Flavonoids, due to being the larger polyphenol class,(7,47,92) are the most abundant polyphenols in the human diet (8,105) and said to account for two-thirds of the phenolic intake.(7,8,12)   Flavonoids are therefore components common and plentiful in the daily diet (100,106,107) with most human beings consuming some flavonoids daily.(108)

Polyphenols have long been considered anti-nutritive,(109,110) non-nutritive (35,111) and non-essential (112-114) dietary components as their lack in the diet, unlike established nutrients, does not cause deficiency symptoms and diseases.(92,114)   These bitter and astringent bioactive compounds are found aversive by the consumer on consumption and thus the reason why the agricultural and food industries remove these compounds from plants through selective breeding and a variety of debittering processes as standard practice.(104)   However, a great interest in them has arisen.(35,97)   Polyphenols, and hence flavonoids, are of prime consideration in nutrition and health due to their powerful antioxidant capacity (AC).(115)   They may act as antioxidants and support the body's tissues against oxidative stress (8,94) and consequently have preventative qualities for resultant disease.(34,115)

As flavonoids are part of the dietary intake,(116) their impact on human health is consequently of importance,(108) particularly because they are not synthesized by the human body.(10) Providing that the absorption of flavonoids from the gut is efficient, flavonoid intake could present important pharmacological levels in body fluids and tissues.(97) The health effects of flavonoids are still largely unknown. A number of different complex mechanisms and pathways may be responsible for their effects.(108) Evidence for their role in the prevention of diseases linked to oxidative stress, particularly CVD, has surfaced sturdily.(100) As antioxidants, they guard cell constituents against oxidative harm and as a consequence they reduce the risk of degenerative disease related to oxidative stress, like CVD.(94) Owing to the above, the view of the dispensability of flavonoids is changing and it has been suggested as heading for essential bioactive compounds (117) with substantial health benefits to persons consuming them regularly.(118) They have the potential to be semi-essential dietary components.(109)

For medical and nutrition practitioners to advocate flavonoid-rich foods as modulators of disease, a sound knowledge of these phytochemicals along with quantification of the dietary intake is required.(7) It is essential to know not only the biological properties,(12,119) the bioavailability and the factors controlling the bioavailability of these ingested polyphenols, but also a person's daily intake. Furthermore, the nature of the major flavonoids consumed, their dietary origins through the intake of particular flavonoid contributing dietary items and the amounts consumed in different diets (12,120) needs to be known to comprehend their role in the protection of human health and the prevention of disease (12,119) and to ascertain their nutritional significance.(12,120)

Hence, while the research itself considered the possibility of determining flavonoid intake through a comprehensive and a resultant abbreviated FFQ, the literature chapters to be presented will attend firstly to the aspects to consider in the assessment and interpretation of dietary flavonoid intake in this chapter. The justification of such assessment in support of heart health protection will be discussed in Chapter 3. Chapter 4 will address the literature concerning the development and Chapter 5 the literature concerning the evaluation of the FFQ as dietary assessment method to establish flavonoid intake while Chapter 6 will attend to short or abbreviated FFQs. These chapters are based on the conceptual framework (Figure 2.1 on the next page) to support and guide the assessment of dietary flavonoid intake in relation to the fears surrounding CHD.

2.2   ASPECTS TO CONSIDER WHEN ASSESSING AND INTERPRETING

This section confers the biology of flavonoids that includes the flavonoid subclasses and types, their distribution in foods, their bioavailability and metabolism and their dietary safety. A comprehensive knowledge of these aspects is required to understand their mechanisms of action (21,97,121,122) in conferring protection against CVD (123) and for these abundant dietary
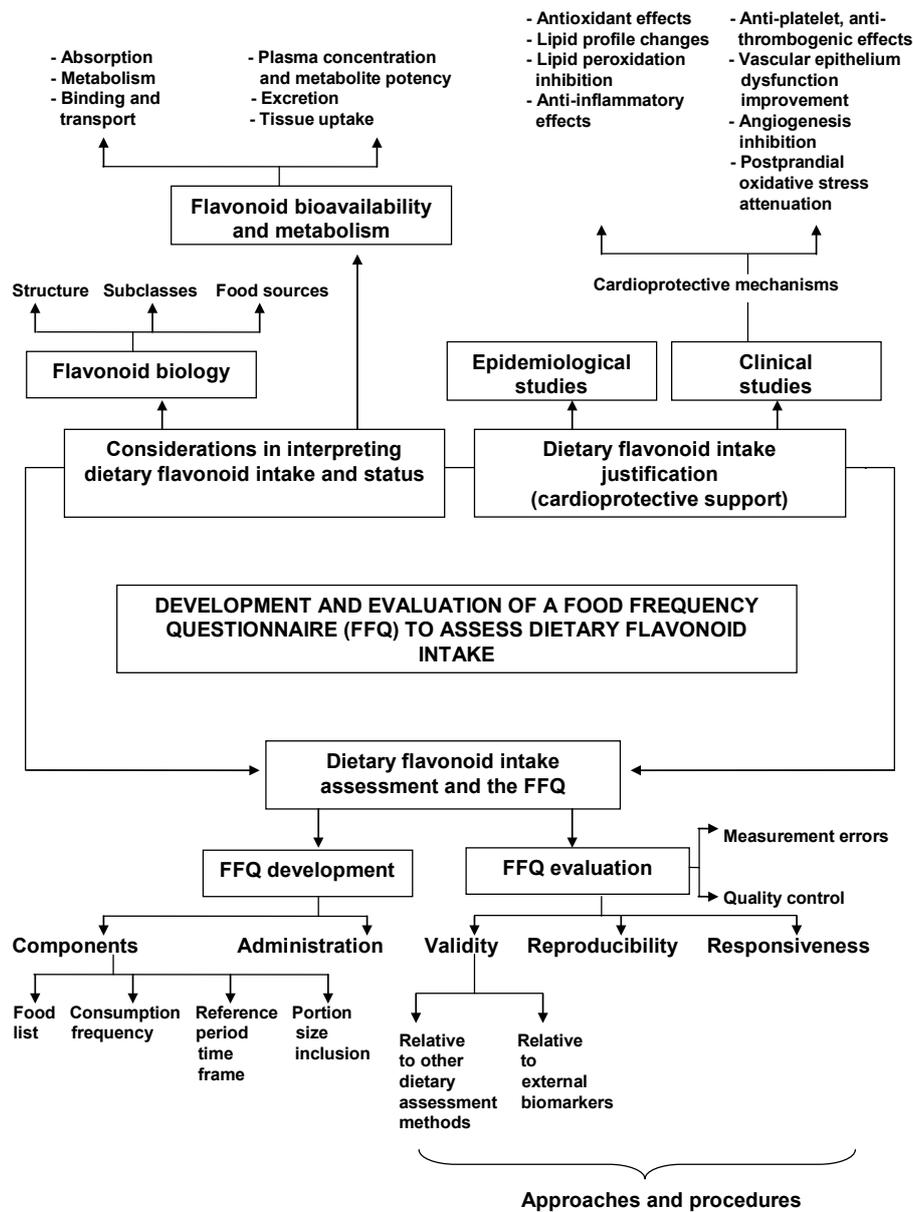
Figure 2.1:   Conceptual Framework for the Research from the Literature

components to obtain a just place in preventive nutrition. However, only modest information is available on their food composition and content, absorption and resultant distribution, metabolism and excretion in humans.(97,120,124-127) Because data is still limited, more studies are needed on the bioavailability in humans,(124) especially to determine dietary habits that can increase it.(124,125) Clarification is needed regarding which polyphenol concentration is biolocally effective and what concentration can be expected from dietary intake.(120,126)

2.2.1 Structural Description of Flavonoids

Flavonoids are benzo-*y*-pyrone derivatives containing phenolic or pyrane rings.(98) The basic chemical flavonoid structure (aglycone) is a common flavan nucleus which generally contains 15 carbon (C) atoms (128) positioned in three phenolic or pyrane rings which are labeled A, B and C.(92,128-130) The two benzene rings, labeled rings A and B, are attached (10) through a C bridge that consists of three Cs that links with an oxygen and two Cs of one of the aromatic rings (the A ring) to form a third six-member oxygenated heterocyclic ring (the C ring) (47,75,92) as illustrated in Figure 2.2. Plant phenolics are consequently usually structurally characterized by this flavan nucleus (98) or C_6-C_3-C_6 skeleton (10,47,48,128,131) or backbone structure.(12)
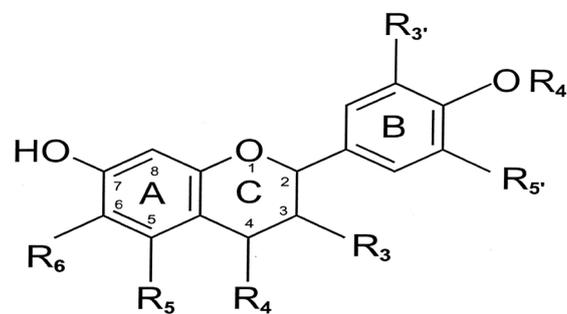
Figure 2.2: **General Schematic Generic Structure and Numbering Pattern of Flavonoids**
Source: Cook and Samman (129)

2.2.2 Subclasses of Flavonoids

The attachment of the B ring or substituent to the C ring divides the flavonoid polyphenol class into flavonoids (B ring bound to C2 position of C ring) (Figure 2.2) and isoflavonoids (B ring bound to C3 position of C ring).(121,132-134) The isoflavonoids are thus structurally isomeric to flavonoids,(135) but distinguished by linkage of the B ring to the C3 position and not the C2 position.(75,109) The isoflavonoids with their predominant isoflavones, genistein and daidzein (100,108) are best known for their estrogenic activity.(10,100) The isoflavones are usually treated separately from the standard flavonoid subclasses (135) and are excluded from this research. Because isoflavones are restricted to the set food source soy (75,95,100,101,108) with much lower concentrations found in legumes,(100,108) their intakes usually only reach a few milligram (mg) per day in Western countries,(75,100,136-138) but are higher in Japan.(75) The growing incorporation of soy extracts into processed foods could contribute to an increased ingestion of isoflavones.(100)

Numerous structural substitution variations within the three-ringed basic structure subdivide the flavonoids into several subclasses.(112,132) Due to the resultant diverse chemical structures,

several hundreds of different flavonoids have been identified and described with more still being added to the list.(75,98,112,134,139) However, only a limited number of these flavonoid structures occur at significant levels in most human diets (8) and are consequently of importance from a nutritional perspective.(108)

Dietary flavonoids are divided into subclasses based on differences in the generic structure of the heterocyclic C ring, the pattern of substitution or functional groups and the oxidation state of this heterocyclic C ring.(8,12,75,92,102,128,140) Variation in the C ring therefore gives rise to the major flavonoid subclasses found.(87,107,112,141,142) These multiple subclasses include the flavonols, flavones, flavanones, flavan-3-ols (flavanols/flavans) or catechins and anthocyanidins. (75,92,93,100,101,130,140) The absence or presence of a hydroxyl (OH) group at position C3 contributes to the formation of the two main subgroups of flavonoids: 3-desoxyflavonoids (flavones and flavanones) and 3-hydroxyflavonoids (flavonols, anthocyanidins and the flavanols or catechins).(134) Ring C may be a heterocyclic pyrone (six membered ring containing one oxygen atom and a ketone functional group), which forms flavonols, flavones and flavanones or pyrane (six membered ring containing five C atoms and one oxygen atom), which forms flavanols (catechins) and anthocyanidins.(9) Although there are many subclasses of flavonoids, only these subclasses are relevant as they are the most important classes of flavonoids (45) and the major flavonoids in commonly consumed foods (75,143) and in the diet.(9) Due to the former and because of their importance to human health,(10,143) these subclasses are also the focus of this research as they represent the total flavonoid intake related to it and the flavonoid subclasses incorporated within the United States Department of Agriculture (USDA) database for the flavonoid content of selected foods.(75)

Within these individual subclasses there is substantial diversity based on the number and positions of the substituents attached to the numerous binding sites of the molecule, that is OH, methyl (CH_3) and glycosyl groups.(108,140) As a result, individual flavonoids within a subclass are identified as they vary in their pattern of substitution of the A and B rings,(128) for example being characterized by specific hydroxylation and/or conjugation patterns of these rings.(92,107) Due to the many substitution patterns that occur in which major substituents (OH or glycosal groups) are themselves substituted (further glycosylated), highly complex structures can sometimes give way (flavanols occurring as oligomers and polymers termed proanthocyanidins and tannins, respectively).(102) Over and above this diversity, flavonoids may be linked with several carbohydrates and organic acids and with one another.(100)

The structural diversity of the flavonoid subclasses appears to determine and affect their physical, chemical and biological properties and activity.(108,134,140) Considering the number of

flavonoids in existence, an effect produced but the desired, may probably be due to a less optimal flavonoid choice made with the compound not reaching its target distribution.(133,134) Each phenolic ring usually contains at least one OH (92) with flavonoids frequently hydroxylated in the positions 3, 5, 7, 3', 4' and 5' (133,134) (Figure 2.2). The polyphenolic structure of flavonoids, encompassing several OH groups on the aromatic rings,(100,103) is related to their biological property as being potent antioxidants.(48,98,130) The flavonoid subclasses with the individual flavonoids within each subclass and their chemical structure characteristics and typical rich food sources are presented in Table 2.1.

Table 2.1: Flavonoid Subclasses, Chemical Structure Characteristics, Individual Flavonoids within each Subclass and Typical Rich Food Sources
Adapted from Beecher,(92) Hollman et al.,(112) Cook and Samman,(129) Rice-Evans et al.(132) and Dwyer and Peterson(143)

Flavonoid subclass	B ring catechol group (3', 4'-di-hydoxyl groups)[a]	C ring unsaturation[a]	C ring functional groups[a,b]	Prominent individual flavonoids	Typical rich food sources
Flavonols	Quercetin Myricetin	C2-C3 double bond	C3-hydroxyl group C4-oxo (or carbonyl) group	Quercetin Kaempferol Myricetin Isorhamnetin	Vegetables (onions, broccoli); Fruits (apples, berries,cherries); Beverages (red wine, tea)
Flavones	Luteolin	C2-C3 double bond	Lack C3-hydroxyl group C4-oxo (or carbonyl) group	Apigenin Luteolin	Herbs (parsley, thyme) Some vegetables (celery, green pepper)
Flavanones	Eriodictyol	Lack C2-C3 double bond	Lack C3-hydroxyl group C4-oxo (or carbonyl) group	Hesperetin Naringenin Eriodictyol	Fruits (citrus)
Flavanols	Catechol group	Lack C2-C3 double bond	C3-hydroxyl group Lack C4-oxo (or carbonyl) group	Catechins (Epicatechin; Epicatechin gallate; Epigallocatechin; Epigallocatechin gallate)	Fruits (apples); Beverages (tea)
Anthocyanidins	Catechol group	Lack C2-C3 double bond	C3-hydroxyl group Lack C4-oxo (or carbonyl) group	Cyanidin	Fruits (red, purple and blue berries, cherries, grapes)

[a] Presence of structural features related to antioxidant capacity (98,128,132,144-146)
[b] The term 4-oxo-flavonoids is used to describe flavonols, flavones and flavanones, which carry a carbonyl group (=0) on C4 of ring C.(9)

2.2.3   Food Sources and Flavonoid Content

Knowledge of the flavonoid content of the dietary provision is required to comprehend their health implications.(135)   Flavonoids occur almost all over in plant foods,(34,47,134,141,147) including edible plants and in foodstuffs derived from such edible plants,(97) and are generally coupled with the plant cell walls.(148)   They are abundantly present in fruits, vegetables and beverages,(8,34, 45-48,149) such as tea, (red) wine (8,12,34,45-48,75,98,149) and fruit juices,(8,12) cocoa (47,75, 98) and chocolate.(8,12,75)   Besides these common dietary sources, flavonoids are also found in herbs.(75)

Fruits and beverages represent the main dietary flavonoid sources (8,94-96,100) with vegetables (8,95) and herbs (75) being less significant sources. Fruits and fruit juices are among the pertinent dietary flavonoid contributors because of the high content in most fruits (93,125) and the relatively large portions (100 to 200 gram [g]) consumed.(93)   Beverages are expected to be a key dietary source for those persons consuming them regularly.(8)   Differences in the overall flavonoid intake in the diverse diets consumed nationally are said to be largely due to the variations in beverage consumption.(128)

Flavonoids are widely dispersed in foods, however, not evenly.(8,84,92)   A plant-derived food or beverage will contain various of the flavonoid subclasses (135,142) while mixed diets will contain all the subclasses.(135)   Although there are many foods that contain more than one subclass,(135) specific foods or groups of foods often contain a high concentration of one or more of the subclasses.(84,92)   For instance, while quercetin is found in all plant products, others are present in specific foods like the flavanones in citrus fruit.(100)   The South African herbal tea, rooibos (*Aspalathus linearis*), is increasingly enjoyed as healthy alternative to *Camellia sinensis* teas. Rooibos contains bioactive phytochemicals commonly and not commonly found in foods. Its major compounds are the flavonols found in common foods and the flavone isomers (orientin and isoorientin) uncommon to the diet, in addition to the unique dihydrochalcone, aspalathin.(63)   The levels of polyphenols may vary a great deal among diets depending on the type and quantity of plant foods in the diet.(147)   Within a single plant genus, great variability may occur as a result of genetic influences, environmental circumstances, plant growth stages, etc.(102,147)

As important as the total flavonoid content in food is, the content of the subclasses present may even be more critical when considered from a health perspective.(92)   Humans are, however, not likely to ingest dietary flavonoids singly because of the wide and varied flavonoid provision in food.(129)   There may be synergistic effects of the numerous nutrients and components present in foods that necessitate attention to intake of foods rather than the single components.(150)

The flavonol subclass is widely distributed in plant foods (10,75,151) and thus profusely present in foods (48,100) and the diet and regularly consumed by humans.(124) Quercetin is the most studied flavonoid (93,108,111,151-153) and the most prevalent and common flavonol in foods and the diet.(8,10,12,47,94,101,107,108,111,141,143,152) Flavonols are, however, generally present in foods in relatively low concentrations (75,100) with their content in the diet generally low. (12,151) Quercetin occurs in numerous fruits and vegetables (8,10) and predominates in vegetables,(10,135) particularly in the leaves,(10) with onions a rich source.(94,100,119,153) Apples, onions, tea and wine are the prominent dietary sources of quercetin.(93) Other common dietary flavonols include isorhamnetin (onion), kaempferol (broccoli) and myricetin (berries) (108) (Table 2.1).

The subclass flavones are not as widespread as the flavonols in fruits and vegetables.(75,100,101) Flavones are not common to fruit, but are present in some vegetables,(10) such as celery (48,75) and green pepper,(153) but are prominent in herbs, particularly parsley, rosemary and thyme.(10) The common dietary flavones are apigenin and luteolin (10,75,108,143) (Table 2.1). Their intake is, however, limited (12,108) since they are present in substantial amounts in only a small number of consumed edible plant foods.(108) Flavones contribute to the taste and can also contribute to the colour provision of plant tissue if they are present in high concentrations.(10)

Although many foods contain flavonols and flavones (with onions and tea the major dietary sources of these flavonoid subclasses),(47) only a few contain the subclass flavanones.(10) This subclass occurs almost exclusively only in citrus fruits (Table 2.1) in high concentrations (8,48,75,101,108,132,143) where they contribute to the flavour of citrus,(10) in particular the bitterness of grapefruit and lemon and their fruit juices.(104) The main flavanones are hesperetin in oranges, naringenin in grapefruit and eriodictyol in lemons (100,101,151) (Table 2.1). The solid parts of citrus fruit, mainly the albedo (white inner spongy part) and the membranes separating the segments, have a high flavanone content.(100,101) The intake of flavonones is most likely higher in areas where citrus fruits are cultivated, as citrus fruits are practically the exclusive supply of flavanones.(100)

The anthocyanin subclass is mainly associated with fruits.(10,100) Anthocyanins are glycosides of anthocyanidins with an appended sugar moiety.(104,135) The anthocyanins are the coloured flavonoids, with the colour being pH dependent.(10,100) The colour is usually red at pH 3.5 and turns to blue as the pH increases.(10) Grapes and highly pigmented fruit,(75,119) including strawberries and other berries,(8,48,75) are good sources of anthocyanins (or anthocyanidin glycosides) (8,48,75,119) (Table 2.1). The anthocyanin content in fruits is generally proportional to its colour intensity and the value increases as the fruit ripens. Although mainly present in the fruit

skin, they also occur in the fruit flesh.(100) Large amounts of anthocyanins may be present in diets incorporating large servings of red fruits like black grapes and berries.(151) Cyanidin is the most common anthocyanin in foods (75,94,100,135) (Table 2.1). Anthocyanins frequently occur as complex mixtures (10) due to esterification with several organic acids and phenolic acids and forming complexes with other flavonoids ('copigmentation').(100) Information on the consumption of these oligomers and polymers is still rather limited and attributed to the absence of accurate data on their food content.(115)

Flavans (or flavan-3-ols or flavanols) are the complicated flavonoid subclass in structure and naming (10) and are referred to as catechins (monomeric form), proanthocyanidins (oligomeric polymeric form) and tannins (polymeric form).(10,75,100) The oligomers of the flavanols are referred to as the procyanidins.(28) The main flavanols are the catechins.(8,12) They are present as monoflavans, biflavans and triflavans and are rarely glycosylated, but may be esterified with gallic acid.(10) Catechin oligomers comprise two to five monomeric units of catechins and catechin polymers over five units of monomeric catechins.(154) In plants, or through food processing, many food flavanols are polymerized into these large molecules. The proanthocyanidins or condensed tannins are thus oligomers and polymers, respectively, of flavanols that may also contain gallates (92) and are formed by catechins that are joined together by links between the C4 and C8 (or C6) positions (101) (Figure 2.2). Although flavanols are present in most fruit,(10,94) with apricots a rich source,(100) tea (green and black), red wine,(28,48,75,100,108,119) grapes and grape juice (28) along with cocoa and chocolate (28,75,93,100,101) are the major dietary sources of catechins. They are responsible for the astringent or bitter taste of these dietary items.(95) As catechins are the main components of tea, they are commonly referred to as the tea flavonoids.(155) The flavanols (catechins and proanthocyanidins) and anthocyanins, as well as their oxidation products, are said to be the most abundant flavonoids in the diet.(8)

Phenolic compounds occur in food in both bound and unbound forms, mainly glycosides, polymeric forms and esters.(142) Natural flavonoids are generally present in a glycosylated form (97,100,106,112,127,156) and occur as *O*-glycosides,(12,92,106,127) the so-called glycosides (112,132,139) that have a glycosidic moiety attached.(21) Food thus usually contain a low content of aglycones compared to glycosides.(157) Free flavonoids or those that are not bound to a sugar molecule are termed aglycones.(75,113)

More than 80 different sugars have been found bound to flavonoids in plants.(112) These sugars can be monosaccharides, disaccharides or oligosaccharides.(148) One parent flavonoid compound, or aglycone, can be linked to a number of different sugars.(112) This additionally

contributes to the complex structure of flavonoids and the numerous individual flavonoids that have been identified (92) and further intricates the quantification in foods.(112) The number of attached sugars is usually one but can be two or three.   There are a number of potential positions of substitution on the polyphenol structure.(12) The OH functional groups, commonly in position C3 (106,127) or C7 (132-134) (Figure 2.2), are potential sites for links to carbohydrates,(75) which include glucose, rhamnose, glucorhamnose, xylose, galactose and arabinose.(109,133,134)   D-glucose is the most frequent sugar residue attached (12,100,101,108,148) along with L-rhamnose. (12,100,101,108) Direct attachment of a sugar to an aromatic C also occur.(148) The main and most widespread glycoside form of quercetin, which is the most abundant flavonol in vegetables and fruits,(97) is quercetin-3-rutinoside, also termed quercetin-3-rhamnoglucoside or rutin.(97,108) This glycoside form of quercetin is the predominant flavonoid in tea and apples.(147)

As most plants store flavonoids attached to a hydrophilic moiety like sugar, it makes the flavonoid less reactive, more water-soluble and more easily handled by the plant.(99,132) The aglycones (the forms without attached sugar moieties) occur less commonly in plants (47,108) but small amounts of aglycones can occur in foods and thus also the diet.(158) Unlike the other classes of flavonoids, flavanols (catechins) are not glycosylated and occur in plants and most foods as aglycones.(75,93,100,120)   With the exception of the various hydroxylation and glycosylation patterns, the possible members of the flavonoid family can be extended (with the exception of the flavanols) through conjugation,(92) sulfation and methoxylation (134) forming numerous flavonoid derivatives.(75,133) Linkage with other compounds, such as carboxylic and organic acids, amines and lipids, and with other phenols also occur.(148)

Most, if not all, of the possible flavonoid compounds are removed in the production process of fats and oils.(10) The commercial techniques that refine oil seeds do away with all the flavonoids naturally present in the oils.(87) The grain production process generally removes most of the grain flavonoids. Flavonols, flavones, flavanols and anthocyanins are all present in grains. It is advantageous to do away with the flavonoids in grain food production as they can precipitate with the proteins present and result in browning to occur.(10) In milling, grain is processed so that some or all of the bran (an important flavonoid source) and oxidants are removed to improve the taste and shelflife.(87) Since most grain phenolics are found bound to cell wall materials in the outer layers (bran) and the grain milling process removes the outer layers containing these biological compounds,(159,160) it leaves refined grains largely phytochemical depleted compared to whole grains.(137) The flavonoid content of some foods including grains and exotic fruits has not been widely analyzed.(100)   No flavonoid content of finished grain products is currently included in the USDA flavonoid database (161) with the exception of commercially prepared whole-wheat bread (nutrient data bank (NDB) number: 18075) with zero flavanol content.

Animal foods are not an acknowledged source of flavonoids.(10) Flavonoids present in these foods originate from the plants that animals feed on rather than being internally biosynthesized.(48) It is not anticipated that milk and dairy products would contain flavonoids due to the mammalian metabolism of flavonoids.(10) Meat and dairy foods are thus devoid of flavonoids.(87) Although sugar is devoid of flavonoids,(87) sugars and sweets, like honey and chocolate, contain flavonoids as they contain plant constituents. Even jams have sufficient flavonoids that survive the jam-making process.(10)

Herbs and spices, in the quantities usually added to flavour food, most likely only increase flavonoid intake slightly, particularly in the case of spices, as only chili powder has high quercetin content. Flavonols and flavones are not present in commonly used spices like pepper and curry.(153) However, herbs like parsley and dill, which are customarily included in traditional Greek dishes, although added in small amounts, greatly add to the flavonol and flavone consumption and the flavonoid content of the diet due to frequent consumption.(137) A further supply of polyphenols is natural or synthetic phenolic food additives, which are added to foods for colour (anthocyanin extracts prepared from grape skin extracts), flavour (vanillin) or as preservative (rosemary). The resultant added flavonoid contributions differ greatly depending on the compound used.(75)

The assessment of the individual flavonoids present in foods and their quantification is analytically complex (112) as flavonoids occur in foods joined to various glycosides.(112,162) Because reference standards of the flavonoid glycosides are not commercially offered, flavonoids in food samples are hydrolyzed to the aglycone form in order to be identified and quantified against appropriate aglycone reference standards.(162) Aglycone reference standards are available for many, but not all of the aglycones, to allow for their quantification.(75) Acid or enzymatic hydrolysis is utilized to separate the sugars from the flavonoids with enzymatic hydrolysis the preferred method. Acid hydrolysis seems to destroy some of the flavonoids present resulting in an underestimation of the content present. The flavonoid compounds on extraction are generally separated, identified and quantified.(143) Various analytical methods are used for the quantification which differ in their specificity (75) and include a reverse-phase high-performance liquid chromatography (RP-HPLC) method,(75,112,143) mass spectrometry (MS) and diode array spectrometry (DAD)(143) with the latter methods used most frequently.(75) The flavonoid amounts in foods reported in databases are thus based on the aglycone form of each flavonoid (92,100,163) after the removal of the glycosides from the parent molecule through hydrolysis.(100)

The USDA currently assembles and maintains a database for the flavonoid content of selected foods. The database that incorporates the five predominant subclasses (flavonols, flavones, flavanones, flavanols and anthocyanidins), with the exclusion of the isoflavones, has been available since March 2003 on the USDA website.(75) The USDA released an updated and expanded version in 2007 for the content of these five flavonoid subclasses and their 19 most abundant component flavonoids comprising 385 selected foods and beverages in various preparation forms,(161) including among others, raw and processed fruits and juices, various vegetables, herbs and edible leaves, tea, wine and beer.(105) However, further research is required to include absent food sources (94) to expand the database.

A comprehensive food composition database is crucial, as it will permit the daily flavonoid intake to be assessed more expansively. The dietary intake could then be correlated with the prevalence of certain diseases or early markers of these diseases in epidemiological studies.(100) Advancement in epidemiological research on the association between polyphenol intake and disease, is in the main held back by the absence of complete and comprehensive food composition data. Although food content data is available for polyphenols and flavonoids, particularly on the USDA's website, content values for some polyphenol types and foods only eaten in particular countries are still not available.(138) As limited data are available for the flavonoid content of country-specific food and beverage items, Lyons-Wall et al. (164) sourced Australian food and beverage item values from published international data.

Specific flavonoid values (quercetin, myricetin and kaempferol) determined for foods in the United States (US) (fruits, vegetables and beverages) were found to be similar to those reported in European foods.(153) Flavonoid concentrations in onions, lettuce and tomatoes purchased in the United Kingdom (UK) corresponded well with concentrations in foods of Dutch origin.(165) For the purpose of this research, the USDA database for the flavonoid content of selected foods (161) was used to assess the daily total flavonoid intakes due to the absence of such a database for SA.

2.2.4   Factors Affecting the Food Content

Intrinsic factors inherent to the food itself and extrinsic agronomic and environmental factors affect the flavonoid content in foods.(9) The intrinsic factors that affect the content are the plant genotype (variety and species) and the distribution within the plant.(9,48,75,127) Variety, for instance, affects the flavonol content of tomatoes (normal-sized *Bond* fruit has a higher total flavonol content per gram than normal-sized *Havanera* fruit).(166) Varietal differences though found in the quercetin content of lettuce cultivars are even greater than that found with tomato cultivars. Different varieties of celery contain variable levels of luteolin and apigenin.(156)

Marked flavonoid content variations have been observed for different cabbage and radish cultivars.(117) Significant differences have been observed in the total flavonoid and total anthocyanin contents among strawberry genotypes, emphasizing that genetic background is a determining factor in manipulating the flavonoid content of the fruit. These findings emphasize the need to determine and differentiate the content of fruits, particulary those utilized for human feeding trials. Furthermore, the findings substantiate the significance of the breeding strategies presently utilized to enhance the content of specific bioactive compounds, given that higher levels of micronutrients and phytochemicals in fruits may be an important dietary tool to increase (antioxidant) intake, particularly in the case of low fruit intake.(167) The distribution pattern relies on the access to and the degree of (sun)light exposure as the higher oxidized flavonoid formation is accelerated by light.(9,48)

Agronomic as well as environmental factors, in addition to the genetic background, establish the flavonoid content of the plant at the time of harvest.(28) Geographical or regional origins and the environmental conditions (temperature, light, ultraviolet (UV) radiation and moisture) may affect the plant flavonoid composition.(75,127) Differences between the floral and geographical origins of honey samples accounted for the differences found in their flavonoid (apigenin and kaempferol) contents.(127) Light exposure has a large influence on the formation of most flavonoids.(100) The flavonoid concentration is highest in external and/or aerial tissues (skin and leaves) since (sun)light stimulates the biosynthesis (9,100) with accumulation in the skin and greater concentrations in foods with a high skin to volume ratio (9) implicating fruit size as a factor.(166) This explains the higher flavonol content of cherry tomatoes than of standard tomatoes as they have different ratios of skin to whole fruit.(100,166) Only negligible amounts of flavonols are present in the parts of plants below the soil surface, with the exception of onions.(9) Seasonal influences can as a result affect the levels where the flavonoid content in summer cultivated vegetables can be three to five times higher than of similar vegetables cultivated in other seasons, yet seasonal influences may not affect all vegetables and fruits.(9)

The seasonal influence is extended to climatic influences through increased light and sunlight exposure.(9). Hence, cultivating plants in glasshouses, which filter UV light, reduce the flavonol level in plants through relatively low UV light exposure. Field-grown tomatoes found in warm sunny climates in countries like Spain, Israel and SA, were found to contain four- to five-fold more flavonols than those from England and Scotland, where greenhouses are used for plant cultivation.(166) Hertog et al. (45,111) found the seasonal variability low and averaged the content of foods measured over three seasons in The Netherlands for the content indication. Because seasonal variation was low, year average catechin values were used in the Dutch National Food Consumption Survey of Arts et al.(168) The flavonol content of Spanish-grown cherry tomatoes

assessed over a period of 13 months did not fluctuate markedly, indicating that the growing conditions in Spain bring about the accumulation of a relatively high flavonol content in cherry tomatoes throughout most of the year.(166) However, the intake of flavonoids may vary between seasons, being highest in summer when the consumption of vegetables is highest.(169)

Besides sunlight exposure discussed above, the cultivation procedure also influences the content.(170) Lima et al. (170) noticed that samples of organically grown Chinese cabbage (*Brassica rapa* (syn. *B. campestris*) spp. *pekinensis*) and maize (*Zea mays* L.), tended to have a higher total phenolic level than the traditionally cultivated. The difference in the total phenolic content was however insignificant indicating that organically grown products with respect to total phenolic content are not better than the conventionally grown.(170) A study by Grinder-Pedersen et al. (116) investigated the influence of conventional versus organic growth conditions on the contents of selected dietary flavonoids and on urinary excretion in a human crossover intervention trial. The organically produced diet contained a markedly higher quercetin level in comparison to the conventionally produced diet. There was a tendency towards a higher isorhamnetin level in the conventionally produced diet and a higher kaempferol level in the organically produced diet.

As flavonoids are produced in the ripening period,(9,116,131) it is expected that organically grown plants, due to their longer ripening period, would have a higher level of these compounds.(116) Thus, the time of harvesting will greatly contribute to the variability in the flavonoid content as was found for the flavonoid content of leafy vegetables and red onions.(171) It is likely that crop growing conditions could have an effect on the absorption and bioavailability of polyphenolic compounds through effects on cell wall structure, but the urinary excretions of flavonoids as a percentage of the ingestion were comparable after both interventions in the study of Grinder-Pedersen et al.(116)

The plant genetics and variety (intrinsic factors) and the agronomic and environmental factors during growth (soil type, sunlight exposure, rainfall, field or greenhouse growth, etc.) alongside the maturity/ripeness at the time of harvest, undeniably determine the content of phytochemicals present at harvesting.(75,100,172) However, postharvest handling plays a major role at this stage (172) where processing and storage affect the final food content.(28,75,100) Even though much must still be learned about the flavonoid content in different foods, even less is known about the effects of processing on the flavonoid content.(162) Various enzymatic and chemical reactions occur throughout postharvest food storage and processing of which the structures of the resultant products are still not fully understood. The concentrations in food of these specific polyphenol derived molecules are usually not known and their properties are possibly dissimilar to those of their precursors.(102)

Preparation of foods, in particular of vegetables, for ingestion can cause loss in flavonoid content, despite the fact that flavonoids are rather stable compounds resistant to oxygen, heat and moderate degrees of acidity.(10) Increasing the temperature above 60°C will lower the phenolic amount, as certain phenolics decompose at high temperatures. At high temperures, certain phenolics may combine with other plant components.(131) Because flavonoids usually occur in higher amounts in the outer than in the inner parts of fruit and vegetables,(100,102,166,173) peeling, skinning, trimming and leaf selection can greatly remove and lower their flavonoid contents.(10,100,162) When different processing methods for onions were contrasted, the only marked losses of quercetin and kaempferol occurred during the pre-processing when the onion was trimmed, peeled and chopped (39%), with a greater loss when several layers were peeled off compared to only one as a result of loss of more outer flavonoid-rich onion layers.(162)

The method of cooking used in domestic food processing has an impact on the phenolic levels (9) with cooking in boiling water seemingly having a major influence on the phenolic level of food.(170) Boiling foods seems to result in the greatest reduction (156,162) followed by microwave cooking and frying that cause reductions (9,156) although less so for frying.(156) Cooking lowered the quercetin content of both tomatoes and onions with higher losses found following boiling and microwaving than after frying. This could be due to flavonoid breakdown during cooking and/or conjugated quercetin being extracted from tomato and onion tissues by hot water more efficiently than with hot sunflower oil.(156) Frying onions extensively only resulted in a 21% loss of flavonols.(156) Further cooking of onions, green beans and peas on pre-processing and blanching, by boiling in water, cooking in a microwave oven and frying, only had small effects on the quercetin and kaempferol contents.(162)

The loss of flavonoids during food handling procedures and cooking may be explained by the ease with which they escape from the cellular compartment of foods. This refers to prepared vegetables with ruptured cell walls, the food enzyme changes that occur, the resultant high surface area (92) along with the leaching action into the cooking water,(9) their solubility in water (92) and possibly chemical or thermal degradation.(9) The major biochemical process is enzymatic oxidation. This begins as soon as the integrity of the cell is broken. Other types of enzymes, such as esterases, glycosidases and decarboxylases, may also catalyse transformations and degradations of polyphenolic compounds. The resulting browning is usually not desirable, especially in postharvest storage of fresh fruits or juice and puree technology, but may be advantageous for some products (raisins, cocoa and tea).(102) The preferable culinary preparation method would be to steam cook vegetables, which avoids leaching.(100) Ninfali et al. (117) found that steamed Brassica vegetables retained about 80% of the phenolic level of the raw vegetables. Warm holding of

processed onions, green beans and peas at 60°C for up to two hours were shown not to influence the quercetin or the kaempferol content.(162)

The food flavonoid content can be greatly influenced by commercial food processing,(27) which affects food flavonoids through losses occurring during thermal processing and through transformation during processing.(92) The total catechin contents determined in commercially canned products were far lower than those in the equivalent fresh products and after home cooking of these items according to standard recipes.(102) Canned peeled plum and cherry tomatoes both contain very low levels of flavonols in comparison to the fresh fruit. However, in contrast to fresh tomatoes, most tomato-based commercial products (tomato puree and juice) contain large amounts of free flavonols.(166) Peterson and Dwyer (10) estimated that food preparation and processing of fresh fruits and vegetables may lower flavonoid content by 50% as a result of parts of the plant rich in them being discarded and leaching into water. Taking into account previous work done, Hertog et al. (111) indicated that the flavone and flavonol loss with average cooking methods (as in home cooking) to be only a small proportion (<20%) when compared to that of rigorous food processing (as in commercial food processsing) (50%). Where catechins predominate in foods, like fruit, that are usually consumed raw, the catechin loss due to home preparation is not an important issue.(174) Drying at room temperature may enhance enzymatic degradation and thus lower the amounts of phenolics.(131)

The production of black tea and red wine are two of the best examples of processing transformation that causes large changes in food flavonoids.(92,102) In the oxidative enzymatic processes and atmospheric conditions that occur during black tea production ('enzymatic fermentation' or heating), the flavanols are converted into more complex condensed structures, theaflavins (dimers) and primarily thearubigins (polymers) (92,100,101) with a reduction in the flavanol concentration and a parallel increase in the thearubigin concentration.(92) In other words, during the oxidation of the polyphenols that occurs to obtain black tea, the levels of the simple compounds decrease while the levels of the condensed compounds increase.(2) These flavanol-derived or condensed compounds or thearubigins are relatively large molecular weight compounds and are termed derived tannins. They are found largely in black and oolong teas (92) and contribute to the distinctive dark brown colour of black tea as a consequence of the non-coloured flavonoids that develop into pigmented species.(102) Black tea contains catechins, thearubigins and theaflavins, which are oxidation products of catechins that develop during enzymatic oxidation by polyphenol oxidase in fresh tea leaves.(175) The phenolic patterns of black and green teas are therefore very diverse, with black tea conceivably regarded as the oxidized form of green tea.(2)

The ingestion of tea flavonoids can vary greatly depending on the kind of tea or tea products consumed and the preparation method (brewing). Teas produced in Europe, which go through extensive fermentation, have lower catechin and higher thearubigin levels than teas prepared for the US.(9) The size of the tea leaves (particle size) that form the extraction surface also seems to be noteworthy.(9,106)   Particle size for the most part accounts for the differences found in flavonoid content of teas prepared from tea bags and those from loose leaves. Teas prepared from tea bags have the higher content. The time tea is brewed can greatly affect the flavonoid content. Increasing the brewing time enhances the extraction of flavonoids from black tea, but the level does not seem to increase further after ten minutes of brewing.(106) Chemical reactions of polyphenols are rather essential in (red) wine production, since they account for the colour and taste changes that occur during the aging of wine.(102)

Cocoa and chocolate represent food and beverage products of which the flavonoid content varies greatly because of several factors, including the manufacturing processes. As with most plants, genetic, agronomic and environmental factors can greatly determine the cocoa contents present at the time of harvest with postharvest handling playing a further crucial role.(172)   Time and temperature, along with other manufacturing processes like roasting, alkali treatment, etc. can have adverse effects on the flavonoid content in chocolate.(21,28) The Dutch process using an alkalizing agent to remove the bitter taste of chocolate results in a notable reduction in the flavonoid content.(27) Lastly, the recipe formulation for the prepared food or beverage product influences the quantity of cocoa and flavonoids present.(172) Not all chocolates are thus equal sources of flavonoids. Dark chocolate is produced with a higher percentage of cocoa bean liquor than milk chocolate, and, consequently, it commonly has a higher flavonoid content.(21)

The effect of storage is unclear concerning either increasing, decreasing or having no effect on flavonoid levels in foods,(9) although storage may influence the levels of flavonoids that are readily oxidized.(100) Price et al. (176) found that the decline in the total content and the composition of quercetin glucosides during normal commercial storage of onions was rather modest.

The various factors indicated above, that affect the food flavonoid content, make it difficult to absolutely quantify the food content and thus the resulting dietary intake.(7)   It must be remembered that the growing conditions and the conditions of transport and processing and storage, impact the content of the resultant supermarket food purchases,(156) which provides the content that would be consumed. Interactions with other components within the food matrix may affect the properties of polyphenols. This is likely to influence the absorption of polyphenols and should be taken cognizance of when bioavailability is considered. Interactions of high molecular weight polyphenol structures, like tannins, with food proteins and digestibility enzymes are

acknowledged for reducing protein digestibility and can be expected to influence polyphenol bioavailability.(102) Consequently, flavonoid bioavailability would probably not equate to the flavonoid intake.

2.3 FLAVONOID BIOAVAILABILITY AND METABOLISM

Knowledge of the processes and the factors implicated in the absorption and metabolism of flavonoids has been incomplete for some time, but the understanding has improved (108) and appears to depend on the type of flavonoid ingested.(98) The matter is, however, far from concluded (98,158) as knowledge gaps still exist on flavonoid uptake and metabolism, distribution and excretion after consumption of common foods (99,122) and in particular the biological effects of the metabolites formed.(99) This section attempts to describe their bioavailability focusing on the enzymatic hydrolysis of the naturally occurring glycosides at the various sites along the digestive tract and the resultant aglycones with their metabolic fate. Much of this information, as pointed out by Walle,(158) was gathered from research done with quercetin while only limited information is available on the absorption and bioavailability of most of the other flavonoids in humans.

2.3.1 Intestinal Absorption

Most flavonoid subclasses, with the exception of the flavanols/catechins, are naturally present in plants in their glycoside forms.(8,107,141) The digestion and absorption of flavonoids possibly begin with flavonoid aglycones perhaps absorbed in the stomach.(75) Because catechins are not glycosylated in nature (93) and only found as aglycones,(120) these flavonoids can be absorbed directly into the bloodstream.(93) Attachment of a sugar to a phenolic compound increases the water solubility and severely reduces passive diffusion, whereas phenolic aglycones are hydrophobic (9,99) and can diffuse passively through biological membranes.(9,48,99,139) The molecular size and solubility of the catechins may be the determining properties for their absorption.(120) Hence, only unbound forms can be absorbed directly.(142)

The sugar moieties of the flavonoid glycosides are cleaved during the absorption process (10,108) and this deglycosylation seems to result from two ß-endoglycosidase enzymes in the small intestine.(8,98,108,157) These enzymes, able to cleave flavonoid glycosides, are lactase phlorizin hydrolase (LPH), also termed lactase, a membrane-bound enzyme found in the epithelial cells (enterocytes) in the luminal side of the brush border of the small intestine wall (157,177) and a less well described cystosolic broad substrate specificity ß-glycosidase (BSßG) within the enterocytes.(157) Because LPH is a membrane-bound enzyme, it can hydrolyze flavonoid glycosides in the gut lumen whereby it can release aglycones into the intestinal lumen before absorption.(8,99,152,177) This allows for passive dispersal of the more hydrophobic aglycone

across the intestine brush border to occur.(8,12,177) Humans are different to other mammals, as high levels of LPH can persist into adulthood.(177) While only 5% of Europeans have LPH deficiency in adulthood, 90% of African adults are implicated in having a deficiency (12) that may have a major inference for the South African black population where lactase deficiency reaches an incidence of 78%.(178)

As the other ß-glycosidases in the small intestine act intracellularly, their action would require transport of the intact flavonoid glycoside into the enterocytes.(177) The flavonoid glucosides might be drawn into the enterocyte by the glucose moiety (179) through the transport action of the absorptive sodium-dependent glucose transporter (SGLT1) found in the small intestine brush-border membrane.(177,179,180) The multidrug resistance-associated proteins (MRP) 2 and 3 (MRP2 and MRP3), and probably also other MRP forms, are further possible membrane transporters.(158) The ratio between these efflux (MRP2) and absorption (MRP3) transporters would be a determining factor for absorption and bioavailability.(181) The monocarboxylate transporter (MCT) has also been implicated.(182) The existence of a specific transport system for transfer across the digestive wall remains to be determined.(97,179) This hydrolysis or deglycosylation of the flavonoid glycoside link by enzymes (ß-glycosidases) is likely to be the first phase of the metabolism of flavonoids (8,9,99,183) and is independent of the metabolism by the colonic microflora.(157)

If absorption does not occur in the small intestine, the flavonoids will be subjected to microbial metabolism and extensive degradation in the colon (177) with the colon microflora playing an important role in the bioconversion of unabsorbed glycosylated flavonoids to their aglycones.(48,108,141) The aglycones may then be absorbed partly or may undergo further bacterial bioconversion.(100,158) Although it is known that bacterial glycosidases in the large intestine are able to release flavonoid aglycones,(133,134) the enzymes involved in the hydrolysis of the flavonoid glycosides remain to be confirmed.(108) Microbial action of the ß-glucosidases, ß-rhamnosidases and esterases may be involved.(99) There is growing evidence to confirm that the human intestinal microflora are equipped with several hydrolytic enzymes that can degrade a number of flavonoid glycoside compounds.(9) Because of the high concentration gradient of flavonoids between the cecal contents and the circulating blood, a passive absorption of flavonoids could occur.(97) Dietary glycosides are thus not absorbed intact or rather do not enter the systemic circulation intact in humans.(158) As the aglycone probably has a greater biological influence than the glycoside, deglycosylation via a ß-glycosidase activity would be a crucial step in the metabolism.(157) Deglycosylation of the flavonoids allows for a site of conjugation,(98,157) which would result in its swift removal and excretion in the bile and urine.(157) Colonic bacteria

can break up the heterocyclic ring and convert the aglycone flavonoids to phenolic acids (8,9,100) that may be absorbed, metabolized further by the bacteria or excreted.(9)

Flavonoid absorption normally reaches only a few percent of the oral dose as indicated by the measured blood levels of intact flavonoids and their conjugates.(128) For instance, an average absorption of around 5% was determined for the cocoa aglycone flavanols catechin and epicatechin (EC).(184)   The flavonoid subclasses flavonols, flavanones and flavanols are sufficiently absorbed to present a likely influence on cardiovascular markers *in vivo*.(75) The levels determined *in vivo* of intact anthocyanidins and proanthocyanidins are in general considered to be inadequate to afford an expected biological effect. Their biological effects *in vivo* occur at higher levels and may be brought about by their secondary metabolites.(75)

2.3.2   Factors influencing the Intestinal Absorption

Awareness of the factors that influence polyphenolic absorption can provide considerable insight into methods for efficient use of these components.(184)

2.3.2.1   Flavonoid Structure

Flavonoid glycosylation, esterification and molecular weight are generally taken into account when considering flavonoid absorption.(95) For instance, as catechins are not glycosylated they do not require deglycosylation before absorption (8,181) and consequently even stomach absorption is suggested.(139)   Furthermore, it seems that it is the nature (99,119,180) and not the position (180,185) of the sugar moiety in the flavonoid structure, which influences its absorption.   It is generally accepted that glycosides with attached rhamnose are absorbed less quickly and less efficiently than are aglycones and glucosides.(100,180)   Whereas polyphenols with attached glucose (or possibly arabinose or xylose) are possible substrates for endogenous human enzymes, attached rhamnose is not a substrate for the ß-glycosidases and is only hydrolyzed by the colon microflora enzyme activity.(8,12,98)

Anthocyanins appear to have poor absorption efficiency,(151,158) which may be related to their structure. Their glycosides do not seem to be readily hydrolyzed to their aglycones.(158) Thus anthocyanins may possibly have a specific absorption or metabolism mechanism.(100)   Large molecular weight polyphenols, with their degrees of polymerisation, like proanthocyanidins, tea theaflavins (95) and even catechin monomers,(115) are very poorly or not absorbed in the gut.(95) They are not depolymerized in the stomach and enter the small intestine intact, where they are almost not absorbed as a result of their high molecular weight.(115) They require degradation to smaller low molecular weight compounds for their absorption across the intestinal epithelium. Besides for the hydrolysis of the flavonoid glycosides, the colonic bacteria are also involved in the degradation of polymers (98) into more bioavailable low molecular weight phenolic acids.(115)

2.3.2.2   *Individual Intestinal Variability*

Individual differences in the human intestine physiology may result in between-person variations in flavonoid absorption and be responsible for the varying extent of the absorbed levels found.(9,184) The carriers and enzymes involved in the absorption and metabolism may for instance be involved.(100) Diet-mediated alterations on the gut physiology may influence individual variability including the intestinal absorption variability, such as gastric emptying time, acid secretion and pH, intestinal fermentations and transit time, enzyme secretion and biliary excretion.(100,184)

2.3.2.3 *Food Factors*

2.3.2.3.1   Food dosage

The food dose is obviously important.(186) As expected from the higher catechin content in green tea, Van het Hof et al. (46) and Leenen et al. (187) found the plasma catechin concentrations to be markedly higher after drinking green tea in comparison to black tea.   In agreement with the changes found in the plasma total catechin levels, green tea consumption produced a significantly higher urinary catechin excretion, which is in agreement with the difference in the catechin content of the teas.(46) While a study found the increase in plasma EC concentrations in subjects after the ingestion of flavonoid-rich chocolate to be dose dependent,(21) another study observed a linear relationship between the consumption of procyanidin-rich chocolate and the plasma procyanidin concentration.(123)   In a study evaluating the effect of strawberry genotypes on postprandial antioxidant status in healthy subjects, the smallest increases in plasma total antioxidant capacity (TAC) occurred on consumption of the nutritionally inferior berry cultivars,(167) which supports the importance of food dosage.

2.3.2.3.2   Food matrix

Food can influence the absorption of many nutrients.(188,189) The food matrix in which flavonoids are delivered, is therefore important.(186) The effects of the food matrix on flavonoid absorption (184) and bioavailability,(100) have not been investigated extensively.(100,184)   As a result, relatively little is known about the effect the food matrix has.(184)

2.3.2.3.3   Flavonoid and other phytochemical factors

One of the important unanswered questions is whether flavonoids are equally bioavailable from different foods.(190) Phenolic compounds could have synergist or antagonist effects when present in complex mixtures.(175) The influence of one dietary component on the uptake of another is important because foods are composite mixtures of coexisting nutritional components.(191) Differences in the cell wall structures, the glycoside location in cells and their binding to cell constituents, influence the release of flavonoids from foods in the gut.(192)   There is still uncertainty about which of the various forms of flavonoids are absorbed more efficiently.(9) As the flavonoid composition of a food may affect flavonoid absorption, it needs to be taken into account

when investigating a food's biological activity. Any synergistic or antagonistic effects the various flavonoids present may demonstrate toward each other, may affect their absorption.(28)

Hollman et al. (193) approximated that the bioavailability of quercetin from apples was about one-third of that from onions. De Vries et al.,(194) in a feeding trial that provided black tea and fried onions to healthy subjects, found that though quercetin was rapidly absorbed, the quercetin absorption from tea was only half that of onions. The reason could most likely be the difference in the type of quercetin conjugate present in the two foods. The absorption of quercetin rutinoside, a prominent quercetin compound in tea, is probably less than that of quercetin glusoside, the prominent compound in onions.(194) Plasma quercetin concentrations were also significantly higher following a combined fried onion and tomato (fresh cherry tomatoes) meal (flavonoid/carotenoid meal) compared to that occurring on consuming fried onions only (flavonoid meal).(191) There is support that some flavanols are better absorbed than others.(172) Catechins from chocolate were indicated to be more bioavailable than catechins from wine.(123) With more information available, it may in future be possible to compare and contrast the relative absorption of the different flavonoids.(151)

2.3.2.3.4   Interactions between flavonoids and nutrients

There is limited evidence available on the magnitude with which flavonoids interact with other dietary components, also nutrients in the diet, before and after absorption.(172) It is assumed that the intake of any food may impact the absorption.(100) Direct interactions between flavonoids and some food constituents can occur, like the presence of alcohol that may solubilize (124) and proteins and polysaccharides that may bind (100,124) these components and influence the absorption.

2.3.2.3.4.1   *Dietary protein*

Dietary components, namely protein (and iron), may hinder polyphenol absorption by forming complexes with the polyphenols (98). Polyphenols have a great affinity for proteins due to their aromatic nucleus and OH groups.(97) The interactions of polyphenols with proteins might be involved in their reduced bioavailability. Protein-polyphenol complexes could withstand gastric hydrolysis and consequently not be available for absorption in the gut.(142) Proteins in the food itself, the gastrointestinal tract and the bloodstream may possibly influence the biological effects of polyhydroxylated flavonoids.(98)

Due to the finding that some polyphenols form complexes with proteins, it was proposed that adding milk to black tea lowers the bioavailability of tea polyphenols.(120) The UK has a high saturated fatty acid intake.(130) Despite of their high tea consumption (five to six cups versus one to two cups per day) the UK still has one of the highest CHD mortality rates in the

world.(130,165,195) It was considered that in the practice in the UK of milk addition to tea, binding of tea flavonoids to milk proteins may result, which will impact the absorption and bioavailability.(130,165,195,196) This was confirmed in a human dietary intervention trial by Serafini et al.,(59) which found that milk addition to black tea (100 milliliter (mL) added milk to 300 mL tea; milk ratio of 1:4), did not bring about the *in vivo* increase in antioxidant potential that was found when black tea was consumed with no milk added. The inhibition of this effect by milk was associated with the binding of tea polyphenols by milk proteins rendering the complex unavailable for absorption in the upper gastrointestinal tract.(59) However, in other intervention trials, milk addition to black tea had no influence on the plasma levels of quercetin or kaempferol (196) and no influence on the plasma levels of catechins (195) in healthy individuals. Milk addition to green tea had no influence on the bioavailability of catechins.(195) Addition of milk (standard addition of 10-15% milk) to black tea or green tea did not influence the increases found in plasma antioxidant activity. The difference in the amount of milk added to tea might be an explanation for the inconsistency between the study results.(187) Based on the above, it is apparent that tea polyphenols are swiftly absorbed after drinking tea with milk and that milk do not interfere with polyphenol bioavailability.(48,195)

There is possibly a milk interfering effect on the bioavailability and likely biological effects of cocoa flavanols and procyanidins.(20) In a crossover experimental trial, the plasma EC content and the plasma TAC significantly increased one hour after the consumption of dark chocolate (100 g), but these effects were greatly reduced after the consumption of dark chocolate with milk (100 g dark chocolate with 200 mL full-fat milk) or milk chocolate (200 g). Volunteers consumed twice as much milk chocolate as dark chocolate to obtain an equivalent intake of antioxidants based on the TAC.(197) The researchers declared that eating dark chocolate, but not milk chocolate or dark chocolate with milk, increased the TAC of human plasma. They speculated that milk, either in the manufacturing process or during ingestion, may impair the absorption of EC from chocolate or inhibit the *in vivo* antioxidant activity of chocolate. The trial results emphasized the likelihood that the *in vivo* antioxidant activity of flavonoids could be reduced by other dietary components so that food combinations may interfere with the absorption and resultant health effects of flavonoids.(197) The researchers (197) indicated that this finding could have implications beyond chocolate ingestion if dairy products do impair the health benefits of dietary flavanols. As a result, dietary habits need to be taken into consideration when planning studies to investigate the relation between flavonoid-rich foods, antioxidant activity and degenerative diseases. This concern was queried by other investigators (198) who did not come across an impaired effect by milk addition to chocolate on plasma AC. The findings of one such investigator, Schroeter et al.,(198) showed that the inclusion of milk in cocoa products (as a cocoa beverage with either whole milk or water) did not impair the absorption and biological activity (as an increase in plasma AC) of monomeric

flavanols from cocoa products. These investigators indicated that it is essential to control for the composition of the matrix in which the flavanols are provided, to compare the absorption of EC from chocolate consumed in the absence and presence of milk.(198)

2.3.2.3.4.2   *Dietary fibre*

Dietary fibre is usually present in plant foods. It stimulates intestinal fermentation which could manipulate the formation of specific microbial flavonoid metabolites. Aspects, like the effect of the diet composition on the microflora and the microflora composition itself on microbial flavonoid metabolite production, should therefore also be considered.(100)   The colonic microflora composition in particular could clarify between-person variability in flavonoid bioavailability.(8)

2.3.2.3.4.3   *Dietary carbohydrate*

Schramm et al. (184) found that the absorption of flavanols can be greatly enhanced by simultaneous carbohydrate ingestion. Increased flavanol (flavanol aglycones, catechin and EC) concentrations on absorption occurred with the accompanying consumption of bread and sugar compared to when cocoa was consumed alone with limited influence of either protein- or lipid-rich meals.

2.3.2.3.4.4   *Ethanol*

Ethanol could play a noteworthy indirect role in the absorption efficiency of phenolic compounds. Phenolic compounds are barely soluble in water, but are soluble in ethanol.(199) In an intervention by Ghiselli et al.,(199) beer consumption brought about a marked increase in plasma TAC, whereas a low and insignificant increase in plasma TAC occurred after dealcoholized beer drinking. The summed plasma phenolic acids measured tended to increase in the case of beer (not dealcoholized beer) consumption although the removal of ethanol did not influence the original phenolic content of the beer.(199) According to these researchers,(199) beer containing ethanol was able to present its phenolic compounds to the body fluids more effectively than dealcoholized beer and facilitated the transfer of the AC from beer to the body fluids, possibly through increased absorption of the phenolic compounds. Ethanol removed from beer as dealcoholized beer, impaired the absorption of the phenolic compounds.

Some investigators proposed that the presence of alcohol in red wine could enhance the intestinal absorption of flavonoids by increasing flavonoid solubility.(100)   Plasma catechin metabolite concentrations were, however, comparable after drinking red wine or dealcoholized red wine in a human intervention by Donovan et al.(186) Donovan et al. (122) found a higher urinary excretion of catechin metabolites after red wine consumption compared to dealcoholized red wine consumption. They indicated that by increasing the elimination in urine, it suggested that ethanol enhanced the elimination rate of catechins by possibly reducing their elimination half-life in plasma and perhaps having a diuretic effect.(122)

2.3.3   Intestinal and Colonic Metabolism

The intestinal lumen, enterocytes and liver are important sites in the flavonoid metabolism.(108) Knowledge of the metabolism and excretion of flavonoids once absorbed is mostly obtained from animal rather than human studies,(120,121,158,141) with the pathway followed similar to that of the metabolism of drugs.(8,12) Flavonoids after hydrolysis to free aglycones and absorbed, will be metabolized by phase I or phase II enzymes.(99) The metabolism of flavonoids was primarily believed to be the result of cytochrome P450 (CYP) enzyme action (phase I metabolizing enzymes) in correspondence with the metabolism of drugs and other foreign compounds (xenobiotics).(158) Flavonoids though are good substrates and inducers of phase II enzymes, which indicate that they are identified by the body as foreign and probable toxic compounds, most likely because of their polyhydroxylated structure and redox activity.(93) In other words, phase I metabolizing enzymes (including cytochrome P450) catalyse the xenobiotic, and the phase I metabolites formed is subjected to phase II metabolism and detoxified by the phase II enzymes involved in conjugation reactions.(139) However, while human liver microsomal CYP-mediated oxidative metabolism of flavonoids has been found to occur,(99,158) its importance *in vivo* has not been fully established. It is expected that the phase II activities, namely conjugation, competes with the oxidative metabolism.(158)

2.3.3.1   Intestinal Metabolism

Absorbed flavonoids are subjected to three main types of conjugation, which are glucuronidation, sulfation and methylation (8,12,95,100), or a combination (8,12) in the gut mucosa and inner tissues,(95,120) like the liver (98) and to a lesser extent in the kidneys.(10,120,141) This gives rise mostly to glucuronidated or sulphated and to a lesser extent methylated compounds.(95,98,120) For instance, flavanols are quickly metabolized which result in the formation of a range of *O*-glucuronidated, *O*-sulfated and *O*-methylated conjugates of the parent aglycone.(28) The phase II metabolism enzymes implicated in the metabolism are first of all UDP glucuronosyl transferase (UDPGT, UGT) and its family of related enzymes. These have a great capacity in the liver and are responsible for the glucuronidation of polyphenols to glucuronic acid. In addition a small group of widely distributed phenol sulfotransferases (SULT) is responsible for the sulfation and catechol-*O*-methyltransferase (COMT) that occur in various tissues responsible for the methylation of polyphenols.(8,12) After absorption, the OH groups of the intact molecule are usually conjugated with glucuronic acid or sulphate.(10,120,141) The majority of flavonoids in the small intestine and liver therefore form glucuronide and sulphate conjugates,(183) whereas flavonoids with a catechol-moiety (or dihydroxylated (di-OH) B ring) (Table 2.1) are expected to be substrates for COMT forming *O*-methylated conjugates.(182,183) It appears that the methylation process may be important when rather high quantities of quercetin are ingested.(124)

Although the liver is important in the metabolism of xenobiotics, the small intestine and kidney contributions should not be underrated.(99)   The small intestine in particular expresses metabolizing enzymes and therefore has the ability to perform metabolic transformations during the course of absorption.(99,100,113)   It is commonly agreed that the conjugation pathway for flavonoids starts with the conjugation of a glucuronide moiety in the intestinal cells (100,200) and then in the liver.(100)   The liver can broaden the conjugation of flavonoids by the addition of a sulfate group, a methyl group, or both.(200) Flavonoid conjugations with glucuronide and sulphate moieties thus largely occur within the enterocyte (181) and in the liver.(121,141) Glucuronidation occurs foremost at higher concentrations and sulphation at low concentrations, as this pathway can easily become saturated.(99)   In addition, *O*-methylation also occurs.(121,141)   Large amounts are also, for the most part, metabolized in the liver with small amounts metabolized in the intestinal mucosa.(8)

These additions directly after absorption, whether in the epithelial cells or liver, effectively metabolize the absorbed flavonoids before the systemic circulation so that the conjugated forms principally circulate in the plasma and are excreted in the urine,(186) whether quercetin,(124,185) flavonol (108,126) or catechin (186,201) conjugates providing support for the effective occurrence of the phase II metabolism in humans.   The metabolism of flavonoids to polar conjugates accelerates their excretion and clarifies why their plasma levels do not accumulate (186) and probably decrease their toxicity.(200)   Substantial amounts of aglycones have been found in human plasma on consumption of gram-level flavonoid doses which may result, because the metabolic enzymes were not able to metabolize the total amount before circulation when administered at such high levels.(186)

2.3.3.2   *Colonic Metabolism*

In addition to the phase II metabolism, colonic micro-organisms metabolize polyphenols that reach the colon.(12,33,95)   For the fraction of unabsorbed flavonoid glycosides, like the rhamnoglycosides (10,48,121,141) and aglycones (133,134) that reach the colon, together with the conjugated flavonoids that are excreted in the bile, colon degradation may occur.(10,121,141) When excreted in the bile, the flavonoids re-enter the gut and are metabolized by the intestinal bacteria with the resulting metabolites of conjugate hydrolysis (or the degradation products) either reabsorbed to enter the enterohepatic recycling (9,100,120) that maintains a longer flavonoid presence within the body,(100) or excreted.(9) The compounds may be reabsorbed and recycled if the intestinal microflora is effective at removing the sulphate and glucuronide residues.(186) The intestinal microflora possess ß-glucuronidases that have the ability to liberate free aglycones from conjugated metabolites secreted in the bile.(100)

The colon microbacterial enzymes have considerable catalytic and hydrolytic potential, with deconjugation reactions (8,128) and cleavage of the heterocyclic C ring (128) occurring readily. The colonic bacteria hydrolyze the flavonoid glycosides (10,121,141) and the flavonoid conjugates (the flavonoid glucuronides and sulfates), which enables absorption of released aglycones, (10,120,121,141) or split the heterocyclic C ring. They degrade the flavonoids into numerous simple or low molecular weight phenolic compounds,(9,10,120,121,141) such as phenolic acids and other ring scission products,(100,121) and in addition all the way to carbon dioxide (CO_2).(158) Dietary flavonoids are very vulnerable to ring cleavage and only a small fraction of the liberated aglycone will be available for colonic absorption.(121)

Most ingested flavonoids and the major resultant metabolites are degraded to a variety of phenolic acids.(12,95,128) The yields of these phenolic acids, which represent the major polyphenol metabolites formed, can be high.(95) The resulting phenolic acids may be absorbed,(120,121) but are prone to secondary reactions like ß-oxidation, demethylation and dehydroxylation,(121) along with conjugation,(10,100) and can then be re-excreted into the bile, excreted in the urine or even metabolized further by the colonic bacteria.(10) A high amount of substrate will therefore reach the colon (95) and the body tissue exposure to these microbial metabolites or phenolic acids will consequently be high.(33,95) It has been pointed out that proanthocyanidins, procyanidins and the related catechin monomers are not notably susceptible to phase I or II metabolism in the small intestine.(202) They are almost fully subjected to colonic degradation into more bioavailable low molecular weight phenolic acids.(28,115,202) This highlights the proposition of microbial metabolites contributing to the biological effects *in vivo* of cocoa and chocolate polyphenols.(28,115)

Consequently polyphenols are comprehensively transformed either in the intestinal tissues, once they are absorbed through the gut barrier, or, for the non-absorbed portion and the portion re-excreted in the bile, by the colonic bacteria.(95) Despite limited metabolic data, the available evidence suggests that flavonoids are structurally changed *in vivo*. Whether phenolic acids or flavonoid isomers dominate is not clear.(98) Plasma pools is hence undoubtedly in a state of flux as a result of the joint effects of transport through the gut wall into the bloodstream and removal by metabolism and excretion.(203) Although the metabolites are still largely unidentified and therefore not accounted for, their presence probably makes the plasma polyphenolic concentration higher.(8) The extensive metabolism of flavonoids thus cause the chemical forms present in foods (mainly glycosides, except for catechins and proanthocyanidins which are present as aglycones) to be greatly dissimilar to the *in vivo* metabolites.(93)

2.3.4 Plasma Binding and Transport

The circulating flavonoids are their conjugated derivatives that are bound to plasma proteins, with albumin the primary protein responsible for binding.(100) For instance, due to the high affinity of quercetin for albumin,(97) its circulating metabolites retained the property to bind to albumin.(97,124) Furthermore many aglycones would be bound to serum albumin due to their low polarity.(133,134) The physiological concentration of albumin is most likely high enough to support extensive binding. However, the opinion is that cellular uptake is proportional to the unbound levels of the metabolites.(100)

The ligand-albumin complex formed raised some deliberation with regard to its biological activity versus that of the flavonoid metabolite in free form.(100) It is possible that plasma binding would reduce the levels and activity of the aglycones (158) and/or that the polyphenols in these complexes are biologically less powerful.(204) For instance, some chemical groups with reducing capacity (OH groups or catechol group) (Table 2.1) could be blocked by a protein amino acid chain.(204) Hence, as polyphenols are bound to plasma proteins, they would be less likely to be able to preserve low density lipoprotein (LDL).(128) Van het Hof et al. (46) recovered more than 50% of the consumed catechins in their intervention in the plasma protein fraction with the catechin concentration highest in the high density lipoprotein (HDL) and increasingly lower in the LDL and then the very low density lipoprotein (VLDL) fractions. Of further importance is the accumulation of polyphenols and their metabolites between the aqueous and lipid phases, which is supportive of the aqueous phase because of their water-solubility and binding to albumin.(100)

2.3.5 Plasma Concentrations and Elimination Half-Lives

Evidence for the absorption of flavonoids has been provided by determining their levels in the plasma and urine after ingestion of either pure compounds or consumption of foods with known content of flavonoids along with, for instance, measuring the AC of the plasma.(12) Plasma levels obtained after flavonoid ingestion varies greatly; in accordance with the compound nature and the food source.(100) In other words, the bioavailability of flavonoids varies to a great extent between the different subclasses and compounds (75,108) that are also grounds for substantial differences in kinetics of uptake and elimination of the different flavonoids.(195)

Due to this extensive metabolism of flavonoids, very little natural flavonoid aglycones are found in the plasma.(158) Whereas plasma levels of flavonoids on the whole are not found,(121) the concentrations of their conjugated metabolites,(158) which form the main circulating compounds,(100) are found. High plasma levels are not found.(120) Plasma levels are relatively low, reaching only 1 to 5 micromolar (µM) concentrations.(75) Intakes of monomeric flavonols, flavones and flavanols are low. Their plasma levels are seldom above 1 µM/liter (L) due to limited

absorption and swift elimination. Flavanones have a better bioavailability profile and their plasma levels can reach 5 µM/L. However, the presence of flavanones in food is confined to citrus fruit.(100) The levels of unconjugated flavonoids are also seldom above 1 µM.(205) The data available indicate that the plasma levels of flavonoids and/or their metabolites are potentially sufficient enough to confer biological activity and effects in humans.(75,121,183) This provides support for assessing the dietary intake of flavonoids despite the numerous factors possibly influencing the absorption.

The plasma half-life of all flavonoids is fairly short (less than 24 hours).(21,75) The rapid excretion is brought about by the conjugation of the aglycone to glucuronide and sulphate groups.(8) The elimination half-lives of quercetin metabolites ranges from 11 to 28 hours (151) or about 24 to 25 hours,(119,141,180,192,203) which affects its bioavailability. Flavonols quickly accummulate in plasma with peak levels being attained within approximately two hours in most instances.(124,203,206) Within 24 hours the flavonol levels decline markedly (124,206) and are either undetectable or present in trace amounts.(203)

The long half-life elimination of quercetin suggests that quercetin will accumulate in the plasma (119,141,151,192) as it is eliminated quite slowly throughout the day.(141,151,192) The half-life elimination of quercetin is furthermore not dependent on the type of quercetin glycoside.(119) Quercetin therefore seem to be adequately bioavailable to contribute as an antioxidant *in vivo*.(119,192) The consumption of quercetin-rich foods (onions, apples and tea) (192) could therefore result in a noteworthy plasma quercetin level that could make a contribution to the plasma AC.(119,192) For instance, quercetin tended to accumulate in the plasma on repeated tea consumption (every two hours each day for a total of eight cups daily), with the plasma quercetin concentration at the end of the third day higher than that after the first day of tea consumption agreeing with the observed longer half-life for quercetin.(196) The periodic consumption of cooked onion slices three times a day for a week favoured accumulation of quercetin conjugates in the plasma with the concentration after the trial higher than before the trial.(207) As the bioavailability of quercetin from apples, and of pure quercetin rutinoside, was both 30% comparative to that of onions, the plasma levels of those who regularly consume onions may reach higher levels.(192) The plasma accumulation of quercetin with repeated consumption is important,(151) as recurring ingestion of quercetin-containing foods throughout the day will not only contribute to accumulation of quercetin in the blood,(4,107,141,192) but also increase the AC of blood plasma.(107,141,192)

Catechin bioavailability compared to other flavonoids differs markedly as they are generally eliminated very fast (151,208) with a much shorter plasma half-life of only two to three hours. (122,208,209) Consistent with the relatively short plasma half-life, flavanols reach their peak

concentration in the plasma within about two hours (28,195,210-213) and are rapidly cleared from the plasma within the first four or more hours (21,122,195,210,211) after ingestion, as the water solubility of catechins is high.(184) When measured in fasted blood samples, the contribution of catechins to the antioxidant status may therefore be underestimated due to their plasma clearance compared to the value during the day.(195,214)

Considering the elimination half-life of catechins, it appears that they have no chance to accumulate in the plasma.(151) The rapid absorption and elimination of catechins in blood imply that upholding a steady catechin concentration in the blood, requires frequent repeated intakes of polyphenols very close together over time to retain metabolite accumulation in the plasma.(46,195) This was observed in the trial of Van het Hof et al. (46) for tea catechins with volunteers consuming tea every two hours. Therefore, if flavonoid-containing foods are ingested before full removal of the flavonoids from the preceding meal, increasingly higher plasma levels could be the result.(186) Warden et al. (189) found an increase in plasma catechin concentrations over baseline levels after black tea consumption at four times points over six hours (to mimic the tea intake of a regular tea drinker during the day) and in response to each successive dose of tea reaching a plateau at between 5 and 8 hours, but which returned to baseline levels by 24 hours.

In the trial of Van het Hof et al.,(46) repeated green tea or black tea ingestion (eight cups with one cup every two hours) during the day quickly increased the total catechin level, and a steady state level was obtained before the end of the day (after five cups of tea). Plasma catechin levels decreased overnight when no tea was consumed; however, there was a steady and significant increase in the plasma catechin levels in the first morning blood sample. The catechin level at the end of the third day was markedly higher than those of the two preceding days suggesting that blood catechin levels increase upon recurring tea consumption.(46) On the other hand, chronic ingestion of high levels may cause a compensatory decrease in absorption suggestive of a potential steady state mechanism at the gut level.(98)

With reference to the elimination half-lives, it appears that flavanones have no chance of accumulating in the plasma even with repeated ingestion. Not much is known about the bioavailability of flavanones in plasma, or their plasma metabolites.(151) Their elimination half-lives are relatively short at approximately one to two hours, reaching 1.5 to 2.5 hours.(108,151) The flavanone plasma concentrations therefore reflect short-term intake and probably not long-term intake.(118) The bioavailability of anthocyanins appears to be low (151,215) or they are very rapidly absorbed and excreted (151) or some of their metabolites not yet known well absorbed.(100) Their bioavailability may thus be underestimated.(151)

For most flavonoids absorbed in the small intestine, the plasma level quickly increases on consumption and then decreases due to the short elimination half-lives of about one to two hours.(8,12,95) The data presented on the bioavailability of flavonoids only considers the occurrence of intact polyphenols in the blood (the ingested compound or it conjugates). The wide range of metabolites formed by the colonic microflora are generally not considered (209) of which the half-life is believed to be longer.(8)

2.3.6 Plasma Circulation and Metabolite Potency

Non-conjugated polyphenols are almost not present in the plasma.(95,97,124) Unlike the administration of drug dosages, it is not expected that food polyphenols would saturate these metabolic pathways and as a result, the circulating species would be expected to be conjugated with no unconjugated species.(8,12) The conjugation mechanisms are extremely effective as the parent aglycones are in general either not present in the blood or only present in a low concentration after ingestion.(28,100,124) A small fraction of some flavonoids escapes conjugation in the intestine and consequently is found in both the conjugated and unconjugated forms so that blood contains a combination of flavonoids.(183) Because the intact parent polyphenols are usually absent or low in the plasma they will not, for instance account for the increase in the AC of the plasma on their own, as the metabolites also contribute.(8)

Flavonoids, as indicated, are significantly metabolized *in vivo* with notable affects on the structure of the flavonoid compounds. These plasma compounds formed can be rather divergent in structure in comparison to the parent compounds obtained via the original dietary source.(175,183,216). These plasma compounds, and not the forms present in foods, demand consideration pertaining to the biological effects of flavonoids.(97,186) These distinctively different metabolic forms will notably impact the physical properties of flavonoids, like their lipophilicity,(28) making them more water-soluble (93,113) affecting their biological activities *in vivo*, like their AC.(28,93,183,216) The biological activities of the flavonoid conjugates or metabolites are important,(158) as this would produce the biological activity *in vivo*.(99,142) If flavonoid metabolites have biological activities somewhat near to what has been identified for free flavonoid aglycones *in vitro*, their occurrence in the plasma would be particularly beneficial.(186) The flavonoid conjugates or metabolites would potentially retain some of the activity of the flavonoid,(158,207) but research indicates that the flavonoid metabolites may have biological potency different to that of their native compounds. This has important propositions for the preventive and therapeutic usefulness of these compounds.(98)

In most cases the metabolites preserve some of the reducing potential of the parent compound. Numeorous phenolic acids formed in the colon still have free phenolic groups.(8) Evidence

supports that scission of the flavonoid heterocyte forms compounds that retain radical scavenging capacity.(98)   Because the radical scavenging OH groups of the aglycone are blocked by methylation, sulfation or glururonidation, it modifies the ability to delocalize electrons.(75,124) This would predict that the antioxidant effect of conjugates could be different from that of the aglycone.(124) In general, the flavonoid metabolites have less powerful antioxidant activity than that of the aglycone itself.(75,113,124) Although the conjugated flavonoid derivatives and phenolic acids demonstrate lower AC than the flavonoid aglycones, the presence of such metabolites will still exert physiological effects to decrease the susceptibility of plasma to oxidative damage (98,124) possibly in synergism with other plasma compounds.(124) The circulating derivatives should contribute to the antioxidant pool in the blood and thus slow down arteriosclerotic processes.(113)   However, glucoronidation or sulfation modifies the hydrophobicity that may impact the antioxidant effect of the conjugates in comparison to the aglycone (124) in the arteriosclerotic processes. This may be ascribed to the difference in the polarity with the parent compound being a lipophilic antioxidant that will interact with the polar head of phospholipid bilayers in contrast to its glycosides or conjugated derivatives being more water-soluble and prone to accumulate in the water phase.(113) Evidence of the changes in biological measurements after flavonoid ingestion (12,95) indicates that the metabolites produced in the tissues and/or by the colon bacteria, exert biological effects which contribute to their health effects and the prevention of disease.(12)

Progress in explaining the role of flavonoids in human health and disease has been held back by the absence of sensitive analytical methodology to analyze plasma levels of flavonoids and their metabolites after ingestion of the doses present in foods.(186) Information on the bioactivity of the different conjugated derivatives and microbial metabolites is thus limited.(20,95,120) Study of the latter is vital to better comprehend the health benefits of dietary polyphenols.(95) In addition, because the metabolites are bound to albumin, the question arises as to whether bound molecules exert the same effects as the free forms.(97)

2.3.7   Excretion

Most of the ingested flavonoids are excreted either unchanged (129,217) or as flavonoid metabolites (75,100,129,181,217) in the urine and the faeces.(129) Collectively, observations propose that intact flavonoids are detected minimally in the urine due to their widespread biotransformation by the intestinal tissues and/or intestinal microflora present.(98) Various studies indicate that only a small portion of the native flavonoid ingestion, in particular quercetin, is excreted in the urine, ranging from about 0.2% to 1.4% up to 4%.(116,126,192,203,218). In an intervention conducted by Donovan et al.,(122) only a small fraction (3-10%) of ingested catechins was recovered in the urine on red wine consumption. Very low concentrations of unmetabolized

catechins of the total catechins consumed were found in the urine and faeces even after drinking multiple regular doses of black tea throughout the day.(189) Catechins are absorbed quickly by the digestive tract and are, on absorption, considerably metabolized and/or degraded that may begin in the enterocytes during absorption. This would explain the overall low percentage excretion.(189)

The phase II metabolites formed (like glucoronide conjugates) are rapidly eliminated (139) and follow two pathways of excretion that is through the biliary tract or via the urine.(75,100,181) Through conjugation, sugar-like molecules are joined to the flavonoid that increases it's polarity (10) and therefore the biliary and urinary excretion.(10,95) This not only explains its swift elimination, but also reduces any potential toxicity.(95) Both the flavonoid glucuronides and the sulfates are hydrophilic compounds that are rapidly excreted in the bile back into the gut and urine.(9,10,99,120,121,144) This is a metabolic detoxification process associated with numerous xenobiotics that limits the occurrence of harmful effects. By increasing their hydrophilicity their biliary and urinary excretion is made easier.(100,113) Flavonoids are mainly excreted in the urine (134) and, although the biliary route is important (75) the extent of the biliary excretion has not actually been assessed.(95)

The urinary excretion of the different metabolites (186) and phenolic acids (115,120) seem to occur at different elimination rates.(115,186) The excretion rates vary because the half-life of flavonoids differs considerably (between 2 and 20 hours), depending on the flavonoid.(181) Catechin metabolites that are conjugated with only sulfate, are the most quickly eliminated,(122,186) followed by the methylated metabolites.(186) The unmethylated metabolites attain the longest half-life, indicating that some of these metabolites are eliminated more slowly.(186) The increased proportion of sulphated catechin metabolites in urine indicates that these metabolites are preferentially excreted in urine.(122) Urinary excretion is an important pathway not only for flavanols, but also for flavanones.(75)

The individual flavonoid levels in faecal water are rather low (µM or less).(205) It is thought that fecal excretion may be a reflection of a very high oral dose.(158) However, Warden et al. (189) reported a significant increase in the urine and the fecal catechin concentrations over baseline levels after multiple regular doses of black tea consumption. The quantity excreted in the faeces only represented a very small percentage of the ingested dose, possibly due to biotransformation by the gut flora. These researchers found wide variability in the between-person fecal catechin concentrations, which may be due to subject differences in the number and species of the normal gut flora.(189)

A major fraction of quercetin is excreted by the lungs as CO_2.(108) This accentuates the bacteria in the colon as being the final step of elimination of quercetin and probably numerous other flavonoids.(158) The A ring degradation is thought to be oxidized to CO_2.(99)

2.3.8   Tissue Uptake

Although evidence is available on the absorption and metabolism of polyphenols in the gastrointestinal tract,(120) there is a lack of information on the tissue uptake and the resulting accumulation in human tissues,(93,100) even in animal tissues.(100) Not much is known about the flavonoids that can be taken up or not in tissues. Considering the efficiency of such uptake and the accumulation or permanence of these compounds or their conjugate and phenolic derivatives in the tissues with chronic ingestion, the forms that accumulate in the tissues are largely unknown.(28,120) Data on the accumulation and storage in the body is of great significance, since some of the biological effects of dietary polyphenols rely on their usual circulating level (their AC).(120) Knowledge of the bioavailability of the metabolites at tissue level may be of far greater significance than knowledge of their plasma levels.(100)   Besides, the human health effects originating from flavonoid ingestion may ultimately be supported by their levels in cells and tissues and an individual's genetic makeup.(219)

A substantial portion of the polyphenols consumed (75 to 99%) is not excreted in the urine.(8) Through the systemic circulation, dose distributions into the tissues can occur with development of biological actions at numerous sites.(158) Nevertheless, this may not necessarily apply to dietary flavonoids as several of them may put forth their preventative effects in the epithelial cells with cellular uptake (transfer across the membrane and accumulation in the cell).(158,205) Nonetheless, animal studies have indicated that some polyphenol metabolites accumulate in a wide range of tissues with even some likely target tissue uptake depending on the compound.(100,220) For instance, animal studies indicated that catechins can accumulate in tissues with long-term consumption as found in consecutive cycle exposures of tea administration to rats.(220) Animal studies indicate that the blood and tissue metabolites may differ due to the specific tissue uptake, metabolism and/or elimination.(100) The possibility exists that some cells or tissues may accumulate and store flavonoid conjugates to higher, more biologically active levels as a result of high-affinity binding to receptors or cellular targets.(139) In obtaining supportive data for tissue uptake the time of tissue sampling is of great importance as the kinetics of penetration and elimination in the tissues is not known.(100)

Much research needs to be done to clarify if flavonoids can reach their numerous projected sites of actions in humans.(158) Chronic or long-term ingestion of flavonoid-rich foods, for instance, does not result in marked accumulation of flavonoids in human plasma.(93) Consequently, it has been

suggested that polyphenols do not accumulate within the body (133,134) and that there are no storage in the body.(75) Even so, considerable amounts of catechins were measured in the colon mucosa and prostate tissues in surgical samples from patients who consumed tea 12 hours before surgery.(100)   Quercetin was furthermore detected in urine samples even after flavonoid-containing foods were avoided for a week that showed that quercetin may be stored.(218) According to findings of animal studies, it is possible for flavonoids to accumulate in organ and muscle tissues with chronic exposure.(220) However, it is still uncertain whether chronic ingestion will bring about comparable tissue accumulations in humans because in acute feeding trials, flavonoids and their metabolites seem to be eliminated shortly after an acute bolus.(28)

The endothelium is possibly a primary site of flavonoid exposure and effect,(100,113) as the local concentration in the gut, particularly the colon,(8,113) should be much higher than in the plasma.(8)   A rather rough representation of flavonoid distribution according to Javanovic and Simic (217) is as follows: 20 to 40% remains in the colon; 60 to 80% enters the circulation and is excreted in the urine; and about 3% enters the cells of several organs (mainly the gut). It is also doubtful that flavonoids will make a remarkable contribution to antioxidant defences in cells and tissues where other endogenous antioxidants, like ascorbate, are normally found.(93) Studies in this area are moreover needed to determine the routes to deliver and the best dosage of certain flavonoid constituents, (in tea) to particular organs. Information gathered on the tissue levels and biological actions of specific polyphenols should be valuable when planning epidemiological studies and human disease prevention trials.(220)

2.4   RISKS OF EXCESSIVE FLAVONOID INTAKES

Due to the attention payed to polyphenols and their protective role against degenerative disease, consumers and food manufacturers have increasingly become interested in flavonoids (145) with a consequential increase in the marketing of polyphenol-containing dietary supplements and polyphenol-rich food products.(94) Flavonoids are widespread components of natural remedies and occur in many medicinal preparations.(10)   In addition, hundreds of herbal supplements containing flavonoids (145) and dietary supplements in the form of a mixture of polyphenols and ascorbic acid, rutin and polyunsaturated fatty acids (PUFAs) (140) are also offered on the market. The consumption of such products would increase flavonoid intake above the common levels of dietary exposure.(94) The consumption of polyphenols is as a result expected to rise due to the use of particularly dietary supplements (182,221) and public health initiatives promoting higher fruit and vegetable consumption.(182)

It is highly unlikely that flavonoids will be ingested in excessive amounts in a diet containing a variety of foods since dietary items originating from plants contain several types of flavonoids in

different quantities.(129) Dietary exposure through food and beverage consumption alone is thus unlikely to cause adverse health effects.   The contributory effect of consuming dietary supplements, in particular antioxidant formulas and herbal mixtures, could result in exposure to potentially excessive and toxic levels (221) assumed to be ingestion of gram quantities.(109) It is at the consumption of these higher doses, above that usually found in a normal diet by possibly some health-conscious persons, that flavonoids may exert potential health hazards that outweigh the possible beneficial health effects. While there is uncertainty about the conditions and the levels of flavonoid intakes that may cause possible health hazards, caution is advised against ingestion at levels above that which would be provided by a typical vegetarian diet.(221)   Until the most appropriate levels of consumption is identified to make sound dietary recommendations for both the general public and populations at risk of developing particular disease, caution is recommended to increase the consumption.(94) The current recommendation to provide the best protection of eating a variety of fruits and vegetables is upheld.(222)

Halliwell (205) indicated that dietary polyphenols are classic xenobiotics, metabolized as such and quickly eliminated from the circulation. While they may aid to protect the digestive tract against injury by reactive species present in foods, or formed within the gut, the consumption of large quantities of polyphenols in fortified foods or supplements should not yet be encouraged while the overall health benefit of flavonoids is indecisive. The unborn fetus is indicated to especially be at risk as flavonoids readily cross the placenta exposing the fetus to high circulating levels that may cause toxic responses in the fetus at levels that may still be safe to the mother. The concern is centred in the rapid cell growth occurring during fetal development that may increase sensitivity to phytochemical exposure.(221)

Epidemiological research provides the strongest support presently accessible on the safety of existing levels of intake for the health effects that have been investigated, but adverse effects are seldom considered in such studies. However, there does not seem to be any safety risks from usual dietary intakes of flavonoid-rich items although safety may be a concern for purified flavonoids, which would be dealt with when generally recognized as safe (GRAS) status for food use of these components is sought.(75)

2.4.1   Anti-nutritional Effects

Very high concentrations of dietary flavonoids have been suggested to have anti-nutritional effects, including impaired mineral absorption, suppression of proteolysis within the digestive tract, reduced glucose uptake and impaired food utilization.(75) After food ingestion, high levels of iron and copper can be found in the gut.(34) Polyphenols may interact with nutrients in the gut lumen by forming stable complexes with for instance non-heme dietary iron.(95) Polyphenolic complexing of

dietary iron in the gut may be a beneficial strategy for reducing iron absorption in patients with iron overload disorders (95) and for persons with high iron status. High plasma levels of ferritin, although not convincingly, have been linked to higher risk of CHD,(94) but polyphenols is generally acknowledged to lower iron bioavailability (anti-nutritional effect).(34)

All major types of polyphenols released during digestion can complex with dietary non-heme iron in the intestinal lumen and inhibit the absorption.(223) These effects engage direct binding of iron by polyphenols in the digestive tract and is the reason why persons at risk of developing iron deficiency (infants, children and pregnant women) are often advised to drink tea and other polyphenol-rich beverages between meals and not during meals (94,95) and to avoid excessive intake of such beverages.(94) Herbal teas, as well as black tea, cocoa and wine, have been found to be inhibitors of iron absorption with black tea polyphenols being more efficient than the polyphenols from the other beverages, which was indicated to be due to their higher content of galloyl esters.(223) It has been suggested that the functional groups important for the inhibitory effect of dietary phenols on iron bioavailability, are the galloyl (trihydroxy-benzene or 3',4',5'-trihydroxy) group,(224) the catechol (94,224) or ortho (o)-di-OH (C3',C4'-di-OH) group, the presence of C5-OH and/or C3-OH in conjunction with a C4-keto group (224) and a larger number of OH groups.(224,225)

The ingestion of excessive amounts of some flavonoids may in theory have a negative influence on the absorption of certain other essential minerals, including zinc, copper and manganese.(75) The ability of high molecular weight polyphenolic structures, like tannins, to interact with proteins is responsible for the astringency perception on intake (resulting from interactions of tannins with salivary proteins) and for inhibition of enzymes and reduced digestibility of dietary proteins.(101,102)   Besides complexing with proteins, polyphenols may also complex with carbohydrates.(103)

2.4.2   Other Potential Health Hazards

Other potential health hazards attributed to excess intakes and the resulting high blood concentrations among others, include flavonoids acting as mutagens, being pro-oxidants that generate free radicals providing risk for genotoxicity/carcinogenicity, inhibition of thyroid hormone synthesis thereby altering key enzyme activities and drug metabolism.(221) The impaired thyroid hormone synthesis has been linked to the inhibition of thyroid peroxidase synthesis causing increased thyroid-stimulating hormone levels, thyroid gland growth, thyroid dysfunction and endemic goiter in populations where flavone (apigenin and luteolin)-concentrated millet is a dietary staple.   The biotransformation of polyphenols and other flavonoids involves phases I and II metabolism enzymes (drug metabolizing enzyme CYP along with COMT, UGT and SULT,

respectively) and phase 3 efflux pumps (active efflux with MRP). As a result of these interactions, polyphenols may inhibit or induce the metabolism of pharmaceutical drugs through the expression of these key enzymes and active efflux pathways.(182) This alteration underscores the need to reflect on probable drug-flavonoid interactions, which can potentially increase or reduce drug potencies.(75)

The radical scavenging property of flavonoids does not in itself imply a fully beneficial effect because after scavenging, a flavonoid radical is formed. A flavonoid radical that is very reactive would propagate rather than interrupt the harmful events initiated by free radical attack.(12,141) In addition, the same flavonoid could behave as an antioxidant and a pro-oxidant facilitating oxidative stress by exacerbating cellular injury by reactive oxygen species (ROS), depending on the structural features, the radical source and the flavonoid concentration.(98,226) Flavonoids demonstrate pro-oxidant activity when a transition metal is available.(34,94,226,227) Pro-oxidant activity increases with increased flavonoid concentration.(226) It has been indicated that polyphenols ingested in large quantities could have pro-oxidant actions.(94) It would appear as if there is an antioxidant optimum, which means that the most active antioxidants are likely to be pro-oxidants when they lie beyond the optimum.(144) This implies that they act as pro-oxdiants at high concentrations and as antioxidants at low concentrations, which may be due to the structural properties or an ability to generate free radicals at high concentration.(228)

The antioxidant/pro-oxidant balance is reliant on the environment (34) with the pro-oxidant activity especially requiring the presence of transition metals (10,34) and biomolecules,(34) along with high flavonoid concentrations.(10) Such pro-oxidant effects have not clearly emerged *in vivo* in humans,(94,229) nor are they likely to.(75,226,227) Due to the limited levels of polyphenols that are achievable *in vivo* after ingestion of polyphenolic-rich foods and beverages,(229) the plasma transition metals (like copper), largely complexed by proteins, except perhaps for certain metal overload diseases,(226,227) and not usually in the free form, and peroxidase being compartmentalized.(75) Concern has, however, been raised about the slow elimination of quercetin, as the prolonged elimination time could allow quercetin to dissociate from its albumin complex in the blood and allow it to interact with cellular proteins and deoxyribonucleic acid (DNA).(221)

2.5   CONCLUDING COMMENTS

Flavonoids are a major group of unique plant phytochemicals, which do not only form part of the daily dietary intake of most individuals, but also have the potential to support human health and prevent disease. Dietary flavonoids comprise five major subclasses each comprising a number of individual flavonoid components that contribute to flavonoids as a phytochemical group and their

dietary intake. It is thus important to differentiate whether the intake of total flavonoids, the flavonoid subclasses or the individual components where assessed when reporting their dietary intake, as it may be misleading to report a dietary flavonoid intake, which was based only on the intake of a few summed subclasses or individual flavonoid components. Dieticians/Nutritionists studying flavonoids should take cognisance of this differentiation, as some past reports on flavonoids referred to flavonoids with the flavonoids not comprising of all five the major subclasses, which can be misleading to those studying flavonoids as a bioactive food ingredient for the first time.

Dietary flavonoids on consumption of plant foods containing them, such as fruits, vegetables, beverages like tea, wine (red) and cocoa or cocoa-containing products along with herbs, undergo extensive intestinal and colonic metabolism in the human body with the flavonoid metabolites and phenolic acids formed greatly different in structural features and metabolic potency compared to the flavonoid structures present in foods and beverages. These flavonoid metabolites and phenolic acids formed, however, still have metabolic potency with evidence of tissue uptake emerging despite them having short elimination half-lives due to the conjugation actions that mainly occur in the gut and liver.

Though numerous factors impact the flavonoid food content and their absorption in the gut, the plasma concentrations of the flavonoid metabolites and phenolic acids are sufficiently high to confer biological activity and numerous resultant health effects in humans, where many provide a cardioprotective potential towards flavonoids. This becomes a very important incentive to assess the dietary flavonoid intake as a total intake based on the summation of the various subclasses and/or the intake of only some of the subclasses or individual flavonoid components. Although adverse health effects have been linked to an excessive intake of flavonoids, a high dietary exposure through consumption of plant foods and beverages alone seems unlikely.

CHAPTER 3

ASSESSMENT OF DIETARY FLAVONOID INTAKE FOR HEART HEALTH PROTECTION

3.1 PREAMBLE

This chapter is devoted to literature that addresses the proposed mechanisms of the relation between dietary flavonoid intake and heart health endpoints (Figure 2.1). These aspects support flavonoids being potentially important therapeutic components for heart health promotion and disease prevention (101) and assessing their dietary intake. The health effects of polyphenols in the prevention of disease depend on not only on their bioavailability, but also their intake.(101) For instance, to prevent or reduce the oxidative damage caused by free radicals, the dietary consumption of phenolics need to be sufficient to contribute to the antioxidant provision.(125) Antioxidant compounds are quickly utilized during scavenging of free radicals and need to be replaced or regenerated by a supply of new dietary-derived compounds.(2) Therefore, to promote a phenol-rich diet the polyphenolic content in the daily diet first of all needs to be assessed.(125)

There are several lines of substantiation that have all added to the mounting support on the preventative role dietary flavonoids play in chronic diseases and, in particular, CVD.(95,158,230-232) These include numerous epidemiological studies, animal studies and clinical observations.(95,158,231,232) Epidemiological studies propose that the intake of numerous dietary flavonoids,(103,186,200,230) as well as consuming dietary items rich in particular flavonoids, such as cocoa-containing foods,(233) tea (45,234-236) and (red) wine,(237-240) have a protective effect against coronary mortality, suggesting that they act as protective components.(186) The association between flavonoid ingestion and improvement in CHD mortality established through epidemiological studies (84,94) is particularly important for chronic disease incidence, as the associated disease symptoms take a long time (often decades) to emerge.(92) Flavonoids, because of their polyphenolic nature, should contribute to the protection against oxidative stress.(95) Because heart disease is associated with oxidative stress,(219,241,242) and flavonoids are strong antioxidants *in vitro*,(93,108) flavonoids have received considerable attention pertaining to this effect in the protection against CVD.(48,93,108) However, not all polyphenols are equal. Some polyphenols, such as the isoflavone phytoestrogens, show specific effects.(95) Nonetheless, almost all the dietary flavonoids are capable of protecting LDL cholesterol from oxidative modification *in vitro*, a process considered to be of importance in the pathogenesis of arteriosclerosis.(230) Their presumed role in the prevention of CVD is furthermore strengthened by the identification of probable mechanisms of action. These biological actions may be generic or, as indicated, specific to a particular phenolic compound.(95)

Much of the support for the biological actions of polyphenols in disease prevention is obtained from animal experiments or *in vitro* experiments on cultured cells, isolated tissues and pure enzymes.(96,100) Although experimental studies on cultured cell lines or animals support a CVD preventive role,(94) there appears to be inconsistencies between the potential health effects as observed *in vitro* and the observed *in vivo* health effects in human subjects.(139) Attention should firstly be given to the dose and method of administration of polyphenols when interpreting the results of such experiments.(95) Many of the biological effects established through these *in vitro* experiments were commonly obtained from experimental studies implemented with exceptionally high doses or concentrations of flavonoids that will result in unphysiologically high levels of polyphenols that far exceed those that can realistically be attained through dietary intake *in vivo* in human plasma and tissues.(94,96,100,181,182,229,243) The biological actions of polyphenols have furthermore commonly been studied *in vitro* by using polyphenols in their native form, as aglycones, or as glycosides as present in food,(95,100,158) rather than on the consequential metabolites. Aglycones and glycosides are components not generally found in the blood due to their rapid and widespread intestinal and hepatic conjugation.(100) To what extent such *in vitro* effects produced by the aglycones or glycosides can be extrapolated to the *in vivo* situation in humans is inadequately understood.(158)

As a result, the *in vivo* absorption and metabolism of flavonoids needs to be considered in establishing their biological actions and health effects.(230) The discrepancies found could be due to poor *in vivo* bioavailability of flavonoids to target tissues.(139) Polyphenols are extensively metabolized in intestinal tissues and by colonic microflora which will alter their specific properties and biological effects due to the conjugated derivatives and microbial metabolites formed. This makes it necessary to identify the biologically active metabolites.(95) The bioavailability of these flavonoid metabolites to target tissues determines the activity *in vivo*.(47) This has rarely been explored.(95) Not much is known about the *in vivo* biological potential of the polyphenol conjugated derivatives that occur in the plasma or tissues, often due to the absence of commercial standards and exact identification.(95,100) Manach et al. (100) indicated that it is not easy to draw conclusions about the effect of conjugation (types and positions of conjugation) on the various polyphenols and their potential biological effects from the few conducted studies. According to Manach et al.,(100) it is clear that the phenolic metabolites are quickly removed from plasma, which indicates that daily consumption of plant products is required to sustain high phenolic metabolite levels in the blood. A better knowledge of these metabolites, particularly the major bioactive metabolites in plasma and tissues, their bioavailability and concentrations, will help to correlate polyphenol intakes and shed light on potential polyphenol health effects. However, this may be a complex accomplishment.(100) Although an understanding of how antioxidants perform

within cells is obviously important, cells in culture are not always reliable models and different cell types in the body can differ widely in their metabolism and consequently their redox responses.(241)

The gut absorption and bioavailability differences of the different components need to be considered to explain the different health benefits. It is currently not possible to indicate which particular phenolic compound is the most protective against the different degenerative diseases. Even if some generic polyphenols are more protective, the effect will be influenced by their absorption in the gut and their bioavailability. As short chain fatty acids may explain some of the health benefits of dietary fibre intake, these conjugated derivatives and microbial metabolites could to some degree be responsible for the health effects of polyphenols. If the active polyphenol fractions can be determined, the best dietary precursors and dietary sources of the precursors can be established and the knowledge used to develop diets with optimal health benefits.(95)

In summary, the contrasting *in vitro* and *in vivo* biological effects may have several explanations.(244) These may include factors like the polyphenol composition, metabolism, bioavailability (the bioavailability of phenolic compounds is limited and is influenced by such factors as nutrient interactions, gut microflora, etc.), tissue distribution and effects on the endogenous antioxidant compounds that have to be considered to comprehend the *in vivo* biological evidence.(32,175,244) *In vitro* results may therefore not reflect what happens *in vivo,*(47,245) and thus cannot be extrapolated from *in vitro* evidence.(32,175) For instance, the AC of a food measured *in vitro* is not necessarily consistent with its effect on the redox balance *in vivo*.(175)

The capacity of a food to transfer its antioxidant activity is linked to several known and unknown chemical/biochemical/physiological characteristics.(175) Considering the phenolic content, red wine would be predicted to produce the highest antioxidant activity *in vivo*. However, the percentage increase with reference to baseline levels in an acute feeding trial of healthy volunteers was lower (21%) in comparison to the increase caused by black (52%) and by green (40%) tea.(32) The researchers (32) speculated that the AC of black tea may have been restored by modification of the ingested condensed polyphenols on digestive breakdown through the release of monomer phenolics, which become available for absorption and bring about increased antioxidant activity in the blood stream. It has been suggested that the black tea theaflavins and thearubigins might be hydrolysed by the acidic gastric juice to produce simple monomer phenolics.(2) Therefore, information obtained from simply measuring antioxidant activities of polyphenols (or foods containing them) *in vitro* without information on their bioavailability, metabolism and resulting antioxidant effectiveness in organisms have limited relevance, according to Collins.(241)

It is thus clear that the conclusive evidence for the protective role of flavonoids against degenerative disease will greatly come from epidemiological and clinical studies.(94) Only the cardioprotective effects of flavonoids established through epidemiological studies, and for the most part human clinical trials, will consequently be considered for the purpose of this section of the literature (a consideration of Scalbert et al. (96)) to support the importance of assessing dietary flavonoid intake for heart health protection. *In vivo* studies do not only provide support for a cardioprotective role of flavonoids, and thereby a promising picture that flavonoids may be useful food compounds,(200) but increasing biological mechanistic information about how polyphenols might reduce CVD.(246) The simplest assays of antioxidant status and oxidative damage are *in vitro* reactions or cell culture assays. They can provide knowledge about mechanistic actions, although extrapolation to *in vivo* activity is risky, because their uptake from the digestive tract and their metabolism is not taken into consideration.(241)

Halliwell (229) cautioned against cell culture studies to determine the effects of reactive species and antioxidants on cellular level, as cells in culture are under oxidative stress and deprived of antioxidants not quite conducive to the situation in the human body. In addition, most cell culture experiments investigating the biological effects of oxidized LDL utilize transition metal-mediated oxidation. Some researchers question this regarding the *in vivo* relevance.(247) *In vivo* studies furthermore provide evidence for biological activities of polyphenols, rather than the simple involvement of polyphenol intake in a healthier lifestyle or association with other confounding factors (246) that have been encountered in epidemiological studies.(105,111,169,232,248) Supplementation with antioxidants *in vivo* and experiments performed with human subjects seem to be the best approach theoretically. Experiments with human volunteers allow the evaluation of bioavailability, and with good biomarkers chosen, can provide an overview of the effectiveness.(241) However, such evidence will improve when validated biomarkers of disease risk that are influenced by polyphenol ingestion are identified. This is possibly a difficult identification task caused by the numerous phenolic compounds found in food.(94) Clinical trials on the health effects of both high and low doses of flavonoids are necessary.(108) The rooibos intervention has contributed to meeting this need (37) and of which the current research serves as an extension.

3.2   CARDIOPROTECTIVE SUPPORT FROM EPIDEMIOLOGICAL STUDIES

Epidemiological studies are necessary to determine the health outcome of long-term exposure to physiologic levels of polyphenol ingestion.(33) The analysis of the flavonoid content in foods has made it possible to explore the association between flavonoid intake and disease incidence in humans through epidemiological studies, as well as the intake of the major food sources of flavonoids and disease occurrence.(130) An epidemiological evaluation of the effects of flavonoids

on chronic disease is needed to uphold the findings from experimental studies.(106) Although epidemiology is efficient in identifying associations between an environmental factor and disease, many relations between dietary intake and disease are quite subtle. It may not be possible to establish whether such rather weak relations are valid, from epidemiology alone,(120) thus requiring corroborative experimental studies. Most epidemiological studies that explored the relationship between flavonoid intake and disease incidence have concentrated on two flavonoid subclasses, the flavonols and flavones, while the other subclasses have been explored in only a few studies.(169) The epidemiological findings could therefore still be thought of as fragmentary as flavonols and flavones, and in addition, catechins only contribute a limited share of the flavonoid ingestion.(94) Most of the studies reported the adjusted relative risk (RR at a 95% confidence interval) for CHD mortality by tertile, quartile or quintile of flavonoid intake and after multivariate-adjustment for CHD risk factors and various probable known demographic, health, dietary and other lifestyle confounders.(249)

3.2.1 Individual and Summed Flavonoid Subclass Intakes

The evidence on the relation between flavonoid intake and CHD incidence is not entirely consistent (246) as epidemiological studies investigating the relation produced diverse results.(250,251) Several epidemiological studies carried out on flavonoid intake (mainly quercetin due to the flavonol and flavone intake estimations) (130) and the risk of CVD, revealed an inverse relationship between intake and mortality risk.(119,250) A reduced CHD mortality with higher intake of total flavonoids (sum of flavonols and flavones) was found in: the Zutphen Elderly Study cohort as extension of the Dutch contribution to the Seven Countries Study on a five-year (68% RR reduction, RR=0.32) (45) and ten-year (53% RR reduction) (252) follow-up; the Seven Countries Study cohort which spanned Finland, Greece, Italy, Japan, Yugoslavia, The Netherlands and the US (50% RR reduction) (253); the Finnish Mobile Clinic Health Examination Survey (RR=0.67 in men and RR=0.73 in women) (11); and the Iowa Women's cohort (Iowa Women's Health Study) of postmenopausal women (38% RR reduction).(250) The Seven Countries Study suggested that flavonoid intake only has a modest effect on CHD mortality rates and that the main determinant remains the intake of saturated fatty acids.(130) The flavonoid intake could explain about 8% of the total variability in CHD mortality rates compared to the intake of saturated fat explaining 73% and smoking 9%. Variation in flavonoid intake across different countries may in part elucidate the different CHD mortality rates across populations.(253) A large prospective study of male US health professionals suggested that the potential benefit of flavonoids (RR=0.63) is limited to men with prevailing CHD.(254) McCarty (255) indicated that these studies to a great extent established the belief that dietary flavonoids could be a involved in coronary risk reduction.

As can be deduced from the above, a number of epidemiological studies seem to support a borderline modest to relatively strong inverse relationship between flavonol and flavone intake and the risk of CHD mortality,(11,45,250,253,256) suggesting that flavonoids may have favourable coronary effects in humans and protect against CVD.(75,98,130,154,169,248) The evidence on the other hand for incident myocardial infarction, which includes non-fatal events,(45,256) is not that evident. Some epidemiological studies, like the Alpha-Tocopherol Beta-Carotene (ATBC) Cancer Prevention Study, observed an inverse relationship between the intake of flavonols and flavones combined and incident non-fatal myocardial infarction events.(256) While other studies, like the Zutphen Elderly Study (45) and the Rotterdam Study (235) in The Netherlands, observed a stronger association in risk reduction related to fatal myocardial infarctions (45,235) or even no relation between the intake and non-fatal acute myocardial infarction as in the large cohort of US male health professionals in the Health Professionals Follow-up Study.(254)

Studies on flavonols report a somewhat stronger protective effect on CHD mortality than on myocardial infarction occurrence (45,257) or a trend towards a protective effect on CHD mortality confined to those with established CVD.(254) According to Tijburg et al. (4) and Hertog,(130) this indicates that flavonoids appear to lower the risk of cardiovascular events that are linked to thrombotic tendencies (fatal CHD) and in those with established CHD rather than on early events like LDL oxidation and lipid accumulation or arteriosclerosis alone. Subjects with a fatal event possibly have more serious arteriosclerosis than do those with no fatal event.(257) In the cohort of the Rotterdam Study, a strong inverse relationship of tea drinking with severe aortic calcification, as observed by radiographic films of the abdomen, was found whereas no relation with mild arteriosclerosis was shown indicating that tea protected against the progression to severe arteriosclerosis.(257) These findings propose that the severity of the underlying CVD may alter the link of flavonoids with coronary events. It is a possibility that flavonoids (in combination with other antioxidants) modulate oxidative stress and prevent severe damage from a myocardial infarction.(235) Consequently, it was proposed that flavonols could influence CHD via platelet aggregation and thrombosis, rather than reducing arteriosclerosis.(168) However, Arts et al. (168) did not observe an effect of catechins on stroke risk, which would be anticipated if platelet aggregation and thrombosis were involved in the underlying pathway. Keli et al.,(258) on the other hand, did find an inverse relationship between tea drinking and stroke risk (69% reduced risk). A high consumption of solid fruit (mainly apples) furthermore predicted independently from tea consumption a non-significant 48% lower stroke risk.

In contrast to these studies proposing benefit, some epidemiological studies like the UK Caerphilly Study of a cohort of Welsh men,(165) the US male health professionals cohort in the Health Professionals Follow-up Study,(254) the Women's Healthy Study of US female health

professionals (232) and the US Nurses' Health Study (259) did not find protective coronary effects with high dietary intakes of flavonols and flavones.(246,250,251,259) On the contrary, the UK Caerphilly Study found a weakly positive trend for CHD mortality rate and flavonol intake (RR=1.1). (165) The researchers of this latter study reflected that the lack of protection of flavonols against coronary artery disease (CAD) found, may be explained by the English habit of adding milk to tea (the foremost source of flavonols for this cohort) with the resultant binding of flavonoids in tea with milk protein that could result in a reduced absorption of flavonoids from the gastrointestinal tract. (33,165,250) This reflection, however, is not consistent with later findings that adding milk to black tea did not influence on the plasma concentrations of quercetin, kaempferol (196) or catechins (195) or even the TAC.(187) It seems likely that another factor than milk is responsible for the lack of association found.(187) Arts and Hollman (33) pointed out that residual confounding by lifestyle factors might have influenced the study findings. In the Caerphilly study tea consumption, as the main source of flavonols, was generally positively linked with a less healthy way of life (smoking and a higher fat intake) and with lower social class, whereas in most other studies tea consumption is associated with a healthier lifestyle (235,257,260) and occasionally higher social class.(235,257) Men with a high intake of flavonols smoked more and were likely to be manual workers in this industrial town (Caerphilly) in South Wales.(165)

As documented by Vita,(246) it is notable that several of these studies that showed less benefit of flavonoids were conducted in the UK where tea drinking is rather high (165) and with relatively well-nourished healthy populace, like US male (254) and female (232,259) health professionals. Vita (246) indicated that baseline flavonoid intake may influence the findings since if a population has a relatively high level of flavonoid intake, then even subjects in the lowest category of flavonoid intake may be obtaining the maximal benefits of flavonoid exposure. According to Vita,(246) this might furnish a reason for the absence of a tea effect found in the UK where the intake is high. Moreover, it is possible that residual confounding masked benefits of flavonoid exposure in these studies.(246) In contrast to tea drinking in most other countries, tea drinking in the UK is positively related to a less healthy way of life (smoking and fat intake) and with lower socio-economic status (165) and such subjects are known to have increased risk for CVD.(246) The intake of flavonoids (flavonols and flavones) at the levels typically consumed, as in the US, can possibly not be considered an established protective factor for CHD.(254)

Mursu et al. (169) pointed out that a limited number of studies have investigated the role of flavanols, flavanones, or anthocyanidins or even the role of all the flavonoid subclasses in CVD. In addition to the prospective studies that reported protective outcomes of flavonols and flavones in relation to fatal or non-fatal CHD and reductions of mortality risk, evidence for a protective role of catechins has also emerged.(75) Catechins are the main flavonoid components of tea (155) and

may be responsible for the proposed beneficial effect of tea.(168) To evaluate the relation between catechin intake and CHD mortality, the Zutphen Elderly Study used data at baseline. Catechin intake in the highest tertile of intake was inversely related with CHD mortality (51% RR reduction), but not with the prevalence of myocardial infarction. However, the study had restricted ability to separate the effects of catechins, flavonols and tea (catechin intake was strongly associated with both tea and flavonol intakes).(168)

In the Kuopio Ischaemic Heart Disease Risk Factor Study of middle-aged Finnish men, inverse associations were found between total flavonoid and the flavanol subclass intakes, as well as an inverse trend with the flavonol subclass intake. The mean common carotid artery intima-media thickness indicated that higher consumption of flavonoids is associated with reduced carotid arteriosclerosis.(169) Case-control studies propose that high flavonoid consumption is protective. A Greek case-control study investigated the effects of the specific subclasses of flavonoids and only found an inverse relationship between quintiles of flavanol intakes and risk of CHD.(261) Furthermore, the Finnish Mobile Clinic Health Examination Survey (262) observed no relation between hesperetin or naringenin and CHD risk. The case control study in Greece did not observe any effects for flavanones and CHD risk.(261) The results of a case-control study in Milan, Italy, indicated that intake of flavonoids is associated with a reduced risk of non-fatal acute myocardial infarction, and that the strongest inverse association was observed for anthocyanidins.(263) The large prospective study of US postmenopausal women indicated that intake of anthocyanidins, anthocyanidins and flavanones, anthocyanidins and flavones were linked to a lower risk of CVD, CHD and total mortality, respectively and various flavonoid-rich foods with mortality reduction.(248)

Despite the limited and sometimes negative epidemiological evidence on the association between flavonoid ingestion and CVD risk and mortality, the overall results of these studies suggest protective effects of the flavonol, flavone and flavanol subclass intakes on the risk of CHD with respect to fatal and non-fatal CHD (75,107,141,147,246) with an apparent quite large benefit of a higher flavonoid intake in some studies.(45,75) It seems that the beneficial effects of flavonoids are mainly aimed at CHD mortality and not morbidity.(128) However, epidemiological studies have the major drawback of residual confounding which must be adjusted for in exposure assessment.(33) Although the findings of epidemiological studies suggest that higher intakes of some flavonoids, in particular of flavonols, flavones and flavanols, are linked with a lower risk of CVD,(33,75) Erdman et al. (75) put forward that the data is not adequate to infer that flavonoids are the active components in the dietary items contributing to the reduced risk. They emphasized that caution should be employed as high correlations between flavonoids and other dietary and lifestyle components exist (105,111,169,232,248) that limits making an accurate component-based analysis required for such conclusions.(75) The epidemiological data, although far from

conclusive, according to Miller and Ruiz-Larrea (109) nonetheless demonstrate a beneficial effect of consuming a diet higher in flavonoids as opposed to lower, and according to Hollmann and Katan (107) and Pietta (128) offer support for a protective role of dietary flavonoids in CHD prevention, which supports regular consumption of dietary items rich in flavonoids.(128)

In support of the above, a meta-analysis using the results of seven prospective cohort studies published before September 2001 involving men and women aged between 30 and 84 years, providing data on approximately 105 000 subjects, found a modest 20% decreased CHD mortality (RR=0.80) between those subjects in the top tertile versus those in the bottom tertile for flavonol intakes. This indicates that flavonol ingestion may be a modifiable dietary risk factor in preventing CHD in free-living populations.(249) The meta-analysis was, however, not able to identify the flavonol intake level above which a protective effect pertaining to a reduction in CHD mortality is conferred (because the mean flavonol intake within the separate studies differed extensively). Although the risk for vascular events in the lowest quintile of flavonoid intake was definitely higher than in the other four quintiles, there was no trend or dose-response or continuous relation observed in risk across the four top quintiles (due to the often small differences among the highest quintile intakes).(249) This pattern suggests that very low intakes of flavonols increases the risk as opposed to high intakes being protective,(249) so that persons with the lowest overall intake of flavonoids have the higher risk of death from CHD.(256)

The meta-analysis (249) did not include the large Women's Health Study by Sesso et al.(232) This study (232) found an apparent L-shaped inverse association between flavonols and flavones and important vascular events (myocardial infarction, stroke and CVD mortality). McCarty,(255) considering the study of Sesso et al.,(232) pointed out that although high flavonoid ingestion emerged as protective in some epidemiological research the response pattern is frequently, as found in the study by Sesso et al.,(232) L-shaped which is more indicative of low consumption being detrimental, than high consumption being protective.

3.2.2   Flavonoid-rich Dietary Source Intakes

Some epidemiological studies investigated food-specific flavonoid-rich sources and their CVD risk effects independent of summed or total combined flavonoid intake.(232) These studies provide further evidence for a cardioprotective effect of higher flavonoid intake and are above all suggestive of tea (45,165,232,250) and red wine (256) consumption and decreased cardiovascular risk.

3.2.2.1   *Tea*

In two cross-sectional epidemiological studies of Japanese men, it was observed that drinking green tea decreased serum total cholesterol.(264,265) Although one of these studies did not

observe that green tea consumption influenced the serum levels of either triglycerides or HDL cholesterol,(264) the other did find beneficial effects on these lipid markers.(265) An inverse relationship was reported between the consumption of green tea and the prevalence of angiographically proven CAD in a case-control study.(149) Although the findings from the few epidemiological studies that evaluated the relation between black tea and CHD, is not conclusive for a beneficial effect of tea consumption on CHD risk,(4,266) some studies showed no effect, or even unfavourable effects. Several studies demonstrated a protective effect of tea drinking on CVD (27,267) with such findings as inverse relationships between tea drinking and CVD (27) and even substantial and significant risk reductions in tea drinkers.(4)

Consuming tea was reported to be related with lower mortality from CHD in prospective studies of Dutch men,(45) Norwegian men and women (234) and patients attending community hospitals in the US,(236) with decreased risk of severe advanced aortic sclerosis (257) and fatal incident myocardial infarction (235) among men and women pooled in The Netherlands and with important vascular events among US female health professionals.(232) A case-control study found a lowered prevalence of CHD among tea drinking Saudis.(260) However, such a protective relationship with tea consumption was not reported in prospective studies of US (254) and Welsh (165) men. In the study of Welsh men, a greater risk for CHD, although non-significant, was found with respect to tea drinking.(165)

Peters et al. (268) performed a meta-analysis of ten cohort studies and seven case-control studies that investigated the relation between the rates of CVD with an increased tea intake in an attempt to resolve the literature inconsistencies. These investigators estimated the occurrence rate of myocardial infarction to decline by 11% with a daily increase in tea intake of three cups (1 cup=237mL).(268) Although it seems that there is a beneficial effect of tea drinking on myocardial infarction, these investigators (268) were very disparaging of their finding and pointed out the limitations of epidemiological study data. They noticed that where tea consumption was raised, the risk for CHD increased in the UK, whereas the risk lowered in other areas, particularly in continental Europe. They could not provide obvious reasons for this inconsistency because tea drinking is very common to both regions.(268)

In summary, epidemiological studies largely seem to propose a possible protective effect of tea drinking against CVD,(4,155) presumably because of the high catechin content.(155). Yet, tea consumption seemed to be unrelated to CHD risk in some studies.(256,267) According to Vita (267) and Peters et al.,(268) there are a few likely explanations for these inconsistent findings. The inconsistent findings between the studies may firstly be due to imprecision in the tea exposure measurement. Some studies, although mentioning that the participants were asked about their tea

consumption frequency, simply refer to tea providing no detail on the type or the preparation of the tea consumed. Tea comprises an assorted group of beverages that include black tea, green tea and ice tea (sweetened and unsweetened) and it might even be understood by subjects to include fruit tea or herbal teas. These varying kinds of tea differ in the type and the quantity of the bioactive components and even, within the same type of tea, differences occur (variable contents of flavonoids are found in different brands of black tea).(260, 266,268)

The catechin content of tea infusions is, in addition to the type of tea used, influenced by the brewing method, which both differ greatly between countries and may partly explain the differences in effect found.(168,260,268) Peters et al. (268) indicated that information on the types of tea, the preparation methods or the differences in tea strength might assist in explaining the regional differences found. Should populations in Europe drink their tea stronger than the North Americans, the biological effect could be greater in European studies. Kris-Etherton and Keen (27) indicated that an important aspect of future research on tea is the attention to the polyphenol profile of the teas being studied. In this respect the effect of green tea may vary less than that of black tea as milk is not added to green tea in Japan.(266) Peters et al.,(268) in re-calculating the summarized risk estimate for CHD or myocardial infarction taking into account the way in which tea is consumed (assumingly only half as strong in the US as in Europe), found that the risk estimate for the US studies decreased only marginally and was still rather different from the continental European summarized risk estimate. This finding suggests that variations in tea strength may only explain a small portion of the regional differences.

According to Vita,(267) a further important consideration is the tea intake itself and where the intake level falls on the dose-effect relationship between tea intake and CVD. A population with a high level (or low and narrow range) of tea consumption may fail to demonstrate a benefit when comparing the intake extremes within the population, because most subjects drink a relatively sufficient (or insufficient) amount of tea. This may explain the absence of effect observed in the UK, as this population drinks a reasonably large amount of tea.(267) To be effective, an epidemiological study must include subjects with a wide range of tea intake,(267) as an inverse association may be masked by a low occurrence of heavy tea drinking.(260) Vita (267) pointed out numerous potential confounding factors in epidemiological studies, which makes insufficient confounder control another likely explanation for the inconsistency in the research findings.(4,268) For instance, in the UK tea drinking is a common practice among those with low socioeconomic status and low social status is related with increased risk for CVD.(267)

3.2.2.2 *Wine*
Wine consumption consistently relates to lower CHD risk. Even cross-cultural comparisons confirm an inverse association between wine drinking and CHD mortality.(130) Renaud and De

Lorgeril,(237) reported that wine drinking was inversely associated with CHD mortality across 17 countries studied. A meta-analysis of 19 epidemiological studies of alcohol intake and CVD morbidity and mortality, strongly favour a protective effect of moderate wine intake (up to 300 mL per day) and risk of vascular events.(239) A further meta-analysis incorporating 26 epidemiological studies suggests a dose-response relationship of wine consumption to the cardiovascular preventive effect on non-fatal vascular end points and cardiovascular mortality. This meta-analysis found a statistically significant inverse relationship between vascular risk (32% RR reduction) and a daily consumption of 150 mL of wine (light-to-moderate wine consumption).(240) This J-shaped relation found suggests that light-to-moderate wine drinkers (one or two drinks per day) have a lower risk than either non-drinkers or occasional drinkers or heavier drinkers.(238,240) For persons consuming three or more drinks daily, the total mortality increases drastically with an increased number of drinks per day constituting the J-shaped curve.(238)

3.2.2.3   Other Dietary Items

The Zutphen Elderly Study provided the first epidemiological evidence of chocolate consumption and CVD. The study found that the cardiovascular mortality and all-cause mortality risks of those men in the highest tertile of cocoa intake (with plain chocolate and chocolate bars contributing to two-thirds of the total intake of cocoa), was 50% and 47% lower, respectively, than for those men in the lower tertile of cocoa intake.(233) High intakes of flavonoid-rich vegetable sources, such as onions (11,45,165,262) and broccoli,(232,250,259) and fruits, such as apples (11,45,232,248, 250,262) or pears or both (248) and apples and oranges,(262) were associated with decreased CVD morbidity and in particular, mortality risk in epidemiological studies. In the SU.VI.MAX Study in France, where the effect of daily antioxidant supplementation on the incidence of major chronic diseases was evaluated, women in the highest tertile of flavonoid-rich food consumption (sum of chocolate, apple, citrus fruit, red fruit, onions, tea and wine) had a lower risk of CVD.(269) Hence, in addition to epidemiological studies relatively consistently showing an inverse relationship between the risk of myocardial infarction mortality and the intake of particular flavonoids, there is convincing evidence that consumption of flavonoid-rich foods (tea and other sources) is related to reduced CVD morbidity and in particular, mortality risk.(94)

3.3   CARDIOPROTECTIVE EFFECTS AND MECHANISMS OF ACTIONS FROM CLINICAL STUDIES

3.3.1   Introduction

There is a great interest in the health effects of bioactive compounds in reducing chronic disease risk and the underlying biological mechanisms responsible for these actions. Bioactive compounds innately have effects more subtle than those of nutrients. Bioactive compounds, for instance,

impact cellular activities that influence disease risk rather than avert deficiency diseases.(3) One of the largest groups of these phytochemicals that may, through altering of chemical and enzymatic processes, provide beneficial health effects is indicated to be the polyphenols and their major subclass, the flavonoids.(7,47,92)

Epidemiological studies have shown that diets rich in flavonoids may be related with reduced incidence of cardiovascular disorders, mainly CHD and myocardial infarction,(140) and in particular mortality from CHD.(270) However, the mechanisms explaining the epidemiological evidence have not been fully described.(140) Although flavonoids have shown anti-arteriogenic prospects in rodent models,(255) the results of epidemiological studies must be supported by data from human intervention feeding trials to ensure an accurate measure of the protective effects so that reliable conclusions can be drawn.(191,271)

In addition to the epidemiological studies,(21) a substantial number of acute and short-term chronic feeding trials with healthy volunteers and at-risk groups have investigated the efficacy of flavonoid-rich plant foods or extracts in reducing markers of CVD risk.(21,212) Human trials aimed at showing a role for dietary flavonoids in heart health have concurrently aimed at identifying the possible mechanistic actions of flavonoids in promoting heart health.(75) This though does not answer the question whether the data gathered is relevant for human disease outcomes, specifically where exposure to polyphenols is chronic, at relatively low levels and dependent on bioavailability and metabolism.(33) The expectation of a varying protective effect depends on the ability of the food to provide flavonoids to the body, and it could reveal differential protective effects of various flavonoids.(211)

Scalbert et al. (94) pointed out that the human clinical trials on dietary flavonoid exposure and biomarkers of oxidative stress and CHD risk factors, revealed inconsistent results. Although several trials indicated favourable effects, some showed that the same biomarkers were not affected by flavonoid intervention.(75) Conquer et al. (272) supplemented quercetin through provision of four capsules daily for 28 days. This provided intakes about 50-fold greater than the dietary intakes related to lowered CHD mortality based on epidemiological research. They did not observe any modifications of serum lipid levels, thrombogenic or other (blood pressure and resting heart rate) cardiovascular risk factors in the quercetin-supplemented subjects. However, their plasma quercetin concentrations were markedly higher (about 23-fold) than those of subjects consuming the control capsules.

It is rather difficult to explain such discrepancies.(75) The reasons for the apparent inconsistencies of individual clinical trials may be related to the many differences in study designs (dose and length

of treatment) (21,75,216,243) and differences in the study subjects (dissimilarity in dietary habits and lifestyle).(216) These may confound study results,(271) in addition to true differences between the sources of flavonoids (75,243) and outcome measures (biomarkers) (21,75) of the studies. The content, subclasses and individual flavonoids found vary between different foodstuffs and this may affect absorption, distribution and the biological effects of the different flavonoids and their metabolites.(243) The resulting complex anti- and pro-oxidant effects, together with large differences in study material and protocols, could explain much of the variation and inconsistencies found in the effects on oxidative damage.(208) It is likely that an effect of antioxidant supplementation on lipid peroxidation is measureable, only when subjects are exposed to increased oxidative stress.(271,273)

Lotito and Frei (93) documented that studies of the effects of flavonoid-containing foods on *ex vivo* oxidation of plasma and LDL obtained from humans before and after acute or short-term chronic interventions, have provided different findings. Even investigations using similar types of food did not find the same results. Lotito and Frei (93) believe there are several factors that may explain these inconsistent findings. Variability in absorption and metabolism of the different flavonoids within the dietary sources may result in the formation of metabolites with dissimilar antioxidant properties. The standard techniques utilized to isolate LDL from blood samples may also contribute. Flavonoids display differences in their distribution and association with plasma constituents, such as the proteins and lipids.(93) Flavonoid metabolites are water-soluble compounds and may not be tightly bound to the lipid-rich LDL fraction and as such may not be able to protect isolated LDL against oxidation.(271) Isolated LDL excludes the effects of antioxidants present in the aqueous phase *in vivo*.(206) Such flavonoids likely to be excluded on LDL isolation, include epigallocatechin gallate (EGCG) from tea and glucoronide and sulphate metabolites of flavonoids, which are inclined to be more hydrophilic than their parent aglycones.(93) Interventions with chocolate and cocoa products have in general observed more consistent antioxidant protection of plasma and LDL, not like the data obtained from tea, wine or fruits. The consistent findings may be ascribed to the food matrix of cocoa products being more lipophilic, uniform and hence improved flavonoid absorption.(93)

Flavonoids differ in chemical structure.(48,169) Although many of them share a common flavan nucleus, the differentiation of flavonoids is not simple. They differ from one another in the position and number of different sugars involved in glycosylation. Acylation may take place at different positions of the flavonoid nucleus, as well as at the glycosyl residues. Further structural diversity arises as a result of the position and number of OH and/or CH_3 group ring substitutions.(145) Because of the differences in chemical structure and as a result differences in their bioavailability, distribution and metabolism,(48,169) the different flavonoid compounds are not equally

physiologically active (145) and could in theory have different biological properties (48,169) and effects on human health.(169,262) A multitude of actions have been demonstrated by flavonoids *in vitro* and *in vivo*.(21) It is apparent that the biochemical activities of flavonoids and their metabolites are closely linked to their chemical structure, which differs in the substitutions on the C atoms of the basic flavonoid structure and the lipid solubility.(48,145,219)

Several researchers have investigated and tried to define the structural characteristics of flavonoids that contribute to these activities.(132,145) Despite abundant data, the structure-activity relationships (SARs) between the antioxidant activities of flavonoids and their chemical structures are still not fully understood.(145,274) The construction of such SARs and quantitative structure-activity relationship (QSARs) of the antioxidant actions of flavonoids is a challenging task and almost all developed models are based on *in vitro* experimental values.(145) Though several SARs are well established, these models are based on the *in vitro* antioxidant activity of flavonoids,(145,154) which cannot be applied to an *in vivo* situation (145) and the SARs consequently excluded from this literature section. Some of the major structural features concerning the antioxidant functioning of flavonoids provided in the published literature were presented in Table 2.1.

In spite of growing support for the *in vitro* efficacy of phenolics, knowledge concerning their effectiveness *in vivo* remains scarce. This may be partly due to limited knowledge of their bioavailability in humans.(275) The best described feature of nearly all the subclasses of flavonoids is their antioxidant activity,(200) which has frequently been investigated and reviewed.(130) Their antioxidant ability was suggested to be their most important biological effect to exert health effects.(93,127) However, the classical concept that polyphenols protect cell components against oxidative damage through free radical scavenging, seems to be an oversimplified understanding of their action.(96,183) Their antioxidant effects are probably not sufficient to explain the suggested protective effects against CVD.(94) Their free radical scavenging abilities and contribution to the body's overall antioxidant defence system could be responsible for certain, but not all, of the observed health benefits.(21,172,183) Thus the prospect that plant polyphenolics may have cardioprotective properties other than, or in addition to, that provided by their well known function to act as antioxidants needs to be considered.(245)

Further biological actions to support their health effects are now being added to the antioxidant action of flavonoids.(108,219) Accumulating evidence suggests that low levels of flavonoids and their metabolites produce various other potential biological actions beyond their antioxidant properties indicating non-antioxidant mechanisms of flavonoid action that support their supposed health effects.(93,147,183,241,262) Flavonoids interact at the cellular and molecular level

(75,140) with cells responding to polyphenols chiefly through direct interactions with receptors or enzymes.(96) This results in altered intracellular signalling pathways and mechanisms,(93,145, 172,183,219) with modification of the cellular redox status,(96) gene expression (93,145,172, 219,276) and even angiogenesis.(35,93,140,277)

The possible tissue accumulation of flavonoids, where they could show biological effects, cannot be excluded.(93) Halliwell et al. (278) proposed that more attention should be given to the antioxidant and other biological protective properties of flavonoids and their metabolites within the gut. Flavonoids might exert direct protective actions within the gut, as some biological effects of flavonoids may not necessitate their absorption through the gut barrier.(95,278) Due to the high concentration of polyphenols in the gut lumen,(95,278) they may have a direct influence on the gut mucosa by protecting it against oxidative damage (or the action of carcinogens).(95)

Since the absorption of phenolic compounds is not complete, the unabsorbed compounds from the diet furthermore enter the colon where they and their products of extensive bacterial fermentation by the gut flora can produce beneficial effects. The gastrointestinal tract is exposed to reactive oxygen, chlorine and nitrogen species, many from the diet and other from the activation of phagocytes in the gut. Flavonoids and other phenolic compounds might therefore provide direct protective actions in the gut by scavenging these reactive species. They could by chelating iron lessen the pro-oxidant actions of colonic iron and perhaps inhibit the free radical generating enzymes cyclooxygenase (COX) and lipoxygenase (LOX).(278)

Despite various studies, the mechanistic actions by which flavonoids affect human health and disease are not entirely clear. Progress in this area has in the main been held back by the lack of quantitative information on the absorption, metabolism and distribution of flavonoids after ingestion of ordinary foods.(186) While flavonoids produce a diverse range of beneficial biological activities,(48) only those related to CHD will be addressed for the purpose of this chapter. The development of arteriosclerosis and thrombosis is a multifactorial process in which oxidative modified lipids and lipoproteins, the inflammatory response, endothelial dysfunction and activated platelets all play key roles.(279) The probable protective role of flavonoids in CVD risk reduction is centred on several reported properties and mechanisms of actions.(112,119,130,169)

Ample attention has been paid to flavonoids conferring promising antioxidant effects (98,127) that may exert an important therapeutic role in protection against CVD, most likely by numerous mechanisms (3) through their capacity to affect oxygen free radicals and lipid peroxidation (127) whereby they inhibit the oxidation of LDL cholesterol to an arteriogenic form.(112,130,232) Flavonoids confer their protective effects against heart disease through anti-inflammatory effects

with reduction of inflammation, which offers further support that they may exert an important therapeutic role in protection against CVD.(3,21,280) Current evidence shows, with some consistency, that flavonoids interact at the cellular and molecular level (75,140) to elicit a variety of biologic effects. This may relate a role in promoting healthy vascular and endothelial function with vasodilator properties reported (21,75,140,281) and a reduction in platelet aggregation activity (21,75,112,130,140,232,281) with a sequential drop in the risk for clot formation.(21) These other potential effects of flavonoids may be more important *in vivo* than the antioxidant ability.(229) It is therefore within reason to propose that the inverse relation found between the intake of flavonoid-rich dietary items and the risk for CVD, is due to several factors, which include flavonoid-induced changes in the antioxidant defence mechanisms, improvements in vascular reactivity, reductions in platelet reactivity and a positive modulation of the immune system.(28) Despite of their anti-inflammatory, anti-aggregative and anti-thrombotic attributes, the clinical evidence may (140) or may not (75) be adequate to substantiate a role for dietary flavonoids in reducing the risk of and treatment of CVD. However, in view of the varied properties of polyphenols, their potential therapeutic use is seriously being considered.(140)

3.3.2 Antioxidant Effects

Oxidative stress is widely accepted as an associative factor in many chronic degenerative diseases,(145,241,242) as either a cause or effect,(145,241) including CVD.(145,219,242) Oxidative stress results from a disproportion between oxidant production and antioxidant defences,(282) when the critical balance between the generation of oxidants and the antioxidant defence is unfavourable (16) due to production of oxidants like free radicals, ROS and reactive nitrogen species (RNS) (219,222) in amounts that exceed cellular antioxidant defences.(222) There is vast evidence in support of the conception that CVD is due partly because of excessive oxidative damage. The role of oxidative damage as a primary cause of CVD and the importance of oxidative stress in arteriosclerosis has in contrast been questioned by some investigators as has the notion that dietary antioxidants can reduce CVD onset and progression.(245,282) This uncertainty is mainly based on the ineffectiveness of antioxidants to reduce cardiovascular morbidity in clinical trials.(282) In defence of the cardioprotective effects of flavonoids, it has been suggested that flavonoids are responsible for many diverse biological outcomes in addition to the antioxidative effect,(245) like improvement in the vascular reactivity and interfering with platelet activation and function.(28)

Improved knowledge on the role of free radicals in the development of degenerative diseases suggest a possible health-promoting role of dietary antioxidants.(32) Antioxidants provide protection against the potential damaging effects of pro-oxidants (22) and can basically be described as chemical compounds or substances that prevent oxidation.(3,22) Antioxidant

compounds must be available in biological systems in high enough amounts to avert an accumulation of pro-oxidant species and oxidative stress.(22) Inadequate antioxidant defences predispose the body to chain oxidation and oxidative cell damage (217) and ultimately oxidant-mediated diseases.(3) For instance, the increased formation of ROS during injury causes depletion of the endogenous scavenging compounds.(200) To ward off chain oxidation and minimize oxidative damage, antioxidants can intervene at numerous points along the chain of events, such as: prevent initiation by blocking cellular free radical generators; scavenge peroxyl radicals by transforming them to hydroperoxides, which are catalyzed by glutathione (GSH) peroxidize; repair biological radicals before they are transformed into steady products; and induce and support enzymatic antioxidants and detoxifying agents.(217)

The antioxidant effects of flavonoids (119,129,219) is only one of various diverse actions these polyphenolic compounds have.(108,219) Numerous studies have detected positive linear and highly significant relationships between the analyzed AC and the total phenolic content of plant materials.(1,93) Many *in vitro* studies have reported that food flavonoids, including anthocyanidins, flavanols, flavonols and flavones, have antioxidant activity.(47,270) There is ample evidence that flavonoids or flavonoid-rich food extracts when added *in vitro* to human plasma or isolated LDL, protect lipids, proteins and endogenous antioxidants from oxidation.(93) If flavonoids are absorbed from foods in adequate quantities, their physiological antioxidant activity could partly explain the epidemiological finding of an inverse relation between the consumption of plant foods and the prevalence of a number of chronic diseases.(211)

Research has indicated numerous plant-derived polyphenol compounds to be more effective antioxidants *in vitro* than vitamins C and E, implying that they as a consequence might contribute notably to beneficial health effects *in vivo*.(101,132) The *in vitro* antioxidant capacities of most flavonoids assessed as scavengers of radicals produced in the aqueous phase, by estimation of the Trolox equivalent antioxidant activity (TEAC) (283,284) or oxygen radical absorbance capacity (ORAC),(226) on the basis of equimolar concentration, has been rated notably higher than that of ascorbic acid and α-tocopherol. For example, the catechin EGCG in black tea exerted a more pronounced effect on extending LDL oxidation lag time than vitamin E at a similar molar concentration.(210)

The reasons suggested for the greater AC include that flavonoids have an elaborate conjugated system to support unpaired electrons, reactive OH groups and less stearic hindrance at the site of the abstraction.(47) Flavonoids contain conjugated ring structures and OH groups that can readily transfer a number of single electrons or H atoms from the OH groups to free radicals.(48,145,285) However, considering the physiological plasma concentrations of EC and its metabolites, the

values reached are about 1/200 of the hydrophilic ascorbate and about 1/150 of the lipophilic vitamin E. In view of such concentrations, EC and associated catechins seem to be less significant physiological antioxidants than ascorbate or vitamin E in humans. The *in vivo* antioxidant activity of a compound depends not only on its plasma or target tissue levels, but its capacity to interact with a radical that is reliant on its redox potential.(211) Whereas the measured AC of plant materials generally reflects their level of antioxidant polyphenols and flavonoids, this is not true for human plasma, which contains only very low levels of flavonoids.(93)

The human body holds various antioxidant physiological defences.(145) All flavonoids are antioxidants (or reducing agents).(95,129,145) Their dietary intake may thus contribute to these defences (145) and consequently play a crucial role in the prevention of CVD by decreasing oxidative stress (214) and protecting cell constituents against oxidative damage.(95) Their antioxidant activity can be ascribed to: the hydrogen (H)- or electron-donating capabilities which links to the reduction potential; the fate of the putative antioxidant-derived radical; the interaction with other antioxidants; and the transition metal-chelating ability.(132)

The antioxidant actions of flavonoids that protect lipids against oxidative damage are mainly attributed to the following:

- Flavonoids can interfere with the propagation reactions of free radicals by participating in free radical scavenging action as chain-breaking antioxidants, which is linked to their ability to transfer electrons to free radicals.(123,129,135,144,145,148,281) Flavonoids can interfere with three or more different free radical-producing systems (200) thereby scavenging OH, peroxyl or synthetic radicals,(98) directly neutralising them and forming products with much lower reactivity, and as a result protect against oxidative damage.(222)
- Flavonoids can chelate redox active metal ions involved in free radical formation that initiates oxidative events.(98,123,129,134,144,145,148,281) The high chemical reactivity of flavonoids is reflected in the ability to catalyse electron transport and scavenge free radicals and the binding affinity to biological polymers and heavy metal ions.(133) Hollman and Katan (141) indicated that the antioxidative and lipid peroxidation inhibiting ability of flavonoids, mainly exist in their radical-scavenging rather than their metal-chelating action.
- Flavonoids generate endogenous antioxidants like α-tocopherol and reduce α-tocopherol radicals.(95,98,145,200)
- Flavonoids inhibit certain enzymes involved in free radical production and activate certain antioxidant enzymes.(144,145,281)

Through these actions, flavonoids may protect LDL from oxidative change guarding against the cytotoxicity of oxidized LDL and inhibit lipid peroxidation by terminating chain reactions in the lipid

phase, which involves peroxyl radicals and hydroperoxides. Herewith it provides protection against arteriosclerosis if they are delivered to the subendothelial space where LDL oxidation eventuates.(98,129) A great deal of attention has been devoted to the antioxidant attributes of flavonoids which affect oxygen free radicals and lipid peroxidation (112) and their beneficial effects on CVD attributed mostly to these antioxidant properties.(98,139)

3.3.2.1 *Free Radical Scavenging and Elimination of Reactive Species*

Biological oxidants important to arteriosclerosis include free radicals and non-radical reactive species. If two free radicals meet, they join their unpaired electrons to form a covalent bond that leads to the formation of non-radical reactive species. Radical reactions with non-radical species form a new radical that can initiate a chain reaction.(286) Free radicals in other words are atoms or molecules that possess one or more unpaired electrons in their atomic structure and are generally highly reactive.(242,287) Free radicals are constantly produced in the body.(145,287) The endogenous production of oxidants, such as free radicals, result from sources like the normal cellular metabolic reactions,(16,287) typically by leakage from the aerobic energy metabolism during mitochondrial respiration.(3,47,222,287) In most living organisms, oxygen is not only required for life, but also responsible for destructive processes and death.(217) Oxygen is a major source of ROS. It has been estimated that about 5% of inhaled oxygen becomes a ROS.(22,242) Oxidation is the transfer of electrons from one to a further atom and represents a necessary action of the energy metabolism. Problems may, however, occur when the electron flow becomes uncoupled (transfer of unpaired single electrons) forming free radicals. ROS, or oxygen-centred free radicals,(128) include the superoxide anion, H peroxide, OH and peroxynitrite radicals.(22,128,242) OH radicals are the most reactive and damaging ROS in biological systems.(98)

Endogenous oxidants or free radicals are produced as a consequence of disease,(16) through oxidative enzymes in infections and inflammation.(222) Exogenous sources of oxidant production include factors like exposure to tobacco smoke, environmental pollutants, certain food components, alcohol,(16) drugs and radiation.(16,222) Oxidation is a chain reaction that can be produced merely by molecular oxygen or by free radical generators like ionising and UV radiation and Fenton chemistry.(217) If not neutralized by antioxidants,(16) these reactive compounds or free radicals, like peroxyl, will initiate chain oxidation (217) reacting with and as a result altering the structure and function of numerous important cellular constituents (16,145) leading to massive chemical changes.(217) The process of lipid peroxidation is an example of a chain reaction generated by a radical obtaining a H atom from a fatty acid side chain containing C atoms with double bonds. Here PUFAs, generate a lipid peroxyl radical that extends the free radical mediated chain of oxidative insult.(286)

Although all molecules are potential targets for ROS (proteins, lipids and DNA where they respectively induce modification, peroxidation and strand breaks),(22,47,98,242) cell membranes, due to the unsaturated lipids they contain with their double bonds, are often targeted.(242) Oxidants in the vessel wall are produced by cellular and extra-cellular sources and enzymatic and non-enzymatic pathways.(286) ROS has been linked to both the pathogenesis and altered physiologic response of arteriosclerosis. It has been suggested that the cholesterol-rich environment enhances vascular production of ROS, like that of superoxide.(288)

RNS seem to be involved in the pathogenesis of CVD.(288) Nitric oxide (NO) is produced by various cells, including endothelial cells and macrophages. In oxidative damage, activated macrophages greatly increase their release of both NO and superoxide anions. These higher concentrations can cause oxidative damage.(200) NO reacts with superoxide in the vascular epithelium to generate peroxynitrite, a highly damaging potent oxidant, which induces LDL oxidation causing irrevocable injury to the cell membrane.(200,288) Such continued generation contributes to maintaining the arteriosclerotic state through production and extension of the fatty streak and consequent plaque formation, which depicts the arteriosclerotic lesion and may contribute to the resultant impaired vascular relaxation.(288) When flavonoids are used as antioxidants, they scavenge free radicals rendering them incapable to react with NO.(200)

As oxidants exert a role in the modulation of arteriosclerosis, it delivers an option for intervention.(3) To reduce the harmful effects of oxidative processes, diverse antioxidants are ingested with foods.(217) As pointed out, flavonoids can prevent damage initiated by free radicals in numerous ways, like the direct elimination of free radicals.(200) Flavonoids act as terminators of free radicals by donating electrons to form stable compounds.(3) By reducing the formation of free radicals, flavonoids act as chain-breaking antioxidants.(289) Flavonoids oxidized by radicals result in a more stable less reactive radical. In other words, flavonoids stabilize ROS by reacting with the reactive part of the radical.(200) Most flavonoids are easily oxidized (133) and exhibit strong antioxidative properties (134) for free radical scavenging (122) as effective electron donors.(133,217) *In vitro* studies have indisputably revealed that flavonoids can eliminate damaging ROS and RNS.(217) This is mainly due to the high reactivity of the OH groups of the flavonoids.(48,98,130) Most flavonoids are effective radical scavengers (141) but flavonoids in particular are very efficient scavengers of the superoxide radical and efficient scavengers of singlet oxygen.(217)

The direct radical scavenging effect of flavonoid action protects the LDL particle, and in theory may have a protective effect against arteriosclerosis.(200) The anti-arteriosclerotic action is probably based on the removal of already formed ROS from the blood and on preventing ROS

formation.(140)   Studies have reported that flavonoids display protective effects against the initiation and further development of arteriosclerosis.(75)   In the initiation stage of lipid peroxidation, free radicals acquire H from PUFA to form lipid radicals.   Flavonoids inhibit lipid peroxidation *in vitro* at the initiation stage (290) by scavenging superoxide and OH radicals and being singlet oxygen quenchers.(217)   Since the conjugated forms of flavonoids are hydrophilic, they could prevent LDL oxidation by scavenging water-soluble free radicals formed by copper through the Fenton reaction and could in so doing reduce the utilization of the LDL antioxidants present in the lipid-water interface.(113)   Whereas some flavonoids can directly eliminate superoxides, other flavonoids can eliminate the highly reactive oxygen-derived radical peroxynitrite.   The catechins and flavones nonetheless appear to be the most potent flavonoids for protecting the body against ROS.(200)   Procyanidin polyphenols offer cardioprotective effects due to their potential to eliminate free radicals and prevent lipid peroxidation.(123)

When antioxidants react with ROS or RNS, the antioxidant is itself usually converted into an 'antioxidant radical'.   The antioxidant radical must react with another antioxidant to reduce its reduction potential and reactivity.   These antioxidant reactions can proceed in a stepwise manner, involving an immense number of antioxidant molecules, until the antioxidant radical is no longer a danger to the cell.   This will occur since the antioxidant radical has been reduced to a product which does not have sufficient reduction potential to react with important cellular components such as lipids, protein and DNA.(16)   The scavenging reactions of flavonoids generate peroxides, which is damaging and has to be scavenged by enzymatic means, like catalase or GSH peroxidase or a flavonoid phenoxyl radical.   An appropriate electron donor (ascorbate) can reduce these radicals to regenerate the parent flavonoid.   Several quinones and other products can be further generated through the flavonoid phenoxyl radicals reacting with each other.   Not much is known about the metabolism and clearance of stable products of the flavonoid phynoxyl radicals.(217)

3.3.2.2   *Transition Metal Ion Chelation and Removal of Potential Initiators*

Free iron and copper are probable sources of ROS formation.(128,145)   As a result, these transition metal ions are usually bound to proteins for both their transport and storage implying, for example that iron is transported by transferrin and stored in tissues as ferritin and hemosiderin and copper is both bound and transported via ceruloplasmin.   Oxidative stress can, however, mobilize these metals from their binding proteins.(22)   Such mobilized iron and copper ions are important pro-oxidants.(34,289)

Oxidative reactions caused by these transition metal ions are warded off by chelating agents and not by chain-breaking antioxidants.(2)   A number of flavonoids have a strong binding capacity for the divalent ions of heavy metals,(133,134) like iron and copper,(34,289) through which they can efficiently chelate these transition metal ions (iron ions in the medium of the macrophages and

copper ions in the cell-free system) responsible for the generation of ROS.(3,34,47,48,98,128,145, 289,291) This ability of flavonoids to form steady complexes with iron and copper ions can greatly reduce the redox potential of these metal ions (34) and render them inactive to partake in free radical formation reactions.(145) In addition to the antioxidant free radical scavenging properties of flavonoids,(3,47) their chelating properties sustain part of their antioxidant action and inhibition of oxidative damage.(34,98)

Polyhydroxylated flavonoids may produce benefit as inhibitors of metal induced oxidation including the Fenton reaction *in vivo*.(98) Flavonoids by inhibiting free radical formation induced by Fenton reactions,(34,128,134,145,289,291) remove a factor responsible for generating free radicals.(200) Variations in the intimal extracellular content of free metal ions, such as copper or iron could theoretically, perhaps due to variation in metal binding proteins, influence the ability to oxidatively modify LDL.(292) Iron chelation is an important factor for moderate antioxidants, or less active scavengers, to increase their lipid peroxidation inhibiting activity. This is the case with flavonols,(144) as quercetin is particularly known for its iron-chelating and -stabilizing properties.(200) Catechins have a strong metal chelating capacity.(293) Some of the health effects of polyphenols may not demand their absorption through the gut barrier of which an iron chelating role in the gut lumen may be one.(95)

3.3.2.3 Modulation of the Endogenous Antioxidant Enzyme Systems and Antioxidants

ROS formation can be prevented by enzymatic means or be neutralized by the antioxidant activity of molecules.(139) Various enzymes and proteins produced in the body function as endogenous antioxidants.(22) A large number of *in vitro* studies have indicated that flavonoids can inhibit and even support numerous enzyme systems, some of which are involved in key pathways that regulate aspects related to cardioprotection, such as platelet aggregation and the inflammatory and immune response.(107,112,121,141)

Plasma protein thiols, GSH and urate are the non-enzymatic endogenous antioxidants (16,128,148,200) of which plasma protein thiols and urate are greatly responsible for the radical trapping capacity of plasma.(128) GSH, found at millimolar concentrations in most cells, is the most important endogenous antioxidant in cells and the key contributor to the redox state of the cell (222) and its antioxidant defence system.(294) The non-enzymatic endogenous antioxidants include ascorbic acid and α-tocopherol.(200)

3.3.2.3.1 Endogenous antioxidant enzyme system support

To protect cells against ROS generation and damage induced by oxidative stress, cells (also vascular cells) are equipped with enzymatic scavenging systems that form part of the endogenous antioxidant system.(16,295) For instance, enzymatic inactivation of free radicals can suppress lipid

peroxidation.(290) The antioxidant action of flavonoids may be attributed to the inhibition and/or induction of the expressions of enzymes, including the antioxidant and the xenobiotic-metabolizing enzymes.(108,241)

3.3.2.3.1.1 _Antioxidant enzymes_

The antioxidant enzymes comprise superoxide dismutase (SOD), GSH peroxidase and catalyse,(16,200,242,295,296) with SOD for the elimination of the superoxide radicals, and catalases and GSH peroxidase for the elimination of H peroxide and organic peroxides.(16,22) GSH exists in cells in both a reduced form and an oxidized form glutathione disulphide (GSSG).(22) GSH peroxidase functions in the conversion of reduced GSH to GSSG.(22) Some flavonoids might mediate their antioxidative function by means of these antioxidative enzymes, like GSH peroxidise.   Such antioxidant enzyme involvement of flavonoids might synergistically enhance their antioxidant action against active oxygen species and lipid peroxides if flavonoids can effectively interact with GSH peroxidase to promote its expression and/or activities. Quercetin and catechin were found in a rat cell culture study to activate GSH peroxidise.(296)

3.3.2.3.1.2 _Detoxification or xenobiotic metabolizing enzymes_

Detoxification enzymes like the members of the glutathione S-transferase (GST) family, nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) and quinone reductase, are indispensable in the endogenous antioxidant defence. These enzymes are commonly referred to as phase II drug-metabolizing enzymes because they catalyse the transformation of toxic metabolites to compounds that are easier excreted.(16,248) Increasing phase II conjugation enzymes allow rapid removal of xenobiotics.(139) Compounds that support GST may reduce oxidative stress. Various flavonoids have been found to reduce oxidative stress by protecting cells against free radical damage through inducing GST expression.(47) Some plant compounds may have a twofold action in supporting the antioxidant defence by donating an electron to ROS or RNS in a conventional redox reaction and then the antioxidant radical, which is produced in the reaction, may in addition activate gene expression of antioxidant and phase II enzymes. The molecular mechanism by which plant compounds can increase antioxidant and phase II enzymes is probably, at least partly, caused by effects on the protein kinases and transcription factors (nuclear transcription factor-kappa B (NF-_k_B)).(16)

3.3.2.3.2 Endogenous antioxidant support

3.3.2.3.2.1 _Glutathione_

An important role of cellular GSH is to eliminate free radicals (222,294) and peroxides formed during normal cellular respiration, which would otherwise give rise to oxidative damage.(222) GSH is involved in regenerating other antioxidants.(294) An altered GSH status can consequently provide imperative information on oxidative stress.(22)

A mechanism operating to reduce oxidative stress entails transactivation of genes encoding enzymes involved in GSH metabolism and synthesis.(222) Moskaug et al. (222) showed that flavonoids increase expression of the GSH synthesizing enzyme (*y*-glutamylcysteine synthetase) *in vitro* and *in vivo* (using a unique transgenic reporter mouse strain) with a simultaneous increase in the intracellular GSH levels in muscles. The results of Moskaug et al. (222) added modulation of intracellular GSH concentrations and as a result the endogenous GSH-mediated cellular antioxidant defence system to the list of possible disease preventing effects of flavonoids. Moskaug et al. (222) pointed out that the cellular GSH modulation brought about in animal studies remains to be determined among humans. This current rooibos intervention trial found that GSH and the GSH:GSSG ratio were both significantly increased after rooibos consumption.(37)

3.3.2.3.2.2   *Urate*

As almost all *in vivo* trials on the TAC of human plasma before and after ingestion of flavonoid-rich foods found acute marked increases,(93,94,154) these findings led to the theory that flavonoids play an important role as antioxidants in human plasma and tissues thereby reducing chronic disease risk.(93) Ingested flavonoid-rich foods found to increase the AC of plasma (94) include: vegetables like fried onions (191,206); fruit juices, such as blueberry and apple juice (1:1 mixture),(297) cranberry juice (298) and Concord grape juice (273); other beverages being red wine,(93,275) green tea (187,271,216) and black tea (187,299); chocolate (123,197,211) and chocolate and cocoa powder dietary supplementation (212); and even meat patties with added green tea extract.(208)

The increase in the plasma AC following the intake of flavonoid-rich foods (93,94,154) may be attributed to the presence of reducing polyphenols and their putative metabolites in the plasma, their protection of other reducing agents such as the endogenous antioxidants or their effect on the uptake of pro-oxidative components like iron.(94) However, the extent of the transient increases observed in the plasma AC on consumption of flavonoid-rich foods generally far exceeds the increases in the plasma concentration of flavonoids and their metabolites.(93) The flavonoid contribution to the plasma AC therefore seems negligible.(188) Only very low (high nanomolar, low µM) levels of flavonoids and their metabolites accumulate in the plasma even after the ingestion of dietary items rich in them (93,188,229,278) and only immediately after the ingestion of these flavonoid-rich items,(188) as the half-lives of flavonoids in human plasma are short.(93) These concentrations seem insufficient to exert beneficial antioxidant effects *in vivo*,(183,229,278) in particular compared to antioxidants like ascorbate, which are present at higher levels (high µM).(183) However, some researchers in this field deem that the low µM levels of flavonoids *in vivo* are high enough for antioxidant action.(217)

Flavonoids are in addition essentially xenobiotics (278) and metabolized as 'typical xenobiotics' (229) in the intestine and liver to flavonoid metabolites (93,94,183) that alter their redox potentials (183) and affect their classical antioxidant (and pro-oxidant) structural nature.(93,94,229,278) The products of flavonoid metabolism, like the glucuronidated and methylated forms, have reduced antioxidant (and pro-oxidant) activity because of blocking of the OH groups involved in these actions.(229,278) The circulating flavonoid metabolites are therefore less efficient scavengers of ROS and RNS compared to their parent aglycone forms.(183) To resolve the large increases in plasma AC after ingestion of flavonoid-rich dietary items accompanied by the small increases in plasma levels of flavonoids, Lotito and Frei (93) in particular sought for explanations.

Lotito and Frei (93), after considering a number of explanations, concluded that dietary flavonoids are not likely to make an appreciable contribution to the AC of human plasma and other extracellular fluids *in vivo,* and that the acute marked increase in plasma AC found after the ingestion of flavonoid-rich items is a likely outcome of a consequential increase in the endogenous antioxidant uric acid. Halliwell et al.,(278) in stressing that reports of substantial increases in plasma antioxidant activity after flavonoid intake must be interpreted cautiously, indicated that such findings might be attributable to changes in urate concentrations. Urate, due to it being present in high concentrations in human plasma (93,276) and it having high reducing and free radical scavenging abilities,(93,188) is a key contributor to the plasma AC.(93,276) Maxwell et al. (276) who developed a predictive model for serum TAC based on individual antioxidant concentrations using multiple linear regression analysis, found uric acid to be the strongest predictor of the TAC.

Assessment of the changes in the plasma levels of urate and ascorbate after ingestion of flavonoid-rich items will provide for assessment of the contributions of the dietary flavonoids. Whereas flavonoid-rich items may also contain vitamin C (fruits and vegetables), they generally do not contain urate or its precursors like inosine.(93) A number of studies found that the consumption of flavonoid-rich foods,(93) such as several non-berry fruits,(188) including apples,(93) increase plasma urate and the TAC. Although the underlying mechanism was not investigated, the data suggested that urate and not necessarily, flavonoids account for the increase in plasma TAC after such consumption. However, fruits like apples do not contain urate or its dietary precursors, inosine and other purines.(93) As fructose is present in most fruits and fructose increases plasma urate levels, Lotito and Frei (93) proposed that fructose in apples is responsible for the increase in plasma urate and thus the AC in human subjects, at least after apple consumption.

In an *in vivo* study by Lotito and Frei,(300) the plasma TAC and urate rapidly and substantially transiently increased after acute apple consumption, with the plasma TAC highly correlated with

the plasma urate. The increase in the plasma urate strongly correlated with the increase in plasma urate after fructose intake, thereby suggesting that apple consumption increases the TAC of human plasma by a fructose-mediated rise in urate.(300) The effect is thought to be mediated through increased activity of the enzymes involved in the degration of purine nucleotides to urate providing for endogenous production of urate. Other carbohydrates in fruit could affect urate formation and the plasma AC. In addition to fructose in fruit, sucrose and sorbitol in fruits are likely to contribute to increased plasma urate and AC. Sucrose, which like fructose is present in high quantities in fruits, undergoes hydrolysis *in vivo* forming fructose and glucose that is absorbed. Sorbitol is a further carbohydrate present in fruits, which is transformed to fructose during its metabolism in the liver.(93)

Tea, coffee and chocolate are rich sources of methylxanthines, including caffeine, theobromine and theophylline. High levels of methylxanthines in these dietary items and their rapid absorption and metabolism to methyl derivatives of uric acid could significantly increase plasma TAC. Although several studies have measured the AC of plasma after tea ingestion, data of plasma urate in these studies are limited.(93) An intervention by Natella et al. (175) reported increases in plasma urate after black tea drinking in healthy subjects, which was paralleled by increases in AC. In contrast, Leenen et al. (187) found no increase in plasma uric acid concentration after black and green tea drinking in humans. An increase in serum uric acid concentration in healthy subjects was observed after acute consumption of red wine,(301) but not in plasma urate of healthy subjects after chronic (four week) dealcoholized red wine consumption.(302) The serum TAC, determined using different assays, of elderly women was markedly increased after the consumption of strawberries, spinach and red wine. The increased urate and vitamin C levels found, could not fully explain the increased serum TAC following the ingestion of these dietary sources. Other antioxidants, rather than urate and vitamin C, accounted for half of the increase found in the serum TAC. These other antioxidants responsible were thought to be the phenolic compounds, including the flavonoids.(303)

Lotito and Frei (93) concluded that the intake of flavonoid-rich dietary items is responsible for the increased plasma AC but that the flavonoids itself make a minor contribution, if any. The increased plasma AC seems largely to be the result of urate formation caused by fructose and other components present in flavonoid-rich dietary items. These investigators further recommended that future studies of the *in vivo* antioxidant effects of flavonoid-rich items, need to regard the fructose, sucrose and sorbitol contents or contents of other components in these items. These may markedly affect plasma urate levels. To rule out postprandial effects on plasma AC not related to flavonoids (93) plasma urate should be determined (300) since the increase in urate level may not necessarily be beneficial.(278)

3.3.2.3.3   Prevention of low-density lipoprotein endogenous antioxidant destruction

LDL contains various lipophilic antioxidants, of which the most prevalent and major lipophilic antioxidant carried is α-tocopherol (289,291) the major form of vitamin E.(291).   All the other antioxidants are far less present in LDL.   It is the key non-enzymatic antioxidant available in the lipid structures of cells.   It is a donor antioxidant (reductant), which consequently increases the resistance of LDL and lipid structures of cells against oxidative modification.(291)   Within the endogenous antioxidant defence system, dietary flavonoids may add to the antioxidant protection,(211) as they may contribute antioxidant activity through protection or enhancement of the endogenous antioxidants (47) like α-tocopherol.(128,217,289)   Due to their *in vivo* antioxidant activity, the absorbed flavonoids and their metabolites may result in a sparing effect on α-tocopherol and ß-carotene.(128)   Flavonoids are known to enhance the absorption of vitamin C and contain vitamin C stabilizing and sparing activities.(148)

Flavonoids protect α-tocopherol in LDL from oxidation by free radicals by being oxidized itself in preference to α-tocopherol (289) or by regenerating oxidized α-tocopherol (217,289) by providing a H atom to the α-tocopheryl radical, which is formed when it donates its own OH H atom to a lipid peroxyl radical to end the chain reaction of lipid peroxidation.(289)   As a result, flavonoids maintain a higher concentration of this important endogenous antioxidant in LDL for longer and delay the start of lipid peroxidation (289); thus protecting to a certain extent the development of arteriosclerosis.(134)   Among the flavonoids, quercetin and the tea catechins have the ability to regenerate α-tocopherol from the α-tocopheroxyl radical.(128)

In a study by Lotito and Fraga,(304) in which human plasma with the presence of ascorbic acid, α-tocoperol and ß-carotene was subjected to *in vitro* oxidation, the addition of catechins effectively prevented lipid peroxidation and α-tocopherol and ß-carotene depletion.   Ascorbic acid was depleted within the first hour of incubation and thus appears to be the first defence in human plasma against oxidation in the presence of a water-soluble initiator.   The catechins were depleted after ascorbic acid, but before the lipid-soluble antioxidants, appearing to be the second barrier against oxidation.   Catechins due to their solubility properties would be located on the surface of the lipoproteins.(304)   Cocoa flavonoids may have a protective effect on vitamin E and other lipohilic antioxidants within LDL because of this surface location.(212)

3.3.2.3.4   Free radical generating enzyme inhibition

Oxidants in the vessel wall may originate from enzymatic pathways.(286)   Many of the enzymes expressed in the vascular endothelial cells, produce ROS and RNS that subsequently induce oxidative stress and vascular pathology.(16,282,286)   These enzymes do not cause oxidative stress directly by themselves, but do so indirectly through generating ROS, which cause the oxidative damage.(16)   These ROS producing enzymes are activated in response to endothelial

cell injury or inflammation. The formation of ROS in vascular endothelial cells induces the expression of ROS sensitive inflammatory genes and the oxidation of LDL.(305) The enzymes generating ROS under pathophysiologic conditions include, among others, a membrane associated NAD(P)H oxidase in vascular smooth muscle cells (VSMCs) and endothelial cells, LOX, COX, xanthine oxidase and myeloperoxidase.(282,286,305) The main enzymatic source of ROS in VSMCs in response to growth factors is NAD(P)H oxidase.(277,282) NAD(P)H oxidase induced oxidative stress is causal in CVD.(282)

Besides directly eliminating free radicals and binding transition metal ions,(145) flavonoids also act as antioxidants through reducing cellular pro-oxidant enzymes involved in generating ROS (128,139,140,145,306) and thereby suppress vascular ROS formation (128,139,140) and cell-mediated oxidation of LDL.(306) The high chemical reactivity of flavonoids produces their numerous biochemical effects and properties including their binding to enzymes.(133) These pro-oxidant enzymes inhibited by flavonoids include, among others NAD(P)H oxidase,(128) LOX, COX (128,139) and xanthine oxidase.(128)

Some of the multitude of enzyme systems flavonoids that are known to modify, are critically involved in the immune function.(148) For instance, the release of arachidonic acid initiates a general inflammatory response. COX and LOX are important inflammatory mediators in the arachidonic acid metabolism.(200) They yield prostaglandins (PGs) that contribute to the inflammatory reactions.(286) The release of PGs accompanies inflammation, which attracts leukocytes to the site of action, produce local pain and increase the body temperature.(133,134) Flavonoids are inhibitors of both the LOX and COX enzymes.(289) The inhibition of these enzymes in PG biosynthesis suppresses the arachidonic acid metabolism explaining the anti-inflammatory result.(148)

LOXs are pro-oxidant enzymes that catalyse enzymatic lipid peroxidation. They are furthermore a resource of free radicals additionally contributing to non-enzymatic lipid peroxidation. Inhibition of the LOXs by flavonoids adds further to their antioxidant behaviour.(145,148) Flavonoids decrease the formation or release of free radicals in the macrophages, which are derived from the activities of LOX and COX.(289) It is unclear whether LOX and COX impact on arteriogenesis via the former immune modulation or the latter lipid peroxidation.(286) Variation in LOX activity, possibly due to genetic or localized factors, could explain differences in the ability of different subjects to modify LDL, and therefore, inhibitors of LOX are sought (292) placing interest on flavonoids for this role.

The blockage of the coronary artery because of arteriosclerotic plaque or vasospasm can reduce myocardial blood flow that if sufficiently extended or severe, can cause myocardial cell injury and necrosis, eventually leading to reduced (consequently fatal) cardiac function. The reintroduction of oxygenated blood into previously ischaemic myocardium (on reperfusion of a blocked coronary artery) can initiate a cascade of events termed 'reperfusion injury' that triggers instant release of inosine, hypoxanthin and xanthine, along with rapid free radical generation and release in the vascular bed.(22) Xanthine dehydrogenase is the form of the enzyme present under physiologic conditions, but its structure is altered to form xanthine oxidase during ischaemic conditions.(200) Tissue ischaemia is the fractional reduction or total obstruction of blood flow through one or more arteries that support specific vascular beds in an organ.(22) Both xanthine dehydrogenase and xanthine oxidase are involved in the conversion of xanthine to uric acid.(200)

In reoxygenation after ischaemia, xanthine oxidase reacts with molecular oxygen, releasing superoxide free radicals,(200) making it a source of oxygen free radicals.(148,200) Xanthine oxidase is considered an important source of superoxide radicals. Inhibition of xanthine oxidase results in a decreased production of both superoxide and uric acid.(307) Many free radical scavengers and antioxidants have been shown to reduce reperfusion injury.(287) Flavonoids that have been found to be inhibitors of xanthine oxidase, include the flavones. The flavones showed a slightly higher inhibitory action than the flavonols.(307) The protective effect of quercetin on the xanthine dehydrogenase/oxidase ratio is linked to its blocking of the dehydrogenase. Xanthine oxidase is produced from xanthine dehydrogenase during ischaemia.(148) Most flavonoid derivatives were furthermore found less active than the original flavonoid compounds.(307)

3.3.3 Modification of Blood Lipid Profile

A largely firmly established major risk factor in CHD is an elevated blood cholesterol level.(4) High plasma LDL cholesterol levels accelerate arteriosclerosis and complex and multifactorial events occur in the artery in reaction to hypercholesterolemia.(292) Given that cholesterol is a key factor in arteriogenesis, reducing the cholesterol level is consequently important in CHD.(308) Modification of the lipid metabolism may also be involved in explaining the proposed protective effects of polyphenols against CVD (94) as found in animal studies, which put forward that flavonoids lower LDL cholesterol levels.(4,94) Simply lowering LDL cholesterol will, however, not be a total solution to ameliorate the arteriosclerotic process.(292)

In a cross-sectional study among Japanese women by Arai et al.,(309) the total ingestion of flavonoids (as flavonols and flavones) was inversely correlated with the plasma levels of total cholesterol and plasma LDL cholesterol after adjustment for various risk factors. As a single component, quercetin was inversely correlated with both total and LDL cholesterol among these

Japanese adult female volunteers. Plasma HDL cholesterol and triglycerides did not correlate with the intake of any flavonoids. These results suggest that a high consumption of flavonoids by Japanese women may contribute to their low incidence of CHD compared with women in other countries.(309) Mennen et al.,(269) however, found no effect of flavonoids, simulated by the consumption of foods rich in flavonoids, on serum total cholesterol of apparently healthy French women.

A cross-sectional study of Japanese men (264) noted an inverse relation between the intake of green tea and serum total cholesterol levels, while no relation was noted with serum HDL cholesterol and triglycerides. Total cholesterol adjusted mean concentrations were lower in men drinking nine or more cups daily than in those drinking zero to two cups daily, but much larger reductions could be anticipated in those with higher total cholesterol levels. Another cross-sectional study of Japanese men (265) noted that increased consumption of green tea (particularly more than 10 cups a day) beneficially influenced various serum lipid markers. The study found decreased serum total cholesterol and triglyceride levels, and the increased HDL cholesterol and decreased LDL and VLDL cholesterol levels found, resulted in a reduced arteriogenic index. The study noted a close relation between high intake of green tea and normalization of these serum concentrations implying that habitual green tea consumption may be preventative against CVD.(265)

Epidemiological studies,(4) in addition to the animal studies,(266) seem to suggest that green tea lowers blood cholesterol levels. However, in Japan high consumption of green tea is generally associated with the traditional Japanese low fat diet, which in itself has lipid lowering effects (4) and frequently includes traditional Japanese foods (fish, vegetables and soybeans), which too have anti-arteriosclerotic properties.(149) Men with higher green tea intake also eat vegetables and fruit more often.(266) Therefore residual confounding by dietary factors cannot altogether be ignored in these studies.(4) Chronic short-term green tea intervention trials of healthy volunteers, however, provided more conflicting results. Some trials showed significant effects, such as two cups (about 400 mL) for 42 days significantly decreased plasma LDL cholesterol level, but did not significantly impact total cholesterol, HDL cholesterol or trigliceride levels,(216) and some showing no significant effects, like six cups (900 mL) for four weeks did not impact serum total, LDL or HDL cholesterol levels.(271)

The cross-sectional studies on black tea drinking and blood lipid levels have not produced conclusive results with most studies showing no significant relation between black tea drinking and these blood lipid markers. The effect of black tea ingestion on the blood lipid profile has been investigated in numerous short-term controlled feeding trials.(4) Several of these trials that

evaluated the possible beneficial effect of black tea intake did not observe any significant modification in the serum/plasma levels of total cholesterol, LDL cholesterol, HDL cholesterol and trigliceride under fasting conditions. This applied in the cases of acute supplementation of a small dose of black tea, either as 200 mL and at a one and two hour interval after administration,(175) or 450 mL at a two hour interval after administration,(299) or on chronic supplementation of four weeks of daily consumption of either five cups (750 mL) by healthy subjects (210) or six cups (900 mL) by healthy subjects (271) and patients with proven stable CAD.(299) A further dietary intervention trial did observe a decrease in total and LDL cholesterol, but there were no effects on HDL cholesterol or triglyceride concentrations, in mildly hyper-cholesterolemic adults after addition of five servings (5 X 180 mL) of black tea per day to the National Cholesterol Education Program (NCEP) Step-1-type diet (prudent diet moderately low in fat, saturated fatty acids and cholesterol) for three weeks. This finding is in conflict with the results of other trials previously mentioned that did not detect an effect of black tea drinking on the blood lipid profile.(310) The data is, however, in agreement with the findings of a large Norwegian cohort study that found black tea intake to be inversely related with serum cholesterol levels after adjustment for various risk factors.(234)

Interventions determining the influence of consuming flavonoid-rich fruit juices, for instance orange juice by subjects with hypercholesterolemia (311) and cranberry juice by healthy subjects,(298) did not find an effect on plasma total cholesterol and LDL cholesterol levels, but orange juice had a HDL cholesterol raising effect,(311) while cranberry juice did not impact HDL cholesterol.(298) Trials evaluating the effect of consuming flavonoid-rich foods like chocolate and/or cocoa powder as drinks among healthy subjects mostly found no effects on the plasma/serum lipid profile with the consumption of semi-sweet chocolate as a test meal,(211) dark chocolate bars daily for two weeks,(213) cocoa powder as a cocoa drink daily for two weeks (231) and cocoa products (dark chocolate and cocoa powder drink) daily for six weeks.(214) Increased HDL cholesterol levels were found after three weeks of daily dark chocolate or enriched dark chocolate consumption (312) and on supplementing the typical American diet with cocoa powder and dark chocolate daily for four weeks.(212) Cocoa polyphenols may thus favourably affect CVD risk status by increasing the concentration of HDL cholesterol.(212,312) Interventions mostly found no notable change in plasma total and LDL cholesterol on red wine drinking for two weeks (275,313,314) and on dealcoholized red wine drinking for four weeks.(302)

To determine the effectiveness of the different flavonoid subclasses and flavonoid-rich dietary items on lipoproteins as a risk factor for CVD, Hooper et al. (251) pursued a systematic review of all published randomized controlled trials related to the above that met their inclusion criteria. Satisfactory evidence was found to propose that chronic ingestion of black tea and chocolate or cocoa have no overall influence on LDL or HDL cholesterol, and green tea, red wine or grapes any

influence on HDL cholesterol. However, green tea reduced LDL cholesterol but to achieve this clinically important LDL reduction, two to five mugs of green tea per day would be required.(251)

Despite the mixed and often less positive results of flavonoids on the *in vivo* plasma lipid profile, it was suggested that the total and LDL cholesterol reducing effects of flavonoids protect CHD and other chronic diseases in concert with the antioxidative effect of flavonoids.(309) The hypothesized mechanistic actions by which polyphenols alter hepatic cholesterol metabolism and plasma lipids include that polyphenols may (a) decrease cholesterol absorption. Lowered cholesterol absorption will reduce the delivery of cholesterol to the liver by chylomicron remnants, which as a result will (b) up regulate the hepatic LDL receptor to compensate for the reduced substrate availability and lower plasma cholesterol. Because of the insufficient availability of lipid components, the degradation of apo-B is enhanced. Further, (c) polyphenols affect apo B secretion rates, microsomal transfer protein (MTP) and acyl-CoA cholesterol acyltransferase (ACAT$_2$) activities, resulting in an altered VLDL particle. Reductions in plasma triglyceride result because of (d) lowered MTP activity and possibly increased lipoprotein lipase (LPL) enzyme activity. Finally, (e) reductions in circulating VLDL particles and plasma triglycerides may further influence the delipidation cascade releasing less LDL in circulation. The lowered substrate availability promotes a reduction in both VLDL synthesis and secretion.(315)

3.3.4 Inhibition of Low Density Lipoprotein Oxidation and Resulting Anti-arteriosclerotic Effects

The underlying arteriosclerotic process of CHD is believed to occur in various stages beginning with LDL cholesterol accumulation and its oxidative modification in the extracellular subendothelium of the arteries.(3,4,292) Damage to LDL by free radicals or ROS, or oxidative modification of LDL owing to oxidative stress generating oxidized LDL, is thought to be an important initiating event and a key contributory factor in CHD pathogenesis with the formation of arteriosclerotic plaque and subsequently CVD.(3,219,292) LDL contains various endogenous antioxidants, including α-tocopherol and ß-carotene. When they have been greatly utilized, oxidation takes place.(289) It is the polyunsaturated lipids in the LDL molecule that are peroxidized either by free radicals released from the cell or by oxidized lipids transferred to them by the cells,(286,292) because they contain bisallylic H atoms.(286) Oxidative modification of LDL will largely take place in the artery wall where lipoproteins are exposed to oxidative stress and antioxidants become used up.(4,292) It would be expected that when the concentration of LDL is high, the relative concentration of oxidized LDL would then be high with decreases in α-tocopherol and ß-carotene as early events representing the initial stages of lipid peroxidation.(292)

Due to the high content of the various antioxidants found in the plasma and in the bulk extracellular space, plasma LDL would only undergo limited oxidative modification. When such modified LDL

enters the intima, it might undergo more intensive oxidative changes brought about by the cells.(292) All cell types (endothelial cells, immune system cells, including macrophages, smooth muscle cells, and platelets) found in the artery can oxidize LDL (140,282,292) at the sites of endothelial damage and result in subendothelial space damage.(282) This may to some extent be mediated by the ability of the cells to secrete free radicals and ROS, such as superoxide anion,(292) and oxidative enzymes.(140) Oxidized LDL becomes captured in the subendothelial space (4) leading to the formation of a fibrous plaque.(282) LDL oxidation leads to the formation of arteriogenic aggregated LDL. Aggregated LDL represents another form of LDL alteration with arteriogenic features as aggregated LDL is taken up by macrophages.(306)

An intrinsic factor that can potentially affect the oxidation of LDL *in vivo*, besides the LDL fatty acid composition providing the substrate for lipid peroxidation (with PUFAs the principal component), is the content of the endogenous and exogenous antioxidants that can increase or decrease its susceptibility to oxidation.(292) To efficiently protect LDL against oxidant stress (292) and enhance its resistance against oxidative modification by radicals formed in the lipophilic phase,(132) dietary interventions can include enhancing the antioxidant content of LDL.(292) Since oxidation of LDL is indicated in the transformation of a native to a modified LDL that contributes to pathogenesis of arteriosclerosis, delaying the onset and slowing the progression of the disease process to reduce the incidence of CHD by antioxidants is considered essential.(11,149,293,316) Many trials have thus been undertaken to identify those natural dietary items which can provide antioxidant defence against LDL oxidation.(306) Dietary supplementation with antioxidants is a probable measure to inhibit oxidative modification to LDL in man.(98,292) It is conceivable that dietary flavonoids, due to their antioxidative properties (98,299) and likely sufficient bioavailability *in vivo* to act as an antioxidant,(119) might demonstrate cardioprotective effects (98,107,141) by preventing LDL oxidation (98,299) and thereby delaying the development of arteriosclerosis (45,250) and ultimately decreasing CHD mortality.(250)

Numerous *in vitro* studies have found that most dietary flavonoids have favourable effects in ameliorating arteriosclerosis.(288,291) The effect of various flavonoids on oxidative alteration of LDL has been assessed in cultured vascular cells and in cell-free systems, most often utilizing copper as pro-oxidant.(4) Several flavonoids when added to LDL isolated from plasma, exhibit effective inhibition of the oxidative modification of LDL, which denotes a key mechanism in arteriosclerosis.(94,228,289,291,306,317-319) Flavonoids extend the lag phase of LDL to be oxidized to a modified form identified by macrophages and/or restrict lipid peroxidation and the generation of lipid peroxidation products for longer.(228,289,291,317,318) Among the tested flavonoids and over the tested concentration range, differing abilities to inhibit LDL oxidation were established, notably related to their structural features (318) along with a dose-dependent

relation.(291)   Food extracts rich in flavonoids provide this protective effect toward *in vitro* LDL oxidation.(107)   Several animal studies reported that the exposure to polyphenols decrease susceptibility of LDL to aggregation,(94) while other animal studies found polyphenol ingestion to decrease the progression of arteriosclerotic lesions developing in the face of hyperlipidemia.(94,247)   In arteriosclerotic apo E deficient mice, the arteriosclerotic lesion development was markedly repressed by flavonoid ingestion.(306)

In plasma, flavonoids are to a large extent conjugated with glucuronide and sulphate groups and are therefore hydropilic and most likely largely discarded during the isolation of LDL before the *ex vivo* oxidation test that would explain the absence of protection in *ex vivo* studies.(94)   Although the research findings on the resistance of LDL to oxidation *ex vivo* before and after ingestion of flavonoid-rich items is contradictory,(93) the ability of flavonoids to reduce *ex vivo* LDL susceptibility to oxidation has been confirmed for the most part.(306,319) Vinson et al. (284) found that a number of individual flavonoids and the common beverages in which they were present (red wine, red grape juice, green and black teas), are not only powerful *in vitro* antioxidants but also increase LDL oxidative resistance after *ex vivo* exposure (measured by increase in lag time) in human plasma.

The evidence for such protective changes in LDL oxidizability in *in vivo* studies is however mixed.(94,278) Some human clinical feeding trials in which protective changes in LDL oxidizability between the high and low flavonoid intake dietary phases were observed, measured a reduced trend or significant reduction in the plasma concentration of lipid peroxidation products, namely conjugated dienes (CDs) and malondialdehyde (MDA) (measured by the thiobarbituric acid reactive substances (TBARS) assay) and/or phospholipid peroxides (F_2-isoprostanes). The dietary phases included acute test meal trials and chronic feeding trials spanning a few days to weeks, often two or four weeks. The acute test meal trials made use of cocoa products as dark chocolate,(123,211) while the chronic feeding trials incorporated black tea,(320) cocoa and cocoa products,(212,214,231,312) red wine (275,313,314) and various fruit juices being blackcurrant and apple juice mixture (218) and Concord grape juice.(273) No significant effect was, however, observed in the intervention of Grinder-Pedersen et al. (116) on MDA after flavonoid intervention for 22 days on strict control of dietary intake compared to the baseline values as a flavonoid reduced diet. In a randomized crossover study conducted by Wiseman,(119) the markers of oxidative damage considered relating to LDL oxidation resistance, did not indicate changes between the low and high flavonoid dietary consumption phases.

The efficiency of the protection of LDL *in vivo* and the consequent reduction of arteriogenesis, depend on several factors that may affect the outcome of human clinical feeding trials. These

include the LDL oxidative state (290,306) and the antioxidant efficiency of the flavonoids,(290) which depend on their absorption,(230) their concentration (an increase of the concentration increases the antioxidant efficiency of flavonoids) (290) and how they interact with the lipoproteins.(230) The LDL oxidative condition is assessed by the equilibrium between the degree of LDL fatty acid unsaturation and cholesterol, which are susceptible to oxidation, the LDL incorporated antioxidants (290,306) and cellular oxygenases.(306) In the study of flavonoid-rich foods and beverages rather than pure flavonoids, the other constituents these dietary sources contain must be considered in the modulation of oxidative damage.(229,278)

The mechanistic actions by which flavonoids attenuate LDL oxidation are uncertain and have not been fully resolved,(289,291) as different mechanisms may be involved through which flavonoids reduce LDL oxidation. Flavonoids may donate H atoms and thus decrease free radical generation via the formation of ROS and/or they may bind metal ions, like copper or iron.(319) Dietary consumption results in macrophages (306) and LDL particles becoming sufficiently enriched with flavonoids to subsequently reduce the oxidative state making LDL less susceptible to oxidation.(291,306) *In vivo*, polyphenols are attached to the LDL particle, and such LDL enriched with polyphenols found to be more resistant to oxidation than native LDL.(306) For instance, the total polyphenol concentration in the LDL fraction was elevated after two weeks of red wine drinking, indicating that the phenolic substances in red wine are absorbed and bind to LDL and thus offer protection to LDL against lipid peroxidation.(313) It seems possible that *in vivo*, these antioxidants will not bind only to the LDL particles, but also bind and accumulate in the vascular wall cells.(306) A study by McAnlis et al. (206) on the consumption of a fried onion meal providing quercetin, detected no quercetin in the LDL, VLDL or HDL fractions, but found quercetin bound to the albumin protein leaving quercetin unable to protect LDL against oxidation. Quercetin may nevertheless still exert an indirect antioxidant effect as a result of incorporation into vascular cells or by protecting the endogenous antioxidants.(206) In addition, the incorporation of polyphenols within LDL results in a marked reduction in it being prone to lipoprotein aggregation (a lipoprotein arteriogenic alteration that leads to the uptake of LDL by macrophages).(306)

Free radical generated oxidation of PUFAs results in the production of lipid peroxides.(132) In the propagation stage, the lipid radical reacts with molecular oxygen from the lipid peroxy radical, which on disintegration produces more free radicals thus sustaining the chain of reactions. Peroxy radical scavengers can break the propagation chain reaction. Flavonoids act by providing H atoms to the peroxy radical, thus inhibiting the oxidation of fatty acids by termination of the free radical chain reaction.(290) The presence of chain-breaking phenolic antioxidants provides a mechanism to interrupt the peroxidation process by reducing the alkoxyl and peroxyl radicals to alkoxides and hydroperoxides, respectively.(132)

Besides the structural features of flavonoids dictating their antioxidant activity,(145,317) their lipophilicity characteristic (145) and their location in the membrane and ability to interact at the membrane water-lipid interface (and capacity to modify membrane dependent processes) (145,317) are further factors influencing their antioxidant activity which could have important applications in human disease accompanied by free radical injury.(317) The major factor influencing the power of flavonoid-lipid interactions seems to be the fat-solubility of the flavonoid compounds.(274) Considering the lipophilicity characteristic, all flavonoids possess antioxidant activity in the hydrophilic environment, but some still show antioxidant activity in a lipophilic environment because native flavonoids are highly soluble in fats and oils and soluble in a water-alcohol mixture.(48) The hydrophobicity of polyphenols is intermediate between that of vitamin C (highly hydrophilic) and α-tocopherol (highly hydrophobic),(100) making it less lipophilic than α-tocopherol.(154)

Consequently the antioxidant effects *in vivo* can either decrease or increase in membranes due to several factors, which are not considered in chemical tests.(317) One of the key features of antioxidants is their solubility and as a result their distribution between different media in the body.(217) Native flavonoids represent the lipid-soluble antioxidants that act primarily in the lipophilic region of cell membranes.(148) However, despite their lipophilic nature, glucuronidation and sulfation consequently make polyphenols more water-soluble,(100,113) which can influence their site of action and their interactions with other antioxidants.(100) Due to their different chemical structures flavonoids and their metabolites can have water-soluble or relatively fat-soluble characteristics.(210,291) Flavonoids are thus capable of entering phospholipid bilayers (274,317) and become associated with lipoproteins.(321)

The LDL particle contains α-tocopherol, the major fat-soluble antioxidant in plasma,(132) which is localized in the lipid membrane within the phospholipid bilayer (47,132,154) and vitamin C which concentrates in the aqueous phase.(47) In contrast, flavonoids are probably mainly located between the two phases owing to their water-solubility (47,154) and expected to act at water-lipid interfaces.(47,100) Because of their amphipathic nature,(210,291) flavonoids could therefore accumulate at the membranous surface (phospholipid structures) of LDL particles (47,154,283) or water-lipid interfaces (113,273) and constitute possible protection. The latter refers to preventing free radical attack and oxidative damages (47,113,273) in both aqueous environments (plasma proteins and the polar surface region of the phospholipid bilayers in lipoproteins or extra particle environment of LDL),(113,210,273,291) acting in a manner comparable to that of ascorbic acid,(210,291) and lipid environments (cellular membranes) (113,273) acting within the LDL particle in a manner comparable to that of α-tocopherol.(210,291) This provides a more effective

antioxidant defence in biological systems.(113,273) Flavonoids would in this way reduce loss of α-tocopherol and thus limit oxidation of the lipids present in LDL. Should the initiation stage and the propagation stage of lipid peroxidation occur as suggested at the surface and the interior of the membranes respectively, then the flavonoids could interfere with the reaction path by hindering the initiation stage,(154) as interactions of flavonoids with lipids are usually confined to the polar region of the lipid bilayer.(274)

However, further factors influence the interaction of flavonoids with lipids in membranes.(317) The effectiveness of the protection by flavonoids depend on the deepness of the membrane penetration that is linked to their structure,(274) their rate of uptake into cells and on their orientation in the membranes.(317) Flavonoids could reach all areas of the bilayer and thus protect the whole bilayer against oxidation. The distribution of flavonoids in the membrane depends on their solubility. The water-solubility of flavonoids depends on the number of OH groups. The most apolar flavonoid will immerse deeper in the hydrophobic innermost part of the bilayer and the polar flavonoids remain closer to the polar water phase.(274) In the auto-oxidation of the rat cerebral membrane experiments of Saija et al.,(317) a necessary element of flavonoids together with their redox properties for the expression of antioxidant activity appeared to be the ability to interact with biomembranes.

Because flavonoids can interact with both the protein and lipid components of biological membranes and penetrate the lipid bilayers, they can consequently alter membrane properties, such as the structure, permeability (274) and fluidity.(317) The ease of flavonoids to influence the structure of the lipid bilayer likely depends on its capability to take on a planar formation.(317) Most flavonoids decrease membrane fluidity. Membrane fluidity changes can modulate membrane enzymes and receptors, as well as the effects of the membrane components. The interaction of flavonoids with lipid bilayers may influence the electric properties of the membranes.(274)

3.3.5 Anti-inflammatory Effects

LDL peroxidation is but one of the important consequences of oxidative stress in arteriosclerosis.(286) Oxidized LDL triggers a surge of inflammatory reactions, which represent an important manifestation of the arteriosclerotic process.(4) LDL modified by oxidation differs from native LDL in a number of ways.(129,292) Due to the oxidative modifications to LDL, it becomes truly immunogenic (292) and potentially arteriogenic to initiate and promote arteriogenesis.(129,141,292) Due to the oxidative changes to LDL, circulating monocytes are attracted to the arterial wall and endothelium (4,286) which transpire immunoreactivity.(247,292) Modified (oxidized) LDL is chemotactic for circulating monocytes recruiting increasingly more monocytes into that same area.(292) Monocyte uptake by modified LDL differentiates into the

formation of monocyte-derived macrophages, which enhances the uptake of excessive amounts of oxidized LDL.(4,279,286,289,292)   The sequestration and removal of modified LDL by macrophages is an important early, protective role of the macrophages in the inflammatory response to reduce the cytotoxic effects of altered LDL on the endothelium and smooth muscle cells.(279,292)

Activated macrophages, however, take up modified LDL continuously and transform them in an uncontrolled manner (140,308) into lipid-engorged foam cell formation in the arterial wall (3,4,140,279,286,289,306,308) and eventually the growth of arteriosclerotic plaque.(279) Native LDL does not initiate foam cell formation in cell culture conditions. It has been proposed that LDL must be altered before it can be incorporated into cells.(308) The accumulating monocytes and macrophages, for instance, cause structural changes to the apolipoprotein B component of LDL.(3) Oxidative damage to LDL, in particular the apolipoprotein B molecule, is considered a key event in the development of arteriosclerosis.(292) Macrophages furthermore express scavenger or acetyl-LDL receptors that recognize the protein of modified LDL permitting them to efficiently engulf oxidized LDL contributing to foam cell formation.(247,289,293)   The macrophage scavenger receptor differs from that of the native LDL receptor in that it cannot be down-regulated.(308)

Furthermore, macrophages generate ROS,(282) which in addition oxidizes LDL (292) and converts oxidized LDL into highly oxidized LDL (282) leading to a vicious cycle of generating ever more oxidized LDL and macrophages (292) and foam cells.(282) The release of ROS by macrophages is part of the body's defence system and this inflammatory condition leads to an increased oxidative burden.(241) LDL can be oxidatively altered by various other cells in addition to the macrophages.(289) Modified LDL inhibits macrophage motility.(292) The oxidant/antioxidant status is a key determinant of LDL oxidation and therefore its attraction by macrophages.(322) The ability of oxidized LDL to accumulate may in part be related to an inability of the macrophages to readily degrade oxidized LDL.(292)

The damaging cycle of modification to LDL, inflammation, further alteration to LDL and inflammation can be sustained in the artery via the presence of modified lipids.(279) Free radicals can attract several inflammatory mediators causing a general inflammatory response and tissue injury.(200)   Modified LDL, in being cytotoxic, facilitates the production of various potent chemotactic, including inflammatory, factors that comprise pro-inflammatory cytokines. These attract adhesion molecules (vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1), growth factors) and other mediators by the macrophages and vascular wall cells (247,292) that expand the inflammatory process (279) and play an important role in the development and progression of the arteriosclerotic plaque.(292)

Because arteriosclerosis involves the recruitment of inflammatory cells into the arterial wall stimulating vascular inflammation,(286) it is considered a chronic disease with a strong inflammatory component (4,140,247,280) or even a chronic inflammatory disease.(279) The inflammatory background is central to the arteriosclerotic plaque formation,(140) but results in the formation of ROS and RNS that add to the vascular inflammation (241,286) and initiate the oxidation of biomolecules (oxidative stress).(286) According to some researchers,(286) this 'oxidative response to inflammation' hypothesis suggests that the oxidative events are a result rather than a cause of arteriosclerosis and explain the poor performance of antioxidant strategies, as part of the oxidative modification hypothesis, in reducing arteriosclerosis and its cardiovascular events in humans. Since CVD is additionally considered an inflammatory disease, interventions that have anti-inflammatory effects may beneficially affect the disease outcome.(3)

Flavonoids seem to regulate cellular actions of the inflammation related cells including macrophages, mast cells, lymphocytes and neutrophils.(280) Flavonoids with anti-inflammatory potential may therefore play a crucial role in the prevention of CVD by decreasing inflammation.(214) The effects of flavonoids on inflammation have been investigated mostly in isolated cells or in experimental animals. However, the pertinence of *in vitro* studies, which used unbiologically plausible high levels of flavonoids that cannot be reached *in vivo,* remains to be fully determined in humans.(4) Cocoa flavonoids have been observed to regulate the immune response,(219) but seemingly not in all studies. Mathur et al.,(214) in a crossover experimental study of six weeks duration, found that cocoa product supplementation in the healthy volunteers did not affect numerous markers of inflammation. EC was not found in the subject's plasma. The absence of effect found on the inflammatory markers was contributed to the short half-life of EC.(214) No effect was observed in C-reactive protein (CRP) as a non-specific marker of generalized inflammation on acute (450 mL) and chronic (900 mL per day for four weeks) black tea consumption by subjects with stable CHD.(323)

Several mechanisms of action are suggested to elucidate the *in vivo* anti-inflammatory effects of flavonoids which, among others, include the inhibition of the eicosanoid-generating enzymes and/or modulation of the production of pro-inflammatory molecules.(280) Inhibition of the arachidonic acid metabolizing and resulting eicosanoid generating enzymes reduces the concentrations of eicosanoid pro-inflammatory factors, such as PGs and leukotrienes,(280) that are bioactive metabolites of arachidonic acid.(21) Flavonoids can modify the actions of these enzymes like COX, LOX and inducible NO found in macrophages.(280) Some phenolic compounds have been found to block both the COX and LOX pathways.(15) Impaired action of these enzymes by flavonoids reduces production of arachidonic acid, PGs, leukotrienes and

peroxynitrates, which are important mediators of inflammation. The inhibition of these enzyme activities by flavonoids is an important cellular mechanism balancing excess inflammation.(280) Schramm et al. (324) reported that dark chocolate procyanidins increased the plasma prostacyclin concentration and reduced the plasma leukotriene concentration which is pro-inflammatory, resulting in a beneficial change in the ratio of the two eicosanoids in apparently healthy subjects. As the ratio of the two eicosanoids influences the pro- versus anti-inflammatory balance, the finding proposed that chocolate procyanidins might influence the inflammatory response via eicosanoid modulation.(21)

Research has furthermore shown that some flavonoids directly modulate pro-inflammatory gene expression resulting in the reduction of the inflammatory response,(280) which seems to engage the decreased expression of the NF-kB.(315) NF-kB is a protein that binds to specific promoter regions on the genome stimulating gene transcription for the formation of various pro-inflammatory proteins as mediators of inflammation,(172,219,280) namely cytokines and adhesion molecules, involved in the onset and development of arteriosclerosis.(172,219) NF-kB remains inactive as inhibitory kappa B-alpha (IkB-alpha) until its phosphorylation. Its phosphorylation leads to the release of the p65/p50 dimer, which binds to nuclear DNA causing gene activation of numerous mediators of inflammation. This response is normally quickly inactivated, but in chronic inflammatory conditions, it continues to be activated. Flavonoids inhibit the activation and action of NF-kB by inhibiting the phosphorylation of IkB-alpha and binding of the p65/p50 dimer to its promoter region of the genome and results in excessive inflammation.(280)

Grape polyphenols, for instance, seem to produce anti-inflammatory effects by reducing the formation of cytokine cellular adhesion molecules on both monocytes and T-lymphocytes. This alteration will decrease the overall uptake of monocytes and T-lymphocytes within the intima and may hinder plaque formation and arteriosclerosis. Decreased expression of NF-kB has been proposed to be involved in this mechanism.(315) Cocoa flavonoids in part regulate the immune response by modulating the oxidant-responsive NF-kB. NO normally inhibits NF-kB. In endothelial dysfunction, which is marked by reduced bioavailability of NO, this inhibition is lost. Excess intracellular ROS in oxidative stress stimulates NF-kB. Data from experimental studies indicate that cocoa flavonoids may prevent the activation of NF-kB and subsequent cytokine transcription by reducing intracellular ROS.(219)

3.3.6 Anti-platelet and Anti-thrombogenic Effects

Platelet-blood vessel interactions seem to be involved in the initiation and progression of arteriosclerosis and thrombosis.(28,129,212,322,325) While healthy vascular endothelium helps to sustain blood fluidity,(28) the injured endothelium becomes dysfunctional and develops more pro-

instead of anti-coagulant properties (3,28,279) that may cause increased platelet adhesion and aggregation on the vessel surface (28,140). Platelet aggregation to dysfunctional endothelium, exposed collagen and macrophages occur.(279)

Activated platelets generate ROS and RNS through enzymatic production (140) and from arachidonic acid, which can be converted into PGs, such as thromboxane (TX) A2 (TXA2), further stimulating thrombogenesis (or into leukotrienes, which can add to the inflammatory response). The platelet-aggregating process recruits additional platelets, which leads to the development of an expanding thrombus.(279) As the inflammatory process persists, the activated leukocytes and intrinsic arterial cells can produce fibrinogenic mediators.(247)

In addition, platelets infiltrate the intima of the arteries following endothelial injury, which sustains the formation of the arteriosclerotic lesions.(322) Sub-clinical episodes of plaque disruption and local mural thrombosis with thrombin activation and consequential healing, may contribute to the progression of arteriosclerosis.(325,326) The frequency of plaque fissures and healing contribute to the development of early lesions to advanced lesions with recurrent episodes gradually leading to vascular occlusion.(325)

In the progression of CHD, blood flow in one or more of the coronary arteries becomes diminished because of arteriosclerotic plaque growth from the inner surface of the arteries, narrowing and obstructing the lumen.(4) Among the haemostatic factors, platelets play a crucial part in coronary thrombosis.(327) When greatly narrowed by arteriosclerotic plaque, the artery is prone to acute platelet-mediated clot obstruction.(4) Activated platelets that adhere to and aggregate at the damaged vascular surface release granules, which contain platelet-derived growth factors and other mitogenic factors like cytokines.(270,279,325) These mitogenic factors play a role in the associated hypertrophy and thrombotic response.(270,325)

Platelet adhesion and aggregation contributes not only to the formation of arteriosclerosis,(200) but plays a critical role in coronary disease progression and the acute coronary syndromes, such as unstable angina, myocardial infarction and sudden ischaemic death, due to plaque rupture with consequent platelet activation and intraluminal thrombus formation that may ultimately lead to occlusion of vessels.(28,142,200,246,267,282,325) The magnitude of the thrombus formation and changes in vascular tone may establish the extent of the ischaemia/infarction.(75,246) Thrombus formation releases serotonin, TXA2 and thrombin. Each of these thrombosis-associated mediators can cause vasoconstriction not only at the site of the thrombus formation, but also further down.(247) Blood flow may ultimately be fully blocked by a blood clot (thrombus) so that the heart muscle tissue further away from the obstruction in the artery is no longer receiving oxygen and

nutrients. The resultant damage to the heart muscle may cause death of the affected tissues (infarction) or be so severe that death results.(4)

There is considerable support that anti-platelet therapy favourably influences thrombosis and the thrombotic complications of CVD.(142,327) Obtaining an optimal platelet function by dietary intake may provide a promising intervention proposition in the control of cardiovascular risk.(142) The potential of polyphenols to lower platelet reactivity could have a huge effect on CVD,(246) although only a portion of coronary deaths result from acute thrombosis in the coronary arteries.(256) Polyphenol inhibition of platelet aggregation that may limit thrombosis formation in the acute phase of myocardial infarction, has been shown *in vitro* and in several animal models.(94) Platelets are generally removed from blood drawn before and at various times after polyphenol administration. Platelet aggregation is then determined *ex vivo* in an aggregometer utilizing various agonists.(142) A number of human studies demonstrated that flavonoids inhibit thrombogenesis (246) and might provide, at least in part, an important mechanistic explanation for the stronger association found between flavonoid ingestion and CHD mortality, than between flavonoid intake and incidence of myocardial infarction in epidemiological studies.(45,142,246)

A review of the available research on the influence of polyphenol supplementation on platelet aggregation in animal models or in humans by Nardini et al.,(142) found that most of the studies observed that polyphenol supplementation either as purified compounds or food extracts provided some evidence for reducing platelet aggregation. Evidence in humans on the anti-thrombotic effects of polyphenols is more limited.(94,142) The nature of the polyphenol source, as phenolic-rich foods, beverages and extracts or purified phenolics, furnishes different outcomes.(142) Data on purified phenolic supplementation supports a marked inhibitory effect on platelet aggregation.(142,328)

An increasing number of plant-based dietary items possibly influence platelet function. These include grape juice, tea, red wine, cocoa and dark chocolate.(28) Nardini et al. (142) found no significant effects reported on platelet aggregation after orange and grapefruit juice intervention, while grape juice, pomegranate juice and armagnac intervention repeatedly resulted in a marked inhibition. The reduction of platelet-caused thrombosis provides a possible mechanistic action for the beneficial health effects of purple grape products, free of alcohol ingestion, in CVD.(329) Tea did not appear to influence platelet activity, with the exception of green tea,(142) although there is some evidence that black tea consumption may decrease platelet aggregation.(4,320) Wine supplementation provided more conflicting results,(142) although standard red wine drinking is linked to reduced platelet aggregation and the prevention of CHD.(237,239) Uncooked onion and kiwi intervention seemed to have an inhibitory action on platelet reactivity.(142)

For intervention trials with polyphenol-rich items, it must be remembered that such foods may also contain non-phenolic active compounds that may additionally influence platelet reactivity.(142) Consumption of flavanols and flavanol-rich foods in particular have been found to inhibit platelet reactivity, adhesion and aggregation, as well as influencing fibrinolysis thus reducing the overall platelet thrombotic tendencies.(20,28) This may result in new approaches for prevention and therapy for CVD to benefit vascular homeostasis.(267) Supplementation with the flavanol catechin and, to a lesser extent, cocoa procyanidins present marked reductions in platelet aggregation.(142) Acute feeding trials have shown that cocoa supplementation inhibits unstimulated and stimulated platelet activation.(245,270) Synergistic relationships may enhance the anti-aggregating effect. It is still not clear if polyphenols themselves, their metabolites or a mixture of both provide the beneficial effect found on the pathogenesis of thrombosis by interfering with platelet reactivity.(142)

Polyphenolic flavonoids have been found to suppress platelet reactivity and aggregation via several different mechanisms.(129,140,243) The inhibitory mechanisms responsible have not been fully elucidated as the effects are associated with complex molecular mechanisms.(140) Experimental evidence show that polyphenols suppress platelet aggregation stimulated by various agonists, signifying that phenolic compounds act at several sites and multiple levels in the complex signalling network or cascade leading to platelet activation. All these pathways are interconnected with outcome the activation of the fibrinogen receptor as the final common step.(142) Collectively supportive evidence is available that phenolic compounds have anti-thrombotic potential that seem to be linked to reduced platelet aggregation, decreased synthesis of pro-thrombotic and pro-inflammatory mediators, reduced expression of adhesion molecules and tissue factor activity as reviewed by De Gaetano and Rotondo (239):

(a) Flavonoids may modulate factors that promote platelet reactivity, like ROS and TX formation.(28) Activated platelets adhering to the vascular endothelium produce free radicals, and thus lipid peroxides.(328) Flavonoids scavenge platelet-derived free radicals (328) and enhance antioxidant defences by sparing endogenous antioxidants thereby decreasing endogenous peroxide formation.(142) Freedman et al.,(329) observed that adding purple grape juice to platelets *ex vivo* decreased platelet aggregation and suppressed platelet production of superoxide anion. Platelet superoxide formation was reduced by incubation with all of the flavonoid fractions.(329) Flavonoids, which are bound to platelet membranes and scavenge free radicals and inhibit lipid peroxidation, are candidates for anti-thrombotic drugs.(328)

Activated platelets bound to the vascular endothelium not only produce free radicals and thus lipid peroxides,(328) but also inhibit the biosynthesis of endothelial-derived factors like NO and prostacyclin.(28,328) The anti-platelet effects of flavanols,(172) and particularly cocoa

and chocolate, may be caused by increased production of NO, which not only brings about vasodilation, but also suppresses platelet aggregation.(219) Flavonoids by scavenging platelet-derived free radicals are likely to protect NO from destruction by superoxide anions.(328) On investigating the effects of purple grape juice on platelet activity, Freedman et al. (329) found that its addition to platelets *ex vivo* and its oral supplementation reduced platelet aggregation and increased platelet NO release. The increased release of platelet derived NO may have supported the observed aggregation suppression.(329) Stimulation of NO synthesis and/or NO release, induces an increase of intraplatelet guanosine 3',5'-cyclic monophosphate, cGMP, a component known to suppress platelet aggregation.(142)

A trial by Schramm et al. (324) provides support that some of the effects of chocolate on platelet function may in part be due to changes in the eicosanoid metabolism. They observed that consumption of cocoa procyanidins (as a flavonoid-rich dark chocolate) increased plasma prostacyclin concentration, while decreasing that of leukotrienes. Prostacyclin inhibits platelet aggregation and is a potent vasodilator, whereas leukotrienes stimulate platelet aggregation, is a vasoconstrictor, and is pro-inflammatory.(21) One possible explanation for the observed anti-platelet effects may be the increased formation of prostacyclin, an eicosanoid that is known to suppress platelet aggregation.(27)

Arachidonic acid, which is released in inflammatory conditions,(200) is converted via COX in the platelets following stimulation (140) to form TXA2.(140,200) TXA2 is a powerful platelet aggregating substance and vasoconstrictor. TXA2 is used as biochemical marker for platelet activation.(140) Its counterpart, 6-keto-PG $F_{1\alpha}$ (PGI_2), is an anti-aggregatory agent and vasodilator primarily produced by vascular endothelial cells and smooth muscle cells. Their urinary metabolite excretion (as TXB_2) can be analyzed as measure of platelet function.(212) The mechanisms of action of flavonoids to inhibit platelet reactivity, aggregation and thrombotic tendency involve, among others, blocking of COX (and thromboxane synthetase along with LOX enzymes) in the arachidonic acid cascade in platelets.(142) Some flavonoids selectively block COX (COX selective inhibitors) or LOX (5-LOX selective inhibitors), and some both enzymes (non-selective inhibitors).(15) These selectives suggest that different SARs influence suppression of the two enzymes by the flavonoids.(15,271) Flavonoids may reduce platelet aggregation by suppressing thromboxane formation, but also through thromboxane receptor antagonism (as TXB_2 formation was parallel suppressed with the platelet aggregation due to the inhibition of TXA2 formation).(330)

Drugs like aspirin, inhibit platelet aggregation.(237) Use of aspirin is a relatively weak preventive anti-thrombotic treatment.(243) Aspirin permanently inactivates COX-1, causing the irreparable loss of the powerful aggregator TXA2 for the lifespan of the platelet (eight to ten days).(28) Ingestion of cocoa causes an aspirin-like result on platelet activity. Regular consumption of the bioactive cocoa components may thus provide therapeutic potential to

reduce thrombotic risk and may complement the platelet suppressive effects of aspirin. Inhibition of platelet reactivity is one mechanism by which cocoa and chocolate could provide cardiovascular benefits.(270) Besides for its flavonoid content, cocoa also contains the methylxanthine theobromine, which may contribute to the observed anti-thrombotic effects.(270,331)

(b) Platelet microparticles are membrane vesicles that are hemostatically active and shed from platelets, endothelial cells and white blood cells during conditions such as stress and agonist activation.(28) These particles have pro-coagulant or platelet activation properties.(28,270) The finding of smaller amounts of microparticles in unstimulated whole blood after cocoa supplementation, supports the inhibitive effects of cocoa components on platelet reactivity.(270)

(c) Platelets undergo a series of intracellular signalling steps on agonist activation with eventual platelet aggregation and coagulation as a result of transformation of the glycoprotein (GP) IIb/IIIa receptor from a resting, non-active state on the platelet surface, into an active platelet activation marker which mediates fibrinogen binding, and P-selectin expression,(28,270) a platelet surface adhesion molecule.(28,140) Rein et al. (245) observed that platelets incubated with procyanidins isolated from cocoa *in vitro*, showed reduced expression of GPIIb/IIa and P-selectin, platelet surface proteins participating in platelet-platelet and platelet-leukocyte interactions, respectively. In an acute feeding trial, Rein et al. (270) evaluated whether consumption of a cocoa beverage as test meal, modulated platelet function in healthy subjects as determined by platelet activation marker expression in response to weak agonists. They observed significantly diminished expression of the GP IIb/IIIa complex and P-selectin in stimulated and unstimulated platelets after the cocoa beverage consumption. In subjects given the control beverages, no significant changes were observed. The EC, which constitutes 30 to 40% of the total flavonoids in cocoa, and oligomeric procyanidins in the cocoa beverage, may be responsible for the findings. The timing of the effects found was concurrent with the absorption kinetics of EC from chocolate.(270)

(d) The protein kinase cascade is actively engaged in regulation of the platelet function.(142) Various protein kinases has been found to be possible targets for flavonoids. Flavonoids have the ability to bind to the adenosine triphosphate (ATP) binding site of various proteins, including the protein kinases, apparently causing three-dimensional structural changes rendering them inactive.(183)

(e) Numerous studies propose that flavanol-rich foods may have the ability to alter fibrinolysis.(28) Flavanols and flavanol-rich foods in particular can modulate fibrinolytic factors like tissue-plasminogen activators (t-PAs). The fibrinolytic system brings about the transformation of plasminogen to plasmin by the plasminogen activators (PAs), tissue-

plasminogen activators (t-PAs) and urinary-type plasminogen activators (u-PAs) or urokinase.(28) Thrombolytic agents are PAs that convert plasminogen, the inactive pro-enzyme of the fibrinolytic system in the blood, to the proteolytic enzyme plasmin.(327) Activated plasmin can then dissolve fibrin within a blood clot, leading to clot dissolution.(28,327)

(f)   It has become apparent that the smaller non-critically stenotic, but vulnerable, plaques that do not obstruct the arterial lumen, are in reality more likely to cause acute myocardial infarction (267,325,326) than the severely fibrotic and stenotic obstructive plaques that have a relatively thick fibrous cap.(325,326) Acute rupture or erosion of vulnerable plaque results in the formation of acute, platelet-rich thrombi that narrows or occludes the arterial lumen to go on to cause ischaemia or infarction.(75,246) The collagenous extracellular matrix (formerly called connective tissue) furnished by the VSMCs determines plaque integrity, or the plaque's fibrous cap strength and stability. Intact ability to form collagen may maintain the strength of the fibrous cap to withstand mechanical strain in stable plaques.(326)

Accumulation of plaque lipid in conjunction with the continuing influx and activation of macrophages in the arterioma,(247,279) supports chronic immune stimulation (326) and local inflammation within the plaque.(279) These may be important factors in thinning of the fibrous cap.(279) Macrophages (247,279,326) and VSMCs activated by inflammatory mediators like the cytokines,(326) can produce proteolytic enzymes (proteinases) at these sites,(247,326) such as metalloproteinases (MMPs) and other proteolytic enzymes.(279,326) These enzymes degrade the collagenous extracellular matrix (247,277,326) rendering the cap thin, weak and susceptible to rupture.(247,326) Cells within arteriosclerotic plaques can express genes encoding these matrix-degrading enzymes.(326) Red wine and green tea-derived polyphenol compounds suppress release of these proteolytic enzymes.(277)

3.3.7   Improvement of Vascular Epithelium Dysfunction

The development of endothelial dysfunction is acknowledged as an initiating early event in arteriosclerosis.(3,219) A possible cause of endothelial dysfunction, which leads to arteriosclerosis, is oxidative stress and a weakened antioxidative defence system (27,239,305) with increased levels of oxidative altered LDL and free radical formation brought about by numerous risk factors, including cigarette smoking.(3,279) Oxidized LDL (140,200,279,292) and the resulting foam cell formation (286) is toxic to the vessel endothelium and injures and damages the endothelial cell integrity.(200,279,292) Mechanical damage to the endothelium, or loss of its functional integrity, disturbs the equilibrium of these cells (140) promoting arteriosclerotic changes,(141,200,239) which disrupt the normal endothelium function.(292)

Within the blood vessel wall, the vascular endothelium with its cells is crucial in the regulation of vascular homeostasis.(27,172,246,267) Normal endothelial cells maintain vascular homeostasis by synthesizing and releasing several regulatory mediators that act within the vessel wall and lumen.(140,172,246,267) These mediators consist of vasodilators and vasoconstrictors (140) that include NO.(27,140) NO is a key endothelial substance that was primarily considered a potent endogenous endothelial-dependent relaxing factor (EDRF) or vasodilator causing vascular smooth muscle relaxation.(140,246,267) It is now known to have anti-inflammatory properties, that prevent binding of leukocytes to the endothelial space,(267,279) and anti-thrombotic properties, that prevent platelet adhesion and platelet aggregation.(140,267,279) When produced in appropriate amounts, NO is a vasodilator and anti-thrombotic, anti-inflammatory and anti-arteriosclerotic substance.   NO inhibits the proliferation of VSMCs and the formation of other non-cellular components related to the matrix of the vascular wall and, thus, is important to lesion development and vascular maintenance. Impairment of NO formation would without doubt have adverse effects on the cardiovascular system.(267)

A consequence of endothelial disturbance is impaired endothelial vasomotor function (292,332) characterized by deregulation of the NO metabolism (213) and loss of NO.(27,219,267) The effective release of NO is reduced,(75) possibly from reduced synthesis of NO,(140,213) but presumably largely as a result of inactivation of NO by superoxide (332) through the formation of peroxynitrite.(219) In arteriosclerosis, expression of superoxide is increased in blood vessels.(332) Endothelial dysfunction itself is characterized by an increase in ROS production in the vascular cells in response to the endothelial cell injury of inflammation.(305) Reaction of ROS with NO leads to NO radical production which causes lipid peroxidation and leads to functional damage in the endothelium.(295) Modified (oxidized) LDL inhibits NO action.(292) The general result being impairment of endothelium-dependent vascular relaxation (213,219) with vessel constriction.(140,219) It has been reported that differences in NO bioavailability may set apart subjects with good vascular health who are less prone to experience a cardiovascular event, from subjects with poor vascular health who are prone to experience a cardiovascular event.(333)

Endothelial dysfunction is pro-arteriogenic (75,149,279) and stimulates the inflammatory (267,279) and pro-thrombotic (172,267) systems in a manner that supports lesion development and progression (267) and clinical manifestation of arteriosclerosis.(75,239,267) Endothelial injury activates macrophages (140) and increases the adhesiveness of the endothelium with regard to leukocytes and platelets.(279)

The injured endothelium responds by up regulating adhesion molecules (3,247) resulting in the overexpression of VCAM-1 and ICAM-1 (3,140,247) that causes leukocyte binding to the vessel

wall to increase.(3,140,200,247) Under normal conditions, blood leukocytes move freely along the endothelial wall,(200) as the normal endothelium does not generally support adherence of white blood cells.(247) Once bound to the endothelium, the leukocytes propagate inflammation and penetrate the intima becoming localized in the endothelium.(247) Increased adhesion molecule expression on the endothelium is responsible for the accumulation of monocytes and T lymphocytes furthering the inflammatory response.(279) The firm binding of leukocytes to the endothelial wall and their immobilization is a major mechanistic action involved in the production and release of oxygen-derived free radicals. Immobilizing the leukocytes stimulates degranulation of the neutrophil.(200) Because of endothelial dysfunction overexpression of TXA2 along with P-selectin, stimulate platelet aggregation.(140)

As the arteriosclerotic process continues macrophages and platelets,(325) together with the resulting foam cells (282) and the dysfunctional endothelium,(3,282,325) release various growth factors (3,270,279,282,325) and cytokines (3,247,279). This leads to abnormal proliferation and migration of VSMCs, the contractile component of blood vessels, into the intima,(3,75,247, 270,279,282,325) which is a process that forms part of the development and progression of the arteriosclerotic lesion, intimal thickening, arterioma development and luminal narrowing.(75,270, 279,325) On injury, VSMCs undergo changes in phenotype, including suppression of VSMC marker genes and activation of a host of proliferative genes. Phenotypically modulated VSMCs increase the formation and growth of extracellular matrix components that play a key role in lesion development.(305) Cycles of smooth muscle cell proliferation coupled with constant inflammation and influx and propagation of monocytes and macrophages, convert fatty streaks to more advanced lesions with the formation of fibrous tissue enlarging and restructuring the lesion. The continued fibrosis yields a fibrous cap that surrounds a lipid-rich core that may be calcified (279,282) as a so-called advanced, complicated lesion. At some point the artery can no longer counterbalance by dilation (a phenomenon termed 'remodelling'). The lesion may then protrude into the lumen and hamper blood flow.(279)

Potential therapies that beneficially influence endothelial function or reverse coronary endothelial dysfunction are consequently of great interest for arteriosclerotic disease progression intervention.(246,334) In addition to their antioxidative,(27,200) anti-inflammatory and anti-aggregator effects, flavonoids appear to improve endothelial dysfunction through favourable effects on endothelial function.(94) Both animal studies with isolated flavonoids (94,255) and clinical feeding trials with foods and food extracts rich in flavonoids reveal improvement in endothelial-dependent vasodilation.(246,255) In particular, ingestion of flavanol-rich dietary items has been found to improve flow-mediated dilation (FMD) (20) on measuring the diameter of the brachial artery vessel in response to stimulated increased blood flow.(267,333)

These flavanol-rich dietary items include beverages of grape products,(246) including wine,(239) and cocoa,(20) as well as black tea.(149,267,299,323) For instance, both acute and short-term black tea consumption (as before and two hours after 450 mL intake and 900 mL intake per day for 30 days, respectively) improved FMD in the brachial artery of subjects with angiographically proven CHD.(299) Similarly in healthy young adults, endothelium-dependent FMD of the brachial artery and thus endothelial function improved following chronic consumption of a flavanol-rich dark chocolate for two weeks and was associated with increased plasma EC concentrations despite no changes in the lipid profiles and oxidative stress measures.(213) In their systematic review of all randomized controlled trials that met their inclusion criteria related to the various flavonoid subclasses and flavonoid-rich dietary sources on FMD as CVD risk factor, Hooper et al. (251) determined that the daily intake of 50 g dark chocolate increases FMD by 4% acutely and by 1.4% chronically, but indicated that such an addition to the diet may negatively affect body weight status and overall diet quality, counteracting the reported CVD benefits.

Endothelial dysfunction is accepted as an initiating early incident in the development of arteriosclerosis and related to reduced availability of the endothelial-derived vasodilator NO.(219). The improvement in endothelial-dependent vasodilation demonstrated by flavonoids could be mediated by enhanced availability of NO in the vascular wall,(20) which is traceable to either increased endothelial NO production and availability,(255) possibly through activation of endothelial NO synthase,(120,213) and/or increased protection of NO against oxidation.(94) It is possible, for example that the increase in plasma EC or catechin levels following flavanol intake stimulate the release of vasoactive substances like NO from the endothelium.(172) EC or catechin can act as selective inhibitors of NO related oxidation and nitration reactions that generate RNS.(219) A probable explanation is that even in nanomolar concentrations obtainable *in vivo* flavonoids can function as effective scavengers of peroxynitrite that mediates oxidative damage, thereby protecting the cofactor tetrahydrobiopterin (BH4), which is required for NO synthase activity, and promoting adequate endothelial formation of NO.(255) Flavonoids therefore influence endothelial function not only as antioxidants, but as modulatory signalling molecules,(281) as flavonoids are believed to modulate FMD via effects on the acute response cell signalling pathways that increase NO formation.(251) Changes in gene expression and in protein patterns are involved that result in reduced arginase activity, which leads to an increased substrate supply for NO synthase.(281) Collectively all these findings suggest that NO mediated endothelial changes can be influenced by ample intakes of flavonoids,(255) and in particular flavanols,(172) possibly through activation of endothelial NO synthase (213) and elevation of NO resulting in vasorelaxation.(281)

3.3.8   Inhibition of Angiogenesis

The angiogenic process is the development of new blood vessels from ones already in existence,(35,277) which can occur in the vascular bed.(277) In adulthood almost all normal tissue lacks angiogenesis. However, endothelial cells maintain the capability to divide in reaction to a physiological stimulus like hypoxia of the arteries.(35) As a compensatory reaction, chronic ischaemia may stimulate the development of new blood vessels.(22) This development of new blood vessels results in increased growth of the arteriosclerotic plaque (140) and accelerates the formation of advanced plaque from arteriosclerosis lesions. These new blood vessels deliver oxygen and nutrients (140,277) to the compromised vascular bed (22), which results in increased growth of the arteriosclerotic plaque.(140)

In vitro studies have indicated that polyphenol compounds found in red wine and green tea inhibits a number of major steps in the angiogenic process.(140,277) Although polyphenols from green tea and red wine show strong angiogenic potential in numerous *in vitro* experiments, few studies have determined their anti-angiogenic potential *in vivo*. Considering extrapolation, the concentration of EGCG, which showed anti-angiogenic ability in a mouse corneal model, is expected to be obtained in blood after moderate ingestion of red wine and green tea. The ability of red wine and green tea polyphenols to suppress angiogenesis contributes in explaining their beneficial effect on CHD following chronic ingestion of moderate amounts of these beverages.(277)

Plant polyphenols apparently interfere in angiogenesis through the regulation of a number of signalling pathways.(35) Major steps in the angiogenic process include proliferation and migration of endothelial cells and VSMCs and the release of two key pro-angiogenic factors which control the angiogenic process, matrix MMPs and vascular endothelial growth factor (VEGF). MMPs are expressed in abundance in arteriosclerotic lesions and play a major role in the degradation of the extracellular matrices thereby promoting migration of endothelial cells and VSMCs. VEGF strongly stimulates endothelial cell migration and proliferation, and therefore play an important role in the new blood vessel development.(277) The molecular mechanism of the *in vivo* anti-angiogenic potential of polyphenolic compounds still have to be elucidated, but may be attributed to their ability to suppress major steps in the angiogenic process like the proliferation and migration of endothelial cells and VSMCs and the expression of VEGF and activation of MMP-2.(35,140,277) Red wine and green tea-derived polyphenol compounds suppress the expression of MMP-2 and the transformation of pro-MMP-2 into active MMP-2 by directly suppressing membrane type 1 MMP (MT1-MMP) activity, thus averting VSMC invasion.(277) The anti-angiogenic effects of flavonoids include direct growth inhibition of the capillary vessel endothelial cells through prevention of the phosphorylation of mitogen activity protein kinase (MAPK).(140)

3.3.9 Attenuating Postprandial Oxidative Stress

In Western societies, a large part of the day is spent in the postprandial state.(281) Postprandial stress, as a subform of nutritional oxidative stress, is a consequence of prolonged postprandial hyperlipidaemia and hypertriglyceridemia,(281) which produce free radicals.(75) Nutritional oxidative stress describes an imbalance between the pro-oxidant load and the antioxidant defence as a result of oxidative overload or inadequate antioxidant supply or availability favouring pro-oxidant reactions. Lipid hydroperoxides present in the dietary intake add to the pro-oxidant load.(281) A large hydroperoxide burden during the absorption of fats may overwhelm GSH pools in some enterocytes, and the capacity of GSH peroxidase may become fleetingly inadequate. This may result in cell injury and inflammatory responses to injury that indirectly support oxidation in target tissues, most notably the gut tissues. Dietary unsaturated fatty acids are incorporated into LDL, and oxidized LDL in addition contributes to the pro-oxidant load.(335)

A mixture of antioxidant compounds seems to be necessary to offer protection against the oxidative effects of postprandial fats and sugars. Postprandial oxidative stress is reduced when dietary antioxidants are provided together with a meal rich in oxidized or oxidizable lipids. The postprandial oxidative load can in all probability be altered by dietary antioxidative components including flavonoids. In hyperlipidemic subjects, epithelium-dependent vasodilation is reduced in the postprandial state, which amplifies postprandial oxidative stress as an important factor in controlling cardiovascular risk. This makes ingestion of dietary flavonoids, such as from wine, cocoa or tea, all the more significant, as flavonoids improves endothelial dysfunction and lowers the susceptibility of LDL lipids to oxidation.(335) Drinking rooibos after consumption of a standardized fat meal notably reduced postprandial oxidative stress in normolipidemic young adults.(336)

3.4 CONCLUDING COMMENTS

CHD is a major chronic degenerative disease of lifestyle in many countries, also SA, which supports preventive efforts, including supplementary dietary efforts, to reduce its incidence. Multiple mechanisms through which flavonoids may contribute as being cardioprotective have emerged. These numerous mechanisms go beyond the well-known property of flavonoids as having antioxidant activity and possibly impacting the blood lipid profile, but signify flavonoids to interact at cellular and molecular level eliciting such biological effects as being anti-inflammatory and anti-thromobogenic and promoting vascular epithelium function. Scholars, who study cardioprotective effects of flavonoids, should take cognisance of the findings of human studies as the *in vivo* effects found result from the flavonoid metabolites and phenolic acids formed on dietary

flavonoid intake, while the effects found under experimental conditions using flavonoid aglycones or glycosides may not fully represent the effects which would occur in the human body.

Although no guideline on dietary intake of flavonoids has been proposed, it is evident that individuals should consume some flavonoids daily or at least incorporate flavonoid-rich items in their dietary intake that has emerged as being cardioprotective through epidemiological, acute or chronic human feeding trials. This makes it important to consider assessing the flavonoid intake of especially those at risk or who has been diagnosed with CHD. A FFQ seems quite suited for this purpose, as the questionnaire food list can be manipulated to represent the dietary sources of flavonoids with some plant foods seemingly being the core dietary flavonoid providers.

CHAPTER 4

DIETARY FLAVONOID INTAKE AND
FOOD FREQUENCY QUESTIONNAIRE AS ASSESSMENT TOOL

4.1 PREAMBLE

Epidemiological studies usually convey the association between the intake of beneficial dietary components and disease risk reduction,(92) as was demonstrated in the previous chapter between dietary flavonoids and heart health protection. Such studies do not only require knowledge and understanding of the bioavailability of the dietary component in support of its reported health protective effects,(119) but also a database of its content in foods and beverages to assess its intake.(92) Assessing flavonoid consumption is a crucial step towards documenting its protective effects against chronic disease risk.(105) However, while available, data on flavonoid intakes are underprovided.(74,153) According to Johannot and Somerset (89) publications on flavonoid intake assessments are limited and the dietary intake assessment methods used in doing so differ greatly. Flavonoid intake assessments are furthermore merely indications of the flavonoid intake (137) and possibly questionable,(147) as it is difficult to plausibly determine total flavonoid intake.(47,107)

Despite flavonoids being important dietary sources of antioxidants,(105) food composition data with respect to flavonoids are incomplete.(92,105,137)   This hampers flavonoid intake assessments.(105)   Not only do incomplete data with respect to the flavonoid food contents exist,(47,107) because of the numerous factors that could cause a large variation in the content (87,137) have not been taken into account,(137) the food composition data are not comprehensive.(105,137) No data were, for instance, available in the USDA flavonoid database for many of the fruits included in the Mediterranean diet. This may in particular underestimate the anthocyanidin content of the traditional Greek plant-based weekly menu as calculated by Vasilopoulou et al.(137) Besides the fact that the food composition information on flavonoids is inadequate, their determination includes various subclasses and individual flavonoids, and the food analytical methods utilized to determine their content vary.(87)

The often contradictory content found in plant foods may be linked to the lack of agreement on the appropriate methods to analyze the different types of polyphenols.(47) A few countries have compiled databases of the flavonoid content of foods.(92) As these databases become more expanded and fully developed and comprehensive in terms of the flavonoid subclasses, the completeness and accuracy of the consumption data for flavonoids will improve. This will advance the precision on the prediction of relationships between flavonoid consumption and the

maintenance of health and the incidence of chronic disease along with the role of certain flavonoid-rich dietary items, such as tea, in this relation.(92)

4.2 DIETARY FLAVONOID INTAKES

Although a number of researchers have calculated the flavonoid intakes of subject groups in several countries,(92) there is still only limited information available on the quantities consumed daily throughout the world.(100,153) This is attributed to the use of partial food composition data and the consideration of only a few flavonoid subclasses as representation of the total intake.(92) The lack of comprehensive food composition data is due to the cost and/or the difficult and time consuming analytical technology involved,(92) the list of compounds to quantify and the large variation in flavonoid food content that hampers a representative sampling approach.(105)

Earlier studies assessed the total flavonoid intake without taking all the flavonoid subclasses into consideration.(137) The total flavonoid intake is still poorly documented as mostly only three flavonols (quercetin, kaempferol and myricetin) and two flavones (apigenin and luteolin) were used to determine flavonoid intake,(10,111,120) which is not quite representative and accurate. Excluding the other flavonoid subclasses can lead to underestimation of the dietary flavonoid intake.(10) Total flavonoid intakes may therefore be assumed greater than that reported in these studies.(9) These intake estimates exclude the catechins (flavanols) and/or the proanthocyanidins. Consumption of these compounds may be of great quantitative importance because they are present in high amounts in tea, wine, cocoa and cocoa products, which are regularly consumed in Western countries.(120) The different flavonoids and foods considered in the flavonoid intake assessments to date, greatly impact the estimated intake variations found among the studies.(105) Improved flavonoid intake estimations can assist investigations into this dietary antioxidant as contributor to disease prevention.(153)

4.2.1   Total Intakes based on Summed Flavonoid Subclass Combinations

Several studies assessed flavonoid intake by using different groupings of flavonoids thought to represent total flavonoid consumption.(261) Some studies that only used the three flavonols (quercetin, kaempferol and myricetin) and two flavones (apigenin and luteolin) to determine the flavonoid intake,(10,11,111,120) are indicated in Addendum A, Table 4.1, which is based on national representative samples and non-representative subsamples. Considering these two subclasses, the consumption is lowest for Finland *[approximate mean intake of 3 mg/day among adults in the Finnish Mobile Clinic Health Examination Survey (11) and 10 mg/day among male smokers in the Alpha-Tocopherol-Beta-Carotene Cancer Prevention Study (256)]*, higher in France *[approximately 14 mg/day among an elderly subject group and 18 mg/day if more detailed information on their vegetable intake is considered (190)]* and somewhat higher *[approximately 20*

to 27 mg/day] in the US among US health professionals (153,232,254) and The Netherlands (from the Dutch National Food Consumption Survey and among male subjects from the Zutphen Elderly Study).(45,111,258)   The flavonoid intake from the five individual flavonoids in the US Iowa Women's Health Study of approximately 14 mg/day,(250) is comparable with the estimate for the French (as 14 to 18 mg/day).(190)   The intake of 17 mg/day found in a Japanese study of adult women (309) is within the range of the flavonol and flavone intakes of the US studies of 14 mg/day (250) to 20 to 25 mg/day.(153,232,254)

Data from the Danish Household Consumption Survey was used as a surrogate information source to assess the mean daily intakes of flavonoids in Denmark, which was found to be in the order of 16 to 32 mg/day for the sum of one flavone (luteolin) and two flavonols (quercetin and kaempferol).(337)   The low intake in Finland of about 3 mg/day is supported in the Seven Countries Study by Hertog et al.,(253) while the higher intake in the other countries is supported by the mean intake of approximately 28 mg/day across the 14 different countries in the study of De Vries et al.(74)   The main reason proposed for the low intake found in Finland is the low consumption of tea and wine.(256,262)   At the time of these studies, the Finns mainly consumed coffee as beverage and they preferred beer and liquors to wine.(262)

The mean flavonol intake of the male participants in the Caerphilly study, South Wales was about 26 mg/day.(165)   The two studies by Hertog et al. (45,111) reported a similar estimated intake for different Dutch population subgroups of 23 mg/day (Dutch National Food Consumption Survey) and 26 mg/day (males in the Zutphen Elderly Study) for the flavonol and flavone subclasses.   The similarity in intake may be due to the high contribution of flavonols to the combined intake of flavonols and flavones.   In the Zutphen Elderly Study,(45) the Finnish Mobile Clinic Health Examination Survey (11) and among US health professionals,(153,232) the flavonol contribution to the intake of these two subclasses was above 95% with the flavones, apigenin and luteolin, making only minor contributions to the intake.

The similarity of the flavone and flavonol intakes for these different populations, namely France, The Netherlands, US, Japan and Denmark, is remarkable, considering their different cultural habits.(92)   However, flavonol consumption have been found to differ widely between countries (253) with some of the lowest levels being reported for Northern European populations, such as Finland,(11,253) while populations from other European countries (The Netherlands),(45,111,258) and the US (153,232,250,254,259) have among the higher reported intakes.(249)   Within countries, individual differences exist as Hirvonen et al. (256) reported more than a fourfold difference in the median flavonol and flavone intakes between the lowest and the highest intake quintiles (3.9 mg/day versus 18.8 mg/day) of Finnish male smokers, while the intake range of these

two subclasses was nil to 41.4 mg/day in the Finnish Mobile Clinic Health Examination Survey (11) and 4.3 to 32.2 mg/day in the Iowa Women's Healthy Study.(250)

The mean intake of the flavanones subclass (flavonoids common to citrus fruits) was, in addition to the flavonol and flavone subclasses, calculated for Denmark and Finland with similar intakes for both these countries for all three of these flavonoid subclasses,(92) of 23 to 46 mg/day for Denmark obtained from the Danish Household Consumption Survey (337) and approximately 24 mg/day for the Finnish Mobile Clinic Health Examination Survey.(262) In a plasma biomarker study on a group of German students their flavonol (only quercetin and kaempferol) and flavanone (naringenin and hesperetin) intake was estimated at about 52 mg/day.(338)

Catechin intake was included in some intake estimations in addition to the flavonol and flavone intake estimates. A study by Arts et al. (174) reported 50 mg/day catechin intakes for adults in The Netherlands. This implies that the total flavonoid intake of the Dutch population may be no less than 75 mg/day, considering the flavonoid intakes from the flavonol and flavone subclasses along with the catechins.(105) A mean flavonol, flavone and catechin consumption of 58 mg/day was determined for the subjects in a national study (MORGEN study) in The Netherlands.(339) The median interquartile intakes for flavonols, flavones and catechins determined in a case-control study in London was 33.2 mg/day, 0.25 mg/day and 81.2 mg/day respectively.(340) The catechins from tea was not considered in the US study among health professionals by Sampson et al.,(153) although their FFQ included tea, as the flavonoid intake was determined based only on the standard three flavonols and two flavones, and in the Japanese study of Arai et al. (309) the flavonoid intake was determined based on one flavone, four flavonols and two isoflavones only.(105) Because consumption data for tea and wines was not available, the intake of catechins was not estimated in the Finnish Mobile Clinic Health Examination Survey.(262)

The consumption of isoflavones (flavonoids common to soy) was calculated in addition to the flavonol and flavone subclasses for a small group of Japanese women providing for a total intake of 63 mg/day of which most of the total intake was provided by isoflavones (approximately 47 mg/day).(309) In the Seven Countries Study, Hertog et al. (253) determined that the average consumption of flavonoids based on the flavonol and flavone subclasses (five individual flavonoids) was about 68 mg/day in Japan, which is similar to the intake of 63 mg/day found in the study of Arai et al.,(309) where the intake of isoflavones in addition to these two standard subclasses were considered. In this study, the main contributory dietary items to the flavonoid intake were vegetables (onions), followed by fruits and green tea. The average consumption of green tea was 206 mL(2 cups)/day. In contrast, tea was the major contributory source to the flavonoid intake in the Seven Countries Study undertaken in the western part of Japan, in which more than 80% of the

flavonoid consumption was provided by about seven cups (700 mL) of green tea per day. The intake of flavonoids in the Japanese diet is predicted to range from 10 to 90 mg/day.(309)

4.2.2   Total Intakes based on all Major Flavonoid Subclasses

Table 4.2 in Addendum A summarizes the total flavonoid intake estimates in relation to the five major flavonoid subclasses in a number of countries (based on national representative samples and non-representative subsamples) and for some, an added intake estimate for isoflavones as the sixth major flavonoid subclass. The mean daily total flavonoid intake estimate for an Australian consumer obtained from a National Nutrition Survey is 454 mg/day considering the intake of the five major subclasses (26 individual flavonoids, excluding isoflavones) (89) compared to a low intake of 129 mg/day for a group of Finnish men in Eastern Finland based on the same flavonoids.(169) An intake of approximately 128 mg/day was found for a small group of young Australian women on excluding the analysis of the subclass anthocyanidins and in addition the isoflavones (based on 15 individual flavonoids).(164) The mean intake determined on the intake of the six major subclasses was 118.6 mg/day for Greek subjects based on a weekly menu calculation representative of the traditional Greek plant-based diet,(137) and the median and mean intakes for Italy obtained from two case-control studies of 121.3 mg/day (341) and 134 mg/day, (263) respectively.

Data from the Danish Household Consumption Survey on specific fruits, vegetables, herbs, beverages and dairy products, which contain the largest amounts of specific non-nutritive components used as a surrogate information source, determined the average daily intake of flavonoids in Denmark to be in the order of 100 mg/day for flavonoids from eight major aglycones representing the flavonols (quercetin and kaempferol), flavones (luteolin), flavanones (hesperetin, naringenin and tangeretin), catechins (epigallocatechin and EGC) and anthocyanins (cyanidin).(337) The mean daily total intake for the US population obtained from the National Health and Nutrition Examination Survey (NHANES) (1999-2002) based on the six major subclasses, averaged 189.7 mg/day.(105,342)

The quintile intakes from the five major flavonoid subclasses (excluding isoflavones) in Greece, obtained from a case-control study among women with and without breast cancer based on median intakes, ranged from 0.3 mg/day for flavones as the lowest (first) median quintile intake to 81.4 mg/day for anthocyanidins as the highest (fifth) median quintile intake.(136) In a case-control study of CHD in Greece, the range was from 69.4 mg/day for the lowest quintile to 235.8 mg/day for the highest quintile of total flavonoid intakes for cases and controls.(261) The median quintile intakes in the Iowa Women's Health Study in the US ranged from 95.8 mg/day in the lowest to

603.3 mg/day in the highest quintile. The flavonoid contributions of onions was not considered in this study, as onions are not included in the food list of the FFQ used.(248)

4.2.3 Varying Intakes and the Prime Food Sources

Mean daily total flavonoid intakes, although limited, as can be deduced from the above studies, indicate differences in consumption among populations (92). This is suggested to be due in part to the different geographical regions (98) and resultant differences in cultures, dietary intakes and food preferences among the country specific populations.(92,98) Based on the consumption of, for instance, only selected subclasses of flavonoids (mostly the flavonol and flavone subclasses), flavonoid intakes ranged across populations. For example, in the Seven Countries Study it ranged from about 20 mg/day (for the US, Denmark and Finland) to more than 70 mg/day (The Netherlands).(92) De Vries et al.,(74) on assessing the intake of only 17 subjects in an across-countries determination, found the lowest consumption (1-9 mg/day) from a South American diet and the highest consumption (75-81 mg/day) from a Scandinavian diet with the average across the 14 countries of 27.6 mg/day for these two standard flavonoid subclasses represented by five flavonoids. Using estimates of flavonoid consumption by adults in different countries, Erdman et al. (75) indicated that the flavonol intake varies from 13 to 64 mg/day, that the flavone intake is low (1-2 mg/day) and that the flavanone intake is in the range of 21 to 29 mg/day (calculated from two German cohorts). Large variations were found for reported intakes of flavanols (catechins), ranging from 4 to 120 mg/day, and an average of 58 mg/day proanthocyanidin for an average American consumer. Very little data for anthocyanin and proanthocyanidin intakes have been published to date.(75)

Great differences in flavonoid intake can occur between individuals, depending on their dietary habits.(124) Hollman and Katan (107) estimated that humans ingest a daily intake of flavonoids that may reach a few hundred mg/day expressed as aglycones. According to Manach et al.,(100) crude estimates of an average intake in Western populations appear to be about 65 to 250 mg/day, while Vaya et al. (319) indicated an intake estimate of 23 to 1000 mg/day. Since it is supposed that the intake of flavonoids may reach 800 mg/day (128) to 1000 mg/day,(217) flavonoids represent an important source of dietary antioxidants.(128)

From the above it appears that dietary habits and food preferences dictate the food sources of flavonoids,(190,342) along with the flavonoid subclasses and amounts of flavonoids that will be consumed.(8,92) Generally the food sources of flavonoids vary somewhat between the different populations.(190,342) Geographical variation accounts for differences in the type of flavonoids ingested, from isoflavonoid-rich food (soybean) in Asia, to catechin derivatives (tea) in the UK.(139) Whereas the isoflavone subclass, for instance, makes a major contribution to the

flavonoid consumption in Japan,(309) it only makes some contribution to the flavonoid consumption in a few other countries, such as in Italy from pulses,(341) but a minimal contribution (about 1 mg/day and less) in other countries, namely Greece (137,261) and the US.(105,342) Variations in the dietary sources can arise between regions within the same country.(9)

When considering total flavonoid intake as the summation of all the flavonoid subclasses, the flavanol (catechin) subclass dominates the intake due to tea (mainly black tea) being the important contributory dietary source across the diverse studies conducted in Australia,(89,164) Finland (169) and the US.(105,248,342) The next main contributory dietary sources after tea as identified in national studies, are oranges and other citrus fruit for Australia (89) and citrus fruit juice for the US.(105,342) While flavanols are the main contributory subclass to the total flavonoid intake in case-control studies conducted in Italy, its intake is closely followed by that of the subclass flavanones (263,341) with the main dietary contributors being red wine, grapes, oranges and other citrus fruits.(341) In the diverse studies conducted in Greece, flavanones are the main subclass contributor to the total flavonoid intake, which is followed by the flavanols (137,261) with the major contributory items being oranges followed by (red) wine.(137) Quercetin is the individual flavonoid contributing largely to the flavonoid intake when only the intake of the subclasses flavonols and flavones were considered across national studies conducted in The Netherlands (45,111,258) and the US (153,232,254) with the major contributory dietary source again being tea (mainly black tea), with onions and apples also pertinent contributors.

Tea is the most prominent provider of flavonoids in the US diet.(342) Determining the dietary flavonoid intake of adults included in NHANES, 1999-2002 Song and Chun (342) found that only 21.3% of US adults indicated drinking tea daily and that the daily total flavonoid intake of these tea drinkers was over 20 times that of the tea non-consumers (697.9 versus 32.6 mg/day). The per capita flavonoid intake from tea was 157 mg/day with the estimated mean daily total flavonoid intake at 189.7 mg/day (including 0.6% isoflavones). Flavanol consumption represented the most highly consumed subclass of the Dutch population investigated by Beecher (92) due to the popularity of tea as beverage.

Besides for the pertinent contributions of onions (11,45,111,137,153,165,232,254,262,338,339) and possibly broccoli (153,232,250,254) to the flavonoid intake, the contributions of vegetable sources are notably lower than that of fruits.(11,153,190,232) Onion, although not consumed in large quantities, is an important dietary quercetin source due to its high content; while tea and wine, which contain relatively low amounts of quercetin, are ingested in large quantities in certain countries.(108)

Countries differ largely in the consumption of tea, red wine, apples and onions, the major contributors to flavonoid intake, and consequently a wide range in the intake of flavonoids can be expected in cross-cultural studies.(253) Even the main dietary sources of only flavonols and flavones varied among the cohorts of the Seven Countries Study, with tea as the major dietary source in the Japanese and Dutch cohorts, red wine in Italy and fruits and vegetables, mainly apples and onions, the major sources in the US, Finland, Greece and the former Yugoslavia.(253)

4.3   DIETARY FLAVONOID INTAKE ASSESSMENT METHODS

A wide range of methods are available to assess dietary intake of individuals (or groups of people).(14,52,343)   Each of these methods has strengths (advantages) and weaknesses (disadvantages) (14,52,343,344) providing for no single perfect method.(344) As these methods vary greatly,(86) they may be appropriate for use in different situations and for different purposes.(52) Though not many consumption studies on flavonoid intake has been published, the dietary intake methods used to determine the intake varied substantially.(89) As can be deduced from Tables 4.1 and 4.2 in Addendum A, the whole spectrum of dietary intake methodologies, such as dietary histories, 24-hour recalls, dietary records and FFQs (14) were used to assess flavonoid intakes.

Accurate assessment of dietary intake based on self-report by free-living subjects, poses several challenges (43,345) and is a complex (52,346) and difficult (347) task. Therefore the specific dietary assessment methods to be used in a particular setting need to be chosen carefully,(346,348) while acknowledging the limitations of each method.(14,346) The selection of an appropriate dietary intake assessment method is generally directed by various considerations. These could include the aims/objectives of the study (91,344,346-348); the foods and/or nutrients of interest (52,344) and the level of specificity needed for describing the foods (344); if current or past diet is the concern; the habituality of the dietary intake (52); the need for individual versus group data (344); and the level of data accuracy required.(52,347,349,350)

Data accuracy refers to the need for absolute values versus relative intake estimations to differentiate the group into high, medium or low consumers,(52,344) which in turn, is guided by the goals of the study (349) that will dictate the survey period length and the amount of detail that subjects need to provide.(52) Further considerations are the time frame of interest (344); the study population characteristics (age, gender, education/literacy, motivation and socio-cultural diversity),(91,344,348,350) as it will impact response rates (52); pre-conditional concerns, for instance, available resources,(344,346,350) like personnel (91,347) (availability of skilled interviewers and skill for coding of the dietary items consumed),(344) finance,(52,91,347) time (91) and even an available accurate, preferably complete food composition nutrient database.(344)

All conventional dietary intake assessment methods involve quantitative methods (52) and rely on the subjects reporting their food and beverage intake information themselves.(343) The need to determine the long-term effects of diet on the development of chronic diseases resulted in the development of dietary assessment methods that measure past and present intake of foods and nutrients (72) and involve recall and record methods.(91) Long-term dietary assessment methods obtain information on usual dietary consumption over the previous months or years and involve recall methods that includes the dietary history interview and FFQ. Short-term dietary assessment methods obtain information on current consumption, which include recalling the consumption of the previous day (using a 24-hour recall) or recording the dietary intake over one or more days (using food or dietary records).(344)

The methods utilized in dietary assessment methodology are therefore either retrospective (24-hour recall, dietary history interview and FFQ) or prospective (like weighed and estimated dietary records).(52) These recall methods (based on interview for the 24-hour recall or dietary history, or on self-administration of the FFQ) and record methods (weighed or estimated dietary records) (91) comprise the generally used dietary assessment methods in surveys and studies of humans.(14,44,86,351) These methods, as mentioned, vary dramatically (86) with each method having its own strengths and weaknesses, among others the ease of administration,(343) subject involvement and cost,(86) which all affect the intended use.(343)

The dietary history, 24-hour recall and dietary record as assessment methods are summarized in Table 4.3 (Addendum A) together with their advantages and disadvantages. The dietary history involves an interview that consists of several components often including a 24-hour recall, a cross-check with a frequency food list (14,44) and sometimes dietary records obtained from the subject.(44) This method attempts to assess usual dietary intake and meal pattern during a defined longer time period.(14,44) The time period may be the present or any past time period. The dietary history method is not appropriate to assess acute intake or to calculate extremes of intake, as it serves to determine regular dietary intake.(44) The dietary history interview normally requires skilled personnel.(71) In the recall assessment method an interviewer probes the subject to identify and quantify each food and beverage consumed during the previous 24-hour period.(14,44) The interview is usually conducted in person or by telephone, but in some situations the subject completes the recall on paper or computer without the aid of an interviewer.(44) Recall interview methods, including the 24-hour recall, are generally time-consuming and costly to administer.(91)

In the dietary or food record or diary method, subjects are asked to record information on the foods and beverages consumed during a specified time period. Subjects are asked to record the

quantities consumed by either weighing and/or measuring the amounts consumed.(44) With 24-hour recalls and dietary records, a sufficient duration of days must be assessed to reflect a subject's usual intake (352) unless the goal is to assess a group mean intake.(86) Obtaining multiple 24-hour recalls or dietary records is generally not feasible in large studies (352) and requires considerable commitment of investigators and subjects.(91) Multiple 24-hour recall interviews may burden subjects contributing to low participation rates.(353) Multiple dietary records involve a high level of commitment from subjects and because of the demand placed on them, it is likely to reduce their compliance.(71,91) Dietary records require subject literacy.(14,352)

Although these assessment methods may produce rather accurate information regarding the dietary intake in the reference time frame, often a single day, investigators are usually interested in longer term intakes of specific nutrients or foods, which tend to differ in intake from day-to-day.(352) Multiple 24-hour recalls and dietary records are costly to administer and analyze in large study groups.(352) The dietary history, 24-hour recall and dietary record methods specifically require a considerable amount of time to be spent on data treatment and coding. Their use in obtaining the dietary intakes of large groups are therefore limited.(71,352) The FFQ as dietary assessment method is discussed below as it is the dietary intake assessment method relevant for assessing total flavonoid intake.

4.4   FOOD FREQUENCY QUESTIONNAIRE AS DIETARY ASSESSMENT METHOD

4.4.1   Description, Uses and Prospects

FFQs are capable of assessing the intake of nutrients and foods or food groups.(14,17,18,355) Although FFQs are quite often developed to assess intakes of foods and food groups, they are most often developed to assess intakes of nutrients.(17,18,356) This spectrum of assessment permits determining a broad range of dietary intakes of current as well as future interest.(355)

A FFQ is described as a questionnaire with a food list and a frequency response where subjects indicate how often each included item was consumed (14,357) during a specific time frame.(14) In a review undertaken of all the dietary studies conducted and published since 1980 in which the development, validation or use of a FFQ was described, Cade et al. (17,18) defined a FFQ as "any list of one or more foods with frequency of intake categories." The basic FFQ therefore typically consists of two components: a food item list and a frequency response section in which the subjects are asked to indicate the typical frequency of consumption of each item included in the list of foods.(14,72,350,357-364) Many FFQs instruct subjects to record the typical portion size consumed for each item (358,360,361,363) and some also ask a few additional general questions often related to cooking methods and additions to foods.(361)

The increasing popularity and widespread use of the FFQ in assessing dietary intake in nutrition research compared to other methods, at least in relation to multiple records or recalls, are attributed to a number of aspects.(86,365) To study large numbers of subjects, dietary assessment methods must be reasonably simple.(38,39) The average FFQ is both easy to administer and easy to complete (71,365-368) and is usually self-administered,(38,39,71,369) which does not require skilled professionals like trained interviewers.(39,370) FFQs require a low level of subject commitment (86) and impose less burden on subjects,(358) which is one of this dietary assessment method's key advantages (371,372) over the other dietary assessment methods.(372) Completing dietary records, for instance, require 30 to 60 minutes/day. Thus completing multiple daily assessments imposes a substantial burden on subjects.(53) FFQs are not only simple to administer, but also quickly completed compared to several other dietary assessment methods.(360) FFQs usually measure intakes of several nutrients (38,71) of both individual and group intakes (71,360) and can assess intake over an extended time period,(368) whether usual, (373) current or past intake.(360)

FFQs assess the typical diet quickly,(374) not only in its administration, but also in its data reviewing and analysis.(43) For subjects to complete dietary records requires at least two hours of training, record-keeping and professional review of the completed records.(43) FFQs are relatively easily coded and analyzed (365,375) and can be scanned for analysis,(43) thereby incurring lower data collection and processing costs.(365,369) The lower cost (39,71,86,367,368,373,376) is attributed to it being cheaper to produce (43) and administer.(44) Compared with other methods of dietary assessment, FFQs have a great advantage in cost,(372,376) in particular against multiple daily dietary records of individual dietary consumption.(372) FFQs are generally indicated to be relatively inexpensive.(38,39,360,373-375,377)

Due to its use convenience,(376,377) FFQs are particularly practical to apply to large numbers of subjects (371,375) making it a feasible and preferred method in large scale studies.(39,368) The FFQ is therefore a dietary tool commonly used in epidemiological studies (371,375) to assess group mean nutrient intakes.(366) Because of within-person variability of a subject's diet, an adequate number of dietary records or 24-hour recalls must be collected for assessing usual dietary intake. However, collection of multiple single dietary records or 24-hour recalls may be difficult and in some cases may not be feasible. FFQs can thus theoretically overcome the problems of high within-person variation in day-to-day dietary intake and such multiple single day assessments.(369) The use of FFQs is generally based on feasibility and logistics like the budget.(369,371)

FFQs have been developed in a variety of ways and used in a wide range of settings and types of dietary studies.(18) In order to study the relation between dietary factors and chronic disease, investigators should obtain the subject's habitual dietary exposure data (364,374,378) over a longer period of time (72,364) rather than during the shorter term.(364) Dietary histories or FFQs have been the methods of choice used in the great majority of reported epidemiological studies to obtain estimates of a subject's dietary intake in relation to the development of various diseases. (371) Self-administered FFQs have of late become the most commonly used assessment method to obtain dietary exposure data in epidemiological studies,(18,71,72,84,356,362,370,371,376, 377,379-383) whether for intake of foods and/or nutrients, (72,380) that is mostly due to its feasibility for large numbers of subjects.(72,368-371) For retrospective studies, a self-administered or interviewer-administered FFQ is probably the only feasible method for recall of dietary habit in the remote past.(369)

FFQs have been developed for and used in various epidemiological settings. To investigate relationships at a single point in time, like in cross-sectional studies of diet and disease incidence, brief questionnaires that measure specific dietary behaviours may be useful.(18) FFQs have been administered in various cross-sectional surveys to assess the means and standard deviations (SDs) of population dietary intakes.(384) A FFQ used in a cross-sectional survey with the aim to compare the intakes of different subgroups of the population should be validated, if possible, for each of the important subgroups within the cross-sectional study.(18) Because FFQs are developed to assess usual dietary intake, they are generally utilized in case-control and cohort studies to determine the relation between diet and disease.(384) In case-control studies, subjects without and with disease are compared relating to what they now report their previous or usual diet was by retrospectively recalling their past diet.(18,354,385) In cohort prospective studies, dietary intake is assessed in a population free of disease at baseline, which is then followed up, and the dietary intakes of subjects who later do or do not develop the disease are compared.(354,385) In cohort studies the FFQ food list must conceivably be comprehensive to recall the whole diet at the onset of the study.(18)

The FFQ has become a means for determining dietary adherence in clinical dietary trials.(351) Dietary modification is viewed as an important factor in the prevention, management and treatment of many chronic health conditions. Clinical trials need to assess the outcome of dietary modification and/or supplementation. A self-administered FFQ is a practical and cost-effective alternative to more accurate dietary assessment methods, for example interviewer-based dietary recalls, dietary records or extensive dietary histories. These methods can be prohibitive in intervention trials due to their cost and high subject burden that contribute to a high drop-out rate.(386) In general, the more accurate the method the higher its cost, the greater the extent of

subject co-operation necessary and the lower the participation rate.(52) FFQs have been used in dietary intervention trials to assess change in dietary intake.(18,41,71,384) In intervention studies, monitoring dietary change is an important consideration.(41) A FFQ used to track changes in diet as a response to some form of intervention must be specific enough to detect changes in the diet and sensitive enough to detect sometimes quite subtle dietary changes.(18)

Consequently, Hebert et al. (86) cautioned that FFQs should not be used to determine dietary intake under conditions for which they were not developed or validated, for instance to assess change due to a dietary intervention. It is crucial to consider the intention of the questionnaire before it is developed to make certain that it is sensitive enough to assess differences in the intake of particularly those nutrients of interest.(17) Dietary records should be collected in dietary intervention studies where assessment of dietary change is required as a result of the intervention.(41) Dietary intakes assessed from these records could then be compared with the FFQ to evaluate the relative validity of the dietary change.(387) Validity and reproducibility analysis of FFQs is quite practical in the situation of an intervention trial.(41) FFQs can also be utilized to screen for subgroups of the population with high or low intake of specific nutrients for inclusion in dietary intervention trials.(383)

FFQs have become a practical measure in diet and health education, like counseling (71,383) and clinical (18,71) situations, to screen for subjects with low or high intake of specific nutrients or to assess dietary intake and monitor adherence to dietary recommendations, for example lowering of dietary lipid intake.(388) In clinical settings, shorter questionnaires that include foods/food groups that discriminate between low and high intakes would be more useful due to it being suitable for administration by staff without specialized nutrition training. Issues of determining absolute intakes may not be as important when used as a general screening tool in clinical settings to discriminate between high and low consumers of certain foods or nutrients, but for identifying those 'at risk', the FFQ should be specific and sensitive.(18)

Cade et al.,(17) in their review of the design, validation and utilization of FFQs, found that FFQs are widely used to determine group or population levels of intakes and for ranking individual dietary intakes. They found that a great number were intended to determine absolute intakes to provide quantitative information on individual intakes. The FFQ has hence become a frequent means of assessing absolute dietary intakes in research.(351,389) Although FFQs are not regarded as suitable for assessing actual nutrient intake, they have been utilized for categorizing subjects correctly according to intake and for distinguishing subjects at the extremes of intake.(390) In other words, although the absolute intake is an estimate,(72) there is ample evidence that the FFQ can rank individuals by levels of intake.(72,383,384) Semi-quantitative FFQs are adequate for

categorizing individuals according to intake and to distinguish individuals with low intakes from those with high intakes of certain foods or nutrients.(18) The FFQ is able to rank the population into levels of exposure (tertiles, quintiles or quartiles), which are used in the calculation of RR for the development of the disease in question.(72)

However, most FFQs employed in epidemiological studies were not developed to assess phytochemical intake.(47) A number of national studies used semi-quantitative FFQs assessed for validity and reproducibility of nutrient and/or food group intakes to assess flavonoid intakes. Although these FFQs were assessed to ascertain such intakes, they were not evaluated for their capability to determine flavonoid consumption.(249) In four of the seven studies included in the dietary flavonol consumption and CHD mortality meta-analysis of prospective cohort studies by Huxley and Neil,(249) the dietary flavonol intake was retrospectively assessed at baseline using internally validated FFQs against seven-day weighed dietary records in sub-samples of the cohorts. In the other three studies, dietary intake was obtained from an interview with a trained dietician/nutritionist.

It is possible that the questionnaires utilized in each of these studies were not originally developed to assess flavonol consumption as the prospective importance of flavonols to the diet only emerged in the early 1990s.(249) However, these researchers appraised the use of the FFQ in determining flavonoid intakes by correlating the intakes of a few foods rich in flavonoids (apples, broccoli, orange juice, tea and red wine) between the FFQ and the reference method (dietary records).(74,153,165,232,248,254,339) Johannot and Somerset (89) indicated that due to the various dietary flavonoid sources and the wide range of flavonoids found in foods, dietary records provide more accurate flavonoid consumption data than FFQs, since the latter mostly have not been validated for flavonoid consumption and individual dietary flavonoid sources can be excluded from the FFQ food list.

Yochum et al. (250) assessed the intake of five major flavonoids (quercetin, kaempferol, myricetin, apigenin and luteolin) using a 127-item semi-quantitative FFQ, but onions and berries, two potentially important sources of flavonoids, were not included in the FFQ food list. Mink et al. (248) used the FFQ of the 1984 Nurses' Health Study in the Iowa Women's Health Study to determine total flavonoid intake. This FFQ includes information of individual dietary items with high flavonoid content (tea, red wine, chocolate, blueberries and strawberries), but onions are not included. The less detailed questionnaire used by Commenges et al. (190) to determine the flavonol and flavone intakes in the Paquid Study, did not record the intake of a number of vegetables rich in flavonoid contents including that of onions.

Johannot and Somerset (89) indicated that dietary assessment tools validated specifically for flavonoid intakes would assist improved international comparisons of flavonoid intake. The lack of association found between flavonoid intake and health benefits may be real, but an explanation may be the lack of an adequate measure to assess the intake.(47) Hertog et al. (165) argued that although the FFQ used in their study was not originally developed to assess flavonol intake, comparisons with seven-day weighed dietary records and its reproducibility suggest that the FFQ determines flavonol intake with satisfactory precision. Hirvonen et al. (256) found satisfactory correlations in the comparisons between the FFQ used in the Alpha-Tocopherol-Beta-Carotone Cancer Prevention Study and the dietary records completed, and its reproducibility in a sub-sample of men in determining flavonol and flavone intakes. Use of FFQs to estimate individual flavonoid intakes seems justified (165,391) and may be judged quite suitable for the exclusive assessment of dietary flavonoid intake.

It is a rarity to find FFQs developed to assess flavonoid in the literature. De Vries et al. (74) used a 74-item FFQ specifically developed for asking about the habitual consumption of flavonoid-rich foods in determining the intake of flavonols and flavones of 17 individuals from 14 countries, but indicated that the FFQ lacked data of a few flavonoid-containing foods, among others citrus fruits. The researchers were not too concerned, as citrus fruits were not expected to contribute significantly to the intake of these flavonoid subclasses. One study was established where a semi-quantitative FFQ was developed and used to determine the daily intake of flavonoids and evaluated against four non-consecutive day dietary records as the reference method or golden standard for validity, and repeated after two weeks for reproducibility in a group of 45 Flemish dieticians (15% participation rate). This FFQ was specifically developed to obtain data on the intake of total flavonoids and subclasses of flavonoids during the previous month. A list of 86 flavonoid-containing foods was incorporated in the FFQ with the consumption frequencies the standard nine-frequency categories ranging from 'almost never' to 'more than six a day'.(84)

A further study was conducted where 49 volunteers completed a modified 159-item FFQ containing six standard response frequencies ('rarely/never' to 'three or more per day') and three portion size response categories (small, medium and large) to estimate the daily intake of quercetin and naringenin. Statistically significant correlations were found between the urinary excretion of these individual flavonoid aglycones as reference methods, and the quercetin and naringenin intake estimates obtained from the modified FFQ covering the past year.(391)

4.4.2   Components, Development and Administration

It has been established that the FFQ has great appeal as a dietary assessment method, which is credited to its simplicity of administration, assessment of consumption over a prolonged time frame and low cost.(392) To obtain a FFQ for use, an existing FFQ can either be acquired,(357,392) a

FFQ adapted from an existing previously developed FFQ to provide a modified version or a new FFQ developed from basic principles and validated.(18,387,392) Before selecting, modifying or developing a FFQ, consideration should be given to its purpose. FFQs can be developed to assess food or food group intakes with most, developed to assess nutrient intakes.(18) In the selection of a FFQ for use, consideration should be given to selecting the most valid, accurate and subject-appropriate FFQ and whether individual level data or group level data are needed.(392)

When modifying an existing questionnaire for use, aspects like its original purpose, the target group for which it was developed and if a previous validation was carried out and was acceptable, have to be taken into account.(18) If additional information concerning a specific food or nutrient is needed, the development of a specially designed questionnaire to address the food/nutrient of interest should be considered and validated.(392) Developing a FFQ is a demanding task.(374) FFQs vary in the way they are developed and hence show large differences in design features.(85) Aspects to be considered in the development of a FFQ include assembling an appropriate food list,(374,393) number of dietary items to be included in the food list,(85) inclusion of portion sizes,(85,393) the design of the questionnaire format,(374) assigning nutrient values to each line item through establishment of a nutrient database and preparation of the software to analyze the data.(374,393)

The major criticisms towards FFQs are the larger measurement error compared with other dietary assessment methods and having less specificity,(392) or as Brown (367) expressed it, that some FFQs are imprecise and too vague. Although the FFQ has the ability to provide accurate assessment of nutrient intake appropriate for use in epidemiological studies,(365) Brown (367) uses FFQ restrictions that include the limited number of foods that can be asked about in the questionnaire food list, the detail about food preparation that is not obtained and the subject difficulty in reporting a usual frequency of intake and portion sizes (365) to support their FFQ criticism, which is related to the potential sources of error of FFQs. The capability to accurately estimate usual food intake by means of a FFQ depends both on the food list being representative of the subject's typical diet and the ability of the subject to accurately recall and quantify the intake.(394) These aspects need to be addressed in the development of a new FFQ. FFQs therefore need to reflect the specific dietary habits of the study population (379) with the food list providing the representation.

4.4.2.1   Food List and Appropriate Items

A crucial issue in the development of a FFQ is the selection list of dietary items to form the food list.(395-397) The compilation of the food list is critical to a FFQ being capable of collecting sound and reliable data.(18,344) The food list in the FFQ addresses specific dietary items consumed by the subjects under study or food groups of interest (361) and are therefore tailored to the dietary

intake of the population for whom the questionnaire is developed.(398) Although FFQs have several common features, they are culture specific and differ, as they are tailored to the customary food consumption patterns of the country or region.(71,387) A major disadvantage of the FFQ is that its data collection is limited by the food list (350,387) and the completeness with which it represents the nutrient or nutrients of interest (350) and reflects a subject's typical diet.(14,357) Thus, it is important that all relevant dietary items be included to avoid underestimation.(350) A population's diet, however, consists of too many different dietary sources and varied brands and preparation methods to be totally captured by a FFQ with a fixed food list.(18)

4.4.2.1.1   Number of items

Initially FFQs were rather short, with a small number of dietary items that were chosen to investigate a single hypothesis. If the questionnaire is brief, the restricted number of included items can attend to only one or two specific hypotheses, which are not sufficient for epidemiological studies. FFQs have hence become much more extensive for application in epidemiological studies, where several diet-disease relations may be investigated.(354) It is therefore vital to consider the ultimate use intention of the FFQ when deciding which and how many items will be sufficient to include in the food list.(29) The items selected for inclusion are usually chosen for the specific purpose of a study and may not assess total diet.(358)

Dietary intakes of the subjects as assessed through a FFQ can be presented as estimates of absolute values or by the intake ranking within a group.(395) If the purpose is to account for as much of the absolute dietary intake as possible, for example to generate a point estimate of the actual level of intake, then the list of items included need to be broad (29) as most nutrients are present in numerous foods.(396) Except if the purpose of the FFQ is very particular (17,18) with the food list including only those items that contribute to the specific nutrient(s) of interest,(350) a comprehensive food list is otherwise advantageous.(17,18) It is, however, not easy to compile a comprehensive list including sufficient but not too many items so that subjects having very diverse dietary habits can find the items they consume.(344)

Since dietary habits differ greatly depending on the ethnic, social and cultural background of subjects, FFQs must be customized so that the items assembled in the FFQ represent the dietary habits of the target group.(344,399) With the FFQ approach, the food list and associated nutrient content values of the included items in the questionnaire can be decided on by the researcher in order to make the questionnaire representative to the dietary habits of the study group. Such tailoring of the food list and nutrient content values on a FFQ for a specific group should increase the validity and reliability of the dietary intake estimates from the FFQ.(400)

Although, the more items included, the more precise the estimate will be,(29) limitations in the questionnaire length will permit inclusion of only a subset of items.(396) Such complete dietary assessment may not be necessary in epidemiological studies.(29) Many investigators (14,29, 357,395) have emphasized that when the purpose is to investigate the effect of a nutrient on disease rates, classifying the subjects as accurately as possible on some absolute level is not as important as preserving the relation of the subjects to each other.

In epidemiology the need is principally to assess a subject's exposure to a specific nutrient (or nutrients) to determine the effect of variation in intake of that nutrient among the study subjects on disease risk.(29,396) Consequently the required number of items included may be considerably less as it only needs to provide differentiation of the subjects within the study group with regard to their intake of the nutrient of interest.(29) Therefore, the choice of items for inclusion in a FFQ can be made according to a food's contribution to the total intake of a group or to the differences in intake among the subjects. Absolute intake may be useful for comparisons with an external standard, whereas relative rank assessments may be more useful for comparisons within a group. In many research settings, particularly when associations with a health outcome are being investigated, it is useful to rank subjects and define groups by centile rank for intake of a given nutrient.(395)

The number of items incorporated in the food list of a FFQ tends to vary widely.(18) The reviews of Cade et al. (17,18) found that the number ranged from 5 to 350 with the median 79 items (18) and the mean 88 items.(17) The 21 FFQs that were developed and validated in Japan and included in the report of Wakai,(399) ranged from 9 to 169 items, a range quite similar to that reported in an earlier review by Anderson (52) of 9 to 199 items. Comprehensive FFQs developed to determine the intake of a larger number of nutrients usually consist of between 50 and 150 or more items.(344) Generally, over 100 items are required to assess nutrient intake comprehensively.(387) Current popular FFQs include well over 100 food items.(354) Semi-quantitative FFQs typically contain questions on frequency consumption of 60 to 130 items.(369)

A quantitative review of studies validating FFQs found that the number of items in the food list is the main determinant in positioning subjects according to their intake.(85) In analyzing the design characteristics of FFQs in comparison to their validity, Molag et al. (85) established that FFQs with longer food lists (200 items) had higher validation correlation coefficients and are thus better able to rank subjects according to intake for most nutrients than FFQs with shorter food lists (100 items). Molag et al. (85) pointed out that their results should be used as motivation not to shorten the food list length too much when developing FFQs to rank subjects according to nutrient intake.

These findings were more apparent for protein and total fat, which are obtained from various dietary sources.(85)

Comprehensive FFQs have more worth than simply increasing validity.(399) Since foods contributing to nutrient intake vary depending on the target nutrients they possess,(399) an extended food list enables researchers to assess the intakes of a wide range of individual foods, food groups and nutrients in the diet.(18,350,399) A restricted food list is only able to assess the intakes of a few specific nutrients (18,350,399) and preclude investigations of other foods or nutrients should interest for these arise after data collection.(350) A comprehensive food list is prudent if the study is etiological in nature and if it is imperative to assess the total diet. It is imperative when assessing energy intake, the nutrient of interest is highly correlated with other nutrients or other nutrients may interact with the nutrient of interest and the data may have long-term usage.(18) FFQs including long food lists, however, impose a heavier burden on subjects because they require more time to complete. The cost/benefit ratio must be considered together with the study aims in the decision-making process.(399)

There are nonetheless situations for which the intended use of the FFQ may be very specific and for which a FFQ with a comprehensive food list may be not be necessary or even unreasonable. It appears that there is little to be achieved by needlessly extending the number of included dietary items when developing a FFQ.(18) A short or brief questionnaire may suffice for a FFQ with a specific purpose and not intended to assess the total diet, if it determines dietary intake to the required accuracy.(18) For instance, this applies when assessing the intake of one or several specific nutrients,(344) the intake of foods rich in a specific nutrient (vitamin A), or fruit and vegetable intake or if only a crude assessment of dietary intake is required.(18) A number of short or brief FFQs have been developed successfully (18) to assess of intake of nutrients (calcium and total fat) and foods (fruit and vegetables). In the latter case, the concession for instance is made not to determine energy intake, therefore items other than fruit and vegetables are not included, as these intakes need to be assessed thoroughly. Determining energy intake too would result in a far more extensive questionnaire, increasing the risk that subjects would complete it less accurately.(401)

Several investigators (78,402,403) have shortened longer FFQs and re-validated the resultant questionnaire. Overall agreement between reference dietary methods and modified FFQs did not appear to be worse than with the originally developed FFQs.(18) For example, the 276-item questionnaire developed to assess dietary intake of healthy middle-aged Finnish men, participating in the Alpha-Tocopherol Beta-Carotene Cancer Prevention Study, was adapted to comprise 110 items to assess the diet of Finnish women participating in the Kuopio Breast Cancer Study and

further developed to comprise of 181 items for use among pregnant Finnish women to assess their total diet.(390)

4.4.2.1.2   Item selection

The food list must for practical reasons (restricted time) be limited.(29) Thus each dietary item must be carefully selected for inclusion to create a maximally informative food list.(395) The estimation of macronutrient intakes, which are derived in part from nearly all foods, will require more items to be included than will the estimation of micronutrients (vitamin A), which is concentrated in fewer foods. Many of the foods, which are indicators of vitamin A, for instance, are also indicators of vitamin C and dietary fibre. Therefore, even if more than one nutrient is of interest, the number of items included may still be limited.(29) Byers et al. (29) believes that a relatively short list of dietary items (perhaps 15 to 20) may possibly be all that is required for the assessment of a single specific nutrient for epidemiological purposes.

The food list should unquestionably cover a large proportion of the dietary items that provide the nutrient(s) of interest and are consumed by the target subjects.(397) An item with substantial nutrient content that is consumed by most subjects in similar amounts will be an important contributor to the absolute nutrient amount the subjects consume. However, information on the frequency of consumption of the item would be of little use in ranking the subjects by nutrient intake if the consumption of the item is similar among the subjects.(395) Willett (357) cites an example in which everyone eats a carrot daily. In this case, enquiring about carrots would not be helpful in distinguishing high from low beta-carotene consumers, even if carrots were the largest source of beta-carotene in the diet. Because nutrient intake is the product of the nutrient content and the frequency of consumption of a specified amount, items of modest nutrient content consumed frequently by a fraction of the subject group can be as important as infrequently consumed items with high nutrient content. It is usually not possible on the bases of a limited food list to estimate the absolute intake of all nutrients for a subject and to determine the subject relative intake ranking together.(395)

Three approaches have been used to identify the items to be included in the food list when developing FFQs.(396,399,404,405) The food list in FFQs being developed are generally compiled by including those items contributing most to the total population intake and/or to the between-person variation in intake.(85) These approaches determine the relative importance of the various items as indicators of nutrient exposure and for subject ranking according to intake with the resultant items to be retained for an abbreviated questionnaire to assess dietary intake.(29) For an item to either contribute to absolute intake or to differentiate between subject intakes, it must be consumed reasonably often by an appreciable number of the population and contain a

substantial amount of the nutrient of interest. The intake of the item must also differ from person to person.(18)

The most common approach applied has been that of Block et al. (355) which involves selecting those items that contain high levels of the nutrient of interest that have been found to account for the greatest proportion of intake of that nutrient in population-based surveys.(404) The further approach to identify important items in predicting nutrient intake is to source those items for inclusion that are important for explaining the between-person variation in intake by utilizing the stepwise multiple regression analysis as proposed by Byers et al. (29) and Willett.(38,357) The stepwise regression approach has frequently been adopted as the primary means of compiling abbreviated food questionnaires.(29,38,72,393,395,360,396,406)

The first logical approach to constructing a food list for a FFQ would be to include, considering the population studied, the likely major dietary sources of the nutrients studied.(29) In this 'experienced-based' approach,(399) commonly eaten foods and composite dishes to be included for the population of interest could be identified by the expert opinion of experienced dieticians and/or epidemiologists,(29,392,399) by consulting previous dietary surveys conducted for other purposes or by a pilot study.(18,29,388,392) In compiling a food list for a FFQ using this approach the first undertaking should be to obtain recent population-specific dietary intake data that can be utilized for determining the items and portion sizes to incorporate.(392) Recent dietary data is required because of the consumption of new foods.(18) Ideally investigators can generate a data resource by obtaining dietary recall or record data in the population of interest and using the data collected to select the items and portion sizes to be included in the FFQ.(392)

The establishment of specific criteria for including items on the questionnaire is nevertheless still critical.(388) In the earlier studies of Willett et al.,(38,405) the researchers with the help of an experienced dietician, prepared an extensive food list comprising those items identified as potentially important sources of the specified nutrients and thought to be commonly consumed. Afterwards, some items consumed infrequently in small scale pilot testing, were eliminated and the list reduced. Block et al. (355) based the selection of the items for their food list on population dietary consumption data of the nutrient contributors.

Other investigators have applied population dietary data on nutrient contribution to determine items for incorporation. For instance, Kassam-Khamis et al. (407) used data collected in previous studies on the foods and dishes consumed by South Asian communities to compile the initial food list of their FFQ for administration among women of South Asian ethnic origin in Britain. Bingham and Day (408) chose the list of foods to be included in each category on their FFQ from those

items found to be commonly consumed by a previously studied population. Warneke et al. (409) included the most regularly consumed fruit, fruit juice and vegetables as obtained from dietary information provided by African-American adults to the 1994 Continuing Survey of Food Intakes by Individuals and subsequent interviews with produce managers in neighbourhood grocery stores of commonly purchased fruit, fruit juice and vegetables by the target population. Subar et al. (82) selected the fruits and vegetables for their survey on the basis of those listed in the standard Block et al. questionnaire, and on information from the produce industry regarding the top selling fruits and vegetables. In addition to these included most popular fruits and vegetables, they lengthened their fruit and vegetable food list to address ethnic diversity. Satia et al. (345) selected the foods for their antioxidant FFQ based on the most commonly consumed antioxidant-rich foods using NHANES data, the published literature and consensus among five white and African American nutritionists.

Although the judgement of nutrition experts based on experience is useful, quantitative data on the dietary intake of the study population would further help the selection of the most appropriate items.(355) This first quantitative procedure employs the method of Block and colleagues (355) of a 'data-based' approach.(399) Typically, the item selection is made by utilizing information on dietary habits, which is available for a group of subjects thought to be representative of the population to be included in the study to be carried out.(396,406) The dietary information may be obtained from a comprehensive questionnaire administered once to a particular cohort of interest or from a population-based survey.(396) These items initially reported are combined to signify the usual foods and beverages consumed, representative of the study area and/or group, to assess total dietary intake.(383)

In this first procedure, the items are chosen according to their percentage contribution to the overall absolute intake of a specific nutrient determined as the amount of the nutrient from the given item consumed by the total population, divided by the amount of that nutrient from all items consumed by the population.(29,355,403) The items can then be positioned based on their relative contribution of each to the total nutrient intake from the highest to the lowest, by the percentage contribution of each item, and the cumulative percentage contribution determined.(355,403) To meet this first condition for incorporation, only those items that have an accumulative percentage contribution equal to at least 80% (397) or 90% (355) for any single nutrient, are typically retained. Because only one or two items often provide more than 10% of the total intake of a specific nutrient,(406) the FFQ food list in the study of, for instance, McPherson et al. (400) included those items that were identified to have been commonly consumed by persons from Starr County and that contributed at least 1% of the total nutrient intake.

The percentage contribution of each item to the total intake of various nutrients were computed in a number of studies to select the important dietary sources contributing to the total nutrient intake for inclusion in a shortened questionnaire.(72,360,383,397,403,406,410,411)   Based on this percentage contribution of each item to the total intake, Tsubono et al. (397) developed a FFQ with 138 items that can assess the intake of 15 nutrients. Although this approach identifies the items that are important contributors of absolute total nutrient intake, it does not necessarily include the items most important in discriminating subjects' nutrient intake, which refers to the items most important in positioning subjects according to intake.(395,406) Because the differences in dietary intake among subjects are more important than the absolute subject intakes in many epidemiological studies, identification of the items that determine between-person variation in nutrient intake could assist in selecting a food list for a FFQ.(395)

A principal limitation of FFQs is that items, central to contributing to between-person variability in intake of specific nutrients, can be excluded from the food list, which potentially confounds the estimation of intake of that nutrient by the FFQ. Associations between the preventative effect of a food or a nutrient on a specific disease are more likely to be identified, when between-person variability in dietary intake are larger and less likely to be identified when consumption among the subjects is similar even if the food contains a high content of the specific nutrient.   The identification of foods that contribute to between-person variation in dietary consumption is, as a result, an important part of any investigation of relations between food or nutrient intake and disease.(411) Ishihara et al. (411) found that spinach, which is a very rich source of folate, was one of the largest contributors to mean folate intake. However, spinach did not contribute to the between-person variation in folate intake because nearly every subject consumed it. In contrast, green tea contributed greatly to both individual intake and to between-person variation in intake because consumption was possibly strongly influence by individual liking.

The third approach or second quantitative procedure is the 'short-version' approach in which a long FFQ is shortened by omitting items. In this approach, the dietary intakes of target nutrients determined in the long FFQ version are used as the dataset instead of those derived from dietary records, as in the database approach. Items for the short version are chosen from the food list of the long version, based on the between-person variations in nutrient intakes that can be explained by some items.(399) To identify the items important for explaining the variance in intake between persons or predicting individual nutrient intake and ranking of individuals, stepwise multiple regression analysis on a dietary dataset can be applied to identify those items that discriminate most between the subjects.(18,29,38,383,393,399,405,406,411)   This can be done by incorporating the entire list of items,(29) a limited number of items (395) or only those contributing equal to or above a specific percentage of the mean total dietary intake (406,412) in the regression

models. Hence, different models (29,38) can be used to identify the items needed to explain the between-person dietary variability.(72) When items are stepped into the regression analysis based on their mean percentage contribution to total nutrient intake rather than by their individual correlation with the total nutrient intake, the findings according to Byers et al.,(29) who used the multiple regression approach, were nearly identical.

In this forward stepwise regression approach, the individual items that contain the nutrient are utilized with the nutrient contribution of each of these items for each subject defined the independent/predictor variable, and the total nutrient intake per subject defined the dependent/outcome variable.(29,38,403) Stepwise regression algorithms are approximate means of maximizing R^2, which is called the percent between-person variation explained. R^2 is used as a measure of how well the selected foods capture the between-person variability.(357,396) Foods, which overall explain about 80% (357,393) to 90% (72,383,403,406) of the between-person variance, are usually included and subsequently considered for inclusion in the food list of the final questionnaire. The final food list in the study of Schaffer et al. (403) included 325 of the more than 800 potential items from the original questionnaire.

Byers et al. (29) found very similar findings for males and females and for three age groups with minimal changes in the rank order of the various items when stepped into multiple regression analysis. Overvad et al. (406) reported similar findings applying the regression analysis separately for men and women and only found a small number of extra items as important predictors of the intake of two or more nutrients in one gender group, which were not included in the analysis for the study group as a whole. The dietary habits of men and women appear to be qualitatively relatively alike. However, the analysis of Overvad et al. (406) found that dietary habits were both quantitatively and qualitatively dissimilar among individuals at different levels of energy intake, which may reveal more homogenous dietary habits in the subgroups compared to the study group as a whole. As a result, this underlines the importance of subgroup analysis. Schaffer et al. (403) established that all items in the food list generated by the regression approach were found within the food list generated by the percentage contribution approach; consequently the analysis of contributions to the variance did not increase the likelihood to compile a more representative food list in their study. Usually items are included in the food list if present in the regression approach for any nutrient.(403) However, a few items can be excluded and/or added to the final questionnaire. Such decisions are based on information like the foods that provide large amounts of the specific nutrients, but which were not incorporated in the regression analysis.(406)

The findings of Byers et al.,(29) supported by that of Stryker et al. (395) and Shahar et al.,(72) indicate that if a specific nutrient is considered, a short list of a few items can explain most of the

between-person variation in intake of the nutrient of interest and that the number of items necessary to explain a given proportion of the between-person variability in nutrient intake, was less than the number required to explain the same proportion of the absolute total intake. A food list that appears to be somewhat incomplete on the basis of the contribution to absolute dietary intake, may still be effective in discriminating between the subjects.(72,395) In the study of Shahar et al.,(72) soft drinks, soda and juices explained 18% to 25% of the between-person variability in energy intake. On the other hand, these beverages contributed only about 4-5% of the total energy intake indicating that they were not a major energy contributor. Nonetheless, they explained a relatively large proportion of between-person variability in energy intake.

Not much appears to be gained by unnecessarily increasing the number of items incorporated in the food list when developing a FFQ.(18) Avoiding redundant items is important in heterogeneous populations. Using the regression approach provides the ability to limit the items in the questionnaire.(72) This approach has major benefits for the development of short or brief questionnaires to assess dietary intakes.(406) However, the contribution of individual items to nutrient intake differs considerably by specific nutrient,(72) providing that a short food list performs much better for some nutrients than others.(395) A greater number of items are still necessary for assessing total energy,(72,406) protein (406) and even potassium (395) intake since these nutrients have many dietary sources.(395,406) Energy intake has to be considered in the data analyses of most epidemiological studies related to dietary intake.(406)

In contrast, nutrients with a limited number of major sources are assessed relatively well via a small number of items, such as vitamin A, vitamin C,(29,395,406) folic acid,(72) beta-carotene, (395) zinc (72) and cholesterol.(395). In the study of Shahar et al.,(72) less than 20 items accounted for more than 90% of the between-person variation in intake of zinc and folic acid. In contrast, 34 to 46 food items were needed to reach the level of 90% for energy. In the study of Overvad et al.,(406) the average number of items identified through multiple regressions, explaining 90% of the between-person variability for any of the nutrients investigated, were 14. In the study of Ishihara et al.,(411) the original long FFQ with 138 items was valid for assessing folate intake, while that with 33 items could rank the subjects by folate intake with the same level of accuracy as the long FFQ. In general, a short list will do well for nutrients like preformed vitamin A, cholesterol, carotene and vitamin B_{12} and worst for total energy intake, total carbohydrates and potassium. For these latter nutrients, the items that are the greatest contributors to the total intake are not the best indicators of the between-person variation in intake.(395)

A further developed variance-based method proposed is called Max_r,(396) which chooses the items that best preserve the relation between individuals by identifying the subset of items

contributing most to between-person nutrient intake variation. Stiegler et al. (413) used the variance-based Max_r method to compile the 82-item food list of a new FFQ developed to assess the intake of fatty acids and antioxidants in school-aged children, based on three-day weighed dietary records.

4.4.2.1.3   Grouping of items

Once the items have been identified, the item compilation order in the questionnaire should be considered. Related dietary items should be grouped or clustered together.(18) Clustering of items in a FFQ should consider the planned use of the questionnaire.(17,18) Decisions on how the items are clustered and organized within a FFQ may influence its completion simplicity.(17) Items within FFQs are normally clustered together in terms of traditional food groups (17,18,414) or as related items with similarity in nutrient profiles.(365,407,414,415) Foods can be clustered on the basis of cultural (365) or culinary (414) usage. Focus group discussions can assist in constructing lists for culturally specific questionnaires or to provide information about which items should be grouped together.(18) Although most food groups possibly contain many distinct foods, the fruit and vegetable food group contain a large number of distinct food types that cannot easily be sub-categorized in FFQs because of important differences in nutrient content between individual foods. This is why FFQ food lists often contain many line items for fruits and vegetables.(82)

The items can also be clustered in a meal-orientated and not a food-orientated basis.(416) Wheeler et al. (394) developed an open-ended, meal-based FFQ format with the columns titled breakfast, lunch, dinner and between-meal snacks that included a completed example page with the intention of improving the quality of nutrient intake data obtained from the FFQ. Feunekes et al. (412) constructed a semi-quantitative FFQ to assess intakes of fat and cholesterol according to meal pattern. It is supposed that such a questionnaire structured around meals, facilitates recall.(415) Although cognitive testing confirmed that many individuals when asked about their usual intakes of, say fruit and vegetables, do so by recalling their typical day from morning to bedtime,(417) the format of the questions pertaining to the order of the items (either as a list or according to meals) does not seem to have a major influence on nutrient estimates from the questionnaire.(394) There was little difference in the potential of two screeners developed by Thompson et al.(418)

Some evidence did emerge that the performance of the All-day screener was better among those with relative irregular fruit and vegetable intake, and that of the By-meal screener better among those with relatively regular fruit and vegetable intake.(418) According to Friedenreich et al.,(415) a questionnaire structured by meals is more difficult, though not infeasible, to implement in a self-administered manner, and less suitable for populations with irregular eating patterns.

4.4.2.1.4   Aggregation of single items

Some combining or aggregation of individual foods into combined single items or questions is both desirable and inevitable in constructing FFQs.(395) Some aggregation of single items might be considered for practical reasons and necessary in the food list to limit the questionnaire length as the food list cannot be endless.(17,18,388) Special attention should be paid to such aggregation of conceptually similar foods into single questions.(83) The key considerations for aggregating items should be based on the ultimate purpose of the questionnaire.(18) Aggregation decisions can be made on the basis of shared attributes of foods,(393) for example similarity of nutritional content per portion eaten (g weights and nutrient densities for the nutrients of interest),(72,393,395,400) as well as logical connection between items,(400) like the manner of serving.(393) All available fruits and vegetables cannot be incorporated in a short questionnaire. Therefore, some selection of items to incorporate and some aggregation of items must be applied.(419) Shahar et al. (72,393) aggregated fruits and vegetables on the basis of their vitamin and mineral contents per portion. Dieticians/ Nutritionists can monitor and evaluate the aggregation process in particular for items as outliers in an aggregated grouping.(72,393)

Aggregation groupings that include several items may cause misclassification of nutrient intake by being too broad in two ways: (a) when the various items in the grouping differ too greatly in content of one or more nutrients; and (b) when the items in the grouping have such different consumption patterns for a subject to recall and integrate it accurately over the category.(395) For these aggregated items average nutrient values must be calculated.(388) In aggregation of items and covered as a single question, postulations about the relative frequencies of intakes and portion sizes of the included items must be made when calculating gram consumption weights or nutrient intakes.(18)

Lengthy questionnaires are often shortened through aggregation of similar items into a single question because of time constraints in conducting research, to reduce the burden on subjects and improve the quality of the response rates.(420) Although aggregation may make a questionnaire easier to complete,(395) aggregation can in fact complicate the question and lengthen the time and effort of completion.(18) In a qualitative approach using a cognitive interviewing method in which subjects express their thought processes while completing a few versions of a FFQ, Subar et al. (374) found that the aggregation of items caused several subjects to have difficulty in working out consumption frequencies for aggregated items, which increased the answering time. Separating items from a single line item to several item lines, increased the number of pages of the questionnaire but seemed not to influence the total time to complete it as a result of better understanding, easier retrieval of information and less computation in forming an answer.(374) Willett (357) supports that researchers use 'multiple, simple, clear' questions instead of 'single,

longer, complex' questions, as the task of the subject is simpler for single line items.(420) The consensus from the group of experts that provided input into the FFQ development, validation and utilization reviewed by Cade et al.,(18) was that single items are better than aggregated foods at least for some items in a questionnaire. Single items are preferred for the food groups of key interest in the research.(17,18)

Aggregation of items has furthermore led to an underestimation of intake.(420) Subjects in the study of Musgrave et al. (358) made it clear during the completion of their questionnaires that there was a greater possibility of omission of a food when items were aggregated, rather than separated. One questionnaire included 29 questions about individual high-fat foods, while the other aggregated these same foods into 14 questions. In comparison to the subjects interviewed using the 29-item single foods questionnaire, the subjects answering the 14-item aggregated foods questionnaire reported lower average intakes of the foods. A considerably higher percentage of subjects responding to the aggregated foods questionnaire reported not consuming the foods.(420)

While it may be better to ask separate questions, increasing the number of items was found to lead to overestimation of intake.(386) When asked separate questions about similar foods, the subject may blend details of food consumption and thereby unintentionally duplicate answers.(420) There are, however, methods available to adjust for this (using summary questions for cross-checking) (18) as part of the quality control procedures to minimize questionnaire error. Serdula et al.,(420) however, was unable to determine which FFQ (separate 29 question high-fat foods questionnaire or the same foods aggregated into a 14 question questionnaire) produced more accurate absolute estimates of intake and therefore which ranked the intake of the subjects more accurately.

4.4.2.1.5   Wording of items

In considering cognition, interpretation and other dietary assessment issues, Wolfe et al. (419) found that when a few items were aggregated together, subjects experienced more difficulty answering when 'and' was used than when 'or' was used in the item grouping. The wording of a question could be misunderstood.(39) For instance, Salvini et al. (39) found that the aggregated item 'spinach and other greens' was answered poorly and was overestimated as, although 'other greens' was intended to cover kale, chard and collards, some subjects understood this to include lettuce. Therefore a more complete and definite list of items should rather be used.(39)

Questions on both individual items and composite or mixed dishes should furthermore be included.(360) Numerous single foods are consumed in daily life, yet various foods are consumed as mixed dishes. It is difficult for subjects not involved in daily cooking to answer questions regarding their daily consumption frequency and intake amount of even single foods. It is even more difficult for them to estimate the intake of single ingredients in meals and mixed dishes

consumed.(360) Wolfe et al. (419) found that responses will not be consistent for small amounts of fruits and vegetables as condiments and in composite dishes without clear instructions about what to include and to exclude. They therefore suggested some specification or detailed instructions concerning items with too small vegetable and fruit amounts to include in the responses and a prompt to consider composite dishes. Subar et al. (374) found respondents were confused as to the comprehension of some types of foods like green salad, which is a mixed dish.

Wolfe et al. (419) found issues concerning interpretation. The subjects experienced difficulty differentiating fruit juices from fruit drinks, causing inconsistent responses. Subar et al. (374) reported confusion regarding the types of drinks to include as fruit drinks. This emphasizes describing items and categories clearly. Because subjects experienced difficulty differentiating between fruit juices and fruit drinks, these questions were reworded by Wolfe et al. (419) directly specifying inclusion of fruit juices (100% fruit juices) and exclusion of fruit drinks. Even if such explanations lengthen the questionnaire, each question still takes only a few minutes to answer.(419)

4.4.2.1.6   Ordering of items

Numerous small problems are found with ordering of the items in the food list.(374) Mistakes may be made in the answers provided to the first few questions as the subject is becoming familiar with the format of the questionnaire. Moreover, towards the end of the questionnaire the accuracy of responses may decline due to boredom or fatigue. Food groups of particular interest should therefore be included near the beginning of the questionnaire, but not at the start. As a result it is better to start with something straightforward and clear placing the more important items just after this. Specific items should be placed before general items.(18)

4.4.2.2   Consumption Frequency

Once the food list has been compiled, the next step is to consider the frequency measure with which each item is consumed.(18) The primary determinant of the total dietary amount consumed is the consumption frequency.(30,387) Subjects are asked to report their usual frequency of intake of the items in the food list over a specified reference time period (53,72,350,361-363) in the past like a week, one or several months.(350) Although FFQs can assess current or past intake,(361) they are generally not developed to assess current intake.(343) The FFQ requires subjects to use long-term memory to conceptualize their intake over a certain time period (421) and therefore estimates longer term usual dietary intake.(86,358,388,393,421)

Cade et al. (18) found that various frequency options were utilized. The frequency responses, in which the subjects are asked to report how often they consumed the items on the food list of the questionnaire, are typically formatted in terms of rates (how many times a day, week, month or

year).(359,421) Response frequencies by which the item was consumed are reported either as an absolute amount or exact frequency (exact number of times per day, per week, or per month) or as categorical frequencies.(362,415)

In the two most extensively utilized FFQs in North America, one developed by Willett's group (Nurses' Health Study) and another by Block's group (Health Habits and History Questionnaire), subjects are asked to report their consumption frequency in terms of categorical frequencies.(362) The FFQ method does require some rather intricate summing and averaging actions in order to turn up a single food frequency consumption estimate,(422) which most subjects have difficulty estimating.(401) Haraldsdóttir et al.,(348) Willett and Lenart (387) and Molag et al. (85) suggested that frequency estimation errors are a more important source of error than portion size estimate errors. The quantification of portion size may be of minor importance compared with frequency estimates, as only small differences were found when utilizing information from estimated portion size questions in comparison to analyzing the same FFQs using measured portions from the subject weighed dietary records.(348) Seasonally consumed items can further be problematic when reporting frequency of intake, as they may be consumed frequently when in season and then not at all when out of season. A separate division can be incorporated that enquires about consumption of seasonal items when in season. The information provided can then be adjusted at analysis to reflect the seasonal availability.(18)

Most self-administered FFQs utilize closed-ended questions with several categories provided as answer options for the consumption frequency (362,407) where subjects complete a frequency grid.(52) Frequency categories should always be continuous (without gaps), otherwise the sensitivity of the questionnaire will be reduced and subjects become annoyed if they cannot find their response frequency.(18) FFQs have incorporated different frequency categories with an intake range of up to 12 response category time divisions (17,18,399); the most common being nine categories.(17) The nine-response category usually range from 'never or less than once per month' to 'six or more per day',(38,362,405,423-425) but other categorical responses are used within the nine frequency response category.(397,413) The number of response frequencies will largely depend on the planned use of the questionnaire.(18) The range of the response frequencies should reflect the involved time frame.(17,18) The frequency categories should emphasize the higher consumption end of the distribution for most items (number of times per week) yet include a less frequent option (less than once a month) that can be used for items eaten infrequently, but make a significant contribution to nutrient intake. Considering the higher consumption end, some FFQs pay attention to items consumed on a weekly basis ignoring those consumed less than once a week, whilst others attend to items consumed daily.(18)

The lowest frequency end of the distribution range usually include 'rarely/seldom/never', (41,364, 365,368,424-429) 'once a month or less',(345) both (24,365,413,423) or a combination of these. (38,362,405,430-432)   Only a few items are consumed more than once a day.   If there are frequency choices of more than once a day, this tends to cause overestimation for some people.(18)   Jackson et al. (365) used two or more times per day as the maximum frequency as only a small number of foods and beverages in their pre-testing were reported as being consumed more often than this.   Wolk et al. (426) considered responses to be inaccurate when the reported frequency of intake exceeded two to three times per day for some foods (pasta, potatoes, rice, seafood, etc.).   Nevertheless, the frequency categories emphasizing the higher frequency end of the distribution include the whole spectrum from two,(345,365,431) three,(41) four,(413,426) six, (24,38,362,405,423-425) seven (397) up to eight (368,429) or more a day.   In applying their cognitive interviewing method, Subar et al. (374) found that it may not be helpful to increase the frequency categories above 'two or more per day' for non-beverage items, but this can be increased for beverage items like coffee, tea and milk to 'six or more times per day'.   In the FFQ used in a study by Patterson et al.,(432) the questions on usual frequency of intake ranged up to 'two or more per day' for foods and 'six or more per day' for beverages.

FFQs can differ by providing the frequency response categories in ascending (consumption from 'never' to 'several per day') or descending (from consumption 'several per day' to 'never') order.(17,18,426)   It seems that the ordering of the predefined categories may have some impact on the frequency response selection.(426,370)   Kuskowska-Wolk et al. (370) investigated the impact of decreasing/increasing frequency categories and found that it does not seem to influence the response selection of all items.   Whilst milk and fat, for instance, were not sensitive to decreasing/increasing frequencies, bread, vegetables and fish were sensitive with the decreasing response frequency category order associated with an increase in the frequency response selection.   A further investigation by Wolk et al. (426) found that compared with the increasing frequency category order, the decreasing order entailed higher mean intake estimates for a number of food groups (3% to 11% for 7 of the 14 food groups) and nutrients (3% to 6% higher for 13 of the 18 nutrients) and for energy (4% higher).

All the response categories need to be assigned an average frequency per the defined frequency category (386,433) and these frequencies then transformed into frequencies per defined period (day) by a scale (24) or more often using coefficients (39,80,386,426) starting with the factor nil for 'never or rarely' (386) for assessment of the dietary intake.   This implies converting average frequency on the FFQ into frequency of intake/day; as one to three/month=2/30=0.07; two to four/week=3/7=0.43; and six or more/day=6.0.(39,386)   Missing frequency responses are usually analyzed as never/seldom answers.(426)

The frequency response format of FFQs can be open-ended by asking subjects to write in the number of times they consumed the various items in terms of an exact consumption frequency either per day, week, month or never,(82,88,390,407,434) rather than in closed-ended pre-defined fixed multiple-choice frequency categories of consumption.(82,347,360,407,434,435) The literature search of the 21 FFQs that were developed and validated in Japan, found three FFQs to have open-ended frequency questions.(399) Open-ended frequencies in FFQs though have been used on a regular basis.(82,88,347,360,384,390,407,434,435) These frequencies are then transformed into frequencies per defined period (day) by using coefficients for assessment of the dietary intake, for example, when a subject responded 'three times a week' as to a particular item, its weekly frequency is converted into a daily one by multiplying it by 3/7.(360)

Only a small number of studies have explored the influence of reporting frequency categories as opposed to exact frequencies on nutrient intake estimates.(362) In theory, an open-ended frequency response format might produce some improvement in reporting precision, since frequency of consumption is a continuous rather than a categorical variable. It is not likely that the overall gain in accuracy will be considerable, because the assessment of how often a dietary item is consumed, is innately a rough calculation.(436) Willett (436) in an invited commentary reported that comparing data from open-ended frequency responses and the same data collapsed into frequency categories with that of dietary records, indicated little loss of information due to the categorizing of the frequency responses. The results of the study of Wheeler et al. (394) suggest that the flexible format FFQ did not appear to reduce reproducibility compared with the fixed format FFQ. A study by Tylavsky and Sharp (437) found an extensive influence on the mean nutrient intakes of subjects and their rank ordering when intakes were obtained using frequency response categories rather than exact frequencies of consumption, but they could not conclude which approach was more accurate. These authors compared nutrient intakes based on open-ended or collapsed frequency response categories without an independent assessment of true intakes to evaluate relative validity.(436)

Jain and McLaughlin (362) designed a study to investigate and compare the influence of utilizing exact versus category frequencies of intake from a FFQ and then addressed its validity. The influence of the two FFQ approaches was determined by recording an exact frequency based questionnaire into categories and re-calculating the nutrient intakes. All comparisons were made by computing only and not by pilot testing the developed categorical FFQ on a new group of subjects. The findings of the study suggested that when evaluated against dietary records, the use of exact frequencies in FFQs provided slightly higher correlation coefficients for most nutrients and slightly better comparability within quartile categories of intake than when exact frequencies were

recorded into frequency categories. Considering absolute nutrient intake estimates per day, recording the FFQ into categories provided markedly different results for several nutrients analyzed, which indicates that even for absolute levels of nutrient intake estimates in this population, the category-based approach was not as accurate as the exact frequency method.(368)

Although the use of the category-based option provides easier questionnaire administration, because the subjects simply have to choose the appropriate categories and it is easier to adapt for scanners than the exact-frequency based FFQ, it is still necessary to evaluate category-based FFQs before use in epidemiological studies to maximize the validity in determining the nutrient intakes of interest.(362) Subar et al. (374) found that utilizing multiple-choice frequency response categories improved questionnaire clarity and lowered errors in comparison to open-ended frequency responses. Subar et al. (374) in applying their cognitive interviewing method found that the open-ended response format, while providing subjects the opportunity to indicate frequency at a level that considered their cognitive preferences, it seemed to be prone to error, particularly with the format and instructions they used. Although the subjects indicated a number representing frequency of consumption, they frequently failed to check the time frame category for day, week, month or year as provided. These researchers indicated that the open-ended format may be more appropriate for an interviewer-administered than for a self-administered questionnaire.(374)

Cade et al. (18) proposed that questions on frequency should be closed rather than open, as this indication decreases coding time and recording errors, and lowers the number of questionnaires that have to be discarded because responses are not complete or cannot be satisfactorily interpreted. Cade et al. (18) and Tylavsky and Sharp (437) further suggested that if it is essential to use open questions, it is best to be used in interviewer-administered FFQs as it is easier to use (437) and it ensures that the questions are completed adequately.(18) Hansson and Galanti (382) in their FFQ application requested the average number of portions/servings per day, week or month for foods that are routinely consumed frequently in the Swedish diet (7 items) and for all beverages,(11 items) while for foods consumed less often (38 items), intake was provided across a range of six precoded frequencies from 'never or less than once per month' to 'once a day or more often' essentially using both options.

Due to the complexity of the forms in use, omitted answers or non-response after self-reports is common in FFQs.(382) Hansson and Galanti (382) studied two strategies to handle such omitted answers in FFQs for assessment of dietary and nutrient intakes. In the first strategy the omitted food list items were interpreted as 'nil consumption'. In the second strategy, the absent answers were replaced with the median frequency of those subjects who provided a frequency consumption

of the specific item. The results indicated insignificant differences between the two strategies. In addition the nil-consumption assumption was supported by follow-up telephone interviews with a sub-sample of subjects who omitted frequency indications. Hansson and Galanti (382) concluded that it is reasonable to interpret and indicate omitted self-reports of intake as 'nil consumption'.

4.4.2.3   Reference Period Time Frame

Past intake is generally assessed in FFQs by asking subjects their frequency of intake of dietary items in the last or even previous few years.(407) Although the most common reference period for the assessment of diet is the past year,(17) shorter reference periods have been assessed frequently, namely past one to two weeks,(88,243,323,409) past month (84,390,400,438) and past three months.(49) Wolfe et al. (419) in their deliberation of the cognition, interpretation and other dietary assessment issues found the reference period time frame an important consideration. Subjects could recall back one month, but stated that recalling over six months or one year would not be easy.

Gibson (349) draws on a number of dietary assessment studies when accentuating the difficulty in obtaining unbiased estimates of retrospective dietary intakes. One factor is the influence of current intake on reporting past intakes that can distort the recall of past dietary intake.(415) Although one month only represents intake in a specific season, trying to average usual intake over more than one season is hard.(419) If this is essential, Wolfe et al. (419) suggested that several brief assessments should be obtained. Wolfe et al. (419) found that determining a time frame and making it clear to subjects is vital for uniformity among and within the subjects. Although the frequency responses are reported per day, week, month, or year, subjects need to be aware of the time frame they are reporting on (frequency per day during the previous month or throughout the previous year). The relevant reference period time frame should consider the metabolism and the physiology or pathophysiology of the dietary factor under study.(357)

4.4.2.4   Portion Size Inclusion

A general criticism against FFQs is that they are too vague and not as accurate as other dietary assessment methods.(367) Once the food list and a measure of the consumption frequency are compiled, the next step to consider is obtaining some indication of the amount consumed of each food list item.(18) FFQs can be developed to obtain dietary information on the quantity in addition to the frequency of the included items consumed over a specific period of time. A reported amount consumed or a standard portion size can be utilized for intake determinations.(439) If portion size consumed is included, subjects are firstly asked how much of the item was consumed and secondly how often it was consumed.(410)

The probable advantages and disadvantages of inclusion of additional questions on portion size in a FFQ should be considered before doing so, as available data suggest that portion size inclusion in FFQs do not greatly contribute to the assessment accuracy of dietary intake, but suggest improvement in the relative validity.(344) If the inclusion is considered it requires knowledge about the portions consumed in the population of interest.(17) The portion sizes should reflect the consumption patterns in the subject group and allocate a sufficient range of portion sizes to enable subjects to report their consumption. This is important to adequately distinguish subjects that may have the same frequency of consumption, but consume different portion sizes. However, even within related groups of subjects the use of small, medium and large as indications for portion sizes may not have the same implication and even more so between different groups of subjects.(18)

According to Cade et al.,(18), the inclusion of an assessment of portion size or not will be influenced by several factors, which include: availability of average portion size data; variability of the portion size data; ability of the subjects to accurately assess portion size; and the accuracy required of the collected data. There are several methods for quantifying the intake frequency in the development of a FFQ.(404,436) These incorporate: (a) ignoring portion sizes (simple FFQ) (436); (b) simply asking how often foods are consumed in terms of a specified standard unit (semi-quantified FFQ),(436) which usually is the medium portion size of the items (404); or (c) asking an additional question about usual portion size, which can be simplified multiple-choice classifications of portion size (404,436) described with words (as applied in the Block questionnaire),(404) pictures or models or open-ended responses to elicit specific consumed quantities from the subject.(436) A non-quantitative simple FFQ asks subjects to only record the frequency of consumption and do not ask them to record portion size information, whereas a quantitative FFQ asks for portion size information.(421,440) As a result, a FFQ can be quantified or semi-quantified. In quantified FFQs the food and beverage portions are quantified in terms of g or mL. In semi-quantified FFQs portion sizes of foods and beverages consumed are presented as small, medium or large.(440)

In epidemiological studies on diet and disease risk (38) and even other dietary studies,(40,399, 427) cost-effective strategies frequently involve the use of simple FFQs, which enquire only about the frequency with which items were consumed over a given reference time frame and no information about portion size.(38,40,427) Information on portion size is necessary if food weights or nutrient intakes are required.(18) In FFQs in which no information is collected on portion sizes, assumptions regarding portion sizes have to be made to facilitate determining food and nutrient intakes.(426) Food weights and nutrient intakes can be calculated in such FFQs by assigning standard portion sizes (40,85,399) like specified 'medium' servings or units derived from

experience with other dietary surveys, published values (40) or existing data on average portion sizes appropriate for the population being studied (18) possibly obtained from dietary records.(40)

Some FFQs (semi-quantitative FFQs) do not include specific questions on portion size, but list each food with a specified standard or 'usual' serving size.(39,364,408,414,434) Subjects must then select a frequency category according to how often they consumed the specified portion size.(18) These standard portion sizes include specific 'medium' servings, defined by natural or household units (cup or spoon).(423) Given the assumption that the contribution of between-person variation to the total variability in portion size is much smaller than the within-person variation, indication of a standard portion size by the researcher may not introduce a large error in the assessment of the dietary intake.(362)

The benefits of utilizing standard rather than reported portion sizes comprise easier reporting as the FFQ is simplified, less effort required from subjects answering the FFQ and hence reduced time for its completion, along with reduced cost of collecting the data.(439) However, combining the frequency with a standard portion size may represent a cognitive challenge for subjects who do not consume the item in the amount specified.(18) Block et al. (355) urged the compilation of portion sizes based on population data as such, 'standard' portion sizes (one-half cup), may reflect assessment convenience and approximation instead of any inherent representation of the portions the subjects actually consume. Use of standard portions applied equally to all subjects simplifies the questionnaire, but will reduce sensitivity if portion sizes vary within the population.(18) In the study of Egami et al.,(441) portion sizes were not asked (simple FFQ) except for rice, alcoholic beverages and coffee in order to simplify the FFQ, and standard portion sizes were assumed for the other dietary items to calculate the dietary intake.

Individual portion size estimates instead of a standard portion, can also be included in a FFQ.(348) Providing subjects the opportunity to indicate their portion size is of more benefit than utilizing average portion sizes.(17,18) Asking subjects to report their portion size as 'small', 'medium' or 'large' (41,52,394) are intended to simplify the recording process and overcome the variable ability of subjects to quantify portion size.(394) This approach of probing relative portion size (small, medium or large) contributes personal variation in food choices and quantity towards the age-gender component.(355) Subjects can firstly be asked to indicate their regular or usual portion size relative to a stated standard portion size (the questionnaire is semi-quantitative).(412) This format requires the subjects to record their usual portion size based on the responses small, medium or large with respect to the reference standard medium portion size stated on the questionnaire for each listed item.(18,42,43, 88,345,355,383,402,410,431,442) For instance, the standard portion size at one serving is indicated for each item, and subjects are asked if their usual

portion is larger (greater than 1.5 times), the same, or smaller (less than 0.5) than the standard.(383) A modification of this relative portion size format includes that subjects are asked to describe their portion size of each food item eaten simply as small, medium or large relative to that of other people of the same age and gender (instead of quantifying portion size in terms of household measures).(394) In the two most broadly administered FFQs in North America, one developed by Willett's group (Nurses' Health Study) and another by Block's group (Health Habits and History Questionnaire) subjects are asked to report the habitual amounts of foods consumed in comparison to a given reference pre-defined portion size ('medium') and portion size questions, respectively.(85)

Portion size options can further be listed in three quantitative groups (with g of food) identified as small, medium and large (347) allowing choice of a more appropriate portion size. Subjects can indicate the portion size category that best fit their portion.(443) Although each set of portion sizes usually contains three differing sizes of the item as a small, medium or large portion,(371) Riboli et al. (435) provided sets of four varying portion sizes as photographs or drawings of food and dishes in a booklet from which subjects could select. In a study by Tsubono et al.,(383) subjects selected their actual use from coloured photographs of three different portion sizes. In a further modification of this format, Wolk et al. (426) classified the relative portion sizes as small, medium or large of some foods and additionally asked subjects to provide responses to open-ended questions about consumption quantities of other foods (slices of bread, glasses of milk, cups of coffee and numbers of apples, oranges) in a self-administered FFQ. Cade et al. (17,18) indicated suited methods as those using specified small, medium and large options and portion size estimation utilizing photographs, as used by the researchers above. Questions relating to portion size (similar to frequency), according to Cade et al.,(18) should be closed and not open. In an approach using cognitive interviewing, Subar et al. (374) found that providing a range of portion sizes within the columns as small, medium and large seemed easiest for subjects to comprehend and complete. Some subjects purposefully ignored the medium portion size, as a specified standard unit in FFQs, since the amount indicated did not correspond to a 'medium' size as perceived by them.

Data on portion size may be thus be assigned by the researcher (85,350) (as in a and b above) or obtained from the subject (350) through inclusion of portion size questions (85) (as in c above) where the subjects are required to estimate or describe their portion sizes of the foods consumed.(348) Portion sizes can be described or estimated in different ways, which include descriptions, household measures like spoons, food models and/or photos.(85,348) Where possible the portion sizes should be specified using common natural units,(39,364,383, 405,414,425) as in the case for most fruits.(425) For foods without natural portion sizes, the options include amounts suggested by dietician/nutritionist experience,(39) portions commonly

used as obtained from previously collected dietary records,(364,405) which are the median portions observed in the dietary records,(383) or if available, weight and volume measures of the portions commonly consumed in the study population.(414)

Where information on the usual portion sizes of a population is lacking, appropriate portion sizes can be established by work with focus groups.(18) National dietary guidelines that include serving sizes can be matched, to quantify a serving or then portion,(417) like those identified in the Food Guide Pyramid (53) and in the '5 A Day' program regarding fruit and vegetable servings.(42) The portion size indication is often in natural or household units (430) using household measures like tablespoons, teaspoons and cups,(444) as these are familiar measuring devices.(425) Most researchers are in agreement that for foods which come in natural units (slices, pieces, cups, etc.), questions on the number of units consumed are practical and easy to include, but they have different views when it concerns other foods, which are not that easy to quantify.(348)

In order to make the dietary assessment process as easy and fast as possible, no visual aids or food models for determining portion sizes may be presented.(445) However, to aid the re-collection of the amount consumed and allow for more accurate measurements across the wide ranges of food intakes, portion size aids may be of assistance.(446) To aid the ability to provide descriptions in obtaining portion sizes,(348) various measurement aids can be utilized to assist the subject to quantify the amount of food or portion size consumed.(18,344) Series of two-dimensional measurement aids (14) like drawings of actual foods,(344) drawings of utensils,(88) abstract shapes, computer graphics, etc.,(344) can be used in addition to food photographs (18,344,348,446,447) as well as series of three-dimensional measurement aids,(14) such as actual food samples, food replicas,(344) food models (18,344,348,365,400,445-447) and standard household measures (344, 348,365,400,445,447) and even a measuring tape.(365)

The idea of 'usual' portion size seems rather complex. Therefore, estimating portion sizes, even with the aid of measurement devices, remains an intricate process in which perception, conceptualization and memory play a role.(344) Studies have found that subjects have difficulty indicating portion sizes even with the provision of models or other portion size aids.(409) Regarding these different portion size measurement aids, there is little decisive support on which type is of greater benefit. Therefore practical reasons might determine the choice. Two-dimensional aids have the benefit of being easily copied, making them suitable for inclusion in a questionnaire and appropriate for dietary assessment in large epidemiological studies.(344) Cade et al. (18) support the use of photographs to obtain portion size. Although pictures of foods would make it easier to estimate portion sizes, it is not viable to embed a number of printed portion size

pictures into the response options for each food (448) and high quality colour photos substantially increase the FFQ cost.(348)

Cade et al. (18) in reviewing all dietary studies conducted or published since 1980 found that 22% of FFQs did not make use of portion size information, 42% specified a portion size and 36% provided subjects the opportunity to describe their portion size. The literature search of the 21 FFQs that were developed and validated in Japan, found that information on portion or serving sizes was collected in 12 FFQs with an additional 6 FFQs that included questions of the portion sizes of selected foods only.(399)

In calculating the food and/or nutrient intake from FFQs, numeric adjustments need to be made to convert the portion size according to the subject's estimate of the portion size as small, medium or large. The converting factors used in most studies in relation to the reference standard medium portion size of one, are one-half (½) of the reference standard medium portion size for small and one and one-half (1½) the size of the reference standard for large.(383,394,404,410) Decarli et al. (449) in their portion size analysis considered 0.7 and 1.3 times the average portion size for the small and large portion sizes, respectively. Bingham and Day (408) counted two for a large portion and Wolk et al. (426) one and one-half to two for a large portion depending on the kind of food.

Age- and gender-specific portion size estimates derived from, for instance national data, have been used in the calculations.(18,49,41,450) It may be fitting to utilize such gender-specific 'typical' portion weights instead of 'standard' portion weights to assess dietary intake from frequency data as differences in portion weights have been found between the genders and by age group and socio-economic status.(17) Using age- and gender-specific portion size values, may enhance the accuracy of the estimated dietary intake. Cummings et al. (404) on the other hand found that the simple numeric adjustments of portion size as indicated above and applying qualitative age- and gender-specific portion size estimates to small, medium and large portion size indications produced virtually identical results.

A possible result of the relatively large day-to-day variation in portion sizes consumed is that subjects may not find it easy to report their 'usual' portion size of a item.(348) Haraldsdóttir et al. (348) found that actual portion sizes consumed differ from day-to-day with within-person coefficients of variation of 34% to 40%. FFQs generally enquire about usual portion size, which may not be difficult to report for foods in natural units like pieces of fruit, but can be problematic and highly variable for foods like meat, pasta, vegetables and beverages and even more so for varied, composite multiple ingredient dishes, which are homemade or bought pre-prepared.(367)

Quantification of portion sizes is therefore one of the error sources in obtaining accurate dietary intake data.(344)

Cade et al. (18) reported that subjects experience difficulty in determining portion sizes of foods when reporting about foods previously consumed and even when faced with exhibited foods. Errors in portion size estimation may affect overall dietary assessment and increase the risk for misclassification bias, with some subjects erroneously categorized as having high or low intake.(393)   Systematic error may occur as a result of consistently assigning portion sizes. Systematic bias would be less likely to result when subjects are asked to indicate their portion sizes.(348) Haraldsdóttir et al. (348) found that comparison of the mean nutrient intake estimates utilizing standard portion size or reported portion size information as the two assessment methods, led to significant differences in nutrient intakes. For each nutrient the mean intakes were lower when standard portion size data (mean and median portion sizes derived from national surveys and from foods commonly consumed) replaced reported portion size data (subjects asked to quantify their dietary intakes using numerous household measures and two- and three-dimensional measurement aids).

It seems that the utilization of standard portion size information may underestimate intake of some nutrients. Although the mean nutrient intake of the groups was lower on utilizing standard portion sizes, the correlations between the two methods of portion size incorporation were high. However, when one method consistently under- or overestimates intake in comparison to another method, a high correlation between the two methods is probable.(348)

A study (426) that investigated the influence of adding portion size to the FFQ for only those foods which mostly come in natural units (slices, pieces and cups) found mixed effects both on dietary items (from 30% decrease for eggs to 76% increase for coffee) and on calculated nutrients (from -7% for beta-carotene to +19% for vitamin C) indicating that food and nutrient estimates based on questionnaires excluding portion sizes, may differ from those based on FFQs incorporating portion sizes. It is likely that the over- or underestimation may reveal some difficulties in comparing the portion size presented as standard with the portion that is actually consumed,(39) especially when an inappropriate standard is offered (a portion size dissimilar to the reported mean portion size).(426)   However, this does not shed light on the overestimation of most vegetables, since mean and median portions are in general about one-half cup, which corresponds with the FFQ standard portion size.(39)

The effect of excluding portion estimate errors by substituting estimated portion sizes with measured portions from weighed dietary records in a FFQ was calculated by Haraldsdóttir et

al.(348) The effect of this substitution was minimal with corrections made to only eight of the total of 92 items taken up in the FFQ food list. Correlations between food group intakes from the FFQ and the weighed dietary records improved somewhat while the mean food group intakes did not change much. Potatoes were the one kind of food where corrections could be applied for nearly all subjects and even this correction did not enhance the relative validity evaluations considerably. The minimal effect on the portion corrections encountered means that frequency errors are more critical than portion estimate errors.(348) One study (404) in which subjects rated their portion sizes as small, medium or large, found that these indications produced estimates that correlated better with that of seven-day dietary records than did estimates obtained from subject ratings of their portion sizes in weight or volume. Haraldsdóttir et al. (348) found no systematic differences between men and women in their capability to choose the correct portion size photo. Most subjects (85-95%) chose the correct photo or a neighbouring portion size photo. The errors, however, indicated a distinct trend. Subjects who chose photos of small portion sizes were inclined to underestimate their actual portion sizes, whereas those who chose the large portion photos were inclined to overestimate. However, the method of determining portion size is less important as portion sizes differ less among subjects than do frequency of consumption.(387)

Willett (436) concluded from several studies that examined the effect of including additional portion size data in FFQs on correlations with dietary recalls or records that in general it provided little if any gain in validity. Cade et al. (17) reported that correlation coefficients between FFQs and the reference method were highest when subjects could indicate their own portion size (correlation coefficients 0.5-0.6) compared with no portion size indication (use of average/median portion weights to assess intakes) (correlation coefficients 0.2-0.5) or a portion size specified (correlation coefficients 0.4-0.5) on the questionnaire.(18) Block et al. (355) indicated that some estimate of portion size provided more valid estimates of intake than it being omitted. Cummings et al. (404) indicated that simple quantitative estimates of portion size (small, medium or large) produced a somewhat more accurate calcium intake than classification of all portions as medium. Considering repeatability, correlation coefficients were on the whole higher when subjects could indicate their own portion sizes.(18)

However, it was difficult for Molag et al.,(85) from their review on the design features of FFQs in relation to their validity, to judge the effect of utilizing portion size questions. The incorporation of portion sizes did not influence the ranking of the various nutrients similarly, that is ranking was worse for protein and vitamin C assessed by FFQs that utilized portion size questions in the place of standard portions, and ranking improved for alcohol when FFQs incorporated portion size questions. The authors indicated that the reasons for these findings might include that for certain foods like vegetables, it is difficult to quantify how much was eaten particularly when they are part

of composite dishes, and that it might be easier to indicate the volume and number of alcoholic drinks consumed. Although it seems that allowing subjects to indicate their own portion sizes improves the validity of FFQ estimates,(18) and suggesting that greater attention should be paid to improve portion size estimates in the development process,(85) this effort only leads to marginal increases in the validity and reproducibility of the assessments (18) and does not improve it for all nutrients.(85)

According to Cade et al.,(18) allowing subjects to indicate their known portion size, which the authors acknowledged is not easy to do, possibly does not justify the extra cost and time involved in the development due to such inclusion. It should be considered that accurate quantification of portion size might be of less importance than accurate frequency estimations.(85) Moreover, FFQs extended in length by portion size category inclusion resulted in a significant lower response rate than the shorter basic form.(370) However, markedly less blank answers were found for item consumption frequencies in questionnaires that incorporated portion sizes. It was speculated that questions about portion sizes for some dietary items made it easier for subjects to select frequency categories or possibly these questionnaires were just studied more attentively.(370)

4.4.2.5   Other Aspects

4.4.2.5.1   Inclusion of open-ended questions

To be fully inclusive, an open-ended question section or blank space can be provided with empty lines in the FFQ where subjects can list any additional items consumed frequently (17,355,402,419) or infrequently consumed items that might contribute significantly to the overall diet,(350) but were not already included in the food list (17,350,355,402,419,423) and report the frequency of consumption and typical portion consumed of these foods.(405) The nutrient contributions from these additions are then added to the estimated daily intake.(402) This provides for capturing additional foods of importance to a specific subject or demographic group.(355) The open-ended or 'other' questioning can be included at the end of each food group, category or section of food clustered together in the questionnaire (29,53,384,390,409,415,419) [labeled 'other fruits,' 'other vegetables,' etc. (29,53) or labeled 'other fruit not mentioned', 'other vegetables not mentioned', etc. (409)] or at the end of the questionnaire.(362,371,405,430) Numerous studies have indicated using FFQs that included such open-ended questioning.(29,41,53,355,362,371, 379,384,390,402,405,409,430)

Wolfe et al. (419) and Gibson (14) do not recommend this provision. Wolfe et al. (419) found that subjects could not remember which items had already been asked about in the food list and therefore would possibly include such items again in an 'other' category, causing duplication and falsely increasing the dietary intake. For instance, many subjects included items they had

consumed only in salads again, when asked about them specifically or in the 'other vegetables' option creating duplication.

4.4.2.5.2   Acquiring additional information

Additional questions could be added that can be placed in additional information sections or at the end of the food frequency section to obtain further information on, for instance, methods of food preparation and cooking,(18) such as types of fat and/or margarine used for frying and for cooking (38) to adjust fat intake,(18) and dietary supplements used.(17,18,41) Additional questions could be asked about key sources of the nutrients of interest to improve the accuracy of the data (specific questions about milk) and as cross-check questions to rectify for misreporting of specific food groups.(18)

4.4.2.5.3   Mode of administration

The administration mode of FFQs can either be interviewer- or self-administered (14,18,85,354, 359,361,374) based on the needs of the study.(18) FFQs can be administered by telephone as alternative to face-to-face interviews.(18) FFQs are typically developed to be self-administered by subjects (72,382,451) and are as a result considered to be a fairly inexpensive dietary assessment method.(357,360) Cade et al. (18) found that 67% of the questionnaires validated were self-administered.

Self-administered FFQs require literate subjects and may result in inconsistent interpretation and lower than anticipated response and completion rates, each of which might affect the accuracy of the data.(361) Self-administered questionnaires consequently require more careful preparation and pre-testing. Problems associated with self-administered FFQs are incomplete answers and that whole pages may be missed and not completed.(18) If self-administered FFQs are used, Cade et al. (17,18) proposed that the questionnaire responses should be assessed for completeness soon after the FFQ has been completed so that any uncertainty can be elucidated and incomplete answers can be kept to a minimum. Caan et al. (361) found that using a nutritionist to query doubtful answers on a self-administered FFQ, enhanced agreement with the dietary records used as reference method.

Cade et al. (18) stated that correlation coefficients between interviewer- versus self-administered FFQs and reference assessment methods were better for interviewer-administered than for self-administered questionnaires for several nutrients. Correlation coefficients for repeatability of interviewer- versus self-administered FFQs were better for interviewer-administered FFQs for certain nutrients. If practical, Cade et al. (17,18) suggested using interviewer-administered in preference to self-administered FFQs. Friedenreich et al. (415) indicated the interviewer-administered FFQ as the best retrospective dietary assessment method, as it permits any

queriable responses to be checked between the interviewer and the subject. In contrast, Molag et al. (85) found no significant differences in correlation coefficients between interviewer- and self-administered FFQs pertaining to the nutrients studied (energy, total fat, protein, carbohydrate, dietary fibre, calcium, vitamin C and alcohol).

Clear instructions on the completion of the FFQ should be given at the beginning of the questionnaire if it is to be self-administered.(18,357) Completion is usually enhanced if accompanied by relevant examples (18,43,357,442) that illustrate the manner of response.(355)

4.4.2.5.4   Administration time

When selecting a FFQ as method for dietary assessment, practical concerns related to its administration should be considered, such as when subject burden is an issue, the time required for a subject to complete the FFQ is important.(88) Block et al. (355) advised that self-administered FFQs should be relatively brief and be completed within approximately 20 to 25 minutes, although some subjects would take longer to do so. For instance, the FFQ interview in the study of Feunekes et al. (412) took on average 25 minutes to complete and the range for completion was from 15 to 55 minutes. Several studies indicated that it took the subjects on average 20 to 30 minutes (365,407,412,441,444) or 40 minutes to one hour (49,91,356) and sometimes longer (one to two hours) (435) to complete the questionnaire, but completing the questionnaire depends on its scope (portion size inclusions or not) (441) and length and number of questions.(356) Most FFQs are usually completed within approximately 30 minutes,(14,43) including interviewer-administered FFQs.(365,407,444)

4.4.2.5.5   Questionnaire pre-testing

Finally, every questionnaire should be thoroughly pre-tested or cognitively tested before the main study in a sample of the population of interest.(17,18) A final draft of the questionnaire can be pre-tested (83,345,347,365,393,445,452) usually on a small convenience sample (345,347) of 10 to 20 (83,412,345,393) and up to 40 (445) subjects different from but representing the validation study population.(83,347) The sample should comprise a few (about 7-8) professionals (dieticians/nutritionists) (412,452) and somewhat more lay persons (83,393,412,445) for evaluation and feedback (393) to be able to make the necessary questionnaire modifications.(345,393) While the ease of administration can be field tested in both groups,(393) the professional panel can assist to check the questionnaire comprehensiveness (452) as representation of its content validity that refers to the extent to which the full content of the construct being measured is captured.(453,454)

The field testing of the FFQ is important to ascertain aspects regarding the food list, like the clarity and interpretation of the food names, the clarity of the portion size indicators and clear instructions

and interpretation of questions for ease of completion and recording of responses.(17,18,83) Such face validity assessment is a necessary step in measuring the validity of any new dietary questionnaire (455) to ensure that the target population, in which it is to be used, understands what is being questioned and is able to provide the answers required.(18,392) Modifications have been made to the food list,(355) as well as the included items to quantitatively estimate the consumed foods (347,445) following pre-testing to develop the final form.(347)

4.4.3   Computation of Food and Nutrient Intakes

FFQs list selected foods and beverages in the food list along with intake frequencies and often portion sizes or serving units of these listed items.(399) Information on the consumption frequency and portion size of each of the items in the food list as reported by the subjects allows for an estimation of their food and/or nutrient intakes.(350) Frequency weights must be assigned across the intake frequency categories to assess either daily or weekly consumption (for daily/once a day=1).(18) A portion size database needs to be incorporated. The edible food portion should be taken into account for the portion weight or unit. Portion size data (in g) can be obtained from either published values, data from studies using weighed records or estimates of specific portion sizes, preferably by the subject group being studied.(18)

To transform mean daily FFQ intake data obtained from the consumed frequency and portion or unit weight estimates of the food list intakes to nutrient values, a suited nutrient database needs to be available. The limitations of the food composition database need to be taken into consideration, particularly the extent of missing values and how they would be attended to.(18) The primary source of nutrient content can be supplemented with other published data and information from manufacturers when necessary.(405) The nutrient content of composite dishes can be estimated from recipes that include foods for which the nutrient composition is known. For such estimations, the weights and/or volumes of the recipe ingredients in the mixed dishes must be known.(18) For foods of which the nutrient content is not known (no published available content), the content can be approximated by using amounts in similar foods (410) and imputing these values where necessary to avoid assigning missing nutrient values.(43) Preferably samples of such foods should be collected and the food analyzed chemically.(18)

If single dietary items are aggregated as a line item in a questionnaire, then a collective nutritional value for the grouping needs to be computed. Assigning nutritional values to each aggregate line of grouped items, should consider the relative frequency and portion size consumption of the singly included items (18) based on a weighted mean of the intake of the study group, as the weighted mean of all the single foods aggregated into a line could differ from the nutritional values of the most frequently consumed item in the line aggregation.(393)

Dietary record and FFQ data (mean daily item intake) can both be converted to nutrient intake using computerized programs incorporating food composition tables.(379)   In the case of data collected through dietary records the food items consumed need to be coded, usually by dieticians/nutritionists (435) due to the open-ended format.(440)   In weighed dietary records exact quantities of foods and beverages consumed are recorded in grams. In estimated dietary records, household measurements are usually used to record amounts of foods and beverages consumed. These household intake amounts then have to be converted to grams.(381)   The mean daily individual nutritional intakes are usually calculated as the average of the number of recording days using equal weighting for each of the days.(379,456)

In the case of the data collected through FFQs, the daily total nutrient intake is calculated by multiplying the nutrient content of the selected unit or portion size (either standard or individual) consumed obtained from food composition data, by the consumption frequency using the calculated frequency weight or coefficient value of each item and summing these nutrient values for all dietary items, to generate total daily exposure to the dietary component.(18,39,40,41,52,76, 350,365,379,383,399,405,425,433,434,443,456,457)

An aspect to consider in the computation of FFQ food and nutrient intakes is missing data, which can be treated in a number of ways.(18) According to Cade et al.,(18) questionnaires with a large percentage of incomplete questions should firstly be disqualified. The limit for such incomplete questions should be decided in advance and will depend on the purpose of the study and level of accuracy required. For questionnaires not exceeding this limit for incomplete data, a value of nil (food not eaten) may be noted or alternatively an average obtained for the population could be substituted.(18) Items not specified in a FFQ, that is the frequency of consumption not reported, are often coded as not consumed.(457)

It should be kept in mind that the nutrient estimates obtained from FFQs provide approximate indications of the usual nutrient intake of a subject (426) and should not be considered accurate indications of their usual intake.(456) As noted by Briefel et al.,(456) to determine a subject's nutrient intake, one must know the type and source of the items the subject consumed as single foods and as components of composite dishes, the preparation of the food, how much of each item was consumed and the nutrient content of each of these items. The FFQ is a short-cut assessment that does not consider all the information needed to determine absolute nutrient intake.(456)

Firstly, the FFQ might not obtain information on specific dietary items as foods are often aggregated into broad categories and subjects are asked how frequently they consume these aggregated categories of foods. The nutritional provision of these line items are furthermore averaged or weighted across all the items in the aggregated food grouping based on consumption amounts derived from estimated consumption frequencies and portion sizes of these items. Secondly, subjects are typically asked to indicate how much they consume in broad portion size categories of small, medium and large, of which frequently only the medium portion is defined, and are not asked to indicate the portion size they truly eat. Quantities given do not necessarily correspond to the medium portion for the subject's age and gender and no food aids, as food models or measurement aids, are used to assess how much was truly consumed. Nutrient intake calculations are furthermore not only based on estimated portion sizes of the items consumed, but on estimated frequencies of intake. All of the above reflects not what the subject actually consumed, but an average.(456) A study of Wolk et al. (426) revealed that food and nutrient estimates based on the FFQ depend heavily on the questionnaire design (in particular increasing/decreasing frequency categories and addition of portion sizes). Briefel et al. (456) argue that estimating nutrient intake from FFQs is markedly imprecise and hence adjustments to the data to assure mathematical fit would be inappropriate.

4.5   CONCLUDING COMMENTS

A number of aspects need to be considered in the development of a FFQ that makes the activity a demanding task. Particular attention should be paid to the compilation of the food list that relates to which dietary items to include (though several approaches are available to assist the selection), the number of items to be included, their clustering, aggregation or not, wording and ordering and the consumption frequency indications, which can either be provided as fixed close-ended category responses or open-ended for exact consumption frequency indication.

A decision must be made as to whether indication of the portion size consumed needs to be incorporated or not. In addition, a decision must be made on whether it is necessary to obtain additional information on the dietary items included in the food list, the mode of administration (interviewer- or self-administered) considered along with its administration time, which will greatly depend on the scope of the questionnaire. Ultimately, it should not be too demanding to complete. The computation of the foods, food groups or nutrient intake it was developed for with assessment in mind, should also be established. It is important that the questionnaire be pre-tested for content and face-related evidence of validity before its use in the field. The food, food group(s) or nutrient(s) of interest to be assessed and the research for which the questionnaire is to be used, will guide many of the decisions to be made in the questionnaire developmental process as was experienced in this research.

CHAPTER 5

EVALUATION OF A FOOD FREQUENCY QUESTIONNAIRE FOR TECHNICAL COMPLIANCE

5.1   PREAMBLE

Establishing the 'true intake' of free-living subjects is difficult.(418)   It cannot be assumed that a FFQ will capture the dietary intake entirely.   It is consequently important to judge the extent to which a FFQ actually assesses the 'true' dietary intake.(41)   To achieve this, the reported intake on the FFQ and the 'true intake' assessed on different methods as reference can be compared through the use of the reference data as representation of the 'true intake' and applying statistical procedures.(418)   The results obtained from a FFQ as method to assess dietary exposure in a target subject group will be far more trustworthy when quantitative information supporting its validity and reproducibility is offered.(41)   Scientists commonly use these two characteristics to evaluate the measurement properties of dietary assessment methods.(39,387)   The accuracy relates to the relative validity and reproducibility.(71)   Reproducibility or reliability is the minimum quality required for an assessment method.(39)   Even rapid and inexpensive methods to determine dietary intake, like FFQs, should provide reasonably valid and reproducible assessments of individual dietary intake.(405)   When a new FFQ is developed, or an existing one is used in a new setting, its validity and reproducibility should be evaluated.(357,387)   Although several studies have investigated the comparability (relative validity) and reproducibility of FFQs, only a few have investigated the potential of a FFQ with respect to its sensitivity, specificity and predictive values (383) and its responsiveness.(386)   In general, FFQs are as standard evaluated against dietary records by means of a few replicate dietary records per subject with statistical correction for the within-person day-to-day variation in dietary intake (458) and sometimes with biomarkers.(344)

5.2   VALIDITY

Once a dietary assessment method is selected, the validity of the selected method must be determined.(343,349)   Validity portrays the capability of a method to determine what it is supposed to measure, that is how accurately ('how well') it does so.(6,39,349,416)   When relating validity to physiological measurements, the term 'accuracy' is generally utilized when considering whether the analytical test is determining what it is expected to assess.(459)   In research to interpret dietary intake results with greater confidence, validity refers to the extent to which a dietary assessment method (instrument) demonstrates that it accurately assesses what it is designed or intended to record in terms of aspects of the subject's dietary intake,(14,76,343,347,349,460,461) referred to as quantified validity.(461)   Validity therefore either means that the findings of a measure of

exposure or outcome offer an agreeable representation of the truth (external validity), or that the findings truly represent that exposure or outcome (internal validity). Measures of sensitivity, specificity and predictive value in dietary assessment signify internal validity.(344)

FFQs are commonly used to obtain estimates of individuals' dietary intake and to relate the dietary intake obtained to the development of various diseases. However, the data gathered from a FFQ can deliver estimates of dietary intake that are in error.(462) The validity of a FFQ is not self-evident. It is limited with respect to the items included in the food list and the degree to which portion sizes and frequency of consumption are quantified.(379) Validation of the FFQ method against a more reliable source of information is essential,(18,462) as erroneous information collected may provide untruthful relations between dietary components and disease incidence or disease-related biomarkers.(18) In the context of a FFQ, validity refers to the truthfulness of the database generated with the database the total information collected using the structure of the questionnaire.(5) Several terms are used to refer to investigations that collect dietary data from a subset of study subjects by using two different dietary assessment methods and which compare the data of the one assessment method to the other, with the aim to determine the level of comparability or the relationship between the two methods. The terms used to describe such studies include 'validation', 'calibration' and 'standardization'.(55,58, 373,377)

5.2.1 Aim of Validation Studies

All dietary validation or calibration studies may not have the same primary aim.(377) Dietary validation studies are generally conducted to assess the extent to which a method agrees with a 'gold standard' or other methods of assessing dietary intake.(18,347) To determine the relation or extent of agreement, the relation between the dietary intake obtained from the (test) method (FFQ) and the true usual intake (reference method) is generally determined by using correlations. This evaluation is necessary if the FFQ has been modified largely from earlier versions or is to be used in a subject group from which limited information has been obtained. If the correlations are low, the main study may have to be delayed until improvements are made in the FFQ design or in the way in which it is presented to the study subjects.(377)

For correct interpretation of epidemiological study findings that utilize FFQs as the dietary assessment method, it is crucial to know the relation between the intakes obtained from the FFQ and the true usual intake, which often is the mean intakes as reported in, for instance, multiple dietary records.(377) Studies comparing the dietary intakes obtained from FFQs with that obtained from other dietary assessment methods (mean intakes obtained from multiple dietary records or recalls) are commonly called validation studies.(442,462) In epidemiological studies this relation is ascertained through a dietary standardization sub-study conducted in a sample of the study

subjects within the context of the larger epidemiological dietary survey and commonly referred to as a validation or calibration study.(373,377)   The objective of a calibration study may be to quantify the level of measurement error or bias, associated with use of the less-detailed method (FFQ,) in relation to the more-detailed method.(18,55)   This allows for adjustment (calibration) of the results or dietary intake for the entire population of the main epidemiological study, considering the measurement error at the sub-study group level derived from the less detailed method.(18,55,373)

Many calibration studies are conducted to estimate the slope of the regression of intake from the FFQ on usual intake, a variable that is important in assessing the error or bias that may result from use of the questionnaire.(377)   Bias due to measurement error in dietary assessment methods, has implications for nutrition epidemiology as it can influence FFQ-based RR estimates.(463,464) The information from the calibration study can be used to adjust the RR estimate from the main epidemiological study for the measurement error associated with use of the FFQ (373,377) by applying established regression calibration statistical approaches.(464)   All dietary assessment methods have systematic intake-related and random subject-specific biases.(464)   As a result of the relatively low correlations between dietary questionnaire assessments and longer term dietary intake values, RR estimates, indicating an association between the intake of dietary components and the occurrence of disease, tend to be underestimated ('attenuated').(463)   An alternative reference method, fundamentally unbiased and that has error structures not related to those in the dietary assessment method, like specific reference biomarkers, may satisfy these requirements.(464)   Concurrent use of additional methods of dietary assessment, including biochemical markers, will provide improved data for calibration studies.(55)   However, biomarkers with a known quantitative relation to absolute dietary intake levels are available only for some nutrients.(463)

Another aim of calibration studies may be to estimate the sample size required in the main study. It entails the total number (N) of disease cases that must be observed in the main study to achieve 90% power for detecting a probable effect.   This estimate is important because the sample size required for an investigation depends heavily on the degree of measurement error associated with the FFQ.   Statistical formulas for calculating N have been established.(377)   The use of dietary assessment methods as reference may be responsible for the attenuation of RR estimates in FFQ-based epidemiological studies.(464)

In nutrition-intervention research, it is also necessary to attend to the responsiveness of dietary assessment methods to changes in the nutrient intake or dietary behaviour investigated.(19) Methods for measuring changes in food and nutrient intakes over time are needed both for

estimating compliance in intervention trials and for monitoring food and nutrient intakes of large population groups.(55)

5.2.2 Types of Validity Relevant to Dietary Intake Assessment

Criterion validity comprises the comparison of values from a measure with a 'gold standard' measure with accepted validity.(465) When a new analytical measure is being tested, a criterion measure or gold standard if commonly available. In such situations, comparison of the new method to the standard method provides an indication of its criterion validity. In many other instances, for example in the assessment of human characteristics, a criterion measure or gold standard does not exist.(459) Because habitual diets of free-living subjects cannot be assessed without error, the 'true dietary intake' or absolute validity cannot be judged.(14,460) Determining the absolute validity of any dietary assessment method is not inherently achievable as there is no fully accurate method of establishing the true dietary intake of free-living subjects.(416) This is only possible in confined settings allowing for direct observation (343,350,354,416) or obtaining duplicate meals.(416) In assessing dietary intake there is thus no 'ideal' method or gold standard available to determine the true intake (91,398,461) as a whole (18) or the usual intake.(407,434) No dietary assessment method fully meets the requirements of an optimal method as, although each has strengths, each is hampered by its own weaknesses (Addendum A, Table 4.3).

The different dietary assessment methods also do not have the same error structures.(344) As a result no gold standard reference method exists to serve for perfectly assessing the validity of a dietary assessment method (91,344) like FFQs.(18,41,461) This implies that dietary validation studies are not true validation studies because the validity of the reference assessment methods themselves are not known.(14,373,387) Only the comparability between the findings of the two methods can inherently be assessed as neither dietary assessment method is valid.(343) Consequently it is generally agreed that in theory, absolute (criterion) validity cannot be determined (91) besides for comparison of the dietary intake with an independent marker of such intake as a biomarker concentration.(86)

In this context, investigators must make sure that all pertinent aspects of the construct or domain under investigation is presented (content validity), and that the new method relates to other methods in the implied way to prove that it is truly determining what it is expected to assess (construct validity).(459) Due to the lack of a method to assess true dietary intake or a reference criterion to determine absolute validity of a dietary intake assessment method, like that of a FFQ, evidence of relative (construct) validity is accepted.(6,91,349,368,461) In determining relative validity, the new dietary assessment method (test method) is compared to an existing generally accepted suitable method (reference method).(14,91,349) Absolute validity accepts that the

reference method can establish the true dietary intake, while relative validity recognizes that the reference method itself is prone to error.(14) In this approach investigators either establish the relative validity of the dietary assessment method or calibrate it by comparison with another dietary assessment method or with other methods that do not have the same error structures.(387)

As indicated relative or inter-method validity of a new dietary assessment measure (test method) is commonly undertaken by comparison with a reference measure.(86) The validity of a FFQ, which means its ability to assess the dietary exposure accurately, is usually evaluated by comparing its results with other dietary methods with different characteristics and limitations.(461) The selected reference method is generally an accepted suitable existing, but different, method chosen for its ability to measure similar dietary aspects over the same time frame,(349) but which has greater evidence of validity (6) and is therefore judged to be superior.(6,41,462) This implies it has the ability to assess the dietary aspect more accurately and precisely,(86,349) and has a record of consistently comparing well with other acceptable dietary assessment methods,(6) but inevitably has its own strengths and weaknesses.(86)

In such inter-method validation, to establish if the findings obtained from the methods are comparable that one method can be replaced by the other, one of the methods is typically a short and simple or less detailed and a less established method (FFQ). This method (test method) is then compared with another assessment method, which is more detailed or complex and time consuming and more established.(55,416) The more detailed or complex and more established method (reference method) is believed to provide more accurate estimates of the dietary aspects under investigation within the study group.(55) Therefore, the extent of the comparability between the methods is utilized to portray the relative validity of the test method or the extent to which it yields the truth that the reference method is considered to provide.(14) However, since each of the methods has its own inherent weaknesses, it is generally not known which of the two assessment methods actually provides the most precise estimate of the 'true' dietary intake.(343,416)

The method is usually compared (validated) against some other method which has greater acceptance, but of which the validity most possibly has not been determined.(6,416) Such comparison with other methods provides divergent results and does not inherently indicate which result is correct.(343,354) Validation of dietary assessment methods is as a result considered if the findings can reasonably represent the true dietary intake.(344) Because no single dietary assessment tool is a 'gold standard' method of dietary intake (not even a dietary record) most validity testing have, according to Kristal et al.,(43) focused on what may best be described as convergent validity.(19,76) This means that the method operates in a similar way or alike to other

methods determining the same construct.(453,454) As a result, indirect validation of dietary assessment methods has mostly been undertaken.(416)

The relative validity of food or food group intake estimates by FFQ is less frequently reported than that of nutrient intakes. Knowledge of this aspect is, however, necessary as it points out those dietary items or questions in the FFQ food list that should be focused on to make improvements.(356) FFQs have been found to be sufficiently valid and reproducible methods of the relative intake of most dietary items.(39)

5.2.3 Validity Evaluation Relative to Dietary Assessment Methods as Reference

5.2.3.1 Validity Approaches

The FFQ has two features relative to its data: (a) the list of questions or food list, which is the structure used to generate the dataset, and (b) the data or input that is gathered that forms the dataset.(438) Validation studies of FFQs accordingly play two related but distinctive roles. The first possibly considered as general validation and revision of the FFQ, namely making sure that all dietary items commonly consumed by the study group are included in the food list and that the questionnaire is clearly worded and easily understood by the study subjects. The further role of a validation study then shifts to the calibration phase, which is needed to quantify the FFQ measurement errors. In this calibration phase of the validation study, the subject's 'true' dietary intake is generally estimated with multiple dietary records or 24-hour recalls, and the relation between the mean dietary record values for a subject and his or her FFQ dietary values (in the sub-study) is then assessed and forms the basis for measurement error corrections (in the main study).(466)

Reference to problems with the structure of the FFQ to generate the dataset is scarce in the literature, while problems with the gathered data are routinely reported. Therefore, it is more accurate to describe the validity and reproducibility of the structure and the validity and reproducibility of the data, instead of discussing the validity and reproducibility of the FFQ.(438) The data gathered through the use of FFQs will be the focus of this validity evaluation literature discussion, as well as in the research itself as it is the data gathered which is submitted to validation and the often reported. In the literature, it features as validation studies of FFQs that report the extent to which the FFQ reveals the 'true' dietary intake or the association between the assessed and 'true' dietary intake (calibration).(466)

5.2.3.2 Dietary Assessment Reference Method Selection

A vital step in the validation process is choosing the most suitable reference method against which to evaluate the test method.(18,387) The possible reference dietary assessment methods for FFQs are weighed dietary records, unweighed or estimated dietary records, 24-hour recalls (17,18,

55,85) or dietary history interviews.(55,85) FFQs are generally validated or calibrated against multiple repeated dietary records or 24-hour recalls (85,365,393,425) or both (58) and in addition sometimes non-dietary methods, like biochemical indicators,(344,365,393) as the gold standard.(393) A dietary record is used somewhat more often as reference method for comparison.(58,344,467) Recalls are labour intensive in that they involve a trained interviewer.(468) Cade et al.,(18) in their review in which the development, validation or use of FFQs was described, found that 75% of studies validated a FFQ against another dietary assessment method of which 26% used the estimated dietary record/diary, 25% weighed records, 22% the 24-hour recall, 6% the dietary history interview, 12% another FFQ and 19% a biomarker (although the use of biochemical measurements (biomarkers) of nutrients in blood and other tissues is increasing), and 64% of the validation studies used only one reference method.(17)

The selection of appropriate combinations of test and reference methods for relative validity depends on a number of aspects (349):

- Accuracy and precision: In the validation of dietary assessment methods, the reference method selected should be as accurate and as precise as possible.(38,349) The validity of a new method is therefore usually evaluated against what is considered a 'gold standard'.(434) Dietary records, which provide more accurate quantities of the items consumed, will generally be favoured for assessing absolute intake.(372)

- Errors: Evaluating validity requires the use of independent methods associated with the construct or domain being investigated.(459) This necessitates the reference method being different from the test method.(38) In the validation of dietary assessment methods, the focal point is that the measurement errors associated with the reference method as comparison, is as unrelated as practicable to those of the test method.(14,18,38,387) In other words, the measurement errors of the FFQ should not greatly correspond to those of the reference method to minimize overestimation of validity.(372) The theory behind validation studies is that the error sources linked to FFQs are different to those linked to short-term dietary recall and recording methods.(442) Therefore, in the case of FFQs, a reference method that does not depend on memory and conceptualization of portion sizes is the preferred selection.(387)

- Other aspects: Sources of variance, that include subject gender, age, ethnicity, socio-economic status and the use of vitamin and mineral supplements, along with season, day of the week and training, should be taken into account during administration of both the test and reference methods.(14)

To avoid bias due to associated error with the use of two similar methods, dietary records or recalls are generally used as the reference methods in validation studies of FFQs.(433) The use of

methods aimed at the assessment of past intake or recall methods (dietary history interview and 24-hour recall), depends on memory (18,91,372) and conceptualization skills (18) of the subject. It is suggested that the agreement of the FFQ relative to such methods can be enhanced due to similarity in measurement error in both methods, since many of the cognitive processes are alike in these methods.(91,372) Subject error in perceived portion sizes could bias recall and FFQ intake estimates similarly.(442) Although the FFQ and dietary history interview are both recall methods, they differ considerably.(91) Although the 24-hour recall is less burdensome for the subject than dietary recording, and less prone to influence the actual dietary intake of the subject, their error sources tend to be more similar to those of a FFQ (dependence on memory, conceptualization of portion sizes and misrepresentation of diet). However, when co-operation or literacy of study subjects is a concern, 24-hour recalls may be the more suited choice.(18)

Although the major drawback of the dietary recall method is its memory dependence,(54) the FFQ depends on longer term memory of subjects and their skill to conceptualize and average dietary intake over a longer period. Other methods may be used to estimate portion sizes than those incorporated in the questionnaire. Hence, it seems agreeable that there is limited correspondence between the measurement errors in intake assessments obtained from the FFQ and those obtained from 24-hour recalls. This implicates that the correlations are possibly not greatly enhanced.(356) Van Assema et al. (398) noted that their validation results were possibly positively biased because the screener and the reference FFQ were both frequency type methods. Validation comparison to a more comprehensive FFQ is generally regarded to be a less suited reference method for a short FFQ than the dietary record.(398)

Validation studies of dietary assessment methods for epidemiological studies have generally utilized dietary records as the reference method.(467) Dietary records, as weighed or estimated, are the preferred reference method choice for a validation study,(347,440,451) including validation of FFQs,(18,390,424) as they are considered the gold standard dietary assessment method.(440) Because the FFQ will certainly be the less optimal method,(39) it must be judged against a dietary assessment method considered to be superior such as a series of dietary records to estimate the actual intake.(39,41) Multiple dietary records collected over time are treated as an appropriate 'gold standard' for evaluating validity of FFQs.(350,371) Among the available dietary assessment methods for validating a FFQ, dietary records probably have the least associated errors with FFQs.(387,424) The FFQ captures general, longer term dietary intakes (43) where the subject must report usual dietary intake restricted to the dietary items provided in the food list (442,469) in relation to conceptualization of their portion sizes and the skill of determining frequency of intake across a longer time frame.(442) As the FFQ has pre-coded items and portion sizes, speed of completing and processing the dietary intake data is achieved.(469)

The major error sources linked to FFQs are interpretation of questions, fixed and limited dietary items, memory of items consumed and conceptualization of portion size and consumption frequency. These error sources are not greatly associated with that of the dietary record method (387) since they minimally depend on the memory of the subjects.(38,54,381,387,440,442,451) Dietary records are foremost open-ended (381,442,469) involving recording of dietary items concomitantly as they are consumed (14,381,451) and greater precision is provided in determining intake amounts (14,381) as they are not dependent on the ability of the subject to accurately recall portion sizes.(440) They allow direct measurement of food quantities or portion sizes consumed,(38,442) as they are weighed or estimated at consumption.(14,381,469) It captures in great detail specific types, preparation methods and portion sizes of that consumed over a short time period.(14,43) In other words, compared to the FFQ, dietary records do not depend on the subject's memory and ability to review and summarize their dietary intake to recall their habitual or usual consumption over a specific time period,(71) as all dietary items are recorded at the time of consumption and subjects are requested to measure and report actual intake on the dietary record.(375,381) However, it requires active participation of the subject.(14,451) Subject burden with the dietary record method is high (440,469) since it requires recording every time a dietary item is consumed,(469) especially with longer dietary recording periods.(440) Such dietary assessments, aimed at determining current intake, are consequently likely to meddle in with the subject's daily habits and cause a distortion of intake.(14,18) The cost of the dietary record method should furthermore be considered as it requires trained coders for the data capturing.(469)

Although considered as the 'gold standard' reference method,(405) dietary records can either be weighed or estimated.(14,381) Gibson (14), Cade et al. (17) and Willett and Lenart (387) suggest that weighed dietary records should be the reference method in validation studies. The main gain is that the most important error sources of the two methods are not the same and not likely to be associated (associated errors cause overestimation of validity). As indicated, they are not dependent on memory and allow the amounts of foods and beverages to be quantified each time they are consumed.(372) Since portions are actually weighed they have the least associated errors with FFQs. If the FFQ results are compared with weighed records, the lack of agreement found could in part be explained by the within-person variation in intake that exists in the shorter but more accurate reference method. The FFQ estimates usual intake without being greatly affected by random measurement error (the within-person day-to-day variation of the weighed records).(18) Thus, the validity of a FFQ is generally evaluated in relation to weighed recorded intakes, which is proposed to be the 'silver' standard.(407) Results obtained from estimated dietary records are closest to those obtained from the weighed record compared to other dietary assessment methods.(380)

Because recalls and records are short-term, they characterize the current diet most adequately (14,54,381); but these methods when administered over an adequate duration of days, also offer a measure to determine the validity of longer term assessment methods, like the FFQ. (14,86) While it would possibly be easy to determine the validity of a method developed to ascertain dietary intake over a short time frame (few days), it would be more intricate to determine the validity of a method ascertaining usual dietary intake over a longer time frame (one or more years).(435)

5.2.3.3   Validation Procedure

5.2.3.3.1   Participating subjects

Validation studies of FFQs are hard to conduct due to problems acquiring an adequately large and representative sub-sample of the subject group in which the FFQ is to be administered and the absence of a 'gold standard' dietary assessment reference method.(461) Since the performance of a questionnaire depends on the characteristics of the actual study subjects, a validation study is ideally conducted as a sub-study with a subset of subjects being representative of the main study or target group subjects.(17,18, 373,379)

The validation study subjects should optimally be a random sample of the study group in which the questionnaire is to be administered.(14,470)   However, since dietary records (weighed or estimated) would be a considerable burden in terms of time, volunteers who understand the objective of the research are used.(364) On the other hand, subjects who volunteer to take part may respond differently to a FFQ than non-volunteers.(18) It is possible that those who participate are more aware of and more interested in diet and disease than those who would participate in an epidemiological study where the FFQ is used alone.(461) Klipstein-Grobusch et al. (470) indicated that if the main focus of the study is on a different aspect, such as blood pressure reduction as in their study, the subjects may be less focused on their diet than subjects recruited specifically for a validation study.   The resource of these validation subjects and their characteristics should be described in the validation report.(18) Subject dietary diversity should be considered, as lack of dietary diversity in the subject sample may provide for a high agreement between a FFQ and dietary records, as was found in the study of McPherson et al. (400) for estimates of energy, fats and cholesterol consumption.

5.2.3.3.2   Subject sample size

The sample size in validation studies is usually small because they are logistically difficult and costly to carry out.(58) Cade et al. (17,18) in their reviews of FFQs undertaken found a wide range of sample sizes ranging from 6 to 3750, with a median sample size of 110 subjects (18) and a mean sample size of 255 subjects.(17) The sample size of the validation study did not seem to greatly affect the study results. The review found that correlation coefficients comparing the FFQ

with a reference method were no better for larger than smaller sample sizes.(17) Cade et al. (18) reviewed the test and reference method dietary intake correlation coefficients, and having obtained an adequate number of days of dietary information to acceptably characterize an individual's diet, found a sample size of 100 to 200 subjects to be sufficient. However, they indicated that some studies do not manage to achieve this and that the sample size used inevitably depends on the available resources.

Willett and Lenart (387) found a sufficiently large sample size to be at least 100 to 200 subjects. The statistical method used to assess validity and reproducibility will influence the sample size. For example, a sample size large enough for the Bland-Altman method to allow the limits of agreement to be estimated precisely, necessitates at least 50 subjects, but preferable more (100 or more).(18) Most validation studies include both genders.(17)

5.2.3.3.3   Number of reference method days

Substantial day-to-day variation exists in dietary consumption,(471) which complicates obtaining an accurate assessment of the 'usual' intake of foods and nutrients. Variation in dietary intake occurs as each person differs in terms of the everyday items and quantities consumed. Such variability impacts the number of days necessary to assess 'usual' dietary intake accurately.(472) Although within-person dietary variation among subjects is high, one solution to overcome this problem of day-to-day variation in intake and to reduce random measurement errors due to day-to-day variability in dietary intake, is to obtain an adequate number of days of dietary intake to represent the true intake of each subject.(411,435,471) When used as a reference method, dietary records or 24-hour recalls should thus be obtained for an adequate number of days to assess usual average intake for most nutrients (17,18,452) and avoid imprecision in the estimate of a subject's longer term intake (466) and attenuation of the correlation coefficient between a nutrient and another factor.(471)

Dietary intake estimates may be required for various time frames (few or more days or months, a year or longer) for a group or for an individual.(473) However, the number of dietary assessment days required varies substantially from nutrient to nutrient because of their markedly different magnitudes of within-person variation; for nutrients with relatively small or lower ratios of within- to between-person variation (food energy, total fat and carbohydrate), the number of days required diminishes rapidly and may require only a few days of record or recall.(373,471,473) When the within- to between-person variation in intake is larger (vitamin A), an increased number of days are required (373,411,473) to obtain a reasonably accurate reference intake. Fewer recording days would be needed for larger groups to estimate mean nutrient intake, and considerably more recording days for smaller groups to estimate individual nutrient intake at the same level of statistical precision. The level of accuracy required also differs according to the nutrient under

study. For instance, higher accuracy may be required in determining the intake of vitamin C, which is not stored in the human body for long periods, than in determining the intake of vitamin A, which is stored for long periods.(473)

Cade et al. (17) found that between two and 28 days of weighed intake were collected in studies, which used a weighed record as the reference method, and one to 28 days of recalls collected in studies, which used the 24-hour recall. Ishihara et al. (411) found the number of dietary record days needed to estimate the intake within 20% of the true intake mean within their subjects with high within-person variation in intake, to be about 17 to 19 days. Cade et al. (18) indicated that to adequately assess an individual's diet, 14 to 28 days are generally required. There is some evidence indicating that increasing the recording period of the reference method provides a better representation of the usual diet, which is more comparable to the information gathered by the FFQ.(452) Molag et al. (85) did find that for most nutrients, correlation coefficients were markedly higher when the reference method was applied over a higher total number of reference days of eight to 14 days (medium reference period) than for one to seven days (short reference period). However, large numbers of measurements per subject are expensive (346,471) and often not feasible.(471) Large numbers of recording days would not be logistically suitable for most research and clinical settings.(445) The dietary record and the recall require substantial review/interview or processing time to collect information and process it into a format ready for analysis.(373)

Cade et al. (18) reported that few studies managed to achieve this with most using between two to five replicates (days) per subject.(14,18) Although calculations can be made using available dietary intake datasets to establish the optimal number of dietary recording days necessary for use in a validation study, Stram et al. (466) utilized calculations considering the cost ratio and the variance ratio in day-to-day nutrient intake and in true dietary intake for a number of nutrients to establish the 'optimal' number of dietary recording days to use in a cost-efficient dietary validation study. They judged that, in most validation studies, the optimal number of days required would seldom be more than four to five dietary records per subject and that in many cases two to three would suffice. Rosner and Willett (471) discussed the use of multiple dietary records in estimating correlation coefficients between exposure variables, like the diet, which are measured with error, and other related variables, and generally found no justification for obtaining more than two to five replicates per subject, assuming that the study cost is proportional to the number of measurements. Rosner and Willett (471) moreover support the strategy of maximizing the number of subjects and minimizing the number of replicates per subject, which may have advantages in addition to statistical efficiency including easier recruitment of subjects and the establishment of a sub-sample more representative of the study group.

Willett et al. (38) only found modest improvements in nutrient correlation coefficients between their FFQ and the dietary records when the records were increased from that of 14 to 28 days. Molag et al. (85) found that correlations did not improve on extending the reference method dietary collection beyond 15 days or more (long reference period). Palaniappan et al. (472) used within- and between-subject variation in intakes for foods and nutrients in adjusted and unadjusted models to establish the number of days necessary to accurately assess food group and nutrient intakes by various degrees by two different methods. The variance ratios found between the two models firstly indicated the need to control for confounders (gender, age, smoking, education, season and family size) to obtain reliable intake estimates of most foods and nutrients. The variance ratios were in general found to be higher for food-groupings than for nutrients, but not for the fruit (including juice) and milk food groups. These higher variance ratios meant that more days would be necessary to assess food intake than nutrient intake. Using both within- and between-person variances in their computation for establishing the number of days, they still found that about two to six days would suffice to estimate nutrient intakes with reasonable precision.(472)

Maintaining dietary records for longer than seven days might theoretically improve the estimate of usual nutrient intake, but might impose a greater subject burden of record keeping.(404) Several researchers have found three days to be an acceptable compromise over seven days that may be an advantage in terms of the gathered data.(474) Gibson (349) reported a study on elderly subjects in which regression analysis showed that records from the first two days of record keeping were more valid for assessing group comparisons than those from the last three days, because of deterioration in accuracy of recording. Usable records during the last three days (day five to seven) were from the more highly educated subjects, resulting in a sample bias. According to Gibson,(349) these results question the apparent validity of the seven-day record to assess usual intake. Molag et al. (85) found that for all the nutrients they considered in terms of the number of successive days which dietary records were kept, the correlation coefficients were lower when the reference method comprised dietary records kept for more than five successive days.

As alternative, deattenuated (or corrected) correlation coefficients can be used, which estimate the true association between two continuous variables after removing the effect of random measurement error, which is the within-person variation in dietary intake. The calculation of corrected correlations can be useful in evaluating the validity of a FFQ when assessing longer term intake due to variability in day-to-day intake. The correlation between the questionnaire and true usual diet could be represented by a large number of days per subject of accurately assessed intake (dietary records or 24-hour recalls). Alternatively, it is possible to obtain a small number of daily assessments per subject to measure within- and between-person variance and then to

calculate a corrected correlation coefficient to represent the correlation between a single FFQ and true dietary intake (average intake) over a large number of days of diet recording. In this case, the attenuation factor is determined by the correlation between the observed and true mean nutrient intake and number of days, considering the within- to between-subject variance ratio.(471)

This approach of employing corrected correlations, based on a small number of dietary recording days, can have major advantages in terms of cost and feasibility compared with the alternative of obtaining large numbers of recording days per subject. The use of corrected correlation coefficients may provide more valid conclusions in situations in which the burden of obtaining extended recording days would eliminate many subjects, and thus reduce the generalizability of findings, and alter the behaviour of the subjects. The corrected correlation coefficient will usually be of more interest than the crude correlation coefficient unless sufficient replicates per subject are obtained to reduce the effect of within-person variation.(471) In practice, it may be better to collect an adequate number of 'independent' replicate dietary records or 24-hour recalls allowing estimation of the variance and then using this information to statistically adjust the comparison of the FFQ and reference method.(18)

5.2.3.3.4   Selection of reference method days

Because individual's day-to-day dietary intake varies considerably, day-to-day variation should be taken into account when selecting the days for dietary assessment. Representation of weekend days and seasons should be considered in the selected days.(474) If the difference between weekend day and weekday intakes is large enough, a stratified selection of days must be considered so that each subject has at least one record from a weekend day and from a weekday.(466) The selected days may be successive days or non-consecutive random assigned days.(416,474) A random sample of a sufficient number of selected days would probably be the most suited to allow estimation of the variance components,(18,474) but it is costly to implement.(474) Randomized sampling of all days of the week would be necessary to give true representation, but seasonal patterns in the usage of particular foods will still exist.(475) Sampling based on concerns like intake on weekends being different from that on weekdays and seasons, provided group intake estimates similar to those provided by a simple random sample.(474)

Larkin et al. (474) conducted a study to determine whether three days of dietary records from randomly selected days or from successive days, more closely estimated the energy and nutrient intake values obtained from 16 days over a one-year period. Although the random day sample produced markedly smaller absolute differences in intake estimates over the consecutive day sample, the group means for energy and nutrient intakes were similar. Thus, the random day sampling may be preferred for describing dietary intake of individuals and small groups when the gain in reliability is worth the cost. Based on the findings, both methods were suited for use in

large groups. Participant convenience often contributes to their study compliance and on-going participation.(474) According to Larkin et al.,(474) researchers must weigh the slightly improved accuracy advantage of random over consecutive day recording against the probable increase in cost and inconvenience. On the individual level or for a small group, the random selection of days is preferable, but the gain in precision must be considered against the cost and inconvenience. For a large group the decision is not that important according to the findings of Larkin et al.(474)

However, Nes et al. (71) found that intakes estimated from a record of non-consecutive days correlate better with 'usual' intake than intakes estimated from consecutive days. Less within-person variation in dietary intake is found across consecutive day intakes. This non-independence of intake on successive days requires sampling days at random intervals when possible.(385) Consecutive day (mainly three or four days) (54,71,361,364,377) and non-consecutive day (400,472) selections occur in dietary assessment studies. Gibson (14) stated that if data can only be collected on consecutive days, dietary intake data of three days should be obtained.

Incorporation of weekday and weekend days were considered in numerous studies (54,377, 400,408,425,443) to ensure a well-matched ratio of weekend- to weekdays.(380,386,408) Some studies considered selection of different days to make sure that all the days of the week were included (14,364,380,386) and there was coverage of all seasons.(364)

5.2.3.3.5   Reference period

In validation studies, the dietary intake should be assessed over the same time period.(14,18,476) The test method and the reference method chosen should not only measure similar dietary aspects, but cover the same time frame, and the assessment period should correspond to this time frame (current, past or usual intake).(17,18,349) If required, the time frame relating to the association between the biological variation and variation in dietary intake should be considered.(17,18,387) The completion of the two methods must be carefully considered so that completion of the reference method does not influence responses to the test method. For instance, the process of measuring and recording dietary intakes may sensitize subjects with respect to their dietary consumption so that they complete the second method more precisely than the first.(349,387) Too long a time interval between the two methods, however, may introduce seasonal effects on dietary intake.(38)

5.2.3.3.6   Completion sequence of test and reference methods

Validation study results might be biased because whatever method is conducted first could influence the completion of the following method.(398) Completing the dietary assessment using the reference method first may in itself draw subjects' attention to their diets.(18) Brown et al. (476) pointed out that because their subjects completed the dietary records for the most part in

their study before the FFQ, it is likely that measuring, weighing and recording of the dietary intake modified or improved estimates provided on the FFQ. To minimize this effect, the sequence of administration should ideally be assessment of the test method (FFQ) ahead of the assessment of the reference method. Subjects would in the main investigation in which the test method is to be used come across it free from any other dietary assessment, and the validation process should imitate this.(18) A number of validation studies (71,80,347,398,451,452) indicated applying this sequence of administering the test method to the subjects before the reference method, while several others did not apply this sequence.(80,346,375,476)

5.2.3.3.7   Interval between test and reference method administration

The interval period between the administrations of the test and the reference methods should be chosen to reduce any training effect and memory influence of one method on the other.(371) A short time interval, less than 24 hours, between the two methods indicate that the methods were not independent of one another.(349) Short periods of a month (371,476) and 10 to 15 days (451) or about two weeks (422) have occurred between such administrations.

5.2.3.3.8   Subject training

The existing view is that study subjects must be trained to complete dietary records (381,448) that can occur in a group setting.(435) The dietary record should thus be introduced and explained before the first recording period.(470) Subjects are carefully instructed by a suitably trained or qualified professional on how to record food and beverage consumption (41,400,404,442) and to do so while they are eating and drinking on the recording days.(452) The recording instructions entail a complete description of items consumed, including brand names and food preparation methods,(41,54,452) as well as portion size estimates (41,452) usually in a specially designed booklet in the form of a diary (411,442,470) that may be accompanied by an instruction booklet with examples.(442,470) Subjects are instructed to report portion size results as weighed or as household measures.(411) Utensils, like measuring cups and spoons along with rulers, are used and often provided to enhance accuracy in reporting estimated amounts consumed.(54,404,411) Foods can be recorded in common terms like the number of units.(404) Additional training points usually covered include recording of composite dishes (recipes) or food prepared by others.(41) Subjects are in a similar way instructed on how to complete the FFQ.(361,435)

5.2.3.3.9   Review of subject dietary information

A further practice is that records are reviewed directly with the subject by a suitably trained or qualified professional after completion, to ensure completeness, clarity and accurateness of food descriptions and portion sizes.(41,43,379,381,435,448,451,470,474,477) Subjects may be probed for foods consumed, but not recorded.(477) It is easier to directly review the dietary records while subjects are present to answer any arising questions,(470) but further follow-up with subjects may

occur if additional information is required.(381,442) Edits, corrections and additions are then made by the reviewers, sometimes on specially designated pages in the dietary record.(477)

In just about the same way, FFQs are checked with subjects after receipt for completeness, clarity and accurateness that reviews missing responses, consistency, range and other response errors as FFQs are often entirely self-administered (91,347,382,435) and the reported information supplemented (91) and corrected (379) whenever feasible. In the study of Grootenhuis et al.,(91) half of the subjects had to be contacted telephonically to obtain additional information. Review of self-administered FFQs therefore seems to be fitting. In comparison to self-administered FFQs, far fewer interviewer-administered FFQs produced correlations below 0.50 in validation studies. Because application of an interviewer-administered dietary assessment method is time-consuming and costly, a mixed approach can be used by obtaining the trained professional to review the self-administered FFQ.(361)

Caan et al. (361) evaluated whether utilizing nutritionist review of a self-administered FFQ could enhance the reported information on a self-administered FFQ. They found that reviewing the FFQ information increased the correlations and percentage agreement into quintile categories with the dietary records for all nutrients, but not calcium. However, such reviewing aimed to achieve precision of reported intake for a specific nutrient or food group, lead to overestimation of its dietary intake. In this study of Caan et al.,(361) nutritionist review specifically probing to acquire more exact information on fat consumption, increased the estimated energy intake, while overestimating absolute fat intake, producing an even greater overestimation of percentage energy from fat than was found before the review process. Error could be induced if the same trained professional reviewed both the dietary record and the FFQ.(361) Caan et al. (361) considered this doubtful, since dietary record retrievals focus on obtaining adequate information on indicated foods to allow truthful coding and analysis, while review of the FFQ focuses on probes for items in the food list that might have been overlooked or the frequency of intake overestimated.

5.2.3.4 *Statistical Methods for Data Validation*
A number of statistical methods are available to evaluate the validity of a method (or instrument) relative to another.(18,387) Using more than one method adds credibility to the findings,(18) as there is no agreement on which statistical procedures are best for evaluating the validity of dietary assessment methods.(461) Evaluating the extent of the comparability between the test and reference method(s), or the relative validity of the FFQ, takes place at two levels: those assessing agreement on an aggregate or group level and those assessing agreement at individual (within-pair) level.(349,378) The statistical methods to assess agreement between methods include comparisons based on continuous and categorized data.(371)

For assessment of agreement at group level, the mean intakes determined by the test method (FFQ) and recorded by the reference method (recorded intake) are compared (349) to determine the ability of the test method to assess the absolute intake at group level.(378) This is determined as the difference between the means (349,369,378,470) and resultant percentage relative difference (379,470) between the two methods of assessment to convey the average tendency of subjects to over- or underestimate their dietary intake by FFQ intake to recorded intake.(470)

Most studies evaluate validity at the individual level of which the main approaches focus on the assessment of the extent of individual within-pair agreements (379) by assessing the capacity of the FFQ to correctly position subjects according to the dietary intake.(349,378) This is done by ranking them along the distribution of intake from low to high (349,378,379,461,470) and by using cross-classification in categories by the two methods.(371,379,461,470) In epidemiological research, positioning of subjects with respect to dietary intake (ranking and cross-classification into categories of dietary intake) may be more important than estimating absolute intake and would thus be the most useful approach to evaluate the validity of a FFQ developed for epidemiological research.(39,403) For epidemiological and clinical research, the ability to position or rank subjects by relative levels of dietary intake permits the evaluation of aetiological hypothesis and interactions. However, for clinical and public health purposes, as well as for the refinement of aetiological hypotheses, the estimation of absolute levels of dietary intake is important,(355) as an accurate estimate of the absolute amount consumed is required to compile recommendations and compare studies.(39)

Statistical approaches and methods generally used, to determine the relative validity of the data gathered through a FFQ and another dietary intake assessment method, include among others assessing the difference between mean intakes (Student's paired t-test or Wilcoxon's signed rank test) and studying method associations through ranking by relative levels of intake (correlation and regression analyses) (80,349,379,470) and contingency table (80) or cross-classification (379,470) of intake. Correlation coefficients (Pearson or Spearman) followed by the Kappa (K) statistic (cross-classification) and the Student's t-test (difference between means) are the main statistics used in these studies.(17) Correlation coefficients and cross-classification are usually considered as an assessment of the precision by which the FFQ can position subjects by dietary intake level.(470) According to Cade et al.,(17) correlation and regression should not be the only methods used, but should be accompanied by the Bland and Altman analysis to assess the data validity. Sensitivity, specificity and predictive values may be of value for binary data.(18)

Alternatively or in addition to the above, other and more advanced statistical methods (statistical equation models) for validation studies can be used, which consider the measurement errors

ascribed to FFQs, but these require expert statistical advice.(17,18) Due to the reservations about application of the correlation coefficient in validation studies,(478) MacIntyre et al. (479) hoped that the advanced structural equation models, that require sophisticated software and expert statistical knowledge to calculate, would provide an alternative to the use of the Spearman rank order correlation coefficient. Application of a structural equation model, by which the mean intake estimates obtained from the seven-day weighed records was compared to the quantified FFQ estimates, provided results close to those provided by the Spearman correlation.

5.2.3.4.1   Paired difference tests

To determine the ability of the test method to reflect group means, the agreement or comparability of the two methods at group level are evaluated by calculating and comparing the mean amount for the intakes obtained from the test and reference methods as indication of absolute agreement,(91,349,383) and calculating the percentage relative difference in the mean daily dietary intakes between the two methods.(91,461) The extent of the under- or overestimation by the FFQ can be described as the difference in dietary intake as assessed by the FFQ minus that assessed by the reference method.(445) Now the null hypothesis of no difference between the group intakes of each of the two methods can be tested,(355) namely whether the intake assessed by the FFQ and the reference method differed significantly from zero.(378) Absolute differences between the two methods significantly different from zero indicate that the FFQ cannot accurately assess individual intakes.(445)

The Student's paired samples *t*-test can be used for normally distributed data to test if the two means are statistically different at some pre-determined probability level (95% confidence limits).(18,91,347,349,469) However, dietary data distributions are usually skewed and therefore less likely to be normally distributed or parametric, and non-parametric tests may be more appropriate to use.(18) Here the median intakes (50th percentile) and selected percentile points (25th and 75th percentiles) are used to quantify the average intakes and their variability.(349,461) In such cases, the Wilcoxon's signed rank test based on comparability of the medians is appropriate for testing statistical differences.(18,349,461) Interpreting the relative validity results at group intake level can reflect systematic (fixed or constant) bias in the test method (91,356,442) reported in some studies as the instrument effect.(54)

5.2.3.4.2   Crude correlation coefficients

In epidemiological studies of the relation between dietary intake and chronic disease, ranking of dietary intake is generally considered (14,381,444) as this may be more critical than accuracy in intake to reflect the diet-disease relationship.(350,381) FFQs are generally considered more worthy for ranking subjects according to food and nutrient intakes, than for quantifying actual food and nutrient amounts consumed.(362,387) FFQs must have the ability to position subjects along

the intake distribution so that subjects with low intakes can be identified from those with high intakes.(387,461)   Consequently the validation of FFQs is usually assessed with respect to its capability to accurately position subjects based on their reported dietary intakes from the FFQ and true usual dietary intake, which is defined by the mean intake reported in, for instance, a series of dietary records.(377,444)   Correlation coefficients are by far the most widely and commonly reported statistical method to assess validity (and reproducibility) of questionnaire data (18,349,425,462) and were found to be used in 83% of the validation studies reviewed by Cade et al.(18)

Assessment of individual dietary intake agreement between FFQs and a reference method (dietary records and recalls) when assessed as a correlation coefficient, like the Pearson correlation coefficient, have been expressed as precision.(42,442)   This indication is flawed because correlation coefficients do not determine the agreement between two methods, only the degree to which the data gathered by the methods are related.(17)   Correlation coefficients estimate the strength or degree of the linear relationship between the dietary intakes as determined by the test and reference dietary assessment methods (91,355) and convey the relative validity for the ranking of subjects based on their dietary intake.(378)   As correlations are used to determine the strength of the association between two estimates, the higher the correlation established thus the better the validation.(462)   Although the correlation coefficient was criticized as a means to establish absolute agreement, this objection may not apply as FFQs are developed to rank subjects rather than to determine their absolute intake.(461)   Although the use of the correlation coefficient for validation purposes is criticized,(17,18,379) it has attractive properties, such as that it reflects the questionnaire's capacity to rank subjects according to exposure (more important than absolute agreement) and facilitates comparison with other validation studies that used questionnaires.(379) Correlation coefficients have the performance benefit that they are not influenced by systematic over- or underestimation of one method in relation to the other, as they reflect the correspondence in positioning between the methods.(435)

Before determining the Pearson correlation coefficient, the distribution of the dietary intake has to be checked for normality.(383,461)   The normality of a distribution can be checked by using tests, like the Komogorov-Smirnov test (347,445) or Skewness/Kurtosis test.(451)   If the data do not have a normal distribution and are skewed, logarithmic transformations need to be performed to improve or approximate the distribution towards normality, before calculation of the Pearson correlation coefficient between the dietary method intakes.(43,54,76,379,383,435, 442,461,470,480,481) This is often the case as most dietary intakes are right or positively-skewed toward higher values.(41,83,345,365,378,405,410,442,446)   Log-transformation, however, do not normalize all dietary intake distributions.(461)   In the study of Masson et al.,(461) log-

transformation of the data did not remove the impact of outlaying data points on the Pearson correlation coefficients. Skewness greater than one, reflecting data distributions that differ significantly from normal distributions, were found for intakes of some nutrients. Because a normal distribution is not relevant to all nutrient intakes, the Pearson correlation coefficient may provide misleading results.(461) Non-parametric statistical methods should be considered for such skewed distributions for dietary intake data in validation analysis,(356,461,482,483) like the Spearman rank order correlation coefficient.(356,379,444,461) Highly skewed distributions (vitamin A intake) may require such a non-parametric approach.(475)

Masson et al. (461) indicated that the Spearman coefficient might be more appropriate because it utilizes rank order and are as a result not as sensitive to outlaying data points as the Pearson coefficient. Therefore the usual methods of evaluating correspondence in positioning between a FFQ and a reference method is to determine the Pearson correlation coefficients after log-transformation of the data, since several nutrient distributions will be skewed towards higher values and/or the Spearman rank order correlation coefficients for data that do not have a normal distribution.(83,461) For ease of interpretation, the correlation results are generally reported back-transformed on the logarithmic transformation to normalize the distributions.(42) The results of the analysis performed on the transformed data may not be appreciably different from those on the crude values.(480)

Validation work has mostly used standard methods of correlation based on the traditional null hypothesis of no correspondence between dietary assessment methods.(484) However, although the dietary methods are different, they are expected to assess the same aspect of dietary intake, making the null hypothesis of no expected correlation, incorrect since a positive correlation is to be expected.(368) It would therefore be more reasonable to test the probability that the correlation coefficient differs from approximately one rather than nil.(474,484) Herbert and Miller (478) proposed a unique statistical approach to attend to the issue of inter-method correspondence based on the null hypothesis that the dietary methods should correspond, since they assess the intake of the same dietary aspect.(484) Although Herbert and Miller (478) used a null hypothesis of 0.95 (H_o: ρ=0.95) as standard, Simon et al. (484) in their application of the methodology, believed that this standard was too stringent. They chose a null hypothesis of 0.70,(H_o: ρ=0.70) as they believed it to be a satisfactory correlation estimate between two dietary methods and more in line with past validation studies of dietary methods that declared 'agreement' between any two methods, when the correlation coefficient lies in the interval 0.50 to 0.70.(387,484) This methodology does not seem to be used generally in dietary intake assessment research.(484)

5.2.3.4.3   Adjusted correlation coefficients

Relative validity between dietary estimates of records or recalls and FFQs can be assessed by calculating crude or unadjusted and adjusted correlation coefficients.(41,371,383,411,470) Associations between nutrient intakes from each method have often been adjusted for energy,(41, 43,371,379,383,403,405,411,435,470) in addition age,(358,383,405,469,470) gender (358,379, 383,403,405,433,470) and other subject characteristics like occupation.(358,433,469) Applying adjustments for subject characteristics were not considered in studies where the subjects, for instance, were a homogenous group (358) and have inconsistent results on the nutrient correlations, by having no effect on the correlations (433) and decreasing the correlations.(403)

5.2.3.4.3.1   Energy-adjustment

In epidemiological studies and dietary intervention trials, the effects caused by specific nutrients must not be associated with total energy intake. If subjects with disease tend to over report their dietary consumption, a nutrient may be correlated with the disease outcome measures because of this error in reporting overall intake. Analysing the association between dietary components and disease risk should be based on an iso-energetic principle as in controlled trials, which are usually designed to be iso-energetic. Fortunately, depending on the extent to which the total energy intake of a subject changes during a specific period, a change in energy intake will be accompanied by a change in energy balance and thus body weight. Subject energy intake cannot change by more than about 10% without noticeable change in physical activity or body size. Such a change in energy balance that resulted in a change in body weight would have an influence on numerous physiologic and health outcomes. Thus, any findings with regard to the specific dietary component being studied would be difficult to interpret because the effects of energy balance could not be distinguished from the effects of the specific component.(485)

Self-administered FFQs are usually semi-quantitative because only fixed or subjective portion sizes of small, medium or large are obtained. The combination of being semi-quantitative and having missing dietary items limits the accuracy of the estimated dietary intakes.(354) However, evidence supports overestimation of dietary intake when using comprehensive FFQs as assessment method.(350,486) The direction of bias by dietary records and recalls as assessment methods is somewhat uncertain. Underreporting seems the most common bias with dietary records or recalls.(486) Underreporting or low energy reporting is a compelling concern in obtaining valid dietary intake estimates (487) and interpreting dietary estimates.(488)

The likelihood that only one factor explains the energy underreporting of subjects in dietary surveys is not plausible. It is most probably explained by a combination of several factors.(488) Evidence furthermore suggests that energy underreporting does not impact foods and nutrients similarly, and possibly transpires in only some subjects.(488) Macdiarmid and Blundell (487) classified the

subjects who admitted underreporting into two groups: those for whom recording of their dietary intake was inconvenient and those who misreported for reasons of embarrassment or guilt. The former group included those who either intentionally or reluctantly altered their diet due to the effort, inconvenience or difficult circumstances linked to weighing and recording all dietary items consumed.(487) Although potentially the most accurate method to assess dietary intake, the completion of weighed dietary records does require greater effort than other dietary assessment methods, which previously presented a barrier.(488) The tendency to omit dietary items from weighed dietary records makes incomplete recording a primary explanation for underreporting.(488) Macdiarmid and Blundell (487) indicated that it should not be too surprising that dietary records inhibit or modify consumption, as it is used as behaviour modification procedure in the treatment of obesity.

The latter group includes those reluctant to report eating foods known to be unhealthy, which is due to the health consciousness trend. Women are furthermore more likely than men to underreport with the health consciousness trend, which is also thought to be of more relevance to women. Obesity is indicative to underreporting but the impact of education and social class in this matter is less clear. In addition, the survey period may possibly overlap with a period of low energy intake. Alternatively, subjects may change their dietary intake during the period of the survey; however, this is an unlikely explanation.(488) Underestimation challenges the credibility of the reported data and if known or suspected bias in reporting exists, the energy and nutrient intakes are likely to be incorrect.(489) The use of nutritional biomarkers would be useful since they confirm the validity of what was actually consumed. However, biomarkers are of no assistance in situations where subjects have altered their usual diet to escape inconvenience or embarrassment.(487)

The intake of several nutrients and other food components correlate with total energy intake (86,485) simply because they provide energy or because subjects who ingest more total energy generally eat more of almost all kinds of food that contribute to the intake of most dietary components.(485) Intakes of particular nutrients, specifically macronutrients, which are related with total energy intake, will tend to be non-causally associated with disease risk if total energy is associated with the disease risk.(485) Control for confounding by total energy continues to be a concern when evaluating the effect of such a dietary component. Energy-adjustment provides determining a correlation independent of total energy intake, partly adjusting for differences in intake that may be related to body size or activity levels.(485) Because nutrient intakes are often simply reflected by the total amount of food consumed, many researchers adjust nutrient intakes by total energy intake.(86) Agreement between methods seems to increase with use of energy-adjustment for nutrients correlated with energy intake,(350,372) as energy-adjustment reduces the

measurement error in reported intake (372) because subjects tend to overestimate their intake on FFQs.(350)

There is no assurance that the findings would be positive for nutrients that do not contribute to energy intake.(449,481) Such nutrients that are not energy-dependent or have a low relation with total energy intake that are often excluded from energy-adjustment, include cholesterol,(86,424) vitamin C,(86) the antioxidant vitamins,(490) calcium and iron.(424) Nutrient intakes from vitamin or mineral dietary supplements are not related to energy intake.(485) Because most of the food groups correlate with total energy intake, energy-adjustment is used with food group data.(390) By adjusting energy intake, the effect of energy underreporting is weakened in the analysis of both nutrient and food intakes, although as food groups are not influenced similarly it is not the full answer.(488) Short and dietary specific questionnaires, like those determining calcium intake, will not provide for estimating daily energy intake.(88)

On the whole adjusting for energy do not provide a consistent effect on correlation coefficients.(86) In some studies (371,380,386,481) adjustment for energy increased the correlation coefficients between the FFQ and the other dietary assessment methods, sometimes only inconsequential,(365) while in others,(430) energy-adjustment did not affect the correlation coefficients. When nutrient intakes are correlated with energy intake, energy-adjustment should minimize the associated measurement error between energy and nutrient intake and thus increase the association between the two dietary assessment methods.(41,365)   If everything is underreported proportionately, energy-adjustment procedures would be preferred in the analysis. If there are differential biases in the reported intakes, however, energy-adjustment procedures would not solve the bias but possibly aggravate it.(489) Energy-adjustment may, for instance, reduce the between-subject variability in intake and thus the correlation coefficients.(365) FFQs are generally developed without consideration to adjust for energy intake in the data analysis. If required, the FFQ should be comprehensive enough to establish energy intake.(17) Bellach and Kohlmeier (491) by injecting errors into a dataset concluded after applying several energy-adjustment methods to the dataset that recall bias cannot be corrected by energy-adjustment. Many investigators (38,405,444,476,492) found that correlations for micronutrients were minimally affected by energy-adjustment, as they are only weakly correlated with energy intake.(38,405)

Since relations with dietary intakes not associated with energy intake are critical in epidemiological analysis, it would be of worth to evaluate the validity (and reproducibility) based on the crude absolute dietary intakes, as well as the energy-adjusted dietary intakes rather than just the evaluation of the crude intakes in validation studies.(485) Several statistical methods of energy-adjustment (nutrient residual, standard multivariate, energy decomposition or partition and

multivariate nutrient density) are available (485,493) of which quite a few are disease-risk models and formulations to account for the impact of total energy intake in epidemiological analysis of dietary intakes.(485) Several investigators have used the residual method to calculate energy-adjusted correlation coefficients.(41,346,371,379,362,383,390,411,435,442) Adjustments for energy comprise such assumptions as, that portion size and dietary intakes are all reported with the same degree of precision and that omissions or biases in the dietary reporting are proportional to the amounts of food consumed.(351) Gibson (14) as a result indicated that correction for energy intake is appropriate only when underreporting occurs at the total diet level and recommends that more research be undertaken to determine when energy-adjustment is suited.

5.2.3.4.3.2 *Adjustment for correction of day-to-day variation in dietary intake*

Adjusted correlation coefficients can be computed for correction of measurement error (termed deattenuated correlation coefficients) (383,444) by considering the influence of within-person day-to-day intake variation determined by short-term period coverage dietary records or recalls on the correlations between these methods and longer term period coverage FFQs.(39,383) Variation in day-to-day intakes of numerous nutrients is substantial.(494) A single day of dietary data reflects intake of some foods even less well than it does of nutrients.(39) Dietary intake obtained from a single day, as with dietary records and recalls, may thus not produce a good estimate of average intake over a longer period (462) and will misrepresent a subject's usual dietary intake.(39) Day-to-day variability in dietary intake can thus seriously affect the validity of short-term methods, like dietary records, as measures of longer term intake.(471)

In order to obtain habitual or usual dietary intake, the recording period must be increased from several days to weeks, depending on the nutrient studied (494) with several dietary records obtained and the questionnaire validated against the average of these, on the assumption that this average is accurate enough to represent the true average intake.(462) Studies of the day-to-day variability in dietary intake have shown that dietary records can contain considerable random error, even when averaged over several days.(495) Such within-person variation in food and nutrient intakes indicate that a single or a few days of dietary recording is not a completely suited method to assess usual intake.(471) It can reduce correlations between questionnaires of longer term intake and reference methods of short-term intake.(471,495) The underestimation is called attenuation.(481) Obtaining the within- and between-person variation in dietary intake assessed by records, can be applied to correct correlations between such short-term methods and longer term methods as FFQs.(471) Correlation coefficients provided as deattenuation, adjust for random measurement errors in dietary records and recalls due to within-person variation in short-term dietary assessment methods based on a small number of intake assessment days under the measurement model, in which measurement errors have a mean of zero, a common correlation and are independent of the true dietary intake.(442)

Formulas have been proposed to adjust correlation coefficients for the imprecision provided by within-person variation or random error in intake of the reference method.(471,496) To correct for variability in the day-to-day variation of dietary intake assessments, correlation coefficients are deattenuated using the variance ratio (within-person or intra-individual variance divided by between-person or inter-individual variance calculated from the number of replicate measurements).(14,471,496) Analysis of variance (ANOVA) can be used to separate the within and between-person variance components of dietary records and recalls (treating them as units of observation, such as four recalls) and the results used to deattenuate the Pearson correlation coefficients according to the provided formulas.(442)

Correction of crude correlations between dietary records and questionnaires for the within-person variability in the dietary records provides more accurate information concerning the validity of the questionnaire. Considerable improvement in the correlation coefficients is seen for foods with high within-person variability, like most vegetables, some fruits and other dietary items.(39) Due to their relatively large day-to-day variability in intake, the effects of deattenuation are most pronounced for cholesterol and vitamins C (379,470) and A.(379) For lower correlations (possibly below 0.30 or 0.40) attenuation will be so great that it will not be that easy to detect relations.(18) These correlation coefficients adjusted for within-person variability based on statistical formulas that assume that the errors on FFQs and reference methods are unrelated (a postulation known not to be true) are at present used, as there are no other acceptable methods for evaluating the validity of FFQs for nearly all nutrients.(392) These methods although used,(39,41,411,414,476) seem not to be used habitually.(462)

5.2.3.4.4   Regression analysis

Regression can be utilized to calibrate one method compared with another with regression analysis undertaken in 4% of the validation studies reviewed by Cade et al.(18) Calibration can quantify and adjust measurement error in the study, if the reference method used as the basis for calibration contains errors that are not related to that of the test method used.(51) While correlation is a useful indication of the validity of a questionnaire, it is more informative to obtain a regression equation between the questionnaire and the reference method.(462) Validation studies generally compare two datasets for strength of association that are dependent on the extent of the variation within the datasets.   Correlations, however, do not report on the extent of misclassification.(51) Due to the absence of a gold standard for validation, the best alternative to quantify and adjust for measurement error in a study is to calibrate one method against another believed to be more accurate for the same use.(410) Regression relations are used in many calibration studies to estimate the slope of the regression of intake from the FFQ on usual intake, which is useful to determine the nature of the measurement error or bias in the

questionnaire.(377,462) Calibration will assist in quantifying the difference between the methods and will allow adjustment of one to the levels of the other and adjustment over time of any time-, season- or measurement-related differences.(410)

Linear regression models are compiled with the FFQ dietary intake as dependent variable and the reference method dietary intake as independent variable.(378) To evaluate the validity of dietary intake from a FFQ with blood levels, linear regression can be used by assigning each subject their biochemical value and including it as the dependent variable in the regression model.(352) Regression analysis may be particularly valuable when validity is evaluated using biomarkers.(14) If a regression of questionnaire versus average dietary record intake estimates is presented, then optimally the slope of the regression line between the two should intercept the Y-axis at the zero point and show a slope of approximately one.(410,462) If the linear regression coefficient is not significantly different from one and the intercept not significantly different from zero, the dietary methods are providing similar data and either can be used as it indicates that the questionnaire is 'unbiased', thus on average the questionnaire intake will equal the average dietary intake.(462)

Two-sided *t*-tests can be utilized to identify whether regression coefficients differed from one (proportional bias).(378) Regression slopes of less than one are often found.(462) Regression slopes below one show systematic flatness of the regression lines of Y on X, a statistical occurrence called 'regression to the mean' (91) or flattened slope syndrome.(417) Different explanations exist for this flattened slope effect. One understanding is that reporting bias is involved.(462) Subjects with high intakes who are inclined to eat a lot, tend to underestimate and underreport their intake on a questionnaire, whereas those with low intakes who eat little, tend to overestimate and over report their intake.(417,462) This feature of an underestimation at high intakes and an overestimation at low intakes is a common characteristic of FFQs.(91,445) This explanation is consistent with the perception that people with extreme eating habits would like to be thought of as behaving somewhat nearer to the norm.(462) Because the SDs of dietary intakes assessed by two methods are often different, it is generally difficult to assess the ability of a questionnaire to provide a ranking or relative categorization of subjects on the basis of a regression coefficient.(387)

Estimates of regression slopes and intercepts, as with correlation coefficients, can be affected by within-person variation in day-to-day dietary intake. Statistical models exist for obtaining estimates of the regression slope that can be used for correction. Repeat assessments using both questionnaire and dietary records are necessary and the frequent absence of repeat assessments may explain why investigators have not routinely used this statistical relation method.(462) Knowledge of the regression relation between dietary intake reported on a FFQ and true average

intake assists in interpreting the results from epidemiological studies and in planning such studies,(462) as regression analysis has direct application in correcting RR estimates for measurement error.(387)

5.2.3.4.5   Category agreement or cross-classification

If the dietary data collected are continuous, a correlation coefficient could be used to evaluate validity at individual level, whereas if the dietary data used are ordered in categories, the percentages of subjects positioned into the same/opposite category could be determined.(461) Epidemiological studies usually address differences in risk of disease on positioning subjects by relative categories of dietary intake as measures of disease association,(38,364,373,470) rather than by specific quantitative or absolute intake values.(364,373) Thus information on the capability of the questionnaire to correctly assign subjects, based on their dietary intake, in categories is important to obtain correct disease risk associations and disease risk estimates.(470)   For instance, comparing the highest and lowest categories of nutrient intake has been used as a measure of association between the nutrient and disease risk.(373)

Many validation studies therefore categorize the intakes of each method and use the agreement in the compiled contingency table (80) or cross-classification analysis of the two methods (14,38) to assess validity at the individual level. Different classifications are commonly used, such as tertiles, quartiles or quintiles, determined from each method's own distribution of dietary intake.(14,18,91, 355,356,371,398,435,444,445,461,470)

Evaluating the capability of both methods to assign subjects in the same or different categories of dietary intake by cross-classification category analysis of the intakes can assess method agreement. The correspondence between the categorical distributions in the contingency table obtained between the two methods is determined to assess the proportion of subjects correctly categorized and grossly misclassified.(18,91,435,445,470) Method agreement and disagreement is presented as the overall proportion or percentage of subjects who are respectively classified correctly in the same category by both methods (identical or exact agreement), the adjacent (+/-1) category and, in extreme cases, opposite categories (top and bottom) (gross misclassification) of the distribution for the two different methods.(18,91,461,469,470) Subjects can also be categorized on the basis of their dietary intakes and their biomarker concentrations.(469)

The K-statistic can be used to compare findings involving small numbers of ordered categories,(17,18) like frequencies of consumption determined by two dietary assessment methods, but it is not relevant for continuous data, except if the data is categorized into a number of ordered groupings.(18) The K-statistic, a measure of exact agreement for ordered categorical data corrected for chance,(18,355,444) can determine how well subjects are cross-classified by

two methods by calculating the number of subjects categorized in the same, the same or adjacent, and in opposite categories.(444) The *K*-statistic is therefore an indication of observed correspondence beyond that probable because of chance.(497) Some of the agreement reflected in the percentage agreement value is due to chance. This is taken into consideration by the *K*-statistic, which indicates how much correspondence is left after removing the expected correspondence as a result of chance.(497) A *K*-statistic of one indicates perfect agreement; for agreement equal to chance, *K* is nil. For instance, if *K* is 0.68, it means that the correspondence between the test and reference methods represented 68% of the possible correspondence beyond chance.(497) Cohen's weighted *K*-statistic (*Kw*) allows for misclassification and reflects agreement plus or minus one category as full agreement.(355) Weighted in this way, the *K*-statistic allows for less than perfect agreement (355) and may be a more suitable indicator of performance (461) and more realistic for most epidemiological purposes.(355)

The percentages misclassified in the cross-tabulation indicate the possible impact of measurement error.(461) However, as cross-classification can group subjects with very similar intakes into different categories and those with widely differing intakes into one category if they are close to the cut-off value, correspondence between the two methods should not be anticipated, especially in studies with small numbers of subjects, in which misclassification of only a few subjects can cause a large difference in the percentages.(461) Since the results of the *K* test rely on the number of categories for comparison, the number of categories can bias the interpretation of the results.(400)

The category classification of the subjects by the test method (FFQ) can be used in a surrogate application. The dietary intakes of the subjects as grouped within the test method categories are obtained from their reference method dietary intakes and these intakes used to derive 'true mean intakes' for each category of subjects as determined by the test method. In other words, 'true mean values' based on the more detailed reference method are ascribed to the category grouping by the test method. This methodology of assigning true mean values to the test method categories now refers to the test method categories as surrogate categories and the test method as the surrogate method.(14,387,347,445) The true mean values assigned to the categories of the surrogate method (FFQ) can be contrasted (347,445) by one-way ANOVA followed by Tukey's *post hoc* tests (14,445) or ANOVA followed by Bonferroni *post-hoc* tests.(347) The assigned actual mean values for the categories of the surrogate method should show a successive increase with significant differences between the category intakes demonstrating discriminative power of the FFQ, suggesting that the FFQ can differentiate between subject groups at different levels of intake.(14,347,445) Differences between the categories in cross-classification occur as a result of the true variation in dietary intake and the measurement error associated with the FFQ. This

surrogate method application allows evaluation of misclassification and error in the intake estimates.(83)

5.2.3.4.6   Limits of agreement or Bland and Altman method

Despite the wide use of correlation coefficients to validate dietary assessment methods, the use of correlation analysis has been criticized by some investigators due to a number of limitations of its use of comparing two methods.(38,498) Correlation coefficients do not indicate the extent to which the methods produce the same results (agreement between the two methods), but rather of the extent to which they are linearly related and hence cannot detect systematic error or bias between the two methods.(368,498) Correlation coefficients depend on the range of the values in the datasets.(498) As the test and reference methods used assess the same aspect a positive correlation is to be expected.(14) Another disadvantage of correlation coefficients is that they are not that suited when comparing findings from dissimilar study groups as they are dependent on the true between-subject variation in the study group and are therefore not comparable between study groups with different variations in their dietary intake.(368) Correlation coefficients therefore only provide a limited indication of the level of correspondence between two methods.(498)

Cade et al. (17,18) suggest that correlation coefficients should never be used singly to evaluate the validity of continuous data, but be supplemented by the Bland-Altman method. However, their review found that the Bland-Altman method was only used in a few studies reporting on FFQ validation.(17) The Bland-Altman method determines in graphical form how well two methods across the range of intakes are likely to agree for a subject, by plotting the mean of the two methods (horizontal or X-axis of the plot) against the differences between the intakes as obtained by the two methods (vertical or Y-axis of the plot) and the 95% limits of agreement for the subjects calculated at ±2 SD units from the mean difference. The limits of agreement (mean difference ±2 SDs) provide an appraisal of the difference between the two methods.(498)

Agreement of mean intakes between two methods does not rule out the likelihood of a non-constant bias. High intakes may be overestimated and low intakes underestimated, or *vice versa*, leaving the correspondence of the mean intake unchanged. The Bland-Altman method examines the likelihood of non-constant bias through computing the differences between the two methods for each subject and the mean values.(498) The Bland-Altman method determines if there is any systematic difference between the methods (bias) and to what extent the two methods agree (limits of agreement). The overall mean difference reveals if one method is inclined to under- or overestimate and the limits of agreement (mean difference ±2 SDs) show how well the methods agree.(18) If there is no bias in the test method, the differences between the two methods will be scattered and clustered along the horizontal line of equality and the mean difference would be close to zero. Bias occurs if the data of the test method falls largely either above or below the line

of equality.(14) It also indicates whether the difference between the methods is the same across the intake ranges, and whether the extent of the correspondence is different for high intakes in comparison to low intakes.(18) For example, there is the occurrence that as the dietary intake increases, the differences between the datasets of the two methods increase.(14)

5.2.3.4.7   Sensitivity, specificity and predictive values and receiver operator characteristic curve

To establish the accuracy of assessment methods further, the sensitivity, specificity, negative predictive values (NPV) and positive predictive values (PPV) can be determined against the setting of a cut-off value or target dietary intake,(83,347,383,445,468) such as nutrient dietary reference intakes,(347,383,445) percentage energy from fat (442) or an adequate daily target serving number of fruit and vegetables.(83,468,482) *Sensitivity* refers to the proportion of subjects meeting the target having adequate intakes by the reference method and the test method (FFQ).(83,347,445) This reflects the number of subjects with an adequate intake by both the reference and the test methods as a percentage of those subjects who have adequate intakes by the reference method.(482) *Specificity* refers to the proportion of subjects not meeting or below the target on the basis of the reference method that fall short on the test method (FFQ).(83,347,445) In other words, the number of subjects with an intake less than the target by both the reference and test methods, as a percentage of those actually having less than the target by the reference method.(482) A PPV reflects the proportion of subjects meeting the target on the FFQ and the reference method. A NPV reflects the proportion of subjects not meeting the target on the test method (FFQ), who are 'truly negatives' on the reference method as well.(83,347,445)

Receiver operating characteristic (ROC) curves can be drawn to further compare the agreement of the FFQ against the reference method. The ROC curves demonstrate the discriminative capability of the test method to detect subjects with dietary intakes above or below the target, by plotting the association between the sensitivity (vertical axis) or 'true' positive rate and the false-positive rate (100% - specificity, horizontal axis). A straight crosswise line from the upper right to the lower left reflects no discriminative capability. A curve lying further upward and to the left reflects a better test result. A FFQ performing well in the context of sensitivity, specificity and positive and negative predictive values can be utilized to function as a screening tool.(383,442) These analyses, however, do not currently have much bearing for flavonoid intake estimate evaluations due to the absence of a dietary reference intake.

5.2.3.5 *Validation Results*

No ideal mean difference, limits of agreement, correlation coefficient or regression slope has been suggested, as it will depend on the objectives of the study.(18) That dissimilar methods like FFQs and dietary records result in alike mean intakes and subject ranking, provides reassurance that these methods are estimating the same dietary aspect.(373) However, validity based on

comparisons of FFQs with multiple dietary records or 24-hour recalls is usually not good.(448) Willett and Lenart (387) noted that validation studies demonstrate that the FFQ as dietary assessment method is remarkably robust.

Jain et al. (371) found that the mean daily nutrient intakes of the FFQ, expressed as a percentage of that determined by the dietary records as the 'gold standard', were generally within 15% of each other. Although the FFQ intakes were higher than that of the dietary records, there was no appreciable difference in the nutrient intakes. Patterson et al. (442) found the mean intakes assessed by the FFQ to be within 10% of those derived from the dietary record for 21 out of the 30 investigated nutrients.

Correlations between FFQ- and recall-derived nutrient intake estimates are often less than 0.40 and rarely larger than 0.60 (448) and between FFQ- and seven-day dietary record-derived nutrient intakes about 0.50 for most nutrients.(469) The correlations considered 'good enough' by most investigators range from 0.40 to 0.70.(392) Correlations for most nutrients fall within the range 0.40 to 0.60,(499) although the range of 0.50 to 0.60 has been indicated suggestive of fairly good concurrence between the test and the reference methods (435) and proof that the FFQ has the capability to rank subjects.(469) Brunner et al. (469) proposed that correlations between the nutrient estimates from FFQs and from dietary records of about 0.50 provides substantial support that the FFQ has the capability to effectively position or rank subjects. Willett (30) suggested that when FFQs are compared with multiple dietary records correlation coefficients could reach 0.60 to 0.70, but that correlations of 0.80 to 0.90 are doubtful. The median correlation coefficients Wakai (399) found between the dietary intakes derived from dietary records and that estimated with FFQs developed in Japan for most nutrients, including energy, ranged from 0.40 to 0.59. It should be noted that the associations of FFQ estimates with both anthropometrical measures and dietary biomarkers are generally weaker. This is likely due to the complex and indirect relationships between dietary intake and dietary biomarkers.(448)

Correlations differ by the nutrient under consideration.(499) Molag et al. (85) reported that for all nutrients considered the correlation coefficients between FFQs and reference methods ranged from 0.45 for energy and protein to 0.74 for alcohol. If the between-person variation in a food group is large, the correlation coefficient for validity will be increased. This may be true for alcoholic beverages, as some subjects drink no alcohol, while others consume it heavily.(399) Cade et al. (17) reported that the correlations for the nutrients from the studies reviewed, was highest for calcium (range 0.20 to 0.89, median 0.56) and total fat (range -0.16 to 0.86, median 0.51) and lowest for vitamin A (range 0.03 to 0.83, median 0.37) and vegetable intake (range 0.16 to 0.72, median 0.38). Masson et al. (461) found the correlation coefficients for retinol and beta-

carotene equivalents to be lower than that for other nutrients that may reflect high within-person variability in intake of these nutrients. It is not easy to assess the intake of vegetables and related nutrients, like vitamin A, using a FFQ. The median correlations found for fruit was 0.51 (range -0.01 to 0.71) and for vitamin C 0.50 (range 0.14 to 0.84).(17)

Wakai (399) in studying the validation of FFQs developed in Japan, found the validity of reported consumption of food groups to be high (median of the correlation coefficients for all studies equal to or above 0.60) for rice, bread, milk or milk plus dairy products, fruit and alcoholic beverages; moderate (0.40 to 0.59) for pulses, fish and shellfish, meat, eggs and green-yellow vegetables; and fair (0.30 to 0.39) for confectionary, fats and oils, total vegetables and vegetables other than green-yellow vegetables. The coefficients were always higher for fruits than for vegetables, which may be due to the inclusion of less items in the fruit group food list.(399) Questionnaires tend to have a long food list of vegetables that may or may not consider vegetables ingested as ingredients in composite dishes.(17) Numerous reasons may explain the misreporting of vegetables that include double counting of items with regard to inclusions in mixed dishes (500) and social desirability bias.(370,500) It might in addition be easier for subjects to report intake frequencies and portion sizes of fruits (as consumption units), opposed to the intake of vegetables, where fruits are more often consumed raw instead of cooked.(399)

Cade et al. (17) indicated that correlation coefficients between FFQs and reference methods were higher for interviewer-administered than for self-administered questionnaires for some nutrients. Using interviewers may have the gain that it provides for direct checking by the interviewer for improper or unlikely responses. In this context the cost of using interviewers, ensuring standardized training procedures and that their presence may present social desirability bias in responses from the subjects must be weighed up.(17) The length of the time frame of the FFQ was considered by Cade et al.(17) They found that FFQs recalling the diary intake of the past month produced slightly higher correlations with the reference method than those recalling the dietary intake of the past year. The studies, which employed FFQs to assess dietary intake a number of years ago, indicate that caution is necessary.(17)

Wakai (399) explored questionnaire length in their review of validation studies of Japanese developed FFQs. In long FFQs (with 97 or more food items), the correlation coefficients were slightly higher and encompassed a narrower range than those of short FFQs (with fewer than 70 items). In long FFQs, the median correlation coefficients for nutrients in an individual FFQ ranged from 0.42 to 0.52, while it ranged from 0.31 to 0.45 in short FFQs. It was suggested that it may be more difficult to accurately assess complicated modern Japanese diets, which can include traditional Japanese, Western and Chinese foods and dishes, in shorter questionnaires, which will

negatively impact correlation coefficients.(399) Cade et al. (17) indicated that the number of items in a FFQ food list did seem to influence the correlation coefficient. They found somewhat higher correlation coefficients for FFQs with the highest number of items compared to those with the smallest number.(17)

FFQs that are more recent contain about 100 to 150 food items and are capable of attaining correlations in the order of 0.60 to 0.70 in comparison with multiple dietary records. This degree of validity is obtainable in the self- and interviewer-administered format.(30) Self-defined portion size FFQs provide higher mean correlations.(17) Correlation coefficients of newly developed FFQs and FFQs modified from other questionnaires were also investigated. Newly developed questionnaires had higher correlations for some nutrients (energy as 0.49 versus 0.44 and fat as 0.52 versus 0.49), while adapted questionnaires had higher correlations than newly developed questionnaires for other nutrients (vitamin C as 0.50 versus 0.44 and vitamin A as 0.41 versus 0.34) and no differences between the two questionnaires for others (calcium as 0.54 versus 0.55 and iron as 0.45 versus 0.44).(17)   Given these results, the statement of Willett (30) must be taken into consideration that FFQ validation evaluations have mostly been undertaken among subjects with better than average education and level of co-operation. In addition, statistical adjustment for total energy intake and day-to-day variability in intake seems to improve correlation findings.(448) Molag et al. (85) found energy-adjusted correlation coefficients to be 0.02 to 0.08 higher for most nutrients, besides vitamin C (0.05 lower).

Masson et al. (461) propose that studies evaluating the relative validity of nutrient intake estimates by FFQs as the assessment method, should report both the Spearman rank order correlation coefficient and the percentage classification into the same/opposite tertile or quartile categories of the assessment methods with the *Kw* values as summary consideration of the cross-tabulation. For assessing nutrients of interest in epidemiological studies, Spearman correlation coefficients above 0.50, more than 50% of subjects correctly categorized and less than 10% of subjects grossly misclassified into tertiles and *Kw* values above 0.40 are recommended. Values of Cohen's *Kw* statistic over 0.80 indicate very good correspondence, between 0.61 and 0.80 good correspondence, between 0.41 and 0.60 moderate correspondence, 0.21 to 0.40 fair correspondence, and 0.20 and less poor correspondence.(461)

In FFQ validation studies the FFQ is compared with a different dietary assessment method which has its own weaknesses.(18,461) These other methods used for comparison are not necessarily more accurate methods of dietary assessment, which implicate that if there is disagreement between the methods the statistical analysis cannot identify which method is correct or even whether absolute or even relative intakes was accurately assessed. It can only indicate whether

the methods provide comparable data or not.(18) According to Cade et al.,(17) the results of a validation study are seldom used to modify actual study results.

5.2.4 Validity Evaluation Relative to External Biomarkers as Reference Method

5.2.4.1 Biomarker Validation

Validation requires that the trueness be known. This is fundamentally unattainable for the assessment of dietary intake, as it presents great practical difficulty to determine true dietary intake.(460) There is as a result no real gold standard for assessing dietary intake (494) that can indisputably be used to validate potential dietary assessment methods performed in free-living subjects.(408) Because a gold standard is not available,(375,494) the common evaluation approach for dietary assessment methods is to establish relative validity as the comparability of the test method to a more detailed and apparently more accurate assessment method as reference measure,(481,494,501) which is assumed to be unbiased and having different errors to those in the test method.(481) It is important that the method errors are as independent as achievable, as good agreement between the results of the reference and test methods do not automatically indicate validity. It may simply indicate that the errors in both methods are alike.(349) However, it has been suggested that it is doubtful whether dietary assessment methods can meet these requirements (481) as the errors are likely to be correlated.(502) The performance of the FFQ is usually evaluated against reference data from dietary records or 24-hour recalls.(375) Many of the cognitive processes between 24-hour recalls and the FFQ are similar, which brings about similarity in measurement error.(91,372) Evidence suggests that comparison of FFQs with dietary records may overestimate the capability of the FFQ to assess usual dietary intake as the method errors are not independent.(467)

Thus despite common use of dietary records as reference method, its validity is progressively being questioned.(71) The first measurement error issues in using dietary records and FFQs as assessment methods are related to errors in completion and the food composition tables used.(77) Because all traditional dietary assessment methods depend on food consumption, as reported by the subjects themselves, the errors of the method evaluated (test method) may be related to those of the reference method.(465,501) Both dietary records and FFQs are self-administered methods (469) and in addition utilize a common food composition database to analyse the dietary intake.(469,501) The nutritional composition data may be inadequate for some foods and nutrients due to limitations in the database, such as food distribution and processing aspects not considered, to provide precise values of the compositional content and the resulting dietary intake assessment data.(77)

True dietary intake may not be provided by dietary records (440) due to changes in subject intakes during the recording days.(501) The major limitation of dietary records is said to be the tendency of subjects to eat differently when recording their intakes.(381,387,495) Subjects may firstly be more conscious of what they consume, which may alter their food choices and dietary intake.(440) The burden of constantly recording consumption has been found to lead to changes in the usual intake in order to simplify the food and beverage measurement and recording.(440,477) Possible changes in food consumption may therefore be introduced by the recording itself.(71,469) FFQ errors might arise from the misreporting of dietary choice, frequency of consumption, portion size and usual diet, as well as the daily dietary variation in intake.(501) In intervention studies, subjects may alter their dietary intake or reporting behaviour during the monitoring period to appear more compliant with the intervention dietary recommendations.(460)

Measurement errors is not considered such a serious problem when comparing multiple dietary records with memory-based methods, like a FFQ,(502) as different subject demands are made.(444) There is still a need for truly independent methods of dietary intake assessment, (460,502) as accuracy is required to know whether the reported intake is true or not (460) and to avoid attenuation of estimates of relative disease risks caused by biased dietary assessments.(408) This lack of a gold standard as dietary assessment method (465) and the resulting concerns about the acceptability of the dietary assessment methods because of measurement error,(18,349) prompted the search for and use of more objective measures or external variables of dietary intake, as nutritional biochemical indicators or markers, to investigate the accuracy or validity of dietary assessment method data.(343,349,408,460,465,467) Such objective physiologic measures may be the alternative sought after for the dietary assessment gold standard.(350)

Biochemical indicator validation has been adopted as a further or second technique for validating dietary assessment methods (416,483) due to the potential value as independent unbiased measure of dietary intake.(18,349,416,463-465,502) The potential sources of error of these two dietary assessment methods are dissimilar (352,463,464,495) with the errors of the biochemical measures unlikely to be correlated to those inherent of reported dietary intake assessment methods, such as subject bias,(18,465) and are thus truly unrelated or independent,(18,352,353, 372,401,467,480,481,483,495,501) as the biochemical marker and the dietary assessment method do not measure exactly the same aspect.(18) Due to this independence in measurement error, adopted biomarkers may represent a highly potential proposition for evaluating validity of self-reported dietary intake (387,465) as the reference assessment measure (401,480,481,483,494) for criterion-related validation.(353) This reduces the problem of correlated errors between the dietary assessment methods, and the correlation coefficients are not enhanced because of similar errors

in the methods.(483) A further assessment using a biochemical indicator can be extremely valuable, especially when the validity of the dietary record is uncertain.(30)

Nutritional biomarkers included in large-scale population surveys and epidemiological studies, as well as smaller clinical studies are used for numerous purposes. Their major uses are to offer measures of nutritional status prone to less error than dietary assessment data; nutrient status for nutrients with insufficient dietary content data; to obtain an integrated assessment of nutrient status that reflects metabolism; to assess dietary intake for the validation of FFQs and dietary compliance and change in intervention studies.(77) The use of biomarkers as measure of dietary exposure and additionally to validate dietary assessment data provides for improved estimates of the extent to which dietary intake influences disease risk within populations.(501) Use of biomarkers as reflection of biological responses to foods and food components are crucial, as long-term dietary interventions are practically not possible to undertake for various reasons, in particular the expense involved.(503) A biomarker can provide for blinded assessment of compliance in dietary intervention studies where a true placebo is not available (504) as was encountered in the rooibos intervention trial.

Biochemical validation involves comparing intake estimates from the dietary assessment method with coupled biochemical indicators or markers,(14,416) which are sufficiently sensitive (353) and reflect the dietary intake closely enough to act as objective indicators of the intake.(501) Such biochemical validation includes blood, urine,(14,416,501) hair,(14,501) nail (14) or fat tissue (14,501) analyses. Blood samples offer minimal subject burden and are logistically achievable on a large scale.(77) Collecting serum measures is, however, invasive and expensive.(350) Venepuncture blood samples are the practical option for large-scale studies.(77) The kidneys are a control point for the body pool of many dietary components, especially the water-soluble nutrients. As a result, urinary markers representing nutrient exposure are often not suitable because excretion is a point of control. Between-person variation in degradation pathways exists, which could affect inferences of the bodily load or dietary intake. For the urinary content analysis of nutrients and their degradation products, a 24-hour collection may be necessary, which is not practical in epidemiological studies. Urine may be suited for assessing water-soluble nutrients; however, the urinary output is influenced by nutrient saturation of tissues and the dietary intake, which makes urine analysis more suited for nutrients with a consistent intake.(77)

The most well-known biological marker is 24-hour urine nitrogen where dietary intake, if kept consistent over time, shows a fair relationship between daily protein/nitrogen intake and daily nitrogen excretion, provided the person is in nitrogen balance.(501) Correlation between potassium intake and its urinary excretion can also be determined as urine is its major excretion

route.(501)   Subcutaneous adipose tissue samples can be obtained and their fatty acid composition compared to fatty acid intake estimates.(501) Although too expensive for routine use, the doubly labelled water method can be used for assessment of energy expenditure.(501)

Using a biomarker is expensive, invasive (18) and sometimes difficult (collection of 24-hour urine samples),(505) and being nutrient-specific, can only be used to validate one nutrient at a time.(18) Considering these feasibility aspects and aspects like the time relation of exposure and biomarker level,(14,387,502) biomarkers are often not used for validation purposes.(17) As only a few possible markers have been researched,(460,463) many dietary exposures lack adequate biomarkers.(502)

Nearly any analytical measurement that identifies a change in a biochemical process, structure or function that occurs because of an interaction between an environmental agent, including a dietary component, and a biological system can act as a biomarker.(503) Biomarkers should ideally be specific,(502,506) sensitive,(460,502,506) not humanely too invasive (506) and be easy to obtain without interfering with subject dietary intakes.(460) To be sufficiently specific, dietary biomarkers should not have too many other determinants.(502) The numerous interactions which occur within the human body provides an explanation why specificity may be a problem for a nutritional biomarker.(14,507) This is an aspect involved in measuring the TAC.(506) To be sensitive to dietary intake, biomarkers should not be regulated by physiological mechanisms.(460)   For example, although serum vitamin C concentration reflects dietary intake of this vitamin within the physiologic range provided by the typical Western diet, the linear relation disappears at very high intakes because of reduced absorption efficiency from the gut and increased urinary loss.(507)

Different types of biomarkers are linked to the dietary intake of nutrients and non-nutrients, which include biomarkers of exposure (502,506) and of biological effect (506) that includes biomarkers of susceptibility and of disease.(502) An intake or exposure biomarker can be utilized as indication of the internal dose,(77) which is a reflection of the amount of food, nutrient or metabolite present in cells, tissues or body fluids after absorption and metabolism.(77,503) Subject exposure can be assessed by determining the internal dose of a compound in blood or urine.(287) With biomarkers of exposure the differences in absorption and accumulation must be taken into account. Assessment of exposure biomarkers is analytically fairly easy, but their utilization requires knowing the appropriate time for their measurement after ingestion and the variability rates of metabolism and accumulation across tissues and biological fluids.(503)

Biological effect monitoring concerns measurement of the early (susceptibility) or late (disease) effect of a compound on a physiological system, organ or organism.(287) A sound biomarker of

effect has at least the following characteristics: high specificity for the effect of interest, reflection of an early effect, accessibility through non-invasive sampling techniques, easy and inexpensive analysis and low background level in the analytical sample. An established relation between the dietary exposure and the biomarker response, and/or an established relation between the dietary exposure and the induced damage is also a requisite. Biomarkers of exposure and of effect should reveal changes in biological systems.(287)

Although biochemical markers may offer an independent validation of dietary intake,(480) they only serve as a plausible validation reference measure of dietary assessment methods,(77,460,502) as biomarkers are most certainly not gold standards themselves.(77,494,502)   It is frequently proclaimed that a biochemical marker of a nutrient corresponds to the amount present in the diet.(77) It has as a result been suggested that the serum concentration of a nutrient can be considered a gold standard against which data on dietary intake can be compared. It is, however, important to recognize that the complexity of the metabolism may render some blood concentrations less perfect reference measures.(76)   For most nutrients there are numerous explanations why the relation may not be straightforward and why other factors need to be taken cognizance of when relating biomarkers to dietary intake.(77)

Most biochemical indicators will not provide an optimal reference measure of true absolute dietary intake.(18,352) In addition to the numerous factors that may influence the associations, inherent sources of measurement errors occur in both methods (352). Error may first of all be associated with the biochemical method itself.(18,508) There are several laboratory-based concerns linked to the assay quality control (QC) procedures of the laboratory that will analyze the samples, such as reproducibility, within- versus between-technician variability, limits of detection, specificity, etc. that require attention.(14,26,77) In general, re-analysis of 10% of the samples should be sufficient to judge laboratory capacity for QC.(77) Although biomarkers may be less subject to within-person variation and may not possess the inherent errors associated with dietary assessment methods,(504) with the measurement errors in determining plasma concentrations (generated during collection, processing and analysis of blood samples) essentially independent of the measurement errors in dietary assessment estimates,(401,480,483) a measured biomarker concentration may be confounded by many endogenous and exogenous determinants.(14,508)

Inadequate food composition data may limit the application of biomarkers for dietary validation.(502) FFQs seldom take account of the detail of food preparation and often determine intake on aggregate foods as if ingested together. A further limitation of using biomarkers is that they are affected not only by dietary aspects, but by biological factors.(14,77, 387,401,483) Any

aspect that impacts absorption of nutrients may blur relationships between dietary estimates and biologic measures.(77)

The biological variations between subjects with identical dietary intakes need to be considered when using biomarkers for validation.(77,401,480,502) It cannot be contemplated that there will be a close or straightforward relation between the intake of the dietary component and its biomarker.(17) The component may be digested, absorbed, metabolized, distributed over body pools and tissues and excreted differently in different subjects (17,77,354,401,480,483,502,508) all of which bear on the relation between the amount ingested and the biochemical measurement.(18) The biological variability may be determined by genetic (502) and environmental (354,502) factors. These variables, which include gender, body weight status, smoking, dietary supplement use, etc. may confound associations between dietary assessment estimates and biomarkers and may thus need to be adjusted (483) or controlled (401) for through sample selection. The between-person variability in bioavailability may seriously reduce biomarker usefulness.(502) Many biomarkers have relatively high within-person variability.(55) Much of the within-person variability is not well understood.(77) The sources of variation in dietary data collection and in biology would undoubtedly limit the use of biomarkers for validation.(502)

Even when a good biomarker mechanism supports the study of a single dietary component, the biomarker may furthermore have a different time frame.(14,502) Knowledge of the biology of the component's absorption and metabolism is required to know what is being evaluated, for instance very recent compared to longer term ingestion.(77) It is essential that the biomarker information is collected at a time representative of the total time frame of the dietary assessment method (FFQ) and that aspects like diurnal variation and lag periods between intake and excretion are considered.(18) Biochemical markers do, however, have the limitation that they commonly reflect short-term intake of specific dietary components that are hampered by their own sources of variation.(416)

According to Potischman,(77) it should be remembered that when examining a nutritional biomarker what is being reviewed reflects a single occurrence in time, similar to a 24-hour recall. Short-term biomarkers are those that react in response to dietary intake within hours.(77) As acute intake is usually reflected in blood, lower correlations should be expected with measures of longer term intake like the FFQ.(352) Medium-term biomarkers are those that react over weeks or months.(77) Epidemiological studies in general are concerned with the average status or usual longer term intake of the study population. Biomarkers that react over longer time periods would be the most suited for this purpose.(77) Slow component turnover rate (as in adipose tissue) suggests that they might reflect exposure over a longer time period.(14,502) Hair or toenail

samples have been utilized to determine trace element content, which would characterize long-term intake.(14,77) In contrast, serum cholesterol levels fluctuate according to daily consumption of fat and cholesterol and is therefore not that appropriate to reveal usual dietary intake.(494) Biomarkers for validation do not necessarily require time-integrating properties. Repeated measurements within a reference period can be collected.(502)

A nutritional biomarker therefore serves as an integrated measure of the nutrient metabolism.(77) As consequence, associations between dietary intakes and biomarkers are disturbed and attenuated by subject variability (502) and other additional variations.(508) Many dietary components, due to this biological and nutrition variability, lack appropriate biomarkers.(502) Although it has been suggested that a nutrient concentration in biological fluids could be considered a gold standard against which dietary intake data could be assessed,(76) concentration-based biomarkers cannot offer valid reference values as the quantitative association to the dietary intake is not known,(481) as it is influenced by complex nutrient metabolisms (76) and individual characteristics.(481) This renders many blood concentrations imperfect standards,(76) which at best can provide correlates of intake.(481)

It should be taken into consideration that using biomarkers only allows for assessing whether a questionnaire is capable of ranking or positioning subjects according to intake, as biomarkers are not a measure that represents absolute intake.(401) The advantages and disadvantages of biomarkers must be considered for them to be utilized for validation purposes.(77,483) Although there are some good biological measures for some dietary intakes, there is no measure for the assessment of the total dietary intake.(18) For a number of dietary intakes, dietary data could be more suitable than biomarker data for validation.(77) To evaluate the validity of a dietary assessment method only a 'relative' validation can be undertaken; whether the method is compared with results from dietary records or with biomarkers.(344)

Given that nearly all biologic measures of nutrients/metabolites are affected by factors other than dietary intake, it is difficult to establish the threshold of tolerable validity coefficients between self-report diet and biomarkers (deciding on the correlation level required to assign validity).(465) Nutritional biomarkers may be more suitable for comparisons across multiple dietary assessment methods (77,465) so that determining the validity can be based on the relative extent of relation across the methods rather than the absolute extent.(465) It has been suggested that multiple biochemical assessments from each subject should be included whenever appropriate (55) to determine the true biomarker level at an acceptable confidence level.(508) However, the capability to adequately rank subjects according to a usual level based on a single blood sample

measurement is required for epidemiological studies, where repeat blood sampling and analysis are generally not achievable.(508)

Biomarkers are valuable in evaluating validity because the measurement errors are expected to be dissimilar to those of dietary assessment methods.(469) The correlations found between dietary intake estimates and biomarkers are, however, modest to low.(480,495) Modest to low relationships are to be anticipated because biomarker levels are subject to laboratory error and are not influenced by diet and nutrition alone,(387,469,483) but by numerous biologic factors, absorption and metabolism.(387,483) The low associations found occur due to factors connected to the absorption, post-absorptive metabolism, or physiologic regulation of nutrient levels. These factors represent sources of random variations in the marker that are not related to true dietary intake. The effects of these factors may vary systematically in extent between subjects.(495) The lack of association between biomarkers and exposures does not necessarily result from lack of validity of the dietary method, as the variability introduced by these extraneous factors cannot be removed.(502) Correlations between FFQ estimates and exposure biomarkers as reference method seem to be about 0.35 to 0.50.(18)

Suitable biochemical indicators are unfortunately not available for numerous dietary components of interest, also nutrients.(372) In comparison to most nutrients, there is a general absence of biomarkers of either exposure or effect for the non-nutrients. Reliable tables of food compositional data for non-nutrients do not exist or are limited by their accuracy. For several of the non-nutrient components, there is a dearth of information on how much is absorbed, the amount that enters target cells and tissues and in what form (for flavonoids as free, glycosylated, protein-bound or conjugated), and the occurrence of interactions (additive or synergistic) between components. Detection limits are also often too low for studies, which utilize dietary intakes and not high experimental ingestion levels.(506)

5.2.4.2 Statistical Analysis

To evaluate the accuracy of FFQ estimates, the effect caused by dietary assessment errors is usually determined by means of a validation study in a representative sample of the study group.(495) The mean estimated FFQ values have mostly been compared with the mean intake values obtained from a reference dietary assessment method, either multiple dietary records or 24-hour recalls, to conclude the accuracy. The correlation between the questionnaire dietary intake estimates and the dietary intake estimates from the reference method is then determined, with correction for attenuating effects due to random errors in the reference measurement itself. In this approach, it is important that the random errors are independent between the questionnaire and reference method. However, this is not always achieved as the same factors that influence the

FFQ may influence the reference method and the correlation coefficient may be over- or underestimated.(495,509)

The advantage of biomarker assessments is that the random errors resulting with their use are in all probability truly different to those in the questionnaire and the reference method.(495,509) Of primary interest is the relationship between questionnaire estimates and 'true' intakes as reflection of the subject ranking according to their habitual intake of specific food groups or nutrients.(495) In most dietary validation studies, the additional information obtained from the comparison with biomarker results has been reported as a separate additional relationship or correlation coefficient between the questionnaire estimates and biomarker measurements. This correlation, even if rather low, provides support that the questionnaire estimates must have at least some validity.(509)

Information on biomarkers of exposure is generally not available in FFQ validation studies to support data from the reference method.(510) When such information is presented, Kaaks (495) suggested three pair-wise correlations between the FFQ, the reference method, and the biomarker measurements with the 'method of triads' to obtain a quantitative estimate of the questionnaire's validity coefficient (VC). This method assumes that the three dietary intake measurements are linearly related to true intake and have independence of random errors. This innovative estimation is an application of a basic estimating technique in factor and path analysis and does correct for bias because of similar errors in the repeat assessments of the reference method.(495,507)

When this triangular method is used, Heywood cases, where the estimated VCs are greater than one, can arise if the product of two of the three correlations is larger than the third. VCs may not be estimable by means of the method of triads because one of the sample correlations is negative. The sample size commonly used in dietary validation studies is generally not larger than 100 to 200 subjects, which are in many situations not adequate to estimate the VCs with reasonable precision. The bootstrap method can be used to estimate confidence intervals (CIs) for the VCs. The bootstrap method is a non-parametric approach that involves repeated re-sampling from the group of subjects from whom measurements have been obtained. The bootstrap samples are usually chosen to be the sample size of the number of subjects in the dataset. By applying the method of triads to each bootstrap sample, distributions of the three estimated VCs can be obtained that can be used to determine CIs of the estimated variables. Validity studies with larger sample sizes, more accurate biochemical indicators of dietary intake, or both, are needed to estimate the VCs more precisely and reduce the probability that sampling fluctuations will lead to Heywood cases in either the original dataset or in a high proportion of bootstrap analyses. Even in

large validation studies, Heywood cases can arise due to rather small sampling fluctuations if the VC of one of the measurements is close to either one or nil.(509)

5.2.4.3 *Biomarkers of Flavonoid Exposure*

There are two approaches to assessing flavonoid intake, absolute and relative intakes.(75) Assessing absolute intake of dietary components, also that of flavonoids, is difficult.(26) Absolute flavonoid intake can be assessed by calculating the consumed amount from the flavonoid content of all the consumed dietary items. However, to calculate absolute intakes, comprehensive and accurate food composition tables, that cover the whole spectrum of consumed foods and beverages, are required.(75) Yet, the flavonoid content of many dietary items is not known (138,194). The lack of comprehensive food composition data that consist of at least all the pertinent singular consumed dietary items restrict the assessment of an absolute intake.(75,506) Plant phenolics in addition include several compounds with different chemical structures and activities (2) that would make the compilation of comprehensive and accurate food composition tables problematic. Although Radtke et al. (338) indicated that increased information on the flavonoid food composition has improved the assessment of the dietary intake, the intake estimation is still not optimal.(75) The inability to fully estimate the intake of a compound negatively impacts the relation between the assessed intake of that compound and its plasma levels.(108)

Accurate assessment of the dietary intake of polyphenols is not an easy undertaking, not only due to the incomplete food composition data,(138,194) but due to the errors inherent to the dietary assessment methods.(138) Dietary records, 24-hour recalls and FFQs represent the generally used methods for dietary assessment.(511) Flavonoid intake assessment by these dietary methods, for instance, is most of all subject to errors derived from the indication of the amounts consumed.(75) Small assessment errors (amounts consumed) may result in large errors in the estimated intake of some flavonoid subclasses, like the flavonols, which are concentrated in a few foods.(194) The difficulty in assessing the absolute dietary intake or exposure level is one of the contributing reasons for the inconsistent epidemiological study findings on the role of flavonoids in disease prevention.(338) Due to the measurement errors associated with dietary assessment methods, there is a need to acquire more objective methods to determine intake of dietary items and components important to health.(511) With more objective methods like optimal biomarkers, an 'exact' intake can be calculated,(138) as with protein (nitrogen) (138,349,378) and salt (sodium) (in a 24-hour urine sample), along with exact energy expenditure (doubly labelled water technique).(138)

For those dietary components for which intake assessment is difficult, use of biomarkers of exposure may be favoured.(26) Biochemical markers of dietary intake exposure would be rather

useful given the difficulties in determining dietary flavonoid intake such as using the traditional dietary assessment methods, the incomplete compositional data of food and the extent of variability in the composition of many foods.(100,126) Systematic errors for flavonoids are probably much smaller for its biological measurements than for its dietary assessment estimation.(194)

However using biological markers may be more questionable than assessing intake estimates,(26) which is pertinent for flavonoids as there are numerous aspects to be taken into consideration with their use as nutritional biomarkers.(511) The flavonoid subclass selected as nutritional biomarker should firstly be considered.(512) For instance, the flavonoid chemical structure (the phenolic rings, their glycosylation and conjugation, etc.) can impact their bioavailability.(2) An example is that dietary flavonols are consumed as glycosides, which can influence their biological properties, and there are a few forms of catechins and these alterations to the structure impact their absorption and biological properties.(506) The flavonoid subclass selected as biomarker should consider its contribution to the dietary flavonoid intake.(512) In a trial to encourage increased fruit and vegetable consumption, fruit and vegetable intake was significantly increased in the intervention group, but no change occurred in the non-fasting plasma flavonol concentration (summed quercetin, kaempferol and isorhamnetin) evidenced from baseline to follow-up. Flavonols were furthermore only detected in a third of the subjects at both baseline and follow-up.(512) Huxley et al. (512) explained that the overall intake of flavonol-rich foods in their study group was low and that breakfast would not be rich in flavonoids (besides possibly for tea as a source of catechins) compared to the main meal that was not linked to the time of the blood sampling.

Although the absorption, availability and metabolic fate of flavonoids is not fully understood,(2) further modification of flavonoids result due to their metabolism, which occurs via the intestinal mucosa/liver/kidney and via the gut microflora.(506) Following absorption of flavonoids or their breakdown products from the liver and by the microflora, conjugation of the intact flavonoids or breakdown products occur. In other words, gut interactions may alter biomarkers of exposure of the external dietary flavonoid dose and even the internal dose on appearing in the blood.(506) It must be kept in mind that the profile of the flavonoid metabolites found in the blood, urine and target organs due to the digestive and hepatic metabolism and biotransformation, are quite different from that of the original flavonoids in the diet.(2,100)

The bioavailability of a flavonoid compound may differ between foods (511) (as with the bioavailability of quercetin from onions being higher than that from apples).(193) A substantial between-person variation in uptake has been reported.(116,511) A high within-and between-

person variation was found both in intake and plasma concentrations of flavonols (as quercetin) and flavanones (as hesperetin) (338) and in intake and urinary excretion.(116) In the study of Manach et al. (124) the plasma quercetin levels in healthy volunteers after consuming a complex meal rich in plant products, showed a marked increase three hours after the meal in all the subjects with quite homogenous values for eight subjects, but a rather high and a low level were detected in two subjects respectively. Although Grinder-Pedersen et al. (116) found a small fraction of the flavonoid intake (quercetin, kaempferol, hesperetin, naringenin and isorhamnetin) excreted in the urine,(0.60 to 4%) a high between-person variation was found in the urinary excretion of all the investigated flavonoids (urinary excretion of one of the 16 subjects in particular reflected a high amount of the investigated flavonoids in comparison to the other subjects). The range between both individual plasma and urinary flavonols was larger than that found for many dietary nutrients that relate to either a wide range in exposure, or the difference in post-absorptive metabolism or excretion.(126)

Large variations in flavonoid excretion were found in a study of low and high fruit, vegetable and berry study groups.(513) Erlund et al. (118) hypothesized that the large individual plasma concentrations of hesperetin (on drinking orange juice) and naringenin (on drinking orange or grapefruit juice) were caused by differences in the gastrointestinal microflora. It seems that the bioavailability of flavonoids is higher in certain subjects because of individual variability in the intestinal physiology or microflora. Several studies have indicated between-person variation in flavonoid bioavailability, which may be related to physiological factors (differences in body weight status, body composition or gastric functioning), molecular factors (differences in the synthesis or activity of secretory transporters or enzymes involved in biotransformation) and the composition and metabolic activity of the microbes in the distal parts of the gut.(108) Although the flavonoid absorption may differ between subjects, flavonoid biomarkers reveal the true systemic exposure of subjects, which are required to uphold the disease protective outcome of dietary flavonoids.(511)

The general biomarker approaches include the analysis of the original compound or its metabolites in blood and urine.(506) Plasma or urine biomarkers of intake are generally utilized in epidemiological studies to compare subject exposures to different nutrients.(75) Although blood and urine biomarkers for intake exposure may offer a more objective and appropriate method for assessing dietary intake,(511) these biomarkers often do not reflect absolute intake values, but only relative values as they are affected by factors that influence the bioavailability, like between-person genetic and physiological differences in absorption.(75) These aspects along with the individual flavonoids present within different dietary sources that vary between the dietary sources and the bioavailability difference of the flavonoid subclasses,(194) impact the use of biochemical markers for determining dietary flavonoid exposure.(75) Using biomarkers for flavonoid intake

exposure can as a result only represent relative intakes. However, they may provide more accurate comparisons of relative intakes between and among subjects because they do not rely on subjectively collected dietary records.(75) A reliable measurement that reflects the various flavonoid component intakes in either plasma or urine samples has not yet been proposed.(100)

5.2.4.3.1   Direct biomarker approaches of relative flavonoid intakes

A small number of mostly acute or short-term human intervention trials have tried to correlate flavonol and flavanone intakes with their aglycone plasma concentrations or urinary excretion.(100) De Vries et al. (194) and Noroozi et al. (126) investigated whether plasma and/or urine flavonol levels can be utilized as biomarkers of intake exposure while Erlund et al. (118) considered plasma and/or urine flavanone concentrations. For instance, the study of De Vries et al. (194) showed that flavonols as amounts of quercetin and kaempferol in the plasma and urine, reflected amounts of intake and could distinguish between high and low short-term (three day) intake of tea versus onion, implicating plasma concentrations and urinary excretion for biomarker use of intake. Noroozi et al. (126) found that dietary flavonols (quercetin, kaempferol, myricetin and isorhamnetin) appear in the plasma and urine in concentrations quantitatively to intake (two weeks of differing flavonol level diets) supporting potential biomarker use. Flavonols could hence be considered for biomarker use to discriminate between low and high flavonol ingestion. Such analytical results may reflect a more objective indication of dietary intake in epidemiological studies.(9)

Fasting plasma and urine flavanone levels appeared to be poor biomarkers of dietary exposure.(118) Although reflecting their short-lived bioavailability, considerable between-person variations in plasma concentrations of hesperetin and naringenin occurred on orange and grapefruit juice consumption in the study of Erlund et al.,(118) which was ascribed to the subject differences in the gut microflora. In addition, the urinary excretion as a percentage of the intakes fluctuated and was largely dependent on the citrus consumption source from which they were obtained, reflecting a bioavailability influence.(118) Few studies have investigate utilizing plasma concentrations and/or urinary excretion of polyphenols as biomarkers of intake in free-living subjects.(138) Modest correlations were found between dietary intake from seven-day dietary records and fasting plasma flavonol and flavanone levels (flavonols: r=0.30 quercetin, r=0.46 kaempferol; flavanones: r=0.32 hesperidin, r=0.35 naringenin, p<0.05) in a group of healthy women (n=48) following their everyday diet.(338)

Studies thus give the impression that plasma and urine samples seem to reveal short-term flavonol and flavanone intakes and may be suitable biomarkers of short-term intake and possibly of medium-term intake.(9,338) Plasma flavanone concentration probably reflects short-term intake and not long-term intake due to the short half-life.(118) As can be anticipated from the short half-life of some plasma flavonoids, markedly higher correlation coefficients were obtained for the

relation between the flavonol and flavanone intake estimates for the last day before sampling and the fasting plasma levels than for earlier obtained dietary intakes and the fasting plasma levels. However, the difference in correlations, whether using the one-day dietary record or the mean seven-day dietary record results, was moderate.(338)

Aziz et al. (203) found that on following a low flavonol diet for three days and an overnight fast, no flavonols were detected in nil-hour plasma samples of four subjects, while the nil-hour plasma sample of one subject contained conjugated quercetin. No subject had measurable plasma or urine flavanone levels at baseline on a one-week citrus-free diet in the study of Erlund et al.(118) The situation is not that straightforward. Erlund et al. (118) put forward that the difference in the relative urinary excretion of naringenin found from consuming both orange and grapefruit juices once in their study, was probably caused by dose-dependent renal clearance rather than differences in bioavailability. Plasma protein saturation with flavonoids can result in faster clearance because unbound compounds are more readily excreted into the urine. At the flavanone levels in this study, complete plasma protein saturation was unlikely, but if the unbound fraction of flavanones increases with increasing plasma concentration, urinary excretion would also increase at higher intakes.(118) Urine flavanone concentration therefore does not seem a good biomarker of dietary flavanone intake.(118)

Quercetin is the main contributor to the dietary flavonols and this is reflected in both urine and plasma measurements.(126) Accurate assessment of the dietary intake of the pertinent source of quercetin (onions), isn't easy.(118) Quercetin intake calculations are subject to error because of insufficient information on the quercetin content in foods and the variation in the food contents. A biomarker of flavonol intake (plasma quercetin concentration), may thus be most valuable.(218) Some studies have investigated the possibility of plasma or urine quercetin being biomarkers of its intake.(108) Study findings do indicate that plasma quercetin concentration and urinary excretion reflected amounts of intake and responded with changes in intake.(194) Urinary quercetin is possibly a more useful biomarker of absorbed quercetin than of total intake due to large variability in the absorption between the major dietary sources.(218) The between-person variability found in the bioavailability of quercetin from quercetin-rutinoside, the major flavonol in tea, was noteworthy.(118) Because the excreted fraction depends on the absorbed fraction, it makes using urinary excretion problematic to be used as biomarker of intake.(218) On the other hand, low quercetin levels in fasting blood samples and lack of a dose response on juice consumption of quercetin indicated the plasma concentration to be a relatively insensitive biomarker of quercetin intake.(218)

If the urinary excretion of flavonoids is assumed to reflect the intake (exposure), then the flavonol quercetin is of negligible quantitative value as a realistic measure of the intake of fruits and vegetables. Other flavonoids like the citrus flavanones may be more realistic biomarkers because their urinary excretion is higher even when they are present in small quantities in the diet.(116) The flavanones may be a group of flavonoids that may be promising as potential nutritional biomarkers. However, their drawback is that they may be biomarkers of citrus fruit only and not the total fruit and vegetable consumption. Flavonols occur in a wide range of fruits/vegetables (total) and may be a more general biomarker of fruits/vegetable intake, while flavanones are largely present in citrus only.(511)

Since EGC, EC and EGCG are not present in substantial amounts in other foods/beverages besides tea, plasma and urinary levels of these catechins may be useful biomarkers to quantify tea consumption and exposure to tea polyphenols.(201) Duffy et al. (299) in their study found that flavanol ingestion was associated with baseline total plasma catechin concentrations (r=0.37; p=0.022). Tea flavonoids have a rather short elimination half-life in plasma and plasma detection necessitates habitual tea drinking (at least two cups black tea/day).(31) Van het Hof et al. (46) found that although plasma flavonoid levels increased during regular tea drinking (eight cups/day), it decreased markedly overnight (no tea consumption). Plasma flavonoid level as such may not be an appropriate biomarker to assess habitual patterns of black tea drinking.(31)

Although urinary excretion of EGC and EC accompanied the increase in plasma levels on the first dose of tea,(189) urinary levels of EGC and EC might not be appropriate to assess habitual patterns of black tea drinking as over 90% of the total urinary EGC and EC load is excreted within six to eight hours. EGC and EC detection in urine requires collection of 24-hour urine samples and from those subjects who habitually drink at least a cup of tea per day.(31) Hakim et al.,(31) did not attempt to validate the consumption of black tea polyphenols in relation to the plasma levels in their study, although plasma samples were on hand for all the study subjects. Despite these limitations, Widlansky et al. (323) found that plasma EC was related to total flavonoid intake and with endothelial function, which implicates it could be a possible marker of chronic flavonoid intake or flavonoid status. However, catechin analysis may be restricted due to restricted sensitivity of the analytical method.(138)

Biomarkers have not been explored for anthocyanins or proanthocyanidins,(75) possibly as a consequence of their high molecular weight, which makes them barely absorbable and thus degraded by the colonic microflora.(115,514) The phenolic acid microbial metabolites of proanthocyanidins, in particular 3-hydroxyphenylpropanoic acid, may be a possible consideration for use as put forward by Erdman et al.(75) The urinary excretion of this metabolite was markedly

increased after ingestion of chocolate (115) and grape seed extract,(514) which are both rich sources of proanthocyanidins.(75)

Biological markers of fruit and vegetable intake (certain fruit and/or vegetables or specific compounds) may improve the understanding of the health effects of fruits and vegetables in epidemiological studies compared to using dietary assessment methods for this purpose.(513) Dietary flavonoids have been considered as new candidate biomarkers for fruit and vegetable intake because flavonoids occur in nearly all fruits and vegetables (511) and enter the systemic circulation.(513) Since quercetin occurs in numerous fruits and vegetables, its plasma concentration or urinary excretion may be an appropriate flavonoid biomarker representing consumption of these foods.(218) Other urinary or plasma flavonoid biomarkers have been proposed by some researchers to compare fruit and vegetable intakes in populations, due to the correlations observed between these biomarkers and fruit and vegetable intakes.(138)

The relatively close relationship between the habitual fruit and vegetable intake (g/day) and the sum of flavonoids excreted, suggests exploring it as a biomarker of dietary exposure, particularly in those who do not consume tea or red wine, like children.(126) Although Nielsen et al. (513) found a relation between the sum of various flavonoids (quercetin, kaempferol, isorhamnetin, tamarixetin, naringenin, hesperetin and phloretin) measured in 24-hour urine samples and the habitual dietary fruit and vegetable intake (g/day), Mennen et al. (138) did not find a relation between the sum of various flavonoids measured in urine samples and total fruit and vegetable intake, but the sum was associated with fruit and fruit juice ingestion. The polyphenol combination considered in the study (isorhamnetin, hesperitin, naringenin, kaempferol and phloretin) might be considered for biomarker use of total fruit intake and not fruit and vegetable intake,(138) which is according to the researchers to be expected due to the high flavonoid content in fruits.(93,125) Significant differences were found at group level in the urinary excretion of flavonoids between subjects on high and low fruit and vegetable diets, such as urinary excretion of a number of flavanones and flavonols,(511) and at individual level a relation was found between changes in fruit and vegetable consumption and changes in urinary flavonoid excretion.(513)

Urinary excretion of numerous flavonoids may thus be utilized to investigate changes in fruit and vegetable consumption.(511) Besides that urinary excretion of summed flavonoids may be a possible biomarker for fruit and vegetable consumption (total consumption of habitual diet) urinary excretion of individual flavonoids may possibly represent specific food groups (513) and of the ingestion of polyphenol-rich foods or beverages.(138) This refers to the sum of various flavonoids (quercetin, kaempferol, isorhamnetin, tamarixetin, naringenin, hesperetin and phloretin) with fruits, berries and vegetables and hesperetin with fruit juice and quercetin with vegetables.(513) Such relations have been encountered in intervention trials with particular foods where correlations

between urinary excretion of specific polyphenols and the intake of these foods were found in relation to the food content. Even several phenolic compounds in spot urine samples collected may be appropriate biomarkers of polyphenol intake.(138)

The question was raised of whether plasma concentrations are actually accurate biomarkers of flavonoid exposure.(100) For instance, in the case of catechins, the saliva levels are two orders of magnitude higher in relation to those in the plasma, but with the half-life much shorter (10-20 minutes) than that of the plasma. These results suggest that when drinking tea slowly, high concentrations of catechins will be delivered first to the oral cavity and then to the esophagus and gut.(220) Urinary biomarkers may be more appropriate for comparing individual intakes or exposures to flavonoids as their urine levels may better reveal their intake than their levels in fasting plasma.(75,138) Recovery in the urine after ingestion of given amounts of a particular polyphenol allows the comparison of the bioavailability of the different compounds present in diets.(95)

Urinary excretion has often been determined in human studies where the total amount of metabolites excreted for most polyphenols were roughly correlated with maximum plasma concentrations.(100,151) Values ranged from 0.3% to 43% of the intake which demonstrate the great variability in the bioavailability of the different polyphenols.(151) Despite the variability, associations between urinary excretion of specific polyphenols and intake of polyphenol-rich foods have been found.(75) Mennen et al. (138) found that various polyphenols can be detected and measured in urine of free-living subjects. They even proposed that various polyphenols measured in a spot urine sample, can be utilized as biomarkers of polyphenol-rich food intake, such as hesperetin of orange, both naringenin and hesperetin of citrus fruit consumption, etc. Polyphenols measured in the 24-hour urine samples were all associated to those measured in the spot urine sample (r=0.42 to 0.74, p<0.0001 to <0.02).(138)

Urinary data, however, may not be appropriate for compounds excreted mainly through other routes, for instance biliary excretion. If the percentage of the compound excreted into the urine differs greatly between subjects, urinary data may not provide acceptable results because marked between-person variation could confound the results. In epidemiological studies, 24-hour urine samples have seldom been obtained.(108) Low urinary values found could be indicative of pronounced biliary excretion or extensive metabolism.(100) For compounds with a short half-life dietary intake data or 24-hour urinary excretion data may be more appropriate. This indicates that fasting plasma flavanones levels are questionable as biomarkers of intake, but that 24-hour urinary samples may be appropriate as biomarkers of flavanone intake.(108) The results overall propose the probable use of urinary biomarkers to compare intake or exposure to flavonols, flavanones and

flavanols. It is envisaged that urinary biomarkers can be used alongside food composition tables to assess and compare polyphenol intakes in populations.(75) The optimal assessment would be to consider and combine dietary intake with plasma and urine data.(108)

Despite these short-term intervention trials, very few found a dose-response relationship. This is necessary for convincing biomarker evidence.(209) More information on a dose-response relationship of flavonoid excretion is required before the validity of urinary flavonoids as exposure biomarkers can be established.(513) The correlations observed with phenolic compounds (r=0.35 in the study of Nielsen et al. (513)) are all in the same line as with other biomarkers (the carotenoids) suggested to assess fruit and vegetable intake and support utilizing urinary polyphenols as biomarkers for intake of polyphenol-rich fruit.(138) Radtke et al. (338) established the intake of several flavonoids of 48 healthy women from one- or seven-day dietary records. The intake data were associated with fasting plasma flavonoid levels, which were for the one-day records (last day before blood sampling) 0.42, 0.64 and 0.47, which dropped to 0.30, 0.32 and 0.35 for quercetin, hesperitin and naringenin, respectively, for the seven-day dietary records. These correlations approximate those that have been found for nutrients, the plasma levels of which are utilized as biomarkers of exposure.(108)

Another requirement for a biomarker is a certain degree of precision with which intake can be assessed.(194) Establishing the reliability of plasma biomarkers for intake of polyphenols is affected by their fast absorption and elimination on consumption.(75) De Vries et al. (194) found that individual subjects with low or high excretion values after onion consumption had low or high excretion values after tea drinking, indicating consistent differences in quercetin metabolism between subjects. The onion treatment was repeated one to two weeks later to assess the within-subject variation as a measure of reproducibility. The total observed coefficients of variation for quercetin were 30% in plasma and 42% in urine. The extent of these variations relative to actual variations (about 60%) between free-living subjects suggests that levels of quercetin in plasma and urine are suitable as biomarkers of its intake, as the random error in these biomarkers is modest relative to the expected variations due to true variation in intake.(194)

The current knowledge of the metabolism and bioavailability of the flavonoid compounds as a whole is not adequate to explain the fasting plasma levels.(338) There are many flavonoids, but most of the data related to biomarkers of exposure concern quercetin (flavonols) and the catechins (flavanols).(506) Flavonols in plasma and urine reveal short-term ingestion and could be utilized as biomarkers to discriminate between low and high flavonol intake in epidemiological studies, but probably do not reflect longer term intake as the elimination half-life of about 20 hours, indicates that a steady state concentration in plasma is reached after about four days and that plasma

concentrations would reflect intake of only the previous three days. Thus repeated measurements may be required to obtain an estimate that represent longer term intake.(194)

Plasma concentration and urinary excretion are possibly markers of flavonol status in the body rather than of flavonol intake as it takes absorption and bioavailability of different sources into account.(194) The association between medium-term flavonol intake through consuming ordinary foods (habitual dietary intake) and fasting plasma levels has not been determined.(338) Biomarkers of the such intakes are required in epidemiological studies.(338) It is not yet possible to propose a reliable measurement in urine or plasma that could reflect longer term intake of the various polyphenols.(100) Even baseline plasma and urine levels of polyphenols and their metabolites in free-living subjects consuming habitual diets, still needs to be established.(506)

Potischman (77) pointed out that the urinary metabolites of phytochemicals might be useful as biomarkers. The identification and quantification of the flavonoid metabolites in the plasma and urine would provide for a wide array of biomarkers for flavonoid intake.(100) Nonetheless, there is lack of knowledge on the metabolites of most polyphenols in humans. The available methods for assessing these metabolites (HPLC) do not detect and measure all of the metabolites. Appropriate standards to quantify the metabolites are especially difficult to obtain and use. As the biological properties of the polyphenols are modified by metabolism, it makes the prediction of their effect and selecting such effect-related biomarkers difficult.(506) Dietary components interact at some stage during their absorption and metabolism. This interaction whether antagonistic and/or co-operative, like the interaction between antioxidants, makes it difficult to fully comprehend possible related biomarkers. The metabolism of a compound, as in the case of flavonoids, may thus impact the validity or sufficiency of a likely biomarker.(506)

5.2.4.3.2   Indirect biomarker approaches of relative flavonoid intakes

Biomarkers are available that do not produce information on the exact dietary intake, but which are highly related with intake, such as serum carotenoids and vitamin C as biomarkers of fruit and vegetable intake.(138) Research on antioxidant status and chronic disease risk has previously mostly concentrated on biomarkers of exposure to antioxidant nutrients.(26) Although plasma can provide an estimation of the systemic exposure to the dietary component of concern, present biomarker research is focused on the measurement of the exposure in the target tissue for a particular chronic disease. This emphasizes the prospect of tissue-specific effects that could be considered for use as biomarkers of exposure that is pertaining to antioxidants, determining oxidative defence or oxidative stress status with biomarkers that simultaneously determine the effect of exposure to oxidants and the antioxidant protective mechanisms *in vivo* as a surrogate measure for antioxidant exposure estimates.(26)

While direct substantiation of flavonoid bioavailability can be obtained from plasma levels of a flavonoid aglycone or its metabolites, indirect substantiation can be provided by biomarkers like the TAC of the plasma, LDL oxidation susceptibility, immune responsiveness, etc.(21) In other words, the antioxidant influence of single compounds, extracts or foods may be investigated in cell culture experiments, animal studies or human intervention trials by utilizing biomarkers of oxidative stress,(215) which in turn provide indirect evidence of absorption through the gut as, for instance, the increase in the TAC of the plasma after their consumption.(8) These biomarkers provide possibilities for flavonoid intake exposure determinations and will fulfil the need of human studies exploring both the bioavailability of flavonoids along with biomarkers of, for example, their antioxidant effects.(47)

5.2.4.3.2.1 *Biomarkers of exposure*

5.2.4.3.2.1.1   Total antioxidant capacity

Antioxidants positioned in the hydrophilic and lipophilic plasma compartments cooperate in the defence against ROS,(506,515) which are constantly produced in the body because of the normal metabolism and disease. However, when the production of ROS is uncontrolled, it results in oxidative stress and damage to membranes and other cellular components in biological systems.(26,287,515)

The antioxidant enzyme system mainly accounts for the AC of the cell, whereas the low molecular weight dietary antioxidants mostly account for the AC of the plasma, which is altered by the ingestion of dietary antioxidants or by radical overload.(2) Antioxidants within cells, cell membranes and extracellular fluids can assist in defusing excess ROS. Blood has a pertinent role in maintaining the redox balance and protecting the human body against oxidant conditions since it transports and redistributes antioxidants to all parts of the body. Plasma antioxidant status is the outcome of the interactions of the numerous compounds and metabolic interactions in the systemic circulation.(2) Various assays have been developed to measure the plasma TAC.(515)

The overall TAC measure considers the cooperation of all the different antioxidants present in the plasma and body fluids against ROS attack and can therefore be regarded as more representative of the *in vivo* balance between antioxidants (known and unknown, measurable and not measurable) and oxidants which provides more biologically relevant information as an integrated measure than determining the plasma concentrations of single antioxidants.(2,242) The synergism between the various antioxidants supplies better protection against attack by ROS, than any single antioxidant or compound on its own.(2) Cooperative/synergistic interactions exist between the antioxidants present in the hydrophilic and lipophilic plasma compartments. Therefore, assays to define the 'true' TAC of plasma should reflect these interactions.(515) Measurement of the TAC of

biological fluids might be an appropriate biomarker to assess the capacity of the antioxidants present in such fluids to avert oxidative damage in biological systems.(506)   These assays therefore assess the bioavailability of dietary antioxidants and potential oxidative stress.(515) The AC of biological samples can be assessed in clinical trials, which require measuring end-products of free radical damage to endogenous compounds like lipids.(506) The usual assay to determine the AC in the lipid compartment of plasma has been to measure the oxidizability of isolated LDL by, for instance, monitoring lipid peroxidation through CD production.(515)

AC is a responsive and reliable marker that reflects changes of *in vivo* oxidative stress. It can be utilized to assess the influence of different interventions on plasma redox status in subjects with the results expressed as a change in comparison to the basal value.(2) Changes from baseline levels could then be the consequence of changes in the dietary TAC and the intervention.(506) Changes in plasma AC on ingestion of antioxidants, or antioxidant-rich foods, may provide valuable information on the absorption and bioavailability of dietary compounds. In this context, the ability to assess change in plasma AC, after ingestion of polyphenol-rich dietary items, may provide a biomarker for indirectly investigating phenolic bioavailability.(2)

The TAC of biological samples can be determined by various assays,(241,506) which are scavenging assays.(506) The principle behind these assays is that, if antioxidants are consumed by human subjects they should be absorbed, reach the bloodstream and enter the blood cells, enhancing the ability of these cells, as well as the plasma lipids, against oxidative attack when challenged *in vitro* with an oxidant.(241) These plasma assays include the ORAC, TEAC and ferric reducing ability of plasma (FRAP) assays.(241,506,515) Although these assays differ in principle (source of oxidants, working pH and sensitivity) and assay conditions to determine the TAC of plasma,(93) they are based mainly on two approaches (515) that either assess reducing ability (electron transfer) or radical scavenging ability to trap free radicals (H-atom transfer) of the compound or (biological) fluid under investigation.(93,94,241) The outcome is compared to that of a reference substance, generally Trolox (water-soluble derivative of vitamin E).(94)

The first approach involves measuring the reducing capacity by determining the vulnerability of plasma to oxidation, induced by added oxidants that act as pro-oxidants (radical inducers) by monitoring the oxidation of an exogenous oxidizable substrate (probe). The oxidation of the substrate is theoretically repressed by the antioxidants present in the plasma during the induction period. The ORAC assay is commonly used for analysing the TAC in the hydrophilic compartment of plasma/serum. For instance, in the ORAC assay plasma or serum is exposed to the aqueous radical inducer 2,2'-azobis (2,4-aminopropane) dihydrochloride (AAPH) and its vulnerability determined by monitoring the aqueous oxidizable substrate 2',7'-dichlorodihydrofluorescein.(515)

The second approach of radical scavenging to analyse the TAC in the hydrophilic compartment of plasma, is the capacity to reduce a stable and pre-formed radical that does not act as pro-oxidant. The TEAC assay analyses the TAC of plasma by determining the capacity of plasma to reduce the radical cation 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate)(ABTS). The FRAP assay determines the reduction of ferric ion to ferrous ion at low pH, which results in the formation of a colour ferrous-tripyridyltriazine complex.(515) The TAC can be indicated in a number of ways that includes the lag phase, area under the curve, or competition kinetics.(515)

Although the AC determined by these assays reveals the overall content of all major oxidant and antioxidant substances, including non-phenolic compounds like vitamin C,(93) these methods primarily measure the TAC of the hydrophilic compartment of the plasma because the radical inducers and substrates are water-soluble. Fat-soluble antioxidants do not contribute to the antioxidant activity within these experimental conditions. Consequently, these assays are mainly influenced by the water-soluble antioxidants (uric acid, ascorbic acid and protein thiols) with the fat-soluble antioxidants, like α-tocopherol and carotenoids, only making a minor contribution. The negligible contribution of the lipid-soluble antioxidants can be attributed to their far lower presence in plasma in comparison to the water-soluble antioxidants. It should though be acknowledged that the synergistic interactions between the water- and fat-soluble antioxidants could enhance the AC of the fat-soluble antioxidants. The ORAC assay was developed further to incorporate the fat-soluble antioxidants by using a solubility enhancer along with AAPH as radical initiator and fluorescein as oxidizable substrate. Partitioning of the water- and lipid-soluble antioxidants may though not be appropriate since interactions occur among these antioxidants in a biological system.(515)

5.2.4.3.2.1.2 <u>Antioxidants and antioxidant enzyme systems</u>

All body cells are constantly exposed to oxidants from endogenous and exogenous sources, but the human body maintains an antioxidant system in defence. Dietary components, both water- and fat-soluble, make a vital contribution to this system.(26) The body cells furthermore have comprehensive defence mechanisms that avert free radical formation and their damaging effects that comprise enzymes to inactivate peroxides, proteins to bind transition metals and various compounds to control free radicals.(287) Besides using products of radical damage as biomarkers, the biological support of these various defence mechanisms against free radicals and free radical damage can hence be determined.(287) Biomarkers associated with CVD that have been assessed include select oxidant defence enzymes and molecules.(123) The antioxidant defence system can be subdivided in the non-enzymatic antioxidants (GSH, urate and α-tocopherol (287)) and the endogenous enzymatic antioxidant systems (SOD, GSH peroxidize and catalyse).(242,287) Assaying these non-enzymatic antioxidants and antioxidant enzyme systems

in blood samples signify the antioxidant status of a subject.(287) The effect of oxidative stress has, for instance, been evidenced by oxidized GSH.(295)

Blood GSH has been indicated as an important biomarker of lipid peroxidation in heart failure. In heart failure increased lipid peroxidation is associated with abnormalities in intracellular GSH cycling.(294) A change in GSH status can produce valuable information concerning oxidative stress.(22,355) The functions of GSH in the antioxidant defence system include repressing free radicals and regenerating other antioxidants.(294) Exposure to ROS causes reduced GSH to convert to GSSG, an increase in GSSG and a decrease in the ratio of reduced GSH to GSSG.(22) Oxidative stress can be assessed by measuring GSH and GSSG, though it is often expressed as the ratio between the two,(222) which is a measure of the redox state.(22) Blood reduced GSH concentrations (294) and the GSH/GSSG ratio (222) has been suggested as clinical markers in diseases in which oxidative stress is particularly important. In IHD, an increase in oxidative stress leads to an accumulation of GSSG and a reduction in the GSH/GSSG ratio.(22,295) The blood analyses of reduced GSH and GSSG are less expensive and time consuming than analysing the GSH peroxidase enzyme.(294) The selenium dependent enzyme GSH peroxidase is involved in the conversion of reduced GSH to GSSG.(22)

5.2.4.3.2.2 *Biomarkers of effect*

5.2.4.3.2.2.1   Markers of lipid oxidation and of oxidative stress status

There is, as indicated before, convincing evidence that oxidative stress contributes to the development of arteriosclerosis.(287,295) Oxidative stress is basically an imbalance between the generation of free radicals and the capacity of the protective mechanisms in the body to handle these reactive compounds and prevent their damaging effects.(26,145) In oxidative stress, ROS produce their damaging effects due to increased production or an impaired antioxidant defence system.(295) ROS can oxidize various substrates in the body, including lipids. If it is left unchecked, it can contribute to chronic disease development.(26,287)

Measurement of ROS is difficult in any biological sample, particularly in easily obtainable samples like serum or plasma, because of the highly reactive nature of these molecules.(242) Different ROS do not have the same affinities for substrates and the efficiency of a specific antioxidant may be influenced by the ROS present.(26) In human pathologies, the key oxidizing species (26,515) and the major sites of ROS action (515) are rarely known. It is important to consider how oxidative damage may be analyzed in the plasma.(242) A good approach to establish the impact of oxidative stress would be to use more than one biomarker to determine the radical damage.(287)

Besides the more traditional use of antioxidant nutrients as biomarkers of exposure, biomarkers presently focus on oxidative stress status (26) that include specific assays of free radical-mediated damage and/or measurements of the total antioxidant buffering capacity of plasma (or plasma total antioxidant status), which is the net effect of several compounds and systemic interactions in the body.(242) Lipid oxidation is therefore the basis for another method of assessing antioxidant activity.(241)

One of the major groups of products that result from free radical interaction is lipid oxidation products, which are important parameters to assess radical damage.(287) The level of peroxidation of lipids, particularly LDL lipids, is a surrogate marker (biomarker) for the development of arteriosclerosis.(114) Biomarkers of lipid peroxidation can be utilized to determine the role (and optimal intake) of antioxidants in protection against oxidative damage in for instance CVD.(114,120) PUFAs are very vulnerable to interaction with free radicals. Their presence in membranes make them readily available endogenous substrates for peroxidation and free radical chain reactions where one substrate radical may lead to the formation of numerous products of lipid peroxidation.(287) The effects of oxidative stress and the degenerative proliferation reactions in lipid membranes are generally evidenced by the development of oxidized LDL with its numerous oxidative modified lipids and degradation products,(242,295) including alkanes and carbonyl compounds.(287) There are many oxidation products that form from both the peroxidation and fragmentation of the lipid components of LDL, and the modification and oxidative degradation of apolipoprotein B.(292) LDL lipid modification can occur without any changes to apolipoprotein B.(286) Assays that focus on measures of oxidative stress and free radical-mediated damage include damage assays that measure cellular accumulation of peroxides like lipid peroxides (295) or by-products such as MDA (241,295) and F_2-isoprostane (242) of the oxidized LDL.

Oxidatively modified LDL is understood to enhance arteriosclerosis.(26) LDL that underwent some oxidation, but not sufficient for detection by the macrophages for subsequent entrance into the vessel wall (minimally modified LDL), may be present in the circulation.(114) The levels of such oxidized LDL are to a large extent related to the plasma LDL levels and provide an indication of the oxidative damage to LDL particles.(242) Lipid-soluble antioxidants are carried in LDL *in vivo*; therefore the resistance of LDL to peroxidation should portray the antioxidant defence system relative to lipid substrates and fat-soluble antioxidant compounds.(26)

A potential biomarker may hence be the vulnerability of circulating LDL to peroxidation.(114) In this assay, LDL particles are isolated from the blood of antioxidant supplemented subjects and subjected to a pro-oxidant challenge.(114,241) Unlike other assays used to quantify oxidative stress,(26) this *ex vivo* LDL peroxidation assay involves a challenge with an exogenous

oxidant.(26,114) Oxidation of the isolated LDL particles or LDL lipid peroxidation can be generated by incubation with cells in culture (endothelial cells, smooth muscle cells or macrophages) or by incubation with LOX, ROS (superoxide anion), or a heavy metal ion secreted into the medium.(292) Copper is usually used to induce this chemical oxidation of LDL.(26,114,241) LDL particles isolated from blood have a certain amount of associated α-tocopherol. LDL particles are initially protected from oxidation by α-tocopherol and once this is utilized, however, lipid hydroperoxides accumulate.(241) If a flavonoid is present in either these systems, it delays the appearance of the lipid oxidation products. The flavonoid either scavenges free radicals thus protecting α-tocopherol and LDL from oxidation or regenerates α-tocopherol from oxidized α-tocopherol radicals.(241) The appearance of lipid hydroperoxides is monitored (241) in addition to measurement of the lag period before oxidation.(26,241) A prolonged lag time presents resistance of LDL to oxidative modification.(241,302,313)

The measurement of the resistance of isolated LDL to exogenously generated oxidative stress has been widely used as assay (114) and as feasible biomarker of oxidative defence (26) to show that antioxidants affecting lipid peroxidation *ex vivo*, can indeed exert effects *in vivo*.(114) The potential of an antioxidant to defend LDL against *ex vivo* peroxidation implies that it has the ability to decrease such peroxidation *in vivo*, but this is no proof that it can actually do so.(114) Firstly, the resistance of LDL in this assay is determined in the LDL particle itself.(26) The LDL peroxidation involved in arteriosclerosis occurs largely within the vessel wall, not in circulating lipids.(4,292) Moreover, in the body as a whole, the involvement of oxidized LDL in plasma oxidative stress is uncertain, as LDL comprises a small proportion of the plasma.(242) In addition, the endogenous antioxidants present in LDL accounts for the lag period before oxidation.(506) An antioxidant that restrains copper ion-induced LDL oxidation *ex vivo* by binding the copper ions, may not be effective in for instance vessel wall lesions in which LDL oxidation is initiated by a different mechanism. A further consideration is that some antioxidants may protect LDL in the plasma itself but wash out during the analytical procedures used to isolate LDL, which may cause no effect in *ex vivo* studies.(114) Flavonoids and their metabolites might accumulate between lipoproteins and plasma in the circulation and could in particular wash out from LDL during lipoprotein isolation procedures, which are generally lengthy to conduct.(278) Halliwell et al. (278) in particular bestows little credibility in this analysis with reference to flavonoids.

Steady-state levels of lipid peroxidation products in humans, presumably representing an equilibrium between lipid peroxidation and peroxide removal, are important in the development of CVD.(114) A range of methods can determine the levels of peroxidation products in human tissues and body fluids.(114,506) These methods for measuring products of lipid oxidation or markers of lipid peroxidation *in vivo* signify damage assays.(506) Lipid peroxidation results when a

potent oxidant is formed near cell membranes with the unsaturated fatty acids in the membranes, the likely oxidative targets.(295) Such 'whole body' lipid peroxidation (506) has been assessed by direct parameters of lipid peroxidation that include, among others, measuring CDs,(292) hydrocarbon gases (ethane, pentane) (506) and aldehyde compounds.(292,506) Lipid peroxides, which are the peroxidation products of lipids, are generally unstable and require HPLC or gas chromatography-mass spectroscopy (GC-MS) as the validity of commercially available kits are questioned.(242)

Reaction of free radicals with cellular membranes may be the initial step of fatty acid fragmentation that involves rearrangement of the fatty acid double bonds with a greater number of lipid hydroperoxides in the LDL.(292) The formation of these CDs represents the first step in LDL oxidation that initiates the development of oxidized fatty acids (312,313) and an early marker of PUFA peroxidation.(293) According to Mursu et al.,(312) the first antioxidative effects can as a result be determined by only measuring the CD level *in vivo*. They pointed out that the measurement of the CD levels *in vivo* might be more sensitive compared to measurement of other components of lipid peroxidation.

Consequently to this initial step of fatty acid fragmentation with rearrangement of the double bonds and formation of an increased number of lipid peroxides in the LDL, the decomposition of the fatty acid fragmentation follows forming various highly reactive intermediate products.(287,292) Following peroxidation of omega-6 (n-6) and omega-3 (n-3) PUFA's, fairly unstable fatty acid hydroperoxides may be transformed into more stable carbonyls.(287)

One of the degradation products of lipid hydroperoxides are short chain alkanes that are released in exhaled air.(287) Pentane, which is produced from peroxidation of (n-6) PUFAs, and ethane, produced from peroxidation of (n-3) PUFAs, are unstable compounds that are excreted into the breath.(26) Measurement of volatile breath hydrocarbons, primarily pentane and ethane, has been utilized and validated as a method to assess lipid peroxidation both in *in vitro* and *in vivo* studies. The hydrocarbon breath test may be a practical non-invasive assessment for determining lipid peroxidation in humans, but a number of methodological concerns must be safeguarded. One concern relates to the considerable background levels of hydrocarbons in human breath. Exhaled hydrocarbons include hydrocarbon contamination of inhaled air. Therefore levels of exhaled hydrocarbons are not necessarily a representation of the endogenous formation (287) providing for potential error sources in assessing breath hydrocarbons.(26) The analysis of alkanes in exhaled breath is restricted by the complex techniques involved and it being time-consuming to perform.(287)

MDA is a further stable end-product of free radical attack on PUFAs in lipids (22,26,242,294,295) and its analysis serves as a widely used relative non-specific biomarker of lipid peroxidation.(287,293,317) Markedly higher levels of free MDA are found in coronary heart failure patients than in control patients.(294) Increased levels of MDA occur in plasma, urine and other tissue of humans,(287) but are generally measured in the plasma.(26) Although lipid peroxidation can be estimated as the amount of MDA present in the plasma,(241) its measurement serves as a monitor of oxidative stress (241,294) and antioxidant status (antioxidant protection) (241) in humans as plasma MDA levels have been reported to be influenced by changes in antioxidant nutrient status.(26)

MDA is conjugated to thiobarbituric acid (TBA) in the thiobarbituric acid-reactive substances (TBARS) assay and measured by and expressed as TBARS.(287,293,295) The TBARS assay is a spectrophotometric method that determines the chromogens produced by the reaction at high temperature between MDA and TBA (a dye) by fluorescence.(26,242,295) The measurement of plasma TBARS is not considered a reliable biomarker.(278) This widely used test, although technically simple,(26,242) is not easily performed and not reliable in complex biological material and should be utilized carefully in human studies.(295) Besides the problem of the instability of MDA,(241) the assay is prone to interference by compounds of non-peroxidation origin (242) that raised doubt about the specificity of the assay.(241) Background levels of MDA in samples occur and several other compounds are reactive towards TBA with the resultant additional formation of artificial products during the reaction with TBA.(241,287) This absence of sample specificity with the spectrophotometric method, has resulted in the assay becoming rather absolute (26) because many artificial products could be generated and overestimation might occur leading to incorrect interpretation of the results.(506) The assay is influenced by the iron content of the buffer and reagents used. The specificity may be enhanced by HPLC.(242) A HPLC modification of the TBARS assay uses an approach whereby HPLC separates the 'true' malondialdehyde-thiobarbituric acid (MDA-TBA) product from interfering chromogens and then measures it with fluorescence assessments resulting in improved specificity.(241,506)

Lipid oxidation is therefore determined by formation of CDs or TBARS (293) of which both assays are not appreciably suitable for use in humans.(114) A group of PG-like compounds, known as isoprostanes, have been identified and are considered capable biomarkers to assess free radical damage *in vivo*.(287) The measurement of plasma F_2-isoprostanes is regarded a far better biomarker of lipid peroxidation and oxidative stress compared to the above assays.(114,242,278) F_2-isoprostane is a product of free radical initiated oxidation of cell membrane phospholipids or circulating LDL containing arachidonic acid.(114,242,287) After their formation, F_2-isoprostanes disengage from the membrane phospholipids, enter the plasma circulation and become excreted in

the urine.(242)   Different families of isoprostanes are formed from peroxidation of the eicosapentaenoic acid residues (F_3-isoprostanes) and docosahexaenoic acid residues (F_4-isoprostanes).   These families can be identified from F_2-isoprostanes in the mass spectrometer and assaying them may assist in determining rates at which the different PUFA residues peroxidize *in vivo*.   It is widely known that the higher the number of double bonds in a PUFA the greater its vulnerability to oxidation *in vitro*.(114)   The analytical methods available to measure isoprostanes might limit the general use of this method because it requires a specialist centre with GC-MS, which is not routinely available to most investigators.   The analytical methodology is furthermore technically complex requiring meticulous sample pre-treatment, a multiple-step complex process of techniques and thus time-consuming.(26,242,287)   A relatively large volume of plasma and analytical experience is furthermore required, as the process has the potential for errors at many points.(242)

5.2.4.3.2.2.2   Markers of inflammation

Oxidative stress is indicated to play an important role in the inflammatory process initiated in the early stages of arteriosclerosis.(516)   The inflammatory component in the vessel wall is the entrance of monocytes and other inflammatory cells into the sub-endothelial space.(4,286)   There they attain the features of macrophages,(4,279,286,289,292) which then transform into foam cells that generate ROS.(282)   Inflammation therefore leads to elevated oxidative stress.(516)   An elevated level of CRP, an acute-phase protein, is a sensitive marker of inflammation.(214,247) Studies confirm a positive association between circulating markers of inflammation like CRP and measures predictive to develop CVD and unfavourable disease outcomes, which is independent of the arteriosclerotic or ischaemic severity.(247)   A clinical study by Abramson et al. (516) found that oxidative stress as determined by the free oxygen radical test (FORT), which provides an indirect measure of hydroperoxides, showed a strong positive correlation with CRP levels among adults free of CHD.   While basically all patients with infarction as a result of unstable angina have elevated CRP on admission, several population-based studies have additionally established that baseline CRP levels is predictive of future cardiovascular events.(247)

Elevated levels of a number of inflammatory mediators among apparently healthy subjects have been found to have predictive value for future cardiovascular events.(247)   Other proteins which increase during the acute-phase inflammatory response in addition to CRP include fibrinogen and PA inhibitor-1. These inflammatory markers can enhance thrombus formation and clot stability by repressing endogenous fibrinolysis.(247)

For clinical purposes, CRP appears to be the most promising inflammatory biomarker. The clinical appeal of CRP is due to its analytic properties like it being easily measurable in outpatient settings. CRP screening provides an easy method to determine low-grade inflammation which can

contribute to improving global risk detection.(247) As the role of low grade chronic inflammation, as detected by increased levels of CRP, has been identified as an independent predictive factor for heart disease,(280) it may serve as marker for underlying chronic infection, systemic inflammation and arteriosclerosis.(517) As CRP defines risk of an unfavourable arteriosclerotic course, it adds to the prognostic and predictive information provided by traditional risk factors.(247)

5.3   REPRODUCIBILITY

5.3.1   Description

If a test or method, when administered on numerous occasions to the same stable subjects, yields approximately the same outcomes, it is termed reliability in measuring health status and precision in measuring physiological components.(459) Reliability describes the magnitude to which a method provides similar findings on re-applying it in the same situation (consistency).(365,416) Reliability is usually assessed by the test-retest method.(365) 'Reproducibility' can be thought of as 'reliability' (14,17,18,381) and refers to whether or not data can be reproduced.(5) The reproducibility or repeatability of dietary assessment methods, including FFQs, is referred to as the capability of the method to produce the same findings when applied at a later stage.(14,425) This has generally been evaluated by re-administering the same dietary assessment method to the same group of people under similar conditions at two points in time.(14,17,18,381,416) The datasets of the first and second administrations are then compared (416) by determining the association between the two datasets by using correlation coefficients.(17,18)

Cade et al. (18) found that reproducibility was assessed in only 47% of the studies included in their review. Most FFQ reproducibility studies also repeat the FFQ application once. Imperfect reproducibility may be caused by measurement error of the assessment method, changes in dietary habits between the two administrations, or both.(381,415) Part of the measurement error may not be random, but correlated, between the two measurements through using the same method, which would result in high reproducibility.(430) It may be useful to repeat the questionnaire administration as repetition allows separating the effect of changes in dietary habits over time from the (pure) test-retest error of the FFQ. Such information is important for the interpretation and correction of attenuation of associations between dietary intakes as assessed by FFQ and a disease outcome. Repeated assessments provide information on the classification of subjects in longitudinal studies with regard to their dietary intake over time.(430)

5.3.2   Influencing Factors

The reproducibility of a dietary assessment method is influenced by properties of the data collection method itself,(415,483) the time interval between the dietary assessments,(415) actual

changes in dietary intake,(415,483) the subject memory (483) and demographic characteristics that impact subject recall ability.(415) These factors will impact the extent of the correlation coefficient found between the dietary intake datasets.(415) Random variation associated with completing the questionnaire and true changes in regular dietary intakes have been considered the two factors affecting the reproducibility of the FFQ.(14,381) The latter factor should be determined as to whether or not the diet has in fact changed from one administration to the next as reflected by weight changes or intentional behaviour changes.(6)

5.3.2.1 Time Interval between Method Administrations

The time interval between two FFQ administrations influences its reproducibility.(18,440) Reproducibility studies should purposely select the time interval and the season for the administrations,(427) as dissimilar results on the two administrations may reflect an unreliable measure or a reliable measure that is measuring a changed condition.(6) Studies should use the same time frame for multiple questionnaire administrations.(427)

Cade et al. (17,18) found the time interval for repeat administration of a questionnaire to range from two hours to 15 years, the most common time interval being a year with a third of the repeatability studies either between one and six months apart or between six and 12 months apart (34% and 31% respectively). Examining the reproducibility of the FFQs developed in Japan, Wakai (399) found that the period ranged from three days to five years. The time interval between repeat administrations should be selected to limit recall of previous answers and changes over time, and will be guided by the reference time frame of the questionnaire.(17,18) Tsubono et al. (427) summarized studies that compared short-term and long-term reproducibility of different dietary questionnaires and found that the reproducibility was higher in the shorter than the longer term studies and that the studies were not much concerned with the differential effects of seasonal dietary variation and other influencing factors. Although correlation coefficients between the dietary intake datasets decreased as the time interval between the assessments increased, average correlations between 0.50 and 0.75 were still found with time intervals ranging from one to ten years.(415)

Re-administering a questionnaire within a too short interval is not prudent, as it may influence subject responses due to the possibility of memory of the questions and responses previously provided (14,381,440,427) that will increase correlation coefficients.(18) Alternatively, when a longer interval is used, true changes in dietary intake,(14,381,502) as well as variation in response,(427) contribute to reduced reproducibility.(427,502) To minimize the possible effects of real change in usual dietary behaviour (and season) (365,394,425) and any training effect or memory influence to recall earlier responses,(362,365,394) intervals of as short as one week

(24,360), two weeks (403,409,425,433) to one month (346,362,365,394,443) to within six (394) to eight (365) weeks have been chosen for repeated administrations of a questionnaire.

Although a two month recall period may prove to be too short to examine a subject's usual intake,(360) a time interval of three months brought changes in dietary intake, which was consistent with expected seasonal changes in food intake patterns.(394) In this context, Kusama et al. (24) developed their FFQ to assess dietary intake for the past three months to roughly cover one season in a year. Evidence has emerged of poor performance for FFQs in assessing fruit and vegetable intake for longer periods, like the past three months.(518) The results of a study of Wheeler et al. (394) suggest that motivation to complete a FFQ is greatly reduced on the second occasion, when the interval between FFQs is only four to six weeks and to a lesser extent when it is three months. They indicated that researchers who are planning studies, which aim to assess short-term changes in food intake by means of a FFQ after an intervention program, need to be aware of this finding. They proposed assessing the reproducibility of a FFQ over the relevant period before the proposed intervention.(394)

5.3.2.2 Data Collection Method Format and Properties

Correlation coefficients are generally higher for interviewer- rather than self-administered questionnaires.(415) When the FFQ is administered by an interviewer, two aspects of reliability should be distinguished, intra-rater and inter-rater reliability. Intra-rater reliability assesses whether repeat administration of the questionnaire by the same interviewer to the same subject provides the same answers (comparable to reproducibility assessment for self-administered questionnaires). Inter-rater reliability assesses whether different interviewers using the same questionnaire obtain similar answers from the same subjects. The statistical methods applied to the above aspects of reproducibility are the same.(18)

In addition, correlation coefficients are generally higher for studies that assess total past dietary intake and which assess a larger number of food items (more than 100) compared to assessing only limited aspects of the diet. This property indicates the level of detail of the questionnaire. Higher correlations coefficients were on average found for nutrient versus food group investigations.(415) According to Barrett-Connor,(354) any questionnaire that provides no or few options for portion size has less variability and more reproducibility than does a more quantitative recall. However, collecting data on portion size and using a more detailed method for estimating portions (individualized portion sizes compared to the standard portion) appears, from the studies reviewed by Fiedenreich et al.,(415) to increase the correlations between the datasets. Cade et al. (17) found correlation coefficients to be higher in reproducibility studies when subjects could indicate their own portion sizes. The FFQ format appeared to have less effect on estimates of mean nutrient intake than the time interval between the questionnaire administrations.(394)

5.3.2.3 Subject Characteristics

Gender, age, education, income, vegetarian status, diet stability and illness are all factors that may impact method reproducibility.(415) Friedenreich et al. (415) in reviewing subject characteristics and dietary recall, found the following: gender did not appear to influence recall ability; higher education and income improved the ability; increased age was associated with decreased recall repeatability in some studies, but not in others; the higher awareness of diet as exists among vegetarians improved recall; and diet stability over time is a key determinant for improved recall. Subjects who consciously change their dietary habits have consistently been found to have larger reporting errors than those who do not report a change, as changes in the more distant past may have been forgotten.(430)

5.3.2.4 Dietary Factors

Recall repeatability is influenced by a number of factors related to the diet itself, including the frequency and stability of consumption of dietary items, changes in diet and current diet. Recall seems the most reliable for items with high stability of consumption and items eaten rarely as the highest coefficients were observed for items consumed either habitually or rarely.(415) The consumption of regularly consumed items is steady throughout the year. Consumption of these items, which have no maximum or minimum intake points, is likely to be highly reproducible at any given point in time.(13) High comparability between administrations may be brought about by limited dietary diversity as found in the study of McPherson et al.(400) If subjects' diets contain a limited number of items and the same diet is routinely consumed, it is very likely for a FFQ with a defined food list to include all the items forming part of the usual diet.(400) Subjects who changed their diet had poorer recall, except when these changes were made for health reasons.(415)

Time influences dietary consumption because food preferences fluctuate depending upon supply and demand.(438) Consequently, many dietary items are unevenly consumed throughout the year.(13) Since what people eat at a given point in time is affected by factors like supply (availability), price and trends (13) and the memory of dietary intake is predisposed towards the present, the time of data collection could greatly bias reported intake for a large number of items (438) resulting in the questionnaire capturing an atypical snapshot representing current consumption rather than the intended overview of usual consumption.(13) Present dietary intake strongly influences the report of past diet.(415) Friedenreich et al.,(415) taking a number of studies into account, found the highest correlations for nutrients between current and recalled dietary assessments, next between original and recalled assessments, and lowest between original and current assessments. The same pattern was observed for food groups. Based on these findings, Friedenreich et al. (415) indicated that the recalled diet presented a more reliable intake of past diet than did present diet, and that these considered studies did not substantiate the suggestion that current diet could be used as surrogate for past diet. Friedenreich et al. (415) found that the

influence of current diet on recall of past diet was independent of the time interval between the two questionnaires, the method of dietary assessment, the order of administration of the current and recall diet questionnaires and the subject group characteristics. FFQ responses were found to vary with season of FFQ administration, supporting with the view that the present dietary intake influences reporting of usual past intake.(363) In a study of Fowke et al.,(363) subjects indicated higher intakes of various foods in winter, while they indicated higher intakes of various fruits and moderately higher intakes across a range of vegetables in summer. Tsubono et al. (397) found that since their dietary survey was conducted in winter, contributions of foods were underestimated if they were consumed mainly in other seasons.

Hence, seasonal fluctuation in dietary habits may be a source of variation.(427) Dietary items of which the consumption varies (seasonal by definition) have a unique pattern of consumption and it is difficult to reproduce data obtained from them at any given point in time.(13) Seasonal reporting errors may thus contribute to problems with the repeatability and validity of data in FFQs.(438) This may be of particular importance when the seasonal availability of items consumed by the target subject group varies substantially and when a reproducibility study is conducted at two points in time in different seasons.(427)

Considering supply and demand, differences should be anticipated for all seasonal items. The consumption of seasonally consumed items fluctuates throughout the year and has peaks in winter, summer, or particular holidays. Therefore questionnaires that contain a combination of regularly and seasonally consumed items, will likely have problems with reproducibility, the variance depending upon the number of seasonally consumed items included in the questionnaire food list.(13) Seasonal changes are likely to be reflected in reported consumption of fruit, vegetables and beverages.(394) While some fruits and vegetables were found to be consumed on a year round basis, largely due to their availability in canned, frozen and fresh forms, the majority were consumed seasonally. Failure to separate items into regularly consumed or seasonal categories will be a source of variability and thus error in the reproducibility of FFQs.(13)

It is intuitively known that some foods are consumed with greater frequency at certain times of the year than others. This is in particular applicable to fruit and vegetables, which are generally thought of as seasonal foods and might be expected to be consumed with greater frequency when cheaper and more available.(438) A pilot study,(438) to establish the items most often consumed at particular times of the year by the Canadian population, showed that many items not generally thought of as seasonal were consumed seasonally. Of the 117 items investigated, 105 (89%) showed differences between summer and winter consumption. This is important because even though researchers address some items as seasonal, their definitions of seasonable have been

limited to foods that are affected by weather, like fruit. In addition to fruits and vegetables, chocolate was among the items that were found to be unevenly consumed throughout the year.(438)

Although subjects might be asked to estimate their usual intake, the time when the questions are asked will likely affect the data collected for many foods.(438) In the Canadian pilot study,(438) the reported consumption of strawberries, peaches, pears, apricots, plums, melons, cherries and fresh green beans was much higher in summer than in winter. Data about usual consumption of these highly seasonal foods may be seriously biased depending upon when the data is collected. Other foods that showed little or no change between summer and winter consumption included potatoes and orange juice. The consumption of these foods could be assessed at any point in time and the data collected would probably not be affected by the time the questions were asked. Conclusions drawn about the relationship between dietary intake and disease may be biased if these are based on data gathered at certain times of the year when some foods are either highly abundant, due to seasonal availability and reasonable price, or unavailable, due to seasonal shortages and prohibitive prices.(438)

A study by Tsubono et al. (397) revealed that responses on fruit and vegetable intakes differed substantially between their two questionnaires reflecting the seasonal differences in availability of these foods. The time frame for fruits and vegetables was therefore modified as average frequencies during seasons when they were available in the market and several items with large seasonal variation in consumption added to the list.(397) In the study of Fowke et al.,(363) season of FFQ assessment was related with the total fruit consumption determined, mainly through a relation with the citrus fruit intake estimates. Bingham et al. (380) found that seasonal variations contributed to differences in the estimation of vitamin C intake. Fowke et al. (363) found the variations across macro- and micronutrient intakes by season of FFQ administration to be smaller than for foods. The substitution of one fruit, vegetable or meat for another due to seasonal availability may maintain a stable nutrient intake.(363) If seasonal FFQ reporting is overlooked, Fowke et al. (363) found that up to 13% of subjects would be categorized in a different dietary intake exposure category fortunately only to an adjacent category.

When a FFQ is developed, some fruits and vegetables with a large difference in seasonal availability may be included to improve estimates of nutrient intakes.(427) The data of Tsubono et al. (427) suggested that inclusion of too many such items, may decrease the overall reproducibility of the questionnaire so that the potential gain and loss between increased validity and decreased reproducibility should be considered. They found that dietary items with higher initial

reproducibility, greater frequency of consumption and smaller seasonal difference in intake were less likely to reduce in reproducibility in five years.

In their summary of previous studies, Tsubono et al. (427) concluded that the agreements obtained at two weeks would mostly reflect random variation in response and those at five months would reflect random variation plus seasonal difference in intakes. The correlations at one, four and five years (in the same seasons) would mainly reflect random variation plus true dietary change and those at 4.5 and 5.5 years in different seasons, would reflect the effects of all three factors. The short-term, different season correlation at five months was lower than the short-term, same season correlation at one year, while the long-term, different season correlation at 4.5 years did not differ from the long-term, same season correlation at five years.

Thus, seasonal variability in diet may affect the reproducibility of the questionnaire more seriously over the short- than over the long-term. The season for a reproducibility study should be carefully chosen if the questionnaire is developed to assess the variation of diet at a point in time or the change in intakes within a short period. If the questionnaire is intended to study the long-term effect of diet, the choice of the seasons would be of less concern.(427) Failure to achieve high reproducibility coefficients alone does not mean that the data collected is not of high quality for the purpose of measuring consumption at a given point in time. All that is meant would be that the data collected at another point is different.(13)

5.3.3   Assessment and Reproducibility Results

For validation as well as reproducibility studies, results of mean intakes, as well as crude and energy-adjusted correlations, should be presented to permit a full assessment of agreement and correlation between measurements.(415) Reproducibility can be assessed by determining the significance of the difference in absolute mean dietary intakes between the two administrations, for example, by the Wilcoxon's signed rank test.(14,394,443) The agreement of the two FFQ administrations can be assessed by calculating the responses in the same categories of both FFQs as total agreement and misclassification into adjacent categories and extreme opposite categories (lowest and highest) and calculating the simple K and/or Kw values (14,519) with the K values correcting for chance agreement in the proportion similarly classified at each administration of the FFQ.(394) Preferable to the use of correlation coefficients is the Bland-Altman method, which determines the agreement between the administrations across the range of intakes.(14,18) Cade et al. (17,18) found that the difference in absolute intakes was determined in 39% of the studies reviewed and that less than 10% used the Bland-Altman method, which indicated limited use of this method in reproducibility assessment.

Correlation coefficients are mainly used in reproducibility assessment.(14,17,18,415) Cade et al. (17,18) found it to have been used in 90% of the studies. Correlation coefficients provide, in a single number, a measure of the correspondence between two dietary reports (415) and an assessment of the ranking of the subjects. Such subject ranking is of great relevance for RR estimation in epidemiological studies.(6)

Since the correlation between the two datasets is influenced by errors in recall and by random and systematic errors in the original diet measurement, limitations in the use and interpretation of the correlation coefficient must be recognized.(415) The method of using correlation coefficients does not determine correspondence between two questionnaire administrations, but only the degree to which the two administrations are related.(14,18) The strength of the correlation is reliant on the range of values in the subject group (which itself can be partly influenced by the size of the sample) and the characteristics of the subjects in the particular sample used. However, owing to the wide use of correlation for evaluating reproducibility, it may be helpful to use it in conjunction with another more suitable method.(18)

Where correlation coefficients are used, Pearson correlation coefficients should be utilized for normally distributed data and Spearman rank order correlation coefficients for data not normally distributed.(14,17,18) These correlations could be used for the ranking of subjects according to nutrient intake and food group intake.(356,519) Dietary intake values for which the distributions are skewed can be transformed (using $\log_e$ or square root functions) to improve normality prior to statistical testing.(394) Pearson correlation coefficients of log-transformed values can be used to assess FFQ reproducibility.(409)

The Pearson and Spearman correlations are often reported repeatability coefficients in the nutrition literature. They offer an assessment of the positioning of the subjects, but the Pearson correlation is limited to assessing linear relations and more sensitive to outliers than the Spearman correlation. This may present different values for the two correlation coefficients. When outliers in the data are removed, the Pearson correlation approaches the value of the Spearman correlation. Log-transformation has the same outcome. If the two correlation coefficients are similar, the Pearson correlation is generally favoured, as it provides more information in terms of data variability. However, neither coefficient reflects absolute agreement. ICCs can be used as supplementary or alternative to the Pearson and Spearman correlations.(519) Pearson's correlation can be used to signify reliability, but it does not take into account variability related to systematic as opposed to random differences in the results of multiple applications. The effects of learning can produce such systematic changes, for instance. Pearson's correlation is limited to two measurements per

subject. An ICC, which reflects both systematic and random differences in the results, is generally the preferable method of quantifying reliability (459).

Rousson et al. (520) reviewed the assessment of reliability of continuous measurements. These authors argued that product-moment correlation is suitable in a test-retest situation, while the ICC should be utilized for intra-rater and inter-rater reliability. They indicated the former as a conditional correlation between repeated measurements; that is, the correlation between two specific measurements made on the same subject. As it does not consider systematic error, it should be used in a test-retest situation, where the systematic error is due to a learning effect.

Rousson et al. (520) furthermore indicated the ICC as an unconditional correlation between repeated measurements, that is, the correlation between two random measurements made on the same subject, which does consider the amount of systematic error and is appropriate to assess intra-rater and inter-rater reliability.(520) The major difference between the two assessments is the treatment of systematic error, which is frequently the result of a learning effect for test-retest data. Intra-rater and inter-rater reliability depend primarily on a solid training of the raters and on good standardization of the task. Test-retest reliability reflects more directly whether a specific task is able to provide repeatable results or whether it is highly reliant on the situation or on the state of the subjects.(520) The ICCs were calculated to evaluate repeatability between two FFQs in a number of studies.(41,53,365,394,410,442,470) In the study of Parr et al. (519) for food groups and nutrients, the Pearson correlation, the Spearman correlation and the ICC provided similar results.

Since correlations are a function of both the true between-person variation and the accuracy of the questionnaire, its generalizability is limited to populations with similar between-person variations of dietary intake.(387) However, they remain for individual dietary intake, a pertinent assessment.(415) Reproducibility of the FFQ is generally good (365) with moderate to high correlations found for intervals ranging from one week to 12 months.(350) Investigators found the correlation coefficients for most nutrients to range between 0.40 and 0.70 (365) and 0.40 and 0.80 (425) with most values falling between 0.50 and 0.60.(365) Nes et al. (71) found the median correlation coefficient for nutrients in their study to be 0.70 and 19 of the 25 nutrient correlations exceeded 0.60. According to Kristal et al.,(448) FFQs are reproducible with test-retest correlations for most nutrients in the range of 0.50 to 0.90. Cade et al. (18) in their review found the correlations between two FFQ administrations of 0.50 to 0.70 to be common, while Gibson (14) noted correlations of 0.50 to 0.80. Examining the reproducibility of the FFQs developed in Japan, Wakai (399) found that with intervals between nine months to one year, the median correlation coefficient for nutrients between two FFQ administrations ranged from 0.50 to 0.72.

Unadjusted and energy-adjusted correlations between intakes on two FFQ administrations are somewhat higher for short-term repeatability compared with long-term repeatability.(17,18,365) Correlations tend to be somewhat higher for repeat administrations of one month or less apart (17,18) and up to four to eight weeks apart (365) compared with those administered six months to one year apart (17,18) and one year apart.(365) This might be explained by a change in eating habits over time.(365) Energy-adjustment has varying effects on correlation coefficients with studies finding improvement or a modest improvement in some nutrient correlations,(41) but not in other nutrient correlations.(41,365) Adjustment for energy intake may even lower the correlation between the FFQs as was found for retinol, ß-carotene and vitamin C.(365) Correlation coefficients between the two FFQs administrations generally seem to be somewhat higher among the macro- than the micronutrients in some (365) but not all (71) reproducibility studies, with often the lowest correlation for retinol.(365) The lowest correlations are generally found for vegetables, possibly because of their numerous and varied sources.(415) Among the highest correlation coefficients found is that for alcohol intake. Alcohol intake is a salient item, which subjects can recall accurately and reliably.(415)

In most repeatability cases, the second administration provided estimates closer to the mean daily dietary intake from the reference method than the first questionnaire administration. Two likely reasons include that the time covered by the second questionnaire overlapped with the reference method; and that the first FFQ and reference method application may have heightened the subject's awareness of their dietary intake.(39,409) However, the differences between correlations for the first and the second questionnaire estimates with the reference method estimates tended to be small, which suggests that any effect resulting from an increased awareness was also small.(39) For both FFQs, the second administration produced lower dietary intake estimates compared to the first administration. Many researchers have found this downward tendency.(409) However, comparing findings across studies is not credible as factors that might be responsible for decreases like with the reference method, for example, number of intervening 24-hour recalls, the time interval between the administrations and the categories of foods and nutrients investigated are not the same. It is not clear if this portrays true variability in diet, response error, or even both.(409)

5.4   RESPONSIVENESS

5.4.1 Description

Dietary assessment tools for observational research (prospective cohort or case-control studies) are selected based on the criteria of validity and reproducibility.(347) Dietary assessment tools are

required to assess changes in food and nutrient intakes over time, when monitoring food and nutrient intakes of large population groups (55) and providing evidence for changes in food behaviour and compliance in intervention trials on dietary exposure.(55,78) The primary purpose of dietary assessment in nutrition intervention research is to evaluate the delivery and effect of the intervention.(43) In nutrition intervention research, as in a clinical dietary intervention trial, the sensitivity of the dietary assessment method to the changes in the dietary behaviour or nutrient intake under study should consequently be considered.(17,459) Instruments developed to assess differences between aspects are discriminative and those developed to detect change over time evaluative. An evaluative instrument should be able to capture an intervention or exposure effect ('change'), even if the effect is small.(459) This capability has been termed the 'sensitivity' of an instrument.(17,459)   To avoid confusion with the word 'sensitivity' in the medical and epidemiological literature,(459) Guyatt et al. (459) and later Kristal et al. (19) called the measurement property of instrument sensitivity to dietary change, 'responsiveness'.(49) Instrument responsiveness is related to the extent of the change in the subject intervention outcome, which should constitute a clinically important difference. As the variability at baseline increases, larger and larger intervention effects will be required to produce a change. Therefore, responsiveness is inversely proportional not to the within-person SD, but to the between-person variability of the subject changes in outcome over time.(459)

In a pre-test and post-test or in a crossover design, the requirement of the outcome measure is less strict as it only needs to identify within-person change over time.(459)   However, demonstration of instrument capability to capture minimal differences or changes in intake over the time frame of a study or its responsiveness, is not in itself enough to guarantee the worth of an evaluative instrument. The usefulness of such instruments depends on their validity and repeatability,(49,459) being essential determinants of the effectiveness of an instrument developed for discriminative purposes, that is, the instrument really measures what it is supposed to measure and the instrument generates consistent results over time.(41,459) The interpretation of the findings of a study of diet and specific outcomes can be improved by quantitative information on the validity and reproducibility of the method used to assess exposure in the subject group. Validity and reproducibility evaluations of dietary assessment tools are achievable in the setting of a dietary intervention trial and should be undertaken when the tool's performance has not been assessed previously in the target subject group. It cannot simply be assumed that the method used to assess exposure in the target subject group will adequately capture dietary practice.(41) Interventions that focus on changing dietary behaviour can in addition cause subjects to alter their responses to dietary tools, resulting in an overestimation of compliance with the dietary intervention. For instance, list based tools (FFQs) that focus on desirable or undesirable foods are likely to increase compliance bias. Investigation of the learning effect on repeated administration

of the same tool is important in studies designed to measure changes in dietary intake over time.(55)

5.4.2 Deployment

Despite numerous limitations, self-report remains the generally used method for determining dietary intake.(49) Issues like subject motivation, supposed study burden and repeated completion of the same tool can each impact the potential to gather valid and reliable self-reported dietary intake data in nutrition intervention research.(49) Collection of dietary records or 24-hour recalls are used in dietary intervention trials,(41) especially when the objective is to assess more recent dietary intake rather than usual intake over a longer duration. These assessment methods have the benefit of providing more specific and detailed information on dietary intake than do FFQs, which relates to them being able to quantify intake more accurately and to describe intake patterns. However, considering the nutrient(s) of interest, the number of recalls and time period for dietary record collection inflate the study period, subject burden and expense. It is also possible that response rates will decline when subjects must be contacted several times. The FFQ is a cost-effective method when dietary intervention requires a large group of subjects who will be followed longitudinally thereby involving multiple measurements over time.(49)

FFQs are indicated for use to assess dietary intake when a representation of the usual diet is required.(49) However, in spite of its limitation to produce an absolute dietary intake (78,409) and concerns about its measurement errors,(49) FFQs have become an assessment method in dietary intervention trials.(41,49) Although the FFQ cannot be a substitute for recalls or records for assessing actual dietary intake,(78,409) completion of a FFQ before and after an intervention can deliver evidence that changes in food behaviour occurred.(78) Inclusion of a comparison group in nutrition intervention studies, where FFQs are used to measure dietary change, can control for reduced estimates with repeated administration of the FFQ.(409)

Although dietary assessment using FFQs is not exact,(78,409) the capability of dietary methods to quantify differences in intake over time is important to determine, given its importance to the interpretation of findings from dietary intervention trials.(49) However, only a small number of studies have validated the use of the FFQ to assess the baseline diet and dietary change (due to the prescribed intervention) in an intervention trial.(386) There is no 'gold standard' for assessing dietary change.(49) To detect any changes in usual intake during the study, observations of actual food intake both during, and either before or after the trial period, should be obtained and compared to the FFQ as test method (349) to assess the relative validity of dietary change.(41) Actual dietary intake could be based on collection of dietary records or recalls in intervention studies.(41) Because all dietary data collection methods have both strengths and weaknesses, it

is at all time advisable, resources permitting, to use more than one method.(54) In the context of intervention trials, if an intervention of unknown effectiveness is conducted and no change is detected by the assessment method under investigation, it will not be possible, without knowing through other related measures, if the assessment method is unresponsive or the intervention is unsuccessful.(459) In addition, in intensive clinical dietary intervention incorporating large changes in consumption and food preparation techniques, the measurement characteristics of the assessment method used at baseline compared to the assessment method required after intervention can be quite different and less comprehensive, but still indicate successful intervention results. While in a low intensity intervention that may result in only a modest dietary change, a general-purpose assessment tool may not detect a small intervention effect.(19)

It is therefore important to regard the dietary characteristic required to change over the study duration of clinical trials, so that the selected dietary assessment method can efficiently assess change in the expected dietary characteristic.(43) Using two independent approaches of dietary assessment can reduce this problem.(43) Intervention trials should therefore regardless utilize at least two but different dietary assessment methods.(19) For instance, the feasibility of using changes in plasma concentrations of carotenoids,(483,490,521-524), ascorbic acid,(483,490,521, 523,524) folate and homocysteine (523) as markers of responsiveness and to substantiate exposure and compliance of free-living subjects at group level, have been examined alongside dietary intake assessments in several dietary fruit and vegetable intervention trials. Thomson et al. (49) in reporting on the Women's Healthy Eating and Living Study, a dietary intervention trial among breast cancer survivors, recommended using a multi-method approach to assess differences in self-reported dietary intake over time. In addition, validity is generally determined using dietary assessment methods covering relatively short time frames in dietary intervention trials.(349) Intervention studies should report the relative validity, repeatability and responsiveness of the dietary assessment tools as part of the study outcome.(19)

5.4.3 Assessment

The capability of evaluative instruments to identify small but clinically important differences, using large enough sample sizes, is essential to their effectiveness and should be established utilizing an acceptable indicator of responsiveness,(459) which is an aspect of repeatability and an indicator of an instrument's sensitivity to change.(17) For instance, the intervention effect should result in marked changes in intake of the major intervention associated dietary exposure in the intervention group subjects compared with minimal or no dietary change in the comparison (control) group subjects (49,490) signifying between-group differences.(524) Two-sample or independent t tests can reveal significant differences in dietary intake between the intervention and control groups (between-group differences) at the follow-up time point.(522,525) The paired t tests on the other

hand can reveal significant differences in dietary intake between baseline and during trial or follow-up time points in the intervention group.(525) Kristal et al. (525) interpreted the exposure difference between the intervention and comparison groups at follow-up as 'adoption' of the intervention and the difference within the intervention group between the study and follow-up points as 'maintenance' of the intervention.

In determining relative validity, both dietary assessment methods should demonstrate significant differences in exposure between the intervention and control group subjects.(49,386) The intervention effect expressed as the mean change in the intervention group minus the mean change in the control group, which controls for baseline intake of the outcome measure,(432) can provide an indication of over- or underestimation of the test method (FFQ) compared to the reference method (dietary records).(476) The two dietary assessment methods should yield estimates of dietary change that are similar (43) in pre-test and post-test or crossover designs of the exposure intakes, as separately assessed by both the methods. To further estimate the impact of the intervention,(526) the differences in mean dietary exposures that resulted at specific study end points in the intervention, can be subjected as an analysis of longitudinal and repeated cross-sectional data, which can be evaluated by repeated measures ANOVA.(351,470,522) ANOVA can be used for determining significant differences between group means gathered over a few dietary assessment method applications, and the Bonferroni multiple comparisons calculated to recognize the pairs of means with significant differences.(78) Bogers et al. (483) indicated the intervention effect by showing the dietary exposure ingestion and biomarker levels in the intervention group before and after the intervention on assessment by means of the Mann-Whitney U test.

Changes in dietary intakes as determined by each dietary assessment method at specific study end points (476,521) and changes in dietary intakes and in plasma exposure concentrations at specific study end points,(483,490,521) can be correlated to indicate the consistency of ranking persons by the two comparison methods (dietary records and FFQ) for change.(476) Although marked increases in dietary intake and plasma levels can be found in nutrition intervention research, the correlations between changes in the dietary intake and changes in the plasma levels of the dietary exposure may be less striking.(490) As indication of validity, Pearson correlations, Spearman correlations as crude correlations or correlations adjusted for energy and/or measurement error (deattenuated), can be provided.(476,483) Considering the relative validity of a FFQ in this context, positive modest correlations between outcome changes in both dietary assessment methods, and between the dietary assessment method and biomarker of exposure, should be probable.(483)

Responsiveness, as described by Kristal et al.,(19) is the intervention effect divided by its SD, where the intervention effect is the mean change in the dietary intake of the intervention group minus the mean change in the comparison or control group, and the SD of this difference is the square root of the sum of the variances of change in the intervention and control group. The responsive analysis of Guyatt (459) is the minimum 'clinically important' effect divided by the SD of change in the control group. If dietary records are considered as the reference measure, the results of Kristal et al. (19) suggest that FFQs were somewhat less responsive to dietary change in both an intensive clinical and a low-intensity self-help fat-reduction intervention trial. In nutrition intervention research, cognizance should be taken of the dietary and exposure effects not relevant to the study that should remain unchanged throughout the study.(490)

5.4.4 Influencing Factors

To capably determine responsiveness the assessment method must be able to measure both pre- and post-intervention exposure aspects well. If a measure does not capture information on the intervention exposure, it will underestimate the magnitude of the intervention effect and the responsiveness will be low. A general purpose FFQ is likely to be the least responsive to dietary change, as it may not be fully equipped to capture the dietary exposure. Larger random errors will increase the variance of the mean difference between pre- and post-intervention, and thus decrease responsiveness. In contrast, a dietary record will probably be the most responsive method to assess exposure responsiveness. FFQs developed to assess the exposure that is the target of the intervention, will be highly responsive.(19)

In addition, responsiveness will be compromised by bias.(19) Dietary intervention trials cannot be conducted blindly as usual placebo controlled studies, and evaluations of nearly all interventions to promote healthful dietary intakes are based on self-reported dietary intake.(57) Evaluations of dietary intervention that depend on self-reported behaviour, are all subject to bias.(19) The inclination of people to eat differently when recording their consumption is in particular a concern in dietary intervention trials (54) because intervention group subjects may report dietary intakes that appear in compliance with the intervention trials goals, instead of what they actually consumed.(54,57) This will bias their reports toward overestimating the effectiveness of the intervention. This type of error is termed response set bias.(57) Assessment methods subject to response set bias capture larger intervention effects and thus appear more responsive to dietary change.(19) Dietary records and FFQs are both subject to response set bias, as the subjects may eat differently on the dietary recording days and could incorporate their knowledge of wanted dietary behaviours when considering answers about their frequency of consuming specific foods.(19) Even a mild dietary intervention can influence responses to dietary assessment

methods. Determining adherence to dietary change recommendations when based on dietary self-report can be overestimated because of response set biases.(57)

The type of intervention being evaluated will affect responsiveness. In an intensive clinical intervention where the magnitude of dietary change is exceptionally large, almost any dietary assessment method will at least be reasonably responsive, which should be considered in this research as rooibos is a rich source of flavonoids and consumed in a relatively large amount at a time, as it is usually consumed as a beverage. For such studies, it may be advisable to use less responsive methods.(19) Evaluations of the effectiveness of dietary intervention trials may be influenced by social desirability bias.(57) Nes et al. (71) found an inclination of FFQ over-reporting of some desirable foods and underreporting of 'less desirable' foods, but the representation was not consistent (underreporting of vegetables, over-reporting of fats in the first questionnaire).

Research needs to examine the effect of repeated administrations of the same assessment method on subject responses, as this relates to training or learning effects on repeated administration (43,55) that may affect the quality of FFQ and dietary record results.(43) This is particularly important in dietary intervention trials, as such studies are designed to measure changes in dietary intake over time,(43,55) and subjects may have 'learned' during the successive trial periods to report their dietary intake more accurately.(435) Such training or learning effects were found in some studies with increases in correlation coefficient results over time, where subjects may have learned to report their diet more accurately,(43,435) but not in others as the correlation coefficient results for the subsequent completed questionnaires (second and third questionnaires) were similar to those obtained for the first.(356)

5.5 DIETARY ASSESSMENT MEASUREMENT ERRORS

Because usual dietary intake of humans is difficult to assess (368) and assess accurately,(500) errors will occur in the collected data of all the methods used for assessing dietary intake.(18,475) However, despite their popularity, the precision of primarily FFQs is questioned.(376) Although less intensive methods like the FFQ (53) lack accuracy, there are few feasible alternatives to this method in particular for large population studies.(371) Collection and analysis of dietary data is still essential to determine relationships between food use and disease.(475) Dietary measurement error could affect such disease risk assessment.(372,463,464,500) FFQ measurement error reduces the estimates of FFQ-based disease RR determination and decreases the statistical power in FFQ-based epidemiological studies that may cause a relation between dietary intake and disease risk to be unobserved.(372,463,464)

The accuracy limitation of FFQs is highlighted through reported inconsistent associations between dietary components and cancer risk in large prospective epidemiological studies.(372,450) This refers in particular to the finding of a statistically significant relationship between dietary fat intake with breast cancer risk based on dietary records, but not based on FFQs.(450) Inconsistent epidemiological relations between FFQ dietary fibre intake and colorectal cancer may be the result of inaccurate assessment of dietary fibre intake.(375) The inconsistent results of such studies may be due to a real lack of diet-disease relations (372) or instead, methodological limitations of the studies, particularly FFQ measurement error,(372,450) with the resultant poor dietary assessment possibly attenuating the diet-cancer relationship.(450) Some low correlations between well-designed FFQs and biomarkers as gold standard criterion measures, in particular for energy and protein intakes, add to the poor measurement connotation.(450)

Even when an effort is made to assess dietary intake as correctly as possible, there will always be sources of error and it is essential that an effort be made to reduce them. The many components and sources of error across the dietary assessment methods include: incorrect recording of dietary items consumed; incorrect consumed amounts recorded (52) or consumed portion size estimates; assuming usual long-term intakes based on current dietary intakes (51); incorrect consumed frequencies recorded; bias in recording socially desirable foods; day-to-day intake variability; change in usual dietary intake (52) as subjects may eat atypically during a dietary recording period, even though an effort is made to discourage this (349); method related bias in which consistent over- or under representations occur (51); training and experience of interviewers (416); coding errors; and food composition tables used.(52) In addition to these measurement error sources related to the dietary assessment itself (completion errors and food composition tables used), there are additional concerns like the extent of the preparation and processing of foods and the combination of foods eaten together that impact nutrient content and absorption. These aspects add error to the intake estimates obtained through dietary assessment methods and will affect diet-disease associations.(77)

With regard to the FFQ, the questionnaire design affects the collected dietary data.(370) FFQ inaccuracies result from many areas. The fixed food list that restricts the collected dietary data may be incomplete.(451) Single dietary item questions and aggregation of different dietary items into single questions are used on FFQs with higher intake values being obtained for the ungrouped single items than for the aggregated items.(359) There is considerable concern about the accuracy of portion size estimates reported on semi-quantitative FFQs from the rough portion size provisions.(346,359) It is possible that the variability in memory of consumed portion sizes over time is so large that portions sizes cannot be estimated accurately from memory.(359) Most

critically is the error from estimating the usual frequency of food consumption from the set of frequency options. As with portion sizes, food frequencies are not constant over time.(415)

Indicating how much of and how often a specific dietary item was consumed over a broad time period involves a cognitive challenge through complex processes of memory retrieval and integration of meals consumed in the past into a computed average frequency response of use for each item on the FFQ.(363) Assumptions and calculations must be made when the subject's individual portion sizes and frequencies differ from those offered in the questionnaire. People do not pay attention to portion sizes and frequencies when eating.(415) The most accurate frequency responses can be expected when the format of the responses matches the internal representation of the frequency information and subjects are not required to carry out complex arithmetic considerations to express frequencies for combination items.(359) Subjects seem to best estimate relative as opposed to absolute frequencies.(415) Consumption estimations provided on a FFQ are never without error and result in biased estimates of reported consumption portion size and frequency.(401) The potential problems of FFQs need to be identified to clarify the interpretation of the results obtained and reduce the misuse of dietary data derived from FFQs.(369)

Some potential sources of error are shared between dietary assessment methods.(378,495) An error common to both the reference and test methods is firstly the use of the same food composition tables.(18,378,502) The same database should be used for both test and reference methods because significant differences can exist in the nutrient content values of different databases.(388) However, dietary assessment is only as precise as the food composition tables and the coders. Reports of up to 30% intra-coder variation may have important implications for the potential to demonstrate relations between dietary intake and disease occurrence.(354) Differences in collected dietary data are most likely derived from the dietary assessment than from differences in the database when the same database is used. Such differences might be attributed to either the types of or the amounts of food or drink recorded.(366) However, errors due to data entry and analysis mistakes are subject to investigator control.(13) Inaccuracies in food composition tables used for conversion of food consumption amounts into nutrient intakes may translate into similar errors in both assessment methods.(495)

Two dietary assessment methods commonly used as reference standards to evaluate the performance of a FFQ, are multiple dietary records and 24-hour recalls.(377,433) Each of these methods involves more work and cost than a FFQ, but is thought to involve considerably less under- or over-reporting at individual level.(377) The generally accepted superiority of dietary records over FFQs makes them the present practical gold standard for dietary assessment,(18,377,440) as the errors in the two methods are overall viewed as

independent.(387)   As mentioned, dietary records are open-ended, allow a more direct assessment of portion sizes and do not depend on memory.(387)   Errors may relate to reviewer/coder interpretation and coding of the records rather than to the subjects.(422)   The training and instructions provided to the subjects is, however, likely to influence their recording of their dietary intake.(416)   In contrast, the main sources of error in the questionnaire are misinterpretation of questions, restrictions imposed by a fixed food list and recall errors related to memory causing misreporting, which is associated with the ability of the subject to describe usual frequency of intake and the assumption of average portion sizes for most dietary items.(368,387,422)

Unfortunately there are correlated errors between dietary records, recalls and FFQs, such as correlated errors in the databases.(442)   Regarding the FFQ and weighed dietary record assessments, both may be biased in the same direction and to a similar degree by a subject's lack of motivation to record precisely all items consumed over one or more days, or to fill out a FFQ completely about longer term usual dietary intake. When 24-hour recalls are utilized as reference dietary assessment method, the possibility is greater that there will be some degree of correspondence between the errors because both methods depend on the subject's memory and the resultant capacity to produce accurate data on the items and quantities consumed.(495) Such memory-based errors cannot be considered statistically independent.(502)   Under- or over-reporting by the same subjects on dietary assessment methods may be a further source of shared error.(378,383)

Such similar error sources in the measurement of dietary intakes by both methods can produce dependent errors, which may lead to systematic over- and underestimation of dietary intakes in subjects with both the test and the reference methods.(502) The consequence of such correlated or dependent errors between methods would be that falsely elevated correlation coefficients are created (38,502) that increase the apparent comparability of the assessment methods (377,383) leading to wrong assumptions on validity.(38,502)   Errors in the estimation of intake by both methods that were independent would tend to underestimate the effectiveness of the questionnaire.(38)   Dependent errors in recording intake could occur, for instance, if a subject consistently underestimated intakes of or misnamed certain foods in both test and reference methods. Since the test and reference dietary assessment methods are usually quite different, this source of common dependent error is believed to be minimal.(38) In the context of the validity of dietary assessment methods, it necessitates that the errors in the reference method and those in the test method (FFQ) be independent.(372) The advantage of biomarkers is that the errors are in principle independent.   Therefore, biomarkers are valuable to evaluate the validity of dietary assessment methods.(502)

Two broad types of measurement error occurring in the measurement of dietary data, including FFQ collected data, are systematic bias and random error.(344,369,372,475,495,501) The errors that affect dietary method validity are systematic errors, those related with precision or reproducibility are random.(14,349) Systematic bias in intake estimates (all subjects showing the same error, for example, where use of the FFQ leads to similar over-reporting of fruit and vegetable intake in most subjects) will have minimal impact on the ability to detect a relationship, but it will lead to a flawed description of the relationship (475,500) that may compromise the interpretation of RR estimates in epidemiological research.(495) It may shift the location of the distribution and cause over- or underestimation of the percentiles of the nutrient distribution.(369) Subject specific errors may give rise to very high correlations between repeated applications of the same or similar methods.(475) Very little is known about the extent or direction of such systematic errors associated with validity and they are difficult to control.(349) Random bias (random misreport variation within subjects over time, and direction and magnitude of bias varying between subjects) (475,500) on the other hand flattens the observed nutrient distribution (369) and reduces statistical power where an effect may be underestimated, but it does not distort the analytical result.(475,500)

In statistical terms, validity is the absence of systematic error (or bias), as opposed to random error.(502) Any dietary assessment methodology is prone to a degree of mis- or underreporting or random error.(18) Systematic respondent related reporting bias or systematic error in measurement is of greater concern than random error.(56,351,377,435) Systematic bias entails a systematic under- or overestimation bias of intake in a subject or group of subjects.(377,435,475) It can arise from under- or over-reporting of intake (intentional or unintentional) and from error in food composition databases.(475) This will result in falsely high correlation coefficients between dietary assessment methods (reference and test methods).(378) In nutrition research, absence of bias has been translated into adequate assessment of the absolute level of intake for a given nutrient, either for the subject, or for the population group studied.(502) If a FFQ is to be utilized to determine absolute dietary intakes, the degree to which these estimates agree with actual intakes should be evaluated.(351) Kristal et al.,(42) for instance, defined bias as the difference between mean FFQ-based method estimates and a reference method, either multiple dietary records or 24-hour recalls, estimates and the result of significance testing (paired *t*-tests).

Systematic bias in 24-hour recalls may result from subjects' varying systematically in their capacity to recall what foods were consumed the previous day or to describe portion sizes.(495) Even dietary records are not completely unbiased.(377) There is a systematic tendency of subjects to underreport actual dietary intake (351,477,495) that has been explained by either a failure to

record that which has actually been consumed or a tendency to change dietary habits on the recording days (495) due to the measurement process itself,(422,469) which equates to observation bias.(469) Due to the burden brought about by keeping dietary records, subjects decrease the number of dietary items consumed and reduce the complexity of their dietary intake by replacing more complex dietary items with items that are simpler to record. Such changes result in subtle changes in dietary intake.(477) Rebro et al. (477) established this trend even in a study of healthy, motivated volunteers who, at the start of a study, only recorded dietary intake on four non-consecutive days. This instrument or method effect may reduce the capability of studies to ascertain the real representation of usual dietary intake from dietary records. Based on their findings, Rebro et al. (477) suggested that studies utilizing dietary records should avoid prolonged periods of successive recording days (such as a seven-day dietary record) to limit subject changes in dietary habits. One of the most frequent reasons provided by subjects for changing eating habits while keeping dietary records is to reduce the inconvenience of recording dietary intake.(477) The presence of dietary record underreporting is generally considered problematic.(351,470,477)

With regard to FFQs, subjects may systematically misinterpret the description of certain dietary items or they may tend to systematically over- or underestimate consumption frequencies or portion sizes.(502) The FFQ tends to overestimate rather than underestimate dietary intake (40,350,366,425,518) and this is evident for most foods, food groups (350,518) and nutrients.(40) Overestimation, which is well-known in FFQs (386,390,425), can be the result of overestimation of frequency of use (40,386,390) and in problems in evaluating portion size.(346,359,390,401) Over-reporting and overestimation of dietary intake has been linked to the long list of food items and food groups in FFQs,(369,379,425) particularly when the number exceeds 100.(386,425) Mullie et al. (425) as a result limited their semi-quantitative FFQ to 150 items, as incorporating less than 100 items may limit possibilities to determine variations in dietary patterns and 150 items still seemed to be an acceptable subject workload. Warneke et al. (409) found that a seven-item fruit, juice and vegetable intake FFQ, correlated better with reference intakes than did a 31-item FFQ. They indicated that the larger number of items on the 31-item FFQ might have inflated intake reports; however, on the other hand, the investigators speculated that the broad seven-item FFQ categories might have caused the subjects to forget foods not specifically mentioned. Bingham et al. (40) does not quite support overestimation to be linked to the length of the food list given that the two questionnaires they used differed in length. In addition, repetition of a task increases memory and the likelihood of remembrance. This may result in an overestimation.(366)

Cade et al. (18) noted in their review that the mean correlations between FFQ and reference methods are generally lowest for vegetable intakes. The poor agreement relays to vegetables

assessed as a food group and as separate items.(518) FFQs provide support for overestimation of consumption of a number of food categories, but this is particularly true for vegetables.(40,408,518) Bingham et al. (40) found that the higher mean total vegetable intake was due to a higher reported frequency of consumption in the questionnaire methods than actually reported by weighed records. Mean intake of vegetables were reported as 406 and 386 g per day, respectively on the two FFQs, but was 272 g per day from the weighed dietary records. Because of this mean difference of 120 g, vegetables the FFQ nutrient intakes were higher. In a study of Marks et al.,(518) more than 10% of the subjects were grossly misclassified (differing by three quartiles) for all vegetables by the FFQ compared to the weighed dietary record categorization. Kristal et al. (42) indicated that the assessment of usual vegetable intake is imprecise and as a result poor, and that vegetable intake should, whenever possible, be collected by additional measures, such as dietary recalls or even biomarkers of intake, to validate results based on FFQ measures alone.

Cade et al. (18) and other investigators (500) indicate that the misreporting and resultant over-reporting of vegetable intake can be ascribed to numerous reasons with the most possible explanation being subjects responding to some food items more than once when they report mixed food dishes. This is the result of double counting of vegetables consumed as part of a mixed dish that do not represent an entire portion,(18,500) and social desirability bias as the tendency to over-report intake of so-called 'healthy foods' that includes vegetables and fruit.(18,56,500) Within vegetables, there seems to be better agreement between FFQ-based and recall-based methods for salads than for cooked vegetables. One explanation of this could be that salad consumption tends to have a more fixed dietary pattern, as in the case of a salad with dinner. Therefore, they may be recalled with greater accuracy than foods consumed irregularly.(42)

Although overestimation of fruit and vegetable intakes are generally found,(518) self-reported methods are more valid for intakes of fruit and fruit juice than for vegetables.(42,518) Correlations between dietary assessment methods are consistently higher for fruit than for vegetables.(42,518) When the ranking of the subjects is retained, the overestimation of the dietary intake does not present a great concern.(390,425)

Systematic biases include response set biases that occur due to social desirability and social acceptance. Given the heightened concern and rising public interest in diet-disease associations, the mounting availability of information on this topic and the broad public knowledge about dietary guidelines for health, dietary self-report is particularly exposed to social desirability and approval bias. Assessment in such domains associated with correct or wanted responses provide the opportunity for response set biases.(56) For instance, given the public awareness to follow

'correct' dietary behaviours, persons with a high intake of apparently 'unhealthy' food may tend to underreport this intake and those with a low intake of apparently 'healthy' food may tend to over-report this intake.(464)

All dietary assessment methods, whether record or recall, may be vulnerable to biases related to the subject's wishes to portray an enviable image or to obtain approval for certain behaviours. Unfortunately, the social desirability and approval bias would theoretically be strongest in using the FFQ, which aims to determine longer term usual intake. Response set biases that result from social desirability and social approval can attenuate the measurement of the dietary aspect of interest thus affecting RR estimates in epidemiological studies. Even being aware of one's health status and having a belief or perception about a dietary-disease link, provides the opportunity for confounding information.(56)

The outcomes of biased self-report of dietary would influence two aspects of intake: (a) how social desirability is spread in the subject group and its effect across intake levels of the nutritional aspects of interest; and (b) whether social desirability is associated with the study outcome. If social desirability were associated both with the reporting of the dietary data and the study endpoint, there is a great likelihood for confounding bias. If everybody in the study group has the same inclination to report socially desirable dietary intakes, the resultant bias caused would be consistent across the distribution levels of the nutrient analysis.(56) However, bias variation may be introduced if certain subjects have a greater tendency to report socially desirable information as found in the study of Herbert et al.(56)

Evaluations of the effectiveness of dietary intervention trials or programs should be concerned with response set biases like those caused by social desirability,(56,57) as it may compromise evaluation of intervention effectiveness.(57) In intervention trials specific recommendations are made to subjects to change their diet and subjects may bias their dietary self-reports to appear to comply with the intervention conditions.(56,57) Even a modest dietary intervention can influence information reported to dietary assessments.(55,57) An intervention intended at increasing intake of socially desirable dietary items and decreasing intake of undesirable items may be subjected to greater response set biases due to social desirability bias because of the messages conveyed to the general public.(56) Determining adherence to dietary change recommendations, when evaluated on dietary self-report, can be overestimated if intervention group subjects report dietary intakes that correspond to the conditions of the intervention rather than what they actually consumed, as a consequence of response set biases.(55,57) Providing incomplete or false information equate response set biases.(469)

Kristal et al. (57) put forward that response set bias would be less prone to influence dietary recall when information is abstracted from the episodic memory, since biased reporting of recent, specific eating events would entail either purposefully forgetting socially undesirable items or purposefully producing socially desirable dietary information. The potential for bias would theoretically be greater in FFQs (56,57) where subjects have to put together quantitative responses about past dietary intake (57) than for short period assessments, like dietary records or 24-hour recalls in which the subject is asked to report on intake of specific items either while they are consumed or during a well-defined time frame in the very recent past.(56) In the FFQ situation subjects for the most part do not know the actual answers to the questions. In compiling a rational best answer, they could be influenced by awareness of normative or socially desirable behaviour.(57) List-based methods that focus on desirable or undesirable dietary items are likely to increase compliance bias.(55)

Under- or over-reporting of intake may be randomly distributed in a study population as some subjects may underreport, while others over-report which may behave like a random error, not a systematic bias.(475) It is commonly assumed that usually more than 75% of the unexplained variability in FFQ-derived dietary intakes in comparison to 24-hour recalls or dietary records, are attributed to within-person variability which is viewed as random.(56) Generally, the between-person variation is small with the within-person variation exceeding the between-person variation.(40) Random errors result mainly from day-to-day fluctuations in true intake.(464,475, 495) Further random within-person errors are processing variation, recording mistakes, and so forth.(464) However, there is still doubt whether random errors of reference methods are fully independent of those of the questionnaire. It is uncertain whether errors are independent between replicate reference measurements obtained with the same method in the same subjects.(495) In contrast, random errors in an assessment method not related to those in the questionnaire would be inclined to artificially decrease correlations.(378)

Neither the FFQ nor dietary record is fully reliable.(476) Concurrent validity which is determined by assessment against another dietary assessment method merely compares one method with another and do not assess reported intakes against a bias-free standard of intake.(351) Although estimates from FFQs is correlated with 'true' usual intake,(377) it may involve error at the individual level in the FFQ, which may incorporate both random and systematic error components.(435,464) Associations that may exist between two data collection methods can be masked by measurement error that can result in attenuated (decreased) correlation coefficients.(476) Random error, like the day-to-day variation in intake within the same individual, can attenuate coefficients or it can lead to overestimation of the coefficients.(475) Validation requires minimal within-person variation.(393) The conventional approach for controlling this is to collect multiple dietary records or recalls and

pool them for each subject, thereby reducing the within-person random error and obtaining a better estimate of usual intake of the subject.(475)

Many researchers have discussed the problem of within-person variability of dietary intake due to an inadequate number of records or recalls.(369) Correlation coefficients can be adjusted or corrected for such random error or attenuation effect of within-person day-to-day variability in intake to evaluate internal validity of the developed FFQ.(378,411) Due to the day-to-day variability in the dietary items people consume, deattenuated correlations are more representative of true association or relationship between the results of FFQs (usual intake) and dietary records/recalls than are the observed crude (attenuated) correlations.(475,476) Deattenuated correlations provide an indication of the relation that would have been found if the methods could assess dietary intakes without error. The higher the deattenuated correlations, the less the possibility that subjects will be misclassified by the linear ranking.(476)

Random error (mixture of methodological error related to random bias in reporting by the same subject on different days and true variation in intake from day-to-day) has no impact on the estimation of group mean intakes or comparison of mean intake across categories of subjects, except that it increases variance and decreases statistical power. Statistical power can be improved by collecting more data or by adding more subjects. The choice is often based on logistical considerations, as well as sampling issues.(475) In categorical analysis in which subjects are categorized by intervals of intake, random error means that a particular subject may be placed in the wrong interval.(475)

The statistical and mathematical procedures for handling error in collected dietary data and improving dietary intake estimation are still being developed, not that readily available (519) and underutilized.(51) Multiple day dietary records or 24-hour recalls are generally used in validation-calibration sub-studies as 'reference' methods to calibrate FFQs and to adjust findings from nutritional epidemiological studies for measurement error.(372) Beaton et al. (496) realized that the reference method itself is subject to error and described a procedure using the within- and between-person variance components to correct the correlation coefficient based on the mean dietary intake of multiple day dietary recalls or records for each subject. According to Cade et al.,(18) this correctional approach in FFQ validation is not commonly utilized. Among this group of experts, the consensus was that they had reservations about adjustment for measurement error, especially if the adjustment created large changes in the dietary intake estimates. They indicated that if, as a result of a validation study, the dietary results were adjusted for measurement error the details of the adjustment must be provided.(18)

For any assessment method chosen as validation for dietary intake in the absence of a 'gold standard', good comparability does not in reality suggest (criterion) validity. Comparability may simply indicate similarity of measurement errors in the methods.(91,349) Validation is often presented by correlation between the reference and the test method dietary intakes.(17) Intakes of various nutrients are linked. True correlation may therefore result from correlations among measurement errors and from correlated dietary intakes. The fact that the test method has some connection to what it is supposed to measure does not validate it. Validity should be considered in terms of what the test method is not supposed to measure.(76)

The nature, sources and impact of measurement errors nonetheless need to be considered particularly when a new data collection method is developed.(475) Unfortunately, it seems as if some researchers do not fully consider the measurement error associated with a FFQ and report their findings as if the data obtained were without error.(86) Smith (359) put forward that in validity assessment, the criteria should be if the test or comparison method (FFQ) is valid relative to the reference method or standard on the reported accuracy with which foods on a FFQ, item by item, have been reported during the reference period, as most subjects eat foods, not nutrients, and it is foods, not nutrients, that are represented in memory.

Human memory is considered to be both episodic and constructive, which makes it susceptible to random and systematic errors.(363) Memory is a huge issue with recall dietary assessment.(346) Responding to recall, dietary assessment methods engage the cognitive processes of subjects, requiring them to retrieve and report information from memory on their dietary intake.(359,363) All such methods assume that subjects can retrieve the relevant dietary information from memory and report reasonable answers.(359)

Dietary intake assessment literature indicates that reported FFQ data are based on cognition and memory.(376) Four stages in the cognitive processes used in recalling dietary information have been described (415): The first stage involves question comprehension. If the question is understood, the next stage involves information retrieval that requires retrieving from memory whether an item was consumed in the past. Problems with interference (current dietary intake) occur at this stage. At the third stage, the subject makes a judgement or estimation about the retrieved information. Estimations are made when the subject cannot retrieve any information, or if the information recalled is considered inadequate. The subject uses general ('generic') knowledge of his/her diet, either current or past, and proxy past information if the subject cannot accurately remember eating an item. Errors are introduced at this point.(415) Dietary assessment methods imply that the subject is to retrieve either memories of actual dietary experiences or general knowledge about his/her diet.(359)

Cognitive psychological experimental results suggest that generic knowledge about diet contributes to dietary reporting.(359) Recorded and reported items can be classified into three groups: 'matches', reported items that had actually been eaten and recorded; 'intrusions' or 'commissions', reported items that had not been consumed or recorded; and 'omissions', items that were not reported that had been consumed and recorded.(359) Errors of omission and commission were found to be high.(415) If the subject accurately remembers consuming a dietary item or erroneously assumes it was consumed, then the quantity consumed and the frequency of consumption must be considered for the final stage of the response formulation,(415) which introduces further errors.

Cognitive psychological experimental results suggest that reporting precision of episodic memory of diet deteriorates as the time interval between the reference and recall period is increased or the retention interval lengthens.(57,359,415) When a subject is asked to report dietary intake in the very recent pass, their episodic memory is relatively exact. The precision of episodic memory of dietary intake declines as the time interval after the event increases. After only a few days the recall of items ingested is compiled from general knowledge about diet, most possibly based on ideas of one's 'usual' or habitual diet.(57) The reporting performance of subjects asked to recall their dietary intake for a two or four week reference period after a delay of as much as six weeks is poor.(359)

It is hypothesized that subjects at short retention intervals report some specific recent memories, but when the report is delayed, describe their typical diets.(359) Dietary items ingested near the time of the FFQ administration may trigger the memory, such that FFQ responses underscore these ingested items as recently consumed. Items less recently ingested may involve generic or usual judgment of past dietary intakes, causing subjects to report past consumed intakes of these items.(363) Thus with the exception of ceremonial items or those never consumed,(57) recall of usual dietary intake is a process not likely to be quantitatively accurate,(57,448) particularly if the required information is about common items consumed over an extended period,(57) compared to when subjects are required to recall dietary intake in the very recent past, as the episodic memory is relatively precise.(448)

Data converge on the notion that a generic representation of the subject's diet plays a major role in dietary reporting.(359) It is assumed that subjects would tend to assign larger numbers to events that have occurred more frequently and smaller numbers to events that have occurred less frequently.(359) In addition, subjects may indicate what they consider they should have been consuming and not fully what they actually consumed, and they may even innocently twist their

responses. For dietary intake, this bias would be manifested in the over-reporting of 'good' foods (vegetables and fruits) and the underreporting of 'bad' foods (high fat foods).(415)

5.6   MINIMIZING QUESTIONNAIRE ERROR THROUGH QUALITY CONTROL

The ability to determine a relationship between dietary intake and disease depends on the quality of the dietary exposure assessment,(345) which in turn depends on the quality of the dietary assessment method.(72) Determining dietary intake is not easy to accomplish and necessitates either thorough dietary assessment or the measurement of specific biomarkers. These methods are not economical and not in particular practical for the day-to-day assessment of dietary intake in a community setting or in primary care.(505) Various efforts have been undertaken to ease the administration of self-administered FFQs and improve the data quality.(374) Quality improvement includes two aspects: to establish good quality by preventing and reducing errors, and to detect errors. The latter is what traditionally is meant by quality control. These aspects are combined in the concept 'quality assurance'.(527)

Because of the simple nature of FFQs, improvements can be made in only the following ways: by adding more items to the food list or subdividing existing aggregated items in single questions; by gathering more detail on the quantities consumed or portion sizes; by collecting more detailed consumption frequency information; or by adding further questions on details of specific foods.(30) According to Willett,(30) the gains to be made by adding additional dietary items are probably quite limited particularly in a self-administered format where cooperation and mental energy are likely to substantially diminish, if many more than 150 items are listed. Willett,(30) however, indicated that one of the areas where small, but real, improvements could be made is the separation of aggregated items based on careful and thoughtful consideration. For instance, including the same food used in different ways as separate items could be useful when the amounts are quite different, such as, separation between cooked carrots eaten as a separate vegetable and carrot rings in a mixed vegetable dish as the actual amount of cooked carrots could differ quite substantially in the two uses. On the other hand when planning to simplify the questionnaire by aggregating foods into categories instead of using single foods, the error introduced by doing this should be evaluated in advance. (527)

Willett (30) indicated that gathering additional information about usual quantity consumed, seemed to be less helpful to provide substantial enhancement in most cases. The possibility exists that for a few cases, possibly beverages, additional information might be useful. A body of evidence had accumulated indicating that the gain in increased portion size information is extremely small. Two underlying reasons include that frequency explains most of the variation in total amount of a food consumed and that the within-person variation in portion size for nearly all foods is much greater

than the between-person variation in portion size. There is little evidence that expanding the use of frequency categories beyond nine or ten response categories or a continuous open-ended format, provides any substantial further enhancement.(30)

During the collection of dietary intake data, the main areas for quality control, which have in the main been described by Haraldsdóttir,(527) include:

(a) *Planning the dietary survey*: The standard components of the planning stage are important for the quality of a survey and include the selection of the dietary assessment method, the data collection plan, the training of fieldworkers, the preparation of instruction materials, method use pre-testing, checking of the comprehensiveness of the nutrient database as the data for some nutrients, may be insufficient or even unreliable.(527)

(b) *Questionnaire design and comprehension and aspects to improve responses*: Friedenreich et al. (415) and Subar et al. (374) provided several practices based on cognitive processes, which can be used to improve the design and comprehension of the questionnaire and the quality of the responses in recall dietary studies of past diet. These practices can be used to reduce random measurement errors occurring in recall dietary assessment.(415) The first being, attention to the questionnaire design and its pre-testing to improve understanding of the questions and provision of a questionnaire, which is clear, simply worded and understandable to the study subjects. To improve judgement and estimation of the retrieved information, the subject must be aware of the time frame of interest to report their intake responding to this time frame.(415) Attentive training of the subjects in the dietary assessment method through providing oral, as well as written instructions, clear instructions with relevant examples and emphasizing the importance of maintaining usual dietary habits during the study, should take place.(357,381)

In the case of interviewing, efforts to advance the quality of FFQs emphasized cognitive interviewing, aided recall and other memory aids developed to assist subjects better calculate the average frequency of the dietary intake. In cognitive interviewing, the subjects are instructed to verbalize their thought processes as they comprehend questions and formulate responses.(374) Detailed interviewing techniques to aid recall and reduce interference during the information retrieval stage, include placing the subject back in the time period for which the dietary intake is being recalled by recreating the context, and to apply and vary the methods designed to assist information retrieval from memory (memory cues, paraphrasing, concurrent think-aloud methods).(415)

When not fully mindful of their dietary intakes, subjects depend on the mental image of their usual dietary intake to report an estimated average frequency of dietary item use in which the reported frequency of use can be an indication of a point of view rather than actual dietary

consumption.(376) A key difference viewed from a cognitive psychology perspective between the 24-hour recall and the FFQ, is the nature of the cues that are provided to the subject to extract information about what he/she has eaten. With the recall task, the subject supplies the consumed items as cues, whereas in the recognition task, the dietary items are supplied to the subjects as cues on the questionnaire. In each case, the subject is required to report about the contents of the memory in response to the cues.(359)

Besides these practices based on cognitive processes to improve subject responses, many technical advances can assist to enhance measurement of dietary consumption, such as electronic devices for directly recording dietary intake.(14) The Wellnavi (Matsushita Electric Works, Ltd, Osaka, Japan) is a highly developed tool for evaluating subjects' dietary intakes and consists of a hand-held personal digital operating system with camera and mobile telephone card attachment for forwarding the dietary photo data for analysis. College students graduating in food and nutrition at the Okayama University, Japan kept a one-day weighed dietary record and obtained a digital image of all items recorded which were provided to dieticians via a mobile telephone card for analysis. The next day, an unannounced 24-hour recall was obtained from the college students. Both the Wellnavi method and the 24-hour recall showed relatively high correlations for most nutrient intake estimates with the weighed records. Although information on standard additions to foods might need to be improved for a better analysis of some nutrients (sodium), the method was found valid and convenient (it took on average of 16 minutes using the instrument and 37 minutes using the weighed record to record the dietary intake).(528)

In addition subject motivation and sense of commitment to the study must be gained by explaining the importance of the study and the need for an accurate dietary measurement to create honest and improved response formulations, which is essential to reduce several sources of measurement error.(415) Gibson (14) pointed out that the motivation and compliance of the subjects, greatly impacts the performance of a dietary assessment method.

(c)   *Checking of interviewers and other fieldworkers*: To ensure that for the most part fixed procedures are correctly followed and do not undergo a gradual change during the study, interviewers or other involved fieldworkers must be trained and standardized beforehand and their procedures and collected data checked during the data collection period.(14,527) The procedures for regular supervision and checking of the data must be established in advance of the study.(527) This is to prevent that systematic differences in the various task procedures, as the instructions in how to keep dietary records, how to complete questionnaires, wording the interview questions, the extent of probing, etc. followed between fieldworkers do not arise during the study. Re-training sessions may even be required in studies with a prolonged data collection period to ensure compliance with the study procedures.(14,527)

(d) *Use of biomarkers*: Biomarker use in dietary surveys is usually regarded as a validation measure, but the use could equally be as calibration or quality control.(527)

(e) *Handling of collected data*: The need for quality control is probably recognized most in the data handling part of a dietary survey.(527) The quality controls include checking the data for coding and capturing errors. Even double-checking of the data can be carried out by duplicate data coding or entry for a certain percentage of the subjects (10% or 20%) and carefully examining the entire dataset in terms of the frequency distribution for each question and each food code in order to evaluate the sensibleness.(381,527)

For quality control purposes, the completeness and the quality of the FFQs are commonly evaluated by means of omitted or blank items and/or blank item blocks or entire blank sections (grouping of items in the questionnaire, such as food groups). However, different investigators apply different guidelines regarding the number of allowable blank items or item blocks on FFQs. FFQs from subjects that suggest that they did not complete it plausibly are then excluded.(529) Such criteria for exclusion of data should be decided beforehand and be stated with the number and percentage of questionnaires excluded.(17,18) Regarding item blocks or entire sections, FFQs were considered unacceptable if two or more adjacent pages were skipped,(417) one or more item blocks or entire sections were left blank (351,379) or if at least one-half of each food-grouping category had not been completed.(442,530)

Pertaining to allowable blank items, FFQs were considered unacceptable on condition of: 46 or more blank items equivalent to two blank pages (457); 10 or more blank items (38,40,380,423); more than 12 blank items (531); 10 or more of the 61 items blank in the case of females and 70 or more of the 131 items blank in the case of males (532); 30 or more blank items (248,250); more than 60 of the 150 items blank (379); and 70 or more blank items.(254,424) On the contrary, some investigators considered the number of completed items to be the retaining factor, namely 90% of the questions had to be completed for acceptance by Patterson et al. (442) and at least 50% in both the test and re-test administrations by Parr et al. (519). If FFQs were not fully completed, subjects were contacted in some studies (432) to obtain the missing data.

Other specific problems that Caan et al. (529) encountered in the completion of FFQs included unreasonable amounts of intake, like too few or too many foods per day, and too many answers in one category. With regard to the former problem, Goldbohm et al. (379) considered the sum of specific response errors, that is fewer than 35 items eaten at least once a month, by means of an error index, and Bingham et al. (40,380) a consumption frequency indication of four or more per day of selected items as rational for exclusions. In relation to the latter problem, Thompson et al. (417) excluded questionnaires with more than 10 items with multiple frequency responses marked. Schaefer et al. (351) excluded FFQs if

the portions of foods listed were not specified. These investigators excluded those with incomplete dietary record data (less than three days recorded) from the dataset instead of asking subjects to complete another dietary record. However, exclusion of subjects introduces an additional error source into the dataset.(14)

Caan et al. (529) found that the most common error to be omitting portion size or frequency (or both). Omitting portion size was less of a problem than omitting frequency because the subjects checked 'medium' for portion size about 70% of the time. The majority of items originally omitted were those that were either never consumed or consumed infrequently,(529) indicating that the omitted items generally represent the rarely consumed.(504) The most common frequency error was entering check marks instead of numbers in the open-ended frequency column.(529) Caan et al. (529) found that changing the format of their questionnaire that required subjects to now only check one of seven frequency categories, rather than designate a number, caused fewer subjects to misinterpret the completion instructions. The younger subjects were the more likely to respond correctly and the less educated subjects the more likely to respond incorrectly.(529) Caan et al. (529) established that only a small percentage of persons omitted 10 or more items on the questionnaire and that even a substantial number of omitted items did not alter the ability to rank the subjects by nutrient intakes, a goal of most epidemiological studies. If the aim of a study, however, is to estimate absolute levels of nutrient intakes, FFQs with even a small number of omitted items will underestimate total energy intake and most nutrients.(529)

The Schofield equations and Goldberg cut-off could be employed to eliminate subjects reporting an unfeasibly low energy intake,(18,343) although this may not be relevant to FFQs because they are developed to assess the usual dietary intake of subjects over a time frame and are not accurate for a precise assessment of energy intake.(343) Briefly, height and weight measurements are utilized to calculate basal metabolic rate from the Schofield equations. A ratio of estimated energy intake (EI) to predicted basal metabolic rate (BMR) is determined as EI/BMR. The ratio is then compared with the Goldberg cut-off value that corresponds to the lowest value of EI/BMR that reflect the habitual energy expenditure given a sedentary lifestyle.(343)

In the framework of the FFQ, subject exclusion could occur based on energy cut-off values associated with a plausible kilocalorie (kcal)/megajoule (MJ) intake range, set by many investigators at a daily total energy intake less than 600 kcal (2.51 MJ) (43,250,423,432, 442,531) or less than 800 kcal (3.34 MJ) (424,431) and greater than 4 000 kcal (16.74 MJ) (423,424,531) or sometimes greater than 3500 kcal (14.63 MJ) (442) or 5000 kcal (20.9 MJ).(43,248,250,431,432) This suggests that the dietary intake was not completed sufficiently.(432) Considering gender, the daily total energy intake for females are often set at less than 500 kcal (532) or 600 kcal (153,232,417) or higher than 3500 kcal (14.63 MJ)

(153,232,417,532) and for men at less than 800 kcal or more than 4200 kcal (17.57 MJ).(153,254,417,532) However, according to Kushi,(373) virtually all studies with dietary intakes based on semi-quantitative FFQs will have some subjects reporting total energy intake estimates at less than 600 kcal per day and greater than 5000 kcal per day.

(f) *Collection of supplementary information*: To improve the accuracy of the dietary intake assessment (18,30) and apply quality control,(442,527) secondary questions can be incorporated to collect supplementary information.(18,30,442,527) Two types of supplementary information are particularly well-founded during the collection of dietary data for quality control purposes (527): data on weight and height (527) and inclusion of additional questions as source of information on details of dietary items usually consumed, like in what quantity.(30,415)

Collecting data on weight and height can be used to check the credibility of the energy intake.(527) Subjects with unfeasibly low energy intakes should be excluded,(18) as such energy intake estimates suggest that the subjects did not credibly complete the FFQ.(442) The Schofield equations to predict energy intake and the Goldberg cut-off method to identify underreporting, can for instance, be employed for this purpose.(14) Otherwise identifying those with energy intakes outside the plausible intake range set by many investigators as indicated can be used.

Additional questions can be formulated to ask about the typical number of portions/servings of food groups (500) and/or key food sources of nutrients (18) of interest usually consumed per week.(500) Studies have shown that when subjects report their intakes on a long list of individual items like in a FFQ, it tends to yield higher estimates of dietary intake than do dietary records or recalls (369,379) and shorter lists with fewer items.(486) Because subjects loose track of the overall number of items consumed, the sum of the FFQ intakes often represents an overestimation of the true overall frequency.(527) This tendency of overestimating the consumption of single items in FFQs therefore needs to be taken into account.(380) FFQs consequently regularly contain questions concerning the frequency of consumption of entire food groups (368,380).

These summary questions precede or follow questions on intake of specific items.(401) Patterson et al.,(442) for instance, included four summary questions that asked about habitual consumption of fruits, vegetables, fat added to foods and in cooking. Although the sum of the individual item frequencies often represents an overestimation of the overall frequency, it cannot be assumed that this overall frequency is more valid than the sum of the specific frequencies.(527) For alcohol the sum is probably more valid than the overall frequency and for foods like vegetables, where more than one type may be eaten within the same meal, the answer is complex.(527) Such summary questions towards providing additional information

on dietary intake can be used for verification (527) or as cross-check (18,415,500) or adjustment (442,527) questions in FFQs to correct or calibrate for misreporting of the intakes of certain foods and/or food groups,(18,500) and as a result nutrient intakes.(500) The summary question data on the overall intake within the food group can be used to adjust the specific item frequencies according to this overall frequency.(527) This simple method of determining the extent of misreporting and applying some correction for it involves a weighting factor to adjust food frequencies and determining its effect on estimated dietary intakes and on subject's ranking according to the dietary intake.(500)

In order to investigate the relationship between fruit and vegetable consumption as a protective strategy against disease, it is essential to assess the intake as accurately as possible adjusting for any misreporting.(500) Overestimation and over-reporting of total fruit and vegetable intake is a common result in FFQs with a large number or long list of individual fruit and vegetable items.(18,40,82)   Fruit and vegetable intakes may especially be overstated, caused in part by bias associated with desirable behaviour as fruits and vegetables are considered healthy and socially acceptable foods.(56)   A long fruit and vegetable listing may produce inflated estimates that may result from the cognitive difficulty subjects have in estimating past intake of any given food.(82) This, in turn, may contribute to overestimation of the intakes of nutrients like ß-carotene, vitamins A and C and dietary fibre, which is a concern if the aim of the study is to assess absolute subject intakes rather than ranking the subjects.(384)

Krebs-Smith et al. (81) suggested that summing across individual items may not be a valid way to assess intake of total fruit and vegetables and that summary food group questions and adjustment factors may increase precision of estimates. Summary or cross-check questions are therefore often employed for fruits and vegetables by asking the number of servings consumed per week of fruit and vegetables as these tend to be over-reported, particularly if each fruit or vegetable is listed singly in a long list.(18) Two summary questions were introduced as part of the NCI Health Habits and History Questionnaire to correct for overestimation of the nutrients present in fruits and vegetables owing to over-reporting of these intakes. These questions are as example: 'Not counting juices, about how many servings of fruit do you eat per day or per week?' and 'Not counting salad or potatoes, about how many vegetables do you eat per day or per week?' (384) or asked as 'About how many servings of vegetables, overall, do you eat per day or per week not counting salad or potatoes?' and 'About how many servings of fruit do you eat per day or per week, not counting juices?'.(82) Potatoes are generally excluded from the category 'cooked vegetables' as they represent a staple food (not a vegetable) in some countries, like The Netherlands.(483)   In the study of Calvert et al. (500) subjects were asked to exclude

potatoes in their estimate of vegetable servings per week and exclude dried fruit in addition to fruit juices in their estimate of fruit servings per week.

Different food-grouping categories have been used across the studies with a summary question asked for each of these categories. For instance, Negri et al. (492) asked three summary questions for fruit and vegetables to obtain the total number of portions of fruit, raw and cooked vegetables ingested in a week. Kristal et al. (42) divided fruits into juice and all other forms of fruit with vegetables divided into salads/raw, fried potatoes, and all other vegetables. Bogers et al. (401) asked a summary question for each of the categories as fruit (including fresh juice), cooked vegetables (excluding potatoes), raw vegetables, total vegetables (sum of raw and cooked vegetables) and fruit juice.

FFQ portions/servings for consumption of each food-grouping category are calculated as a frequency per week of the various relevant items on the questionnaire. Then all item frequencies are added together to provide a total number of servings per week from the simple summed food-grouping category item frequencies on the questionnaire.(42,483,500) A weighting factor is then calculated for each subject as the reported frequency per week to the summary or cross-check food-grouping intake question divided by the summed frequencies from all items consumed in the FFQ for each food-grouping category.(401,500) If the sum of the frequencies for the singly consumed items are not the same as the answer to the summary question on total consumption, the correction or weighting factor may be used to adjust frequency of intake accordingly (500) and correct for possible over-reporting.(18,500) The intake of the items is then corrected or calibrated by multiplying the intake of each item by the calculated weighting or correction factor.(401) Here the newly estimated amount of foods is used to estimate both the food and nutrient intakes.(18)

To calculate nutrient intakes adjusted by this weighting factor, each subject's weighting is applied to each food item within each food group; that is the fruit weighting is applied to each fruit item individually and so on. This application through which the frequencies for all the individual items within a food-grouping category are corrected proportionally, assumes that all items are misreported to the same extent, as the weighting factor does not take into account the differential misreporting of individual food items within the food-grouping category.(500) If summary questions are utilized to adapt data, details of the methodology and adjusted and unadjusted food and nutrient intake estimates should be made known.(18) Summary or cross-check questions asked on total number of portion/servings of fruit and vegetables consumed per week to correct for over-reporting are discussed in numerous studies.(42,82,356,401,457,486, 492,500) Amanatidis et al. (384) only applied the adjustment approach in a small number of cases in which the difference was greater than 20% between the two reports, as they assumed that there will always be some variability in reporting.

Bogers et al. (401) found the largest discrepancy in intake using the summary question amount and the summed amount of the single items on a group level, to be for cooked vegetables. The summed amount represented the summary amount most closely for fruit juice.(401) Several possible explanations, according to Bogers et al.,(401) can be provided as to why the summary amount is as applicable as the summed estimate for fruits, but not for vegetables. According to them, fruit portion size consumed is easier to report in comparison to vegetables, as fruits are mostly consumed in natural units and the number of different fruits consumed is generally less than the number of different vegetables consumed. In addition, the consumption frequency of fruits may be less difficult to approximate and as a result, the error of the correction factor for fruits would probably be lower than that of vegetables.(401)

Considering the validity of the two different methods, Bogers et al. (401) and Calvert et al. (500) in their studies considered the relative ranking of the subjects by the two different methods, that is the capability to position the subjects based on their fruit and vegetable intake of the summary questions and the sum of the consumed single fruit and vegetable inclusions within the questionnaire categories. Bogers et al. (401) found that the best categorical comparability was for fruits and fruit juice, where 95% and 93% of the subjects were categorized in the same or adjacent quartile, respectively. Again, the lowest category agreement found was for cooked vegetables (83% in the same or adjacent quartile). Calvert et al. (500) at the individual level found that although only 18% reported fruit and 7% reported vegetables similarly between the standard and weighted analysis, most of the correlations of the standard versus the weighting analysis were above 0.90 when the food group weightings were applied individually. Because the standard and weighted analysis used the same data, correlations of this order would be expected. The ranking of the subjects considering their nutrient intakes was not greatly affected, as Calvert et al. (500) found that only between 1% and 3% of the subjects were misclassified by two quartiles for most nutrients besides for vitamins A and C, which grossly misclassified 5% and 8% of the subjects, respectively, according to the two methods of analysis.

Based on biomarkers as reference validation method, Bogers et al. (401) furthermore found that the correlations with biomarkers were consistently higher for the consumption of cooked, raw and total vegetables based on the summary question ('summary estimate') than for the intake estimates obtained by summing all single item inclusions within each category in the questionnaire ('sum estimate'). For fruits, they found the correlations with biomarkers for summary and sum estimates to be similar.

Summary questions have improved nutrient correlations somewhat between multiple days of dietary records and FFQs.(82) Bogers et al. (401) concluded that a few brief, broad summary questions might aid to rank subjects based on their intake of the major fruit and vegetable categories. However, in the case of epidemiological studies on the association between

single dietary items and disease, correcting single items with a correction factor based on frequencies does not seem advisable, given that this may reduce than improve the capability of a FFQ to rank subjects based on their intake of these items.(401) Although the inclusion of cross-check questions has been used successfully as a strategy to identify possible over-reporting of fruits and vegetables,(500) they may not be as effective when used to assess other foods.(18) Tsubono et al. (383) used questions on the total frequency of consumption of five food-groupings (vegetables, fruit, meat, fish and cooked rice) to adjust for overestimation of nutrient intakes because of the long list of items included in the questionnaire food list.

5.7   CONCLUDING COMMENTS

The validity and reproducibility of FFQs are important as these ultimately indicate the questionnaire performance. The relative validity is most often assessed against another presumably more accurate dietary assessment method (24-hour recalls or dietary records) and sometimes, external biomarkers of exposure or of effect as reference methods. The reproducibility is most often assessed by the test-retest method where the time interval, between the two questionnaire administrations, need to be judged carefully. Several statistical methods are employed to determine the validity and reproducibility at the group level and the individual level administration of which the paired difference tests and correlation coefficients (crude and adjusted) are applied most often, with the application of category agreement or cross-classification, and the limits of agreement or Bland-Altman method recommended. FFQs may be employed to assess dietary change, such as in intervention feeding trials, where the questionnaire responsiveness is of importance and needs to be evaluated.

Systematic and random measurement error associated with FFQs may affect their validity and reproducibility and therefore need to be controlled. FFQs that for instance include a long list of fruits and vegetables in the food list may result in their intake being over-reported. To minimize this measurement error in FFQs, additional summary questions can be asked on the usual fruit and vegetable portion consumption, which can be used to adjust the fruit and vegetable intake as obtained from the FFQ, as envisaged for this research, pertaining to the comprehensive FFQ.

In the use of a FFQ as method to assess the dietary flavonoid intake for heart health promotion, its validity and use reproducibility and its responsiveness to dietary change for possible use in flavonoid dietary interventions, need to be evaluated. Biochemical biomarkers of dietary intake exposure are useful in these evaluations due to the difficulties in determining true dietary intake in particular using questionnaires.(100,126) The selection of such biomarkers interrelated with dietary flavonoid intake moreover requires knowledge of the bioavailability and metabolism of

dietary flavonoids, along with their health mechanisms, to equate flavonoid intake to a potential relevant direct and/or indirect biomarker of exposure or effect.

Comprehensive knowledge and understanding about the biology of flavonoids and their effects on health is not only fundamental for preventive nutrition (98) and for assessing the dietary intake, but will assist in the formulation of dietary recommendations for flavonoid intake,(75) which as yet, have not been established.(127) According to Nasaringa Rao (110), there is a need to establish an optimal dietary intake of phytonutrients, as in the case of nutrients, that can provide maximal protection against the non-communicable diseases like CVD.

CHAPTER 6

A BRIEF FOOD FREQUENCY QUESTIONNAIRE
AS A DIETARY ASSESSMENT METHOD

6.1   PREAMBLE

FFQs generally provide an overview of subjects' dietary intakes as they are mostly used in nutritional studies that assess the risk for chronic disease.(72) FFQs suited to calculate the intakes of a wide spectrum of nutrients are, however, not required in all situations.(482) Sometimes a study may have limited scope or resources that may call for brief assessments.(417) When the study objective is to assess the intake of a single nutrient, or a group of dietary items, a modified brief FFQ relatable only to the aspect(s) of interest may be practical.(72)

6.2   DESCRIPTION, DISADVANTAGES AND ADVANTAGES

A comprehensive and complete dietary assessment method would require collecting data on numerous items, but when only one or a few nutrients are of importance, the number of items, which needs to be assessed, becomes greatly less. An abbreviated questionnaire determining the consumption of as little as 15 to 20 dietary items may be all that is necessary to investigate the relationship between the ingestion of one dietary component and a disease outcome.(29) Byers et al. (29) and Stryker et al. (395) observed that a rather small group of dietary items could explain a substantially higher percentage of the between-person variation in intake than the percentage of the absolute total intake. Byers et al. (29) proposed that when only a single nutrient is considered such a small group of dietary items providing for a brief FFQ food list might account for most of the variation in the intake estimation. To explain 90% of the variance in nutrient intake, Byers et al.,(29) for instance found that only 5 items are required for vitamin A, 8 for vitamin C, 17 for fat, 18 for fibre, 19 for protein and 21 for total energy.

In addition, comprehensive FFQs, as well as multiple day dietary records and recalls, require too much time to complete to be practical.(433) Great interest has consequently arisen in developing a means of determining dietary intake of specific nutrients employing minimal cost and time, particularly in large population studies.(29) Because resource limitations often prohibit detailed dietary assessment in such studies, investigators have developed abbreviated measures (418) adequately suited to meet a particular purpose.(417) The ability to assess the intake of several dietary components for a time frame in the past and the ease of administration, have also made reduced or brief FFQ versions particularly attractive for research purposes.(403) Studies have found that reduced questionnaire versions are able to adequately quantify various aspects of the dietary intake.(449)

A major problem in constructing FFQs is the questionnaire length.(497) Block et al. (355) advised that a food list be relatively short to be less burdensome for subjects to complete. In addition to being less burdensome for subjects to complete (482) and easier and less time-consuming to administer,(398,417) a further advantage of such brief or short questionnaires is that the data analysis is less and easier to conduct, as judged against other dietary assessment methods.(417,482) The low subject burden also might make increasing the sample size possible.(418) A further advantage is the possibility of collecting dietary data on a more regular basis since it is less expensive to use than the traditional assessment methods.(482) Longer FFQs, dietary history interviews and multiple 24-hour recalls, in contrast, are less practical because of interviewer and/or subject burden to be repeated on a regular, reasonably inexpensive basis. The time estimated for such brief or short measures to be administered is often less than ten minutes.(446)

However, despite their advantages, these brief dietary questionnaires may not contain enough detail, for example to capture hidden sources of energy and fat,(403) and may have limited value in estimating subjects' exact or absolute dietary intake.(80,418) Block et al. (450) declared that full-length questionnaires are favoured for nutrition research. Neuhouser et al. (431) strongly supported the use of comprehensive dietary assessment methods, and particularly in large cancer prevention trials, and questioned whether abbreviated dietary measures should be used in national nutritional surveillance, epidemiological studies and clinical trials where dietary intake may be an important exposure variable. Their major concerns regarding such brief measures are that they cannot be adjusted for total energy intake and that the intake of only one or more dietary components is considered. Not adjusting for total energy intake can influence study results if energy intake itself is a risk factor for disease, as various nutrients, particularly the macronutrients, are strongly associated with total energy intake. Analysis limited to one dietary aspect also eliminates potentially important analyses of other dietary components or total diet and disease.(431)

Additional concerns are their lower validity and potential bias,(431) as these short methods appear to have only limited value in estimating the absolute intakes of nutrients.(80) As a result, Neuhouser et al. (431) have reservations about using short measures as the sole dietary assessment method in studies where dietary intake may be an exposure variable. Thompson et al. (418) too support that such brief measures are not substitutes for more thorough traditional dietary assessment methods in studies that necessitate information that is more comprehensive or that call for information about several dietary components. A further limitation is the method of administration, as it utilizes self-administration, which requires highly literate subjects without visual

impairments. Interviewer-administration, while too expensive for use in epidemiological studies, can be considered, as they can be engaged with most populations.(403)

Despite their limitations, these short dietary assessment measures are useful for a number of purposes.(418) Valid, short and simple dietary intake assessment measures can quantify food intakes (447) and are responsive to efficiently assess dietary change over time necessary to evaluate the effectiveness of dietary interventions (398,447,497) and nutrition education (398) at group level with respect to target foods,(447,497) such as fruit and vegetable consumption.(483) Such questionnaires can also be used to identify potential subjects for intervention studies.(343) In the evaluation of intervention effectiveness, many subjects often need to be included and therefore, more extensive dietary assessment methods are too time-consuming and expensive.(398)

Therefore, the dietary assessments must be brief and easy to administer in order to lessen the subjects' effort and time (398,483) and so increase participation rates,(483) and sustain the intervention outcome that might result from using demanding dietary intake assessment methods, which may obscure the true intervention impact.(398,483) Most dietary assessment methods require effort from subjects, which can potentially influence adherence with key requirements of the study protocol. In most trials, it is advisable to assess the intake of at least a few dietary components because dietary intake may be a variable influencing the study outcome.(431) It is possible to develop short dietary assessment measures that work sufficiently well for population dietary monitoring (447,505) and clinical work,(505) for example in dietary counselling.(343)

6.3 DEVELOPMENT AND EFFICACY

Abbreviated or brief FFQs are generally developed by altering an existing more complex questionnaire to a simpler format (402) by reducing the comprehensive food list to a more concise food list and applying the percentage contribution analysis to omit the least important dietary sources,(402,403) and/or the stepwise multiple regression analysis to identify the most discriminating dietary sources.(38,395,403,449) Neuhouser et al.,(431) however, has a reservation about selecting a food list only considering the main contributory items to the total intake of the subject group under study, as it may yield low between-person variation in intake. Consequently, the results generated would possibly be unable to discriminate subjects well and rank or position them. Neuhouser et al. (431) considered this one of the contributing factors to the poor performance of the 13-item dietary fat screener they compiled.

Block et al. (402) developed a reduced version (60-item) of the full Block questionnaire (98-item) by omitting certain segments of the questionnaire. Some omissions were the open-ended section

where subjects can report additional foods that they consumed frequently but were not mentioned in the food list, cooking-fat use questions and the two standard fruit and vegetable adjustment questions permitting the use of a less-inflated frequency of total fruit and vegetable consumption. They also omitted the least important dietary sources by excluding those items from the food list that were lower than the top 80% contributory items on all of the 18 single nutrient food lists. The brief version also captured all nutrients as the full-length version and required only 17 minutes to administer by an interviewer compared to the 30 to 35 minutes utilized for the full questionnaire completion. Willet et al. (38) reduced a 120-item questionnaire into a 61-item questionnaire, by eliminating or collapsing nutritionally similar items. The reduced questionnaire was able to account for at least 80% of the total between-person variability, and often far more than 80%, for each of 18 major nutrients.

Decarli et al. (449) investigated the ability of two shortened dietary questionnaires derived from a more comprehensive questionnaire to assess the Mediterranean diet in epidemiological studies. They extracted two lists of items from the original ranked list by retaining the foods explaining at least 75% and 90% of the between-person variability in nutrient consumption using the stepwise multiple regression analysis. Schaffer et al. (403) modified a full in-person FFQ to a shortened telephone interview by utilizing the percentage contribution and the forward stepwise regression analysis. The average shortened telephone interview time was 36 minutes compared to the longer one-hour full in-person FFQ.

The dietary components of interest seem to affect the number of foods to be included in the food list. For some nutrients, for example micronutrients like vitamin C, it seems adequate to collect information about a very small number of food items to obtain a good estimate of both the total intake and the total variability of consumption.(449) Reducing the number of dietary items from 34 to 15 in the study of Cummings et al.,(404) did not greatly influence the relationship of the calcium intake estimates from the frequency questionnaire data with seven-day dietary record data. For these nutrients, it is possible to reproduce the results obtained with a full-length dietary questionnaire using a reduced dietary questionnaire.(449) The situation is different for some nutrients like total energy and macronutrient intakes. For these nutrients, total intake is scattered among many food items, each with very low variability of consumption, thus hampering obtaining a reduced set of food list items.(449)

The reduced version of the interviewer-administered Block questionnaire was validated against three four-day dietary records in a group of middle-aged women, and against two seven-day dietary records obtained 10 to 15 years earlier in a group of older men. The absolute levels of macronutrient intakes assessed by the shortened questionnaire was lower than the dietary record determinations, as would be expected, since intake of only 60 instead of about 100 items was

being considered, but the intakes of most micronutrients were not underestimated. Macronutrient correlations with the dietary records were somewhat lower as a result of using the abbreviated version, but for micronutrients the correlations were not decreased or only slightly lower using the abbreviated version.(402) Each of the two reduced dietary questionnaires created by Decarli et al. (449) to evaluate the Mediterranean diet, produced nutrient intake estimates that related highly with the estimates as provided by the full-length questionnaire.

In the study of Schaffer et al.,(403) correlations were calculated between the nutrient estimates provided by the full-length and abbreviated questionnaire versions with the full-length version utilized as the reference method. The data also indicated that the brief telephone-administered questionnaire provided a ranking of nutrient intake comparable to that provided by the full in-person interview, and very little gross misclassification occurred. However, adjustment for total energy intake and gender were inclined to reduce the correlation coefficients found for most nutrients. The ICCs that evaluate reproducibility ranged from 0.62 for animal protein to 0.83 for folate indicating that this brief telephone-administered questionnaire is also reproducible.(403) These findings suggest that reduced questionnaires have the ability to sufficiently quantify the dietary components related to the purpose for which they were developed.

Inclusion of questions regarding portion size in reduced questionnaires seems to improve their performance. For instance, Cummings et al. (404) found the most truthful indication of daily calcium intake on using the subset of 34-items of the full-length Block questionnaire, where subjects reported the consumption frequency and indicated how their usual portion sizes consumed compared to the medium portion sizes indicated on the questionnaire. The subset of 34-items and subsets of 15- and 10-items, when foods were chosen from the top down in terms of their percentage contribution to calcium intake, yielded similar correlations with estimates from a seven-day dietary record when the 'small-medium-large' approach to portion size was retained. However, when this approach to portion size was ignored and all portions were assumed to be 'medium,' the 35-item subset without portion size correlated less well with the seven-day dietary records than did the 10-item subset with portion size.(402) Omitting variable portion size from the reduced interviewer-administered Block questionnaire also resulted in lower correlations, while the inclusion of variable portion size improved correlations with actual reference data.(402) In the study of Thompson et al.,(418) the improved ability of the complete screeners to assess median fruit and vegetable intakes versus the reduced versions appeared to be largely due to the inclusion of the questions considering portion size.

6.4 MODIFIED BRIEF QUESTIONNAIRES FOR ASSESSMENT OF SPECIFIC DIETARY ASPECTS

In this discussion of modified brief FFQs the various simple and rapid tools often referred to as screeners and indexes are excluded, the latter being used for among other purposes as nutritional screening and counselling tools that comprise a dietary scoring technique. As the intent of brief questionnaires is to be a substitute measure for more detailed and comprehensive dietary assessment methods,(431) the validity and reproducibility of these brief questionnaires need to be addressed.

6.4.1 Estimating Food Group Intakes

In certain circumstances the use of brief questionnaires to determine intake of food groups of importance are sufficient, as in the case of fruit and vegetables. Simple dietary screening tools that can assess consumption of fruit, vegetables and fat are required to determine if public health intervention strategies are effective in increasing fruit and vegetable intakes and in reducing fat intake. A small number of studies have evaluated short FFQs developed to assess the intake of food items.(482)

6.4.1.1 Fruit and Vegetable Food Groups

Quick, simple methods to specifically determine fruit, fruit juice and vegetable intake have been utilized to monitor changes in fruit and vegetable intake over time, and to evaluate interventions promoting increased fruit and vegetable intake for disease prevention,(409,468,486,533) which are key dietary behaviours important to public health.(446) Even though fruit and vegetable intake data obtained via the use of comprehensive assessment methods present more accurate absolute intake estimates at the national and population level, short fruit and vegetable consumption measures are practical for utilization as public health tools.(417) For instance, a brief assessment measure consisting of seven inclusive categories to determine fruit and vegetable intake was developed by the NCI and national 5 A Day program associates and is used as standard screener to track changes in fruit and vegetable intake and to measure such program and intervention effectiveness.(417) This brief 7-item fruit and vegetable FFQ was developed to correct the overestimation that occurs when subjects respond to a long list of various dietary items, particularly socially acceptable foods like fruits and vegetables.(421) Simple dietary methods may in addition be used to determine fruit and vegetable intake in a primary health care setting,(505) as screening tool for dietary therapy and for dietary assessment in epidemiological studies.(446) Simplified tools that only assess fruit and vegetable intake, may be feasible markers of diet quality.(52)

Kim et al. (446) reviewed ten brief measures developed to assess fruit and vegetable consumption levels based on self-report, to identify measure features that contribute to greater validity and/or reproducibility and may improve the performance of such methods. These measures met their

criteria of 'brief', which entailed the inclusion of a maximum of 16 fruit and vegetable items as either individual or grouped food. All the measures were FFQs with the exception of two. These measures were directed via face-to-face interviews, telephone interviews or were self-administered.(446)

Validation studies of the measures included in the review were based on comparisons with full-length FFQs, multiple dietary records or 24-hour recalls. In numerous of the validation studies that considered total fruit and vegetable intakes, the mean/median daily intakes as derived from the reference methods were markedly under- or overestimated by the short measures.(446) Thompson et al. (417) stated that the use of short measures without appropriate adjustment is sub-optimal for estimating median intakes of fruit and vegetables and determining whether recommended intakes are being met. Serdula et al.,(486) however, in their evaluation of the application of a brief (6-item) telephone-administered questionnaire to assess fruit and vegetables consumption in five diverse subject groups, generally found the mean daily fruit and vegetable consumption determined by the brief questionnaire to be similar to intake estimates obtained by multiple dietary records or recalls and lower than the intake estimates obtained by comprehensive FFQs. This study, however, did not assess agreement between methods at an individual level.(83)

Ten of the 13 validation studies included in the review by Kim et al. (446) utilized Spearman and/or Pearson correlation coefficients to evaluate the agreement in the individual total fruit and vegetable intakes obtained. None of these correlations were adjusted based on total energy intake. They (446) indicated that in general there seemed to be good subject ranking agreement between the brief measures and the reference methods in estimating fruit and vegetable intakes. A study by Warneke et al.,(409) for instance, indicated that a FFQ using seven broad food categories correlated more highly with the reference 24-hour dietary recall telephone interview intakes than a 31-item FFQ indicating individual fruit and vegetable items among predominantly African-American women. Thompson et al. (418) in their evaluation of the ability of two short measures (an 'All-Day' screener with portion size and frequency questions about nine food items and a similar 'By-Meal' screener, besides that two of the nine food items are asked about in terms of mealtime) and a FFQ to assess fruit and vegetable intake, in contrast found both the screeners to be adequate to determine median intakes of these foods in US populations, but that they might perform less well in ranking the subjects. The use of a deattentuation procedure to correct for random within-person day-to-day variation in intakes were not reported in all the validation studies.(446) Thompson et al. (417) in their evaluation of the performance of two brief measures, a 7-item standard questionnaire on consumption over the past month and a new 16-item questionnaire and a complete FFQ in measuring total fruit and vegetable consumption, found that the attenuation coefficients for the FFQ and short measures were comparable. Thompson et al. (417) also stated that short

measures and FFQs might be similar in performance for estimating RRs in relations between fruit and vegetable consumption and disease.

Short FFQs (less than 15 items) developed to determine fruit and vegetable intake have been validated using biomarkers as independent reference method.(483) Several studies found good correspondence between brief questionnaires only assessing fruit and vegetable consumption and nutrient biomarkers.(533) A high consumption of fruit and vegetables provide high antioxidant and high potassium intakes that can be utilized as biomarkers.(505) Cappuccio et al. (505) studied the associations between a simple 2-item fruit and vegetable intake questionnaire and plasma vitamin C and urinary potassium excretion as biomarkers. After controlling for confounders, those who reported a daily consumption of adequate portions (5 and more) of fruit and vegetables had higher plasma vitamin C and urinary potassium excretion expressed as both total daily excretion and as potassium/creatinine ratio than those reporting low portion (2.5 and less) consumption per day. There was a low, but significant association between the daily intake of fruit and vegetables as assessed by the questionnaire and the plasma levels of vitamin C and with urinary potassium excretion of the subjects. Coyne et al. (533) found that the responses to two rather simple fruit and vegetable intake questions, where subjects report their usual daily total consumption of these dietary items as the total number of servings consumed per day, were positively and significantly associated with serum carotenoids and red-cell folate as reference biomarkers of fruit and vegetable consumption. Block et al. (534) investigated the relation between fruit and vegetable consumption as assessed by the abbreviated CLUE II FFQ (60-item scan able version of the Block/NCI FFQ) of which the frequency of the included ten vegetable and six fruit items were summed, to determine total fruit and vegetable intake and several plasma antioxidants, and found both the plasma carotenoid and vitamin C levels to be higher in those with the higher fruit and vegetable consumption.

Reproducibility was only considered for three of the brief fruit and vegetable intake measures taken up in the review by Kim et al.(446) Reproducibility of the short FFQ used in the study of Bogers et al. (483) was 0.80 for total fruit intake and 0.73 for total vegetable intake after one month.

The number of items included in the food list and the inclusion of questions regarding portion size seems to improve the performance of brief questionnaires estimating fruit and vegetable intakes. Thompson et al. (417) found that the agreement between true intake (two 24-hour recalls) and their brief measures was higher for the new 16-item questionnaire than the 7-item standard measure. The correlations of total fruit and vegetable serving intakes with specific and total carotenoid levels were also in general higher for a 36-item FFQ than for a 2-item measure that assessed the number of portions/servings of fruit and of vegetables generally consumed per day, and a 7-item measure

where subjects had to indicate how many times they had consumed each item in the previous week.(465)

Plesko et al. (421) ascribed the inability of the standard 7-item FFQ to accurately assess the fruit and vegetable consumption of the subjects in their study against two-day dietary records to the non-quantitative format of the FFQ. They indicated that asking about portion size would improve the accuracy of the brief questionnaire. Kim et al. (446) found that in comparison to more intensive dietary assessment methods, short measures with a relatively longer list of fruit and vegetable items in the food lists and that include questions on portion sizes and composite dishes, presented higher Pearson and/or Spearman correlation coefficients for fruit and vegetable intake assessments and closer estimates of mean/median total fruit and vegetables intake assessments against these methods. Their review puts forward that the incorporation of a reasonable number of representative fruit and vegetable items, and consideration of questions on consumed portion size and consumption of composite dishes, may improve the validity of short fruit and vegetable measures.

6.4.1.2 Fruit, Vegetable and Other Food Groups

Increasing fruit and vegetable consumption and reducing fat consumption is part of effective public health intervention strategies.(482) Andersen et al. (482) evaluated the ability of a short 27-item FFQ against 14 days of weighed dietary records to distinguish subjects with low fruit and vegetable intake and subjects with high versus low fat intake as fat spread use. They found the FFQ suitable to screen for such subjects in intervention programs. The capability of the FFQ to identify those having insufficient fruit and vegetable intakes based on the weighed record data was above 90% and the correlation coefficient between the amounts of fat used as bread spread from the two methods was 0.79.

Ling et al. (83) assessed the ability of a 16-item FFQ developed for monitoring cereal food, fruit and vegetable intakes in Chinese Singaporeans, but also for screening for low intakes and ranking subjects into broad categories of intake. Intake estimates obtained from the 16-item FFQ were compared with those obtained from three 24-hour dietary recalls. They found that the short questionnaire could be utilized to screen for low consumers in intervention programs (gross misclassification was rare), to determine mean food group intake in large subject groups (difference in mean food group intake assessed by the two methods were not significantly different) and to rank subjects for food group intakes (unadjusted Pearson correlation coefficients ranged from a mean of 0.63 for vegetables to 0.79 for cereal foods and 0.84 for fruit with only slight reductions following adjustment). According to Kim et al.,(446) inclusion of dietary items presenting the dietary intake and locally consumed foods of the subject groups investigated, pre-testing of the included items for clearness, with resulting ease of cognition and completion, and use

of portion size photographs, are all contributory factors to the encouraging validation findings of this brief measure of Ling et al.(83)

6.4.2   Estimating Single Nutrient Intakes

Brief questionnaires may also be suited to assess the intakes of nutrients, like calcium, that have a relatively stable intake (404) and minimal seasonal influence on the intake.(358) Musgrave et al. (358) found no seasonal difference in calcium intake between winter and summer, yet it appeared that larger intakes of cheese, crackers and eggnog transpired in winter and of strawberries, rhubarb and greens in summer.

Cummings et al. (404) created a shortened 34-item modified version of the 98-item Block FFQ to solely assess calcium intake, whilst not overburdening the subject. The number of items in the original food list was reduced to represent only those items that made the greatest contribution to calcium intake. The 34 selected items represented 85% of the calcium intake of adult participants in NHANES II. The short FFQ (with portion sizes rated as small, medium or large) produced a mean calcium intake estimate which compared well with the mean estimate from seven-day dietary records, and the estimates from both methods also related well.(404) Musgrave et al. (358) produced a shortened FFQ to assess calcium intake by first selecting 65 dietary items high in calcium based on total calcium content and on frequency of consumption. They reduced the number to 53 and then revised it to 39 items considering frequency of ingestion of items with lower total calcium content but making a noteworthy contribution to overall calcium intake. Portion sizes were also incorporated in this short FFQ. The mean daily calcium intakes derived from the reduced FFQ and four-day dietary records were not significantly different. More subjects rather under- than overestimated their calcium intake on the short FFQ.

Yang et al. (535) shortened a 79-food grouping calcium-specific semi-quantitative FFQ developed and evaluated it for use with adolescents, as the length of the original questionnaire limited its application as a self-assessment tool in community settings. Stepwise regression analysis was used for the food list reduction to select those foods that best explained the between-person variation in total calcium intake based on the data from the longer FFQ. The questionnaire was reduced to 15 items, which took about five minutes to complete. The simple 15-item calcium measure intakes correlated significantly with bone mass evaluations and were highly reproducible.

Sebring et al. (88) investigated the performance of three FFQs of differing length and varying format to assess dietary calcium intake in apparently healthy adults participating in an outpatient trial of calcium supplementation. The subjects completed a dietary history questionnaire (DHQ) of consumption over the past 12 months listing 124 separate items (36 pages), a calcium questionnaire listing 87 items (3 pages) and a 25-item short calcium questionnaire (1 page) on

consumption during a typical week incorporating only the key dietary calcium sources. Mean daily calcium intake derived from the dietary records differed significantly from the mean daily calcium intake derived from the DHQ and the questionnaire, but not from that derived from the short questionnaire. Correlation coefficients between the dietary record and the questionnaire estimates were also lowest for the DHQ and highest for the short questionnaire.(88)

Mean calcium intake derived from analysis of a brief FFQ of 41-items modified from the 98-item Block FFQ did not differ at three assessments, initially, at two months and at six months representing the intake of the previous six months. Pearson correlation coefficients of paired calcium intake estimates over these assessment periods ranged from 0.70 to 0.77 for the participants. When subjects were categorized into quartiles of calcium intake estimates, misclassification into extreme quartiles was 1%.(78)

The number of items included in the food list seems to improve the performance of brief questionnaires estimating calcium intakes to some extent. In the study of Cummings et al.,(404) the absolute value of the mean estimates of daily calcium intake was reduced by successive reduction in the number of items on the food list (34 to 15 and then to 10 items) to only represent those items that make the greatest contribution to calcium intake in older women. The reduction only had a relatively small effect on the correlation with the results of seven-day records. Therefore, for solely assessing calcium intake, brief FFQ versions may perform comparatively well at determining dietary calcium intake in comparison to other more in-depth dietary assessment methods (78,88,404,535) and can be used in some epidemiological studies (404,535) and community-based programs for research, assessment and evaluation purposes (535) and for clinical purposes (404) without overburdening subjects.(78,535)

Short questionnaires that assess fat intake have also been developed. Kemppainen et al. (80) developed an interviewer-administered short 21-item questionnaire that incorporated the main sources of dietary fat in the Finnish diet. The short questionnaire very slightly underestimated the mean intakes of total fat, fatty acids and cholesterol when compared with three-day dietary records as reference method. A strong correlation was found between total fat intake estimates based on the questionnaire and the dietary records. The 21-item questionnaire proved to be accurate in estimating the intake of total fat and the different fatty acids at group level.(80)

Neuhouser et al. (431) were not that positive about their 13-item dietary screener to estimate fat and saturated fat intakes to differentiate groups of subjects with low (or high) fat intake in the Prostate Cancer Prevention Trial in comparison to a 109-item FFQ sensitive to sources of fat commonly consumed and multiple 24-hour dietary recalls. Their study did not find the 13-item screener to be an appropriate substitute for a comprehensive FFQ, as the screener captured less

than 40% of the absolute fat in the diet from the comprehensive FFQ and the multiple dietary recall estimates. Although the correlations between the screener and the multiple 24-hour recalls as reference method were modest, these correlations were markedly higher for the comprehensive FFQ and the 24-hour recalls. Neuhouser et al. (431) indicated that the results of the study propose that utilization of brief dietary measures as the only assessment method may lead to loss of information regarding important dietary exposures (fat intake) contributing to lost prospects to gain knowledge and insight into dietary factors and cancer risk.

A self-administered FFQ comprising of 20 items to estimate protein intake was compared against four-day dietary records and three 24-hour recalls as reference methods in two separate subject groups. The FFQ and the two reference methods provided comparable information. The mean intake estimates and the distribution of the estimates were very similar across the FFQ and the two reference methods, with moderately significant correlations between the FFQ intake and the two reference method intakes.(451)

A brief interviewer-administered FFQ to assess lutein and zeaxanthin intake was evaluated against seven-day dietary records and plasma lutein and zeaxanthin concentrations as reference methods. The mean dietary lutein and zeaxanthin intakes did not differ significantly between the methods, and the FFQ intakes significantly correlated with the plasma lutein and zeaxanthin concentrations.(452) Frankenfeld et al. (410) found the isoflavone intake estimates of post-menopausal women obtained from a 20-item self-administered soy-specific FFQ and a general FFQ that incorporated tofu and soymilk in its food list, highly repeatable on administration one week apart (>0.98 for both questionnaires) and of moderate validity in relation to plasma isoflavone (daidzein and genistein) concentrations based on Pearson's coefficients (0.35 to 0.43).

6.4.3   Estimating Total Diet

PrimeScreen is a 25-item brief FFQ developed to determine nutritional risk for the common adult chronic degenerative diseases of lifestyle in the primary health care setting. It determines the average consumption frequency over the previous year of the most frequently consumed foods of targeted food groups (fruits, vegetables, dairy foods, whole grains, fish, red meat) based on datasets from two large cohort studies.(433) Rifas-Shiman et al. (433) evaluated the comparability of Prime Screen with two reference standards, a full-length 131-item semi-quantitative FFQ and plasma levels of specifically chosen nutrients. For foods, food groups and nutrients the mean correlations were high for both agreements with the FFQ and reproducibility at an interval of two weeks. Correlations for the assessment of selected nutrient intake estimates as obtained from the brief PrimeScreen questionnaire with the plasma levels were comparable to those comparing the full-length FFQ determined estimates with the plasma levels.

6.4.4 Estimating Change in Dietary Intake

Changes in dietary intake are often the main impact indicators in nutrition intervention studies, which make dietary consumption assessments necessary.(494) Change in food intake as reflected by a short 26-item FFQ was estimated by Osler and Heitmann (447) by subtracting the 1993-1994 food intake values from the 1987-1988 intake values. Individual changes in food consumption presented by the dietary history interview were analyzed in the same way. The change in food intake frequency as determined by the short FFQ related well with the change in the consumption data determined by the dietary history interview during the six years of follow up, that is those subjects who reported a less regular food intake by the FFQ had lower mean daily food intakes as assessed by the dietary history interview.

Kinlay et al. (434) developed a brief 12-item questionnaire to assess changes in the intake of total and saturated fat among groups of individuals in attendance at health promotion events. A simple score (the fat-habits-score) calculated from the brief questionnaire responses was compared with intake estimates of total and saturated fat (% total energy) obtained from a standard 180-item FFQ. The differences between the changes in total and saturated fat obtained from the brief questionnaire and the changes assessed by the FFQ were not significantly different, indicating that the simple FFQ can be used to assess group changes in fat intake.(434) Short FFQs thus also appear to be suited to assess dietary change as they seem to be responsive to changes in dietary intake over time.

6.5 CONCLUDING COMMENTS

A number of modified brief FFQs have been developed to assess the intake of a specific dietary aspect of which most relate to the assessment of a single nutrient (calcium) or a few related nutrients (total fat and fatty acids) or a food group (fruit and vegetable grouping). The food list of a modified brief FFQ directly depict the specific dietary aspect under study and it seems that portion size inclusion may enhance the questionnaire performance. A brief FFQ seems appropriate for nutrients of which a small number of dietary items contribute to both the total intake and the variability in the intake.

Despite the subject convenience and use for a number of specific circumstances, such as the assessment of the dietary intervention effectiveness, they may not capture enough detail to present an absolute intake estimate, which may favour the use of comprehensive FFQs where absolute intakes are important or the intake assessment of numerous dietary components are relevant. In the latter case, they may be used in conjunction with a more detailed dietary assessment method as was applied in this research.

CHAPTER 7

RESEARCH DESIGN AND METHODOLOGY

7.1 INTRODUCTION

The following research objectives provided a focus for the scope of the study and the framework for the research design (see Figure 7.1):

(a) To develop a self-administered comprehensive quantitative FFQ to assess the daily total flavonoid intake and to evaluate its validity, reproducibility and responsiveness within the context of the rooibos intervention trial;

(b) To identify those foods and beverages that contributed most to the daily total flavonoid and the between-person variation in intake, according to the estimated dietary record and comprehensive FFQ estimates in order to create an abbreviated FFQ; and

(c) To evaluate the validity, reproducibility and responsiveness of the resultant abbreviated FFQ within the context of the rooibos intervention trial, and its validity and reproducibility in a further participant group administration as appraisal of its external strength.
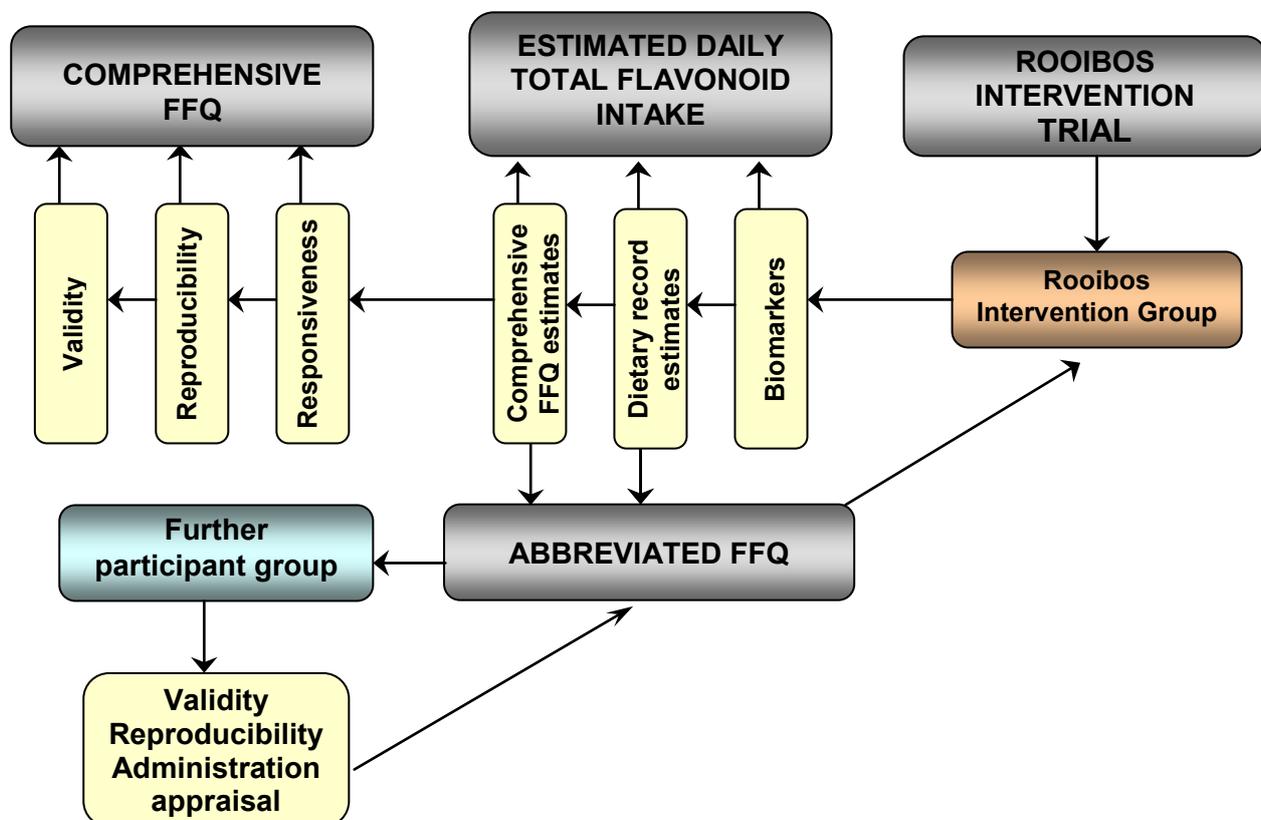
Figure 7.1:   Schematic Research Design

This research firstly comprised the development and evaluation of a comprehensive FFQ to assess the mean daily total dietary flavonoid intake of participants at risk of CHD in a human rooibos intervention trial (top section of Figure 7.1). The food list of this comprehensive FFQ was subsequently reduced to an abbreviated FFQ (middle section of Figure 7.1), which was evaluated within the framework of the intervention trial and in a further participant group administration as opportunity to investigate its external strength and probable generalizability (bottom section and left side of Figure 7.1).(14) The literature resources used in support of the decisions made to carry out these methodological components with their built-in steps, are indicated to uphold the research execution.

The mean daily total flavonoid intakes were assessed as the intake estimations in the research. The flavonoid subclasses included for the summation of the total intake consisted of the flavonols (n=4), flavones (n=2), flavanones (n=3), flavanols (n=6) and anthocyanidins (n=6).(161) King and Young (7) indicated that quantification on total intake would be useful as most flavonoids share related biological properties, such as being antioxidants or reducing agents.(7,94) Humans are furthermore unlikely to consume dietary flavonoids individually due to their diverse and wide distribution in the diet.(129) No particular polyphenol has been pointed out to provide more health benefits than others.(94) Although it is difficult to determine which polyphenols are the most protective against CVD,(148) the consumption of some flavonoid subclasses may be more important from a health perspective than total flavonoid intake.(84) The results of the Iowa Women's Health Study suggested that the intake of certain flavonoid subclasses (in particular the anthocyanidins along with the flavones and flavanones) might be associated with lower CHD and total CVD mortality in postmenopausal women.(248)

Johannot and Somerset (89) indicated that in view of variations in individual flavonoid intakes and that various flavonoids may have specific roles and health implications, data on individual flavonoid intakes should be presented where possible. They indicated that it is currently unclear whether absolute intakes of individual flavonoids or ratios between them are more relevant to human health.(89) Merely rough quantitative intake estimates of flavonoids and its subclasses can currently be made. Food composition data are too limited and the analytical methods too variable to permit more precise quantitative intake estimates.(87) For this research, only the total flavonoid intake was assessed. The intake of a number of flavonoid-rich dietary sources that represent diverse flavonoid subclasses with the individual flavonoid components included in each, were restricted after the baseline or run-in period for the duration of the intervention trial. This made determining the mean daily total flavonoid intake over the intervention trial periods more suited.

7.2   ETHICS APPROVAL OF THE RESEARCH

Ethics approval for the development and evaluation of the comprehensive FFQ, which formed the first phase of this research, was obtained from the Health and Applied Sciences Research Ethics Committee, Cape Peninsula University of Technology (CPUT), as part of the research proposal submission for the human intervention trial titled, *Modulation of blood oxidative stress markers and DNA damage by rooibos in volunteers at risk for coronary heart disease,* (Principal investigator, Prof JL Marnewick) executed in 2007 (Ethics approval: HAS-REC 4.1, 11 May 2007) (Addendum B).

Ethics approval for the second phase of the research, that involved the evaluation of the developed abbreviated FFQ in a further participant group administration, was obtained from the Faculty of Health and Wellness Sciences Research Ethics Committee, CPUT, through submission of a further research proposal, as this phase was considered an extension to the rooibos intervention trial (Ethics approval: HAS-REC 4.1, 7 August 2009). Ethics approval for this research in its entirety, and in particular for this second phase, was obtained from the Committee for Human Research, Faculty of Health Sciences, Stellenbosch University, 7 October 2009 (N09/08/223) (Addendum B), on submission of a collective research proposal. All the eligible participants throughout the entire research provided their oral and, thereafter their written informed consent on reading the participant information leaflet and consent forms prior to the start of each phase of the research. Participation was voluntary and anonymous as each participant received a participant code number on participation in each phase.

7.3   DEVELOPMENT AND EVALUATION OF COMPREHENSIVE FFQ WITHIN ROOIBOS INTERVENTION TRIAL

The human rooibos intervention trial of 14 weeks, executed from end July to beginning November 2007 on receiving ethics approval, serves as the study base for the methodological evaluation of the comprehensive FFQ developed specifically to assess mean daily total flavonoid intake of the participants during the run-in or baseline ('usual') and the experimental (washout, intervention and control) periods of the trial (Table 7.1).

7.3.1   Rooibos Intervention Trial

The intervention trial was advertised internally (institutional website and posters) and externally (radio talk broadcast) to recruit adult volunteers as potential participants among the CPUT employees and inhabitants of the City of Cape Town Metropolitan Municipality of SA. Volunteers were recruited based on the participant inclusion criteria and the demand placed on the participants due to the 14 weeks duration of the trial (Table 7.1).

Table 7.1: Outline of the Research Design for the Rooibos Intervention Trial

Experimental period	Time frame	Estimated dietary record[a] completion	Comprehensive FFQ[b] completion/Biomarker (plasma) collection[c]	Dietary stipulation
Run-in or baseline	2 weeks	3 consecutive days[d] (2 week days and 1 weekend day) alternating for weeks 1 and 2	On completion of trial period	Usual diet
Washout	2 weeks	3 consecutive days (2 week days and 1 weekend day) alternating for weeks 1 and 2	On completion of trial period	Usual diet incorporating flavonoid beverage and minimal flavonoid food source intake restriction[e]
Intervention	6 weeks	3 consecutive days (2 week days and 1 weekend day) alternating for weeks 5 and 6	On completion of trial period	Usual diet incorporating flavonoid beverage and minimal flavonoid food source intake restriction[e] + 6 cups (1200 mL) rooibos/day
Control	4 weeks	3 consecutive days (2 week days and 1 weekend day) alternating for weeks 3 and 4	On completion of trial period	Usual diet incorporating flavonoid beverage and minimal flavonoid food source intake restriction[e] + 6 cups (1200 mL) fluid/water (in place of rooibos)/day

[a] Main reference method (due to provision of a more precise quantification of absolute dietary intake necessary in dietary intervention trials) (18,381)

[b] Test method

[c] Second independent reference method comprising fasting blood sample analysis as biomarkers of exposure and/or of effect

[d] Consecutive recording days were used due to the rapid metabolism of polyphenolic compounds that necessitate polyphenolic consumption from plant products on a daily basis to maintain concentrations in the blood.(100) Fasting blood sample collections on completion of a study period will reflect polyphenolic dietary intake of the day before to possibly usual short-term dietary intake.(338) Collection of these samples immediately after the last days of a study phase will reflect the time window of effect.

[e] Dietary flavonoid intake restriction stipulations comparable in these experimental periods of the intervention trial with the restricted dietary items pertaining to fruits (apples, oranges, grapes - black & red), dark chocolate and dietary items to avoid pertaining to beverages (fruit juices, black & herbal teas, red wine, cocoa drinks) towards flavonoid content and coffee and some soft drinks towards their total antioxidant capacity (TAC) to limit the intake of dietary flavonoids (and the dietary TAC).

7.3.1.1 *Selection of Study Participants*

It was suggested that requiring participants to avoid flavonoid-rich foods and beverages, like tea, could be difficult for some (536) and would require motivated and dedicated volunteers as the trial participants. All interested volunteers attended the trial information and screening session. A self-administered structured demographic, general health and lifestyle questionnaire along with clinical assessments (blood pressure), anthropometric assessments (weight, height, body mass index [BMI], waist circumference) and fasting blood samples were used to screen each volunteer, on written informed consent, for risk factors.

The questionnaire comprising multiple-choice questions requested demographic information on gender, age, educational attainment, marital status, etc., along with general health and lifestyle information (smoking status, physical activity level, family and own medical history). Information was obtained on dietary aspects, such as alcohol consumption, dietary supplement use, number of cups (or small mugs) of tea (black and herbal teas), servings of fruit (as 1 medium-sized fruit and one-half cup cut-up fruit or juice) and vegetables (as one-half cup raw or cooked vegetables and 1 cup raw leafy vegetables) consumed per week.

7.3.1.2   *Participant Inclusion and Exclusion Criteria*

The volunteers accepted into the trial had to have two or more risk factors for CHD which included: hypercholesterolemia (raised cholesterol above 5.2 mmol/L), hypertension (equal or greater than 140/90 mm Hg) or pre-hypertension (120 to 139/80 to 89 mm Hg), but not on any medication for these medical conditions, a family history of CHD, smoking and/or an increased BMI (25 to 30 kg/m^2). In addition the participants' Framingham risk scores (537) were calculated to determine their risk for developing heart disease.(37) Oxidatively stressed participants were selected for the intervention trial. Halliwell (114) proposed selecting such persons for antioxidant supplementation, as there would be no point in conducting an antioxidant intervention trial to obtain further protective effects on oxidative damage with antioxidant supplementation in those whose mean body antioxidant levels are already markedly high.   Møller et al. (215) reported that as even large amounts of dietary antioxidants (using blackcurrant juice as antioxidant mixture) did not decrease the already low steady state oxidative DNA damage in healthy adequately nourished subjects, it is very unlikely that antioxidant supplementation to an adequately nourished population will provide additional protective effect on endogenous oxidative damage.   O'Byrne et al. (273) reported that those experiencing chronic oxidative stress respond quicker and more radically to antioxidant supplementation than do healthy, normolipidemic subjects who are not faced with some abnormal oxidative stress.

Probable participants were required to be between the ages of 30 and 60 years, apparently healthy based on self-report with a stable body weight for six months prior to the trial, no clinical significant abnormalities of the liver, kidneys or blood, including diabetes, renal, hepatic and endocrine disorders besides the CHD risk, not taking any chronic medication, vitamin and/or antioxidant dietary supplements, following a conventional diet without undesirable alcohol consumption (three or more drinks per day), and females not being pregnant or breastfeeding or intending to become pregnant. Subjects were selected without regarding race or gender, and all had to be occasional or habitual tea drinkers willing to commit to dietary changes, if they were placed in the intervention trial.(37) For the purpose of evaluating the validity, reproducibility and responsiveness of the mean daily total flavonoid intake estimates as obtained from the developed comprehensive FFQ (and

resultant abbreviated FFQ), the participants of this clinical intervention trial served as the participant resource for these evaluations.

7.3.2   Rooibos Intervention Trial Design

This experimental study of 14 weeks that entailed a dietary change involving rooibos consumption as intervention to modulate blood oxidative stress markers in volunteers at risk for CHD, followed a crossover pre- and post-measurement single group intervention design, where the subjects concurrently participated in all experimental periods of the trial. Because of the envisaged small sample size, the primary interest in investigating the feasibility of the rooibos intervention and the 14-week duration of the trial, a crossover design was used which allows each subject to serve as their own control.(186,511,521) In a crossover design, the analysis is based on within-person differences (511) with the rationale that the treatment effects can be assessed more accurately due to the limitation of inter-group confounding.(536) The crossover design can thus reduce within-person variation in flavonoid uptake and metabolism (536) and in addition provide assurance that any observed changes did not reflect seasonal changes in consumption patterns (86,521) over the nearly three month duration of the trial. A three-month period roughly covers one season in the year.(24)

Cooney et al. (538) pointed out that short dietary intervention studies should be planned for a particular season of the year as it had been reported that seasonal variation influences the consumption of fruit and vegetables as food groups (363,538) and as a result, the intake of dietary components, like the carotenoids (in particular α- and ß-carotene).(538) As the study progressed from mid-winter to late spring, it was likely that subjects could change their dietary intake over its course. To minimize the seasonal effect and ensure dietary compliance, the participants were not randomized as considered in other intervention trials.(299,312) As a placebo for rooibos is not available (rooibos has a very unique flavour and taste) the investigators and the participants would in addition not have been blind to the nature of the intervention trial. Duffy et al. (299) indicated that in relation to black tea, it is not possible to produce a placebo beverage that convincingly tastes like black tea but lacks tea flavonoids.

Participants first entered a two-week run-in or baseline period where no intervention took place and they followed their usual diet. The following two weeks represented the washout period where participants were instructed not to consume rooibos and had to comply with additional dietary restrictions based on the flavonoid content and/or TAC. This pertained to chiefly beverages and some foods to minimize the potential of confounding by flavonoid consumption as the trial was a study comparing rooibos consumption to water (as placebo) against a background of minimal flavonoid consumption (Table 7.1). To minimize the potential confounding effects of consuming

fluctuating amounts of foods and beverages that are high in dietary flavonoids, all participants had to sustain this flavonoid-restricted diet throughout the entire trial, a protocol similar to that of other favonoid-rich dietary intervention studies.(118,189,202,208,212,214,218,271,275,312) Throughout this washout period, participants began to control their flavonoid-restricted diets prior to the initiation of the intervention. After this washout period, the participants were assigned to a six-week intervention period and instructed to consume six cups (1200 mL) of rooibos per day, while still continuing the flavonoid-restricted diet. A four-week control period followed hereafter, which required the participants to crossover to consuming an equivalent volume (six cups, 1200 mL) of water, as a placebo for rooibos is not available, while still continuing the flavonoid-restricted diet.

The participants received a printed list of the restricted foods (pertaining to fruits, such as apples, oranges, grapes (black and red), and dark chocolate) and beverages to avoid (fruit juices, black and herbal teas, red wine and cocoa drinks due to flavonoid content, and coffee and some soft drinks due to their TAC), as a guideline to limit the intake of dietary flavonoids and the dietary TAC. The flavonoid-restricted diet was designed to be low in flavonoids from sources other than rooibos. Thus, foods that were excluded or limited included those foods known to be rich dietary sources of flavonoids and being consumed in larger quantities at a time.

The food and beverage intake restriction selected correspond to that applied in many other flavonoid-containing dietary intervention trials, although the extent of the restrictions and the study periods of application differed across the studies, such as the trials of Warden et al.,(189) Wan et al.,(212) Engler et al.,(213) Mathur et al.,(214) Erba et al.,(216) and Mursu et al. (312) and with Young et al.,(218) Tsang et al. (275) and Wilms et al. (297) that applied more stringent restrictions. Despite asking the participants to avoid the consumption of certain beverages and restricting their daily portion intake of some foods, due to the high flavonoid content and/or TAC, these free-living subjects were instructed and encouraged to consume and remain on their self-selected diet for the 14-week duration of the trial. This meant that they should not alter their usual diet, as the intervention diet must be matched to the control diet in every respect with the exception of the bioactive compound,(3) which in this trial, was the total flavonoid intake. In addition they were encouraged to refrain from any habitual lifestyle changes, like physical activity level, throughout the trial period.(37) However, in a crossover design, carry-over effects can affect the results.(536) The two-week washout and the four-week control periods were considered sufficient to prevent a carry-over effect of flavonoids as most flavonoids have short elimination half-lives.(21,75) A two-week washout dietary depletion regime of flavonoids was used by Young et al.,(208) while in the study of Wilms et al. (297) a five-day wash-out period was used.

The trial participants were instructed not to drink any tea, herbal tea or rooibos, aside from the rooibos as supplied in the intervention period. The rooibos used was the fermented, traditional type of superior grade supplied by Rooibos Ltd (Clanwilliam, SA). All participants followed a standard recipe on how to prepare it as beverage (1 tea bag per 200 mL freshly boiled tap water, with a brewing time of 5 minutes). As applied by Duffy et al.,(299) the trial participants consumed their rooibos as desired (with or without added milk or sugar) to increase compliance and mimic their usual practice of drinking rooibos. The full amount of six cups had to be consumed daily throughout the day in the intervention period. Similarly prepared rooibos was institutionally analyzed for its antioxidant activity and total polyphenol/flavonol/flavanol content.(37)

7.3.3   Development of Comprehensive FFQ as Test Method

Most FFQs used to determine flavonoid intake to date was not originally designed to measure flavonoid intake and, therefore, did not in the development have a prior flavonoid-specific hypothesis. Some of the aspects to consider in developing a FFQ are the features of being a valid, reproducible and responsive dietary assessment measure. These features imply: How is the consumption frequency to be determined? Are determining consumption amounts required? What is the time period of interest? Is a group mean or individual intake required? Is absolute or relative intake important? Is information on dietary change needed?(482) The FFQ was designed considering the construction and evaluation directions provided by Block et al.,(355) Willet et al. (38) and Cade et al.,(17,18) and numerous other publications on the FFQ development, validation, reproducibility and field use fundamentals, which are included in the executed methodological components and steps.

When the intake of specific nutrients are the focus of the questionnaire, as for this research being only total flavonoids, the compilation of the food list and frequency categories demand careful consideration.(413) In the intervention trial, the absolute levels of daily total flavonoid intake had to be estimated as accurately as possible to obtain a truthful group intake estimate within each period of the trial. The developed FFQ therefore had to be sensitive enough to detect differences in flavonoid intake of the participant group between the different trial periods. Although rooibos contains unique flavonoids,(539) the metabolic end products are quickly removed from the circulatory system due to their rapid turnover rate,(540) which would not have been suited to fully substantiate the rooibos consumption through the fasting blood samples required for the lipid profile analysis within the rooibos intervention trial. The dietary intake became the means to substantiate the rooibos consumption as reflected by the change in the mean daily total flavonoid intake over the trial periods.

A sample page of the developed FFQ (from the final abbreviated FFQ) is included as Addendum C, showing its outlay as to the food list, the portion size and consumption frequency indications to estimate the mean daily total flavonoid intake. The food list has different food group categories and explanatory notes introducing each of these categories as to which items to consider for the intake estimation and which not. The outlay and format of the abbreviated FFQ is the same as that of the comprehensive FFQ except for the reduction in the number of food group categories and the items included in the food list of each of the food group categories.

7.3.3.1   Selection and Description of Items for Food List

Stiegler et al. (413) stated that the data collected on the intake of the nutrient(s) of interest must be at a high level of accuracy, yet it is advantageous if the FFQ could be concise. The validity of a FFQ is, however, influenced by the adequacy of the food list.(422) All plant foods and beverages containing appreciable and less appreciable amounts of flavonoids were included in the food list and not only those foods and beverages that contain appreciable amounts. No matter how high the concentration of a certain bioactive compound is in a dietary item, if the amount of that item consumed is low, the contribution of the bioactive compound from that item to the dietary intake would be low.(125) Even if containing less appreciable amounts of flavonoids or possibly consumed infrequently, the food or beverage item was included, as its flavonoid contribution might be important to ensure that the absolute flavonoid intake of most participants' diets could potentially be determined accurately by the questionnaire, and to correctly classify a participant with respect to the flavonoid intake. No plant foods were arbitrarily omitted from the questionnaire food list. This was considered imperative, as awarding flavonoid levels to dietary items had been identified a limitation because the full spectrum of prepared and processed foods are not covered in the USDA database for the flavonoid content of selected foods (Release 2.1).(161)

A FFQ with a comprehensive food list was compiled by selecting those foods and beverages in the USDA database,(161) which was used for the mean daily total flavonoid intake estimations. The food list was representative of the diet of South Africans, by using the South African food consumption studies report of the Department of Health as compiled by Nel and Steyn (90) and FoodFinder 3, the nutrient analysis software program of the South African Medical Research Council,(541) with the addition of a few dietary sources identified to reflect the South African dietary consumption not included in the database. A vital aspect in designing a new questionnaire is that the food list be customized for the specific country or region.(422) The food list was therefore modified by changing an American food name to the South African equivalent, like squash, summer, zucchini (NDB number: 11477 for raw and 11478 for cooked) (161) to baby marrow. In addition, properties such as cultivar, colour, maturity and with or without skin or peel have been reported to make a marked difference in flavonoid content. These properties are only partly considered in flavonoid intake assessments (105) due to incomplete information to provide

for specific food flavonoid contents. These properties could not be accounted for in the flavonoid intake estimation of this research besides for determining the consumption of certain fruits with or without skin or peel (indicated shortly).

The most comprehensive food list possible was compiled to make the flavonoid assignments more precise. This is especially important as bioactive compounds, like flavonoids, typically occur in small amounts (mg per 100 g) in foods.(3) A comprehensive list of foods and beverages were included in the questionnaire, not only to cover the potential diverse range of items to be consumed by the participants that might contribute to their total flavonoid intake, but also to account for the current unknown subset of items likely to contribute to between-person variation in total flavonoid intake. It is important to incorporate the dietary items that contribute to between-person differences in intake, since Ishihara et al. (411) surprisingly found that green tea best predicted between-person variation in total folate intake in their study. Byers et al. (29) pointed out that if the questionnaire purpose is to account for as much as possible of the absolute intake, then the food list included needs to be broad.

As flavonoids are not present in animal products (10,87) (meats, dairy products, fish and shellfish) or not present in significant amounts,(190) these food categories were not included in the FFQ. Cereals, oil seeds and oils are generally excluded in dietary flavonoid intake determinations (10) as, although flavonoids are present in small quantities only in cereals,(542) the grain production process, as well as the production process of fats and oils, remove most if not all the possible flavonoid compounds.(10,87) Although sweets made with fruits or natural flavours and colours may contain flavonoids,(10) they were not included in the dietary intake determination. As indicated previously, the isoflavone class of flavonoids was not included in the dietary determination since the main food source, soy,(12) is not commonly consumed by South Africans.(90) The intake from dietary supplements was excluded in the analysis, because this research focused on intake from food and beverages only and it served as an exclusion criteria for the intervention trial participants.

The selection of the food group category and individual food item inclusions in the comprehensive FFQ food list were based on exclusion of animal foods and those plant foods as indicated above and considering that the main flavonoid dietary sources are fruits and beverages (tea, fruit juice, cocoa, red wine and beer),(8,100) along with vegetables.(48) The FFQ was organized into 14 categories (A to N of the FFQ) based on grouping conceptually similar items and/or culinary usage items together in a category:

- Category A: fruit (n=7) and vegetable (n=2) juices;
- Category B: fresh fruit (n=19 extended to 25 considering varieties such as black, red, white or green grapes) and preparation (with or without skin or peel);

- Category C: canned fruit (n=6);
- Category D: dried fruit (n=6 extended to 7 considering processing, seedless or seeded raisins);
- Category E: vegetables as side dish, main soup ingredient, vegetarian dish (whole portion/serving) (n=23 extended to 41 considering raw, cooked and canned options);
- Category F: vegetables as ingredient in mixed dishes (stews, bredies, quiches, pastas, etc.), pizza, soups, burgers, sandwiches and salads (n=23 extended to 43 considering raw, cooked and canned options);
- Category G: legumes (n=4);
- Category H: condiments and sauces (n=6);
- Category I: nuts (n=9);
- Category J: honey and jam (n=6);
- Category K: chocolate (n=2);
- Category L: alcoholic beverages (n=3 extended to 5 considering types with wine as red or white);
- Category M: other beverages (n=5 extended to 10 considering types, plain or flavoured);
- Category N: herbs added to foods (n=10 in fresh or dried form);
- Total of 131 extended to 183.

All the dietary items included within each of these categories were placed in alphabetical order. At the end of each category participants could list any additional items within each of the categories they consumed but which were not included in the food list, despite Gibson (14) not recommending the use of these open-ended questions. These open-ended questions represented the only write-in items due to lack of information received on additions to the compiled food list in the pre-testing of the questionnaire.

In conducting cognitive, think-aloud interviews, Thompson et al. (418) found more precise wording as an area to improve the performance of short instruments for assessing fruit and vegetable intake, which was considered in introducing each of the food categories. As introduction to each food group category, explanations were included as to which foods to include/exclude based on making important contributions to the flavonoid intake (like exclusion of very small amounts of dried fruit included in products like breakfast cereals) (Addendum C), as previously applied in FFQs to assess consumption of fruit.(83) Such inclusion/exclusion indications for each category were considered crucial by Horwath,(422) who indicated it necessary to reach a balance between adequate coverage of foods eaten by participants and minimal participant burden. Thompson et al. (418) found that their participants did not understand the 100% fruit juice question and erroneously included fruit-flavoured drinks in their responses. They therefore reworded this question by providing examples of drinks to include and not include, which was applied in this FFQ in introducing the fruit juice category (Addendum C).

FFQs have their own unique problems that complicate estimation of flavonoid intake. For instance, items are aggregated together within single line grouped food items. This forces the adoption of assumptions about the frequency and amount of foods consumed within each aggregate food group and the average flavonoid value computed for the food group based on these assumptions. Other quantitative problems with FFQs are the categorization of processed food with non-processed foods, and categorization of several species of different genera or even the same genus together.(87)

A limitation found in determining carotenoid intake using a FFQ was the aggregation of some vegetables and fruits within single line items in the FFQ. Within one line item, only one or two of the fruits or vegetables incorporated might be high contributors of a given carotenoid.(502) This holds true for flavonoid intake estimations. Single foods aggregated into food groups, like leafy and green vegetables, may need to be ungrouped into individual foods, as these have very different flavonoid profiles.(89) As in other FFQs,(407,526) individual items were not aggregated into broad single line grouped items as typically applied,(89,526) but were listed as separate items, besides fruit juices, contributing to the long food list. Separating aggregated items from each other might be needed in FFQs to make more precise quantitative estimates of flavonoid intakes (87) due to the widely differing flavonoid content of particularly fruit and vegetable items.(391)

Fruit juice is a major dietary source of flavonoids (543) and a commonly consumed item among the South African population.(90) In the USDA database,(161) the flavonoid content is provided only for pure single and not for blended fruit juices. In order to determine the flavonoid intake through the consumption of fruit juices (Category A of the FFQ), information regarding the available fruit juice types (100% and fruit nectars) and flavours (single or blended) had to be obtained across the different local brands. The managers of two Cape Town-based large retail food stores, known for carrying a variety of processed food products, on being informed about the purpose of the store visits provided permission to obtain the required fruit juice information of the fruit juice products in the stores. A total of 84 different South African manufactured 100% fruit juices were counted in the two stores. In addition four fruit juice and yogurt blends, 11 dairy fruit blends (major ingredient skim milk) and 26 nectars were counted indicating that 100% fruit juices made up the bulk of the South African fruit juice market. Only 100% juices were noted on the FFQs for obtaining fruit juice intake, as the fruit juice inclusion in FFQs for determining fruit and vegetable intakes usually comprise 100% fruit juices.(409)

The ingredient listings of the fruit juices on the product labels only indicated the ingredients that included the different types of fruit juices making up the specific flavour without an indication on how much of each fruit juice type was present in the specific fruit juice flavour. The Manager of the

AgriFoods Technology Station, CPUT, obtained this information from the local fruit juice industry (07/08/2007), which indicated that apple, pear and grape juice are used as base juices (either by itself or in combination) in blended fruit juices. Manufacturers in keeping their product cost to a minimum, use on average about 80% of the base juice and will not use on average more than 20% of other juices in the total juice composition of 100% blended fruit juices. Apple juice is used predominantly in blended juices and was, at the time, the used base juice due to availability and price. The fruit juices were entered into the FFQ with the fruit juices aggregated (Table 7.2). The fruit juice consumption data was captured in the data analysis with the fruit juices aggregated and having the ingredient proportions as follows: the base fruit juice (apple) contributing 80% and the other fruit juice, representing the flavour identified on the fruit juice packaging, 20% of the formulation.

The fruit food list was compiled comprehensive enough to capture most types of fruits the participants could consume during the rooibos intervention trial (initiating any time as from approximately March/April to October/November in the initial planning). The fruit list was not fully complete due to the exclusion of a few fruits that could, due to seasonal availability (apricots being available November and December; cherries being available October to December; litchis available November to February),(544) not be accessible for consumption during the duration of the trial.

As indicated, fruits were subdivided to represent the processing form with the included categories as 100% fruit juice, fresh, canned, and dried fruit (categories A, B, C and D respectively). In addition the consumption of apples and pears with or without skin or peel were ascertained due to these inclusions in the USDA flavonoid database.(161) Guava (*Psidium guajava*) was added to the fruit list (fresh and canned) with the flavonoid content (quercetin, myricetin, kaempferol, luteolin and apigenin) (112.85 mg/100 g) obtained from Miean and Mohamed (131) and included in the data capturing template for incorporation in the data analysis. Sufficient flavonoids survive the jam making process (10) and as a result jams were included as a category along with honey (Category J).

There are limitations to the types of data obtained from FFQs. Concerning fruits and vegetables, no data is gathered on either meal patterns involving fruits and vegetables, consumption of mixed dishes that contain substantial amounts of fruits and vegetables, or food preparation methods. Such information can be very important for etiological studies of diet and disease and for designing and/or evaluating intervention strategies to increase consumption of fruits and vegetables.(73) The vegetable food list was rather comprehensive to capture the vegetables the participants consumed.

Table 7.2: Grouping of 100% Fruit Juices* in FFQ and Data Analysis

Possible fruit juice groupings for line food list	Number fruit juices (n=84)	Major ingredients on product packaging	Fruit juice aggregation in line food list	Nutrient database (NDB) fruit juice [a]
Apple juice Apple juice blends, Pear juice	9 2 1	Apple juice Apple & pear juice/concentrate Pear juice	Apple, including apple & pear, mango & apple	9016 apple juice, canned or bottled, unsweetened, no added ascorbic acid
Berry and mixed berry juice	8	Blackcurrant juice (n=3) Cranberry juice (n=2) Loganberry juice (n=1) Strawberry juice/puree (n=2) Base juice inclusions (n=5)	Berry and mixed berry juice	9016 apple juice, canned or bottled, unsweetened, no added ascorbic acid / 99007 blackcurrant juice / 99110 cranberry juice, raw
Grape juice, red	3	Red grape juice No base juice	Grape, black	99049 as grape juice, black
Grape juice, white	2	White grape juice No base juice	Grape, white	99050 as grape juice, white
Grapefruit juice Ruby grapefruit juice	4	Grapefruit juice Base juice inclusions (n=3)	Grapefruit and ruby grapefruit	9016 apple juice, canned or bottled, unsweetened, without added ascorbic acid / 9128 grapefruit juice, white, raw
Orange juice Orange juice blends (naartjie & orange, peach & orange, breakfast punch) Naartjie juice	7 8 1	Orange juice Base juice inclusions (n=6) Orange juice Base juice inclusions (n=7) Naartjie juice Base juice inclusion	Orange & orange juice blends (mango & orange, peach & orange, breakfast punch)	9016 apple juice, canned or bottled, unsweetened, without added ascorbic acid / 9206 orange juice, raw
Tropical and tropical fruit juice blends (granadilla, guava, litchi, mango, pineapple)	34	Base & orange juice (n=16) Base juice (n=9) & mango juice/pulp (n=7) or pineapple juice (n=2) Orange juice & mango puree (n=2) Base & litchi juice (n=4) Base juice & guava puree (n=3) [Mango juice, puree or concentrate inclusions (n=22)]	Tropical and tropical fruit juice blends, including granadilla, kiwi, mango, paw-paw, pineapple	9016 as apple juice, canned or bottled, unsweetened, without added ascorbic acid / 9206 as orange juice, raw
Other 100% pure fruit juices (apricot, peach)[b]	5	Base juice & apricot puree (n=2) Peach juice (n=1) Base & peach juice (n=1) Base juice & peach puree (n=1)		

* Fruit juices observed across two Cape Town-based large retail food stores. Only 100% pure single fruit juices and blends were captured. Fruit juice and yogurt blends, dairy fruit blends and nectars containing only a percentage (40% or 50%) fruit juice were excluded.

[a] Nutrient database (NDB) fruit juice numbers are used in the data analysis and obtained from the USDA database for the flavonoid content of selected foods, release 2.1.(161)

[b] Specified by participants in the open-ended question section at the end of Category A: Fruit and vegetable juices of the questionnaire.

Vegetables were subdivided to represent the types, the processing form (raw and cooked) and the vegetables included as ingredients in mixed dishes with vegetable juice as a sub-section included within the fruit and vegetable juice category (Category A). According to Willett,(30) including the same food used in different ways as separate items can be useful when the amounts consumed are quite different; such as the actual amount of a vegetable consumed separately as single whole portion versus that of the same vegetable consumed as an ingredient in a composite dish which can differ quite substantially. The vegetable category was thus foremost split into two representing vegetables as side dish/main soup ingredient/main salad ingredient/vegetarian dish (Category E as single whole portions) and vegetables as ingredient in mixed dishes (stews, bredies, quiches, pastas, etc.), pizzas, soups, burgers, sandwiches and salad (not main ingredient) (Category F).

The estimation of total flavonoid intake is difficult (7,107) because limited data is available on the dietary contents.(107)   Therefore to obtain more accurate estimates of the flavonoid intake, refinements are needed to separate foods so that more precise quantitative intake estimates can be made.(87) Vegetables may be consumed as a whole portion or appear in many composite dishes, as they are often used as ingredients in such dishes. Onion intake, for instance, can be represented as vegetable rings or in soups.(232) Onions, in particular in mixed dishes, (ready-made dishes and home-cooked food) may be an important dietary source of flavonoids.(162) However, obtaining accurate intake reports for such foods eaten both alone and in mixed dishes is particularly problematic.(18) Although counting food ingredients from mixed dishes always poses a challenge when assessing dietary intake, not undertaking it represents a potential loss of dietary information.(424)

Underestimation of vegetable (418) and fruit and vegetable (468) intake primarily occur because subjects do not consider or forget vegetable use in mixtures and as a result underreport their intake.(418,468) Small portions of vegetables eaten as part of mixed dishes, like soups and casseroles, are not likely to be reported unless, for instance, the interviewer engages in lengthy and detailed probing, which could be burdensome for both study staff and participants.(73) Multiple small amounts of vegetables eaten as part of mixed dishes can add up to significant intakes.(83) Thompson and co-workers (417) found that the use of fruit and vegetables in mixtures (all uses, however small) accounted for more than 13% of the total fruits and vegetables reported by their study participants on 24-hour recalls.   O'Brien et al. (545) pointed out that not disassociating fruit and vegetable inclusions in composite dishes in a food list is a limitation of the FFQ when it comes to estimating fruit and, in particular, vegetable intake. They found that vegetable inclusions in composite dishes made a noteworthy contribution (26%) to the mean total vegetable intake.

Considering the use and composition of multiple mixed dishes, which cannot be readily captured using current FFQs, are among the measures Kristal et al. (448) indicated to improve FFQ-type dietary measures. The mix of vegetable varieties present in combination dishes may provide different types and amounts of flavonoids that can be accounted for when considering the use and composition of mixed dishes. Mixed recipe foods consist of animal and plant ingredients in which flavonoids are present in some ingredients (plant ingredients potentially containing flavonoids) and absent in others (animal ingredients as flavonoid devoid).(87) FFQs may ask subjects to report either a combined frequency of a particular food eaten both alone and in mixed dishes. Alternatively, they may ask the subject to report separate frequencies for foods eaten alone or in combination.(18) The latter can be accomplished by indicating the vegetable consumption as individual foods and as components of mixed or combination dishes. The first approach is considered cognitively complex and difficult for people not involved in cooking, but the second approach may lead to double counting and overestimation of intake.(18) When asked separate questions about similar foods, the subject may blend details of food consumption and thereby unintentionally duplicate answers.(420) To avoid double counting in the vegetable intake assessment, which was noted important,(162) the participants were guided to this category split in the instructions for completion of the FFQ.

In the FFQ, vegetable consumption representing a main dish was thus asked separately from vegetable consumption from mixed dishes in two split vegetable categories. In introducing the latter vegetable category, the participants were guided to consider vegetables consumed as part of mixed dishes, like vegetables in stews, quiches, pastas, burgers, sandwiches, salads, etc. and to break down these dishes in their component ingredients. The food list incorporated a spectrum of vegetables in the latter listing to account for vegetables found in food mixtures. Participants therefore had to indicate the consumption of vegetables as individual whole portion food items and as a component of mixed dishes, as a listing of mixed dishes could not be drafted since these are only partly indicated in the food consumption data available for South Africans.(90) Even if available, the food list for this category would have become infinitely long and recipe vegetable ingredient deviations may nevertheless have occurred. Flavonoid levels were as a result collected on specific vegetables as mixed dish inclusions. To not further lengthen the food list, a fruit category representing fruits included in mixed dishes (fruit salads and desserts) were not included. O'Brien et al. (545) found that fruit inclusions in composite dishes make a small contribution (4%) to the mean total fruit intake and thus seem to be a less important source when allowing for composite dish ingredients.

Tomatoes may provide a more sizable contribution to the daily intake of flavonoids, particularly the flavonol intake, in the Western diet than was previously realized due to the widespread use of tomato-derived products in dishes, like pizza and lasagna, besides the addition of tomato sauce to many foods.(166) Canned tomato paste/puree was included in both the vegetable food listings (categories E and F) and tomato sauce in the condiments and sauces category (Category H) along with a few other sauces (chili and pasta, marinara) and vinegar.

Chocolate too was included (Category K), as it is a very rich flavonoid source and a minor consumption may contribute appreciably to the total flavonoid intake.(8) Plain chocolate and chocolate bars (chocolate confectionary) contributed two thirds of the total intake of cocoa among users as measured in the Zutphen Elderly Study among 470 elderly men. Cocoa sandwich filling, cocoa desserts, drinks and some miscellaneous items contributed to their remaining cocoa intake.(233)

While the alcoholic beverage category (Category L) listed wine (red and white), beer and apple cider, the 'other beverages' category (Category M) listed cocoa drinks and various teas. Black tea is the commonly consumed tea in SA with consumption of the other teas, besides possibly rooibos,(90) negligible. However, various teas as black, green and herbal (plain and flavoured) were included in the food list despite possible negligible intake. Tea comprises a heterogeneous group of beverages, including fermented black tea, unfermented green tea, sweetened or unsweetened ice tea, and it might even be understood to include fruit tea or herbal teas.(268) According to Peters et al.,(268) it may be expected that study subjects would give a summary answer for any kind of tea if they are asked only about their tea consumption without more detailed questions, which might disturb the compilation of the food list of the Abbreviated FFQ. Further flavonoid dietary sources included were legumes (Category D), nuts (Category I) and herbs (Category N).(8)

The amounts of herbs and spices normally added to food would probably only increase the flavonoid intake marginally.(153) Uncertainty exists whether the quantity in the diet is enough to have an influence on the antioxidant protection of the body.(546) In a Danish study, the contributions made by the consumption of herbs to the average daily flavonoid intake was not considered, as it was viewed negligible on an average daily basis.(547) However, the concentrations of phenolics are much higher in aromatic herbs than vegetables. Aromatic herbs represent a reservoir of phenolic compounds concentrated in just a few gram of material (117) and can represent one of the most promising sources (548) and simplest ways (117) to increase the phenolic content of the daily diet. The consumption of a few gram of some herbs would be sufficient to contribute noticeably to the average daily flavonoid intake.(547)

The importance of herbs and spices is possibly best illustrated in the National Health and Medical Research Council (NHMRC) dietary guidelines for older Australian adults that include a food variety checklist. This checklist was used to assess intake of phytochemical dense foods. Herbs and spices make up 11 out of the 64 foods (17.2%) in the checklist.(549) Only limited information is available on the consumption of herbs and spices by South Africans. The report by Nel and Steyn (90) based on dietary intake assessment via 24-hour recalls, indicate that although only 0.01% of the South African adult population on a particular day may be consuming herbs and spices where the average portion per day for those persons consuming herbs and spices is 3.5 g.

Herbs were included in the FFQ, in particular, as Tapsell et al. (549) indicated that herbs and spices constitute an important dietary source in the intake of flavonoids. Herbs and spices (along with ready-to-eat meals containing onions) were included in a FFQ determining the habitual consumption of flavonoid-rich foods by de Vries et al.,(74) while Wilms et al. (297) in their quercetin-rich dietary intervention trial asked volunteers to minimize intake of herbs and spices as part of the flavonoid-restriction dietary regime. While Sultana and Anwar (173) detected no flavonols (quercetin, kaempferol and myricetin) in garlic (*Allium sativum*), Miean and Mohamed (131) detected that garlic (*Allium sativum*) contains 639 mg/kilogram (kg) myricetin, 47 mg/kg quercetin and 217 mg/kg apigenin. The discrepancy may be due to characteristics of the cultivar or variety examined along with different growing conditions.(131) The flavonol content of garlic chives (NDB number: 99053) is included in the USDA flavonoid database, but garlic as food source is not included (161) and its consumption was not considered in the data analysis. In contrast to herbs, the concentrations of flavonoids in most spices are relatively low (550) and limited data is available on the individual flavonoids in spices.(110) The participant intake of herbs and not of spices was thus obtained as the last food list category of the FFQ.

A balance is necessary between adequate coverage of foods eaten by participants and minimal participant burden.(422) The food list of the comprehensive FFQ developed comprised 131 items extended to 183 to cover the different types, varieties, preparation and processing forms of the included food and beverage items. The open-ended section at the end of each of the 14 food/beverage category sections of the questionnaire allowed for additional write-in items not included in the food list. This broad detailed flavonoid-related food list might be considered a strength of the developed comprehensive FFQ, as it should aid to account for as much of the absolute dietary intake as possible as anticipated by Byers et al.(29)

Willett (30) indicated that in a self-administered format, co-operation and mental energy were likely to substantially diminish if many more than 150 items were used. This FFQ, therefore, might be extensive enough to cover the dietary items to be consumed by the participants, but still close to the maximum length to be reasonably well completed. Comprehensive FFQs typically ask about many different fruits and vegetables to estimate intake of micronutrients and other compounds.(42) The bulk (66%-72%) of the food list of this comprehensive FFQ included the various fruit and vegetable items considering their different forms spread across 6 of the 14 categories.

The major difference between the comprehensive FFQ developed for the rooibos intervention trial and other FFQs, besides the flavonoid food/beverage intake emphasis, is the split vegetable intake assessment as eaten alone and in mixed dishes, and the incorporated herb assessment. The incorporation of these items to the regular food list should increase the capability of the questionnaire to estimate a more precise absolute total flavonoid intake.

7.3.3.2   *Consumption Frequency and Reference Time Period*

The validity of a FFQ is influenced by the ability of the subjects to report their pattern of intake.(422) If specific nutrients are to be assessed, the FFQ should not only be developed to include dietary sources high in these, but provide adequate options for the frequency of consumption to reflect day-to-day variations in intake.(440) The nutrient amount contributed by any food item takes into account not only its nutrient composition, but also the frequency of its consumption and portion size.(355) The frequency of consumption was left open-ended versus the provision of different category responses, as according to Willett (436) it might provide some enhanced precision in reporting because the frequency of use is a continuous rather than a categorical variable. The open-ended format was particularly important for those dietary items consumed a few times per day and which are rich sources of flavonoids. These centered on the consumption of beverages. An exact intake frequency compared to category intake distributions would provide for a better flavonoid intake estimate and between-person variation in intake, which could not be built-in in the food list compilation. This is particularly important towards the higher end of the intake distribution. Wolk et al. (426) found the mean number of self-reported cups of coffee per meal to be about two (mean number of cups 1.82 to 1.89), which may be applicable to tea consumption indicating that a high daily consumption may be reached (6 or more times per day) by some participants, considering consumption with meals and in-between meals. Studies in which the consumption of tea was addressed came across a range of tea drinkers ranging from non-drinkers to heavy use drinkers.(236,260)

The same study of Wolk et al. (426) in which open-ended questions were asked about quantities consumed during one meal attributed to one food frequency, found a wide range of self-reported consumption of bananas, apples, citrus fruits and glasses of juice per meal. These are all dietary sources that would make a contribution to the flavonoid intake and would require a more precise intake determination per participant to allow for between-person variation in intake. Maintaining an open-ended format allowed for using the same frequency time frame throughout the different food list categories included in the questionnaire. Providing different frequency response categories for the different categories in the FFQ (foods versus beverages) probably contributed to response errors in its completion, as it was reported that subjects failed to check for time frame indications when completing consumption frequency in a FFQ.(374)

The open-ended format required participants to enter a number in the daily column (as the number of times the item was consumed per day) and in the weekly or per two weekly columns (as the number of days the item was consumed per week or two weeks) with the time frame being the past two weeks. This designated the frequency with which the dietary items were consumed over the past two weeks. The division, between the number of times per day and then the number of days per week or per two weeks over the past two weeks, was made so that the participants were not required to make their total intake quantification of per week or per two weeks over the past two weeks, but were guided for the quantification to be made within the FFQ analysis. This frequency response format was thought to reduce guessing of the total consumption frequency. The open-ended responses were coded as whole numbers and the quantification indicated the past fortnightly frequency of consumption.

According to de Vries and co-workers,(74) the within-subject coefficient of variation in flavonoid intake of 63% in their study suggests that the number of days needed to estimate the individual intake within 20% of the actual intake 95% of the time, is 38 days. A high correlation coefficient was found between the reported flavonoid intakes in a FFQ determining flavonoid intake over 30 days and three-day dietary records.(74) Because this phase of the research was an intervention trial, the timing of the dietary assessment relative to the treatment, however, had to be considered.(86) A period of two weeks was selected for the FFQ time frame to determine the flavonoid intake in each of the rooibos intervention trial periods (baseline, washout, intervention and control) to validate the FFQ with the flavonoid exposure and effect biomarkers obtained as part of the intervention trial. The time frame was chosen to represent the relationship between the variation in intake and the biological variation, as Cade et al. (17,18) indicated that in order to validate the test method (FFQ), the time span of the reference method (exposure biomarkers and the estimated dietary records) in relation to the period of the test method (FFQ), needs to be taken

into consideration and in theory should assess diet over the same time span,(18) which for the intention of this research was current.

As the phenolic metabolites are rapidly eliminated from the plasma,(100) due to their short elimination half-life periods, these biomarkers probably do not reflect long-term intake,(118,338) but short-term intake and possibly medium-term intake.(338) As quercetin is a principal phenolic constituent of plants and quantitatively an important dietary flavonoid,(7) its plasma concentration or urinary excretion may be a potential useful biomarker of habitual intake of flavonoid-containing foods.(218) Based on the elimination half-life of quercetin, a 'steady state' of quercetin in the plasma should be reached well within a week, with two weeks sufficient to increase plasma concentrations.(297) Duffy et al.,(243) for instance, estimated their intervention study baseline dietary flavonoid intake by a one week FFQ. Two weeks of increased fruit and vegetable intake have brought about changes in urinary flavonoid excretion (509) and two weeks of flavonoid-rich dark chocolate consumption an increased plasma EC concentration.(213) As a result, a relatively short duration period of two weeks was chosen for the FFQ to estimate flavonoid intakes, based on the literature addressing the time-course to reach a 'steady state' in plasma flavonoid concentration. According to Kris-Etherton et al.,(3) diets should be fed for long enough duration to assure that a biological effect is observed and sustained. When the methods under comparison refer to different periods of consumption, discrepancies may be expected between food frequency data, which assess usual intake and record or recall data, which measure a shorter, time specific intake.(350) The FFQ was thus developed to assess the flavonoid intake of the past two weeks to coincide with a short, time specific period that relates foremost to the window of effect and in addition the dietary record completion within the intervention trial (see Table 7.1).

7.3.3.3   *Choice of Reference Portion Size and Portion Size Estimation*

With the aim of improving the quantification of the total flavonoid intake in the FFQ, portion size options were included. Although the portion size of each food and beverage item was asked, the usefulness of portion size inclusions have been questioned,(85,436) as it increases the number of questions related to the food list (370,397) and imposes a greater burden on the subjects (397, 422) without possibly improving the validity of the nutrient estimates (85,357,397,436). Ranka et al. (391) suggested that portion size should be included in FFQs developed to assess flavonoid intake, as their FFQ provided higher correlations between the intake estimates of quercetin and naringenin and the respective urinary excretions on portion size inclusion.

The portion size format of the FFQ required participants to specify their portion size as small, medium or large in designated columns, which each came with pre-specified portion size or unit quantifications for each of the items in the food list to guide the participants to indicate their portion consumed. For most of the items in the food list, additional guidance was provided as to the

reference medium amount in a further descriptive column, such as one-half cup cut-up fruit representing one medium fruit unit (551) in the fresh fruit category (Category B), and one cup representing a small mug (552) in the 'other beverages' category (Category M) (Addendum C). The medium portion sizes mostly matched those medium food quantities indicated in the Food Quantities Manual of the National Research Programme for Nutritional Intervention, Medical Research Council (MRC), SA (552) with consideration of those identified in the summarized South African food consumption studies report (90) and dietary guidelines, like the Food Guide Pyramid for fruit and vegetable intakes.(551)

The portion size consumption quantifications as small, medium and large related to the medium size portion indication with a small portion as one-half and a large portion as one and one-half the medium portion. This was applied for the most part of the food list items in numerous FFQ quantifications (24,383,394,497,404,410) except where small and large quantity descriptions were indicated in the Food Quantities Manual, MRC, SA,(552) which greatly occurred in relation to whole unit indications for fruit and some vegetables (tomato and potato). The listed portion sizes were described using familiar household measures, for example cup, tablespoon, teaspoon and others, and not vague description terms, i.e., bowl descriptions, as propagated by Briefel et al. (456).

Across the fruit categories, a medium portion/serving was indicated as one-half cup 100% fruit juice (Category A), a whole (1 unit) fruit (Category B), one-half cup canned fruit (also represented as number of canned halves or rings) (Category C) or one-quarter cup dried fruit (also represented as number of dried halves, rings, units and other) (Category D). For vegetables as side dish or main ingredient as whole portion (Category E), a medium portion/serving was indicated as one-half cup raw or cooked vegetables and one cup raw leafy vegetables. A medium portion/serving equated to one-half cup for legumes (Category G), one level dessertspoon for condiments and sauces (Category H), one-quarter cup (or two heaped tablespoons) for nuts (Category I), one heaped teaspoon (or about 1 level dessertspoon) for honey and jam (Category J), six blocks (40g) for chocolate (Category K), one can or white/red wine glass for alcoholic beverages (Category L) and one cup for other beverages (Category M).

The medium portion fruit and vegetable amounts were for the most part the same as the amount indications of a serving as presented by the USDA and generally used,(504,521,522) while one-half cup (4 fluid ounces [fl oz] with 1 fl oz = 29.57 mL) was used for 100% fruit juice. This was presented by Popkin et al. (553) in the proposed guidance system for beverage consumption, to allow for a more practical quantification of a small portion as one-half and a large portion as one and one-half the medium portion (equating to a quarter cup, one-half cup and one cup,

respectively) than using the usual three-quarter cup (178 mL or 180 mL) fruit juice as medium portion measure.(504,521,554) Small, medium and large visual sketches of a number of fruits in season (n=8) and some vegetables (n=2), as obtained from Steyn and Senekal,(555) were supplied as an appendix to the participants to assist them in selecting their fruit or vegetable unit portion consumed.

Despite these portions not reflecting the portions actually consumed the participants, by indicating their portion consumed as small, medium or large, could add personal variability in their amounts consumed.(355) Such portion selection may accommodate gender disparity in amounts consumed.(422) Some flavonoid-rich foods are consumed in small quantities (chocolate and onions), whereas others, especially beverages (tea and red wine) are consumed in larger quantities.(269) Such differences in intake were reflected in the portion size quantification descriptors. The equivalent in gram of all the household measure portion indications (small, medium and large) for each of the items in the food list were obtained from the Food Quantities Manual, MRC, SA (552) and used to calculate the flavonoid intake contributions.

7.3.3.3.1 Determination of vegetable ingredient amounts in composite dishes

Due to time constraints and for logistical reasons, the fractional portion sizes assigned to each vegetable type and its preparation (raw and cooked) included in the food list of those consumed as part of mixed dishes (Category F), were not determined from such actual individual inclusions across the wide spectrum of South African composite dish recipe formulations. To simplify the FFQ completion, similar reference amounts for the small, medium and large amounts consumed as part of mixed dishes, were assigned across all the vegetable items in this food list, with the allocation of a heaped teaspoon for a small amount, a heaped dessertspoon for a medium amount and a heaped tablespoon for a large amount. These amount allocations allowed for the best portion descriptors to meet the one-half of the medium portion for a small amount and one and one-half the medium portion for a large amount, considering the weight indications of these household measures in the Food Quantities Manual, MRC, SA (552) across all the vegetable inclusions in this category of the FFQ.

Although the FFQ portion size indications across all the vegetable item inclusions for the mixed dishes food list were standard in the FFQ (heaped teaspoon, heaped dessertspoon or heaped tablespoon), the portion weight allocations used in the analysis for these portion size indications were different, as each weight allocation corresponded directly to that assigned to the household measures of that specific vegetable as indicated in the Food Quantities Manual.(552)

7.3.3.3.2   Determination of herb amounts

A list of those herbs containing flavonoids, and which are common recipe ingredients in South African published cookbooks, was compiled (Table 7.3). Six miscellaneous South African recipe books published over time (556-561) were screened and all recipes that contain these herbs identified. The recipe naming, the number of portions and the herb amount included in each of these recipes was noted and the average herb amount used per portion calculated for each recipe for fresh and dried herbs. The frequencies of the calculated amounts used per portion for each of the herbs were determined. Considering the frequency allocations per portion amount, the highest frequencies were identified to group the herb use into three categories to represent lightly, mildly and strongly flavoured.

Table 7.3:   Volume and Mass of Flavoured Herb Options for Determining the Flavonoid Contributions

Herb	Source	Nutrient Database Code[a]	Total no. recipes (n=512)[b]	Lightly flavoured (Small amount)			Mildly flavoured (Medium amount)			Strongly flavoured (Large amount)		
				[b]No. of recipes	Vol. (mL)[b]	Mass (g)[a]	[b]No. of recipes	Vol. (mL)[b]	Mass (g)[a]	[b]No. of recipes	Vol. (mL)[b]	Mass (g)[a]
Basil	Fr.	02044	26	21	5.00	0.83	3	15.0	2.50	2	30.0	5.00
	Dr./Gr.	02003	61	24	0.50	0.10	32	1.0	0.20	5	2.5	0.50
Chives	Fr.	11156	22	10	2.50	0.50	7	5.0	1.00	5	15.0	3.00
Coriander	Fr.	11165	31	7	0.50	0.03	19	1.0	0.06	5	2.0	0.13
Marjoram	Fr.	02023	2	0	N/D	-	2	4.0	N/A	0	N/D	–
	Dr.	02023	10	4	0.50	0.10	6	1.0	0.20	0	N/D	–
Mint	Fr.	02064	8	4	2.00	0.20	2	5.0	0.50	2	7.0	0.70
Oregano	Fr.	99115	8	4	1.50	N/A	1	3.0	N/A	3	4.0	N/A
	Dr./Gr.	02027	89	69	0.50	0.20	14	1.0	0.40	6	2.5	1.00
Rosemary	Fr.	02063	7	3	1.00	0.20	2	1.5	0.30	2	4.0	0.80
	Dr.	02036	22	8	0.25	0.05	10	0.5	0.10	4	1.0	0.20
Thyme	Fr.	02049	6	2	0.50	0.10	1	1.0	0.20	3	4.0	0.80
	Dr./Gr.	02042	50	25	0.25	0.05	17	0.5	0.10	8	1.0	0.20
Parsley	Fr.	MRC 8157[c]	164	68	3.00	1.00	58	7.5	2.00	38	16.0	4.00
	Dr.	02029	6	0	N/D	-	4	0.5	0.03	2	1.0	0.07

 NOTE: N/D = Not determined;   N/A = Not available;   Fr. = Fresh   Dr. = Dried;   Gr. = Ground

 a Source: (562)

 b Recipes were obtained from six miscellaneous South African recipe books published over time.(556-561)

 c Source: Food Quantities Manual of the National Research Programme for Nutritional Intervention, Medical Research Council (MRC), South Africa.(552)

Considering the higher frequency allocations that allowed for selecting the three flavour categories, 512 recipes were maintained and their portion amounts used for the allocated three categories of herb use. The volume information (in mL) obtained was converted to mass indications (in g) with

the assistance of the USDA nutrient database for standard reference, release 13.(562) Table 7.3 presents the different portion volumes and thus weights allocated to each herb in this food list. To determine the flavonoid contribution from herb use, guidance was included on their use in recipe formulations in the food list to assist the participants to complete the FFQ (Addendum C).

7.3.3.4 Pre-testing of Comprehensive FFQ

Joachim (5) indicated that the questionnaire is the tool used as format to collect the data that will be captured in the database. The questionnaire determines the data recorded, as it impacts the clarity and accuracy with which the dietary information is collected and then captured in the database.(5) It is vital that the performance of a new questionnaire regarding its length and format, be suitable for the administrators and subject group of interest. It must be pre-tested or piloted with a sample from this group to identify and rectify any problems with the content and format (422,563) with the pre-testing or piloting forming part of the FFQ development work.

The FFQ developed was evaluated for content- and face-related evidence of validity (peer reviewed by seven professionals knowledgeable on various aspects related to the FFQ) and pre-tested on adult volunteers (n=12) who had similar demographic characteristics to the trial participant group, but were not participants in the trial, to ensure face validity. The content validity of the FFQ, focusing in particular on the food and beverage inclusions in the food list, was evaluated by a senior researcher and a laboratory manager qualified in the field of biochemistry and involved in antioxidant research and familiar with dietary flavonoid sources, two food and food science lecturers, a qualified dietician and a food scientist, familiar with the dietary habits of South Africans. To guide the FFQ evaluation, a detailed explanation accompanied each of the questionnaire construction or format aspects. The developmental aspects that were addressed included the following: clarity of instructions; comprehensiveness and adequacy of food list; meanings of food names; clarity of food descriptions; portion size indications; method of reporting responses; ease of understanding and completion (capability of being self-administered) and completion time (relatively brief to be completed by most participants in 45 minutes).

In the pre-testing, two preliminary versions of the questionnaire for the vegetable category were provided to the panelists to address improvement of the format. The one version combined the vegetables as side dish/main ingredient (whole portion) and the vegetable as ingredient in mixed dishes, while in the other version the two vegetable categories were separated. The panelists indicated that separation of the two categories was preferred as it reduced bewilderment in the vegetable provision visualization when combined and for simplicity in completion, but that the separation of the vegetable categories must be highlighted to those completing the questionnaire. This indication was taken up in the instructions for completing the questionnaire. Pre-testing of the FFQ showed that the food list adequately covered the food and beverages consumed, as the

panelists could not think of other items missing from the food list and they had no difficulty to select their portion size. The set of visual sketches compiled on some fruit and vegetable portions were found useful and appropriate to complete the FFQ. The ticking of the items consumed in the food list of each category of the questionnaire and then answering the questions related to the portion and frequency with which these items were consumed, were considered very helpful. This completion procedure was followed to minimize fatigue and boredom in completing the FFQ because its food list was comprehensive.

Although the panelists did not consider it difficult to fill in the FFQ open frequency responses, misunderstanding did occur. For example, the question about the number of days per week or per two weeks in the past fortnight the food item was consumed, was misunderstood by some, as an answer was reported for both these column indications. Some participants neglected the indication 'one' if an item was consumed only once per day. The panelists suggested that, although the FFQ was to be self-administered, the provided instructions to complete the frequency response indications in the questionnaire should still be verbally explained to users to minimize misunderstanding. These indications, in addition to the separation of the vegetable categories, were the major outcomes of the questionnaire pre-testing. The compiled instructions to complete the FFQ were verbally explained to the participants in the rooibos intervention trial. Jones (563) in their pre-testing found that the production of a training package or instruction manual was needed to minimize misunderstanding amongst users. The panelists considered it easier to complete the questionnaire using a ruler due to the row print format per item of the food list, as the print format was not that large. This advice thus accompanied the completion of the FFQ in the rooibos intervention trial. It took the panelists on average about 30 minutes to complete the whole FFQ.

7.3.4   Multiple Estimated Dietary Records as Reference Method

In the intervention trial, multiple estimated dietary records were used to assess the mean daily total flavonoid intakes which were used as reference method to evaluate the intake estimates as obtained from the developed FFQ (test method) due to the proposed accuracy of dietary records to assess absolute dietary intake.(381) The primary use of dietary records is to provide a more valid and reproducible assessment of dietary intake,(564) since it measures the actual dietary consumption of a subject.(381,390) Because dietary intake, and in particular the intakes of most vitamins and trace elements, vary from day-to-day multiple dietary records were obtained to represent the best overall mean daily dietary intake (354) of flavonoids. FFQs are less sensitive than are dietary records or recalls to capture subtle changes in dietary intake that would lead to an underestimation of the adoption of the intervention trial dietary instruction and overestimation of the maintenance.(525)

Dietary records are suitable to assess change in dietary intake (275,381) and dietary intervention compliance (273,275) and could be used in this trial to monitor the dietary flavonoid intake for changes in intake during the trial periods along with changes in the selected biomarkers of exposure and of effect. Compliance to the dietary restrictions and rooibos consumption was monitored through the reported food and beverage intakes in the estimated dietary records.(37) These records served to estimate the mean daily energy intake (as evidence of body weight maintenance), macronutrient intakes for determining the fat energy contributions (as evidence of blood lipoprotein and cholesterol profile maintenance),(565) antioxidant intakes (vitamin C, vitamin E, ß-carotene and selenium as evidence of a stable exogenous antioxidant provision besides for the dietary flavonoid provision) and folic acid intake (as evidence of a stable supply to support the homocysteine metabolism as an increased total plasma homocysteine concentration had been associated with an increased CHD risk and folate intake one of its most important determinants) (565,566) of the participants throughout the trial periods, as further measure that no other major change in dietary intake occurred besides for variation in the mean daily total flavonoid intake throughout the trial.

The participants completed both the reference and test measures during all periods of the intervention trial as was indicated in Table 7.1. Dietary records and FFQs are both commonly used in experimental and observational studies as methods to assess dietary intake (536) and multiple dietary records as reference method, since it is generally considered the optimal method of dietary assessment when the aim is to assess usual nutrient intake for individuals, provided a number of recording days are incorporated and the time period covered is long enough.(381,422) The dietary record was furthermore chosen as the reference or comparison evaluation method, since its errors are largely independent to those associated with the FFQ.(387,390) Errors with FFQs are the central role of memory and the perception of consumption frequency and portion sizes.(387) In dietary records the role of memory is limited because recordings are carried out at the time when eating and drinking occur.(84,381) The main limitation presented by the dietary record method is the potential of subjects to alter their eating behaviour as a result of the recording process.(381,422) Multiple dietary records and the developed FFQ were consequently used as the measures to assess the mean daily total flavonoid intake in the intervention trial.

Estimated and not weighed dietary records were chosen as the dietary intake methodology and as reference method for the developed FFQ evaluation. Mass indications were not considered, as South Africans are accustomed to recipe units provided by measure rather than by weight. Weighed dietary records may work well in countries where subjects are accustomed to recipe units by weight, rather than by measure.(354)

Variability in dietary intake determines the length of time over which dietary information needs to be obtained in order to get a meaningful assessment of intake.(564) In the design of a validation study, the aspect whether it is better to obtain many dietary records from a moderate number of subjects or a small number of dietary records from a larger number of subjects, need to be considered. Many researchers choose the first option so that they can characterize the usual intake for each subject as accurately as possible.(377) Short periods of two to three days of dietary recording are sufficient because if continued over an extended period, subjects become less interested and counter-productive.(564) Three days of estimated dietary records were completed each week of the two weeks for the baseline and washout periods of the trial, and each of the two weeks at the end of the intervention and control periods providing six days of dietary records for each trial period. These records were completed for three consecutive days comprising two week days and one weekend day (see Table 7.1). The maximum of three consecutive days for the recording periods was aimed at reducing boredom, fatigue and omissions and, thus, increasing the accuracy of record keeping.(422) Each three-day week of dietary records included different chosen days (Sunday/Monday/Tuesday followed by Thursday/Friday/Saturday) to account for day-to-day variability in intake (386) and to ensure that a more representative sampling of days of the week,(470,484) along with an appropriate ratio of week- to weekend days (41,80,83,358,383,386,400,408) occurred during the period of dietary monitoring.

Due to their short elimination half-lives (approximately 1.5 to 5.5 hours) some flavonoids have no chance to accumulate in the plasma.(151) This indicates that to accumulate and uphold a high concentration in the plasma, repeated consumption over time (8) on a daily basis (100) is required. In other words, high plasma metabolite concentrations could be reached and maintained, but with regular and frequent consumption of dietary plant sources.(100) It was assumed that the concentrations of individual flavonoids and their biologically active conjugates/metabolites on occasional intake may not be high enough to explain the low mortality rates from CVD in Mediterranean countries, but that accumulation may occur on regular intakes which may result in a more prominent formation of flavonoid metabolites, which would probably result in greater flavonoid concentrations and biological activity to be functional.(200)

This is supported by studies conducted by Manach et al.(97,124) In an experimental study of Manach et al. (97) conjugated metabolites were recovered in the plasma of a group of rats adapted to a daily quercetin or rutin intake and by decreasing the daily quercetin or rutin amounts fed, the levels of conjugated metabolites in the plasma reduced significantly. The results of their study indicated that the conjugated compounds formed in the adapted group were sensitive to a reduction in flavonoid intake. In further work of Manach et al. (124) with ten healthy volunteers, a marked increase occurred in the plasma concentration of conjugated derivatives of quercetin after

consumption of a meal rich in plant products. However, the plasma concentrations returned to quite low basal levels after 20 hours without new quercetin ingestion. These results implied that the beneficial effects of flavonols depend on regular consumption of rich dietary sources. Radtke et al. (338) furthermore estimated the intake of several flavonoids from one- or seven-day dietary records from 48 female students. As expected from the short elimination half-life of some plasma flavonoids, Radtke et al. (338) found markedly higher correlation coefficients for the relationship between the intake estimates for the last day before blood sampling and the fasting plasma concentrations than for the mean of the seven-day dietary record intakes (0.42, 0.64, 0.47 which dropped to 0.30, 0.32, 0.35 for quercetin, hesperitin and naringenin, respectively).

Although recording of dietary consumption for more than one or two consecutive days is a well-known behaviour modification method to reduce dietary intake,(354) consecutive recording days were used in the intervention trial due to the rapid metabolism and plasma elimination of polyphenolic compounds (100,151) that necessitate polyphenolic consumption from plant products on a daily basis to maintain high metabolite concentrations in the blood.(100) Trial participants were asked to consume six cups of rooibos herbal tea daily for the 6-week duration of the intervention period and to daily restrict or avoid the consumption of a number of flavonoid-rich dietary sources for the 12-week duration of the trial that followed the run-in period. This made biochemically and nutritionally more sense to request the participants to record their dietary intake over a consecutive period than selecting a number of random days to do so.

Fasting blood sample collections on completion of a study period would reflect polyphenolic dietary intake of the day before to possibly usual short-term dietary intake.(338) Collection of these samples to determine flavonoid exposure and effect immediately after the last days of a trial period with the recording days scheduled consecutively within this time frame, will reflect the time window of effect. Despite the marked difference in the correlation coefficients found by Radtke et al. (338) between the one-day or the seven-day dietary records and the fasting plasma values, these investigators considered the difference in the correlation coefficients moderate and concluded that single fasting plasma values not only reflect dietary intake on the day before blood sampling, but is also a measure for intake estimates over one week.

7.3.5 Completion of the Dietary Assessment Methods

The participants were informed about the completion of the FFQ and the estimated dietary records during each of the trial periods at the intervention outset as part of the informed consent procedure. The participants themselves completed the FFQs during each subsequent visit to the institution at the end of each trial period and the dietary records at home to the end of each trial period. At each visit, the participants completed the FFQ after which it was checked, the completed dietary records

were collected and reviewed, and the dietary record booklets provided with the set dates for completion for the next period of the trial. Based on the design of the intervention trial, the participants were asked to fill out the FFQ at the end of each of the four trial periods after they had completed the dietary records for that trial period to account for the timing of the assessment relative to the intervention. Because the responses to the FFQs had to be concordant with the period during which the multiple dietary records were collected, the reference method was administered prior to the test method. A pattern of completing the dietary record followed by the FFQ was pursued over the whole duration of the trial. These conditions were favourable for a more accurate completion of the FFQs due to the recent dietary recording involved that may provide for recall bias and a learning effect due to the repeated occasions of the dietary record and FFQ administrations.

7.3.5.1 Comprehensive FFQ

Directions on completing the FFQ included printed instructions on the FFQ, which was accompanied by a step-by-step completion example (Addendum C), and oral guidance on the completion. The participants were personally instructed by the researcher in an approximate 10-minute group training session on the procedure to complete the questionnaire based on the printed directions on the FFQ at the end of the baseline/start of the washout trial period. The FFQs were completed during group sessions in the mornings when the blood samples were drawn at the end of each trial period at the institutional venues under guidance of the researcher. Two Consumer Science: Food and Nutrition graduate students who attended a two-day training session on dietary recording and analysis assisted the researcher. The research staff answered questions regarding any FFQ completion uncertainties. The participants could take as much time as needed to complete the seven-page questionnaire.

Participants were instructed to complete the questionnaire by reporting what they had consumed during the past two weeks of each trial period. Participants were required to tick those items in the food list of each of the 14 categories and then mark the portion size (small, medium or large) that corresponded best to their consumption of the items. The portion size consumed of some fruits and vegetables could be obtained from the appendix illustrating the visual portion size sketches of these fruits and vegetables. A written response was required for only those open-ended questions referring to frequency of consumption over the two-week reference period of possible items consumed, but not included in each category food list. Items not consumed in the past two weeks were not marked. All questionnaires were checked after completion for completeness (unanswered questions) and improper responses (unclear, doubtful or unusual, for example frequency range) by the researcher and the two trained graduate students. Where necessary these reviewers edited the response information. The comprehensive FFQ took on average 30 minutes to complete.

7.3.5.2 Estimated Dietary Records

Participants were comprehensively instructed by the researcher at the onset of the intervention trial in a 30- to 45-minute group training session on the procedure for completing the estimated dietary records in which they were provided time to ask questions. They recorded detailed descriptions on the type of food/beverage item consumed per meal and in-between meals and, if applicable, item brand names, including method of food/beverage preparation, all additions to foods/beverages and the portion size of each of the food/beverage items consumed. Subjects were asked to record all food and beverage items at the time of consumption. Emphasis was placed on the need to record all items consumed throughout the day (88) at the time of eating and drinking and not from memory at the end of the day.(422) For completion of the dietary records, the participants were not required to weigh foods but were asked to estimate/measure the volume of the food and beverages that they consumed to help assess their portion sizes. Portion sizes were described in household measures. A household measuring jug (250 mL capacity, 25 mL graduations) and a set of measuring spoons (1, 2, 5 and 10 mL) and a ruler, for measurement of food dimensions were provided to each participant. The appendix illustrating visual portion size sketches of some fruits and vegetables was also supplied to facilitate quantifying the portion sizes consumed.

The consumption of mixed dishes of several ingredients was broken down into their component ingredients and the relative proportion amounts of each ingredient consumed provided in the dietary records. In addition, participants could provide recipes from which the appropriate amount of each component ingredient was calculated as would routinely be done in analyzing complex dishes per their consumed portion indication. This facilitated the estimation of the vegetable intakes in casseroles, soups, salads, etc. The dietary record is more accurate when participants are trained by dieticians in how to record intakes and estimate intake quantity.(354) Each participant received a printed dietary record instruction booklet providing all the instructions discussed in the training session to facilitate the recording during the intervention trial. The participants were encouraged to study the printed instructions in the dietary record instruction booklet. As in numerous other studies,(71,88,405,482) the participants were repeatedly reminded to continue to eat as usual, to record their normal dietary intake, to avoid any temptations to change their diets in order to simplify the recording, and that they were not expected to change or "improve" their diets as this would impact the purpose of the intervention trial.

The estimated dietary record was open-ended (unstructured) so that participants could record the required dietary information at the time of consumption. The dietary record template in the format of blank diary forms, but each containing four columns labeled "Time", "What did you eat/drink?", "How was it prepared?" and "How much did you eat/drink?" and row lines for ease of completion, was bound in a booklet (that included two pages for each day with additional pages if required). An

instruction booklet (where the procedure for recording the intake of one day was demonstrated as an example) was provided, which was obtained from the Nutrition Intervention Research Unit, MRC, SA with permission from a Senior Specialist Scientist in the Unit. The researchers had used the dietary record successfully in studies requiring adult dietary intake data. A two-day training session provided by the specialist scientist of the MRC was attended in order to train the project team that included four Consumer Science: Food and Nutrition graduates on how to accurately complete dietary records, as well as how to check and analyze dietary records effectively. Each team member had to keep her own estimated dietary records for a period of three consecutive days, which was then practically used and discussed as part of the training session.

Each participant kept estimated dietary records for a total of six days (twice for three consecutive days) over each of the two weeks of dietary monitoring for each trial period. To ensure that the dietary records were completed properly, the researchers carefully reviewed each record, when the records were handed in, for completeness of description of the foods and beverages consumed, as well as the amounts consumed. If any information was incomplete, the participants were asked to provide further detail and to resolve discrepancies where necessary. Any dietary record information found incomplete (items consumed away from home not recorded as was found with beverage intake like red wine consumption with the evening meal) was edited by the research staff. Participants were contacted telephonically during the capturing of the dietary record data to provide further detail for clarification, if found necessary.

7.3.6   Data Treatment and Daily Total Flavonoid Intake Determination

Each food and beverage item in the food list of the FFQ was pre-coded using the NDB number of the best matched item in the USDA flavonoid database (161) and independently quantified in weight (g) in accordance with the three portion size indications (small, medium and large) prior to its use for incorporation in the specially designed standard Microsoft (MS) Excel template for capturing the FFQ data. The portion size weight estimates used in these calculations were primarily obtained from the Food Quantities Manual, MRC, SA.(552) All items in the FFQ food list included just one food or beverage. This meant that no weighted average of flavonoid values for aggregated items needed to be calculated based on consumption amounts of these individual foods. The questionnaire data was entered and checked by two trained graduate students in the FFQ data file using the standard template designed.

After each trial period when the estimated dietary records were collected and reviewed, the participant daily energy, macro- and micronutrient intake analyses were computed first from the dietary records using the FoodFinder Dietary Analysis Program. This is a dietary analysis software system developed by the MRC, SA based on nutrient values obtained from international tables and

local foods and beverages.(541) The data entering for this software analysis was completed by an experienced dietician to minimize variability in the data interpretation across the intervention trial periods. All food and beverage items recorded in the records and the quantity eaten were directly selected in the software program data file. The energy, macro- and micronutrient intake analyses was deemed necessary to screen whether the participants were following their usual diet over the intervention trial periods and to verify the dietary flavonoid intake as major dietary determinant on the trial biochemical results.

The weight quantification of the portion size indications of each food or beverage recorded was derived from this software program for computing the flavonoid intake analysis from the estimated dietary records. Exact recipes of mixed dishes and home-prepared items were recorded so that precise vegetable, herb and fruit ingredients and portion sizes could be computed. For each participant, the plant food items consumed in each dietary record were matched with the most appropriate item in the coupled USDA flavonoid database.(161) The quantity of that item consumed (as derived from the nutrient analyses) was entered per dietary record for each trial period over the entire trial duration in the dietary record data file using a further standard template designed based on that of the FFQ. To minimize variability in interpretation and ensure quality of the data and that discrepancies and errors were corrected in the flavonoid intake analysis, each graduate student independently captured 50% of the FFQs and dietary records for the flavonoid intake analysis and then reviewed each other's data captured.

A computer software program using MS Excel was written for analyzing the participant FFQ and estimated dietary record food and beverage consumption data in terms of the mean daily total flavonoid intake per trial period. To translate the food and beverage consumption into total flavonoid intake all food and beverage items, included in the FFQ food list, were coupled via the USDA NDB numbers to the best-matched items in the USDA flavonoid database (161) for each through the specially designed FFQ template. The USDA database for the flavonoid content of selected foods, release 2.1 (161) was used to compute the dietary record and comprehensive FFQ dietary flavonoid intake, as no such database exists for the flavonoid contents of South African foods and beverages. Johannot and Somerset (89) used this approach in the Australian study in the absence of adequate local data for a comprehensive dataset. Although the applicability of the USDA flavonoid database is a limitation in this research, the database is a generally sound and reliable source.(136) The USDA flavonoid database presents the content of the most profuse individual flavonoid compounds in 234 selected food and beverage items.(161) The USDA flavonoid database was transferred to Microsoft Excel spreadsheets in order to calculate the daily total flavonoid intakes of the participants based on their completed FFQs and dietary records electronically through linkage to the designed data analysis templates.

Total flavonoid intake was calculated by means of the program in four parts from the FFQ by firstly multiplying the frequency of the food and beverage consumption over 14 days by the selected portion weights to obtain the total weight of each food and beverage consumed, which were then used to calculate the weight of each item consumed per day. These consumed daily food and beverage weights were converted to flavonoid intakes based on the food and beverage content per 100 g and summed to obtain an average intake per day using the appropriate food and beverage NDB number. The developed software template food list of the FFQ was used for the estimated dietary record template.

To generate total flavonoid intake estimates from a dietary record, the portion weight of each of the consumed foods and beverages were entered in the template and the daily flavonoid intake calculated in three parts using its program. The flavonoid content of each item selected in the food list was first calculated based on the amount consumed from the flavonoid food or beverage content per 100 g, which was then summed across all the items to obtain a total daily intake. Thereafter the participant daily flavonoid intake from the dietary records was calculated as the mean of the six recording days using equal weighting for each of the six days. The daily total flavonoid intakes for the FFQ and the dietary records represent the summation of the total intakes of all the individual flavonoids across the five major flavonoid subclasses.

The participant total daily flavonoid intake analyses from the estimated dietary records and the comprehensive FFQ were computed using the USDA flavonoid database (161) with a small number of additional foods added to the database using published content information (content of fresh guava (131) added). In addition some theoretical food content determinations were added considering processing and/or preparation methods, which expanded the database. In their use of the USDA flavonoid database, Chun et al. (105) expanded the database through considering those foods, which differed in processing and/or preparation to that included in the database. Pre-coding of these foods occurred by adding a yield indication to the USDA NDB number for incorporation in the comprehensive FFQ and for the analysis of the data. The content of rooibos herbal tea (institutionally analyzed) was added, with rooibos included in the food list of the comprehensive FFQ (Category M: Other beverages of the FFQ).

The food and beverage items in the comprehensive FFQ template and the estimated dietary records were matched to the best-related USDA NDB number and the flavonoid content of the matching USDA food or beverage assigned to the FFQ and dietary record item. In matching the food and beverage consumption items to the USDA flavonoid database there were some instances where the data were available, but the food form or preparation method did not match, as was

encountered by Mink et al.(248) The following aspects were, hence, considered regarding the food and beverage inclusions, which required application steps in the developed software analyses program templates. Foods or beverages consumed raw excludes cooking so that all the flavonoids present in these foods would be ingested.(87)

Flavonoids are predominantly found in the leaves and the outer part of plants (162) and are relatively stable compounds, being resistant to heat and oxygen.(10) Pre-preparation and processing methods will to some extent cause a reduction in the original flavonoid content.(10,87) Therefore, food that is home prepared or commercially processed may show a loss in the original flavonoid content (87) owing mostly to the removal of portions of the plant and leaching into water.(10) Because flavonoids are heat stable, losses due to such thermal processes as cooking and frying are low (111) with sizeable amounts of flavonoids still recovered after cooking.(166) As a preliminary study found flavonoids to be heat stable, with losses less than 20%, Hertog et al. (111) did not correct for loss of flavonoids due to preparation in their study. Based on the observation that flavonoid losses due to preparation were negligible, correction factors of flavonoid levels were not applied by Vasilipoulou et al. (137) when calculating the daily average flavonoid intake from a traditional Greek plant-based weekly menu. However, Peterson and Dwyer (10) indicated that the content might be reduced on extensive processing by up to 50% in plants rich in flavonoids.

The USDA flavonoid database provides the food forms as fresh/raw or cooked.(161) The line food vegetable items listed in the FFQ were indicated as fresh/raw or cooked that included all the cooking methods (boiling, steaming, frying, baking or roasting). To minimize this further limitation that the large variation in food preparation and processing methods were not fully accounted for in the flavonoid intake calculation analyses, use of weight yield adjustments associated with cooking was applied to the flavonoid content values, where items were consumed cooked and only the raw food contents were provided in the database. Cade et al. (18) stated that data on weight losses associated with cooking (water evaporation) should be recorded to ensure accurate nutrient density of the portion size consumed. Sampson et al.,(153) due to the losses of quercetin in cooking, frying and other forms of processing being small, set the quercetin levels in cooked foods equal to those in raw foods, but accounting for weight changes due to processing. Mink et al. (248) made adjustments for weight changes as a result of processing from available flavonoid concentration data for similar foods of this research, without consideration of the retention factor method for cooked foods. Although there are actual retention factors available for most nutrients by cooking of specific foods and dishes, there is no such data available for other food constituents for which Bognár,(567) as a preliminary recommendation, suggested using a retention factor of 1.0.

Food yields,(568) hence, had to be applied to a number of raw vegetables and legumes in the USDA flavonoid database consumed in a cooked form to adapt the flavonoid content of the raw items. The raw vegetable yields considered on average when boiled, steamed and pressure-cooked were as follows: carrots (92%), cauliflower (97%), eggplant (94%), peppers (green, red, yellow) (96%), potatoes (98%), pumpkin/butternut (92%), sweet potato (100%) and onions (85%). The canned food yield (67%) was used for beetroot for which only the raw food flavonoid content was provided. The flavonoid content of some canned and dried fruits and dried herbs are not included in the USDA flavonoid database.(161)

To obtain flavonoid content values for these items, the flavonoid content for the canned and dried foods were adjusted on the basis of the moisture content as applied by other investigators.(105,248,342,569) The water content of some fresh and canned/dried fruits and some fresh and dried herbs, were used as a guide to obtain the flavonoid content values based on the flavonoid content of the fresh/raw fruits and fresh herbs respectively, to create further item choices in the database. The water content as indicated in the USDA NDB (570) for fresh and dried/canned fruit (Table 7.4) and fresh/dried herbs (Table 7.5) was used for these ratio calculations.

Table 7.4: Water Content of Raw and Dried or Canned Fruit for Flavonoid Content Calculations[a]

Fruit	NDB number for raw fruit	Raw fruit water content (g/100 g)	NDB number for dried fruit	Dried fruit water content (g/100 g)
Apple	09004	86.67	09011	31.76
Apricot	09021	86.35	09032	30.89
Peach	09236	88.87	09246	31.80
Pear	09252	83.71	09259	26.69
Fig	09089	79.11	09094	30.05
Mango	09176	81.71	–	30.24[b]
Fruit	NDB number for raw fruit	Raw fruit water content (g/100 g)	NDB number for canned fruit	Canned fruit water content (g/100 g)
Apple	09004	86.67	09007	82.36
Guava	09139	80.80/86.10[c]	–	77.60[c]
Pineapple	09266	86.46	09354	83.51
Pear	09252	83.71	09258	80.35

[a] Source: National Nutrient Database (NDB) for Standard Reference (570)
[b] Average of other dried fruits
[c] Source: Food Finder[TM]3, 2002 (541)

Table 7.5: **Water Content of Fresh and Dried Herbs for Flavonoid Content Calculation of Some Dried Herbs** [a]

Herb	NDB number for fresh herb	Fresh herb water content (g/100 g)	NDB number for dried herb	Dried herb water content (g/100 g)
Basil	02044	90.96	02003	6.43
Mint	02064	85.55	02066	11.3
Rosemary	02063	67.77	02036	9.31
Thyme	02049	65.11	02042	7.79

[a] Source: National Nutrient Database (NDB) for Standard Reference (570)

The flavonoid content of a dried fruit roll was entered as 50% dried apricot and 50% dried peach. The flavonoid content of sun-dried tomato had to be calculated using the water content of raw/fresh and dried tomato. The water content of the raw tomato (NDB number: 11529) was 94.5 g and the sun dried tomato (NDB number: 11955) 14.56 g (per 100 g), as obtained from the USDA NDB.(570)

The flavonoid content in the USDA flavonoid database for legumes, such as lentils, chickpeas and black beans, are indicated for the raw and not the cooked product.(161) Their food yield amounts (289%, 207% and 238%, respectively) were used to convert the cooked amounts consumed to the raw amounts.(568) The flavonoid content of raw green peas (NDB number: 11304) and its water content (78.86 g/100g) and that for raw mature seeds (11.27 g/100g; NDB number: 16085) were used as a ratio to determine the flavonoid content per 100 g for split peas, raw. The food yield of 247% was used to convert the cooked amount of split peas consumed to the raw amount (568) and the flavonoid content calculated based on the water content calculation as only the flavonoid content of raw green peas are included in the database. Beans, canned in tomato sauce was entered as 68% broadbeans, canned (NDB number: 16054) and 32% tomato products, canned, puree (NDB number: 11547) based on the food yield of canned beans (drained solids as 68% and drained liquid as 32%).(568)

Some of the beverages consumed by South Africans are not included in the USDA flavonoid database. The flavonoid content for a similar beverage item, from the same botanical group, was used as proxy if no data was available, as was applied by Pellegrini and co-workers (569) in assigning a TAC to foods in their study, which had not been analyzed. Proxy values utilized were for instant, pure and blended coffee entered as filter/brewed coffee and light beer entered as regular beer. For these beverages the following codes in the USDA flavonoid database were used: instant coffee and pure coffee, blended was entered as filter/brewed coffee (NDB number: 14209);

rooibos ice tea was entered as rooibos herbal tea using its analyzed flavonoid content; and beer, light was entered as beer, regular (NDB number: 14003). It appears that hot tea and brewed ice tea (cooling traditionally brewed tea) at comparable leaf concentrations have very similar flavonoid concentrations in the infusions.(571)

Chutney and chili sauce in the condiments and sauces group (Category H), was eliminated from the data analysis as the flavonoid contents for these two items could not be obtained. Spices, although they may contain low amounts of flavonoids, were not included in the analysis, as the flavonoid contents for a number of spices are not reported in the USDA flavonoid database. Only peppers, hot chili, green/red (NDB number: 11670) were retained in the analysis because of the reported content in the flavonoid database. As a few chocolate confectionary intakes were recorded in the estimated dietary records, an additional data space had to be added for cocoa powder (NDB number: 14192) as it was not included in the original template. Cocoa drinks were included (in the other beverages Category (M)). For instance, in the case of chocolate cake, a cocoa consumption of 3 g was entered per portion/slice (572) in the dietary record template.

7.3.7 Application of Quality Assurance Procedures in Intervention Trial Dietary Data Collection

A number of aspects, as advocated by Friedenreich et al.,(415) were considered and addressed with the intention of improving the quality of and reducing sources of random error in the determined total daily flavonoid intake data obtained from the FFQ and the dietary records. These included aspects like the careful consideration of the questionnaire design and pre-testing (415) and checking and reviewing of the participant completion of the FFQ and dietary records, respectively for missing data or any ambiguity in responses and then editing of errors found.(72,78,403,418,571) Participant motivation and commitment to the study, along with honest responses were considered, as Friedenreich et al. (415) indicated that these features may potentially lessen error. The participants were constantly reminded of the importance of the trial and the resultant need for an accurate assessment of their dietary intake. Participants were informed that recommendations on flavonoid intakes have not been established (127) and that the research staff could therefore not credibly comment on the calculated daily total flavonoid intakes. This information was put forward to achieve a further feature proposed by Friedenreich et al. (415) of creating a non-judgemental atmosphere for the dietary intake reporting and to minimize biased reporting of intake in a socially desirable direction, as the major scope of the FFQ food list included fruits and vegetables.

As a further measure of quality control, participants were asked to report on cross-check questions regarding the general consumption of certain dietary items as an additional source of information, as suggested by Friedenreich et al.(415) Participants were asked three summary questions

pertaining to the weekly serving consumption of fruit, vegetables and tea to permit the dietary analysis to use a less-inflated frequency of specifically total fruit and total vegetable intake as advocated by Block et al.(402) Applying the adjusted frequency obtained from summary questions on the total fruit and vegetable intake had been found to reduce the overestimated intake of several nutrients, such as vitamins A and C and fibre, linked to the completion of extensive lists of fruits and vegetables as frequently found in FFQs.(402)

The participants were asked to include potatoes in their estimate of vegetable servings per week (with one-half cup raw or cooked vegetables and 1 cup raw leafy vegetables as 1 serving) and include fruit juices in their estimate of fruit servings per week, but exclude the intake of dried and canned fruits (with 1 medium-sized fruit and one-half cup cut-up fruit or juice as 1 serving). These summary questions were drafted, based on the standpoint of Amanatidis et al. (384) that consideration should be given to what subjects might think of when answering such summary questions as they, for instance, might only think of fresh fruit when answering the summary question on the total fruit serving intake. The participants were asked to consider all types of tea (black and herbal) consumed in their estimate of their weekly consumption (with a cup or small mug as 1 serving). In the FFQ vegetable, fruit and tea servings were quantified by summing the frequency of consumption across all vegetable items (Category E, excluding composite dish inclusions as Category F), fruit items (categories A and B, excluding dried and canned fruits as categories C and D) and teas (Category M) at the portion sizes specified on the FFQ. Intake frequency was computed as total fruit, vegetable and tea servings per week. As the medium portion indications in the FFQ corresponded with the serving size indication provided for the weekly serving intake summations, its weekly calculated reported intake could be judged against that of the weekly summation and the determined ratio used as factor to adjust the mean daily total flavonoid intakes as calculated from the FFQs for each of these categories.

The often-used criteria to exclude persons based on the number of omitted items and reported implausible energy intakes outside set ranges (232,248,250,254,417,423,424,529,531) in their FFQs, could not be applied in this study. In the case of omitted items in the FFQ, the researchers reviewed the completed FFQs to obtain missing data. The scope of the FFQ did not allow for quantifying total energy intake. Consideration was therefore given to the exclusion of participants from further analysis, who had relatively extreme reported energy intakes (531) on their dietary records, because this would suggest that the dietary intake recording was not completed in a reliable manner.(432) The reported implausible energy intakes were considered as a daily energy intake less than 600 kcal (2510 kJ) or more than 3500 kcal (14.6 MJ) for females [as applied by Sampson et al.,(153) Sesso et al. (232) and Thompson et al. (417)] and less than 800 kcal (3347

kJ) or more than 4200 kcal (17.6 MJ) for males [as applied by Sampson et al.,(153) Rimm et al. (254) and Thompson et al. (417)] on their dietary records.

As applied by Riboli et al.,(435) the dietary data analyses were first analyzed for quality control in particular for distributions and outliers of absolute values. Outliers were checked by returning to the FFQs or estimated dietary records. Corrections were made when appropriate in the data capturing, such as a participant who mistakenly indicated consuming red wine (a rich source of flavonoids) instead of white wine in the FFQ, as the two items are listed one below the other in food list. Errors attributed to data input or management was corrected when detected.

7.3.8   Evaluation of the Comprehensive FFQ

The usefulness of a measure is usually determined in terms of its validity and reliability.(347,563) However, if an intervention impact or ability to detect change is to be assessed, the measure responsiveness (or sensitivity) becomes of value.(459) All four trial periods were used to assess the validity and responsiveness of the developed comprehensive FFQ as measure of the mean daily total flavonoid intake.   The validity performance evaluations were cross-sectional comparisons between the mean daily total flavonoid intake estimates of the comprehensive FFQ against that of the estimated dietary records and selected biomarkers of exposure and of effect as the reference methods.   The responsiveness evaluation was a longitudinal comparison to determine the ability of the comprehensive FFQ to assess group changes in daily total flavonoid intake over the trial periods.   The reproducibility of the comprehensive FFQ was ascertained across the washout and control trial periods, as the intervention guidelines were comparable in these two experimental periods, namely the usual diet incorporating flavonoid beverage and minimal flavonoid food source intake restrictions.

MS Excel was used to capture and calculate the participant daily total flavonoid intakes and the MedCalc Software, Belgium (MedCalc version 9.4.2.0) was utilized to perform the statistical analyses. Participant-specific daily total flavonoid intakes from the six estimated dietary records were averaged and the mean daily total intake over the past two weeks computed from the FFQ to determine the overall participant mean daily intake estimate from each method per trial period. Summary statistics as means, medians, SDs and ranges were computed independently for the mean daily total flavonoid intakes determined from the participant questionnaires and their dietary records preceding the evaluative comparisons of these dietary intake measures for determining mean daily total flavonoid intake. Similar to most other FFQ evaluations, the usual statistical procedures of assessing FFQ validity and reproducibility against a reference method were applied to allow for interpretation of the findings. The responsiveness was also assessed as part of the evaluation due to the use of the FFQ within the context of an intervention trial. The statistical

procedures were executed after the calculated mean daily total flavonoid intakes were tested for normality and if not normally distributed the appropriate non-parametric statistical methods were used for the analyses. Statistical significance was accepted at p<0.05 in the hypothesis testing.

7.3.8.1 *Dietary Intake Reference Method Validation*

Validation of the FFQ method is important as it evaluates the degree to which the questionnaire measures items (foods or nutrients), which it was developed to assess.(17) Since no 'gold standard' is available for dietary intake assessment and criterion validity cannot be evaluated,(91) relative validity was evaluated by comparing the FFQ dataset with the estimated dietary record dataset as reference method. Many validation studies have compared the results of one dietary method with another dietary method, presumed more accurate, of the same subjects.(502) Dietary records, which provide more precise quantification of foods consumed, is usually relatively better for assessing absolute intake.(381) The validity of the FFQ was determined by a two-way comparison. On the one hand the comparison is between the FFQ and multiple dietary records (three consecutive day dietary records over two adjoining weeks) of the participants and on the other hand with their biochemical (plasma) flavonoid measurements of exposure and effect (only evaluated during the baseline trial period). Most FFQs are validated against another dietary assessment method, while some are validated against one or more biomarkers.(17)

The ability of the comprehensive FFQ to properly characterize the dietary flavonoid intake was judged by two major functions, namely, its ability to assess the mean absolute daily total intake and the ability to rank the participants according to their mean daily total intake. These aspects were considered important in its evaluation, as it could accordingly be judged for use in intervention research, for nutritional surveillance and for consideration in epidemiological research. Intervention research and nutritional surveillance depend on accurate estimates of the distributions of true dietary intake, especially the central tendencies (mean or median intakes) via the dietary assessment method.(418) Central tendencies and distributions of dietary intake are important in the provision of quantitative recommendations for dietary intake in public health.(373) Such complete quantification of as much as possible of the dietary nutrient of interest may not be required in epidemiological studies.(29) Most epidemiological studies, investigating dietary intake and chronic disease aetiology, require dietary information, which allows differentiating or ranking the subjects within the study group with regard to their intake of the nutrient of interest.(29,373,406) Characterization that centers on the correlation coefficient (418) and/or grouping of subjects into three to five levels of dietary intake,(406) may be all that is necessary. The ability to assess absolute mean nutrient intake and the intake distribution to rank subjects are two important functions of FFQs often considered and evaluated.(355,369) As it is useful to know the variation and error of dietary intake,(573) this was attended to via the above and further statistical analyses. In the framework of present flavonoid research, a measure with the ability to

rank subjects by flavonoid consumption is needed to study the relation between dietary flavonoids and chronic disease (74) in addition to a measure with the ability to assess absolute and relative intakes for use in further flavonoid intervention trials.

Several statistical methods pertaining to the group level and the individual level mean daily total flavonoid intake estimates were used to compare the dataset of the FFQs to that from the estimated dietary records used as reference, namely:

(a) *At group level*: Group level data are suited to compare mean nutrient intakes.(392) The absolute mean daily total flavonoid intake estimates at group level were computed from both the dietary records and the FFQs. To test if the daily group level intake estimates were statistically significantly ($p<0.05$) different between the two assessment methods, the group level intake estimate of the FFQs was compared to that of the dietary records as first measure of the relative validity evaluation. The mean and SD of the FFQ was compared with that of the dietary records as reference method using the paired samples *t*-test for normally distributed trial period data. The median and 25th and 75th percentiles were computed for each method and the non-parametric Wilcoxon's signed rank test performed where trial period data was not normally distributed.

By comparing the mean absolute intake estimates of both assessment methods and estimating the difference in the means of the two methods, the existence of systematic bias was determined. Overestimation was calculated as the mean difference in flavonoid intake between the assessment methods and the relative difference (%) with the dietary record mean intake estimate as the reference. As people eat various plant foods from various environmental surroundings, the mean flavonoid intake was used as it is thought the most apt as intake estimate.(248)

(b) *At individual level*: To evaluate the individual level of agreement between the participant mean daily total flavonoid intakes, correlation analysis was firstly performed as relative validity measure to assess the linear and rank order agreement between the intakes derived from the two assessment methods. The Pearson product-moment correlation coefficient (unadjusted) was used to assess the ability of the FFQ to rank the participants equally to their dietary records.

Before calculating the Pearson correlation coefficient, the distribution of the flavonoid intake in each trial period was checked and where the scores did not suffice characteristics of a normal distribution, correlation coefficients between the FFQ and dietary record intakes were assessed by applying a logarithmic transformation to improve normality. The adjusted Pearson correlation coefficient along with the Spearman's rank order correlation coefficient was reported. Although Pearson correlation coefficients measure the strength of linear

association between two variables not the agreement between them,(498) they were calculated (or on log transformed values) to allow comparison with other dietary intake validation studies.

The correlation coefficients to compare individual intakes between the two methods were supplemented with cross-classification of the participants into tertiles and quintiles of mean daily total flavonoid intake and compiling Bland-Altman plots to assess agreement between the two assessment methods. The flavonoid intake distributions of both assessment methods were ranked and then divided into three and additionally five equal parts. These tertile and quintile cut-off points were determined separately for the FFQ and the dietary records. The number and percentage of the participants categorized correctly within each part by both the assessment methods were tabulated. The *Kw* statistic was calculated to correct for chance agreement in order to assess if the participants were categorized similarly (classified in the same or adjacent part) by the two different assessment methods and not grossly misclassified (disagreement between the two assessments beyond an adjacent part).

To further assess the agreement between the data derived from the FFQ and the dietary records, Bland-Altman plots were constructed by plotting the differences between the two assessment methods for each participant against the mean of the two assessment methods for each participant and the 95% limits of agreement identified.(498) As recommended by Cade et al.,(17) correlation coefficients in conjunction with the Bland-Altman method were used to measure the agreement between the FFQ and the dietary records, which was supplemented by the categorization of the participants in tertiles and quintiles.

Due to the error introduced by within-person variability in dietary intake, linear associations of dietary intake assessments may underestimate the underlying association.(400) Day-to-day variation in intake plays a minor role in FFQs, which usually estimate dietary intake over a period of time, i.e., several weeks.(31) However, with reference to dietary records a large number of recording days are needed to estimate usual longer term intake. To control for day-to-day variation in dietary intake, correlation coefficients between the FFQ and a small number of days of dietary records can be corrected or deattentuated if the ratio of the within- to between-person variation of the dietary component is known.(400,458) The availability of multiple dietary records (n=6) within each trial period allowed correction of the correlation coefficients for error attenuation using the within- to between-person variance ratio method of Beaton et al.,(496) as six dietary records for each trial period may not have been adequate to account for the complete within-person variation.

No further correlation coefficient adjustments were made. Because intake of most foods, food groups and nutrients is positively associated with total energy intake, such intake values are often energy-adjusted.(136) The energy-adjusted correlations are generally higher than the energy-unadjusted correlations (37) due to the variation in energy intake and its associated measurement error being minimized by energy-adjustment.(41) Despite the comprehensive FFQ not being equipped for estimation of total energy intake, the effect of energy-adjustment of total flavonoid intake may have been minimal for the following reasons. Adjustment for energy does not appreciably alter the correlation coefficients for micronutrients,(38,444,476) as they are usually weakly associated with total energy intake,(38) whereas for most macronutrients they improve after adjustment for energy intake.(444) For instance, energy adjustment made little difference in the magnitude of the coefficients of vitamin intakes, which are minimally correlated with energy intake,(38) nor cholesterol intake, which is not an energy-producing nutrient (400) (which is applicable to flavonoids) and carotene intake which is not related to energy intake.(408) In the study of Bosetti et al. (341) energy-adjusted and unadjusted flavonoid intake estimates yielded similar results so that only the crude flavonoid estimates were reported. The mean total flavonoid intake (5 major subclasses) from four-day dietary records based on household measures of middle-aged eastern Finnish men partaking in the population, based Kuopio Ischaemic Heart Disease Risk Factor Study, too yielded an energy adjusted intake (128.5±206.7 mg/day) very similar to the crude intake (128.5±207.7 mg/day).(169)

Correlation coefficients can be adjusted for gender and age. In analyzing the data from a validation study, it is important to adjust nutrient intakes for such covariates that are controlled in epidemiological studies. For micronutrients, which are less strongly correlated with total energy intake, adjustment for both gender and age and for energy intake produced relatively small and inconsistent changes in the correlation coefficients.(405) Most studies do not report results for subgroups as the subgroups are usually small within the small validation study sample size, which exclude estimation of gender- or age-specific, deattentuated coefficients with reasonable precision.(441) When the subjects in a study are rather homogenous with respect to a covariate, like within a narrow age range, the adjustment effect may be modest.(435)

Due to the sample size of the intervention trial, such covariate adjustments were excluded in the correlation determinations. However, adjustment for measurement error on the baseline dataset of the FFQ was undertaken to correct for systematic FFQ over-reporting bias in view of the three summation questions on typical participant weekly fruit, vegetable and tea serving consumption. The adjusted mean daily total flavonoid intake estimates of the baseline FFQ on incorporation of these summation question consumption frequencies were evaluated against the baseline dietary record mean daily total flavonoid intake estimates at the group and the individual level.

7.3.8.2 Biomarker Validation

Owing to the complexity in assessing accurate dietary intakes of free-living subjects (108,194,249) and the current inadequacy of the USDA flavonoid database to fully cover the extent in the compositional variability of many foods,(126) (variation due to diverse food preparation and processing methods not accounted for,(108) and the content of many foods still unknown (194,249)), biochemical biomarkers were proposed as particularly valuable to substantiate flavonoid intake.(108,126) Biomarkers are helpful to evaluate the validity of a FFQ in addition to using dietary records of which the validity is uncertain.(14) Large errors in flavonoid intake estimates may result, given that flavonoids are found concentrated in some dietary sources. Small errors in reporting the intakes of such flavonoid-rich sources may result in large errors in the estimated intake.(194,249) Using biomarkers to determine the phenolic exposure was an important consideration as such analytical measures may provide a more accurate estimate of dietary intake than using dietary intake assessment measures with food composition tables.(9) As the research staff and the participants were aware of the nature of the rooibos intervention trial, biomarkers would be of particular importance, since biomarkers would be sensitive to variations in the total quantity of flavonoids consumed and would therefore provide an objective measure of the dietary compliance over the intervention trial periods. Halliwell (114) proposed that any intervention trial should be accompanied by one or more relevant biomarker measurements at intervals during the trial.

Although several anthropometric, clinical and biochemical analyses were undertaken by trained staff as part of the rooibos intervention trial,(37) the decisive factors considered for the biomarker selection of flavonoid exposure and of effect for this research inferred causation relating to biologic plausibility and a possible dose-responsive effect, was as put forward by Halliwell.(114) It is analytically complex to measure the individual flavonoids and their metabolites, as well as their combined role in the body and correlating these values to the time course of the flavonoids and their metabolites in the plasma.(128) Thus to determine the individual flavonoids and their metabolites, the plasma total polyphenols was assayed as a measure of the flavonoid intake/exposure,(214,554) the *in vivo* antioxidant effect of the intake of flavonoid-containing foods and beverages evidenced by measuring the TAC or antioxidant status of the plasma,(128,214) along with the plasma CDs and TBARS as predictors of lipid peroxidation and as measure of oxidative stress.(128) Although the metabolism of the flavonoid compounds remain elusive, ample evidence supports that most flavonoid compounds are sufficiently absorbed to exert a marked reduction in various indices of plasma oxidant status.(98) Application of the CD and TBARS (MDA, measured as TBARS) assays as plasma indices of oxidative stress demonstrate the bioavailability and antioxidant efficacy of flavonoids in humans (98) with CDs an early marker of PUFA peroxidation (293) and TBARS used as rather non-specific marker of whole body lipid

peroxidation.(293,506) The assessments were done by the Oxidative Stress Research Centre, CPUT. All assays were done in triplicate or duplicate as specifically outlined.

Fasting (10-12 hours) blood samples were drawn from the participants by two registered nurses on completion of each intervention trial period during the morning visit (07:00-10:00 am) when the estimated dietary records for the trial periods were collected and reviewed and the FFQ for the trial period completed and checked. Peripheral venous blood samples (30 mL) were collected into one SST tube and two (EDTA)-treated (ethylenediaminetetraacetic acid) (0.1%) tubes (BD vacutainers, Plymouth, UK) for serum and plasma. Samples were protected from light throughout handling and transported on ice to the laboratory for processing the same day. Blood samples were centrifuged at 1000 X g at 4^oC for 10 minutes for plasma separation. Samples were stored at -80^0C until analyzed.(37)

Extraction and measurement of the total plasma polyphenols was determined by the Folin-Ciocalteu reaction and the spectrophotometric assay for phenols described by Singleton and Rossi (574) with a gallic acid standard, with the results expressed as mg gallic acid per L plasma. The polyphenols react with the Folin-ciocalteu phenol reagent at alkaline pH and form a blue colour measured at wavelength of 765 nm (nanometer). The plasma TAC was determined using the FRAP, ORAC and TEAC assays. The FRAP assay, applying the method of Benzie and Strain,(575) measures the ferric reducing ability of plasma or the ferric ion to ferrous ion reduction at low pH, which causes a coloured intense blue ferrous-tripyridyltriazine complex to develop with an absorption maximum at 593 nm with the FRAP values obtained by comparing the change in absorbance at 593 nm in the test sample (plasma) with those containing ferrous ion in known concentration. The rate-limiting factor of ferrous-tripyridyltriazine, or colour, formation is the reducing ability of the plasma. The units for the plasma TAC as assessed via the FRAP assay is in µmol ascorbic acid equivalents (AAE)/L plasma.

The method described by Ou et al. (576) was used for the ORAC assay. In this assay fluorescein is used as the oxidizable substrate or fluorescent probe, which is challenged by AAPH as peroxyl radical initiator and Trolox used as the control standard. AAPH decomposes at 37^oC giving rise to carbon-centered radicals that react with oxygen yielding peroxyl radicals. The loss of fluorescein fluorescence is an index of the oxidative damage with the protection against the fluorescein fluorescence loss by antioxidants reflected in the ORAC assay, as measure of the antioxidant capacity. The ORAC value represents the area under the quenching curve of fluorescein initiated by AAPH in the presence of plasma antioxidants relative to that of Trolox. Using the method of Re et al.,(577) the TAC of the plasma samples was determined by the TEAC assay. This is a further spectrophotometric detection method based on the ability of the antioxidant molecules in the

plasma to quench the radical cation generated by oxidation of ABTS by potassium persulfate, and measured by the extent of the decolourization or inhibition of the radical cation at 734 nm relative to the reactivity of Trolox, as standard reference compound. Units for both the plasma TAC, as assessed via the ORAC and the TEAC assays, are expressed in µmol Trolox activity/equivalents (TE)/L plasma.

Lipid peroxides are formed in cell membranes from PUFAs as a result of exposure to oxidants and free radicals. The measurement of the end products of lipid peroxidation as indication of the extent of the lipid oxidation provides useful markers of endogenous oxidation or of susceptibility to oxidation.(578) Lipid oxidation can be estimated by the amount of CDs (293) and MDA (26,241,504) present in the plasma. The plasma CDs were estimated according to the method of Recknagel and Glende,(579) while the plasma MDA was measured according to the method of Yagi,(580) which is based on the reaction of TBA with MDA, an end product of lipid peroxidation. The plasma CD hydroperoxide accumulation was measured spectrophotometrically on determining UV light absorbance in the wavelength range 230 to 235 nm to assess LDL oxidation, as unsaturated fatty acid peroxidation is accompanied by an increase in UV absorbance at this wavelength.(579) In the TBARS assay, which is a spectrophotometric assay, heating the plasma sample at low pH produces a reaction between TBA and MDA, which produces a pink chromogen that is measured by detection absorbance at 532 nm.(580) The results of the CD and TBARS assays are expressed as peroxide equivalents (nanomol (nmol)/mL and µmol/L, respectively).

The individual-level data were used for assessing the mean daily total flavonoid intake in relation to each specific biological exposure or disease outcome biomarker. Correlation coefficients were calculated to assess the association between the total flavonoid intake estimates calculated from the comprehensive FFQ and the plasma measure values for the baseline period only of the rooibos intervention trial.

7.3.8.3   Reproducibility

Repeatability is usually assessed by administering the same FFQ twice to the same group of subjects determining the association between the two response datasets.(17) Since this was an intervention study the evaluation of the reproducibility of the FFQ had to take into account the intervention trial periods. The FFQ reproducibility was determined by identifying its consistency in determining the participant mean daily total flavonoid intake over the washout and the control periods of the intervention, as these two trial periods required the same dietary flavonoid restriction applications in conjunction with continuation of the usual participant diet.

A multiple analytic approach was used to assess the reproducibility of the flavonoid intake estimates from the FFQ completed at the end of the washout trial period and the FFQ completed at

the end of the control trial period after an interval of six weeks. An important factor influencing reproducibility is the time interval between the two FFQs. If the interval is short, the participant may remember responses to the first FFQ and the reproducibility will be overestimated, and if the interval is long, the dietary pattern may change, resulting in a large error in reproducibility.(387) In the intervention trial a time interval of six weeks occurred due to the timing of the assessment relative to the trial periods, which would limit these influences. The level of stability between the FFQs covering the washout and the control trial periods was assessed considering the within-person variation between the two FFQ applications (test-retest method) calculated as paired differences in the participant group intakes, the relationship between the two applications in ranking and categorizing the participants in the intake distributions and the limits of agreement between the participant intakes of the two FFQ applications.

7.3.8.4   Responsiveness

The sensitivity of the FFQ to reflect evidence of changed dietary conditions during the intervention trial periods was used as evidence of its responsiveness. Because of the repeated measurements design in this longitudinal intervention trial, four sets of cross-sectional data on the dietary intakes were available to compare the mean daily total flavonoid intakes across the intervention trial periods and determine if they reflected changed dietary conditions. Four sets of biochemical analyses were available to reflect the exposure manipulation.(502) The plasma total phenolic levels as biomarker measure of the dietary flavonoid exposure (214,554) and the plasma CD and TBARS levels as biomarker measures of effect were compared across the trial periods, as reflection of the changed dietary exposure through low-level exposure, absence of exposure, and exposure to the dietary component.(506) In other words, although the FFQ as dietary measure may reflect the dietary exposure manipulation, its ability to reflect differences in the exposure manipulation for the different trial periods were investigated for support by true exposure and effect (biomarker) differences between the trial periods.

Kristal et al.,(19) furthermore, pointed out that the type of intervention being evaluated would affect the responsiveness. In an intensive clinical intervention, the magnitude of dietary change may be so large that almost any dietary assessment measure will be at least reasonably responsive.(19) According to Kristal et al.,(19) it may be desirable to use less responsive measures in such studies. As the above scenario may be true for the intervention trial due to the large contribution the consumption and avoidance of tea (black and herbal teas) was expected to make to the mean total flavonoid intakes during the trial periods, all flavonoid intake contributions from tea were removed across all the trial periods to determine if the FFQ would be responsive to the other flavonoid intake restrictions placed on the trial periods in comparison to the baseline trial period.

Repeated measures ANOVA was used to assess whether the means of the daily total flavonoid intakes and the means of the biochemcial analyses of the participants across the trial periods differed significantly (p<0.05). When the ANOVA was positive, the Bonferroni correction for multiple comparisons was performed, which considers pair wise comparisons of the different trial periods for identification of the trial period contrasts in the total flavonoid intakes and the selected biomarkers.

As a change in dietary intake (as was required in this trial through dietary flavonoid food and beverage source intake restrictions) may lead to less or more food being consumed, the nutrient intake and the nutritional status of the participants may change which may change the levels of oxidation markers in the blood.(536) The energy, macronutrients (total fat, fatty acids, cholesterol, carbohydrate and dietary fibre), vitamin C, vitamin E, ß-carotene, selenium and folic acid intakes of the participants were assessed from the completed estimated dietary records for each of the trial periods using FoodFinder 3.(541) The latter nutrient analysis accounts for dietary changes other than mean daily total flavonoid intake. Willett et al. (485) indicated that if an individual is to change his or her intake of a specific nutrient reasonably, it must be done primarily by changing the composition of the diet rather than by changing the total amount of food consumed or the total energy intake, as was expected from the participants in this trial. Therefore body weight should not change substantially unless the level of physical activity changes considerably.(485) Therefore after completion of the trial each participant was again weighed on a platform electronic scale without shoes and in light clothing to the nearest 0.1 kg. Body weight was measured twice and if an inconsistency occurred in the weight measures, a third measure was obtained.

7.4 DEVELOPMENT AND EVALUATION OF ABBREVIATED FFQ AS FURTHER TEST METHOD

7.4.1 Development

A long list of foods and beverages were included in the comprehensive FFQ to cover the diverse array of dietary items possibly consumed by the trial participants. Even dietary items with low flavonoid content and items possibly infrequently consumed were included, on account of their potential contribution to the absolute intake and possible role in contributing to the between-person variation in intake. However, in the development of FFQs, consideration is given to limiting the food list by only including those items of which the nutrient content and the frequency of intake add appreciably to the absolute intake of the nutrient of interest and items that contribute most to the between-person variation in intake.(49)

Modification of the comprehensive FFQ occurred after its use in the rooibos intervention trial to obtain a simpler and less expensive tool for assessing mean daily total flavonoid intake by using a

well-defined food list. De Vries et al. (74) indicated that a brief FFQ might be a suitable method for estimating flavonoid intake, especially for ranking individuals by intake. In most determinations undertaken to estimate flavonoid intake, a few specific foods were the principal dietary sources and major contributors to the flavonoid intake.(512) The sources included, amongst others, tea (black), red wine, apples, onions,(9,253) citrus fruit (89,137,341) and citrus fruit juice.(342)

In assessing dietary intake, information is needed about the main dietary sources and the within- and between-person variation in intake.(74) To identify the foods/beverages to be included in the abbreviated or brief FFQ, a two-step procedure was used that firstly entailed applying the percentage contribution approach (355) and then the stepwise multiple regression analysis (18,38,405) to the datasets. The datasets of the estimated dietary records completed during the baseline period of the intervention trial and the FFQs completed for this period were independently used for the analyses. Firstly, the percentage contribution of each food and beverage to the total daily flavonoid intake was assessed to identify those items making the highest contributions to the mean daily total flavonoid intake in each dataset. Secondly, stepwise multiple regression analysis was applied to the datasets to identify those items most predictive of the between-person variability in daily intake.(395,403,411,501,581) These modifications to a FFQ will shorten its food list, as well as the number of main food categories, but retain its features.(403)

To determine the dietary sources contributing most to the absolute intake, the percentage contribution of each item to the daily total flavonoid intake of all the participants combined was computed using the baseline trial period dietary records and FFQs. All dietary items consumed over the two-week time frame were obtained and included in the analyses. The percentage contributions were calculated by dividing the contribution of each item by the total flavonoid intake estimate. The items were then ranked for each dataset in the order of their percentage contribution to the total consumption until the sum of the items reached 90% or more of the total participant intake, according to the procedure of Block et al.(355)

Because of the small number of participants relative to the number of dietary items consumed, only the 20 or so items contributing most based on their percentage contribution, were considered for the stepwise multiple regression analyses as applied by Stryker et al.(395) Stryker et al. (395) limited their list of candidate dietary items to only the 20 that provided the largest absolute nutrient amounts and did not include the full list of possible items, as the analysis would likely have included these items, as well as some that might enter on the basis of chance alone. In addition, only these 20 or so items were considered, as it has been shown that only one or two foods provide more than 10% of the total intake of a given nutrient.(406) For any particular nutrient, most of the variance in intake can be accounted for by about 20 dietary sources.(29) These top items

were re-ranked by stepwise regression to assess the contribution of each food to the variation in intake among the participants. Different models can be used, for instance incorporating only foods contributing equal to or greater than 0.1% to the mean intake (412) or nearly all foods, even foods providing less than one percent of the total intake.(406) Agreement in the models is very close for nutrients whose sources are a relatively few discrete items.(395) In multiple stepwise regression analysis, the dietary items are chosen by statistical variable selection with the individual dietary items incorporated as the independent variables, and the total intake of the nutrient as the dependent variable.(38,449) The increase in percentage of the variance for intake is explained by the addition of an item to the progressively longer list of items (cumulative R^2) which serves as the measure of the between-person variation in nutrient intake.(395)

In the process of compiling a short list of dietary items for the nutrient estimate of interest, additional criteria may be used or other reasons considered in adding items in order to fully explain between-person variations in the intakes of nutrients or to determine absolute intakes and therefore the basic list may be extended.(412) In the final compilation of the abbreviated FFQ, additional considerations were applied to the selected food list by firstly considering seasonally available items for inclusion. Inclusion of many seasonal foods in a questionnaire may influence its reproducibility, which will depend greatly on when the data was collected.(5) A further aspect considered in the compilation of the final food list was the addition of dietary items, based on matching item selections, which may contribute to the absolute total flavonoid intake, but were not selected in the analyses for inclusion in the food list. The detail of these above-mentioned manipulations to compile the final food list is discussed as part of the study results.

7.4.2 Evaluation

The validity of the abbreviated FFQ and its reproducibility was evaluated within the context of the rooibos intervention trial, but also in a further participant group that consisted of a convenient sample of adult volunteers from different geographic locations in order to obtain a broader assessment of its probable validity and reproducibility or external strength and probable generalizability. Kim et al. (446) reviewed brief survey measures to assess fruit and vegetable intakes, and indicated that to evaluate the ability of these measures to estimate absolute and relative intakes, mean or median differences in addition to correlation coefficients, from comparisons of intakes with dietary reference methods, should be reported along with the sensitivity, specificity and predictive values which, besides for the latter, was considered for the validation evaluation of the abbreviated FFQ as especially fruit comprise a major part of its food list. These researchers, furthermore, indicated that correlation coefficients and mean or median differences derived from the test-retest reproducibility testing should be reported,(446) which was determined in the reproducibility evaluation of the abbreviated FFQ.

7.4.2.1 Evaluation within Rooibos Intervention Trial

The same statistical procedures applied for the validity, reproducibility and responsiveness evaluation of the comprehensive FFQ in the rooibos intervention trial was used in the evaluation of the abbreviated FFQ in the trial except for the summary question adjustment to the FFQ dataset of the baseline period of the trial. The validity evaluation of the abbreviated FFQ again occurred against the estimated dietary records and the exposure biomarkers as reference methods.

The comprehensive full-length FFQ did not serve as surrogate reference standard to the abbreviated FFQ, as its dataset was obtained from that of the full-length questionnaire. The accuracy of the abbreviated FFQ can to a great extent be overestimated, as the selection of the abbreviated FFQ food list was based on the comprehensive FFQ and estimated dietary record datasets of the intervention trial. In determining the actual performance of the abbreviated FFQ, it was administered in a further independent participant sample and evaluated against their completed multiple day estimated dietary records, as this is considered a more optimal validation design.(429)

7.4.2.2 Evaluation within a Further Participant Group Administration

Tsubono et al. (397) pointed out that a food list selection for a FFQ based on quantitative data from the target population does not necessarily mean that the questionnaire is valid. An independent study is required to assess its validity whereby the intake estimations from the questionnaire as administered in another study sample can be compared with that calculated from a more extensive dietary assessment method (dietary records) obtained in this study sample. Further testing and validation of the abbreviated FFQ was thus needed to know how its changes, the reduction in the food list, might affect the consumption estimates (419) and to verify its generalizability.(411) The aim of this external study was to assess the agreement and/or disagreement between the abbreviated FFQ as test method and estimated dietary records as reference method in another, but independent participant group.

7.4.2.2.1 Participant group

In the selection of the participant group for an independent evaluation of the mean daily total flavonoid intake estimates as assessed by the abbreviated FFQ, an attempt was made to obtain a wide variability in intake of the dietary component of interest and not to achieve representativeness in its intake, as advocated by Mullie et al. (425) and Bolton-Smith et al.,(480) respectively. To achieve this, a study group could be drawn from contrasting cities.(480) In this study, a heterogeneous participant sample (n=100; Dependent sample t-Test sample size calculation with standardized effect=0.298, Alpha=0.05, Power=0.8002, n=91) in terms of dietary exposure to flavonoid food and beverage sources was considered. In particular, tea and red wine drinking were considered to provide variation in intake as a measure to maximize exposure contrasts, and

to rank participants across the variation in intake.(373) The mean daily total flavonoid intake of only persons from an urban location was studied in the rooibos intervention trial.

To extend the generalizability of the abbreviated FFQ and its validation results within the intervention trial, the abbreviated FFQ was administered to persons from four different geographic locations, including rural environmental locations, with presumably different dietary intakes to urban environmental locations.   Using geographic information systems (GIS) is at present considered in the study of the food environment and access to food to determine the local food environmental (agricultural and built) influence on food choices, eating behaviour and the known individual influences of food affordability, taste, etc.(582,583) This is in the context of this research supported by a Canadian study that established that in an area where the number of places that sold wine had increased, the wine consumption of the residents in the area increased.(584)

Although the group of participants was apparently healthy, free-living male and female adults, they represented a diverse group with differing risk factors for CHD and from differing urban and rural geographic locations and environments where flavonoid intake may differ. Clanwilliam as a rural and rooibos herbal tea producing area, Paarl as a semi-urban and wine producing area, the City of Cape Town as urban area (probable higher exposure areas) and Graaff-Reinet as rural area not producing tea or wine (probable lower exposure area) (Table 7.6). The participants provided written informed consent and met the inclusion criteria of being asymptomatic, but at low and intermediate CHD risk (one or more risk factors) (585) based on questionnaire self-report of risk factors.(586)

The participants were recruited by inviting them to participate in a study in which a newly developed brief questionnaire, to estimate mean daily total flavonoid intake, was being tested. The researcher and recruitment assistants directly recruited participation through oral invitation considering the inclusion and exclusion criteria (Table 7.6). The volunteers who responded positively on being informed of the purpose of the research and provided verbal informed consent, enrolled at each of the four geographic sites that constituted the participant subsets. They were informed that they would complete the newly developed brief questionnaire (abbreviated FFQ)

Table 7.6: Participants and Participant Recruitment for Validity and Reproducibility Evaluation of Abbreviated FFQ in the Further Administration

Participating area description	Selection of participating area	Invited volunteer participants[a]	Participant recruitment		Assembly venue
			Method(s)	Responsible person(s)	
Clanwilliam: Famous for rooibos herbal tea & orange production situated in sparsely populated West Coast district, Western Cape Province (SA) about 230 kilometers (km) from Cape Town[b]	Rooibos herbal tea producing as probable higher flavonoid intake area	South African Rooibos Council employees and members	Group presentation[f] / Individual oral invitations (using group presentation information)	Researcher assisted by Client Liaison Officer, Rooibos Ltd	Rooibos Ltd venue
Paarl: Largest town Cape Winelands with deep viticulture heritage situated in Western Cape Province, SA about 60 km northeast of Cape Town (now considered an urban unit due to its population growth)[c]	Wine producing as probable higher flavonoid intake area	DISTELL LTD Paarl employees and friends	Individual oral invitations (using group presentation information)	Researcher assisted by employee of DISTELL LTD Paarl	DISTELL LTD Paarl venue
City of Cape Town: Second-most densely inhabited city in SA and capital of the Western Cape Province and regional manufacturing center, part of City of Cape Town metropolitan municipality[d]	Urban as probable higher flavonoid intake area	Cape Peninsula University of Technology employees	Individual oral invitations (using group presentation information)	Researcher	Institutional lecture venue
Graaff-Reinet: Known for game farming & stock (merino sheep & angora [mohair] goats) situated in semi-arid desert (Great Karoo) on western frontier of Eastern Cape Province about 790 km from Cape Town[e]	Rural (not producing rooibos herbal tea or wine) as probable lower flavonoid intake area	Neighbouring businesses employees	Individual oral invitations (using group presentation information)	Researcher assisted by a local dentist, teacher and financial adviser	Dentistry staff room, school staff room, financial business meeting room in close proximity to each other, located between neighbouring businesses

[a] Participants: Apparently healthy adult volunteers (n=100) meeting inclusion criteria of asymptomatic, low and intermediate coronary heart disease (CHD) risk (one or more risk factors) (585) based on self-report of risk factors: age (men 45+ years & women 55+ years),(551) dietary intake (no daily fruit & vegetable intake), body weight status (above optimal, overweight or obese), waist circumference (abdominal fat distribution), physically inactive (no regular exercise), smoking habit, personal/family history of increased blood pressure (hypertension) and/or increased blood glucose (diabetes mellitus) and/or increased blood cholesterol (CHD),(586) stress at work/home (587). Participants should not alter customary diet for duration of study. Participants excluded: pregnancy/lactation; treated/untreated symptom(s) of coronary heart, other diagnosed disease, particularly chronic disease; on a special diet; food allergies.

[b] Source: South African History Online (588)

[c] Source: Wikimedia Foundation, Inc. (589)

[d] Source: South Africa Travel (590)

[e] Source: Wikimedia Foundation, Inc. (591)

[f] Presentation provided to South African Rooibos Council employees and members

twice, about two weeks apart, and that it would take about 10 minutes of their time. However, they would have to complete two sets of three consecutive day estimated dietary records on receiving training to do so at the initial assembly, when completing the first abbreviated FFQ. They were informed that they will complete a demographic and CHD risk factor questionnaire and sign the consent forms for voluntary and anonymous participation, on receiving a participant study code, which would take about 45 minutes of their time. Recruitment ceased after 100 volunteers were recruited with approximately 25 from each of the participating locations.

The participants recruited had to meet at least one of the following eligibility criteria based on self-report of risk factors via a self-administered structured questionnaire that collected information on demographic, lifestyle and health factors:

- Age (men over 45 years and women over 55 years)(551),
- Dietary intake (daily fruit and vegetable intake not present),
- Body weight status (above optimal representing overweight or obese),
- Waist circumference (indicative of abdominal fat distribution),
- Physically inactive (do not participate in regular weekly moderate or strenuous exercise),
- Smoking habit,
- Personal/family history of increased blood pressure (hypertension) and/or increased blood glucose (diabetes mellitus) and/or increased blood cholesterol (CHD),(586) but not receiving medical treatment for any of these conditions besides for advised lifestyle changes,
- Stress at work/home.(587)

The selection of the risk factors was based mainly on the work of Yusuf et al. (586) that identified the nine risk factors that accounted for around 90% of the population attributable risks in men and women, old and young, and in all regions of the world for an initial acute myocardial infarction, which included: adverse factors of smoking, abnormal lipids, raised blood pressure/hypertension (self-reported history), elevated blood glucose level/diabetes (self-reported history), abdominal obesity and psychosocial factors; protective factors of daily consumption of fruit and vegetables, regular alcohol consumption (3 or more times a week) and regular physical activity (4 or more hours a week moderate or strenuous physical exercise). For each of the risk factor questions in the questionnaire, the multiple-choice responses to each question were based on the response provisions used of Yusuf et al. (586) with the psychosocial risk factor descriptor and responses obtained from an accompanying report by Rosengren et al. (587) on the same study.

Advancing age and body weight status (obesity) were considered in the questionnaire, as these factors are related to CVD risk.(585) With age, oxidative damage to cell components (including lipids) accumulates and contributes to the degeneration of the various cells and to the pathogenesis of CVD.(94) One of the major American Heart Association (AHA) dietary guidelines

indicate achieving and maintaining a healthy body weight (565) although the relation of BMI to risk of myocardial infarction is weaker than that of abdominal obesity.(586) A measuring tape was available to participants to use to assist them in answering the question on their waist circumference (female: larger than 88 cm; male: larger than 102 cm), which was used as measure of abdominal obesity.(592)

In addition to the participants having to reside in the specific area and describing themselves as healthy, they had to be able to communicate (read and write via the English or Afrikaans languages) their dietary intake and be agreeable not to alter their customary diet for the duration of the study. Participants excluded for participation included those with medically treated/untreated symptom(s) of coronary heart and other diagnosed disease, particularly chronic disease, those on a special diet, those with food allergies and females being pregnant and/or lactating. Nutritional assessment requires motivated and compliant individuals (347) that cannot be assured by a randomly selected sample. The AHA guidelines stipulate limiting alcohol consumption to no more than one drink per day for women and two drinks per day for men.(565) In the participant recruitment, this was considered to elude inviting those that may partake in heavy drinking habits with heavy drinking described as a usual daily intake of three or more standard-sized drinks as characterized by Klatsky et al.(593)

7.4.2.2.2   Completion of test and reference dietary methods

According to Joachim,(5) the time and duration of the data collection greatly impact the potential to collect data that accurately reflect the consumption over time and that this time issue is particularly important when the data collection involves questioning about many seasonal foods. Although the seasonal availability of fruit was addressed in the food list of the abbreviated FFQ, the seasonal intake of fruit may impact the mean daily flavonoid intake estimates of the dietary records versus that of the abbreviated FFQ. The potential seasonal variation in food intake, especially of fruits and vegetables,(363,510,538) was considered in the research design and this phase of the study conducted between the end of spring and the beginning of summer (end of October to beginning of November), when citrus fruits were reaching the end of their seasonal availability and the summer fruits were not quite entering the market.

As in the rooibos intervention trial, the researcher guided the participants to complete the abbreviated FFQ and instructed the participants in detail on how to complete the estimated dietary records in group information sessions held at the various sites. The same general study design, as in the rooibos intervention trial, was followed with the participants being asked to complete six estimated dietary records, as the reference method, to assess the dietary intake for the following two weeks that comprised two sets of three consecutive day dietary records on two weekdays and one weekend day alternating over the two week period to provide a more representative sampling

of days so that six different days of the week were studied and an appropriate ratio of weekend to weekdays were included over the two week analytical period.

In the group information session the participants were first asked to fill out the abbreviated FFQ with respect to their dietary intake of those included items in the two weeks preceding the abbreviated FFQ completion. The period between completion of the abbreviated FFQ and commencing the first subsequent day dietary records was two to three days. The completion of the dietary records occurred during the two-week period between the completion of the first and the second FFQ. The completion of the second self-administered FFQ was carried out within two weeks after completion of the first FFQ and within two to three days after completion of the last set of dietary records. In order to cancel any potential training effects, the participants completed the FFQ first before the period of dietary recording and the abbreviated FFQ re-administered two weeks after its initial completion. The evaluation analyses were conducted using both pre- and post-measures as applied by Hebert et al.(56) The former corresponded with the use of the FFQ most likely encountered in an epidemiological study (unaffected by training from responding to multiple dietary assessments) and the latter so that the responses to the second FFQ could be concordant with the period during which the multiple dietary records were collected. The abbreviated FFQ took on average about 10 minutes to complete.

The completed estimated dietary records were collected from the participants' within three days after completion of the last consecutive day dietary records at the sites during the follow-up group session, where they completed their second abbreviated FFQ. As a measure of quality control, each FFQ was checked and the dietary records reviewed. Any imprecisions and uncertainties encountered in the reviews were clarified with the individual participants.

7.4.2.3.3   <u>Flavonoid intake and data analysis</u>

The data collected from the abbreviated FFQ was not assessed against data collected from a comprehensive FFQ itself, but against data collected from estimated dietary records in terms of its validity evaluation and against data collected from its repeated administration in terms of its reproducibility evaluation. As emphasized by Joachim,(5) it is not the structure of the abbreviated FFQ that is tested and judged, but its data collection.

Four further trained Consumer Science: Food and Nutrition graduates randomly assisted in checking the two completed abbreviated FFQs and reviewing the completed dietary records on collection and captured the data of each into separate computer-based file templates for the analyses. The abbreviated FFQ information was directly captured in a template specifically developed for this purpose that incorporated the weight quantification of each of the three portion size (small, medium, large) indications of the foods and beverages included in the food list (Food

Quantities Manual, MRC, SA (552)) and the food and beverage item assigned the best matched USDA NDB number.(161) Before the information of the dietary records could be captured into the original template and based on the comprehensive FFQ, the graduate students first had to quantify the household amount or common measure amount (unit) consumed of each item indicated in the dietary records in a weight/mass indication. The weight quantification of the portion size indications of each food or beverage recorded was derived from the published weight indications of common household measures or standard units using the Food Quantities Manual, MRC, SA.(552) The allocated calculated weight amount of each item consumed on the dietary records was captured for the food or beverage item, as matched to that in the software template system utilizing the USDA flavonoid database.

The researcher and one of the trained graduate students that assisted in the rooibos intervention trial data analyses, checked the quantifications of the household measures in the dietary records and all data captured in the respective software template systems for the abbreviated FFQ and the dietary records to minimize variability in interpretation (395) and to provide consistency in the analyses (594) between the rooibos intervention trial dietary data and this external participant administration of the abbreviated FFQ.

Summary statistics were calculated on the daily total flavonoid intake data of both the abbreviated FFQ administrations and the dietary records. The validity of the abbreviated FFQ was evaluated against estimated dietary records and its reproducibility through a second questionnaire administration two weeks apart (test-retest method). Biomarkers of flavonoid exposure were not used as reference method in this phase of the study as several biomarkers were used for the validation through the rooibos intervention trial. The abbreviated FFQ responsiveness was not evaluated in this study phase, as this was not an intervention study.

To evaluate the performance of the abbreviated FFQ in comparison to a more precise quantification, the data obtained from the FFQ was compared to that of the estimated dietary records as reference method to determine if the abbreviated FFQ provided a valid measure (construct validity) of mean daily total flavonoid intake by applying the same statistical methods to that of the rooibos intervention trial.

The validation of the first and the second abbreviated FFQ administrations was assessed against the data derived from the dietary records, as the first FFQ administration selection would represent the use of the FFQ likely to be encountered in its field use,(56) while selection of the second administration of the FFQ covered the same period as that covered by the dietary records.(56,423)

Through test-retest administration two weeks apart, the reproducibility was determined of the abbreviated FFQ. An important factor influencing reproducibility is the time interval between the two FFQs. If the time interval is short, the subject may remember the first FFQ responses which will cause overestimation of the reproducibility, and if the time interval is long, the subject dietary pattern may change resulting in a large error and underestimation of the reproducibility.(387) In the present study a time interval of two weeks was applied as in some studies,(403,409,425,433) whereas in other studies (24,360) an interval as short as one week occurred which was considered long enough to minimize response recall and short enough to avoid true changes in dietary intake. The reproducibility was also evaluated using the same statistical methods to that of the rooibos intervention trial. The dietary intake data obtained was non-normally distributed.

<div align="center">

CHAPTER 8

RESULTS AND FINDINGS

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8.1 INTRODUCTION

The research results are categorized and will be presented as follows:

- *The evaluation of the developed comprehensive FFQ* as test method by depicting its validity (dietary records as reference method), reproducibility and responsiveness as applied within the rooibos intervention trial periods (section 8.2);

- *The compilation of a food list for the abbreviated FFQ* as further test method based on the identification of those dietary items that contributed most to the trial participants' absolute daily total flavonoid intake estimate and to the between-person variation in intake as obtained from the dietary records as reference method and the comprehensive FFQ as test method (section 8.3);

- *The evaluation of the validity, reproducibility and responsiveness* of the resultant abbreviated FFQ in the context of the rooibos intervention trial periods (section 8.4);

- *The evaluation of the validity and responsiveness* of both the comprehensive and the abbreviated FFQ datasets against biomarkers of exposure and effect as reference methods within the rooibos intervention trial evaluation (section 8.5);

- *The evaluation of the validity and reproducibility* of the abbreviated FFQ in a further participant group administration as measure of its external strength or probable generalizability (section 8.6).

- A *confirmation of the dietary items* included in the abbreviated FFQ reduced food list using the dietary record dataset of this further participant group by identifying those dietary items that contributed most to these participants' absolute daily total flavonoid intake estimate and to their between-person variation in intake (section 8.6.5).

Descriptive statistics and the other assessment results of the dietary records and the FFQs as applied within the rooibos intervention trial are provided within the same tables for ease of comparison of the estimated daily flavonoid intakes and the research results across the employed dietary assessment methods.

8.2   EVALUATION OF COMPREHENSIVE FFQ AS TEST METHOD WITHIN ROOIBOS INTERVENTION TRIAL GROUP

8.2.1   Sample Profile

Eighty-three adults responded to the trial advertisement and 64 provided written informed consent to be screened for participation, on attending the trial information and screening session. Fasting blood samples and anthropometric measurements were obtained from these subjects who also completed the self-administered demographic, general health and lifestyle questionnaire. The persons who withdrew at this stage mostly did so due to the scope of the participant involvement in the intervention and doubt in their own motivation and commitment to adhere to and complete the trial schedule.

Forty-three eligible participants entered the intervention trial on meeting the inclusion criteria of being asymptomatic but at an intermediate to high CHD risk on risk factor assessment (595) that included having two or more risk factors for CHD.(37) Three participants (one female and two males) voluntarily withdrew during the trial that was conducted at the Cape Town and Bellville campus grounds of the institution because of personal reasons. A total of 40 subjects (26 females and 14 males) finally completed the intervention trial and provided complete dietary assessment data. The baseline CHD risk factor profile of the 40 participants is captured in Table 8.1.

Table 8.1:   Coronary Heart Disease Risk Factor Profile of Rooibos Intervention Trial Participants (n=40)

Variable	Mean±SD	Variable	Mean±SD
Age (years)	46.80±9.70	Diastolic blood pressure (mmHg)[a]	84.70±9.10
Body mass index (kg/m^2)[a]	28.40±5.50	Fasting glucose (mmol/L)[a]	5.25±0.63
Waist circumference (cm)[a]	89.32±14.18	Physically active (regular physical activity)	47.5%
Waist circumference, at risk[b]	30.0%	Smokers, current	12.5%
Total cholesterol (mmol/L)[a]	5.68±1.30	Smokers, former	62.5%
Systolic blood pressure (mmHg)[a]	135±15.70	Family history of cardiovascular disease	40.0%
		Framingham score[c] (% risk)	5.92±4.11

[a] Measurement procedures described in Marnewick et al. (37)

[b] Waist circumference, as measure of abdominal obesity.(393): Females larger than 88 cm; Males larger than 102 cm.

[c] The Framingham risk scoring (537) calculates the participants' risk of developing heart disease.(37)

The participants practiced a wide range of occupations mostly representing the International Standard Classification of Occupations (ISCO) major occupational groups (596) of legislators,

senior officials and managers (17.5%), professionals (32.5%), technicians and associate professionals (20%) and clerks (20%), with nearly three quarters (72.5%) of them having a post Grade 12 educational qualification. The participant educational level and the fact that they were informed of this first of its kind research in SA, investigating the potential of the popular local herbal tea, rooibos, in heart health promotion and the major contribution they would make through participation to this knowledge, may have contributed to subject motivation and the resultant low drop-out rate.

8.2.2 Group Daily Energy and Nutrient Intakes

None of the participants were excluded from the trial due to extreme total energy intakes, or because of missing items on the FFQ, as the FFQs along with the dietary records, were thoroughly reviewed after completion. 'Extreme' was defined as being outside the biological plausible daily ranges of 600 to 3500 kcal (2.5 to 14.6 MJ) among women, and 800 to 4200 kcal (3.3 to 17.6 MJ) among men.(153,417) The participants were instructed to maintain their habitual lifestyle pattern, in particular their dietary habits, during the entire trial duration, with the exception of the flavonoid intake restrictions during the washout, intervention and control trial periods.

The body weight of the participants remained unchanged (p>0.05) throughout the trial (trial-start = 78.8±16.6 kg; trial-end = 79.2±17 kg), indicating that the subjects sustained a stable energy balance.(271) Their systolic (trial-start = 133.4±16.9 mm Hg; trial-end = 137.3±21.2 mm Hg) and diastolic (trial-start = 83.6±8.2 mm Hg; trial-end = 86.1±12.2 mm Hg) blood pressures,(37) possibly reflects some stability in their habitual lifestyle. In addition, no statistically significant (p>0.05) differences were found in the participant energy intake (body weight maintenance), macronutrient intakes or fat energy contributions (blood lipoprotein and cholesterol profile maintenance), antioxidant intakes (stable exogenous antioxidant provision, besides for the dietary flavonoid provision) and folic acid intake (stable supply to support the homocysteine metabolism) (565) between the various trial periods.

The participant mean daily energy, total fat, saturated fatty acid, protein, carbohydrate, dietary fibre, cholesterol, vitamin C, vitamin E, ß-carotene, selenium and folic acid intakes for each trial period is summarized in Table 8.2. This reflects a fat energy contribution profile indicative of CVD risk across the study periods (total fat energy contribution: >35%; saturated fatty acid energy contribution >7%).(597) The stability of the participants' body weight and dietary intake, besides for the varying total flavonoid intakes in corroboration with the changed serum total polyphenol concentrations during the rooibos intervention trial, are measures indicative of the participant compliance during the trial.

Table 8.2: **Means and Standard Deviations of Dietary Variable Intake Estimates from Dietary Records during Rooibos Intervention and Comparisons between Trial Study Periods (n=40)**

Dietary record variables	Mean±SD for rooibos intervention trial periods				p-value[a]
	Baseline	Washout	Rooibos	Control	
Energy (kJ)[b]	8537±2404	8671±2530	8097±2206	8052±2394	0.565
Total fat (g)[b]	79.7±27.1	85.8±32.3	79.1±24.2	82.3±32.0	0.722
Energy from total fat (%)	34.2±5.5	36.5±7.1	36.2±4.7	36.5±8.9	0.382
Saturated fatty acids (g)	25.7±9.8	29.2±11.3	26.8±9.3	27.6±10.8	0.499
Energy from SFAs[b] (%)	11.0±2.1	12.3±2.4	12.3± 2.4	12.3±3.3	0.067
Total protein (g)	81.1±24	81.3±26.2	81.7±27.0	76.2±25.1	0.751
Total carbohydrate (g)	213.2±63.9	213.7±72.9	197.1±58.8	192.5±68.8	0.358
Total dietary fibre (g)	20.2±8	20.3±9.1	17.6±7.7	16.3±7.1	0.062
Cholesterol (mg)[b]	268.9±105.8	267.1±113.1	275.1±99.4	255.7±97.4	0.867
Vitamin C (mg)	118.9±83.2	112.7±77.3	88.5±67.1	84.4±67.0	0.093
Vitamin E (mg)	14.7±5.5	15.3±0.2	12.8±5.5	14.2±14.6	0.707
ß-carotene (µg)[b]	3580.2±3059.3	2720.2±2752.6	2427.3±2027	2326.9±2355.3	0.124
Selenium (µg)	55.1±23.4	58.7±28	56.4±22.9	56.8±28.7	0.942
Folate (µg)	247.7±92.3	248.7±106.6	221.2±83.5	242.2±170.7	0.704

[a] ANOVA, F-ratio significance level

[b] Kilojoules = kJ; Gram = g; Milligram = mg; Microgram = µg; SFAs = Saturated fatty acids

8.2.3 Group Daily Total Flavonoid Intake Estimates

For each dietary assessment method (dietary records and FFQ), the mean (geometric mean where flavonoid intakes were not normally distributed), SD and median along with the lowest and highest values and the 25th and 75th percentile values (mg/day) across the intake distribution range, for each of the rooibos intervention trial periods, are indicated in Table 8.3.

Not surprisingly, the estimated mean for daily total flavonoid intakes at the baseline and intervention trial periods, was substantially higher than the intake estimates for the washout and control periods as assessed by both the dietary methods. Considering the SD and the lowest and the highest values as indicative of the mean daily total flavonoid intake range, the distributions as estimated by the FFQ were somewhat wider than that estimated by the dietary records for each trial period. For instance, the mean daily total flavonoid intake as estimated with the different methods was respectively 343.5±305.6 mg/day (range: 15.5-1253.6 mg/day) at baseline for the dietary records and 390.2±519.5 mg/day (range: 11.8-2206.4 mg/day) for the comprehensive FFQ.

Table 8.3: **Central Tendencies, Standard Deviation, Percentile Scores for Daily Total Flavonoid Intake Estimates (mg/day) and Comparisons within Rooibos Intervention Trial Periods (n=40)**

Rooibos intervention trial periods	Arith-metic mean	Geo-metric mean	SD (Standard deviation)	Lowest value	Highest value	Median	Percentiles		Differences in group intakes
							25th	75th	
Estimated Dietary Records (**DR**)									
Baseline[a]	343.49	233.99	305.55	15.49	1253.60	250.80	113.41	427.27	d; h; i: ND
Washout[a]	31.29	23.20	24.79	1.89	111.06	24.13	13.69	40.41	e; j: ND
Intervention[b]	343.18	–	89.96	160.56	540.54	354.68	314.94	384.23	f; k: ND
Control[a]	27.43	20.13	24.07	4.9	93.77	16.41	12.15	35.61	g; l: ND
Unadjusted Comprehensive (**UN**) FFQ									
Baseline[a]	390.16	162.83	519.51	11.78	2206.39	135.72	55.27	580.31	d: ND
Washout[a]	36.55	25.52	25.35	2.14	153.97	28.33	12.30	42.88	e: ND
Intervention[b]	345.09	–	96.79	117.88	596.44	353.93	313.43	381.59	f: ND
Control[a]	28.54	20.88	32.52	0.86	146.41	21.87	13.62	37.30	g: ND
Adjusted Comprehensive (**ADJ**) FFQ[c]									
Baseline[a]	495.12	163.05	709.06	10.00	3010.00	143.47	42.58	712.35	h: ND
Abbreviated (**ABR**) FFQ									
Baseline[a]	350.92	120.32	504.87	4.63	2173.95	106.49	39.51	465.91	i: ND
Washout[a]	16.87	10.17	21.47	1.07	124.59	10.56	4.83	19.35	**j: SD**
Intervention[b]	318.02	–	99.71	112.96	590.77	340.92	285.97	355.67	k: ND
Control[a]	14.97	9.81	13.14	0.27	56.93	10.55	6.10	21.23	**l: SD**

[a] Back-transformed data after logarithmetic transformation

[b] Normal data distribution

[c] Comprehensive FFQ intakes on *adjustment* of the collective consumption FFQ responses after applying weighting factors based on three inclusive frequency summary questions on weekly consumption servings of total fruit, vegetables and tea

Differences in group daily total flavonoid intakes between the estimated dietary records and the specific FFQ as test method per trial period:

<u>NOTE</u>: **d** = Baseline DR vs Baseline UN-FFQ; **e** = Washout DR vs Washout UN-FFQ; **f** = Intervention DR vs Intervention UN-FFQ; **g** = Control DR vs Control UN-FFQ; **h** = Baseline DR vs Baseline ADJ-FFQ; **i** = Baseline DR vs Baseline ABR-FFQ; **j** = Washout DR vs Washout ABR-FFQ; **k** = Intervention DR vs Intervention ABR-FFQ **l** = Control DR vs Control ABR-FFQ ND = No significant difference (p>0.05) **SD** = Significant (p<0.0001)

8.2.4 Validity Evaluation against Dietary Records as Reference Method

The statistical analyses were performed in two stages. In the first stage, the agreement between the dietary records as reference method and the developed comprehensive FFQ as test method were compared by using the intake estimates of each. In the second stage, the agreement in the baseline trial period intakes was evaluated using the dietary records and adjusted comprehensive FFQ intakes after applying weighting factors to the collective consumption FFQ responses based on three inclusive frequency summary questions on weekly total fruit, vegetable and tea consumption servings. This was necessary in order to prevent potential overestimation due to falsely high frequencies of use of the items in these food group categories to weight down the answers within these categories provided in the FFQ.

Although the statistical analyses were performed in two stages, various statistical approaches were used to evaluate the validity of the comprehensive FFQ. In the first approach, the mean/median daily total flavonoid intake estimates obtained at group level with the FFQ was compared with the mean/median intake estimates of the dietary records as the reference values. In intervention studies where the effect of the intervention needs to be determined (the percentage of persons complying), and in evaluation studies where subjects need to be classified according to compliance with dietary recommendations, establishing rather accurate absolute intakes are essential.(398)

In the second approach, the ability of the FFQ to classify the individual participants within the intake distribution was largely assessed, as the main use of FFQs is in ranking or categorizing (tertiles or quintiles) subjects (52,72) rather than to survey the group mean.(17,40) It is considered particularly constructive to check the validity of a FFQ at varying levels of precision at both group and individual levels using different approaches because of the observation that a FFQ can produce good group means, but have unsatisfactory validity at the individual level, or *vice versa*.(83)   Because the estimated flavonoid intakes from the dietary records and the questionnaires were generally skewed towards higher values as found in other studies,(111) non-parametric methods were selected for the statistical analyses as performed in numerous other nutrient intake validation studies.(71,153,403,463,508)   Non-normal and highly skewed distributions of intake data often occur in nutrition studies.(38,41,49,362,403,423,463,508,598)

8.2.4.1   Group Level Validation

The difference in the group mean daily total flavonoid intake estimates as obtained from the multiple dietary records and the comprehensive FFQ was computed at each trial period to examine the quantitative efficiency of the FFQ as set down by Feunekes et al.(412) This bias or difference between the methods (43,598) was calculated as the intake assessed with the test method (FFQ) minus the intake assessed with the reference method (dietary records).(412,598)   This is a measure of the tendency of the FFQ to over- or underreport the dietary intake in comparison with the dietary records.(43) Paired difference tests were used to examine the statistical significance of differences found in the group intakes between the two dietary assessment methods.

The absolute group mean (arithmetic) daily total intake estimates of the six dietary records and the FFQ were comparable with negligible mean differences in the intakes for the baseline trial period, and in particular the intervention and control trial periods, with the FFQ estimate 13.6% higher (390.2 vs. 343.5 mg/day), 0.6% higher (345.1 vs. 343.2 mg/day) and 4% higher (28.5 vs. 27.4 mg/day) than that of the dietary records in each of these trial periods, respectively. The absolute group mean (arithmetic) daily total intake estimate for the washout trial period for the FFQ (36.6 mg/day) was roughly comparable (16.8% higher) to that of the dietary records (31.3 mg/day). None of the group daily total intakes were significantly (p>0.05) different (Table 8.3) based on the

paired samples *t*-test for the intervention trial period utilizing the group mean due to the normal distribution of the data (p=0.836) and the Wilcoxon's signed rank test for the baseline (p=0.778), washout (p=0.582) and control (p=0.405) periods of the trial utilizing the group medians and interquartile ranges due to the non-normal distribution of the data.

8.2.4.2 *Individual Level Validation*

8.2.4.2.1   Linear agreement

The relative validity at individual level was firstly evaluated by applying correlation coefficients to determine the associations between the participants' mean daily total flavonoid intake estimates from the dietary records and their FFQs in each of the intervention trial periods. Natural log-transformation was used to better approximate normal distribution (353,379,390,423,441,463) and specifically to calculate the Pearson's product moment correlation coefficient as parametric method as applied by other investigators in this field,(71,153,370,403) in addition to the application of the non-parametric Spearman rank order correlation coefficient. The log-transformed Pearson correlation coefficients are provided as measure of association (502) for comparison in published studies (444) due to its broad use. These correlation coefficients between the individual participants' absolute mean daily total flavonoid intakes, as calculated from their estimated dietary records and their FFQ at each of the trial periods, are indicated in Table 8.4. Since the day-to-day variation of intakes of many nutrients is substantial,(38) and to correct for this within-subject variation using dietary records,(38,458) deattentuated Pearson and Spearman correlation coefficients were computed, which take the within- to between-person variation into account.(496)

Analysis of the daily total flavonoid intakes generally show moderately to strong linear associations between the absolute values obtained from the six days of dietary records and those from the FFQ, besides for the application within the washout period of the trial. The Spearman correlation coefficients ranged from 0.33 (p<0.05) for the washout period to 0.69 (p<0.0001) for the control period of the trial with that of the baseline period (0.58, p<0.001) intermediate. The Pearson correlation coefficient for the intervention trial period was 0.81 (p<0.0001). All the relationships including that found within the washout period of the trial as indicated correlated significantly. The Spearman correlation coefficients did not differ appreciably from the log-transformed Pearson parametric statistic presented except for the trial washout period where the latter relationship lost its significance (p=0.175) (Table 8.4). Deattentuation of the correlation coefficients between the FFQ and the dietary records, which take within- to between-person variation in intake into account, improved the correlations to a small extent. Only slight improvements were found in the applications in the intervention and control trial periods, where the linear associations found were already particularly strong (0.81 and 0.74, respectively) and highly significant (p<0.0001).

Table 8.4: **Correlations between Mean Daily Total Flavonoid Intake Estimates of Dietary Records and FFQs for Rooibos Intervention Trial Periods (n=40)**

Rooibos intervention trial period	Correlation coefficients				Deattenuation coefficients	
	Pearson (Log transformed)	p-value	Spearman	p-value	Pearson (Log transformed)	Spearman
Estimated Dietary Records and Unadjusted Comprehensive FFQ						
Baseline	0.620	<0.0001	0.575	0.0003	0.707	0.656
Washout	0.219	0.1745	0.334	0.0371	0.341	0.223
Intervention[a]	0.809[a]	<0.0001	–	–	0.810[a]	–
Control	0.740	<0.0001	0.688	<0.0001	0.743	0.690
Estimated Dietary Records and Adjusted Comprehensive FFQ[b]						
Baseline	0.557	0.0002	0.500	0.0018	0.617	0.554
Estimated Dietary Records and Abbreviated FFQ						
Baseline	0.617	<0.0001	0.587	0.0002	0.618	0.587
Washout	0.183	0.2581	0.272	0.0897	0.289	0.430
Intervention[a]	0.814[a]	<0.0001	–	–	0.912[a]	–
Control	0.602	<0.0001	0.593	0.0002	0.968	0.954

[a] Parametric statistic on account of normal distribution of the data.

[b] Comprehensive FFQ intakes on *adjustment* of the collective consumption FFQ responses after applying weighting factors based on three inclusive frequency summary questions on weekly consumption servings of total fruit, vegetables and tea.

8.2.4.2.2   Agreement by cross-classification

As a further description of agreement between the mean daily total flavonoid intake estimates of the dietary records and the comprehensive FFQ, the proportion of subjects grossly misclassified into the extreme quintile or tertile categories of intake was calculated (412) and the level of inter-test agreement between the two dietary methods assessed using the *Kw* statistic.(594) These results are presented in Table 8.5.

Misclassifications were generally low across the rooibos intervention trial periods with few participants grossly misclassified into the extreme quintiles or tertiles, besides for the washout period of the trial. The percentage of participants classified in the same quintile or tertile of intake by both the assessment methods were above 50% (n>20) in all the trial periods besides for the washout trial period quintile classification, where only 45% of the participants (n=18) were classified in the same quintile (not presented in Table 8.5). The level of inter-test agreement between the two dietary methods calculated using the *Kw* statistic was below 0.2 for the washout period of the trial, signifying poor agreement. However, it was above 0.4 for the baseline, intervention and control periods of the intervention trial, signifying moderate to substantial agreement beyond chance. Considering the quintile category classification, the inter-test agreement using the *Kw* statistic for the intervention trial period was 0.76, which signifies substantial agreement beyond that explained by chance.

Table 8.5:   Gross Misclassification of Mean Daily Total Flavonoid Intake Estimates in Rooibos Intervention Trial Dietary Records and FFQs and Level of Agreement as Measured by Weighted Kappa Statistic (n=40)

Classification categories	Rooibos intervention trial periods							
	Baseline		Washout		Intervention		Control	
	Gross mis-classification (%)(n)[a]	Weighted Kappa[b]	Gross mis-classification (%)(n)[a]	Weighted Kappa[b]	Gross mis-classification (%)(n)[a]	Weighted Kappa[b]	Gross mis-classification (%)(n)[a]	Weighted Kappa[b]
Estimated Dietary Records and Unadjusted Comprehensive FFQ								
Quintiles	7.5 (3)	0.524	22.5 (9)	0.199	5.0 (2)	0.762	12.5 (5)	0.511
Tertiles	2.5 (1)	0.453	10.0 (4)	0.128	0.0 (0)	0.479	2.5 (1)	0.464
Estimated Dietary Records and Adjusted Comprehensive FFQ[c]								
Quintiles	7.5 (3)	0.493						
Tertiles	2.5 (1)	0.529						
Estimated Dietary Records and Abbreviated FFQ								
Quintiles	10 (4)	0.469	17.5 (7)	0.055	5 (2)	0.712	12.5 (5)	0.453
Tertiles	5 (2)	0.424	12.5 (5)	0.042	0 (0)	0.604	0 (0)	0.515

[a] Proportion (%) of subjects classified in extreme quintiles/tertiles

[b] Weighted Kappa (*Kw*): Poor agreement=≤0.2; Fair agreement= >0.2 -0.4; Moderate to substantial agreement= >0.4-0.6; Good or substantial agreement beyond chance= >0.6.(459,495)

[c] Comprehensive FFQ intakes on *adjustment* of the collective consumption FFQ responses after applying weighting factors based on three inclusive frequency summary questions on weekly consumption servings of total fruit, vegetables and tea types.

8.2.4.2.3   Limits of agreement

To assess the agreement between the comprehensive FFQ and the estimated dietary record computed mean daily total flavonoid intake estimates at the individual level, the limits of agreement, as described by Bland and Altman,(498) were checked graphically by plotting the difference between the method mean intake estimations against the mean estimations considering both the methods for each of the trial periods (Figures 8.1 to 8.4). In each plot the solid line, the line of equality or correspondence, indicates the mean difference between the two methods and the dashed lines indicate plus or minus two SDs (95% confidence interval) of the difference. Considering these indications on the Bland-Altman plots, the agreement overall was good with all but one participant (washout trial period) (Figure 8.2) or two participants (baseline, intervention and control trial periods) (Figures 8.1, 8.3 and 8.4) within the 95% limits of agreement. The mean difference was +46.7 mg/day with the limits of agreement +806.2 mg/day and -712.8 mg/day for the baseline trial period (Figure 8.1), +5.3 mg/day with the limits of agreement of +73.2 and -62.6 mg/day for the washout trial period (Figure 8.2), +1.9 mg/day with the limits of agreement of +115.7 and -111.9 mg/day for the intervention trial period (Figure 8.3) and +1.1 mg/day with the limits of agreement of +37.5 and -35.3 mg/day for the control trial period (Figure 8.4).

Although the plotted points were rather equally distributed above and below the line of identity (solid line) with no distortion in the scatter of the differences in one or the other direction in the

washout trial period (Figure 8.2), this was not entirely the case in the other trial periods. In the baseline and the intervention trial periods (Figures 8.1 and 8.3), the scatter of the differences between the methods did not agree equally across the range of mean values. At intakes of above 500 mg/day in both these trial periods, a tendency towards positive differences or overestimation of the comprehensive FFQ against the dietary records emerged. In the control trial period with its more limited mean daily total flavonoid intake range, the differences between the intake estimates from the FFQ and the dietary records showed a trend of negative differences towards the higher mean intakes and a positive difference at the highest mean intakes (Figure 8.4). This seems to support an emerging overestimation at higher intakes as found in the baseline and intervention trial periods.
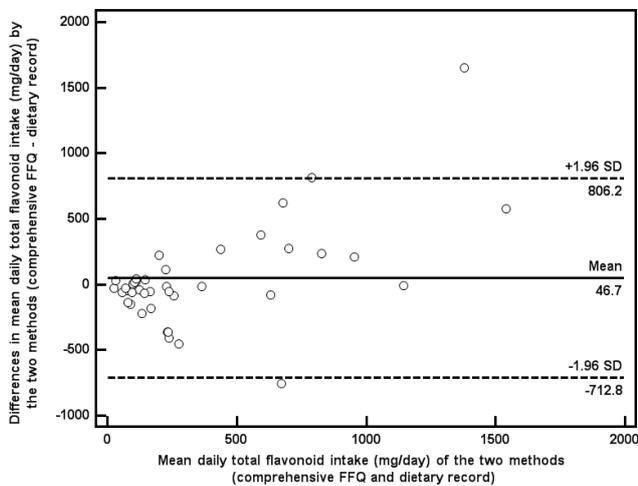
Figure 8.1: **Bland-Altman Plot of Agreement between Flavonoid Intakes of Comprehensive FFQ and Dietary Records within Baseline Period**
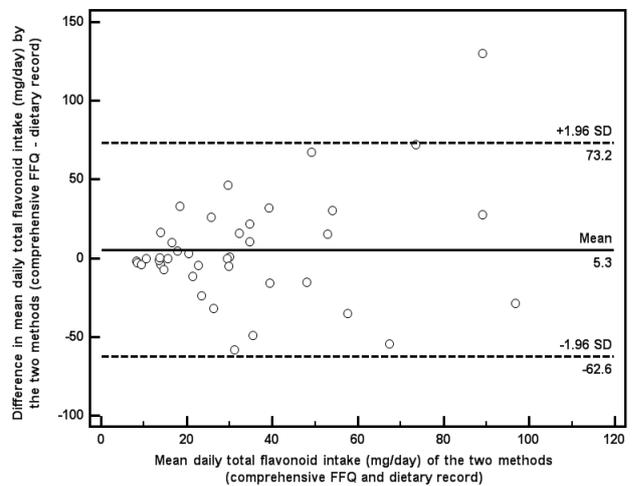
Figure 8.2: **Bland-Altman Plot of Agreement between Flavonoid Intakes of Comprehensive FFQ and Dietary Records within Washout Period**
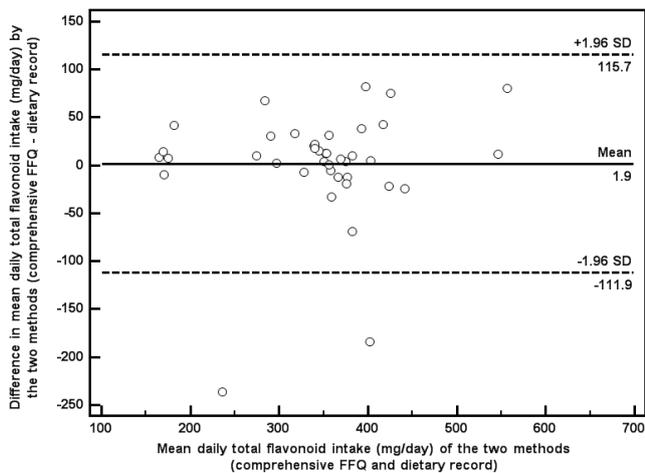
Figure 8.3: **Bland-Altman Plot of Agreement between Flavonoid Intakes of Comprehensive FFQ and Dietary Records within Intervention Period**
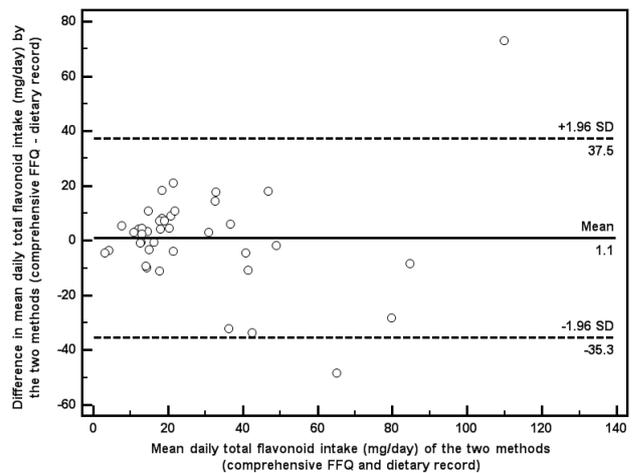
Figure 8.4: **Bland-Altman Plot of Agreement between Flavonoid Intakes of Comprehensive FFQ and Dietary Records within Control Period**

8.2.5 Validity Evaluation of Baseline Questionnaire based on Adjustment derived from Summary Questions against Baseline Dietary Records as Reference Method

The bulk of the comprehensive FFQ food list was made up of fruit and vegetable items. To avoid consumption overestimation that FFQs appear to provide (380,402,504) and which are attributed to the usual long list of items in the FFQ food list,(369,379,384) and in particular when incorporating numerous fruits and vegetables in the FFQ food list,(18,40,82,384) summary questions were applied. These questions were asked on the weekly total fruit and vegetable, and in addition total tea, portion/serving intakes to weight down the collective consumption responses in the FFQ (71) to each of these food categories.

Corrected mean daily total flavonoid intakes were calculated for the comprehensive FFQ using a weighting factor based on the collective consumption frequency for each in the FFQ and the weekly provided frequencies for the total fruit, vegetable and tea summary questions. This adjustment, as data quality assurance procedure, was applied only to the comprehensive FFQ of the baseline period of the rooibos intervention trial, as this was the single trial period where the participants followed their usual dietary intake without intervention dietary stipulations to which the weekly fruit, vegetable and tea portion/serving intakes of the summary questions could be related. The impact of correcting the total fruit, vegetable and tea consumption frequencies of the baseline comprehensive FFQ using the summary questions were determined by assessment of the participant group mean daily total flavonoid intake and the agreement of the individual participant intakes in comparison to the baseline dietary records.

8.2.5.1 Daily Total Flavonoid Intake Estimates based on Adjusted FFQ

The mean, SD and median, as well as the lowest, highest, 25th and 75th percentile values across the daily total flavonoid intake distribution range for the baseline trial period comprehensive FFQ estimates based on the afore-mentioned intake adjustment, are indicated in Table 8.3 (values of adjusted comprehensive FFQ). Needless to say, the estimated mean daily total flavonoid intakes (mg/day) of the adjusted comprehensive FFQ for each of these indices are closer to that of the unadjusted comprehensive FFQ than the estimated dietary records (see Table 8.3), as these adjusted comprehensive FFQ calculations were obtained from the unadjusted comprehensive FFQ data.

Adjustment of the mean daily total flavonoid intake increased the mean daily total flavonoid intakes contributing to further possible overestimation and not reducing it, as can be seen in particular the arithmetic mean, the highest value and the 75th percentile. This overestimation is considered to be due to the summary question portion/serving intake provided by the participants for their weekly tea intake and not the summary question portion/serving intakes provided by the participants for

their weekly fruit and vegetable intakes. Whereas the mean weekly portions/servings of fruits and vegetables were found to be lower using the summary question method (7.5 and 7.7 weekly portions/servings, respectively) versus the FFQ method (18.9 and 16.3 weekly servings/portions, respectively), the mean weekly portions/servings of tea were found to be higher based on the summary question method (19.2 weekly portions/servings) versus the FFQ method (13.8 weekly portions/servings). Both the fruit and vegetable indications in the FFQ food list therefore followed the trend of a collective overestimation of the fruit (152% or 11.4 weekly portions/servings) and vegetable (112% or 8.6 weekly portions/servings) intakes as indicated in the comprehensive FFQ food list in comparison to the summary question weekly intake estimates, where the weekly fruit portion/serving intake overestimation was somewhat higher than that of the weekly vegetable portion/serving intake.

The weekly tea portion/serving intake estimate through the summary question provided for an intake overestimation (39% or 5.4 weekly portions/servings) in comparison to the comprehensive FFQ weekly tea portion/serving intake indications in the food list. Adjusting the mean daily total flavonoid intake based on the three summary questions, weekly portion/serving intakes of fruit, vegetables and tea slightly reduced the lowest mean daily total flavonoid intake (from 11.8 mg/day to 10 mg/day), as at such a low intake level the contribution of tea as total flavonoid source would be minimal. The highest mean daily total flavonoid intake value increased (from 2206.4 to 3010 mg/day) and at this high intake level it is expected that the consumption of tea would be a major contributory source to the intake and that adjustment on its weekly summary question value (39%) would have increased this value.

8.2.5.2 Group Level Validation

The relative validity of the adjusted baseline comprehensive FFQ dataset at group level was assessed by comparing its mean daily total flavonoid intake estimate with that of the baseline estimated dietary records. Although the mean (arithmetic) absolute daily total flavonoid intake estimate computed from the adjusted comprehensive FFQ was much higher (by 44.14%) than that computed from the dietary records, the difference in the absolute median and interquartile range daily intakes estimated by the questionnaire and by the six dietary records were not significantly (p>0.05) different (see Table 8.3) based on the results of the Wilcoxon's signed ranked test (p=0.702) for non-parametric data.

8.2.5.3 Individual Level Validation

The relative validity of the individual mean daily total flavonoid intakes, as calculated from the adjusted comprehensive FFQ and the estimated dietary records for the baseline period of the trial, was evaluated by the Spearman correlation coefficient because the datasets were not normally distributed and the Pearson's correlation coefficient based on the log-transformed data. Since the

day-to-day variation of intakes of many nutrients is substantial (38) and to correct for this within-subject variation using dietary records,(38,458) the Pearson and Spearman correlation coefficients were statistically adjusted to reduce the within- to between-person variation (496) (see Table 8.4). The Spearman correlation coefficient was 0.5 (p=0.0018) and the log-transformed Pearson's correlation coefficient 0.56 (p=0.0002) generally showing modest linear associations yet these correlations were both significant (p<0.05). The Spearman correlation coefficient did not differ appreciably from the log-transformed Pearson parametric statistic presented. The deattenuated Pearson's and Spearman correlation coefficients were 0.62 and 0.55, respectively. Deattenuation of the correlation coefficients between the adjusted comprehensive FFQ and the dietary records, which take within- to between-person variation in intake into account, appreciably improved the Pearson's correlation coefficient to a moderately strong level.

Agreement between the comprehensive FFQ corrected intakes and the estimated dietary record intakes was examined by categorizing the individual participants into quintiles and tertiles of intake according to each assessment method. The proportion of subjects grossly misclassified within the mean daily total flavonoid intake estimate quintile and tertile distributions of the dietary records and the adjusted comprehensive FFQ was 7.5% (3 participants) and 2.5% (1 participant), respectively with the *Kw* coefficients of agreement indicating moderate to substantial agreement (0.41 to 0.60) for both the quintile (*Kw*=0.493) and the tertile (*Kw*=0.529) groupings across the two dietary methods (see Table 8.5). The percentage participants classified in the same quintile or tertile of intake by both the adjusted comprehensive FFQ and the dietary records were 62.5% (n=25) and 80% (n=32), respectively (data not presented in Table 8.5).

Figure 8.5 shows the Bland-Altman plot for the adjusted comprehensive FFQ and estimated dietary record datasets within the baseline period of the rooibos intervention trial. Although the plotted points were predominantly within the 95% limits of agreement, the tendency of the data points to cluster around the zero value (zero difference), reduced with an increased intake illustrating that the adjusted comprehensive FFQ did not measure the intake well in participants with higher mean daily total flavonoid intakes. At intakes of 750 mg/day and above, the plotted difference points were distributed above the line of identity (solid line) representing a tendency of positive differences or overestimation of the adjusted comprehensive FFQ against the dietary records. In comparison to the unadjusted comprehensive FFQ Bland-Altman plot within the baseline trial period with the mean difference at +46.8 mg/day and the limits of agreement at +806.2 mg/day and -712.7 mg/day (Figure 8.1), the comprehensive FFQ adjusted for overestimation showing poorer agreement with the dietary records. Its mean difference was +151.6 mg/day and the limits of agreement at +1234 mg/day and -930.7 mg/day (Figure 8.5).
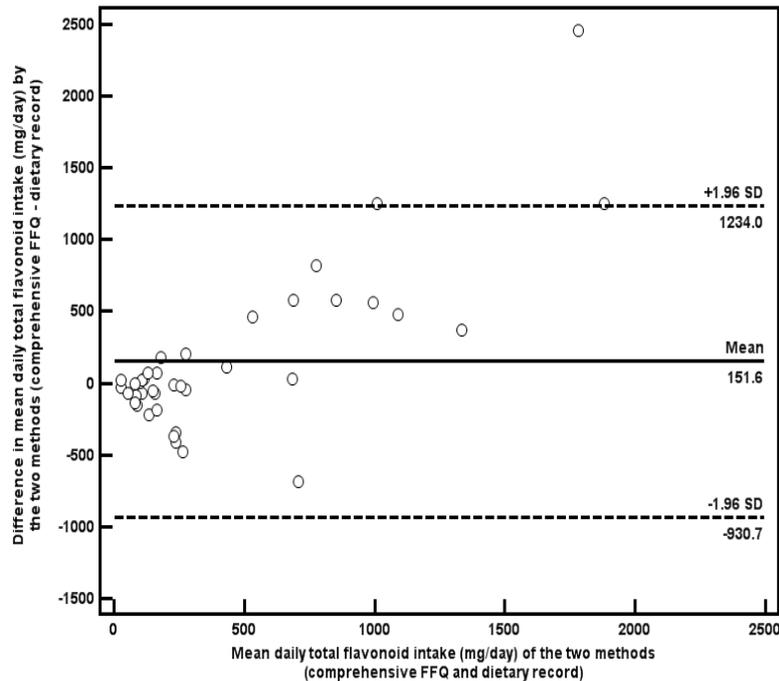
Figure 8.5: Bland-Altman Plot of Agreement between Flavonoid Intakes of Adjusted Comprehensive FFQ and Dietary Records within Baseline Period

8.2.6 Reproducibility Evaluation

To assess the reproducibility of the comprehensive FFQ within the rooibos intervention trial, the mean daily total flavonoid intake estimates in the washout trial period was considered against the control trial period by the questionnaire, as the stipulations for the flavonoid intake restrictions were alike for these two trial periods. At group level, the reproducibility was determined as the difference between the mean daily intakes at these two occasions and expressed as percentage intake of the washout trial period administration as the first administration occasion. A difference in mean daily total flavonoid intake of about 22% (+21.92%) between the comprehensive FFQ administered in the washout and the control trial periods two months apart was found, with the intake in the control trial period or the second occasion administration (28.54 mg/d) lower than that of the first administration in the washout trial period (36.55 mg/d) (see Table 8.3). The difference in the median and interquartile range intakes as determined by the Wilcoxon's signed rank test was not significant (p=0.0599).

The log-transformed Pearson's product moment and the Spearman rank order correlation coefficients were considered as measure of the comprehensive FFQ reproducibility at individual level. Both these correlation coefficients were moderately strong and significant at 0.44 (p=0.0044) and 0.55 (p=0.007), respectively with the Spearman correlation coefficient higher than that of the log-transformed Pearson correlation coefficient.   To evaluate the reproducibility of the

comprehensive FFQ to rank individuals by categories on mean daily total flavonoid intake, the participants' were classified into quintiles and tertiles of intake using each FFQ at a time. Although the reproducibility assessment of the comprehensive FFQ indicated the median paired differences between the two measurements to be non-significant (p>0.05), and the percentage of participants grossly misclassified between the two FFQ administrations considering the interquintile and intertertile ranges low (12.5% or 5 participants and 5% or 2 participants, respectively), both these ranges only reflect fair agreement (0.21 to 0.40) on the *Kw* statistic (0.361 and 0.291, respectively). Considering the quintile and tertile agreements, 65% (n=26) and 80% (n=32) of the participants were classified in the same quintile or tertile at the two administration occasions, respectively.

The Bland-Altman plot of the comprehensive FFQ administration within the washout and control trial periods showed good agreement with all but three of the participants within the 95% limits of agreement. The mean difference between the two administrations was 8 mg/day with agreeable limits at +69.1 mg/day and -53.1 mg/day for the upper and the lower limits, respectively (Figure 8.6).
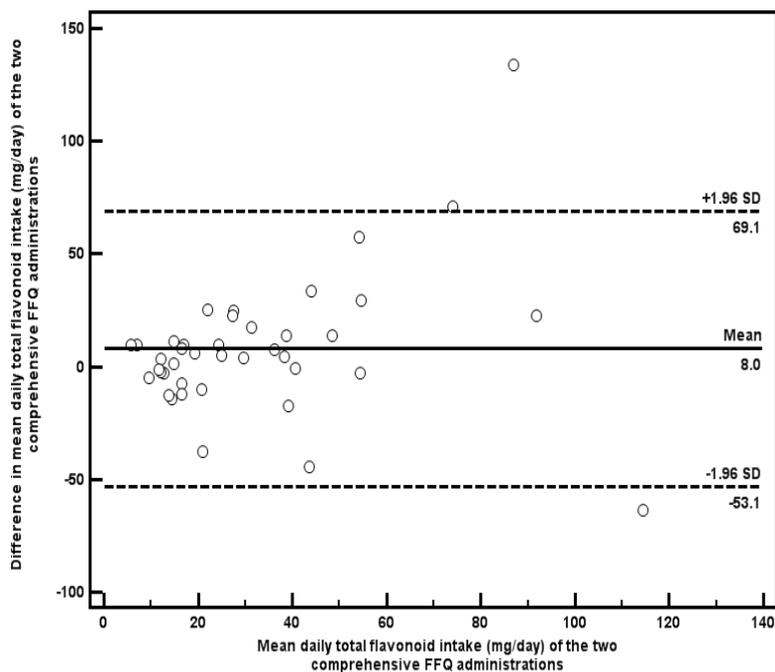
Figure 8.6: **Bland-Altman Plot of Agreement between Flavonoid Intakes of Comprehensive FFQ Administrations within Washout and Control Periods**

8.2.7 Responsiveness Evaluation

Responsiveness was used as a measure to evaluate the sensitivity of the instrument, in this case the comprehensive FFQ, to change as pointed out by Thomson et al.(49) As can be seen in

Tables 8.3 and 8.6, the mean daily total flavonoid intake at baseline decreased noticeably in the washout trial period, which could be presumed to be the result of the application of the intervention trial flavonoid intake dietary restriction stipulations by the participants. The intake then increased substantially in the intervention trial period due to the rooibos consumption that occurred in this trial period. Thereafter the intake decreased markedly in the control trial period as a result of the consumption of water in the place of the rooibos.

Table 8.6: **Mean Daily Total Flavonoid Intake Estimates (mg/day) of FFQ and Biomarker of Exposure and Effect Levels within Rooibos Intervention Trial as Measure of Responsiveness to Dietary Intake Changes (n=40)**

Responsiveness variables	Mean±SD for rooibos intervention trial periods				Significant differences[a]
	Baseline (1)	Washout (2)	Intervention (3)	Control (4)	
Mean daily total flavonoid intake (mg/day)					
Unadjusted Comprehensive FFQ	390.16±519.51	36.55±25.35	345.09±96.79	28.54±32.52	(1) – (2)(4) (2) – (3) (3) – (4)
Unadjusted Comprehensive FFQ excluding all tea consumption	68.82±80.15	36.55±32.52	33.54±32.21	28.37±24.74	(1) – (2)(3)(4)
Abbreviated FFQ	350.92±504.87	16.87±21.47	318.02±99.71	14.97±13.14	(1) – (2)(4) (2) – (3) (3) – (4)
Abbreviated FFQ excluding all tea consumption	38.80±71.74	15.54±20.85	14.38±14.16	13.46±12.47	(1) – (2)(3)(4)
Plasma biomarkers of total flavonoid intake exposure					
Total polyphenols (mg/L)[b]	85.7±11.04	72.3±10.21	89.8±14.15	79.8±16.89	(1) – (2) (2) – (3)(4) (3) – (4)
Plasma biomarkers of effect as measures of lipid peroxidation					
Conjugated dienes (CDs) (nmol/mL)[b]	121.3±24.3	134.7±20.6	108.8±21.1	167.3±29.5	(1) – (2)(3)(4) (2) – (3)(4) (3) – (4)
Malondialdehyde[c] (MDA) (µmol/L)[b]	1.95±0.74	1.65±0.73	0.86±0.26	1.88±0.58	(1) – (2)(3) (2) – (3) (3) – (4)

[a] Bonferroni correction for multiple comparisons for identification of the pair-wise contrasts on overall significance ($p < 0.001$) in the repeated measures analysis of variance (ANOVA)

[b] milligram = mg; liter = L; nanomol = nmol; milliliter = mL; micromol = µmol

[c] As measured by thiobarbituric acid reactive substances (TBARS)

Because of the repeated measurements design of the rooibos intervention trial, the treatment effect on the outcome variable, being the mean daily total flavonoid intakes, could be examined by the repeated measures ANOVA. The repeated measures ANOVA was used to determine any differences between the mean daily total flavonoid intakes as assessed by the comprehensive FFQ

across the rooibos intervention trial periods. After observing an overall significant (p<0.001) difference in the repeated measures ANOVA, the contrasts between the multiple mean daily total flavonoid intake values of the different trial periods were compared applying the Bonferroni correction for multiple comparisons for identification of the pair-wise contrasts. Significant differences (p<0.001) were revealed in the mean daily total flavonoid intakes between the baseline and the intervention trial periods in comparison to the washout and control trial periods, but not between the baseline and intervention trial periods and the washout and control trial periods (Table 8.6).

Kristal et al. (19) pointed out that the type of intervention being evaluated would affect the responsiveness. For instance, in an intensive clinical intervention the magnitude of dietary change may be so large that almost any dietary assessment tool will be at least reasonably responsive. According to Kristal et al.,(19) it may be desirable to use a less responsive tool in such studies. As the above scenario was true for the intervention trial due to the large contribution the consumption and avoidance of tea made to the mean total flavonoid intakes during the trial periods, all flavonoid intake contributions from tea were removed across all the trial periods (Table 8.7) to determine if the FFQ for the purpose of this research, would be responsive to the other flavonoid intake restrictions placed on the trial periods in comparison to the baseline trial period. After observing an overall significant (p<0.001) difference in the repeated measures ANOVA, the Bonferroni correction for multiple comparisons for identification of the pair-wise contrasts was performed, which produced significant (p<0.001) differences for the mean daily total flavonoid intakes of the baseline trial period versus that of the washout, intervention and control periods but no differences between the latter three trial period intakes (Table 8.6).

Table 8.7: **Descriptive Statistics for Daily Total Flavonoid Intake Estimates (mg/day) for FFQ Dietary Assessment at Rooibos Intervention Trial Periods with Flavonoid Intake with Tea Consumption removed (n=40)**

Rooibos intervention trial periods	Arith-metic Mean	Standard Deviation	Lowest value	Highest value	Median	Percentiles	
						25th	75th
Unadjusted Comprehensive FFQ							
Baseline	68.82	80.15	3.45	440.88	49.12	23.54	69.26
Washout	36.55	32.52	2.14	153.97	28.35	12.32	42.96
Intervention	33.54	32.21	1.90	175.82	24.50	16.71	41.40
Control	28.37	24.74	0.86	146.41	21.89	14.14	37.32
Abbreviated FFQ							
Baseline	38.80	71.74	0.94	423.58	21.28	9.65	33.77
Washout	15.54	20.85	0.12	122.20	9.15	4.59	18.18
Intervention	14.38	14.16	0.00	64.86	10.42	5.83	20.68
Control	13.46	12.47	0.27	56.93	9.12	5.40	18.45

8.3   FOOD LIST COMPILATION OF ABBREVIATED FFQ

Both the estimated dietary record and the comprehensive FFQ datasets of the rooibos baseline trial period were used to identify the dietary items to form the reduced food list of the abbreviated FFQ. All the reported dietary items from the multiple dietary records and also the comprehensive FFQ were first independently ranked on the basis of the percentage that each reported item contributed to the total participant group summed mean daily total flavonoid intake of each dataset. Thereafter, the stepwise multiple regression analysis approach was applied to the subset of top 24 items identified by the percentage contributing the most to the total absolute group intake in each dataset to identify the items that contributed largely to the between-person variability in intake or the most discriminating items in each dataset.(405,581)

Considering the percentage contribution approach, the largest dietary contributors of both the datasets to the mean daily total flavonoid sum were black tea ranked first providing approximately 68% of the intakes in both the datasets and rooibos ranked second providing 16.7% of the intake in the dietary record dataset and 13% in the comprehensive FFQ dataset. The following major dietary contributors to the daily total flavonoid intake in both the datasets were red wine, oranges, apples, berry and mixed berry fruit juice, green tea, bananas and naartjies (mandarins), although with some differences in the percentage contribution ranking besides for green tea and bananas, which ranked 8th and 9th, respectively, in both of the datasets. The only difference in the top 10 contributing dietary items to both the datasets were tropical fruit juice and tropical fruit juice blends that ranked 6th in the dietary record dataset (contributing 0.91%) and 13th in the comprehensive FFQ dataset (contributing 0.45%), and orange juice and orange juice blends ranked 10th in the comprehensive FFQ dataset (contributing 0.65%) and 21st in the dietary record dataset (contributing 0.15%). These nine dietary items ranked among the top ten major contributors to the total flavonoid intake in both the datasets contributed cumulatively to 94.3% of the total dietary record intake estimates and 94.5% of the total comprehensive FFQ intake estimates. The top identified dietary items in the dietary record and the comprehensive FFQ datasets, considering the percentage contribution approach and their ranking contribution to the total intake, are presented in Table 8.8.

As the next step, the top 24 items in each dataset identified by the percentage contribution approach that contributed most to the absolute intake were re-ranked by employing the stepwise multiple regression analysis to identify the specific items that accounted for most of the between-

Table 8.8: **Percentage Contribution, Ranking, Variance Analyses of Top Food and Beverage Items to Rooibos Baseline Trial Period Estimated Dietary Record and Comprehensive FFQ Summed Mean Daily Total Flavonoid Intake Estimate**

Food/Beverage item	Baseline estimated dietary records			Baseline comprehensive FFQ			Included in food list of abbreviated FFQ[d]
	Percentage contribution (%)[a]	Rank[a]	Stepwise regression analysis[b]	Percentage contribution (%)[a]	Rank[a]	Stepwise regression analysis[c]	
Tea, black brewed	67.98	1	<0.0001	68.10	1	<0.0001	Yes
Rooibos, brewed	16.69	2	<0.0001	13.02	2	<0.0001	Yes
Wine, red	3.20	3	<0.0001	2.34	5	<0.0001	Yes
Oranges, raw	2.60	4	<0.0001	3.29	3	<0.0001	No[f]
Apples, raw	1.18	5	0.0134	1.83	6	<0.0001	Yes
Juice, tropical and tropical blends	0.91	6	0.0002	0.45	13	–[e]	Yes
Juice, berry and mixed berry	0.85	7	<0.0001	2.40	4	<0.0001	Yes
Tea, green brewed	0.61	8	<0.0001	1.16	8	<0.0001	Yes
Bananas, raw	0.60	9	–[e]	0.88	9	0.0002	Yes
Naartjies, raw	0.57	10	0.0406	1.49	7	<0.0001	No[f]
Parsley, dried	0.50	11	0.0002	Outside top 20 ranking			Yes
Guava	0.43	12	–[e]	0.24	15	–[e]	No[f]
Apple cider	0.31	13	–[e]	0.13	19	0.0394	Yes
Juice, apple and apple blends	0.27	14	–[e]	0.48	12	–[e]	Yes
Pear halves, dried	0.24	15	–[e]	0.14	18	–[e]	Yes
Strawberry, raw	0.20	16	0.0001	Outside top 20 ranking			No[f]
Pears, raw	0.18	17	–[e]	0.48	11	<0.0001	Yes
Beer, regular, all	0.17	18	–[e]	Outside top 20 ranking			No[g]
Onions, raw	0.17	19	–[e]	Outside top 20 ranking			No[g]
Coffee, brewed	0.16	20	–[e]	Outside top 20 ranking			No[g]
Juice, orange and orange blends	0.15	21	–[e]	0.65	10	<0.0001	Yes
Onions, white cooked in mixed dish	0.14	22	–[e]	0.20	16	0.0122	Yes
Chocolate, milk	0.14	23	–[e]	0.13	20	–[e]	Yes
Tomato, raw	Outside top 20 ranking			0.11	23	0.0002	No[g]

[a] Ranked on basis of percentage of summed mean daily total flavonoid intake of each dataset

[b] Estimated dietary record items capturing the between-person variation: R^2 = 0.9994 as the percent between-person variation explained

[c] Comprehensive FFQ items capturing between-person variation: R^2 = 0.9999 as the percentage between-person variation explained.

[d] Abbreviated FFQ food list: N=16: Other beverages (tea and herbal tea), n=3 with black, green, rooibos; Alcoholic beverages, n=2 with apple cider and red wine; Fruit juices, n=4 with apple, berry, orange, tropical juice blends; Fruit fresh, n=3 with apples, pears, bananas; Fruit dried, n=1 with pears; Vegetables, n=1 as onions cooked as mixed dish ingredient; Herbs, n=1 as parsley dried; Chocolate, n=1 as milk chocolate.

[e] Item not extracted in the stepwise multiple regression analysis to account for the between-person variability in intake

[f] Item not included due to seasonal availability

[g] Item not included as it was not a contributor to absolute intake or between-person variability in intake in either of the datasets, but giving predilection to the between-person variability in intake and the dietary record dataset.

person variability in intake.(395,411)   A list of items from the original ranked dietary record and comprehensive FFQ lists of 24 dietary items were extracted, retaining the foods explaining at least 99% of the intake intervariability in both with the cumulative R-square (R^2) statistic for the two lists so obtained reaching a value of 0.99 in both.   The models explaining 99% of the intake intervariability identified a total of 11 items in the dietary record dataset and 14 items in the comprehensive FFQ dataset, which were important predictors of the between-person variation in intake.   Of the 11 items in the dietary records and of the 14 items in the comprehensive FFQs that accounted for 99% of the variance for the daily total flavonoid intake within these datasets 10, in the case of the dietary record dataset, and 11, in the case of the comprehensive FFQ dataset, were included among the top 11 contributors to the absolute intake of each dataset (at a cumulative percentage of 94.8% and 95.6%, respectively in each dataset).   In other words, for the mean daily total flavonoid intake, 10 items accounted for 94.8% of the absolute intake and 11 items for 99% of the total variance in intake of the dietary record intake estimates, while 11 items accounted for 95.6% of the absolute intake and 14 items for 99% of the total variance in intake of the comprehensive FFQ intake estimates.

Eight of these foods were included in both extraction lists. Black tea, rooibos, red wine, oranges, apples, berry and mixed berry fruit juice, green tea and naartjies were the eight dietary items extracted within both lists. These specific items thus did not only contribute considerably to the between-person variation in intake in both the datasets, but all occurred among the top contributors to the absolute intake in both the datasets.

The items included in the abbreviated FFQ food list were a mixture of those items that occurred among the top contributors to the absolute intake and/or those that contributed considerably to the between-person variation in intake. Seasonally available items were excluded, as it was believed that they might restrict the use of the abbreviated FFQ. As a result, oranges and naartjies were excluded from the food list although both these items were among the highest contributors to the absolute intake and contributed considerably to the between-person variation in intake in both the datasets.

Hertog et al. (111) found that seasonal variability (over spring, summer and winter) in flavonoid intake was low because the consumption of the major contributor's tea, apples and onions did not vary between the seasons. Fowke et al. (363) stated that subjects reported the greatest consumption of watermelon in the fall that was attributed to its late-summer availability. Such consumption based on seasonal availability may have an impact on the consumption of dietary components like flavonoids. Knekt et al.,(262) during a follow-up study in Finland, found a change in flavonoid intakes that they ascribed to the increase in consumption of fruit and vegetables. Such changed fruit and vegetable intakes will impact the consumption of dietary components like

flavonoids. These study results supported the thought that retaining oranges and naartjies in the abbreviated FFQ food list would impact its general usability, as the seasonal availability of oranges are from May to September and naartjies April to July with the peak availability for these citrus fruits from June to August (3 months) and May (1 month), respectively.(544) However, Stryker et al. (395) found that omission of the top five foods contributing to absolute intake, markedly decreased the effectiveness of a list of 20 foods to assess nutrient intake.

As the exclusion of oranges and naartjies in the food list reduced the cumulative percentage contribution by 3.2% in the dietary record dataset and 4.8% in the comprehensive FFQ dataset, and an abbreviated food list of about 15 to 20 items was envisaged, the food list was extended to make up for the flavonoid intake reduction brought about by the exclusion of oranges and naartjies. Those items that were identified in the datasets to either be among the top 20 item contributors to the absolute intake or contributed to the between-person variation in intake in either of the datasets were included thereby giving predilection to the between-person variation in intake and dietary record dataset and again excluding identified seasonally available foods among these items. As a result, guava and strawberry were excluded from the food list despite meeting the above inclusion criteria.

Based on the above, a food list of 16 items extended to 23 was compiled for the abbreviated FFQ. The items included are: black tea, green tea and rooibos (plain and flavoured) with the ice tea versions added to each of these items (n=3 extended to 6) due to their local availability and accepted flavonoid content ("other beverages"); apple cider and red wine (alcoholic beverages: n=2); apple, berry, orange and tropical fruit juice and such fruit juice blends (fruit juices: n=4); apples, bananas and pears (fruit, fresh: n=3 extended to 5 by specifying apples and pears with and without skin/peel to strengthen the between-person variability in absolute intake); dried pear halves (fruit, dried: n=1); onions, cooked as mixed dish ingredient (vegetables: n=1 extended to 2 by adding onion, cooked as side or main dish ingredient/whole portion, as such consumption will make a contribution to the mean daily total flavonoid intake); dried parsley (herbs: n=1); milk chocolate (chocolate: n=1 to which dark chocolate was added and extended to 2) (see Addendum C, abbreviated FFQ).

Dark chocolate was added to the food list as it was not nutritionally logical to include milk chocolate, although consumed more commonly than dark chocolate, but having substantially lower total flavonoid content than dark chocolate.(21,155) Arts et al. (155) established that dark chocolate has a total catechin content of about 53.5 mg per 100 g compared to milk chocolate that contains about 15.9 mg per 100 g.(120) Dark chocolate seems to be potentially more beneficial to human health than milk chocolate (120) and further substantiates its addition. Van Assema et al. (494) in considering that over time changes might occur in dietary habits, that new products might

be introduced to the food market and that the key food sources are subject to change, revised their originally developed food list to assess total fat intake. Six of the 10 questions added in the revision to the original food list assessed intake of chocolate and chocolate products, a food group not represented in the original questionnaire. This revision further substantiated expanding the chocolate inclusions by adding dark chocolate to the abbreviated FFQ food list.

Dark chocolate itself was ranked 32nd in the dietary record dataset and 33rd in the comprehensive FFQ dataset contributing 0.06% to the absolute mean daily total flavonoid intake sum of both the datasets. In the process of compiling a short food list for the dietary estimate of interest, additional criteria may be used or other reasons considered in adding items to fully determine absolute intakes and/or explain between-person variations in the intake. Therefore the basic list may be extended (405,412) as was done with the food list extension of this developed abbreviated FFQ. The 16 dietary items included in the food list together accounted for 94% (93.95%) of the dietary record and 92% (92.41%) of the comprehensive FFQ intake estimates, providing overall for a cumulative total of approximately 93%, as achieving absolute intake is specifically important for intervention trials.(398)

8.4   EVALUATION OF ABBREVIATED FFQ WITHIN ROOIBOS INTERVENTION TRIAL GROUP

8.4.1   Daily Total Flavonoid Intake Estimates

The group means (arithmetic and geometric), SDs, medians, lowest and highest values and the 25th and 75th percentiles of the daily total flavonoid intakes were computed for the abbreviated FFQ within each of the rooibos intervention trial periods. The comprehensive FFQ dataset was used for determining these values, but only considering the intake contributions of those dietary items included in the abbreviated FFQ food list. The mean daily total flavonoid intake estimations across the trial periods were lower on the whole for the abbreviated FFQ determinations compared to the comprehensive FFQ and the dietary record estimates with the exception of the baseline trial period dietary record computation (2.2% higher) (see Table 8.3). This is to be understood due the inclusion of only 16 dietary items extended to 23 items in the abbreviated FFQ food list compared to the inclusion of all items consumed in the full comprehensive FFQ and the estimated dietary records.

The mean daily total intake estimate of the baseline trial period as determined by the abbreviated FFQ (350.9 mg/day), although higher (2.2%), was very close to the intake estimate of the dietary records (343.4 mg/day) for this trial period. The abbreviated FFQ captured 89.94% of the computed full comprehensive FFQ mean daily total flavonoid intake for the baseline trial period. The mean daily total flavonoid intake captured by the abbreviated FFQ for the intervention trial

period was very close to that determined by the comprehensive FFQ (92.2%) and the dietary records (92.7%). In the other trial periods, the abbreviated FFQ captured roughly half of the mean daily total flavonoid intake as determined by the comprehensive FFQ and the dietary records, 59.1% and 53.9%, respectively, for the washout and 40.9% and 54.6%, respectively, for the control trial period (see Table 8.3).

8.4.2 Validity Evaluation against Dietary Records

The between-method agreement of the abbreviated FFQ was assessed at group level by comparing its computed group mean daily total flavonoid intake estimate with that of the estimated dietary records within each of the trial periods. The results indicated that the abbreviated FFQ and the dietary records agreed remarkably on average intake, with negligible mean difference (+2.16%) at the baseline trial period. Mean differences in intakes assessed by both the methods were comparable in the intervention trial period (-7.33%), but differed greatly and to the same degree for the washout (-46.09%) and control (-45.42%) trial periods (see Table 8.3). The statistical agreement of these group intake results were investigated using the Wilcoxon's signed rank test for the baseline, washout and control trial periods due to the non-normal distribution of the data and the paired samples t-test for the intervention trial period due to the normal distribution of the data. Although no significant ($p > 0.05$) differences were found for the intakes as assessed by the abbreviated FFQ and the dietary records for the baseline ($p = 0.2213$, Wilcoxon's signed rank test) and intervention ($p = 0.6154$, paired samples t-test) trial periods, significant differences occurred between the medians and interquartile ranges as estimated for the washout ($p = 0.0001$, Wilcoxon) and control ($p < 0.0001$, Wilcoxon) trial periods (see Table 8.3).

The ability of the abbreviated FFQ to rank the participant intakes against that of the estimated dietary records were tested using the Pearson correlation coefficient for the intervention trial period and the log-transformed Pearson and Spearman correlation coefficients for the other trial periods. These correlation coefficients were adjusted for attenuation due to within- and between-person variation (496) in the dietary record data. The data showed a significant degree of association for the baseline, intervention and control trial periods, but not for the washout trial period. The log-transformed Pearson correlation coefficients were slightly higher than the Spearman correlation coefficients in the baseline period as 0.62 ($p < 0.001$) versus 0.59 ($p = 0.0002$) and the control period as 0.6 ($p < 0.0001$) versus 0.59 ($p = 0.0002$). These correlation relationships were moderately strong (ranging from 0.59 to 0.62), except for the particularly strong relationship (0.81) found in the intervention period with the Pearson correlation coefficient. The Spearman correlation coefficient (0.27, $p = 0.0897$) was higher than that of the log-transformed Pearson correlation coefficient (0.18, $p = 0.2581$) in the washout trial period. Both these correlations were low (about 0.2) and non-

significant (p>0.05). Deattenuations of the correlation coefficients improved the correlations of the washout and in particular the control trial periods (see Table 8.4).

The ability of the abbreviated FFQ to correctly classify the participants into broad categories of intake were tested by cross-classifying the participants by both the questionnaire and the estimated dietary records into quintiles and tertiles of intake and considering those classified in the extreme opposite categories of intake by both the methods. In the baseline, intervention and washout trial periods, only a small number of participants were grossly misclassified into the extreme opposite quintile or tertile of intakes by both methods (see Table 8.5). The level of quintile and tertile agreement between the two methods as assessed using the *Kw* statistic were found to be moderate (0.41 to 0.60) for the baseline (0.469 and 0.424, respectively) and the control (0.453 and 0.515, respectively) trial periods and good (0.61 to 0.80) for the intervention trial period (0.712 and 0.604, respectively). Both the quintile and the tertile level agreements (0.055 and 0.042, respectively) between the two methods as assessed using the *Kw* statistic were poor (<0.20) for the washout trial period (see Table 8.5). The percentage of participants classified in the same quintile or tertile of intake by both the abbreviated FFQ and the dietary records were above 50% within each of the rooibos intervention trial periods, except for the quintile agreement (40% agreement) of the washout trial period (data not presented in Table 8.5).

To examine the agreement in the estimates of the daily mean total flavonoid intake of the abbreviated FFQ and the dietary records within the rooibos intervention trial, the Bland-Altman method (498) of plotting the differences between the participant mean estimates computed by these two methods against the average of the two methods were applied (Figures 8.7 to 8.10). The agreement overall was good, with all but one (in the washout trial period) (Figure 8.8), two (baseline and intervention trial periods) (Figures 8.7 and 8.9) and four (in the control trial period) (Figure 8.10) of the participants within the 95% limits of agreement.

The mean difference was +7.4 mg/day with the limits of agreement +764.8 mg/day and -750 mg/day for the baseline trial period (Figure 8.7), -14.4 mg/day with the limits of agreement of +43 and -71.8 mg/day for the washout trial period (Figure 8.8), -25.2 mg/day with the limits of agreement of +89.7 and -140 mg/day for the intervention trial period (Figure 8.9), and -12.5 mg/day with the limits of agreement of +21.3 and -46.2 mg/day for the control trial period (Figure 8.10). In the baseline and the intervention trial periods the scatter of the differences between the methods did not agree equally across the range of mean values. At intakes of above 500 mg/day in both trial periods, a tendency of positive differences between the intake estimates of the abbreviated FFQ and the dietary records or overestimation emerged. In the washout and control trial periods, with the more restricted intake range, the differences between the estimates from the abbreviated FFQ and the dietary records showed a negative tendency at higher mean intakes.
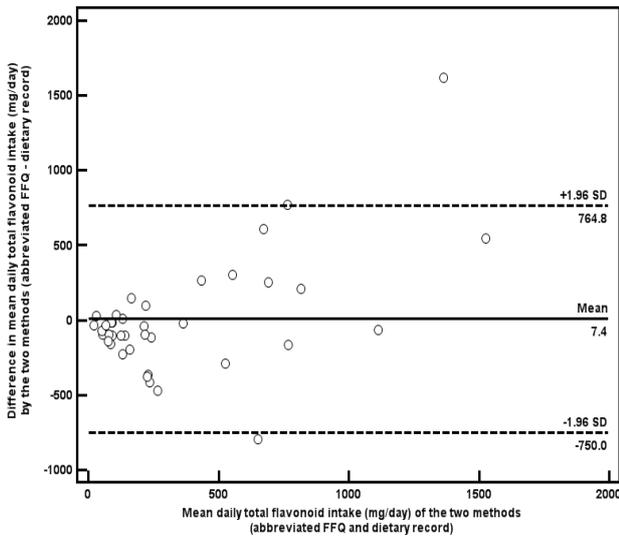
Figure 8.7: **Bland-Altman Plot of Agreement between Flavonoid Intakes of Abbreviated FFQ and Dietary Records within Baseline Period**
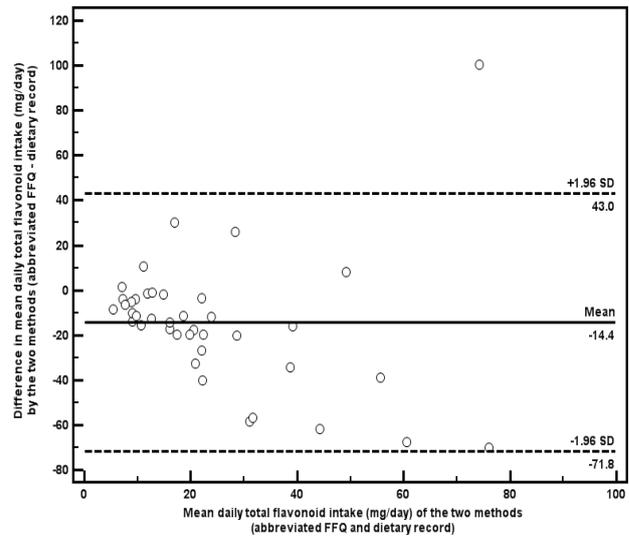
Figure 8.8: **Bland-Altman Plot of Agreement between Flavonoid Intakes of Abbreviated FFQ and Dietary Records within Washout Period**
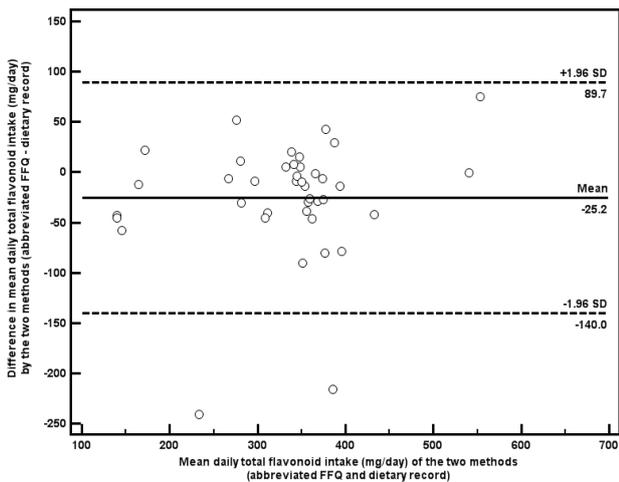
Figure 8.9: **Bland-Altman Plot of Agreement between Flavonoid Intakes of Abbreviated FFQ and Dietary Records within Intervention Period**
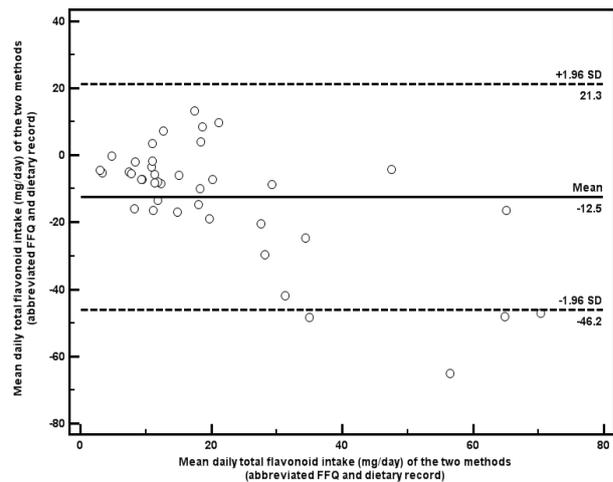
Figure 8.10: **Bland-Altman Plot of Agreement between Flavonoid Intakes of Abbreviated FFQ and Dietary Records within Control Period**

8.4.3 Reproducibility Evaluation

As for the comprehensive FFQ, the reproducibility of the abbreviated FFQ was evaluated by determining the agreement in the group daily total flavonoid intakes and the ability to rank the participant intakes by comparing the individual intake values and the intake values classified into quintiles and tertiles of intake. The latter intake was obtained from the abbreviated FFQ at the washout trial period and compared with that obtained from the abbreviated FFQ at the control trial

period eight weeks apart due to the dietary flavonoid intake restrictions being similar between these two trial periods.

Although the group mean daily flavonoid intake as determined from the abbreviated FFQ was 12.7% higher at the washout trial period (16.87 mg/day) compared to that of the control trial period (14.97 mg/day) (see Table 8.3), the group median and interquartile ranges were not significantly (p=0.5633) different as determined by the Wilcoxon's signed rank test for non-normally distributed data. The log-transformed Pearson product moment and the Spearman rank order correlation coefficients between the participant daily mean total flavonoid intake estimates as calculated from the abbreviated FFQs at the washout and the control trial periods, were both moderately strong and significant (r=0.4858, p=0.0015 and r=0.575, p=0.0003 respectively) with the Spearman correlation coefficient higher than that of the log-transformed Pearson correlation coefficient.

The proportion (%) of subjects classified into the extreme quintiles and tertiles of daily total flavonoid intakes between the two trial periods were 17.5% (n=7) considering the interquintile range and considering the intertertile range 2.5% (n=1). However, both the quintile and tertile cross-classifications reflect poor inter-test agreement (<0.2) between the two dietary assessment trial periods on the *Kw* statistic (0.159 and 0.018, respectively), despite that 52.5% (n=25) and 70% (n=28) of the participants were classified correctly in the same quintile or tertile, respectively, by the abbreviated FFQ administrations in these two trial periods.

The mean difference in the mean daily total flavonoid intake estimates of the abbreviated FFQ applications within the washout and control trial periods was negligible (+1.96 mg/day) with values for the upper (+43.3 mg/day) and the lower (-39.5 mg/day) agreement limits. All the plotted points, with the exception of one participant, were within the 95% limits of agreement with the data points clustering around the line of identity (Figure 8.11).
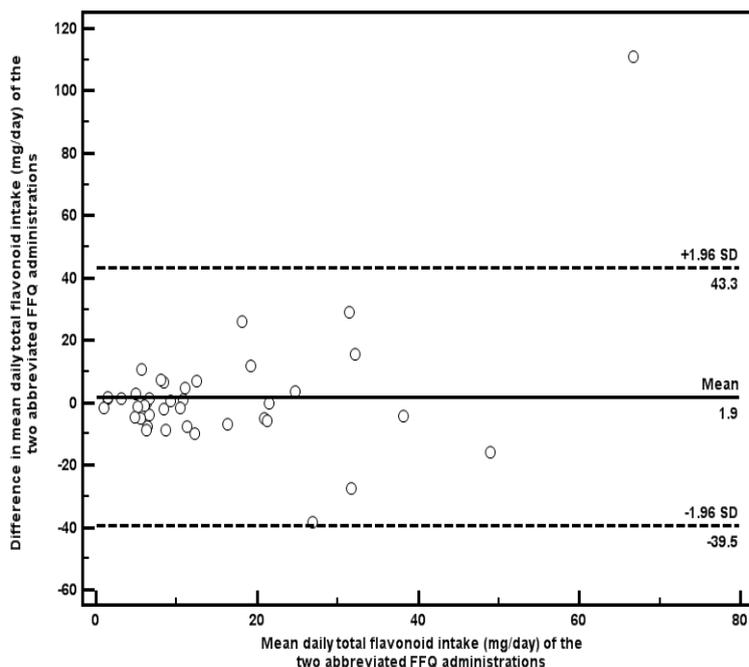
Figure 8.11: Bland-Altman Plot of Agreement between Flavonoid Intakes of Abbreviated FFQ Administrations within Washout and Control Periods

8.4.4   Responsiveness Evaluation

In accordance with Table 8.3, the mean daily total flavonoid intake at baseline noticeably decreased in the washout trial period, which could presumably be attributed to the participants carrying out the intervention trial flavonoid intake restrictions.   The intake then increased substantially in the intervention trial period due to the rooibos consumption that occurred in this trial period.   Thereafter, the intake noticeably decreased in the control trial period as a result of the consumption of water in the place of the rooibos. The repeated measures ANOVA was applied to determine any differences between the mean daily total flavonoid intakes as assessed by the abbreviated FFQ across the rooibos intervention trial periods. After observing an overall significant ($p<0.001$) difference in the repeated measures ANOVA, the Bonferroni correction for multiple comparisons for identification of the pair-wise contrasts between the different trial periods was performed, which revealed significant ($p<0.001$) differences in the mean daily total flavonoid intakes between the baseline trial period and the washout and control trial periods, the washout trial period and the baseline and intervention trial periods, the intervention trial period and the washout and control trial periods, and the control trial period and the baseline and intervention trial periods (see Table 8.6).

Due to the expected large contribution the consumption and avoidance of tea may make to the mean total flavonoid intakes during the trial periods, all tea flavonoid intake contributions were removed across all the trial periods (see Table 8.7) to determine if the abbreviated FFQ would be

responsive to the other flavonoid intake restrictions positioned on the trial periods in comparison to the baseline trial period. After observing an overall significant (p<0.001) difference in the repeated measures ANOVA, the Bonferroni multiple comparisons for identification of the pair-wise contrasts between the different trial periods was performed. Here significant differences (p<0.05) were found for the mean daily total flavonoid intakes of the baseline trial period versus that of the washout, intervention and control periods, but not between the intakes of the latter three periods (see Table 8.6).

8.5 EVALUATION OF COMPREHENSIVE AND ABBREVIATED FFQs WITHIN ROOIBOS INTERVENTION TRIAL GROUP AGAINST BIOMARKERS AT BASELINE PERIOD

8.5.1 Validity Evaluation

Linear associations were examined between the participant plasma levels of the selected biomarkers of exposure and of effect with their mean daily total flavonoid intakes from the unadjusted comprehensive FFQ and its resultant abbreviated FFQ as obtained for the baseline period of the rooibos intervention trial. Fasting plasma total polyphenols and the TAC served as biomarkers of flavonoid exposure and plasma CDs and TBARS as biomarkers of the dietary flavonoid intake effect (Table 8.9).

Table 8.9:   Associations between FFQ Mean Daily Total Flavonoid Intakes and Selected Biomarkers of Exposure and Effect for Baseline Period of Rooibos Intervention Trial (n=40)

Rooibos baseline trial period biomarkers	Rooibos baseline trial period mean daily total flavonoid intakes			
	Unadjusted comprehensive FFQ		Abbreviated FFQ	
	r	p-value	r	p-value
Plasma biomarkers of total flavonoid intake exposure				
Plasma total polyphenols (mg/L)[ab]	0.019	0.9077	0.028	0.8619
Ferric reducing ability of plasma (FRAP) (µmol AAE)/L)[ab]	0.198	0.2208	0.169	0.2965
Oxygen radical absorbance capacity (ORAC) (µmol TE)/L)[ab]	0.178	0.2677	0.171	0.2927
Trolox equivalent antioxidant capacity (TEAC) (µmol TE/L)[b]	0.217	0.1796	0.187	0.2470
Plasma biomarkers of effect as measures of lipid peroxidation				
Conjugated dienes (CDs) (nmol/mL)[ac]	0.048	0.7689	0.061	0.7073
Malondialdehyde (MDA)[d] (µmol/L)[c]	-0.287	0.0726	-0.261	0.1040

[a] milligram = mg; liter = L; micromol = µmol; ascorbic acid equivalents = AAE; Trolox equivalents = TE; nanomol = nmol; milliliter = mL

[b] Pearson correlation coefficients

[c] Log-transformed Pearson correlation coefficients

[d] Malondialdehyde measured as thiobarbituric acid reactive substances (TBARS)

A weak negative and non-significant (p>0.05) correlation was observed between the mean daily total flavonoid intake estimates of both questionnaires and the plasma TBARS (r= -0.29, p>0.05 for the comprehensive FFQ; r= -0.26, p>0.05 for the resultant abbreviated FFQ) and even weaker and non-significant (p>0.05) positive correlations between the mean daily total flavonoid intake estimates of both questionnaires and the plasma TAC determinations (r=0.2, p>0.05 for both the comprehensive FFQ and resultant abbreviated FFQ on the FRAP, ORAC and TEAC assay determinations) (Table 8.9). These correlations found are in line with the weak correlations often reported between dietary intake estimates and biomarkers and does not implicate a lack of validity.

8.5.2 Responsiveness Evaluation

As no significant (p > 0.05) difference was observed between the plasma TAC as determined by either the FRAP, ORAC or TEAC assays between the intervention trial period and that of the washout and/or the control trial periods (37) as determined by the repeated measures ANOVA Bonferroni comparisons for identification of pair-wise contrasts, the TAC as biomarker of exposure was not considered for the responsiveness evaluation. The repeated measures ANOVA and the Bonferroni identification of the pair-wise contrasts between the different trial periods revealed significant (p<0.001 for each) differences in the plasma total polyphenols, the CDs and the TBARS, in particular, between the intervention trial period versus that of the washout and control trial periods. The plasma total polyphenol level was highest in the intervention trial period followed closely by that of the baseline trial period where the mean daily total flavonoid intakes were also higher with the lower plasma levels in the washout and control trial periods where the mean daily total flavonoid intakes were the lowest. In contrast, the plasma CD level and the plasma TBARS level were both significantly (p<0.0001) lower in the intervention trial period compared to the other trial periods including that of the baseline period (see Table 8.6), where 65% and 45.7% of the control trial period and 80.8% and 52.1% of the washout trial period values, respectively were found. The changes in the biomarker levels relate to the changes in the mean daily total flavonoid intake estimates across the trial periods as assessed by the FFQs. This supports FFQs being responsive to changed conditions of dietary flavonoid intake.

8.6 EVALUATION OF ABBREVIATED FFQ WITHIN FURTHER PARTICIPANT GROUP

8.6.1 Sample Profile

Ninety-nine free-living subjects were approached to voluntarily participate in this phase of the study. All agreed to participate on being informed about the research details. No subject was following a specific diet at the time of the research, although some subjects indicated paying attention to their dietary intake mainly for health reasons. Of the group of volunteers who enrolled, two subjects withdrew due to changes in their eating habits during the execution of the study. One

withdrew due to the completion of the dietary records which she claimed had her eating more than usual and one due to not completing the dietary records because of the illness of her husband and eating differently. The data of another seven subjects were excluded from the analyses, as they did not submit all the required dietary records.

The convenient sample for this validation study therefore was comprised of 90 subjects who willingly answered the self-administered FFQ at both opportunities and completed all six the dietary records in the language of their choice (English or Afrikaans). The follow-up rate was 90.9%, which is considered high since keeping dietary records for a number of days and completing a questionnaire twice according to Erkkola et al. (390) requires motivation. The sample of 90 subjects is a reasonable size for a validation study.(387) The number of participants per the geographical area was: 28 for Cape Town; 20 for Paarl; 16 for Clanwilliam; and 26 for Graaff-Reinet.

The subjects represented the population for which the questionnaire was designed as all matched at least one or more of the investigated risk factors for CHD (advanced age, smoking, stressed at work, stressed at home, waist circumference indicative of abdominal obesity, personal history of raised blood pressure, personal history of elevated blood glucose, personal history of abnormal blood lipids and/or family history of CHD) and/or did not comply with the protective factors (daily fruit and vegetable consumption, being physically active and moderate weekly alcohol use) to various extents. Most participants had either one (22.2%) or two (24.4%) risk factors followed by three (17.8%) and four (12.2%) risk factors. Ten (11.1%) of the participants had no risk factors, but neither of these participants met all three the protective factors. Only two participants met all three the protective factors.

Table 8.10 presents an indication of the participant risk and protective factor occurrence for CHD. While 74.4% of the participants indicated to have never smoked and 65.6% indicated being physically active, only about a third (33.3%) indicated consuming fruit and vegetables daily and very few (17.8%) consuming alcohol three or more times per week. Most of the participants either considered themselves having an optimal or normal body weight status (47.8%) or being overweight (40%). While 39.3% of the women reported having a waist circumference larger than 88 cm, 48.3% of the men indicated having a waist circumference greater than 102 cm that is indicative of abdominal obesity.(592)

Overall approximately 36.1% of those employed reported being stressed at work most of the time, while only 13.3% reported being stressed at home most of the time. While many of the participants reported having a family history of CHD (58.9%), hypertension (62.2%) or diabetes mellitus (45.6%), some participants reported a personal history of abnormal blood lipids (17.8%), raised

blood pressure (24.4%) or blood glucose (12.2%) levels and were therefore watching their dietary intakes as they were not taking any prescription medication.

Table 8.10: **Profile and Coronary Heart Disease Risk Factor Characteristics of Further Participant Group (n=90)**

Demographic and CHD risk factor characteristics		% (n)	Demographic and CHD risk factor characteristics		% (n)
Gender[a]	Female	67.8 (61)	Stressed at work[a]	Never	11.1 (10)
	Male	32.2 (29)		Some times	48.9 (44)
				Most of the time	21.1 (19)
				Permanently / Always	12.2 (11)
				Not applicable	6.7 (6)
Age[a]	< 45 years	66.6 (60)	Stressed at home[a]	Never	25.6 (23)
	46 - 55 years	20.0 (18)		Some times	61.1 (55)
	56 - 65 years	6.7 (6)		Most of the time	8.9 (8)
	66 - 70 years	– (0)		Permanently / Always	4.4 (4)
	> 70 years	6.7 (6)			
Education	Gr. 11 (& below)	11.1 (10)	Body weight status[a]	Underweight	5.5 (5)
	Gr. 12 (Matric)	25.6 (23)		Normal body weight	47.8 (43)
	Gr. 12 & Certificate	11.1 (10)		Slightly overweight	40.0 (36)
	Gr. 12 & Diploma	23.3 (21)		Obese	6.7 (6)
	Gr. 12 & Degree	24.4 (22)	Waist circumference (abdominal obesity)[a]	Female: <88 cm	41.1 (37)
	Postgraduate	4.4 (4)		Female: > 88 cm	26.7 (24)
				Male: <102 cm	16.7 (15)
				Male: >102 cm	15.5 (14)
Occupation	White collar[b]	75.5 (68)	Health history: Raised blood pressure[a]	Yes	24.4 (22)
	Blue collar[b]	17.8 (16)		No	75.6 (68)
	Retired	6.7 (6)			
Weekly fruit & vegetable consumption[c]	Every day of week	33.3 (30)	Health history: Elevated blood glucose[a]	Yes	12.2 (11)
	Most days (4+)	35.6 (32)		No	87.8 (79)
	Few days (3 or less)	31.1 (28)			
Weekly alcohol use[c]	Less than 3X p.w.	82.2 (74)	Health history: Abnormal lipids[a]	Yes	17.8 (16)
	3+ times p.w.	17.8 (16)		No	82.2 (74)
Smoking status[a]	Non-smoker	74.4 (67)	Family health history: Hypertension	Yes	62.2 (56)
	Current smoker[d]	23.3 (21)		No	37.8 (34)
	Former smoker[d]	2.2 (2)			
Physically active[c]	Yes[e]	65.6 (59)	Family health history: Diabetes mellitus	Yes	45.6 (41)
	No	34.4 (31)		No	54.4 (49)
			Family health history: CHD	Yes	58.9 (53)
				No	41.1 (37)

NOTE: Gr. = Grade; p.w. = per week; CHD = Coronary heart disease

[a] As adverse factors smoking, raised blood pressure (self-reported history), elevated blood glucose (self-reported history), abnormal lipids (self-reported history), abdominal obesity,(586) psychosocial factors,(587) family history of CHD,(595) along with gender and advancing age (men over 45 years and women over 55 years) (551)

[b] White-collar employees as professional staff, technical staff, administrative staff and other staff with functionaries medium and high versus blue collar workers with lower functionaries performing skilled and unskilled labour (599)

[c] As protective factors, daily consumption of fruit and vegetables, regular alcohol consumption (three or more times a week) and regular physical activity (four or more hours a week moderate or strenuous physical exercise) (586)

[d] Current smoker as persons who smoked in the past 12 months and those who quit smoking within the past year; Former smoker as persons who quit smoking more than a year ago (586)

[e] Being physically active meant regular moderate exercise (e.g., walking, cycling or gardening) or strenuous exercise (e.g., jogging, football and vigorous swimming) for four hours or more a week (586)

The socio-demographic characteristics of the participants were provided. The sample was predominantly female (67.8%) with most (66.6%) participants within the age grouping younger than 45 years. The participants represented a wide range of occupations mostly representing the ISCO major occupational groups of legislators, senior officials and managers (12.2%), professionals (8.9%), technicians and associate professionals (21.1%), clerks (21.1%) and shop workers and shop and market sales workers (12.2%), that were classified to represent either the white or the blue collar occupational grouping,(599) with the education level relatively high as nearly two thirds (63.3%) of the participants obtained a post Grade 12 educational qualification (Table 4.10).

8.6.2   Daily Total Flavonoid Intake Estimates

The further participant group mean (arithmetic and geometric) and the 25th and 75th percentile daily total flavonoid intakes for both the abbreviated FFQ and the estimated dietary record administrations are greatly alike. However, the dietary record values were slightly lower than that of both the abbreviated FFQ administrations, and the second abbreviated FFQ administration was slightly higher than those computed from the first abbreviated FFQ administration and the dietary records. The mean daily total flavonoid intake and standard deviation as estimated from the different dietary assessment methods was respectively: 234.74±259.23 mg/day (range 6.33–1442.80 mg/day) for the first abbreviated FFQ administration; 223.40±235.10 mg/day (range 1.86–1297.36 mg/day) for the estimated dietary records; and 245.63±277.48 mg/day (range 6.25–1791.77 mg/day) for the second abbreviated FFQ administration (Table 8.11).

Table 8.11:   **Central Tendencies, Standard Deviation, Percentile Scores for Daily Total Flavonoid Intake Estimates (mg/day)[a] for Dietary Assessment Methods and Comparisons within Further Participant Group (n=90)**

Dietary assessment methods	Arith-metic Mean	Geo-metric Mean	SD Standard Deviation	Lowest value	Highest value	Median	Percentiles		Differences assessment methods[b]
							25th	75th	
Estimated dietary records (DR)	223.40	119.65	235.10	1.86	1297.36	163.55	53.40	291.92	ND
Abbreviated FFQ (Before)	234.74	132.92	259.23	6.33	1442.80	139.58	58.70	305.46	ND
Abbreviated FFQ (After)	245.63	132.54	277.48	6.25	1791.77	152.07	55.72	310.04	ND

[a] Back-transformed data after logarithmetic transformation

[b] Comparisons for differences: DR vs FFQ-Before; DR vs FFQ-After; FFQ-Before vs FFQ-After; ND = No significant difference (p>0.05)

8.6.3 Validity Evaluation of Abbreviated FFQ

The validity of the abbreviated FFQ administration in the further participant group was also evaluated by implementing several statistical analyses as suggested in the literature.(18,412) These analyses were computed to compare the daily total flavonoid intake estimates of the first and the second or repeat abbreviated FFQ administrations with that of the estimated dietary records as the reference method as representation of the minimal (before the dietary record collection) and maximal (after the dietary record collection) estimates of the true validity.(387) In the first analysis, the quantitative efficiency of the abbreviated FFQ was evaluated by comparing its daily total flavonoid intake estimates with that of the dietary records by using the group intake estimates as measure to survey a population mean intake.(40) Although the mean daily total flavonoid intake estimates were comparable, the abbreviated FFQ provided a somewhat higher intake estimate than the dietary records. The first abbreviated FFQ administration estimate was five percent (5.08%) higher and the second repeat abbreviated FFQ administration estimate ten percent (9.95%) higher than the dietary record estimate. No statistically ($p>0.05$) significant differences were found between the daily median and interquartile range total flavonoid intake estimates of either the first or the second repeat abbreviated FFQ administration and the dietary records (Table 8.11) on using the non-parametric Wilcoxon's signed rank test for this comparison ($p=0.162$ and $p=0.144$, respectively).

In the second analysis, the relations between the two methods were investigated by determining the ability of the abbreviated FFQ as the test method to rank and to categorize the individual participant intake estimates into its intake distributions alongside that of the estimated dietary records. For these comparisons, the non-parametric Spearman rank order correlation coefficient and the *Kw* statistic was used, respectively. The Pearson correlation coefficient on log-(natural) transformation of the data to a achieve normal distribution was additionally computed to determine the degree of association between the results of the abbreviated FFQ and the dietary records to rank the individual participant mean daily total flavonoid intakes. The correlation relationships for both the first and the second or repeat abbreviated FFQ administration intake estimates with the dietary record intake estimates were particularly strong with the Spearman and the log-transformed Pearson correlation coefficients statistically significant ($p<0.0001$ in all cases) and equaling 0.9 (0.92 and 0.91 for the Spearman correlation coefficients and 0.91 and 0.92 for the log-transformed Pearson correlation coefficients for the administration before and after the dietary records, respectively).

Deattenuation correlation coefficients were also calculated using the dietary record data to include the within- and between-person variation (496) because moderate to high day-to-day variability in

dietary intake might obscure correlations between the FFQ and the reference method.(417) Deattenuation of the correlation coefficients between the questionnaire and the dietary records resulted in slightly stronger associations, but did not change the results appreciably. The Spearman correlation coefficients did not differ appreciably from the parametric Pearson correlation statistic in this further participant group administration and these correlations from the Pearson correlation coefficients adjusted for attenuation effects resulting from measurement error (Table 8.12).

Table 8.12: Agreement between Daily Total Flavonoid Intake Estimates of Dietary Records and Abbreviated FFQ in Further Participant Group (n=90)

Statistical analyses	FFQ before		FFQ after	
	Correlation coefficient	p-value	Correlation coefficient	p-value
Correlation coefficients[a]				
Log-transformed Pearson	0.912	<0.0001	0.915	<0.0001
Spearman	0.917	<0.0001	0.905	<0.0001
Deattenuated correlation coefficients				
Log-transformed Pearson	0.819		0.940	
Spearman	0.924		0.930	
Joint category classification				
Category levels	Gross mis-classification % (n)	Weighted Kappa[b]	Gross mis-classification % (n)	Weighted Kappa[b]
Quintile	0 (0)	0.666	1.11 (1)	0.598
Tertile	0 (0)	0.790	0 (0)	0.532

[a] Non-parametric statistic on account of the skewed data distribution

[b] Weighted Kappa (*Kw*): ≤0.2 = poor agreement; >0.2-0.4 = fair agreement; >0.4-0.6 = moderate to substantial agreement; >0.6 = good or substantial agreement beyond chance (459,495)

Employing both quintile and tertile classification of intakes, very little misclassification (participants in the lowest quintile/tertile on the dietary records, but the highest quintile/tertile on the abbreviated FFQ, or *vice versa*) was found with only one participant greatly misclassified at the extreme quintiles on the second or repeat abbreviated FFQ administration. While the *Kw* statistic indicated good or substantial agreement beyond chance (k>0.60) for the first abbreviated FFQ administration, moderate to substantial agreement (0.41 to 0.60) was indicated for the second abbreviated FFQ administration. For both the first and the second or repeat abbreviated FFQ administration and the dietary records 76.7% of the participants were classified in the same quintile of intake, while 94.4% and 90% of the participants were classified in the same tertile for the dietary records and the first and the second or repeat FFQ administration, respectively (data not presented in Table 8.12).

In general, the distribution of the points on the Bland-Altman plots related to the participant abbreviated FFQ computed daily total flavonoid intake estimates against that of the dietary records for the first and repeat abbreviated FFQ administrations were well within the 95% limits of agreement with very few participants above or below the limits of agreement (Figures 8.12 and 8.13). More subjects fell above the upper limit of agreement than below the lower limit of agreement (4 versus 3 participants) for the first questionnaire administration, whereas the participant distribution above and below the limits were equal (two participants in each case) with the repeat administration. The Bland-Altman plots further indicated that there was good agreement between the two methods as evidenced by the symmetry of the data around the line of identity, but with a greater tendency of positive values at higher intakes emerging at intakes approaching 750 mg/day in both administrations. Although a greater symmetry of the data around the line of identity occurred within the repeat questionnaire administration, its mean difference (+22.2 mg/day) and upper (+252.9 mg/day) and lower (-208.4 mg/day) limits of agreement (Figure 8.13) were larger than that of the first questionnaire administration (+11.3, +190 and -167.3 mg/day, respectively) (Figure 8.12).
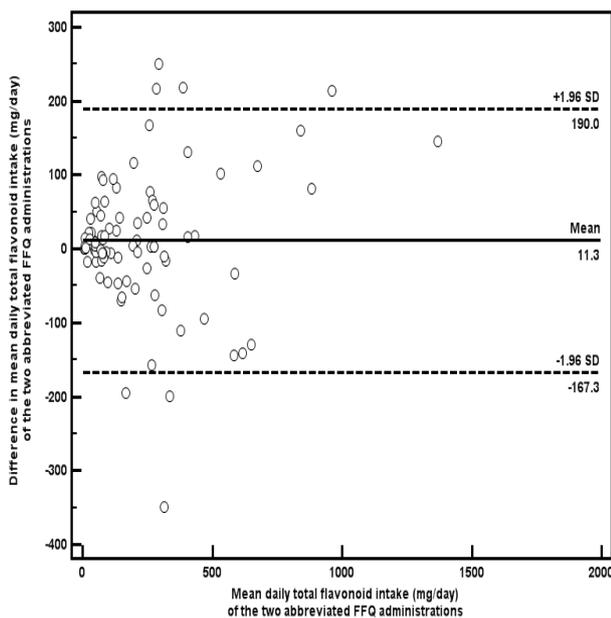
Figure 8.12: **Bland-Altman Plot of Agreement between Flavonoid Intakes of First Abbreviated FFQ and Dietary Records within Further Participant Group**
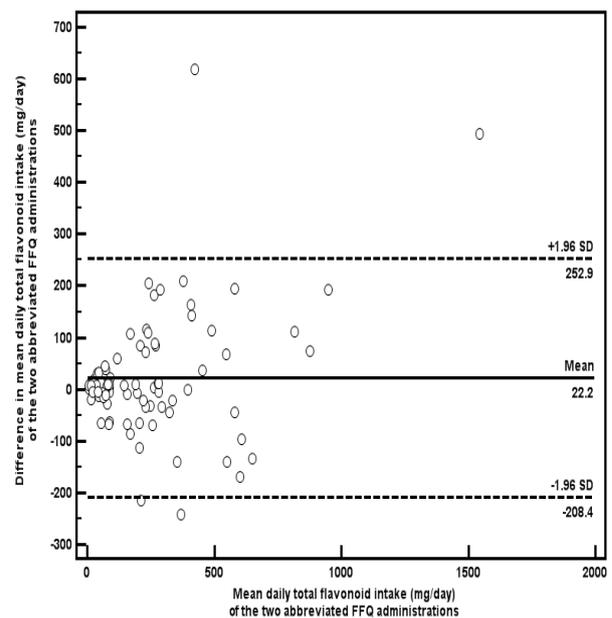
Figure 8.13: **Bland-Altman Plot of Agreement between Flavonoid Intakes of Repeat Abbreviated FFQ and Dietary Records within Further Participant Group**

8.6.4 Reproducibility Evaluation

The relative difference in the intakes as assessed by the first and the repeat abbreviated FFQ administration was used as initial measure of its reproducibility. The mean daily total flavonoid

intake as assessed by the abbreviated FFQ, approximately two weeks apart, were similar, with the value computed from the abbreviated FFQ administered before the dietary records were completed (234.74 mg/day) only slightly lower (-4.43%) than that of the abbreviated FFQ administered after the dietary record completion (245.63 mg/day) (see Table 8.11). Although the paired difference between the medians (+8.9%) and the interquartile ranges (-5.07% for the 25th and +1.5% for the 75th) between the first and second measurements were small, the interquartile ranges *per se* were again large (see Table 8.12). There was, as a result, no significant difference (p=0.6919, Wilcoxon's signed rank test) between the median and interquartile range intakes of the two abbreviated FFQs.

The log-transformed Pearson's and the Spearman correlation coefficients were used to describe the reproducibility of the data in the ability to rank the individual participants between the two questionnaire applications, which both showed strong significant (p<0.0001) associations of 0.9 (0.9109 and 0.895, respectively). The log-transformed Pearson correlation coefficient showed almost the same value as the Spearman correlation coefficient. The reproducibility assessment of the abbreviated FFQ indicated the median paired difference between the two measurements to be non-significantly (p>0.05) different from zero, with strong correlation coefficients between the two administrations. The percentage of participants who were greatly misclassified between the paired intakes of the two FFQ administrations considering the interquintile range was 2.22% (n=2) and the intertertile range nil. The two administrations reflect moderate agreement (0.41-0.60) on the *Kw* statistic for both the cross-classifications (0.569 and 0.567, respectively). Most (76.7%) of the participants were classified within the same quintile and the majority (91.1%) within the same tertile intake range considering the two FFQ administrations.

The distribution of the points on the Bland-Altman plot for the first and repeat administration of the abbreviated FFQ computed daily total flavonoid intake estimates within this further participant group were well within the 95% limits of agreement with very few participants above or below (two participants in each case) the limits of agreement (Figure 4.14). The Bland-Altman plot further indicated that there was good agreement between the two administrations as evidenced by the symmetry of the data around the line of identity with the difference in mean intakes at the higher intake levels approaching zero and the line of equality or correspondence.
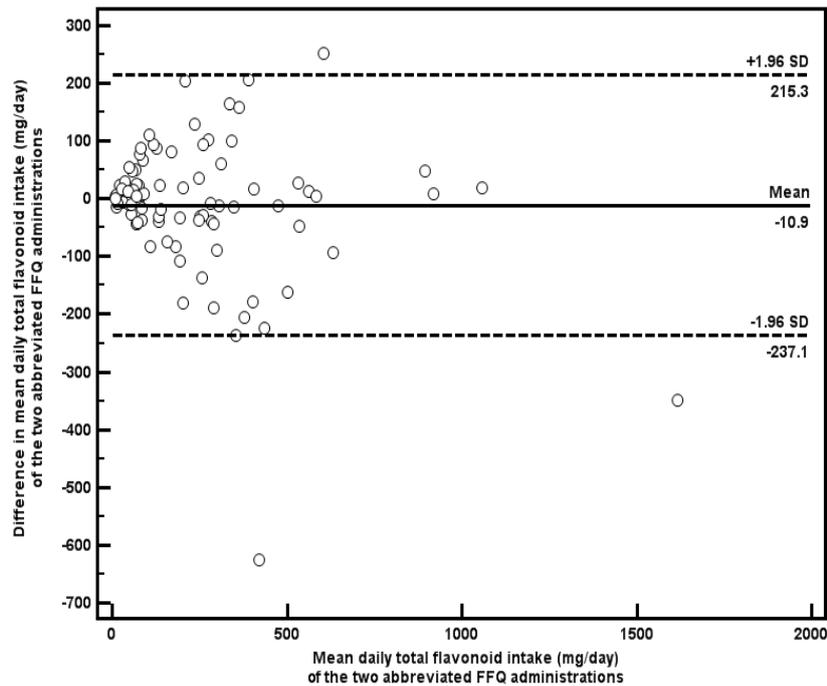
Figure 8.14: **Bland-Altman Plot of Agreement between Flavonoid Intakes of First and Repeat Abbreviated FFQ Administrations within Further Participant Group**

8.6.5   Confirmation of Dietary Items in Reduced Food List of Abbreviated FFQ

The contributions of the recorded dietary items to the sum of the participant absolute mean daily total flavonoid intake estimates and the between-person variance in intake were investigated using the estimated dietary records to verify the dietary items incorporated within the abbreviated FFQ food list. The percentage contribution of each dietary item consumed during the six days of dietary recording over the two weeks to the summed total flavonoid intake was computed and 20 foods contributing most to the absolute intake ranked based on their percentage contribution identified. The top 24 items, selected according to their absolute contribution from the dietary records, were re-ranked in stepwise multiple regression analysis to assess the degree to which consumption of these specific items explained the between-person variability in intake. The items that best predicted variation in intake was black tea and rooibos, which contributed 71.15% of the mean daily total flavonoid intake (Table 8.13), although about 10% less than in the determinations for the compilation of the abbreviated FFQ food list (84.67% for the dietary records and 81.12% for the comprehensive FFQ as reported in Table 8.8).

The items contributing most to the total flavonoid intake in this analysis were greatly those items included in the reduced food list of the abbreviated FFQ (n=14 items as black tea, rooibos, green tea, apples, red wine, bananas, orange juice, and orange juice blends, tropical juice and tropical

juice blends, berry and mixed berry juice, pears, cooked onion, apple cider, apple juice and apple juice blends, dark chocolate) and those identified items but excluded from the reduced food list due to seasonal availability implications (n=4 items as oranges, strawberry, guava, naartjies). Milk chocolate, ranked 24th in the percentage contribution analysis, was just short of inclusion among the top 20 foods (Tables 8.8 and 8.13). The only differences were: dried parsley and dried pear halves included in the reduced food list, which were not included within the top 24 ranked contributory items (ranked 30 and 32, respectively based on the percentage contribution) in this analysis; beer, raw onions (as mixed dish ingredient) and white wine which were included in the top 21 contributory items (ranked 10, 11, 17, respectively), but they were excluded from the reduced food list as they only contributed to the top ranked foods in the dietary records (beer and onions, raw ranked 18 and 19 respectively) and not in the top 20 ranked foods of the comprehensive FFQ and were not identified as discriminating items in the stepwise regression analysis of the dietary records and the comprehensive FFQ (see Table 8.8).

Among the top 24 items selected according to the absolute contribution, most were, as previously found in the compilation of the reduced food list of the abbreviated FFQ items, contributing to the between-person variance in intake (discriminating items). The stepwise multiple regression analysis extracted a total of 21 foods as predictors of 99% of the between-person variability in the total flavonoid intake. These included black tea, rooibos, green tea, apples, red wine, bananas, orange juice and orange juice blends, tropical juice and tropical juice blends, berry and mixed berry juice, pears, apple cider, dark and also milk chocolate included in the abbreviated FFQ food list.

Again those identified items excluded from the reduced food list due to seasonal availability implications (oranges, strawberry, guava, naartjies) (Tables 8.8 and 8.13), and in addition beer, raw onions (as mixed dish ingredient) and white wine (ranked 10, 11, 17, respectively) along with coffee, instant pure (ranked 23) were included. Cooked onions and apple juice and apple juice blends, although included in the reduced food list based on their percentage contribution to the total flavonoid intake of the dietary records and the comprehensive FFQ, were also not identified as discriminating items in the previous stepwise multiple regression analysis (Table 8.8) as in this analysis based on the dietary records. Based on this confirmation analysis, a new abbreviated FFQ with a food list of 21 items could be developed that accounted for 97.64% of the absolute mean daily total flavonoid intake and 99% of the total variance in the intake (Table 8.13). Of these, 17 (80.95%) of the items were identified for inclusion in the abbreviated FFQ food list, but only 13 incorporated due to the seasonal availability concern of some identified fruits (oranges, strawberries, guavas and naartjies) (see Tables 8.8 and 8.13).

Table 8.13: **Percentage Contribution, Ranking and Variance Analyses of Top 20 Food and Beverage Items in Estimated Dietary Record Summed Mean Daily Total Flavonoid Intake Estimate of Further Participant Group (n=90)**

Top contributory food and beverage items in estimated dietary records	Percentage contribution[a]	Rank[a]	Stepwise regression analysis[b]
Food and beverage items in abbreviated FFQ[c]			
Tea: black brewed (including iced tea)	56.44	1	<0.0001
Rooibos, brewed (including iced tea)	14.71	2	<0.0001
Tea: green brewed (including iced tea)	12.56	3	<0.0001
Apples: raw	3.06	4	<0.0001
Wine: red	2.32	5	<0.0001
Bananas: raw	0.98	7	<0.0001
Juice: orange and orange blends	0.86	9	<0.0001
Juice: tropical and tropical blends	0.54	12	<0.0001
Juice: berry and mixed berry	0.50	13	<0.0001
Pears: raw	0.44	14	<0.0001
Onions: cooked (main and side dish)	0.30	15	Not included
Apple cider	0.26	16	0.0002
Juice: apple and apple blends	0.17	19	Not included
Chocolate: dark	0.17	20	<0.0001
Chocolate: milk	0.11	24	0.0005
Parsley: dried	0.07	30	Not incorporated[d]
Pear halves: dried	0.06	32	Not incorporated[d]
Cumulative percentage total	93.55		
Food items not included in abbreviated FFQ (seasonal availability[c])			
Oranges: raw	1.85	6	<0.0001
Strawberry: raw	0.87	8	<0.0001
Guava	0.18	18	0.0103
Naartjies: raw	0.15	21	0.0002
Cumulative percentage total	3.05		
Food and beverage items not included in abbreviated FFQ (not in previous analyses[c])			
Beer: regular, all	0.73	10	<0.0001
Onions: raw (mixed dish ingredient)	0.57	11	<0.0001
Wine: white	0.23	17	<0.0001
Coffee: instant pure	0.11	23	0.0047
Cumulative percentage total	1.64		

[a] Ranked on the basis of the percentage that each reported dietary item contributed to the total participant group summed mean daily total flavonoid intake of each dataset

[b] Estimated dietary record items capturing the between-person variation: $R^2 = 0.9998$ as the percent between-person variation explained

[c] With reference to Table 8.8, not included in these analyses

[d] Not incorporated into model due to ranking

CHAPTER 9

DISCUSSION OF RESULTS

9.1 INTRODUCTION

It should be noted that given the wide variation in the design and methodology among the numerous FFQ validation studies published in terms of the characteristics of the questionnaires, the dietary reference methods used and the characteristics of the study subjects, the results obtained among the various studies cannot be compared directly.(41,490) In the absence of studies that are to a great extent similar, consideration was given to the research findings to determine, if they were of comparable magnitude to findings reported in the literature. Such comparisons, although uncontrolled and possibly flawed, can direct reflections.

9.2 REALISM OF ASSESSED DAILY TOTAL FLAVONOID INTAKES BY DIETARY ASSESSMENT METHODS

The computed mean daily total flavonoid intake estimates were based on the summed intake of the five major flavonoid subclasses (flavonols, flavones, flavanones, flavanols and anthocyanidins) incorporating 26 commonly occurring individual flavonoid compounds,(161) for the rooibos intervention trial baseline period (comprehensive FFQ: 390.16 mg/day; abbreviated FFQ: 350.92 mg/day; dietary records 343.49 mg/day) and in the further participant group administration (first administration abbreviated FFQ: 234.74 mg/day; second administration abbreviated FFQ: 245.63 mg/day; dietary records: 223.40 mg/day). These mean daily total flavonoid intake estimates were greatly lower than that reported for an adult Australian consumer (454 mg/day) by Johannot and Somerset (89) based on a 24-hour recall national nutrition survey analysis.

Although the USDA flavonoid database is the most advanced food composition data available for assessing flavonoid intake, the database is still not comprehensive, which may contribute to an underestimation of the flavonoid intake,(150) as foods not included in the database are assumed to contain no flavonoids.(248) The foods and beverages (mostly alcoholic of nature) consumed by participants in this research that were not captured in the mean daily total flavonoid intake analyses, but which could have contributed to the intake estimates, include such items as: hummus, peanut butter, fig jam, gherkins, seeds (sunflower and pumpkin), basil pesto, balsamic vinegar and garlic, consumed in small amounts (often as mixed dish ingredients); and dried figs, canned guava, canned blueberries, granadilla, sherry, muscadel and brandy consumed in larger amounts (as whole portions).

The intake estimates of the rooibos intervention baseline trial period and the further participant group administration were somewhat higher, particularly for the rooibos intervention baseline trial period, due to the primarily tea drinking participant group (percentage contribution of black tea and rooibos to the total flavonoid intake), than that reported by Mullie et al. (84) in their validation study in which 45 Flemish dieticians participated and the computed intake from the NHANES, 1999–2002, for the US adult population.(105,342)

The total flavonoid intake estimates in the validation study of Mullie et al. (84) was 166 and 158.3 mg/day for their first and second FFQ applications, respectively, and 203 mg/day for the dietary records. An intake of 189.7 mg/day was computed for the US adult population from the NHANES, 1999 – 2002, based on 24-hour recalls.(105,432) The mean daily total flavonoid intake estimates within the rooibos intervention baseline trial period and in the further participant group administration were, however, much higher than that reported in a case-control study of acute myocardial infarction, Milan, Italy of 134 mg/day,(263) the Kuopio Ischaemic Heart Disease Risk Factor Study for middle-aged Finnish men in Eastern Finland of 129 mg/day (169) and analysis of a weekly menu representative of the traditional Greek plant-based diet of 118.6 mg/day.(137)

The total daily flavonoid intake estimates obtained in this research demonstrate reasonable agreement with reported intakes, as indicated above as well as intake indications in the literature, though some individuals may have mean daily total flavonoid intakes lower or higher than intake indications in the literature. According to Manach et al.,(100) crude estimates of an average intake in Western populations appear to be about 65 to 250 mg/day, while Vaya et al. (319) indicated an intake estimate of 23 to 1000 mg/day. Pietta (128) and Jovanovic and Simic,(217) respectively, indicated that the intake might reach 800 and 1000 mg/day.

In this research, the lowest computed mean daily total flavonoid intake estimates were 11.78, 4.63 and 15.49 mg/day for the comprehensive FFQ, the abbreviated FFQ and the dietary records, respectively. In the rooibos intervention baseline trial period the highest intake estimates for these assessment methods were 2206.39, 2173.95 and 1253.6 mg/day. In the further participant group administration, the lowest computed mean daily total flavonoid intake estimates were 6.33, 6.25 and 1.86 mg/day for the administration of the first abbreviated FFQ, the second abbreviated FFQ and the dietary records, respectively, with the highest intake estimates for these assessment methods 1442.80, 1791.77 and 1297.36 mg/day, respectively. In the validation study of Mullie et al.,(84) the mean daily total flavonoid intake range was 30 to 769.1 mg/day for the first FFQ administration and 19 to 1027 mg/day for the dietary records.

Although the mean daily total flavonoid intake and intake range distributions were generally similar between the two dietary assessment methods, the distributions as estimated by the questionnaire were somewhat wider as has the distributions reported for other nutrient assessments using questionnaires.(38) It was pointed out that allowing for individual portion size estimates for all items in questionnaires, as was applied in this research, produces greater variability in nutrient intakes than when standard portion sizes are used for many of the food list items.(422) As expected, the SD was large regardless of the dietary assessment method as found for such nutrients as vitamin A and cholesterol.(86) Vitamin A and cholesterol are found in large amounts in a few foods, which on consumption or not, can cause substantial variation in intake of these nutrients within subjects over time.(86,444) As certain dietary items are rich sources of vitamin A or cholesterol, flavonoids also occur in large amounts in a limited number of dietary items, which on consumption or not, can cause substantial variability in intake within subjects over time and to between-subject variation in intake, contributing to the high within- and between-subject variation found in flavonoid intakes.(338)

De Vries et al. (74) found a large variation in the consumption of flavonols and flavones for their study participants with the within-person variation less than the between-person variation, which is supported by the findings of this research. In the rooibos intervention trial phase of the study, the within-person and the between-person coefficients of variation (%) was 48.62% and 103.48%, respectively, and in the further participant group administration, 54.43% and 123.21%, respectively. This provided for a ratio of the within-person and between-person variance components of 0.47 and 0.44 in the two study phases, respectively, while the ratio was 0.57 in the study of De Vries et al. (74) that was calculated from the three-day weighed dietary records of the Dutch subjects (n=8).

An attempt was made to draw the further participant group from different geographical areas in order to attain a wide range in the daily dietary intake.(480) This could be considered successful, as the mean daily total flavonoid intake range between the lowest and the highest intake values of both the first and the second abbreviated FFQ administrations and the dietary records of less than 10 mg/day (6.33, 6.25 and 1.86 mg/day, respectively) to over 1000 mg/day (1442.80, 1791.77 and 1297.36 mg/day, respectively) were large. On the other hand, this may be considered unsuccessful, as the intake ranges determined for the baseline period of the rooibos intervention trial dietary records and the comprehensive FFQ were large and of the same to an even larger magnitude (dietary records: 15.49 to 1253.60 mg/day; comprehensive FFQ: 11.78 to 2206.39 mg/day).

9.3 RELATIVE VALIDITY OF COMPREHENSIVE AND ABBREVIATED FFQ ASSESSING DAILY TOTAL FLAVONOID INTAKE AGAINST ESTIMATED DIETARY RECORDS

9.3.1 Quantitative Efficiency and Bias Based on Equality at Group Level

The group level difference in intake as assessed by the FFQ, as test method, and the estimated dietary records, as reference method, was calculated as an estimate of the average bias of one method relative to the other. In the case of the results of the rooibos intervention trial period, the paired samples *t*-test was applied, and the Wilcoxon's signed ranked tests was used in the case of the results of the other rooibos trial periods and the further participant group administration to capture differences. Although the comprehensive and the resultant abbreviated FFQ tended to present higher mean daily total intakes than the dietary records, the absolute group level intake estimates by the questionnaire and by the six dietary records were comparable as the results indicated that the group mean/median intakes were not significantly (p>0.05) different. The results, therefore, indicate that good agreement between group means/medians were achieved, as the FFQ and the dietary records on average obtained absolute group intakes with negligible differences. Such approximate and comparable absolute dietary intake estimates between FFQs and reference methods, have been reported previously.(38,345,412)

This equality at group level is a measure of the systematic bias of the dietary assessment method (43) indicating whether on average the FFQ consistently over- or underestimates the intake.(518) Slightly lower FFQ intake estimates have been reported for nutrients compared to the intake estimates from dietary records,(364,375,379,441,442) 24-hour dietary recalls (442,444,445) or dietary histories (368) as the reference method. Although this implicates that lower estimates or underestimation are found, higher mean/median intake estimates by the FFQ are more often found in comparison to dietary records and 24-hour recalls as reference methods, implicating that overestimation is the more general pattern.(6,86,369,379,380,407) This applied to the finding of this research for the comprehensive and the abbreviated FFQs.

FFQs frequently overestimate dietary intakes, as they provide higher intake estimates and substantially larger estimates of between-person variability than the reference methods as shown by the larger SDs of the means of nutrients.(435) Mean intakes as estimated by full-length FFQs in particular, is often higher compared to intake estimates from dietary records,(39,380,390, 405,407,411,425,467,470,484) 24-hour recalls (24,345,365,386,413,465,484) and the dietary history.(412) This overestimation by the FFQ has been reflected by values greater than 100% for nutrient,(24,365,380,386,390,407,411,412,425,435,470,484,510) for food (39,380,390,413,424) and for food group (356,390,413,465) intake estimates.

Given the capability of the abbreviated FFQ to capture about 93% of the mean daily total flavonoid intake, estimates in line with or very close to those derived from the dietary records were expected for administration to the further participant group. However, the abbreviated FFQ did not fall short in capturing the intake, but overestimated it. Several short FFQs have been found to overestimate dietary intakes in comparison to reference methods. For instance, the reduced version of the interviewer-administered Block questionnaire overestimated the intakes of vitamins A and C, which is usually seen with full-length questionnaires.(402) The estimated daily intakes of fat, fatty acids and cholesterol (412) and calcium (404) assessed by FFQs developed for these specific assessments, were found to be higher than those by the dietary history interview and dietary record as the reference methods, respectively, although the mean differences in the intakes between the methods were not very large (412) and not statistically significant.(404)

Van Assema et al. (398) found that even short FFQs developed to assess fruit and vegetable consumption overestimated the daily intake compared to dietary records. These investigators indicated that the subjects could have over-reported the intake because fruit and vegetables are considered nutritious and socially desirable foods. Sebring et al. (88) found that a short 25-item calcium questionnaire estimated mean dietary calcium intake reasonably well compared to dietary records and greatly better than a longer questionnaire listing 87 items. Kemppainen et al. (80) also found that their short 21-item questionnaire very slightly underestimated the intake of total fat, the various types of fatty acids and cholesterol compared to dietary records. However, in the study of Schaffer et al. (403) the median energy intake from the abbreviated telephone version was lower by 23% in women and 17% in men than from the original in-person full-length FFQ. A 13-item dietary screener developed to assess fat and saturated fat intakes used in the Prostate Cancer Prevention Trial, however, was not found to be a sound substitute for a comprehensive FFQ, as the absolute nutrient intake estimates from the screener were about one-third of the estimates from the FFQ and dietary recalls as reference methods.(431)

The differences in mean/median intakes between the FFQ and the reference method (multiple dietary records or recalls) are often within 5%,(365,412,422) typically within 10% (345,405) to 15% (365,412) but also arriving at 20% (405,441) to 30% (345,390,412) higher for some nutrients and foods. Median antioxidant intakes (carotenoids, vitamins C and E) from a questionnaire used in the study of Satia et al. (345) and the average of four dietary recalls were generally comparable, with differences typically between 10% and 30%. In this current research, estimates of the final abbreviated FFQ were within 5 to 10% of the dietary records as reference method. This finding is consistent with the results of most other studies using FFQs as dietary assessment method.

Various explanations have been proposed as to why FFQs tend to produce higher dietary intake estimates (overestimation) compared to dietary records or recalls. It was suggested that the problem may to some extent lie with the dietary records used as reference method, which are known to be prone to underreporting.(407) Regarding the FFQ itself, bias or overestimation seems to be introduced by measurement errors via the length of the questionnaire food list, where a number of dietary items might not have been consumed yet indicated to have been consumed.(386) Asking subjects about their intake on a long list of many separate dietary items, can lead to a higher intake estimate than asking them about a shorter list,(369,379,380) that is questions about food groups.(486) This explanation is particularly relevant to the study of Warneke et al.(409) These investigators found that the mean total fruit and vegetable consumption obtained via a 7-item FFQ of broad food categories was closer to reference estimates than a 31-item individual fruit and vegetable FFQ.

In the current research, the overestimation found for the comprehensive FFQ in the rooibos intervention trial did not differ much from the abbreviated FFQ in the further participant group administration, despite the far longer list of items in its food list. This known overestimation of FFQs may be the result of the long lists of included foods,(444) but also because of sources of error that include subject misinterpretation of specific items,(387) difficulty in conceptualization of portion sizes (387,444) and an accurate consumption frequency.(386,444) The latter is specifically relevant when there are several foods in an aggregated item,(357) which is not applicable to the questionnaire developed for this research. Overestimation difficulty due to the conceptualization of portion size may reveal difficulties in relating the standard portion size indicated with the actual portion consumed.(390) This was also controlled for in this research as the three portion size options for each listed item were obtained from a South African food quantities manual.(552) Moreover, averaging amounts consumed over a longer time frame, as with a FFQ, is subject to estimation misjudgement,(386) which may not be a relevant consideration in this research as the FFQ reference period was limited to the past two weeks.

The FFQ seems to overestimate intakes for several food groups that include rarely consumed foods (413), but in particular fruit and vegetables.(153,390,398,414) The overestimation seems to be magnified for these latter foods as they are perceived to be healthy and socially desirable (39,56,398) contributing to overestimation of plant-derived micronutrient intakes,(469) such as vitamin A,(365,405) vitamin C (365,457) and carotene.(457) The most marked intake overestimates have been reported for vegetables (380,390,408,413) and fruit juices (390,413) amongst other foods of which the fruit juice intake overestimate could be relevant to this research due to its noteworthy contribution to the mean daily total flavonoid intake. Assessment of vegetable intake as part of composite dishes is said to be problematic at all times.(505) In the

case of quercetin, the accurate assessment of onion, the major source of quercetin, is difficult.(108) Subjects may estimate the intake of onions worse than other foods (457) as onions are commonly a 'hidden ingredient' in numerous processed and homemade foods.(108) Although it is probable that the intake estimates of onions include a large margin of error,(108) it is the only vegetable source included in the abbreviated FFQ. Higher mean intakes for alcohol consumption have been found in FFQs (365) with alcoholic beverages forming a section in both the developed FFQs.

According to some investigators (390,398) such overestimation is not necessarily a problem as long as the FFQ is used to rank the subjects based on their intake, as fundamental in epidemiological studies.   However, absolute intakes are important in intervention studies to determine the effect of the intervention on the percentage of persons complying, and in evaluation studies where subjects often need to be classified according to compliance with dietary recommendations.(398)   In such instances, the assessment method bias must be regarded. Kristal et al.,(42) indicated that for some purposes, like where the goal is to assess absolute rather than relative intake, it may be preferred to, for instance, have comprehensive data on which fruit and vegetables are consumed to better plan or evaluate the outcomes of a dietary intervention program.(42)   In the current research, this was accomplished with the development of the abbreviated FFQ.   It nonetheless still overestimated the mean daily total flavonoid intake. Grootenhuis et al.,(91) for instance, found that systematic differences were absent or negligible for all nutrients except for vitamin A. Sevak et al. (444) explained the finding by indicating that only a few foods contain vitamin A in large amounts. Reference methods, like 24-hour recalls or even dietary records, may not include days when such foods are consumed. On the contrary, FFQs may overestimate intakes of vitamin A because of the large number of different fruits and vegetables listed in the food list, recall of usual frequency over a longer time frame and pre-allocation of portion sizes.(444)

In the current research, questionnaire overestimation of fruit juices may be responsible,(390,413) as fruit juices form a large part of the questionnaire food list and flavonoids are found in large quantities in fruit juices. Bingham et al. (40) found that the consumption frequency of orange juice, which is specifically listed in the abbreviated FFQ, was overestimated by their questionnaire versus the dietary records. The consumption of tea, which is the key contributor to the mean daily total flavonoid intake, was furthermore assessed rather crudely in the FFQ as it is reported in terms of three standard portion sizes and not as the actual amount consumed. Stiegler et al. (413) found that the consumption of tea as assessed with the FFQ was lower (by 39%) than the estimates established from the 24-hour dietary recall as reference method.

Fruits make a major contribution to the FFQ food list. Overestimation of fruit intakes, as assessed with FFQs versus that derived from the reference method,(413) may thus be a further contributory factor. Even the consumption of chocolate, an item which is included in the questionnaire food list, have been overestimated, but to a lesser extent (9 g), with the FFQ.(413) As indicated, the self-report of wine (and beer) consumption is thought to be inaccurate,(240) which are items included in the FFQ food list. It has also been reported that nutrient-specific FFQs result in over-reporting of intake.(345) In this research, black tea and rooibos seem to be the responsible dietary items, as determined through direct comparison of the total flavonoid intake estimates on an item-by-item basis of those included in the abbreviated FFQ food list in comparison to the dietary records, which found the black tea and rooibos intake estimates to be the notably higher.

The significant (p≤0.0001) difference found in the daily total flavonoid intakes between the abbreviated FFQ (test method) and dietary records (reference method) in the rooibos intervention trial for the washout and control trial periods can be explained by the trial conditions. During the washout and control trial periods the intake of all teas and herbal teas (black, green and rooibos), along with pure and blended 100% fruit juices, were prohibited. This meant that the abbreviated FFQ could capture the flavonoid intakes as obtained from the consumption of the restricted foods (apples and dark chocolate) and beverages (red wine), as they were all included in the food list but so would the dietary records. Along with these flavonoid intakes, the abbreviated FFQ captures the flavonoid intakes as obtained from the consumption of bananas, fresh pears, dried pears, cooked onions consumed as a food portion and as ingredient in mixed dishes, apple cider, chocolate and dried parsley. Any other food or beverage including oranges, naartjies, guava and strawberry were excluded from the abbreviated FFQ food list based on seasonal availability. However, the consumption of these four fruits and the other consumed foods and beverages would have contributed considerably more to the restricted daily total flavonoid intake on the exclusion of teas, herbal teas and 100% pure or blended fruit juices. The abbreviated FFQ did not capture these four fruits and all other consumed items. This would have contributed to its far and significant (p≤0.0001) lower mean daily flavonoid intake determination in the washout and control periods (46.09% and 45.42%, respectively) in comparison to the dietary records.

9.3.2 Agreement based on Individual Intake Estimate Ranking

Because of methodological differences in validation studies, such as in the participating subject groups and differences in the FFQ food lists, it makes reporting precise comparisons between correlation coefficients established in the different studies difficult,(356,365) as it is virtually impossible to find comparative validation studies. Although FFQs are not as suitable to assess absolute intake as are dietary records, the reasonable correlations observed between the FFQ and the reference assessment method estimates, indicate that subjects can be ranked with sufficient

accuracy,(424) as was confirmed in this research. The comprehensive and abbreviated FFQs were developed to assess mean daily total flavonoid intake and no other dietary component. It can be expected that the intake estimates from these test methods should compare favourably with the intake estimates from the dietary records, as the reference method, in comparison to other validations based on questionnaires, which were designed to assess the total diet or a wide range of nutrient intakes.

Although the correlation coefficients, between the two dietary assessment method intake estimates achieved in the intervention trial on using the comprehensive FFQ as test method, were generally high (0.58 to 0.81), they were within the overall range of those reported in the literature that includes the lower correlation coefficient (0.33) achieved in the washout period of the trial. According to Willett,(30) when comparing FFQs with multiple dietary records, correlation coefficients could reach 0.6 to 0.7 which was generally achieved in this research for the comprehensive and more so for the abbreviated FFQ in the further participant group administration. These correlation coefficients found of greater than 0.50 indicate a steadfast classification according to nutrient intake and that the extent of misclassification brought about by the FFQ would be satisfactory.(444) Masson et al. (461) suggested that to consider the use of a dietary assessment tool in, for instance epidemiological studies, a Spearman correlation coefficient of above 0.5 is desirable. The Pearson correlation coefficients for log-transformed values and the Spearman non-parametric rank order correlation coefficients were not appreciably different from each other in this research, as had been found in other studies.(11,41,86,390,534)

In this research, only the correlation coefficient of the washout period in the rooibos intervention trial was within the 0.3 to 0.4 threshold proposed by Cade et al.(18) Correlations at the 0.3 to 0.4 threshold and exceeding 0.7, as found in this research, has been reported in validations of FFQs against other dietary assessments as reference method. For instance, Nes et al. (71) found correlation coefficients ranging from 0.31 for vitamin C to 0.79 for the energy percentage from carbohydrate with most coefficients (20 out of 25) falling between 0.55 and 0.75 when comparing intakes assessed by a FFQ and dietary records. Correlation coefficients between the dietary history and FFQ as methods to assess intakes of fat and cholesterol ranged from 0.38 for linoleic acid (% of energy) to 0.83 for energy intake, with 0.78 and 0.75 for the intakes of total fat and saturated fatty acids, respectively, in the study of Feunekes et al.(412) Kabagambe et al. (510) found that FFQs performed well in relation to dietary records in estimating intake of fatty acids, with the highest correlations found for linoleic (0.73) and myristic (0.77) acids, along with caffeine (0.80). Segovia-Siapco et al. (386) in their validation study found significant correlations (corrected for measurement error) between dietary recalls and a FFQ as assessment methods to determine

fat intakes, for example 0.77 for polyunsaturated fat, 0.78 for linoleic acid and 0.79 for α-linolenic acid.

This study (386) also found significant correlations for vitamin C (0.96) and for certain minerals like 0.46 to 0.80 for iron, calcium, phosphorus, potassium and magnesium. In the study of Horwath,(422) correlations between the nutrient intake estimates from a FFQ and dietary records ranged from 0.34 for zinc in women to more than 0.75 for protein (0.78), zinc (0.77) and calcium (0.75) in men. In the study of Date et al.,(360) the correlation coefficient values were of a similar range and from 0.35 for calcium to 0.70 for animal protein, with most of the nutrient correlations between 0.5 and 0.7. Egami et al. (441) found a high correlation (0.83) for calcium in their validation study of a FFQ against dietary records. Mullie et al. (84) found high correlation coefficients between dietary records as the reference method and the first and the second FFQ applications (0.72 and 0.70, respectively) for total flavonoid intake.

The abbreviated FFQ correlation coefficients of approximately 0.9 found in the research compare favourably with other validations based on dietary records as reference method and brief questionnaires as test methods that were designed to assess only single or a limited range of dietary intakes, whether foods or nutrients. In the study of Kemppainen et al.,(80) the Pearson correlation coefficient between the total fat intake based on a short questionnaire of 21 items, including the main and commonly consumed sources of dietary fats of the Finnish diet, and dietary records was rather strong (0.8). Cummings et al. (404) evaluated the validity of a 5-item, a 10-item and a 15-item questionnaire developed to assess calcium intake against dietary records. Correlation coefficients for these questionnaires were 0.67, 0.75 and 0.76, respectively.

The comparability of PrimeScreen that includes the most frequently consumed foods in a number of targeted food groups (fruits, vegetables, dairy foods, whole grains, fish and red meat) was compared with the full-length 131-item FFQ. For foods and food groups, the mean correlation coefficient was 0.61 and for nutrients 0.60 for agreement with the FFQ.(433) However, Neuhouser et al. (431) found higher correlation coefficients between the longer 109-item FFQ than between the short 13-item FFQ developed to differentiate groups of subjects with high (or low) fat intake in the Prostate Cancer Prevention Trial and the reference method (repeat 24-hour recalls) for estimating intakes of fat and saturated fat (percentage energy from fat and saturated fat 0.71 and 0.72 for the longer FFQ and 0.50 and 0.53 for the short FFQ, respectively).

The correlations found in this research would be expected to be generally higher than in other studies. For instance, in the study of Willett et al.,(405) the magnitude of the correlations found between the two dietary assessment methods were mostly between 0.40 and 0.70 and overall 0.60

on assessing the intakes of various nutrients over a far longer period of time as the past year. In the current research, the time frame was the past two weeks, which would utilize the episodic memory to recall the very recent past diet and can be designated reasonably precise.(57) A further factor that could contribute to the higher correlations may be the larger number of recording days of dietary assessment (six days of dietary records over a 14-day period), which may have been greatly sufficient to characterize the subjects' diets. Both Serdula et al. (486) and Mullie et al. (425) pointed out that the low correlations they found between the application of their FFQs and the reference methods (dietary recalls and records, respectively) may be as a result of the limited number of days (four days each) used. This may not sufficiently have characterized their study subjects' usual diets in comparison to the lengthy time frames of their FFQs encompassing several months. Mullie et al. (425) pointed out that men comprised their study group and that a further reason for the low correlations could possibly be attributed to the lower awareness of the usual dietary intake found among males and the enhanced competence of women to estimate portion size. In the current research, there was a predominance of female participants.

For a number of reasons, the comprehensive FFQ correlations could have been expected to be lower than in other studies during the washout and control intervention trial periods and specifically in comparison to that of the abbreviated FFQ as applied in the further participant group administration. Not consuming the rich dietary sources of flavonoids that contribute greatly to the total flavonoid intake like the various teas, which are consumed in larger amounts than most other flavonoid-rich dietary sources, along with the other flavonoid intake restrictions, will negatively influence a strong linear flavonoid intake association as the variation in intake will presumably be greatly reduced. Egami et al.,(441) for instance indicated that the consumption of milk as a main food source of calcium and its regular consumption among the Japanese, could contribute to calcium intake being reported accurately and to the high correlation (0.83) found for calcium in their validation study of a FFQ against dietary records.

The bulk of the long food list of the comprehensive FFQ is made up of fruits and vegetables. In the washout and control trial periods the daily total flavonoid intake would mainly have been provided through the intake of fruits and vegetables and mostly by those with lower flavonoid content due to the flavonoid intake restrictions stipulated for the rich fruit sources, along with fruit juices, for the intervention trial. Various studies found that subjects overestimate their intake of fruit and vegetables when they are assessed as the sum of long lists of fruit and vegetable items on the questionnaire.(18,40,42,81,82) In addition, subjects seem to over-report the intake of foods perceived healthful or desirable, like fruit and vegetables.(39,56)

Salvini et al. (39) indicated that this tendency to over-report socially acceptable foods would in all likelihood not be easy to eradicate by altering the questionnaire design. Reducing the comprehensive FFQ food list by eliminating various included fruits and vegetables may have contributed to the generally higher linear correlations found for the abbreviated FFQ. Various studies reported that subject intakes as assessed with short FFQs are closer to the reference intakes than that assessed by more lengthy FFQs. In the study of Warneke et al.,(409) the mean total fruit and vegetable intake was closer to reference intake estimates (24-hour recalls) for the 7-item than the 31-item FFQ where the 7-item FFQ also correlated better with reference estimates than did the 31-item FFQ estimates. Although Sebring et al. (88) found that three FFQs of varying length (124-, 87- and 25-items) all performed reasonably well at estimating dietary calcium intake compared to dietary records as the reference method. The highest correlation among the three questionnaires was observed for the short calcium questionnaire. However, in the study of Resnicow et al. (465) the correlations of fruit and vegetable servings with total and specific serum carotenoid levels were in general higher for the 36-item FFQ than for the 2-item (a measure that enquired about the number of servings of fruit and the number of servings of vegetables typically consumed daily) and 7-item questionnaires.

The dietary items included in the food list of the abbreviated FFQ may be a major contributory factor to the high correlation coefficients found in its applications. The items included in the abbreviated FFQ are commonly consumed foods and beverages in SA.(90) It was reported that recalling the consumption of frequently consumed dietary items may be easier for subjects and such intakes reported more accurately,(39,353) especially those items not subject to seasonal variation,(39) than items consumed infrequently,(42) which are often recalled with the least accuracy.(366) Salvini et al. (39) found that dietary items consumed regularly, that include beverages like tea, orange juice, coffee and others, were inclined to have a lower within-person week-to-week variation in intake. Uncorrected correlation coefficients between dietary assessment methods were in general high for these items and correction for within-person variability improved the coefficient for these items only slightly. For items consumed less often, the correlation coefficients were in contrast greatly reduced by within-person variability. Thus the corrected coefficients provided a better estimate of the true correlation between the questionnaire and long-term intake especially when infrequently consumed items were included.(39) Erkkola et al. (390) found unadjusted Pearson correlation coefficients to be greatly stronger for dietary items consumed regularly.

Moreover, the dietary items included are those commonly consumed and include those beverages and foods, which in previous studies have been found to provide high linear correlations in intakes between two dietary methods when assessing food and beverage intakes. Although correlation

coefficients vary substantially among the different dietary items, the correlations are in particular strong for most of the items in the beverage group,(39) which form a large part of the abbreviated FFQ food list. For instance, food groups found with correlations between two dietary assessment methods equal to and above 0.70 in the study of Marks et al.,(518) included alcoholic beverages and tea, both of which are included in the abbreviated FFQ food list. High correlations for alcohol consumption is a common finding and said to be due to the fact that a proportion of subject values obtained are nil, which leads to a wide range in alcohol intakes and it is generally consumed in standard units.(380) Most persons, due to the relatively high cost of alcoholic beverages like wine with its standard bottle size packaging, can estimate alcohol intakes rather accurately resulting in little response bias except at high consumption levels.(75) The linear correlations found for alcohol intake between FFQs and the reference methods used in dietary validation studies were, for instance, at 0.86,(365) 0.88 for men and 0.94 for women (423) and often 0.90.(380,403,408) High correlations are sometimes only obtained for alcohol consumption in a FFQ.(380)

Assessment of tea intake is accurate too (108) with calibration and repeatability data showing good estimates of tea intake.(457) Correlation coefficients between dietary records and FFQs found for tea intake were 0.83 in the study of Salvini et al. (39) and 0.88 in the study of Marks et al. (518). The intake estimate of citrus juice consumption may be fairly accurate, as people would probably do so regularly.(118) It has been indicated that juices, for instance, tend to be consumed regularly, like a glass of juice with breakfast. Therefore, they may be reported with greater precision than the consumption of irregularly consumed items.(42) This implicates that the errors in estimating the intake of flavanones may be relatively small as they are primarily provided by citrus fruits and juices.(108) Validation and repeatability data show good estimates of apple intake (457) which is included in the questionnaire food list.

The correlations for the abbreviated FFQ may be better due to the exclusion of all vegetable choices in the food list, with the exception of onions. The relative validity for the ranking of subjects according to the consumption of some food groups, like vegetables, is generally poor, while that for fruit generally reasonably good.(356) Several investigators found that correlation coefficients between the FFQ and reference methods were consistently higher for total fruit intake (42,398,428,482) and fruit juice intake (42) than for total vegetable intake.(42,398,428) Even in the customary application of the summation method to assess usual fruit and vegetable intakes, most studies found that the correlations with the reference dietary methods were higher for fruit than for vegetable intakes.(42) According to Van Assema et al.,(398) clear explanations for this occurrence are lacking. The questionnaire overestimation of vegetables, also fruit juices, but not fruits, in comparison to other foods may be responsible.(390) It might be that vegetable consumption is more difficult to report for those who do not regularly do the shopping and cooking, which would

probably be males, and since healthy eating is more of a desirable issue among women, they may be more inclined to over-report their vegetable intake. A further explanation may be that people may find it easy to indicate the number of pieces of fruit they consume, but that there is variability in understanding of what is meant by a portion of vegetables.(398)

It is probable that the intake estimates of onions produce a large margin of error.(108) It is though the only vegetable source included in the abbreviated FFQ. A small between-person variation in consumption frequency and measurement errors in portion size estimation might be likely explanations for the typically low relative validity for estimates of vegetable intake.(356) In addition to the aforementioned long list of fruit and vegetables, it was drastically reduced in the abbreviated FFQ. Many studies have shown that subjects overestimate fruit and vegetable consumption when they are assessed as the sum of long lists of fruit and vegetable items.(18,40,42,81,82) The between-person variance in the mean daily total flavonoid intake due to the consumption of dietary items rich in flavonoids or not (differentiation made by Song and Chun,(342) regarding tea and non-consumer) and the consumption or not of some other dietary items included in the abbreviated FFQ food list, like red wine, would contribute to larger validity correlations between the mean daily total flavonoid intake estimates of different dietary assessment methods (FFQs and dietary records).

Adjustment for random errors associated with the effects of day-to-day or within-person variation in dietary intake almost always increases correlation coefficients.(39,379,386) This trend that deattenuation or correction for the day-to-day variability in dietary intake increases the relative validity or correlation coefficients between two dietary assessment methods, was reported in numerous studies,(345,378,386,390,423) but were not as noticeable in this research. Deattentuation of the Pearson correlation coefficients between the FFQ and the dietary records, which take within- to between-person variation in intake into account, were almost identical to the Spearman correlation coefficients as observed by Block et al. (534) and improved the correlations to a small extent, as was also observed by Martinez et al. (41) and Sevak et al.(444) Only slight improvements were, in particular, found in the comprehensive FFQ applications in the intervention and control trial periods where the linear associations found were already strongly (0.81 and 0.74, respectively) and greatly significant (p<0.0001), as well as in the abbreviated FFQ in the further participant group administration where the linear associations found were particularly strong (0.91) and very significant (p<0.0001). Sevak et al. (444) indicated that the less marked improved deattenuated correlations found in their study may be due to the large number of dietary recalls obtained per subject, which may have reduced the measurement error due to the day-to-day within-person variability in dietary intake. This has relevance to this research. The six dietary

records obtained from each subject within a time frame of two weeks may have reduced the measurement error due to capturing of the day-to-day within-person variation in dietary intake.

The poor linear relationship (log-transformed Pearson and Spearman correlation coefficients of about 0.2) found in ranking the participant mean daily total flavonoid intakes as assessed by the abbreviated FFQ and the dietary records in the washout trial period were reflected in the poor (<0.20) inter-test agreement between the two dietary methods in the washout trial period as assessed using the *Kw* statistic. Considering dietary item intakes between the washout trial period FFQs and the dietary records, differences occurred between the flavonoid contributions from items included in the abbreviated FFQ, which may implicate early compliance difficulties. For instance, while the consumption of 100% pure and blended fruit juices (included in abbreviated FFQ food list) were prohibited in the washout trial period, higher contributions to the flavonoid intakes in the FFQ occurred through 100% pure and blended apple juice and lower contributions through tropical fruit juice and such fruit juice blends like berry and mixed berry fruit juices as compared to the dietary records. As fruit juices are a rich flavonoid source, such intake discrepancies between the FFQs (and as a result in the abbreviated FFQs) and the dietary records in this trial period of flavonoid intake restriction, may have a major affect on participant ranking in the abbreviated FFQs dataset versus the dietary record dataset in particular when considering the restricted daily total flavonoid intake of the participants in this trial period.

9.3.3 Individual Intake Estimate Agreement based on Category Cross-Classification

For nearly all epidemiological studies, FFQs must have the ability to classify subjects correctly based on their dietary intake.(85) Considering the literature, it seems that on average a low level of severe misclassification occurs. This applies to the classification of subject dietary intake estimates into the extreme quintile,(38,71,441,470) quartile (345,371,365,375,380,435,445,500) or tertile (24,91,494) intake category by the FFQ and the reference dietary assessment method. Often a few subjects are grossly misclassified at the extreme categories implicating a high level of correct classification.(365,403) Disagreements as misclassification into extreme categories of intake are often below 10% (24,91,345,380,435,469,500) and even in some studies considered rare as nil or only a small percentage of the subjects fall into extreme opposite categories.(38,71,368,371,375, 412,441,445,470,494)

The cross-classification agreement for macronutrient intakes seems to be better than that for micronutrient intakes (365,444) where validation studies often find the intake classification of vitamin A (425,444,500) and vitamin C (500) as lower. Mullie et al. (84) found that 89% of the 45 dieticians were classified in the same or in an adjacent quartile for the total flavonoid intake estimates of the first administration of their FFQ and the four-day dietary records, and 87% of the

dieticians on the second administration of the FFQ and the dietary records. The high level of correct classification and low level of severe misclassification found in the rooibos intervention trial and the further participant group administration for the mean daily total flavonoid intakes, could possibly be attributed to the better intake agreement found in the intake of beverages, including tea, which on average contributed greatly to the daily total flavonoid intake. Marks et al. (518) found no gross misclassification for the intake of alcoholic beverages, tea and coffee as item estimates obtained from a FFQ and weighed dietary records.

It seems that in comparison to the high level of correct classification and low level of severe misclassification found, a less impressive level of agreement is seen when measured by the K statistic with its levels of agreement for nutrients often found in the range of poor (<0.20) (24,400,494) to possibly moderate (0.41 to 0.60),(24,494) but most often either poor (400) or fair (0.20 to 0.40) (497,594) agreement.

The agreement between the mean daily total flavonoid intake estimates from the estimated dietary records and the resultant abbreviated FFQ by classifying the intake estimates obtained by both methods into quintiles and tertiles, were greatly similar to that of the full-length comprehensive FFQ across the rooibos intervention trial periods with few participants grossly misclassified into the extreme quintiles or quartiles, besides for the washout period of the trial. Although the percentage of participants classified in the same tertile were above 50% for this trial period by both methods (comprehensive FFQ and dietary records; abbreviated FFQ and the dietary records), this was not the case for the quintile agreement as less than 50% of the participants were classified in the same quintile. The level of inter-test agreement between the two dietary assessment methods calculated using the Kw statistic was below 0.20 for this period of the trial indicating rather poor agreement. The poor agreement may be ascribed to the quite low mean daily flavonoid intake estimates of this trial period, which did not allow for much deviation in intake across the intake categories (quintiles), along with the commencement into the trial dietary flavonoid intake restrictions.

The level of inter-test agreement was above 0.40 for the baseline, intervention and control periods of the intervention trial indicating moderate to substantial agreement beyond chance agreement. Moderate to substantial agreement beyond chance (Kw: 0.41 to 0.6) was mainly found in the quintile and tertile groupings calculated for the rooibos intervention trial period mean daily total flavonoid intake datasets and for that of the second abbreviated FFQ and dietary record datasets in the further participant group administration based on the Kw statistic. The highest inter-test agreements using the Kw statistic were obtained for the quintile category classification in the intervention trial period (0.71) and for the first abbreviated FFQ administration in the further participant group (quintile: 0.67; tertile: 0.79, respectively) that shows substantial agreement

beyond that explained by chance as, the *Kw* was greater than 0.6.(497) The comprehensive and resultant abbreviated FFQs developed for this research thus met the guidelines put forward by Masson et al. (461) that a dietary assessment tool considered for use in epidemiological studies should correctly classify more than 50% of subjects and grossly misclassify less than 10% of subjects into thirds and obtain a *Kw* value above 0.4 in its validation evaluation (besides the applications within the washout period of the rooibos intervention trial). This better inter-test agreement found in this research is possibly due to the large number of dietary records (six) obtained per subject within the time frame of 14 days , which may have reduced the measurement error due to the day-to-day within-person variability in dietary intake. Poor comparability in inter-test agreement might occur as a result of great individual day-to-day variability in dietary intake.(497)

9.3.4   Individual Intake Estimate Limits of Agreement

The Bland-Altman plots indicate the relationship between the difference in the mean daily total flavonoid intake and the mean intake range as assessed by either the comprehensive or the abbreviated FFQ within each of the rooibos intervention trial periods, and both the abbreviated FFQ administrations in the further participant group and the estimated dietary records. The intake estimates were well within the 95% limits of agreement with very few exceptions where the intakes were above or below the limits of agreement. In the study of Mullie et al. (84) all but two of the measurements on the Bland Altman plot were within the 95% confidence interval. The mean difference was -37 mg/day with the limits of agreement of +329 and -403 mg/day, respectively, for the first FFQ administration versus the dietary records (n=4). For the repeat FFQ versus the dietary records, the difference was -45.0 mg/day and the limits of agreement +337 and -427 mg/day, respectively.(84) For the further participant group administration in this research the mean difference was +11.3 mg/day with the limits of agreement +190 and -167.3 mg/day, respectively for the first abbreviated FFQ and the mean difference +22.2 mg/day with the limits of agreement +252.9 and -208.4 mg/day, respectively for the second or repeat abbreviated FFQ administration versus the dietary records (n=6). In the baseline period of the rooibos intervention trial the mean difference was +46.8 mg/day with the limits of agreement +806.2 and -712.7 mg/day, respectively for the comprehensive FFQ versus the dietary records. The comprehensive FFQ and abbreviated FFQ showed an acceptable agreement to dietary records for an individual in its standard use.

The plots showed a tendency of the data points to cluster around the line of identity, but the differences tended to be positive with increasing intakes, showing higher estimates or bias from the FFQs as to the dietary records with higher mean daily total flavonoid intakes. This tendency towards positive intake estimates against the dietary records at higher mean daily total intakes emerged with the comprehensive FFQ, particularly in the baseline and intervention trial periods

where intake estimates were higher, the abbreviated FFQ application within the baseline and intervention trial periods, and the abbreviated FFQ administrations in the further participant group. This tendency for an overestimation at higher intakes emerged at intakes of above 500 mg/day in the case of the comprehensive and abbreviated FFQ applications within the rooibos intervention trial and at intakes approaching 750 mg/day for the abbreviated FFQ administrations in the further participant group.

The negative difference between the abbreviated FFQ and the dietary records in the washout and control trial periods with higher intakes was not unexpected within the more limited intake range of these trial periods, where the abbreviated FFQ with its reduced food list possibly did not capture the full food and beverage intake range as that of the dietary records. Adjustment of the comprehensive FFQ for overestimation showed poorer agreement with the dietary records at individual level than the unadjusted FFQ within the baseline period of the rooibos intervention trial. There was a tendency of fewer participants clustered around the zero difference level between the methods at the higher than the lower mean daily total flavonoid intakes. At the higher mean daily total flavonoid intake estimates, the flavonoid contribution brought about by the higher weekly tea consumption indication on the summary questions compared to the questionnaire weekly frequency, was such that the adjustment increased the mean daily total flavonoid intake estimates of particularly these participants and may be accountable for the tendency observed of an overestimation at the higher mean intake estimates (750 mg/day).

9.3.5   Influence of Adjustment Questions on Intake Estimates

A general difficulty encountered in dietary intake assessment using FFQs, is the overestimation of the dietary intake.(402,504) The extent to which this problem was experienced with the present comprehensive FFQ was assessed using three summary questions whereby the participants had to report the number of portions/servings of total fruit, vegetables and tea usually consumed per week. The mean of the summary question indications on the weekly portions/servings of fruit and vegetables consumed was lower than the mean of the collective indications for each obtained from the comprehensive FFQ. This confirms the general finding that FFQs overestimate fruit and vegetable intakes, in particular when obtained from a long food list.(18,40,82,384) The relative over-reporting found for fruit and vegetable intakes may be attributed to either the long list of items on the FFQ or the response to health promotion information.(384) However, the overestimation of the fruit intake in the comprehensive FFQ seemed to be greater than that for the vegetable intake, while it is generally found that overestimation of vegetable intakes in FFQs is greater than that of fruit intakes.(380,390,408,413)

It is commonly believed that the consumption frequency of fruits may be easier to estimate and as a consequence the error of the correction factor for fruits would most likely be less than that of vegetables,(401) which were not confirmed in the research. The collective mean weekly fruit portion/serving intake calculated from the comprehensive FFQ fresh fruit and fruit juice food list indications, was about two and one half times that of the mean of the summary question, while the collective mean weekly vegetable portion/serving intake calculated from the comprehensive FFQ food list was just slightly more than double that of the mean of the vegetable summary question. Amanatidis et al. (384) found major discrepancies in the fruit and vegetable intakes between the summary question estimates and the FFQ indications in their study with the weekly fruit estimates from the FFQ being more than double those from the fruit summary question. The discrepancy for the weekly fruit intake in this research was, somewhat greater than that in the study of Amanatidis et al.(384)

Although the items responsible for the FFQ fruit consumption overestimation could not be investigated in this research, as the summary question was a collective indication on the total weekly fruit group portions/servings that included fresh fruit and fruit juice, the overestimation could possibly be attributed to the fruit juice consumption indications as fruit juice intakes was identified as a possible source of overestimation in FFQs.(390,413) Overestimation of the fruit juice consumption and even the fresh fruit consumption in the comprehensive FFQ compared to the actual weekly consumption will increase the mean daily total flavonoid intake. Fruit juice and fruit are rich dietary flavonoid sources (93,125) and more so than for vegetables of which the intake indications in the FFQ have not been overestimated as much as the fruit juice and fruit intake.

The discrepancy found for the weekly tea portions/servings consumed between the summary question and the comprehensive FFQ food list indications is thought to be responsible for the substantial effect on the absolute mean daily total flavonoid intake estimate when the summary questions were used to correct for the fruit, vegetable and tea intakes from the FFQ. Adjustment based on a summary question pertaining to the weekly tea portion/serving consumption to correct FFQ results should be reconsidered, as summary question adjustment is supposed to weight down the collective responses in the dietary consumption in a FFQ.(71) This discrepancy in particular is thought to be responsible for the substantial effect on the estimate of absolute daily mean total flavonoid intake, when the summary questions were used to correct for overestimates from the FFQ. If absolute estimates of mean daily total flavonoid intakes need to be generated, then the use of a tea consumption summary question and/or correction factor may need to be reassessed. A small disparity in tea intake may have a substantial influence on the total flavonoid intake due to it being a rich flavonoid source (48) and its regular consumption.(260) Cade et al. (18) indicated that such cross-check questions may not be as effective when applied to assess other foods,

although Tsubono et al. (383) used such questions to correct for meat, fish and cooked rice consumption. The finding that correction for over-reporting may not improve or even lower correlations has been attributed to the fact that the correction factor or weighting considers the sum of items and not more appropriate weightings that belongs to each specific item. The sum of item errors may possibly be greater than the estimate error of a single item and, when applied in the correction for over-reporting, would decrease the validity of an estimate of a particular fruit or vegetable.(401)

Despite the substantial effect on the absolute mean daily total flavonoid intake estimate of the comprehensive FFQ, the intake was not significantly different ($p>0.05$) from that obtained from the baseline dietary records. Despite the overestimation, the participants could still be ranked with sufficient accuracy as the effect on ranking the participants was not particularly marked, as the Spearman and log-transformed Pearson correlation coefficients between the unadjusted comprehensive FFQ and the dietary records was 0.58 ($p<0.001$) and 0.62 ($p<0.0001$), respectively, and between the adjusted comprehensive FFQ and the dietary records 0.5 ($p<0.05$) and 0.56 ($p<0.001$), respectively, with all the correlations modestly strong and statistically significant. Ranking was barely affected and the proportion of participants largely misclassified the same for the unadjusted and the adjusted FFQs against the dietary records (quintile: n=3; tertile: n=1, in each case). This indicates moderate to substantial agreement (*Kw* of 0.41 to 0.60) for both these groupings and the two methods could be exchangeable for research, where subjects will be ranked or categorized.(384) However, the reduced quantitative efficiency brought about, limits the adjustment application where absolute intakes are important (intervention studies).(398) Calvert et al. (500) found that the ranking and quartile classification of subjects were not greatly affected on applying food group weightings to their questionnaire dataset.

9.3.6 Influence of Training or Learning Effects on Intake Estimates

In a crossover design where subjects progress from being the experimental to the control group or *vice versa*, measurement error resulting from a training effect of one method on the other can be shielded.(371) In a single group crossover application as in the rooibos intervention trial, measurement error may result from a training effect over time. The sequential administration of several dietary assessment methods to the same subjects raises concern that the completion of one method may influence responses to the other.(38) The participants in each of the rooibos intervention trial periods completed the dietary records before the FFQ. Van't Veer et al. (502) and Brown et al. (476) pointed out that in such an application sequence, the completion of the FFQ might partially be dependent on the earlier dietary reporting, which may have modified or improved the estimates given on the FFQ.

Considering the group mean total daily flavonoid intakes for each trial period in the rooibos intervention trial, it is possible that a learning effect from multiple administrations of the dietary records and FFQs could be interpreted for the first two versus the last two trial periods as put forward by Cullen et al. (53) in their study of multiple administrations of the dietary recalls and FFQs. When the group mean daily total flavonoid intake is considered for each trial period, the difference between the dietary record and FFQ estimates improved from the baseline (13.6% higher) and washout (16.8% higher) periods to the intervention (0.6% higher) and control (4% higher) periods. The comparative intake estimate was somewhat worse for the washout than the baseline trial period, and the control than the intervention trial period. The probable learning effect thus considered for the first two versus the last two trial periods, although there is no way to assess this directly, cannot be considered across the whole trial. Inattentiveness could possibly be considered for the larger mean difference found between the two dietary assessment methods in the washout trial period and overburdening for the control trial period due to it being the last period of the 14-week intervention trial.

In the intervention trial, the correlation coefficients (Pearson, Spearman and de-attenuated) between the dietary records and the comprehensive FFQ in the last two periods of the trial (intervention and control periods), were also higher than the correlation coefficients between the two dietary assessment methods in the baseline period and in particular the washout period of the trial, which may also implicate training or learning effects over time. According to Kristal et al.,(43) training or learning effect probably results after repeated administration of dietary records and FFQs so that subjects would more accurately record their diets on both methods. However, the correlation coefficient in the washout period that occurred after the baseline period of the trial, were far lower between the two dietary assessment methods in the washout period and not higher. The same trend occurred with the correlation coefficients between the two dietary assessment methods in the control period of the trial that occurred after the intervention period, but being lower than that of the intervention period. This is not quite supportive of training or learning effects, but could be more suggestive of a dietary intake attentiveness difficulty in the washout period, as this is the period where the flavonoid intake dietary restrictions started and into a reporting overburdening in the control trial period, as it was the last trial period. Cullen et al.,(53) found lower correlations in their study on further administration of a FFQ with 24-hour dietary recalls and suggested that the lower values either reflect a lower subject attentiveness to their dietary intake or to an overburdening due to the further additional reporting that was required.

Intensive dietary recording could alter awareness of diet and affect the responses to the questionnaire completed after the recording (471) thereby improving the accuracy in completing the questionnaire.(390) Feunekes et al.,(412) picked up a differential bias related to such altered

awareness. In the group in which the dietary history interview took place first, the intake assessed with the FFQ was more similar to the results of the dietary history than in the group that started with the FFQ. Such differential bias may, according to Feunekes et al.,(412) have various causes. When caused by a learning or test effect, it means that results of the FFQ may be improved by additional information obtained beforehand, which may have enhanced the consciousness or ability to recall consumption in the second FFQ application.(412) In general, the second questionnaire application, which is completed after the reference method application, seems to produce estimates more similar to the reference method than the first questionnaire application and lower than the first questionnaire application.(41,71) This might possibly reflect a certain learning effect pertaining to the recording of the consumed foods and their actual amounts eaten.(71)

Concerning the abbreviated FFQ in the further participant group administration, the results of the first FFQ application were biased less than that of the second FFQ application, which covered the same period as the dietary record completions. McKeown et al. (423) found that the second FFQ provided higher estimates of most nutrients compared to the mean of the two dietary records as the reference method. This is not quite supportive of learning or test effects as the process of keeping dietary records should have conceivably enhanced the accuracy in completing the second questionnaire. Because the subjects in the further participant group administration were not familiar with the dietary recording process and had not previously completed a FFQ, learning bias that contributes to overestimating the accuracy of dietary intake values,(403) was limited as no training effect seemed to have emerged over time providing for a somewhat larger quantitative difference in reported intakes to emerge, as found by Hebert et al.(86) Erkkola et al. (390) found a minimal effect of increased awareness based on the correlations found between the food and the nutrient intakes in the first and the second questionnaire applications in their study. The use of a FFQ both before and after the recording period provide for minimal and maximal estimates of true validity,(390) which in this research provides for a difference in mean daily total flavonoid intake of about 5% for the FFQ administration in the further participant group.

Several investigators consistently found higher validity correlations between dietary records and FFQs for the second FFQ than the first FFQ, whether comprehensive FFQs (38,41,43) or short/brief FFQs,(410,424,447) and between 24-hour dietary recalls and FFQs.(365) This finding is not surprising as the second questionnaire inquires directly about the period covered by the references methods, whether dietary records (38) or recalls.(365) Participation in longitudinal studies on diet and health resulting in increased awareness of the dietary intake, could possibly explain better correlation coefficients found for a second FFQ application as put forward by Osler and Heitmann.(447) It is possible that subjects may become more aware of their diets through the

reference method application, hence the higher correlation values for the second questionnaire. (365) Given that the second abbreviated FFQ was collected close in time to the dietary recording (within one week as in the study of Date et al.,(360)) it is conceivable that the intensive dietary recording may have altered the participant awareness of their diet,(471) sensitized them with respect to their food consumption (38) and heightened their awareness of completing the second FFQ. They may have completed the second questionnaire more accurately than the first creating artificially strong correlation coefficients between the second FFQ and the dietary records.(38,41) This lack of independence would result in high correlated errors and an overestimate of validity.(41) It is argued that the expected relative validity of the second FFQ compared to a first FFQ application would be greater because of a learning effect.(423) This was not encountered in the application of the abbreviated FFQ in the further group administration as the correlation coefficients between the first and the second abbreviated FFQ applications and the dietary records are almost identical.

As in this application, Rosner and Willett (471) and Mullie et al. (425) also found the nutrient correlations with a questionnaire completed before the dietary recording similar to those with the questionnaire completed after the dietary recording.(471) McKeown et al. (423) found that the overall relative validity of the second FFQ was not greater than that of the first FFQ. Martinez et al.,(41) although finding a similar range of correlation coefficients across the nutrient intakes and thus a similar correlation average between the first and the second FFQ and dietary records as reference method, only the relative validity of dietary fibre was similar whether using the first or the second FFQ. To eliminate this possible contribution of learned responses when subjects answered questions on the second FFQ, Frankenfeld et al. (410) only reported the data from the first FFQs in their isoflavone intake validity analysis. Because the error sources for the FFQ are least shared with that of dietary records,(387) it is generally assumed that there is independence between these two methods,(41) which reduces possible enhanced agreement as would be found with similar errors in the assessment methods.(14)

9.4 REPRODUCIBILITY OF COMPREHENSIVE AND RESULTANT ABBREVIATED FFQ

Several studies found that the dietary intake estimates on two FFQ administrations agreed well, as was found in this research, and in particular when administered within a shorter time frame, as in the further participant group administration. In the rooibos intervention trial, the difference found in the mean daily total flavonoid intake computed for the washout and the control trial periods for the comprehensive FFQ administration and the resultant abbreviated FFQ application at these two occasions two months apart, was about 22% and 13% respectively (21.92% and 12.7% higher on the second compared to the first occasion, respectively). These values should be seen in the

context of the washout and control trial periods of the rooibos intervention, which demanded restricted total flavonoid intakes where a small difference in the absolute total flavonoid intake estimates would contribute to a large percentage difference in the intakes.

In the further participant group administration the absolute intake agreement level between the two abbreviated FFQ administrations two weeks apart was high, with the difference in the mean intake being about 4% (4.43% higher on the second compared to the first occasion). Schaffer et al. (403) found that the median estimates for all nutrients assessed on their second questionnaire were 90 to 99% of the estimates from the first questionnaire, while Kusama et al.(24) found no difference in the median nutrient intakes for lipid, carbohydrate, fibre, riboflavin and potassium between their two FFQ assessments on a time interval of one week. There was virtually no difference between the total mean daily flavonoid intake of the first and the second FFQ administration (166±146.6 versus 158.3±151.8 mg/day) two weeks apart in the study of Mullie et al.(84) The strong linear associations (correlation coefficients of 0.9) found between the daily total flavonoid intake estimates of first and the second or repeat abbreviated FFQ administrations is supported by the validation study of Mullie et al. (84) who also found strong correlations ($\geq$0.80) between the two FFQ applications for all the individual flavonoids.

Although there were no significant (p>0.05) differences between the paired median and interquartile range daily total flavonoid intake estimates of the comprehensive FFQ administration and the resultant abbreviated FFQ application in the washout and control rooibos trial periods and the two abbreviated FFQ administrations in the further participant group, comparing these occasions all indicate that the intake estimate on the second FFQ occasion, whether the comprehensive or the abbreviated FFQ, was somewhat higher than that of the first occasion. A generally reported occurrence on repeat administration of the same FFQ is that the first questionnaire administration generally gives higher intake estimates of foods and nutrients, than the second questionnaire administration,(365,390,403,407) despite the intakes often not significantly different except in some nutrient cases, like vitamin C.(365)

There is no clear reason why first measurements produce higher estimates than those obtained in subsequent measurements, but is has been proposed that subjects are likely to have a more rational view of their diets and could therefore quantify their intakes better at the second administration of the questionnaire,(365) producing response set bias if subjects had learnt to give more accurate answers in the subsequent measurements.(407) Brown et al.,(476) expressed concern that repeated administration of a FFQ may reduce food intake reporting with time that result in falsely low intakes. Wheeler et al. (394) found a significant decrease in reported intake of approximately 10% for all nutrients regardless of the format used across three FFQs, when the

same questionnaire was completed a second time after an interval of four-to-six weeks. In this current research, however, the opposite occurred with the intake estimate of the second administration somewhat higher than that of the first administration. The completion of the dietary assessment measures may have sensitized the participants to their dietary intakes contributing to the finding of a higher intake estimate on the second questionnaire application.

The test-retest correlation (on average 0.5) of the comprehensive FFQ administration and the abbreviated FFQ application within the rooibos intervention trial washout and control periods for the mean daily total flavonoid intake estimates, showed a moderate, but significant (p<0.05 for each) degree of reproducibility. It is not very useful to compare the reproducibility of a FFQ with those reported for other FFQs, since the reproducibility is determined in different ways and FFQs may have varying degree of correlated error.(430) The correlation coefficients (log-transformed Pearson and Spearman correlation coefficients of 0.44 and 0.55 for the comprehensive FFQ and 0.49 and 0.58 for the abbreviated FFQ, respectively) found in the rooibos trial are of similar magnitude to others that have reported reproducibility correlation coefficients irrespective of the magnitude of the FFQ and the time interval between the questionnaires.

Knekt et al. (262) found a correlation coefficient of 0.53 after 4 to 8 month repeatability for the daily intake of all individual flavonoids combined (sum of four flavonols, two flavones and three flavanones) utilizing a questionnaire listing more than 100 dietary items and composite dishes customary to the Finnish diet. According to Willett and Lenart,(387) correlation coefficients generally ranged from 0.5 to 0.7 for nutrient intakes in reproducibility studies. It is believed that few responses would have been remembered and transferred directly from the long questionnaire within the rooibos trial periods from the washout to the control periods with the intervention period in-between. The study sample tended to consume a wide variety of dietary items, which decreased the possibility of remembrance and as a result response repetition. These reasons were also offered by Bergman et al. (366) for the possible lack of response set bias. A contributory factor to the more moderate than high reproducibility found, could be attributed to the number of questions and specifically the long list of items included in the comprehensive FFQ food list. Matthys et al. (443) pointed out that a high number of questions could introduce answering-fatigue. This latter reason does not seem to be applicable, as the mean total daily flavonoid intake of the control period was higher than that of the washout period.

The abbreviated FFQ administered within the further participant group showed a strong degree of reproducibility with log-transformed Pearson and Spearman correlation coefficients of 0.9 being highly significant (p<0.0001), indicating that the levels of within-person agreement between the two FFQ measurements were high. High correlation coefficients in the order of 0.8 to 0.9 have been

indicated in published reproducibility reports. The Pearson correlation coefficients for macronutrients ranged from 0.88 to 0.97 in the study of Kassam-Khamis et al. (407) utilizing a 207-item FFQ re-administered after three months. Schaffer et al. (403) found ICCs of 0.80 for energy, 0.81 for vitamin B_6 and dietary fibre, 0.82 for carbohydrate and 0.83 for folate between a FFQ with 49 main food questions re-administered at a two-week interval. Egami et al. (441) reported a correlation coefficient of 0.82 for calcium between two administrations of a 97-item FFQ at a one-year interval. Published reports with the same interval time between questionnaires as applied in the further participant group administration show that high correlation coefficients could be achieved.

Date et al. (360) at an interval of one week and using a 122-item interview-administered FFQ, found most nutrient correlation coefficients above 0.7, with 0.78 for calcium. For the same interval and using a 166-item FFQ Kusama et al. (24) reported that 0.8 could be achieved for half of the investigated nutrients with 0.89 achieved for potassium and 0.88 for vitamin B_2. Mullie et al. (425) after a two-week interval found that the correlation coefficients for energy adjusted nutrients between the two FFQs could reach 0.79 for total energy and vitamin C intakes. The reproducibility of a brief dietary screening tool, PrimeScreen, a 25-item FFQ about the average consumption of targeted food groups over the previous year, at an interval of two weeks delivered a mean correlation coefficient of 0.70 for foods and food groups and 0.74 for nutrients.(433) For flavonoid intake specifically, Mullie et al. (84) found the correlation coefficients for all the individual flavonoid compounds equal to or above 0.8 between the two administrations of their 86-item FFQ two weeks apart.

However, whereas true changes in dietary intake can reduce the correlation coefficient, high correlations of within-person measurement errors can result in inflated correlation coefficients. (423) In the further participant group, the repeat administration of the abbreviated FFQ with a reduced food list within a period of two weeks apart, may have caused a possible response transfer from the first to the second FFQ, but this is not thought to be too likely as the first abbreviated FFQ completion was the first encounter of all the participants with a FFQ. Although response set bias may be a likely consideration for the high reproducibility found, the items included in the reduced food list may be accommodating the high reproducibility. Reproducibility correlations comparing food intakes on first and second questionnaire administrations found correlation coefficients to be highest for those dietary items consumed daily and lowest for rarely eaten foods.(390) High reproducibility has specifically been observed for a number of dietary items included in the abbreviated FFQ that obviously contribute greatly to the daily total flavonoid intake. The reproducibility coefficients for these dietary items, which are consumed daily vary between 0.59 for fruit juice, 0.72 for fruit,(390) 0.68 for green tea (266) and 0.77 (390) and 0.90 (39) for tea

intakes. High reproducibility has been observed in particular for alcohol intakes with the correlation coefficients equaling 0.9 and higher, namely 0.88,(394) 0.90 (430) and 0.91 (266) for alcohol and 0.93 for beer (39) intakes.

The mean total daily flavonoid intakes were also classified into quintiles and tertiles to determine the level of agreement between the first and second administrations of the comprehensive FFQ and the abbreviated FFQ within the rooibos intervention trial. As would be expected the percentage grossly misclassified with the comprehensive FFQ (12.5% and 5% for the quintile and tertile agreements, respectively) was similar to that found for the abbreviated FFQ (17.5% and 2.5%, respectively). Considering the *Kw* statistic to allow for agreement by chance alone the agreement between the comprehensive FFQs signified moderate agreement (0.41 to 0.60), while that of the abbreviated FFQs poor agreement (<0.2). Kusama et al. (24) found the *Kw* values for their FFQ administrations with a time interval of one week to range from 0.42 for lipid to 0.65 for potassium, which signify moderate agreement, despite the low number of subjects in their study classified into opposite thirds ranging from nil for carotene, riboflavin, vitamin C and calcium to 4% for retinol. Wheeler et al. (394) indicated that the fact that their FFQs did not show more than moderate agreement (*K* values for the three FFQ groups of 0.40, 0.44 and 0.40 respectively), despite the tertile participant percentage grossly misclassified on each occasion for all three of their FFQ groups being only 4% (range 0-7%), 4% (range 0-13%) and 7% (range 0-12%), respectively, could be due to the reduction in overall reported intake, which was observed between the first and second FFQ administrations in their study.

This explanation may be applicable to the comprehensive FFQ administration and resultant abbreviated FFQ application in the rooibos trial which, respectively, showed moderate and poor agreements as the overall reported intake increased by about 22% and 13% between the two occasions for the comprehensive and the resultant abbreviated FFQs, respectively. Of greater importance is the extremely low mean daily total flavonoid intakes in the two utilized trial periods (37 and 29 mg/day for the comprehensive and 17 and 15 mg/day for the abbreviated FFQ in the washout and control periods, respectively), which did not allow for much intake flexibility, especially for the abbreviated FFQ with its extremely low computed intakes on both occasions where poor inter-test agreement was found. The inter-method agreement between the two administrations of the abbreviated FFQ in the further participant group administration, categorized as moderate (about 0.56 for each), reached the upper range of moderate agreement (*Kw* of 0.41 to 0.60) for both the quintile and tertile cross-classifications.

Good agreement in the mean daily total flavonoid intake estimates, however, does seem to exist between the repeat administrations of the comprehensive and the abbreviated FFQs within the

rooibos intervention washout and control trial periods, as well as the abbreviated FFQ repeat administration in the further participant group. All the respective Bland-Altman scatters plots showed a tendency of the data points to cluster around the line of identity, and even at higher intakes at the zero difference level, along with very few participants above and below the limits of agreement.

9.5 BIOMARKER VALIDITY AND RESPONSIVENESS OF COMPREHENSIVE AND RESULTANT ABBREVIATED FFQ

Although comparison of dietary intake estimates, obtained from a FFQ, with plasma concentrations of the intake exposure provide a measure of the relative validity of the dietary assessment method,(531) the validity of such analytical biomarkers as measure of the dietary exposure level may not be fully suited and the correlations with the dietary intake estimates weak (269,502,508) as a result of several intervening factors.(269) On the one hand, the limitations in dietary assessment methods and the incomplete data in food composition databases restrict precise dietary intake assessment research,(86) also of flavonoid intake.(9) The FFQ as dietary assessment method is known to have several sources of error that include the restrictions imposed by its fixed food list, its estimations of portion sizes and the cognitive challenge of reporting the item consumption frequency over a broad time span,(387) which was restricted to the past two weeks in this research.

Flavonoid concentrations in food furthermore are influenced by numerous genetic (variations in plant type and cultivar), environmental (plant growth, season, light exposure and degree of ripeness) and technological (food preparation and processing) factors.(9,101) These factors influence the dietary flavonoid intake, and ultimately the bioavailability,(9) which are practically not possible to present through a food composition database. Tulipani et al. (167) evaluated the individual effects of selected strawberry genotypes on postprandial antioxidant status in healthy subjects and found that the lowest increases in plasma TAC occurred with the consumption of the nutritionally inferior cultivars considering the anthocyanin content of the berries. These findings underline the importance of the nutritional quality of fruits.(167) The current flavonoid database not only has missing content values for various dietary items that were consumed by some study participants, but it also provides limited differentiation between cooked and raw vegetables, which could affect flavonoid food content and bioavailability.

The consumption of tea, which was the major contributory source to the dietary flavonoid intake, was measured rather crudely in the research, as the strength of the tea brew was not considered. Erdman et al. (75) explained this matter rather well by referring to wine and tea and the potential error in their intake estimates and the resulting attenuation this may cause in epidemiological study

RR estimates because of the greater measurement error for tea consumption. These researchers related the stronger and more consistent cardioprotective effect seen with wine, rather than with tea, to the more accurate intake assessment of wine consumption. They indicated that most individuals could estimate their wine intake reasonably accurately because of its relatively high cost and the standard bottle size that results in little response bias (except at very high intakes). The intake estimates of tea are, however, often crudely indicated as only cups consumed are reported. This results from differences in actual cup size and dilution factors, including ice in iced tea, that not only greatly affect the tea amount consumed but also the tea strength, which makes the amount of dry tea used to make the tea an important indication.(75) Using the USDA flavonoid database, in the absence of a South African flavonoid database, would have introduced inaccuracies in the research total flavonoid intake estimations. Strong correlations between calculated intake of a compound and its plasma levels are thus not usually obtained that is said to reflect the incapacity to estimate the intake of the compound correctly,(108) which may in consideration of the above dietary assessment inaccuracies be relevant to this research.

On the other hand, the biological potency of bioactive compounds itself is influenced by many factors, like the specific dietary source, the quantity ingested and how the bioactive compounds are consumed (alone or with other items forming a further food matrix), while the biological response and resulting clinical outcome in the subjects consuming the compounds will be influenced by their genetic variability in dietary response, demographic profile, clinical characteristics and possibly multiple lifestyle factors.(3) Circulating polyphenol concentrations will potentially be influenced by various dietary and host factors that will not only affect polyphenol intake, but also the absorption and metabolism. Even though homeostatic control of phenolic concentrations has not been described, it was assumed that the linear correlation strength between the mean daily total flavonoid intake estimates and the biomarker exposure levels, like the plasma total polyphenol concentrations and the plasma TAC, would be rather weak due to the varying degrees of influence by the factors indicated above.

The current research found no to rather weak non-significant linear correlations between the mean daily total flavonoid intake estimates and the biomarker exposure levels, that implies it is innately absent for the plasma total polyphenols and weak for the plasma TAC. The contributing factors to the wide variability in both plasma and urine levels of polyphenolic conjugates and metabolites among subjects, might be individual variability in the levels and activities of the small intestinal enzymes and transporters, as well as the distinctive individual compositions of the colonic microflora that provide for variable levels of mucosal uptake and excretion.(202) A great number of rooibos flavonoids are either *C*-glucosides or *O*-rhamnoglucosides, which are poorly absorbed. Rooibos flavonoids will therefore largely be metabolized and absorbed in the colon making the

phase II metabolism and degradation by intestinal bacteria important factors in the absorption.(63) Several studies have found non-detectable baseline circulating concentrations of certain flavonoids, like EC,(123,211) following a 12-hour fasting period in some, but not all, study subjects. The amounts of anthocyanin and its metabolites excreted on consumption of blackcurrant juice, for instance, varied considerably among subjects in the study of Rechner et al.(202)

The subjects were also consuming varied diets in terms of the flavonoid subclasses and the individual flavonoids contained in them during the baseline period of the intervention trial. The type of flavonoid does not only differ among foods,(269) but the distinct phenolic compounds formed on consumption differ in their bioavailability and metabolism (175) and it may be that one type has a stronger biological effect than another,(269) for example the antioxidant activity of the resulting metabolites,(175) though valid data to support this is lacking.(269) The bioavailability of quercetin from tea is lower than from onion.(108) These intervening factors in terms of total flavonoid intake (via the consumption of the various subclasses and the individual flavonoids and the foods in which they are present) and the bioavailability (absorption and post-absorptive metabolism) will most possibly vary systematically between subjects and influence the validity of a marker as reflection of the dietary intake level and correlations with questionnaire estimates may thus stay weak.(502,505) Furthermore, if such biomarker measures are subject to day-to-day fluctuations like dietary measures are, the utilization of a single day blood sample may introduce random error that will reduce the correlation found in comparison to the true correlation.(531)

Total systemic phenols almost certainly reflect the total intake of plant products.(554) Although it is widely indicated that flavonoids contribute to two-thirds of the polyphenolic intake,(7,8,12) this may not be the case. It seems that phenolic acids may make a far larger contribution to the total polyphenolic intake (600,601) than considered earlier. Ovaskainen et al. (601) found that the phenolic acids comprised 75% of the total polyphenolic intake of a national representative sample of Finnish adults, to which coffee and cereal products were the main contributors. Maskarinec et al. (554) explained the lack of relation found between total plasma polyphenols and fruit and vegetable intake in their intervention study to be a result of other dietary sources of polyphenols, like coffee and isoflavones from soy, which may have masked the probable '9-A-Day' intervention effect.

Most human intervention antioxidant supplementation trials have failed to demonstrate an effect of flavonoid supplementation on the TAC. Even if such supplementation resulted in a large increase in the plasma concentration of the particular antioxidant, it resulted in only a modest change in the TAC. This is not unexpected, as the TAC is the sum of many different antioxidants, including those of exogenous or dietary origin and those of endogenous origin.(241) The TAC measurements of

polyphenols and their food extracts have poor predictive values *in vivo,* as to their TAC and other health benefits as polyphenols are comprehensively metabolized in the body.(94) Many of the intermediate and degradation products, due to structural similarities to the parent compounds, in many cases still retain antioxidant effects, although the potency and efficacy may be affected.(63) For instance, the *in vivo* EC metabolites are likely to be present at lower concentrations and to have a reduced antioxidant capacity compared with that of EC itself as the parent compound.(602)

The dietary phenolic content itself cannot be considered as index of the *in vivo* antioxidant activity. The chemical nature of the phenol, the combination of the different phenolics, the different dosages, the food matrix, the interactions with nutrients and the metabolic transformation must be taken into account when evaluating the *in vivo* phenolic bioavailability and activity, like the TAC, of phenol-rich dietary sources.(2,32) As the types of flavonoids differ among foods and one type may have a stronger biological effect than another,(269) this may have greatly determined the plasma TAC of the individual participants, as they followed their usual diets in the baseline period of the trial. It is not surprising that only a very weak (a maximum of 0.2) and non-significant (p>0.05) correlation was found between the mean daily total flavonoid intake estimates of both the comprehensive and the abbreviated FFQs and the plasma TAC as determined by the different TAC assays applied.

The health-promoting effects of flavonoids are generally ascribed to their potent antioxidant activities, but support of their *in vivo* antioxidant effects is conflicting. This may be because maximal plasma levels, even after high flavonoid ingestion, may be low (not enough to produce marked systemic antioxidant effects) and because flavonoid metabolites tend to have lower antioxidant activity.(278) Research results indicate that polyphenol-rich dietary sources are able to transfer their antioxidant capacity to body fluids,(2) as indicated for this research in using the plasma TAC as biomarker of flavonoid exposure. Although no significant (p>0.05) associations were found between the mean daily total flavonoid intake estimates of both the comprehensive and the abbreviated FFQs and the plasma CD and MDA levels, an association of nearly -0.3 was observed for the plasma MDA with that of the plasma CDs innately absent. The plasma CDs represent the early stage of lipid peroxidation of unsaturated fatty acids, while most of the aldehydes that react with TBA in the TBARS assay are derived from already formed peroxides.(578) The weak correlation found for the plasma MDA levels, as measured by the TBARS assay, in comparison to the plasma CD levels in relation to the mean daily total flavonoid intake estimates, may be explained by the intervention study participant group that were selected on meeting the inclusion criteria of being at an intermediate to high CHD risk on risk factor assessment (595) that included having two or more risk factors for CHD (37) to represent oxidatively stressed subjects.

Overall the weak correlations found were comparable to what has been reported for several other nutrients, the plasma levels of which are used as biomarkers of exposure. Adjusted Pearson's correlations of questionnaire-derived antioxidant (carotenoids, vitamins C and E) intakes capturing a one month time frame ranged from 0.10 to 0.33 between the questionnaire and the exposure biomarkers of these dietary antioxidants.(345) Correlations between changes in the between-trial period participant mean daily total flavonoid intakes and changes in the between-trial period participant plasma biomarker concentrations, were not investigated due to the differences in the flavonoid content of even the same foods (as a result of plant genetics, agronomic and environmental factors during growth and postharvest handling), the differences in the flavonoid bioavailability from the various dietary items and the wide variation in between-person bioavailability with the numerous factors influencing the bioavailability. These may have constrained the ability to find such correlations as put forward by McEgliot et al. (522) for changes in carotenoid intakes and changes in serum carotenoid concentrations.

The changed mean daily total flavonoid intake estimates found, as determined by the repeated measures ANOVA (with Bonferroni correction) for the comprehensive and the resultant abbreviated FFQs in the intervention versus the washout and the control trial periods, demonstrated the intended intervention effects and the questionnaire responsiveness. It could distinguish between low and high consumption levels, even on the exclusion of tea as the major dietary contributor to the datasets. This was not fully reflected in the plasma biomarker results across the trial periods, particularly the TAC. A sample obtained after an overnight fast may not be appropriate to assess changes in plasma TAC.(214) As emphasized by Cao et al.,(603) the TAC measured in blood samples taken from subjects who had fasted for at least 10 hours reflect the minimum levels expected in subjects with extended and consistent daily intakes. Using the repeated measures ANOVA as for the dietary intakes, the plasma total polyphenols as biomarkers of exposure demonstrated that a reduction and increase in the dietary consumption of flavonoids was accompanied by a decrease and increase, respectively, in the plasma concentrations of total polyphenols. In contrast, the plasma CDs and TBARS as biomarkers of effect, demonstrated that a reduction and increase in dietary consumption of flavonoids was accompanied by an increase and a decrease in their plasma concentrations, respectively. An intervention study that increased the total fruit and vegetable intakes to nine daily servings in healthy women, observed a treatment effect on the plasma TBARS, although the decrease after three and six months of intervention was not as large as expected.(554)

9.6   FOOD LIST OF ABBREVIATED FFQ

9.6.1   Percentage Contribution and Between-Person Variance of Food List Dietary Items towards Mean Daily Total Flavonoid Intake

The full comprehensive FFQ food list was reduced for the abbreviated FFQ utilizing the percentage contribution and the stepwise multiple regression approaches as applied by other investigators.(29,395,403,449) The final 16-item abbreviated FFQ, extended to 23 items, provided about 93% of the mean daily total flavonoid supply, which can be considered a rational contribution. Several other investigators have also compiled their FFQ food list to together supply about 80% (397,412) to 90% or more (24,360) of the total population intake of the nutrient(s) of interest. The number of items necessary to provide high intake coverage was found to be higher for the intake of energy and macronutrients, than for most micronutrients.(24,395) Kusama et al. (24) found the number of dietary items to provide 90% coverage were 57 for energy, 47 for protein, 51 for lipid, 53 for carbohydrate versus 26 for retinol and 18 for carotene. Stryker et al. (395) found that ten foods accounted for 82% of their sample's intake of preformed vitamin A and vitamin B$_{12}$, yet only 34% of the total energy intake. The present abbreviated FFQ food list was compiled to ensure a high coverage of the mean daily total flavonoid intake. Based on the number of dietary items to achieve this, the results seem to be in agreement with the findings that micronutrients require a low number of items to provide for a high intake coverage. This is not unexpected, as it pointed out that nearly all foods contribute to energy intake, whereas some nutrients have only a few major dietary sources,(395) which seem true for flavonoids.

As found for the Australian (89) and the Dutch (45) populations, the major source of flavonoids in this research was not fruits or vegetables, but black tea, which provided about 76% of the flavonoid intake for the Australians based on flavonoid intake estimates from the major subclasses (89) and 61% of the intake for the Dutch based on flavonoid intake estimates from the flavonol and flavone subclasses.(45) In the current research, black tea provided 68% of the intake in the baseline period of the rooibos intervention trial and 56% of the intake for the further participant group. Worldwide, tea is said to be one of the main dietary sources of antioxidants due to the high concentration of polyphenols in tea, in combination with the regular consumption of this beverage.(260) Song and Chun (342) considered the dietary intake data of the adults included in NHANES, 1999-2002, and found the daily total flavonoid intake to be provided mainly by tea (82.8%). Mullie et al. (84) in their validation study, in which Flemish dieticians participated, found that the total flavonoid intake as assessed by the different dietary assessment methods, were greatly provided by flavanols, which accounted for 75% of the intake, due to the consumption of tea. Black tea may provide over half of the flavonoid intake in Western diets,(604) which was confirmed in this research. Tea, therefore, seems to be the major dietary source for flavonoids in

Western populations (235,257) with black tea the major form of tea consumed (31) and among the general South African population.(90)

Besides for the major contribution of black tea to the dietary flavonoid intake,(45,169,258,338) a number of other foods and beverages have also emerged as notable contributory sources across epidemiological studies on flavonoid, mostly subclass, intakes though to a far lesser extent than tea. The notable dietary sources identified in the studies that investigated flavonoid intake based on the sum of a few subclass intakes, in particular the flavonols and the flavones, are apples (11, 111,153,232,250,253,254,262,309,338) and berries (11,262) for fruits, onions (11,74,111,153,232, 253,254,262,338,390) and sometimes broccoli, particularly in US studies (153,232,250,254) for vegetables and fruit juices (11,262) and wine (190) for beverages and in addition citrus fruits when the intake of the subclass flavavones is additionally considered.(11,338)

The notable dietary sources identified in studies which investigated the daily total flavonoid intake considering all the major flavonoid subclasses, include: as fruits, apples,(89,137,164,248) pears, (248) grapes,(89) strawberries,(248) citrus fruit (89,137,248,342) and citrus fruit juices, like orange juice,(248,342) and other fruit juices;(248) as notable vegetable source, onion; as herb source, parsley (137); as beverage source, wine, particularly red wine,(89,137,164,342) along with dark chocolate and cocoa (164,248). All these dietary sources were identified in this research as notable dietary sources contributing to the mean daily total flavonoid intake. In the Mediterranean countries, wine consumption appears to make an important contribution to the flavonoid intake.(130) In the Seven Countries Study, red wine was, for instance, the main source of intake of the flavonol and flavone flavonoid subclasses in the Italian cohorts.(253)

These items that were among the greatest contributors to the mean daily total flavonoid intake were, furthermore, among the best indicators of the between-person variance in intake. Stryker et al. (395) found that some foods contribute greatly to absolute intake and to the between-person variation in intake, which as, in this research, may be ascribed to the use of the top contributory foods to the total intake to identify the dietary items that contribute to the between-person variability in intake. However, it is not the case for all items that the greatest contributors to the dietary intake are among the best indicators of the between-person variation in intake.(395,411,449)

Ishihara et al. (411) found that some dietary items (spinach, rice and cabbage) that contributed greatly to the total intake of folate, were less important contributors when the between-person variability was taken into account. Spinach, being a very rich source of folate, and one of the highest contributors to mean folate intake, could not explain the between-person variation in their study because nearly all subjects consumed it. Green tea contributed greatly to both individual

intake and to between-person variability, probably because consumption was strongly reliant on individual liking,(411) which in the current research may be applicable to all three major contributors being black tea, rooibos and red wine, where individual preference may be a strong determinant. Song and Chun (342) differentiated between tea consumer and tea non-consumer groups in studying the dietary intake data of NHANES, 1999-2002. The current research found that a relatively low number of foods is necessary to explain 99% of the between-person variation in the daily total flavonoid intake estimates by using the comprehensive FFQ (n=14 items) and the dietary records (n=11 items) in the rooibos intervention trial, and the dietary records (n=21 items) in the further participant group of which the dietary items were similar across these multiple regression analyses extractions.

9.6.2 Nutritional Health Appraisal of Food List Dietary Items

In the context of heart health protection, a specific subclass of flavonoids, namely, the flavanols, has received extensive coverage. Plant-derived, flavanol-rich foods and beverages include various fruits, berries, tea, wine, as well as cocoa and cocoa products.(172) Research on these flavonoid dietary sources and CVD have mainly been conducted on tea, red wine and chocolate,(232) all of which are incorporated in the reduced food list of the abbreviated FFQ. Additional indications of the potential cardioprotective effects of flavonoids can be derived from epidemiological studies on specific flavonoid-rich dietary sources, like tea or wine consumption and CHD risk, although tea and red wine both contain compounds that could, in addition to the flavonoids present, explain the cardioprotective effects found.(130) Several dietary intervention trials in humans and animals suggest that flavanol-rich beverages and foods provide cardioprotective effects, in particular relating to vascular function and platelet reactivity.(172)

Beverages like black and green teas, along with red wine,(32,605) and even cocoa-containing,(605) are commonly consumed throughout the world and known to be rich in the phenolic flavonoid phytochemicals.(32,123,130,605) The consumption of phenolic-rich beverages represent a natural source of these health-promoting compounds (32) and have become an attractive utility in the prevention of CVD.(301) Flavonoid-rich beverages may be an important dietary entity in reducing the risk for certain chronic diseases.(27) The consumption of beverages emerged in this research as a major source contributing to the dietary intake of flavonoids, as half of the items in the abbreviated FFQ food list represent beverages (n=9 of the 16 items) and the other half foods (n=7 of the 16 items). Nutritional health concerns have been raised about some of the dietary items, both food and beverage items, extracted for incorporation in the abbreviated FFQ food list. This necessitated their nutritional health appraisal for inclusion recognition, which could only be conducted by literature support. A nutritional health appraisal of all the dietary items incorporated in the food list of the abbreviated FFQ was undertaken principally in support of the

future use of the abbreviated FFQ, as it would not be acceptable to accentuate or advocate such foods as contributory to flavonoid consumption from a nutritional health viewpoint.

9.6.2.1 Black Tea, Green Tea and Rooibos Herbal Tea

Tea is worldwide one of the most popular consumed beverages (271) and after water,(27,187,571) the most common beverage consumed throughout the world.(27,59,187,260,268,571) While black tea is mostly consumed in the Western world (59,187) and in the whole African continent, it is also consumed in the South and South East Asian countries.(59) Green tea is largely consumed in Asia and the Middle East,(187) with it being the principal beverage in China.(59) Although most Western societies consume black tea with the intakes of other types of tea, like green tea, there is an increasing popularity of this tea,(232) which was demonstrated by its inclusion in the abbreviated FFQ food list, although subsequent to black tea (and rooibos).

Most people in China (210) and Japan (149,210,266) drink large quantities of green tea. CAD incidence and mortality is much lower in China (210) and Japan (149) than in the West (the so-called 'Far East paradox').(210) Nevertheless the high smoking rate in these countries, compared to other industrialized countries, the incidence and mortality of CAD is still low under these circumstances. Genetic differences, the environment or living habits and a low plasma cholesterol concentration could be explanations for the low incidence.(149) The strong antioxidant property of green tea, due to the catechin flavonoid contents,(149,266) may be an explanation for the low prevalence through its preventive effect on the development of CAD.(149) Tea flavonoids may play a significant role in this so-called 'Far East paradox'.(210)

Tea flavonoids are thought to interfere with the arteriosclerotic process (4) and have a protective effect on CVD.(268) Because of the high consumption of tea (268) and CVD being the most common cause of mortality in Western societies,(4) even a modest effect of tea consumption on heart disease risk would be remarkable from a public health point of view.(4,243,268) This is particularly important for Western populations, as the consumption of black tea would be a major dietary source of their flavonoid intake,(257) as was confirmed in this South African research in both the rooibos intervention trial and the further participant group administration.

Based on limited research data, about 150 mg of flavonoids is necessary to initiate an acute antioxidant response and changes in prostacyclin. Some dose-response data indicates an antioxidant effect with about 500 mg of flavonoids. Brewed tea generally provides about 172 mg total flavonoids per 235 mL (brewed for two minutes); hence intake of one cup against three and one-half cups would be necessary to produce acute and chronic physiologic effects, respectively.(27) A meta-analysis of epidemiological studies on tea consumption by Peters et al. (268) concluded that three cups of tea per day might reduce the overall CVD risk by about 11%.

These findings certainly suggest that dietary inclusion of a plant-based beverage, particularly tea, may do well alongside the American Heart Association recommendation that the consumption of plant foods, like fruits and vegetables, should be increased.(267) Tea, as a non-caloric beverage, has been indicated to be an optimal delivery vehicle for certain bioactive compounds.(27)

The indigenous South African herbal tea, rooibos (*Aspalathus linearis*), is increasingly consumed as a healthy replacement to *Camellia sinensis* black and green teas.(63) Rooibos contains no caffeine and a low tannin content.(606) Its popularity has grown locally and internationally.(63) The most common rooibos herbal tea on the market is the traditional fermented product (63) produced through fermentation of the shredded plant leaves and thin stems at ambient temperature, followed by sun-drying. This fermentation process entails extensive oxidation of the phenolic compounds present, which provides for the formation of the rooibos sweetish flavour and red-brown colour.(63,606) Green, or unfermented, rooibos with its higher phenolic contents due to keeping the oxidative changes to a minimum, by mostly quick sun drying,(606) has of late entered the market.(63) Rooibos is generally enjoyed prepared as an infusion with boiled water to release the flavour and colour and is then enjoyed hot with or without milk and sugar added, according to taste. Several ready-to-drink rooibos ice teas have also entered the market (606) and contributed to the extension of the abbreviated FFQ food list to accommodate their inclusion.

The antioxidant activity of the phenolic compounds present in rooibos forms the underlying basis of its health promoting properties. Rooibos contains the unique phenolic flavonoids aspalathin (the most abundant) and nothofagin, with the highest concentrations in green or unfermented rooibos, as well as other phenolic compounds present and not present in common foods. A great number of these rooibos phenolic flavonoids present are *C*-glycosides.(63) In contrast to the vast available literature resources on black and green tea, only a few studies have reported on the potential beneficial health effects of rooibos consumption in particular in humans.(37,68,540)

A study reported by Kreuz et al.,(607) where a rooibos extract was administered orally to pigs, was the first to provide evidence that the *C*-glycosal flavonoid aspalathin is intestinally absorbed as *C*-glycoside, and as cleaved in an aglycone and sugar moiety, with the occurrence of *in vivo* formed aspalathin metabolites in the urine. Work in humans has confirmed that aspalathin is absorbed and bioavailable in humans with aspalathin metabolites also occurring in the urine after the ingestion of rooibos,(540,608) but none in the plasma.(540) The absence of plasma metabolites reflects the low bioavailability of these compounds, in addition to their rapid turnover rate and removal from the circulatory system.(540) Regarding the potential cardioprotective effects in humans, rooibos consumption appears to increase plasma TAC in healthy humans representing a source of dietary antioxidants,(68) to inhibit angiotensin-converting enzyme activity, which converts

angiotensin I to angiotensin II, a potent vasoconstrictor, in healthy volunteers implicating vasodilatory and cardiovascular benefits (69) and to increase GSH concentration in workers occupationally exposed to lead.(70) The rooibos intervention trial, of which this research formed part, itself provided evidence that rooibos consumption improved the plasma lipid profile (decreased serum LDL-cholesterol and increased HDL-cholesterol) and the redox status (reduced levels of plasma CDs and TBARS and increased reduced glutathione and GSH:GSSG ratio) of adults at risk for CHD.(37)

9.6.2.2 Red Wine

Studies on the beneficial health effects of grape flavonoids began far earlier than those of tea and chocolate because of the 'French paradox'.(75) In most countries, a high saturated fat intake is positively related to high CHD mortality.(237) However, the situation is different in France in that the French wine drinkers have a much lower CHD mortality, despite their high saturated fat intake (75,130,237) and serum cholesterol concentration.(237) It was proposed that the cardiovascular protection may in part be provided by the high wine (red) consumption (75,237) that counteracts the untoward effects of saturated fats.(237)

It is now thought that the flavonoids from red wine, and not solely the alcohol, are the primary cardioprotective components.(75) Although some cardioprotective effects of alcoholic beverages are the likely result of the ethanol-induced increase of HDL-cholesterol, reduction of fibrinogen plasma levels and platelet aggregation, the vascular protective effects of wine, observed in the French and in other populations, may be ascribed in part to the antioxidant, vasorelaxant and anti-thrombotic characteristics of the polyphenolic components.(239)

From these results, even though contradictory at times, it seems possible that absorption of polyphenols from wine, or from wine-derived products, may alter the composition and the chemical properties of plasma and its components, ensuing anti-sclerotic and anti-thrombotic effects.(239) According to some researchers,(240) informing patients and their relatives on what lifestyle adaptations might be advantageous to them, besides for emphasizing the management of risk factors, abstainers should be told that in the absence of contraindications and in the context of a healthy lifestyle, low-to-moderate wine drinking may contribute to better health. People who are already regular light-to-moderate wine drinkers should, in addition, be assured to continue. However, the dangers of alcohol abuse should constantly be emphasized, and heavy drinkers be advised to reduce their intake to a moderate level.(240) If no contraindications to alcohol intake are present, a moderate intake of alcohol (one to two drinks daily) may be considered safe.(238) Wine polyphenols as a food ingredient could be beneficial for those persons for whom alcohol is forbidden on account of social, health or religious grounds.(314) Nigdikar et al. (314) reported that one gram of polyphenols extracted from red wine per day is efficient to reduce LDL oxidizability.

In a cohort study of subjects in a northern California prepaid health care program, red, white and other types of wines and combinations of these wines had comparable relations to lower CHD mortality risk with a more encouraging mortality outcome for drinkers of wine, than for drinkers of beer or liquor.(593) A meta-analysis performed on 26 epidemiological studies also found that beer drinking was linked to a lowered risk of vascular events, although at a reduced level than that found for wine.(240)

9.6.2.3 *Dark and Milk Chocolate as Representatives of Cocoa Product Inclusions*

Until the 20th century, the Europeans ascribed numerous health properties to cocoa and chocolate (21,233). Thereafter, the beverage cocoa and chocolate became viewed from a more neutral perspective throughout Europe, whereas in the US it is typically viewed as non-nutritional confectionary food.(21) While cocoa and chocolate are still widely enjoyed as beverages, they are today most commonly consumed by many as confectionary (27) due to the flavour and texture properties.(21)

Cocoa is a potentially rich dietary source of flavonoids (21,27,214) particularly in the subclass flavanols or catechins,(21,120,123,233) and its polymeric compounds proanthocyanidins (also termed procyanidins).(21,120,123) It should be noted, however, that not all chocolates are similar contributors of flavonoids. Dark chocolate contains a higher cocoa bean liquor content than milk chocolate and thus also higher amounts of flavonoids.(21) The quality of the cocoa liquor used in the cocoa product production influences the polyphenol content. Typically the polyphenol content in dark chocolate is about three times more than that present in milk chocolate, due to the larger quantity of cocoa liquor included in dark chocolate in comparison to milk chocolate.(120) The flavonoid content of commercially available chocolate varies considerably with some products practically devoid of flavonoids (0.09 mg procyanidin/g), whereas others are rich in flavonoids (4 mg procyanidin/g).(27)

Cocoa and cocoa products, like chocolate, have a higher total flavanol content on a per weight basis than most other foods and beverages that contain flavanols,(21,605) also per serving of black tea, green tea (200 mL, respectively) or red wine (140 mL).(605) A serving size at 40 g of dark and milk chocolate provides phenolic antioxidants in amounts comparable to that of beverages like tea and red wine and fruits like blueberries, which are acknowledged to be rich in antioxidants. Dark and milk chocolate contain significantly more phenols than other chocolate products.(609) Cocoa also exhibits a larger antioxidant capacity on a per weight basis than most other foods and beverages that contain flavanols.(21,605) On a per serving basis, the antioxidant activity is four to five times stronger than that of black tea, two to three times stronger than green tea, and nearly twice that of red wine.(605) The antioxidant capacity of chocolate is generally

judged to be four times that of tea.(155) These findings propose that cocoa is more favourable to health than teas and red wine due to its higher antioxidant capacity. In addition, gallic acid and EGCG, the key antioxidants in tea, have multiple OH-groups, especially in the B ring, which increases pro-oxidant activity that may suggest that cocoa procyanidins may produce more positive health effects than green tea phenolics.(605) Collectively, the data from studies investigating the plasma catechin absorption recovery on consumption suggest that catechins from chocolate are more bioavailable than catechins from wine.(123)

Chocolate has been reported to contribute appreciably to the total dietary catechin intake, second to black tea in a representative sample of the Dutch population (Dutch National Food Consumption Survey). Chocolate provided 20% of the total catechin intake in this study and tea 55%.(155) However, the absolute consumption levels of tea were much higher than those of chocolate, which resulted in a lower contribution of chocolate to the total catechin intake, despite the fact that the catechin concentration of chocolate is higher than in tea.(174) In younger age groups, in which chocolate is usually favoured to tea, and in countries where tea is less often consumed, chocolate may be an even more significant contributor of catechins than tea.(155,212) Chocolate can, for instance, be an important source of phenolic antioxidants for the European population through their chocolate and chocolate confectionary consumption.(120) In the US diet, chocolate is the third highest per capita daily dietary source of antioxidants after coffee and tea. Milk chocolate is the most popular of the chocolates consumed in the US.(610) As a result, cocoa and chocolate can be important dietary contributors of flavonoids, along with tea.(212)

Collectively, data indicate that the consumption of cocoa and chocolate rich in the flavanol and procyanidin flavonoids could positively influence cardiovascular health (21,27,331) via several *in vivo* biological effects (123) that occur at physiological plasma concentrations of EC on such consumption.(21) The smaller flavanol oligomers (particularly those with EC subunits) seem to have the ability to diffuse across the membrane and into the cell. This ability to diffuse into the cell is important as cocoa products and isolated flavanols and procyanidins have been found to influence enzymes and signaling cascades.(172) Cocoa and chocolate flavanols, consequently, in addition to the evidenced antioxidant protection,(21,27,123,172,219) that might enhance the plasma antioxidant potential,(123,172,211) and decrease LDL susceptibility to oxidation,(331) might have cardioprotective effects ascribed to other cellular mechanisms (21,172) that includes anti-inflammatory,(172,219) anti-platelet (27,28,172,213,219) and vasodilatory (120,219) effects.

Both cocoa powder and chocolate have antioxidant activity (147) and the chocolate fatty acids may alter the fatty acid composition of LDL by making it more resistant to oxidation.(172,312) It has been shown that cocoa and chocolate consumption reduces LDL susceptibility to oxidation *in*

vivo.(231,331) Consumption of chocolate, due to the high monounsaturated and saturated fatty acid contents, may concurrently modify the LDL lipid content by increasing these fatty acid amounts and decreasing the PUFAs, making it more resistant to oxidative damage. It is possible that the monounsaturated and saturated fatty acid uptake on chocolate consumption are accountable for the inhibition of the LDL peroxidation.(312) Besides entering or binding to LDL particles, cocoa flavonoids may decrease oxidation-mediated events in the hydrophilic surroundings of LDL particles or at a cellular level in the arterial wall.(212) A diet that includes chocolate may thus afford some protection against arteriosclerosis.(331) Small, short-term, intervention trials indicated that cocoa-containing foods improved CVD through endothelial function and blood pressure.(233) Although such findings are encouraging, most of these studies utilized chocolate or cocoa that contained much higher levels of flavanols than that present in commercially accessible products. It is not certain whether the observed findings are long-term or short-lived and whether they relate to clinical CVD.(233)

Cocoa and chocolate are typically viewed as negative foods with regard to vascular health and not considered part of a heart-healthy diet.(21,609) Dietary inclusion of cocoa and dark chocolate is a means of contributing to the antioxidant intake, along with other sources of antioxidants, such as fruit, vegetables, tea and wine.(212) However, although a bar of chocolate demonstrates strong antioxidant activity, the health benefits are overshadowed (21) because chocolate is generally considered a non-nutritional confectionary food that contributes to high energy (21,609) and fat (21) intakes. Cocoa butter forms 50% to 57% of the dry weight of cocoa beans and contributes to the melting properties of chocolate.(21) This high cocoa butter content in cocoa and cocoa products is the main reason why these products are not received positively with regard to cardiovascular health.(28)

The predominant fats in chocolate (cocoa butter) are saturated and monounsaturated fatty acids of which the composition is approximately 60% saturated (35% stearic acid, 25% palmitic acid) and 35% monounsaturated (as oleic acid) fat.(21,219) It is as a result of this high saturated fatty acid content (21,312) that it is postulated that cocoa and chocolate have a hypercholesterolemic effect (312) and in particular viewed negatively in relation to the vascular system.(21) Although the lipid and saturated fat content of chocolate is substantial, one third of the lipid (30% of fatty acids) in cocoa butter consists of stearic acid, which produces a neutral response on total and LDL cholesterol in humans.(21,312) Palmitic acid has cholesterol-raising effects; however, the effect is understood to be counteracted by the neutral response of the stearic acid and the slightly cholesterol-lowering response of the oleic acid fatty acids present.(219) Thus, the dietary consumption of a moderate amount of chocolate containing stearic acid is not envisaged to have unfavourable effects in the lipid and lipoprotein profile of its consumers, as long as the total fat and

energy intake is held stable.(21) Consumption of cocoa, or dark chocolate, may as a result possibly have a beneficial effect on serum lipids.(312) Similar to tea, flavonoid-rich chocolate or cocoa consumption may thus be a valuable potential component of a healthy diet when consumed in moderation. Its high energy density (provided by fat and sugar) is the key determining factor in the quantity of flavonoids that can be obtained from chocolate.(27) Cocoa powder, however, may be the considered dietary choice as a cup of hot cocoa has a much lower provision of saturated fats (0.3 g per serving) than a portion of chocolate (8 g per 40 g portion).(21)

The cocoa bean contains several minerals of which some are still present in high quantities in processed chocolate. The quantity retained from the cocoa bean is determined by the amount of cocoa bean solids present in chocolate; therefore, dark chocolate generally has a higher mineral content than milk chocolate.(21) In addition to being a rich source of flavonoids, the presence of these minerals in cocoa and chocolate may make a contribution to their overall dietary intake and affect platelet and vascular health contributing to CVD risk reduction.(21,28) These minerals include magnesium, copper, potassium and calcium.(21) Cocoa intake was positively related with energy intake and with calcium and magnesium intakes in the Zutphen Elderly Study.(233) Although the cocoa bean itself has high phytate contents, the fermentation and heat treatments during its processing results in hydrolysis of the phytates.(21)

According to Engler and Engler,(219) it would, based on the available information, be advisable to encourage individuals to consume a variety of flavonoid-rich beverages and foods, particularly those that contain substantial amounts of the same flavonoids (flavanols) present in cocoa and chocolate. The daily consumption of the 'polymeal,' a combined meal of seven dietary items, including fruits, vegetables, fish, wine, dark chocolate (as 100 g daily), almonds and garlic, was recently suggested as approach to reduce CVD events by 76%.(610) However, the dietary inclusion of cocoa and chocolate must be done sensibly and prudently in a diet that includes a wide variety of foods with the emphasis on the consumption of fruit, vegetables, whole grains, skim milk, reduced-fat dairy products and lean meats, fish and poultry, and underline the balanced principle.(212)

Balance and moderation are key aspects in a prudent diet and must be regarded for dietary items like chocolate,(21,219) which is high in energy and fat.(219) A similar circumstance for moderation can also be made for red wine: too much is as bad as too little.(331) Consuming substantial amounts of chocolate, which provides surplus energy and fat to the diet above maintenance requirements, could contribute to obesity and adversely effect CVD incidence.(21) To avoid such side-effects, excessive chocolate consumption is not recommended (331) and the added sugar and fat from the chocolate ingestion be accounted for by adaptations in the rest of the diet.(609)

Although Mursu et al. (312) found that chocolate ingestion for three weeks caused no weight gain nor any adverse effects on serum lipids in healthy subjects, and in effect may have favourable effects on HDL cholesterol and lipid peroxidation, these researchers indicated that their results should be interpreted with caution due to the proposed health concerns. The daily ingestion of substantial amounts of chocolate, due to the high level of energy and fat content, may according to them increase body weight over the longer term. The outcome in subjects other than healthy subjects, as in hypercholesterolemic and hypertriglyceridemic subjects, may also be different. Chocolate ingestion may increase the energy and fat contribution in the diet and as a result decrease the contribution of carbohydrates and several nutrients in the diet and may thus detrimentally affect the dietary quality and nutrient density.(312) According to Steinberg et al.,(21) the nutrient density of a particular flavonoid-containing dietary item should be considered, together with the gratification of that item, when recommending suitable dietary sources of phytochemicals.

Alonso et al. (611) reported that the feeding trials conducted have not elucidated the outcome of cocoa ingestion in a free-living population consuming standard commercial chocolate products. They stated that feeding trials in general utilize pure dark chocolate, rich in flavonoids, while the chocolate consumed by the general public has comparatively low amounts of cocoa and even lower amounts of flavanols. Moreover, individuals do not ingest chocolate on its own, but in a dietary pattern represented by regular snacking and high-energy food consumption that can promote weight gain and the development of obesity and, as a result, may offset any positive affect of dark chocolate.(611) As chocolate consumption was associated with snacking in their study population, Alonso et al. (611) emphasized that foresight is required when distributing messages to the general public on the merit of this potential vascular health dietary contributor.

9.6.2.4 *Fruit Juice*

To ensure an adequate fibre intake, the intake of whole fruits and vegetables, rather than juice, is recommended (421,553,565) with the '5-A-Day' for better health program advocating one serving of fruit juice to be included towards the five portions of fruits and vegetables per day.(612) It is accepted that fruit and vegetable consumption lowers CVD risk, however, this is not quite the case for fruit juices. The role of fruit juice is often downplayed due to the view that they are nutritionally inferior to whole fruits.(613) However, fruit juices are nutritious in other ways.(311) Fruit juices are rich sources of nutrients that include vitamins, minerals, trace elements and possibly soluble fibre.(613) Considering the kind of fruit juice, various antioxidants and phytochemicals,(421,613) especially flavonoids, may in addition be present.(613) Although processing influences the levels of the phenolic compounds present, fruit juices still hold substantial antioxidant potential.(613) Orange juice, for instance, is a rich source of vitamin C, folate and flavonoids, like hesperidin.(311)

Ruxton et al. (613) in reviewing the evidence whether pure fruit juice protects against CVD concluded that there was compelling support from the epidemiological and clinical study data sourced that fruit juice consumption lowered CVD risk via several plausible mechanisms with the clinical data implicating the antioxidants and phenolic compounds for the risk reduction. It appears that fruit juice may impact favourably on various CVD risk factors, in particular platelet aggregation, LDL oxidation and LDL concentration (in those with hypercholesterolemia).(613) In addition, frequent consumption of oranges and orange juice as rich sources of folate tends to increase plasma folate levels and reduce homocysteine levels.(614) Orange juice has been found to induce hypocholesterolemic responses in animals.(311) Kurowska et al. (311) found that drinking 750 mL (three cups), but not 250 or 500 mL, orange juice daily for four weeks improved the plasma lipoprotein profile of a group of subjects comprising mostly persons with mild-to-moderate hypercholesterolemia. The HDL-cholesterol level increased significantly, while the LDL-HDL cholesterol ratio was markedly reduced. The reduction in the LDL-HDL ratio found with the largest volume of orange juice in their study was totally ascribed to changes in the HDL cholesterol levels. The significant increase in plasma triglycerides, also found in the research, did not surpass the normal range. This led the researchers to believe that it may not be of clinical importance or contribute to increased cardiovascular risk. Due to the resultant increased vitamin C and folate intakes on the consumption of orange juice, the plasma concentrations of these nutrients increased significantly.(311)

These researchers indicated that their results did not imply that large amounts of orange juice (about 20% of daily energy) should be advised for consumption, but rather that the cardioprotective nutrients in quantities similar to those present in 750 mL orange juice should be provided by a variety of dietary items with consideration of the recommendations to consume five to ten or more servings of fruit and vegetables daily.(311) Ruxton et al. (613) noted that moderation in fruit juice intake, as advocated for the daily consumption of red wine (238) and chocolate,(21,219) would be the key until more information is obtained on the potential concerns linked to such consumption, like its probable links with obesity, micronutrient dilution and dental caries/erosion.(613)

9.6.2.5 Apple and Pear as Representatives of Pome Fruit Inclusions

Apples and pears are fruits belonging to the pome botanical type. Apples are among the most popular fruits in the world (615,616) and are generally described as being a healthy food.(617) Apples contain various phytochemicals, including the antioxidant flavonoids quercetin and catechin.(618) Weichselbaum et al. (616) postulated that apples and apple products (fruit juices and other apple products) add to the total polyphenol intake of the UK population. Although there is very limited evidence available regarding apples or apple products and CVD in humans,(616) such available information on pears could not be obtained.

In reviewing the health benefits of apples, Boyer and Liu (618) concluded that in particular animal studies and *in vitro* work show that apples markedly lower lipid peroxidation in both rats and humans and lower cholesterol in humans. Weichselbaum et al. (616) in their review indicated that although only a few human studies are available the findings, despite being inconclusive, indicate that the polyphenols present in apples probably have a beneficial effect on the blood lipid profile in humans. Epidemiological studies have further linked the consumption of apples with lowered risk of CVD.(618) Apple consumption which contributed about 10% of the total ingested flavonoids was related to a lowered mortality risk from CHD in men in the Zutphen Elderly Study, but the relationship was not significant.(45) Apple intake was inversely related with death from CHD in the Iowa Women's Health Study of postmenopausal women.(174) In the Women's Health Study with US female health professionals as the study subjects, apple intake was associated with non-significant reductions in risk of both cardiovascular events and CVD. The women who ingested apples had a 13 to 22% lower CVD risk.(232) The inverse association found between apple consumption and risk of CVD may partially be ascribed to the phenolic compounds and dietary fibre present.(618) Boyer and Liu (618) in concluding their review on apple phytochemicals and their health benefits indicated that the regular consumption of fruits and vegetables, including apples, as part of a healthy diet may contribute to the maintenance of good health and support the prevention of chronic disease.

9.6.2.6 Banana

The banana, though a tropical fruit, has the highest consumption in the world (619) and is also commonly consumed in SA.(90) An acute feeding study investigating the oxidative effects of a banana meal (400 g) determined that the oxidation of plasma lipid and lipoproteins, in particular the susceptibility of LDL to oxidative modification, of healthy volunteers were markedly decreased following the meal. This indicates that banana consumption lowers plasma oxidative stress and increases the resistance of LDL to oxidative modification.(619) In addition to this possible cardioprotective role of bananas, a number of biogenic amines are found in small amounts in some foods like bananas. Bananas contain the neuropharmacological agent 5-hydroxytryptamine. Although the pharmacological affect and health importance of the biogenic amines naturally present in foods is unclear, it is possible that a number of these neuroactive chemicals can add to the dietary outcome on mental and brain function.(110)

9.6.2.7 Onions as Vegetable Representative

Worldwide onions (*Allium cepa* L.) are the second most important horticultural crop. Due to its versatility, it is a frequently used ingredient in numerous dishes, many processed foods, including the so-called 'fast foods', in which it is eaten mostly cooked.(620) Onions are rich in flavonoids, mainly flavonols and anthocyanins, and alkyl cysteine sulphoxides that have perceived health benefits. The beneficial health claims of *Alliums*, with the exception of garlic, have not yet been

investigated thoroughly. While scientific investigation on the beneficial health effects of onion is not greatly advanced, some experimental study work on components of onion and onion extracts has shown positive effects towards CVD related to hypolipidaemic and anti-platelet activity, but how these benefits surface *in vivo* awaits further study.(620)

Both garlic and onion have an inhibitory influence on the arachidonic acid metabolism, namely enzyme inhibition of LOX and COX, with resultant anti-aggregation and anti-thrombotic effects, although the findings indicate that onion in comparison to garlic may be less efficient in promoting these effects in humans.(621) LOX and COX are also pro-inflammatory enzymes. Chronic overproduction of either of these enzymes results in excess inflammation and contributes to chronic pro-inflammatory diseases, like CVD. Limiting the activity of these pro-inflammatory enzymes also ascribes anti-inflammatory effects to onions.(622) Results suggest that the biological potency is retained in raw onion, but reduced or destroyed by heat, which affects its efficacy in CVD, as onion is normally consumed cooked.(621) An acute feeding trial did find a short-lived decrease in biomarkers of oxidative stress, such as improved resistance of lymphocyte DNA strand breakage, but no change in urinary MDA excretion, on consumption of a meal of fried onion by healthy volunteers.(191)

9.6.2.8 *Parsley as Herb Representative*

Herbs and spices have never been considered to contribute to human nutrition (623) because of the small quantities included in the human diet.(624) However, numerous beneficial health attributes are now attributed to these dietary adjuncts.(623,624) Numerous herbs are exceptional sources of antioxidants, particularly phenolic antioxidants, and their dietary intake may therefore greatly add to the daily antioxidant ingestion.(547) The antioxidant capacity of these plant materials seems to be very closely associated with their total phenolic contents.(625) Tapsell (626) has raised the question on where herbs and spices belong in the dietary guidelines for health promotion and the prevention of the major chronic diseases. The review by Tapsell (626) pointed out that herbs and spices deserve to be incorporated as a separate inclusion in the dietary guidelines that should seriously be considered as there is supportive evidence that they afford health protection against oxidative and inflammatory mechanisms, which potentially connect them in the protection against such diseases as CVD. Such properties may provide herbs with far reaching therapeutic potential and nutraceutical value.(623)

The main connection between herbs and health seems to be linked to their provision of compounds with potent antioxidant abilities, which include their high polyphenolic compound content.(626) Ninfali et al. (117) stressed the need to establish herbs as a seasoning agent in the diet as they are able to bring about a substantial increase in the dietary phenolic content and antioxidant capacity. Research has shown that the phenolic concentration and antioxidant

capacity of herbs are generally many-fold higher than that of vegetables,(117) and that at typical ingestion levels, the antioxidant capacity of herbs is similar to that of vegetables.(626) Due to the very high concentrations of phenolic compounds in herbs, the supplementation of the daily diet with herbs will increase the phenolic content and the antioxidant capacity with potential health benefits.(625,626) Dragland et al. (627) indicated that the intake of one gram of herbs in the usual diet may make a pertinent contribution to the total intake of plant antioxidants and can even be a better source of dietary antioxidants than some other food groups. Srinivasan (624) considers these food adjuncts as necessary components of the daily diet and nutrition. Nakatani (548) indicated that the daily intake of herbs and spices might be one of the most promising dietary behaviours leading to a healthier life, because of the important role they play against the major diseases. However, more data is required on the bioavailability and bioactivity of herb antioxidants.(627) In addition to the antioxidant and other attributes of herbs and spices, their use can partially or entirely replace less beneficial ingredients, particularly flavourings, such as salt and sugar use in recipes, and help to lower these intakes.(549)

Herbs are used fresh and dried.(626) In SA dried and fresh parsley (*Petroselinum crispum*) ranks amongst the top five dried and fresh herbs (second and forth, respectively) sold by a countrywide large retail chain food store (personal communication Van de Vyver, 2006). As in the Danish diet,(547) numerous South African dishes contain herbs as recipe ingredient. Some herbs, in particular parsley, contain high levels of flavonoids, especially apigenin glycosides.(547) In a study in Denmark on the average daily consumption of flavonols and flavanones, the calculation did not incorporate the contribution made by fresh herbs, as its intake is minimal on an average daily basis and the consumption is greatly seasonal belonging to summer. In a further study, the same research group (547) found that the intake of a few grams of parsley, dill or spearmint would be enough to contribute appreciably to the average daily Danish flavonoid intake. The intake of flavones in Australia was higher than that determined for Denmark and the USA due to celery and parsley ingestion that was not included in the dietary analysis of the Danish and US studies.(89) Tapsell et al. (547) noted that herbs and spices have an important role in dietary flavonoid intake.

In the past, recipes called for herb addition as only a teaspoon or less, now chefs are adding substantial amounts of herbs to their dishes. Grocery stores have also enlarged the herb section (626) in line with the global trend of increased herb and spice consumption.(549) Most animal studies that found beneficial physiological influences of herbs and spices, did so at levels about five to ten times the average amounts present in Indian diets. Such liberal consumption was proven safe and possibly provide positive effects on specifically the antioxidant status.(623,624) Limited safety evaluation studies have been conducted on herbs despite their widespread consumption for centuries. Though not extensively studied, the safety of some herbs have been

established upon feeding at levels approximating human intake, as well as intakes up to 100-times the customary human intake in animal studies.(624) Although a diet, in which herbs are used generously to flavour food, provides numerous active phytochemicals with biological activity that promote health and protect against disease,(628) it may be unwise to recommend large intakes before the exact biological effects are understood,(547) as safety concerns on some have been expressed.(624) While some herbs are safe in modest amounts, they may become toxic at higher doses.(628) Parsley, for instance, is known to contain furocoumarins (547,629) and is among the high nitrate-accumulating foods.(630)   Although furocoumarins, which are secondary plant metabolites or natural toxicants, present no risk of phototoxic skin reactions on ingestion of amounts through normal dietary habits and UV exposure, extreme habitual intake must be avoided, especially when UV exposure cannot be shunned, as the safety factor between the actual intake and the phototoxic threshold dose is of a small order.(629) Nitrate itself is relatively non-toxic, but is converted by bacterial enzymes in the saliva and digestive tract to the more toxic nitrite on ingestion, which can bind with amines and amides to form carcinogenic compounds. The daily total ingestion of nitrate though is usually far lower than the acceptable daily intake.(630)

Hinneburg et al.,(631) assessed the ability of extracts from selected herbs and spices to reduce iron(III) and inhibit linoleic acid peroxidation. They found that although parsley performed well as an iron chelator, it was not that efficient at reducing the oxidation of linoleic acid. Nielsen et al.,(632) found a significant increase in the activity of the antioxidant enzymes GSH reductase and SOD on the consumption of apigenin-rich parsley and decreases in protein oxidation that might indicate that parsley intervention decreased oxidative stress in some of the volunteers. Experimental animal work by Gadi et al. (633) demonstrated that a crude aqueous parsley extract inhibited *in vivo* platelet aggregation causing a prolongation of bleeding time. The researchers indicated that parsley consumption in normalization of the platelet hyper-aggregation linked to CVD represents a promising strategy for its nutritional prevention. There is only modest data to support a direct cardioprotective effect from herbs and spices and limited authoritative recommendations on the intake.(549)

9.6.3   Confirmation of Food List Dietary Items and Notable Contributors towards Mean Daily Total Flavonoid Intake

In the compilation of a reduced food list to determine the total flavonoid intake of South Africans, it seems plausible to assume that the items included within the abbreviated FFQ food list being black tea, rooibos, green tea, apples, red wine, bananas, orange juice and orange juice blends, tropical fruit juice and tropical fruit juice blends, berry and mixed berry juice, pears, apple cider, milk chocolate and dark chocolate and those identified fruit items, but excluded from the reduced food list due to seasonal availability implications, oranges, strawberry, guava and naartjies are the top contributory items to the total flavonoid intake and contribute significantly to the between-person

variability in the total flavonoid intake covering the mid- to end-year time period. These items were identified in both the reduced food list compilation in the rooibos intervention trial execution, running across the months ending July to early November, and the confirmation of the items included in the food list on completion of dietary records by the further participant group end October to beginning November, which together covered a period of roughly four months, the seasonal period of mid-winter and spring to early summer.

Further items that may contribute to the total flavonoid intake are cooked onions, apple juice and apple juice blends, dried parsley, dried pears, beer, raw onions and white wine of which most of these items (cooked onions in the food list of the comprehensive FFQ and beer, raw onions and white wine of the abbreviated FFQ) also contributed to the between-person variation in total flavonoid intake. The items that contributed greatly to the absolute mean daily total flavonoid intake was black tea and rooibos in both the food list compilation and confirmation analyses (70% and 80%, respectively), with a further three items, apples, red wine and oranges, that contributed one percent or more of the mean daily total flavonoid intake in both these analyses. The rest of the items contributed less than one percent of the total flavonoid intake or more than one percent in either of the analyses, namely berry and mixed berry juice, green tea and naartjies in the comprehensive FFQ administration in the rooibos intervention trial and green tea in the dietary records of the further participant group. This indicates green tea to be a further notable contributory beverage to the South African mean daily total flavonoid intake, though still not to the same extent as rooibos.

CHAPTER 10

CONCLUSIONS AND RECOMMENDATIONS

10.1   INTRODUCTION

The rooibos intervention trial provided the opportunity to explore the assessment of total flavonoid intake using a FFQ along with dietary records. The major goal of this research was to develop an abbreviated FFQ that would provide valid and reproducible information in its assessment application, to determine current daily total flavonoid intake. To achieve this goal, as put forward for most validation studies, entailed a comparison of one dietary assessment method against another. In this research, a comprehensive and resultant abbreviated FFQ served as test method and estimated dietary records as reference method. The administration of the FFQ within the rooibos intervention trial allowed for exploring its use to assess dietary change in total flavonoid intake, which refers to its responsiveness to such change. The intention of this research was not alone to provide new information about the validity, reproducibility and responsiveness of a questionnaire for assessing total flavonoid intake, but simultaneously to pay specific attention to developing the food list for the abbreviated FFQ.

10.2   DEVELOPMENT OF ABBREVIATED FFQ TO ASSESS MEAN DAILY TOTAL FLAVONOID INTAKE

De Vries et al. (74) suggested that flavonoid intake could be assessed by a brief questionnaire, as few dietary sources contribute appreciably to the intake.(9) The comprehensive FFQ with its long expanded food list was initially developed to meet the requirement of assessing the group absolute mean daily flavonoid intake as precisely as possible, as this was of major importance for the outcome of the rooibos intervention trial itself. An extended food list that is able to capture the whole dietary spectrum of flavonoid intakes was important, as the ability to accurately assess absolute dietary intake is not a characteristic ascribed to FFQs.(367,390,392) However, in the case of comprehensive FFQs, participant burden is an anticipated problem since a large amount of writing is required to indicate all foods and beverages consumed in the questionnaire.(394) An abbreviated FFQ with its reduced food list would be of practical worth.

Applying the percentage contribution and stepwise multiple regression analysis approaches to the datasets of the dietary records and comprehensive FFQs of the baseline period of the rooibos intervention trial, and considering a number of additional factors, culminated in an abbreviated FFQ with a food list of 16 items extended to 23. The bulk of food listed included dietary items within the fruit and fruit juice categories, as fruits are generally rich sources of flavonoids.(8) The abbreviated

questionnaire, which included dietary items representing nearly all of the food and beverage categories included in the food list of the comprehensive FFQ, was expected to reproduce at least 93% of the total absolute and 99% of the within-person variability in total flavonoid intake captured by the dietary records and the full FFQ.

In support of the suggestion by De Vries et al.,(74) it can be concluded that dietary flavonoid intake can be assessed by a brief questionnaire containing a limited food list as few dietary items were found to contribute greatly to the absolute intake and that most of these items contributed significantly to the between-person variability in the intake. Flavonoids, as non-nutrients, can thus be added to the list of nutrients of which the intake can be assessed by a short list of dietary items. These nutrients have few major dietary sources and include among others cholesterol, preformed vitamin A and vitamin B_{12} in contrast to other nutrients for which a short food list does not perform as well, since they have many contributory dietary sources.(395) However, for many nutrients the dietary items that are the greatest contributors to the total intake are often not the best indicators of the between-person variance in intake,(395,411) as was found for total flavonoids in this research.

The abbreviated FFQ developed, based on the rooibos participant group dietary record and comprehensive FFQ datasets, was also completed by a further participant group at risk for CHD. This data not only determine the generalizability of its validity and reproducibility to external subjects recording their dietary intake, but also compare the dietary items included in the abbreviated FFQ with that identified in this participant group to contribute to their total and within-person variability in intake through their dietary records. This participant group included subjects from four distant and different geographical areas, as it is known that the geographic location impacts food prices and food availability,(5) thus providing for a wider dietary exposure contrast. The findings of this phase of the research imply that this short questionnaire, which specifically targets total flavonoid intake, can be administered to adult South Africans following a dietary intake similar to a Western diet. The dietary record dataset of this further participant group produced the same beverage and food items for inclusion in the food list based on applying the percentage contribution and stepwise multiple regression analysis approaches as was found for the rooibos intervention trial participant group residing in the urban city area of Cape Town.

Although polyphenols are abundant in the diet (101) and present in all plant foods,(59) the dietary items identified for inclusion in the abbreviated FFQ food list in both the research phases represented items that have been reported to make major contributions to the absolute dietary flavonoid intake. These items include tea and red wine as beverage items, apples as fruit source, onions as vegetable source and chocolate in addition to citrus fruits.(9,75) It can be concluded that many of the beverages and foods identified in this research for inclusion in an abbreviated FFQ

food list in a South African context can possibly be relevant to other populations sharing a Westernized dietary pattern. However, the absolute amounts consumed and the subclass contributions to the total flavonoid intake may differ ultimately. At the very least, the food list identified in this research may provide a valuable initial departing point for other researchers wishing to assess total flavonoid intake through the use of an available FFQ, which includes these identified dietary items in the food list, as it may be suited to provide for an assessment of the total flavonoid intake of populations who have typically Western eating habits. Cade et al. (18) although suggesting that it is not advisable to use a FFQ developed for one country in another country, indicated that such use might be considered if the eating habits are comparable. Neuhouser et al. (73) reminded those designing abbreviated questionnaires that the food list must be comprehensive enough to capture the dietary items that are the major sources of the nutrient(s) of interest, including those foods that are part of mixed dishes. This was accomplished in the food list with its onion inclusion and could provide a starting point for other scientists to pursue with their research.

The findings suggest that the flexible format of the FFQ used in this research, considering the open-ended frequency format and portion size descriptions as small, medium and large to select from, did not reduce the validity and reproducibility. The dataset obtained were comparable to that obtained from six days of careful dietary recording in both the phases of the research. The findings provide evidence that supports the conclusion of Horwath (422) that a reduced FFQ can produce comparable intake estimates to that collected from more demanding, time-consuming and costly (in relation to coding and analysis) dietary assessment methods which besides for their practicality, is indicative of the feasibility of developing an abbreviated FFQ.(445) The abbreviated FFQ can be completed on average in 10 minutes and its data entry can be done quickly and at little expense, whereas the full-length FFQ administration takes about 30 minutes to complete.

10.3 VALIDITY, REPRODUCIBILITY AND RESPONSIVENESS OF COMPREHENSIVE AND RESULTANT ABBREVIATED FFQ TO ASSESS DAILY TOTAL FLAVONOID INTAKE

Producing rather accurate absolute dietary intake levels is important,(422) particularly in nutrition intervention research where subjects need to be classified according to their dietary compliance. (398) Information about the level of dietary intake is required for epidemiological research as FFQs must be able to rank and classify the subjects acceptably based on their dietary intake,(85,422) to set recommendations for dietary intakes and to determine the percentage of a population that meet recommended dietary intake guidelines.(85) To accurately categorize subjects according to intake and for identifying subjects at the extremes of intake, relatively exact quantitative information is needed.(390) This is a feature not characteristically attributed to FFQs.

(398,403,424) Molag et al. (85) in their FFQ validity review concluded that FFQs are not appropriate to assess dietary intake levels precisely. This conclusion was based on their finding that FFQs validated against exposure biomarkers underestimated energy intake on average by 20% and protein intake by 11%.

This research was based on a relatively small number of participants (a group of 40 and in addition 99 adult women and men). The findings provided evidence that a short, self-administered FFQ can be devised and can provide sufficiently accurate information related to both group and individual current daily total flavonoid intake levels. Although the results of the research equate and are even better than that achieved in other validation studies, the findings were obtained from well-motivated volunteer participants in both the research phases. In this research the group mean daily total flavonoid intake estimates obtained from the comprehensive and resultant abbreviated FFQs agreed favourably and were hence greatly interchangeable with those obtained from the dietary records as reference dietary assessment method. The slight overestimation of the intake on the side of the FFQs is a characteristic inherent in FFQs.(6,86,369,379,380,407) The abbreviated FFQ proved to be convincingly successful in assessing an absolute mean total daily flavonoid intake at the group level in the further (external) participant group administration. The latter group was comprised of apparently healthy, independent-living adults that have never recorded their dietary intakes or completed a FFQ before. The intake reported for the first abbreviated FFQ administration was within five percent of the dietary record value. This suggests that despite the good agreement in group means achieved between the two dietary assessment methods, the level of precision was similar to or no better than the results from any other dietary assessment validations, where the group questionnaire means were compared to dietary record values.(422,441)

These findings suggest that the abbreviated FFQ intakes were almost as good as those obtained from six days of dietary records within the two week reference period. The statement of Kemppainen et al. (80) that a short method appears to have only limited value in estimating the absolute intakes of nutrients, therefore, does not hold true for a current estimation of the mean daily total flavonoid intake. However, reducing the number of items on the food list to represent those items that made the greatest contribution to total flavonoid intake and the between-person variation in intake, did not reduce the absolute value of the mean daily flavonoid intake estimates in this research in comparison to that of the dietary records. This is in agreement with the findings of other studies (80,403,404) using a modified or brief FFQ to determine intakes of one or multiple nutrients against the dietary reference method. Schaffer et al. (403) hypothesized that due to the tendency for modified brief questionnaires with reduced food lists to provide lower absolute intake estimates in comparison to comprehensive FFQs with an expanded food list that generally

overestimate dietary intakes, a modified questionnaire may produce improved estimates of true dietary intake. This hypothesis may not quite be applicable to this research, as in its development the abbreviated FFQ was calculated to capture at least 93% of the mean daily total flavonoid intake as would be obtained from multiple dietary records and a comprehensive FFQ.

Sebring et al. (88) ascribed the tendency of their calcium questionnaire to overestimate calcium intake to be related to, in part, the design features of their FFQ which, besides for the long list of calcium-containing foods, comprised an open-ended format for frequency responses that may cause subjects to overestimate their intake. This may well have relevance to this research. As the mean differences in intake between that of the comprehensive and the abbreviated FFQ against dietary records, were negligible based on the Bland-Altman plots, the two methods greatly agreed. The Bland-Altman plots showed no serious systematic difference between the two dietary assessment methods across the range of mean intakes until the higher mean daily intakes are reached, reflecting a non-constant bias. In the rooibos intervention trial, there was a tendency that, as mean intakes exceeded 500 mg/day, the differences between the data from the comprehensive and the abbreviated FFQ and the dietary records became predominantly positive. This was apparent with the abbreviated FFQ administration in the further participant group when the mean intakes approached 1000 mg/day. The data points for the mean intakes lower than these levels, showed a tendency to cluster around the line of equality with the differences between the two methods both negative and positive and the dots fairly equally distributed with no distortion in either direction. The slight bias found between the FFQs and the dietary records is most likely proportional and as it occurs at higher intakes implicate a likelihood of an intake overestimation at these levels.

A reduction in the number of items on the food list of the comprehensive FFQ, to represent those items that made the greatest contribution to the absolute daily total flavonoid intake and the between-person variation in intake, did not negatively influence the correlations with the results of the dietary records, but seemed to influence it positively. The abbreviated FFQ provided participant estimates of intake that correlated highly with the estimates produced by the estimated dietary records in the further participant group administration. Correlation coefficients close to or greater than 0.5, indicate that the extent of misclassification introduced by the FFQ would be acceptable (444) and that the FFQ can be regarded a valid tool (494) with a correlation at 0.7 thus assuming reasonably true intake.(402)

Applying these norms to the correlation results of the rooibos intervention trial and the further participant group administration, it can be concluded that the comprehensive FFQ, but more so the abbreviated FFQ, are valid for assessing daily total flavonoid intakes among apparently healthy

adults at risk for CVD as the correlation coefficients indicated that the participants were ranked similarly to the dietary records by means of the comprehensive FFQ. The ranking of the participants was accomplished with reasonable precision relative to the dietary records with the abbreviated FFQ. It is noteworthy that the participants could be ranked by daily total flavonoid intake based on only 23 foods with a higher level of relative validity than with the more comprehensive FFQ. This finding was also reported by Ishihara et al. (411) for their 33-item versus 138-item FFQ to assess folate intake.

The correlations suggest that there is relatively little loss in the use of the abbreviated FFQ version. Correlation coefficients in dietary assessment reflect the ability of the assessment to discriminate between the dietary intakes of the subjects and thus to categorize them correctly. This ability relies on the ratio of the within- and between-subject variance, which is influenced by both the accuracy of the dietary assessment method (within-variance component) and the between-person variation in the dietary intake (between-variance component).(405,502) The stronger correlations found in this research can be attributed to the six days of dietary recording within the two-week reference periods. This allowed for better representation of the dietary intake as captured by the FFQs and thus better agreement between the two methods.(425,461) A further contributory factor may be the wide range of participant intakes found. This is most possibly due to the differing participant dietary intakes of flavonoid-rich foods and beverages, like practicing a tea drinking habit, or not. According to Block et al.,(402) it is important to bear in mind that the correlations are not with actual dietary intake averaged over all days of the reference period, but with reference data that represent only certain days or 'snapshots' during the reference period. The log-transformed Pearson correlation coefficients were in general not better than the Spearman correlation coefficients. Deattenuation of the results using the within- and between-person variability also did not generally increase the associations.

While most published comparable studies did not determine the participant percentage (gross) misclassification and/or K statistics,(494) 50% or more of the participants in this research, when categorized by the dietary records, fell into the same quintile or same tertile of intake when categorized by the comprehensive or the abbreviated FFQ, with less than 10% of them grossly misclassified. This meets the acceptance levels proposed by Masson et al. (461) when considering the tertile categorization. The Kw statistic in most cases indicated moderate to good agreement between the reported intakes in the FFQ and those in the estimated dietary records in the rooibos intervention trial periods and more so in the further participant group administration. Thus, both the comprehensive and the abbreviated FFQ could effectively categorize the participants according to higher or lower mean daily total flavonoid intakes when compared with the dietary records.

The correlations and *Kw* statistics between the datasets of the comprehensive and resultant FFQs and the estimated dietary records indicate that both the FFQs, and in particular the abbreviated FFQ in the further participant group administration, could rank and categorize the adult participants according to absolute daily total flavonoid intakes acceptably well. The abbreviated FFQ performance was comparable to and even more effective than the longer comprehensive FFQ. Correlations and the category cross-classification of dietary intake are considered the most valuable and appropriate indications of the validity of a dietary assessment method.(403) Validity of the dietary assessment in both these cases relate to the ability of the method to discriminate between subjects with true exposure differences.(502) Both the comprehensive and the resultant abbreviated FFQ could rank and categorize subjects by the level of dietary flavonoid intake to a great extent similar to the supposed superior multiple dietary record assessment. It can be concluded that these FFQs could be used in various applications where ranking or categorizing of such intakes are required in the South African context where a dietary intake represents a Western diet.

As FFQs are generally utilized to rank subjects by dietary intake,(88) the questionnaire appears to work well for this purpose. For instance, in epidemiological studies the ability of a questionnaire to rank or categorize the individual subjects correctly with respect to the level of dietary intake is more important (85,403,405,413) than its ability to accurately quantify the absolute intake values,(413) and thus the absolute group means.(403,405) Applications of short questionnaires with the ability to classify intakes into broad category levels, like low, medium or high intakes, is necessary for nutrition interventions and for classifying subjects in stages of dietary change.(494)

Based on the analyses above and on the group means, this research demonstrated the ability of especially a reduced FFQ to provide reasonably valid absolute estimates of current daily flavonoid intakes. It captured considerable information to adequately quantify such intakes for the purpose of epidemiological research particularly on repeated administration. This supports the indication by Molag et al. (85) that FFQs have the ability to differentiate between subgroup intakes although the extent of the absolute differences in intake may be uncertain. This may be of relevance to this FFQ developed to assess mean total flavonoid intake at the higher intake level estimates.

The reproducibility of the comprehensive and resultant abbreviated FFQ in the rooibos intervention trial, as evaluated by paired difference tests, the correlation coefficients and category agreement or cross-classification overall was satisfactory. The group intakes assessed by the two administrations were not significantly (p>0.05) different with a moderately strong linear and statistically significant (p<0.05) association between the first and the second or repeat FFQ administrations. These evaluations of the abbreviated FFQ in the further participant group

administration was remarkably better, particularly in terms of the strong linear and statistically significant (p<0.0001) association found. This could be due to the short interval of two weeks between the two abbreviated FFQ administrations in this phase of the research. The Bland-Altman plots also demonstrated an acceptable agreement between the comprehensive and resultant FFQs administrations within the washout and control periods of the rooibos intervention trial and the first and repeat abbreviated FFQ administrations within the further participant group. The difference in intake between the FFQs at each administration was small and did not show critical systematic variation or bias over the range of average intakes.

The shorter time interval in the further participant group administration could have overestimated the reproducibility as subjects may have remembered earlier responses.(83) However, although these subjects were notified, as part of the informed consent that the abbreviated FFQ would be completed again after their completion of the dietary records, they were not notified about the actual use of the repeat FFQ to assess its reproducibility. Moreover, long intervals mean that true dietary changes are more likely to occur, which would reduce the apparent repeatability.(83) This seems particularly relevant to flavonoid intakes due to the intake contributions provided by seasonally consumed dietary items. The reproducibility of the FFQ appears satisfactory although caution must be taken with regard to its long-term reproducibility assessment of daily total flavonoid consumption on repeated use.

Prospective data collections (longitudinal studies), as in both intervention and cohort studies, can greatly contribute to the scientific support for the health benefit(s) resulting from a changed dietary condition,(23) as was envisaged for the rooibos intervention trial itself, if the dietary factor and its change is adequately assessed.(23,50) Dietary and other lifestyle factor changes are often recommended in health promotion and disease prevention.(23) This makes dietary change an important aspect in many research and clinical settings.(50) Given the unique opportunity to use the rooibos intervention trial clinical data along with the trial period FFQ daily total flavonoid intake estimates, a multi-method approach was made possible to assess dietary change that included more than a single method of self-reported intake, as propagated by Thomson et al.(49) FFQs used in intervention studies are generally not intended or developed to assess dietary change as a consequence of an intervention.(56) Adequate assessment of dietary change was not only an important issue in the design and data analysis of the rooibos intervention trial itself, but also of the developed FFQ regarding its ability to assess mean daily total flavonoid intake across the trial periods as the subjects were not blinded to the intervention. The changed mean daily total flavonoid intakes determined by the FFQs were consistent with the changed intakes obtained from the dietary records and the changed biomarker clinical data for the intervention trial periods.

Thomson et al. (49) underlined that when several independent assessment methods of dietary intake ascertain change in the anticipated direction, support is provided as to that change in dietary intake, which adequately provides for an unambiguous interpretation of the research outcome. The comprehensive and resultant abbreviated FFQ distinguished between low (in the washout and control trial periods) and high (in the intervention trial period) mean daily total flavonoid intake levels. In addition, the estimated high consumption level at the intervention exposure was accompanied by a changed biomarker of exposure level with increased plasma total polyphenol concentration and changed biomarker of effect levels concerning reduced CD and TBARS levels or oxidative lipid damage *in vivo*. In contrast, the plasma total polyphenol levels decreased and the CD and TBARS levels increased on low dietary intake exposure in the washout and control trial periods. This highlighted the FFQ as responsive measure to changed conditions of total flavonoid intakes.

These results should be interpreted with caution, as other dietary and lifestyle factors may have influenced these biomarker levels. The responsiveness capability of the FFQ was supported by no significant ($p > 0.05$) differences in the participant energy, macronutrient and antioxidant intakes across the trial periods. The participants maintained a stable body weight and blood pressure throughout the trial. On the basis of the group analysis, it was concluded that the comprehensive and the resultant abbreviated FFQ effectively captured the changes in the mean daily total flavonoid intake of the participants, as suggested in the intervention trial dietary guidelines over the trial periods and even on the removal of all tea from the datasets. FFQ use could consequently be considered a viable approach to assess dietary change in mean daily total flavonoid intakes.

The research findings demonstrated that the developed self-administered abbreviated FFQ can provide acceptably accurate and useful information related to current daily total flavonoid intake among adults at risk for CHD with dietary intakes representative of a Western diet. It performed quite well in its relative validity evaluations by comparison to multiple estimated dietary records as the superior dietary assessment method. The reproducibility evaluations at both the group level intake (reasonably similar mean intake estimates and absence of a constant bias) and the individual level intake (ability to rank and classify subjects reasonably similar) in the rooibos intervention trial and in the further participant group administration were equally satisfactory. A questionnaire able to capture intake with a few well-defined food list items could reduce the estimation error and the associated misclassification of dietary intake.(531) This may have been achieved through the food list development work. The possibility that a training or learning effect may have contributed to the relatively high agreements found in the daily total flavonoid intake estimates between the comprehensive and the abbreviated FFQs and the dietary records, was rejected. Further training throughout the research through the dietary assessment did not over

time lead to substantially improved comparability in the dietary reporting between the two dietary assessment methods as has been reported by researchers.(375) It should be noted that it is more difficult to develop a FFQ if the aim is to assess the total diet.(40)

The H_0 stated for the research could be assessed as follows:

(i) 'There is no significant (p>0.05) difference between the daily total flavonoid intake estimates of the comprehensive and resultant abbreviated FFQ as test methods and that of multiple dietary record estimates as reference method in the relative validity evaluation on a group level' was accepted;

(ii) 'There is no significant (p>0.05) difference between the daily total flavonoid intake estimates of the test method (comprehensive and resultant abbreviated FFQ) administrations in the reproducibility evaluation' was accepted, as essentially similar results were obtained on a group level reflecting that reasonably sound quantitative assessments of the dietary intake occurred.

(iii) 'There is no significant (p>0.05) association between the daily total flavonoid intake estimates of the comprehensive and resultant abbreviated FFQs, as test methods, and the multiple dietary records as reference method on the individual level' was rejected;

(iv) 'There is no significant (p>0.05) association between the daily total flavonoid intake estimates of the test methods and the plasma TAC as biomarker of exposure and the plasma TBARS as biomarker of effect (as obtained from the baseline period of the rooibos intervention trial), within the relative validity evaluation on the individual level' in the main accepted although the participants could be weakly ranked for these biomarkers; and

(v) 'There is no significant (p>0.05) difference between the mean daily total flavonoid intake estimates of the comprehensive and resultant abbreviated FFQs in the responsiveness evaluation within the rooibos intervention trial' was rejected, as the FFQs were good measures of the intervention effect.

It can be concluded that the abbreviated FFQ can, therefore, be used as alternative to dietary records to assess the current mean daily total flavonoid intake when appropriate, as it is a shorter, less labour-intensive method. In addition, it is suited for broad use among those South African adults consuming a dietary intake similar to a Western diet.

The generalizability of its developed food list that contributed to absolute and between-person variation in intake was tested and notably confirmed in the further participant group of which the four subgroups covered various geographical areas distant from each other. The independent validation of the comprehensive and abbreviated FFQs for total flavonoid intake exposure by comparison with biomarkers of exposure (reported by the plasma TAC) and of effect (reported by

the levels of the plasma TBARS), provided additional evidence, although weak and non-significant, that the FFQ could be used in studies that require a shorter dietary assessment method.

These findings, thus, fundamentally support the overall conclusion that the abbreviated FFQ could capture the intent of the research.(5) It was sufficiently valid and well reproducible for the purpose it was developed and, in addition, plausibly responsive to changes in dietary flavonoid intake. It could be particularly useful for administration within further clinical interventions pertaining to rooibos consumption, but possibly not limited to rooibos. The results indicate that it could be a reasonably good method on repeated administration for use in related nutritional epidemiological studies, as it was valid for ranking the subjects. This refers especially to nutritional epidemiological studies for relating total flavonoid intakes and health effects among South African adult groups consuming a dietary intake typical of a Western diet. Optimally, the dietary assessment method utilized in chronic disease epidemiology needs to be valid, reproducible and inexpensive and easy to be administered to large groups of people, who should be ranked acceptably according to the dietary component of interest.(407) These criteria were met by the developed abbreviated FFQ in addition to it being sufficiently simple to complete on explanation by first-time users. Its limitation in assessing the total diet must be kept in mind in this regard.

10.4   REFLECTION ON THE RESEARCH AND DEVELOPED FFQ

Although the findings are predominantly positive with the research having definite strengths, it does have shortcomings related to the FFQ developed and the design of the research and its logistics as encountered in other dietary assessment validation efforts.(88,345, 346,353,375,397,528)

10.4.1   Strengths

The strengths of the _research_ include:

- _The less stringent dietary restrictions in the rooibos intervention trial as compared to the stringent dietary protocol used in the grape juice trial of O'Byrne et al. (273);_

These researchers advised use of a well-defined less restrictive diet as the stringent dietary restrictions reduced the intake of ascorbic acid and other phytochemicals in their trial that interact with flavonoids and support their antioxidant properties. This may have limited their study effects.

- _The relatively large study sample sizes in the rooibos intervention trial as a clinical study and the further participant group administration;_

Even the smaller sample size of the rooibos intervention trial did not threaten the internal validity of the research since two different dietary assessment methods were compared in the research and it did not aim to assess the distribution of the intake estimate.(91)

- *The high response rates considering both the research demands and the burden placed on the participants, particularly the large amount of time required to complete the dietary records and the comprehensive FFQ with its long food list, which is unavoidable when detailed dietary information is requested (457);*

- *The controlled participant dietary intake across the rooibos intervention trial periods for energy, macronutrient and antioxidant intake and the participant body weight maintenance as reflection of their dietary compliance;*

This indicated that the effects on the plasma levels could be attributed to the differences in the daily total flavonoid intake alone across the trial periods.(37)

- *The use of a dissimilar dietary assessment method, which has vastly different error sources to evaluate the performance of the FFQ to assess the daily total flavonoid intake estimate, since a reference assessment method that involves errors related to those in the questionnaire (dietary history interview) could lead to inflated indications of validity (422);*

The error sources of the FFQ relate to the restrictions caused by a set food list,(73,422) assumption of portion sizes for most foods and the dependence on memory (422) and the cognitive challenge of reporting foods consumed over a broader time frame.(73) Dietary records on the other hand are open-ended, do not rely on memory and permit a more exact indication of portion sizes, with the error sources related to the likelihood of change in the dietary intake as a result of the assessment process itself and errors in interpretation related to the coding of the records rather than to the subject.(422)

- *The attempts made to obtain detail and accuracy through the dietary assessments as advised by Horwath,(422) as neither the estimated dietary records nor the questionnaire are considered perfect methods of assessing dietary intake*;

- *The use of biochemical indicators in the validity evaluation;*

Many validation studies refrained from using biochemical indicators as more objective and independent reference methods,(17) as there is still much to be learned about which is best to use.(398) The biological markers in the rooibos intervention trial, whilst based on only a single blood sample obtained in the baseline trial period, provided evidence for a correlation. Correlations were weak and non-significant between increased total flavonoid intakes and increased plasma TAC, as measure of the plasma antioxidant status,(128,214) and reduced plasma MDA levels, as predictor of lipid peroxidation and measure of oxidative stress (128). In addition, there was no correlation with the plasma total polyphenol concentration and CD level. These results add to the scarcity of data surrounding flavonoid intake and accompanying plasma measurements as put forward by Crews et al. (506) and Radtke et al.(338) However, lipid

oxidation as measured by the formation of CDs and TBARS, are not considered the best possible methods.(114)

- *The repeat of the analyses in a second further external subgroup of participants that was not only independent of the group for which the FFQ was originally developed, but from diverse and distant geographic areas to assess its external strength and probable generalizability, as also undertaken by Ishihara et al.(411)*

The strengths of the *abbreviated FFQ* include:

- *Its focus on the intake of a phytochemical grouping with phytochemicals presenting a new stance in the support of nutritional health and the prevention of disease;*

- *The improvements applied within its development based on the experiences reported by other developers;*

These include word use to enhance comprehension by the participants,(497) less restricted choice of portion sizes with specific portions size descriptions provided for nearly all listed items, the use of single food list items rather than multi-item aggregated groups, which require participants to consider many contributors,(433) and very importantly, removal of infrequently consumed dietary items from the food list,(497) as it refined the comprehensive FFQ and increased its efficiency of use as abbreviated FFQ (358);

- *Its capability of being self-administered as advisable for some clinical situations and many large-scale epidemiological surveys;*

This is desirable or essential (355,398) especially when resources are limited,(398) while the other dietary assessment methods may be intensely prohibitively either in expense and/or subject burden.(408)

- *Its additional practical advantages (in particularly in relation to the original comprehensive FFQ) like the ease of administration and quick completion with the data entry inexpensively done;*

This stems from its brief food list, which requires no post-administration coding to quantify the daily total flavonoid intake quickly and inexpensively.

- *Its relative validity against multiple dietary records and biomarkers of exposure and effect as reference methods and its responsiveness within the rooibos intervention trial.*

Despite their limitation in assessing absolute intake, the correlations or ranking of subjects on dietary intake found for FFQs render them useful in epidemiological studies.(150,403) Precision in terms of absolute intake at the individual level is often not necessary for epidemiological studies.(83,150) It may be sufficient for a dietary assessment method to discriminate in intake

variation among groups of individuals being high and low consumers,(83) which seems to be a particular pertinent feature of the abbreviated FFQ. This research finding suggests that the abbreviated FFQ will be a particular practical and easy tool to administer to obtain information on total flavonoid intake in chronic disease epidemiology, although the administration should be of recurring nature.

10.4.2　Limitations

The FFQ developed is highly specific as it assesses the intake of one phytochemical grouping over the time frame of two weeks. This may limit its use in such settings as epidemiological research, given that its food list is not suited to assess the total diet and its time frame to assess the usual diet. FFQs by design focus on particular nutrients or food components and are pre-coded with a restricted number of selected specific dietary items in its food list.(86) However, for clear interpretation of the relation between dietary intake and disease, assessment of the total diet is required so that information on the full range of dietary intakes can be obtained.(402) In particular in investigating the development of the chronic diseases of lifestyle (as CHD), the chosen dietary assessment method must optimally cover the intake of the total usual diet, since various dietary items and components are linked to such disease development and prevention,(380,408) and be sufficiently flexible to assess and accommodate dietary changes over time.(408)

The relation of the dietary component of interest to foods, nutrients and other dietary components must be regarded,(492) as confounding, by known and unknown factors, is a concern in studies of diet and disease.(428) Chronic disease develop over a long period of time and the dietary assessment method should be able to assess long-term usual dietary intake in these cases.(407) However, the averaging that is required in the subject having to report dietary intakes over extended periods of time and to average the intakes in his/her head, are subject to bias and quantitatively not the same as asking subjects what they recently consumed,(370) which due to the greater reliance on the episodic memory may support reporting accuracy.(57)

In this research, the participants reported on their dietary intake over the past two weeks which, although supporting quantitative accuracy, does not cover the longer time frame of usually one year for FFQs.(389) Assessing the long-term total dietary intake with its full range of foods, nutrients and non-nutrients cannot be achieved from this FFQ, which supports the indication by Neuhouser et al.,(431) that the use of short dietary assessment methods as the sole assessment method may result in a loss of information regarding important dietary exposures and lost opportunities to enhance knowledge regarding dietary factors and disease risk. However, the purpose of the research was the development of an abbreviated FFQ capable of assessing the

current daily total flavonoid intake in intervention research, as further dietary assessment method to the dietary records and not a FFQ capable of assessing the usual total diet.

Energy intake can be assessed as a means to validate subject reporting.(88) Errors in subject nutrient intake assessments are concurrent with errors in their total energy intake assessment, because over- or underreporting of individual items lead to errors in all dietary constituent estimates. Dietary intake adjustment on total energy intake would thus revoke a large amount of error in the subject dietary intake reporting.(372) Determining energy intake is not possible with the abbreviated questionnaire, which may not be a serious questionnaire limitation as flavonoids are not energy-bearing dietary components.

All the gathered dietary intake data were also obtained from participant self-report. Self-reported dietary intakes are not precise and are subject to both random error and systematic bias, which affects the accuracy of the dietary data obtained.(345,372) The use of one self-report dietary assessment method to test the validity of another self-report assessment method is a weakness,(346) as correlated errors have the potential to bias precision determinations.(73) This error dependency shortcoming between two dietary assessment methods can potentially be overcome by using biological markers, as was employed in the first phase of the research. The information on the risk factors for CHD was mainly based on self-report, particularly in the further participant group administration of the abbreviated FFQ. Self-report of risk factors have been applied in a number of prospective cohort studies to assess the association between flavonol intake and subsequent risk of CHD mortality (249) that may in such studies be considered of greater concern than in this research.

Although the research sample sizes were comparable to other validation study samples reported in the literature,(18) the small number of participating subjects, especially males, did not allow meaningful subgroup investigation in the datasets. Even in the larger and more diverse participant sample of the further abbreviated FFQ administration, and the sample size being calculated to provide a reasonable estimate, a predominant participation of female volunteers still occurred. Subgroup analysis has been neglected in validation studies.(400,494) Van Assema et al. (494) found quite large differences in the findings between certain subgroups in their validation study.

Specific subject characteristics seem to be associated with measurement error (518) that may be accountable for the differences found in the findings of dissimilar groups. Marks et al. (518) not only indicated the need to evaluate FFQ validity in a sample that is representative of the study group in which the FFQ will be utilized, but also the need for a sample size large enough to assess the differences among subgroups. This supports the suggestion of subgroup analyses in validation

studies by Van Assema et al.(494) Marks et al.,(518) found that of all the personal characteristics, gender was the most frequently related with intake estimate errors for food groups.

In relevance to this research, these investigators found that the difference in consumption estimates was greater among women than men for several vegetable food groups, including green leafy vegetables, total vegetables and fruits. The difference was small for the consumption of tea,(518) the major determinant in this research to the absolute and the between-person variation in daily total flavonoid intake. The observation that single gender validation studies tend to have higher correlations is illustrative of the effect of gender on measures of agreement.(518) This may be accounted for by the portion size data collections in FFQs, especially in assigning the same portion sizes for both genders.(518) In their review, Cade et al. (17) reported that there appeared to be differences in portion size between men and women and that correlations in validity evaluations appeared to be highest when subjects indicated their own portion sizes, as was applied in this research. Marks et al. (518) found that the presence of a medical condition and dietary supplement use were further characteristics related with subject food group reporting errors. Older subjects, being more established in their dietary habits, and subjects with a stable diet have been found to report their dietary behaviour more reasonably than, for instance, younger subjects.(17)

This raises a further limitation of this research which is the general limitation of FFQ validation studies of subject selection bias.(356,383,408,411,441,483) However, although the participants in this research may reasonably represent an adult population at risk for CHD in SA, they would be different from other such free-living adults to whom the abbreviated FFQ might be presented. They were not randomly selected from a population in which the questionnaire would be used, as ideally should be applied for obtaining a validation study sample,(407) but volunteers. The research placed considerable demands on the participants, as it minimally required repeated completion of detailed dietary records and a FFQ in both the research phases. In addition, the rooibos intervention trial lasted 14 weeks with occasions of blood sample collections and, therefore, demanded volunteers. Keeping detailed dietary records requires substantial time and proficiency from study subjects (407) and thus sufficiently co-operative subjects very different from a more representative population sample.(422) Thus, most validation evaluations utilize volunteers and not a truly representative subgroup sample of the study population.(407)

It could well be that the selected subjects were in general, as anticipated in other validation studies, possibly more health conscious and thus interested in health (23,378,422,442,476) and healthy eating,(401,442) more conscious of their own diets and what they consumed (441,476) and as a result reported their dietary intakes somewhat better.(378,476,483) It is said that health

conscious individuals are particularly attracted to participate in health oriented research (23) and that those who volunteer for dietary intervention trials may be exposed to and have an interest in nutrition messages.(57) Validity evaluations have been conducted in subjects with better than average education,(347,375,431,442,525) as in this research, and possibly also a high level of motivation (375,379,411,445,482,528) and strong sense of co-operation (347,422) since the requirements of validation studies are many.(483) All these factors possibly result in overestimations of the FFQ validity coefficients in comparison to when administered in a random population subject sample.(422,441,483)

The extensive dietary record keeping and the resulting high level of food awareness,(405) along with the dietary recording training (375) obtained, would tend to favour completing the dietary intake on the questionnaire more accurately than in the case of a more representative subject sample (401,405) that could lead to higher correlations between the FFQ and the reference method estimates.(483) As noted by Hu et al. (414) regarding their validation study, it could be possible that the study participants who were volunteers might be nutritionally slightly more biased than persons who did not volunteer and could have reported their dietary intake, in this case their daily total flavonoid intake, with more accuracy that would consequently have enhanced the inter-method correspondence. However, the research participants may correspond well with other validation study volunteers as postulated by Grootenhuis et al. (91) for their participants.

On the other hand it is known that dietary assessment requires compliant persons (347) and validation studies are distinctive in that numerous dietary assessments are conducted utilizing different methods.(86) Dietary intake is a direct function of the subjects that contribute the data.(5) Hence to obtain indisputable results for dietary validation studies necessitates a much better level of participant compliance than what might be attainable in, for instance, a random sample.(86) In validation studies with non-randomized samples, health-conscious, well-nourished participants with healthier dietary habits, without extremes in dietary intake, might presumably be over represented. (84,405,524) This would minimize the variability in the dietary intake and thus the magnitude of the correlation coefficients,(405) which might not be of great relevance to this research as participant extremes in daily total flavonoid intakes occurred.

Although the participants might have been more health conscious, this bias was possibly lessened by the exclusion of those persons taking dietary supplements. Tsubono et al.,(383) indicated that developing and evaluating a FFQ as a supplementary research project, as it occurred in this research in the rooibos intervention trial, may lead to overestimation of the true accuracy of the questionnaire, although they had no substantiation for this suggestion. To overcome such a possible distortion in the performance of the questionnaire, they suggested that further evaluation

of the questionnaire in independent subject samples should be undertaken, as was done in this research in the further participant group administration. Sebring et al. (88) also put forward that subjects' awareness that they are partaking in a study considering dietary intake may affect their reports of their intake and likely improve their recording. The human relationship itself in validation studies, which are intensive, could also result in more detailed dietary recordings and, accordingly, in valid estimation of individual dietary intakes.(441)

Besides the fact that the research subjects are predominantly a limitation, as the sample is not representative of the proposed population,(432,442,532) but are volunteers selected by virtue of their willingness to participate in, for instance, dietary reporting (432,442) and who may be more highly educated and motivated (375) and would do better in the dietary assessment validation than the population in which the method is to be applied,(379) it is also a strength. The strength lies in this volunteer bias providing participants notably being more highly educated and motivated and therefore being able to follow instruction in dietary intake reporting (375) and thus more likely to keep the dietary records accurately and complete the FFQs more carefully.(442) This is important in a validation study as the reference method, for instance, should be completed as accurately as possible.(482) However, the participation of a volunteer group to represent the population and not a random sample, also result in the findings not being uncritically generalizable to other segments of the proposed population, such as other age groups and ethnicities.(71, 375,528)

In the research, the results were achieved with participants who were predominantly a white and coloured group with high educational levels and, although across a wide age range, a predominantly younger adult age along with more female representation. As in other validation studies,(86) no claim can be made about generalizing the results to a group unlike in demographic characteristics or level of compliance. The research findings might thus not be generalizable, as in practice it may be applicable to the restricted study sample and not to subjects with lower literacy levels and thus lower socio-economic status and subject groups with differing dietary habits, like other ethnic groups, as indicated by other investigators.(445,525) This selective participation of a restricted subject sample reduces the ability to generalize or extrapolate the research findings to other groups and populations.(401,431)

Participant selection bias and non-participation (or non-response) bias may affect validation study results. Due to the invitation to volunteers to participate and the high response rate in both the research phases' reasons for non-participation to shed light on the issue of participant selection bias and non-response bias, cannot be presented. Buzzard and Sievert (55) indicated that the reasons for non-participation should be investigated to identify possible sources of bias, like age and culture, and to account for such biases when reporting the results. How these aspects affect

validation results is unknown.(483) It seems that in many instances those who fully participate in a study and those who do drop out of the study (78,433,476) or do not participate,(433) do not differ much in demographic (78,433,476) or even general health and lifestyle (476) characteristics. Martinez et al.,(41) indicated that it is unlikely that their validation results were biased, because the reasons for non-participation provided in their validation study did not appear to be associated with differences in subject dietary intake or with the ability to complete a questionnaire. It has been established that the background characteristics of the subsample participating in validation studies did not differ much from those of the actual study participants overall.(41,43)

When using relative validation, the administration sequence of the dietary assessment methods is important as the method of administration may affect the findings. The best arrangement is to conduct the test method before the reference method.(18,482) The recording of the dietary intake as the reference method before completing the questionnaire as the test method may sensitize the subjects to their dietary intake so that they complete the FFQ with greater accuracy.(442,482) A relatively large lag time between the recording period and completing the FFQ can lessen this problem.(482) This arrangement was not possible within the rooibos intervention trial design as at times in other validation studies.(88) As it is preferable for subjects to keep dietary records after administration of the FFQ rather than preceding its administration, this arrangement was applied within the further participant group administration that provided a more rational assessment of the performance of the FFQ.

However, since the repeat FFQ and the dietary records were administered within a short lag time of a few days in the further participant group administration, these agreement correlations are possibly overestimated based on the suggestion of Block et al.(450) Comparison of the dietary record results with the abbreviated FFQ results obtained over the same time, reflects the maximal estimates of the true validity.(387) The potential for correlated error in this approach (433) was considered in the comparison of the dietary record results with the abbreviated FFQ results administered before the dietary record reporting, which reflects the minimal estimates of the true validity (387) and the best estimate possible to imitate how subjects would come across the test method in practice.(18)

The USDA database is still incomplete.(105) Chun et al. (105) indicated that in its current development stage, it still could not even fully assess the flavonoid content of the typical American diet. Flavonoid intakes may be underestimated because of missing items, which are high in flavonoids, as the database is a compilation of data available in the literature on the flavonoid content of foods.(248) The USDA food composition database was constructed based on US representative food samples.(105) Because the flavonoid content in products of plant origin

depends highly on the cultivation surroundings, it is difficult to apply such data collected in one country to foods from another.(269) Changes during food preparation and processing are also not fully addressed.(89,105,248) Currently the effect that processing has on the flavonoid content in foods is expressed as the ratio of flavonoid content in processed foods to that in the unprocessed foods.(248) Preparation practices also vary across countries which may make food content extrapolations problematic.(248) The USDA database has, in general, been used to assess the flavonoid intakes in other countries as no information or accurate information is available in many countries to estimate a total flavonoid intake,(136,261) as was applied in this research. Concern over such application of flavonoid food composition tables compiled in one country to another had justifiably been raised.(136,165,263)

10.4.3   Value of Research and Developed Abbreviated FFQ

As new facts emerge, it seems that polyphenols are increasingly becoming more important for the prevention of numerous chronic degenerative diseases.(101) Considering the review of the literature, it is apparent that a regular and presumably daily and adequate flavonoid intake is capable of modulating oxidative stress in humans through several mechanisms in addition to other protective properties and might, therefore, play a crucial role in heart health protection.(59,129) Dietary and other lifestyle modifications, rather than pharmacotherapy, is the considered preferred means to decrease the incidence of CVD.(243) The increased consumption of items rich in antioxidants, micronutrients and phytochemicals has been promoted in this regard.(188) Assessing the intake of flavonoids, and possibly other phytochemicals, are to a great extent becoming important in nutrition to shed light on the intakes and the food sources providing the intake, as means to arrive at dietary intake recommendations.

Information on the intake of flavonoids across countries are scarce (100,153) and most of the assessed intakes were done retrospectively using FFQs that were not developed with a flavonoid specific intake hypothesis in mind.(47) Developed FFQs with a food list compiled specifically considering the dietary sources of flavonoids are scarce. No publication could be found on the development and evaluation of a brief questionnaire with its food list compiled using approaches like the percentage contribution to identify the dietary sources most predictive of the absolute intake, and stepwise multiple regression analysis to identify the dietary sources most predictive of the between-person variability in intake. This research may be the first to have undertaken this task. The internal evaluation of the abbreviated FFQ, within the rooibos intervention trial, and its external evaluation, within the further participant group, makes the development of the current abbreviated FFQ quite unique. This newly developed abbreviated FFQ not only bridges a gap in the scarcity of FFQs aimed at assessing daily total flavonoid intake and adds to the limited pool of

currently available FFQs to assess phytochemical intake, but also allowed for identification and confirmation of the key foods associated with total flavonoid intake.

In the development of the food list, the dietary sources were identified from both the baseline comprehensive FFQs administered in the rooibos intervention trial and the dietary records completed. These identified dietary items for inclusion in the food list were very similar and additionally confirmed in the dietary record completion of the further participant group administration. The developed abbreviated FFQ has advanced sufficiently from the inception of the comprehensive FFQ with its extended food list to exhibit particular strength in its food list to be of value. Here the issue of the contributions of vegetable inclusions in composite dishes and the addition of herbs to food to the total flavonoid intake provides a further edge to the research and the FFQ, as these were specifically addressed. The food list results, that is the identification of the contribution of onions as vegetable ingredient in mixed dishes and dried parsley as herb addition to foods, confirmed that not taking these contributions into consideration as dietary sources of flavonoids, particularly the onion contribution as part of mixed dishes, could introduce bias, although very small, in the total flavonoid intake estimate.

The abbreviated FFQ is to our knowledge, the first attempt to develop and evaluate a FFQ to specifically assess the total flavonoid intake of adult South Africans, which seems to have a generalizable edge among those adult South Africans following a dietary pattern representative of a Western diet. The identification of the dietary sources for inclusion in its food list inherently confirmed the key or main dietary sources of total flavonoid intake among adult South Africans following a predominantly Western diet. These dietary sources may not only be relevant as target foods for increasing total flavonoid intake, but a higher consumption of a number of them singly,(248,262) and in combination,(269) has been associated with lower CHD and total CVD mortality risk reduction.

Although the main aim of this research was to validate a FFQ able to assess total flavonoid intake versus a reference method and not to consider the dietary intake of the participants in general, the research provided an indication of the mean daily total flavonoid intake among South Africans. This research is the first step towards generating baseline data of the flavonoid intake of South African adults. The research adds a South African perspective to the current available international data. Considering the mean daily total flavonoid intake estimate of some participants, the total dietary intake of polyphenols estimated to generally reach 1000 mg/day,(8,96) particularly in persons who eat several daily servings of fruit and vegetables,(100) may be an underestimation based on the flavonoid intake estimate in this research. The total dietary intake of polyphenols may be well over 1000 mg/day as proposed by Lotito and Frei,(93) which they based on

consuming a balanced diet incorporating the recommended nine daily servings of fruits and vegetables and moderate daily amounts of tea, coffee, wine, beer and chocolate. Flavonoids and polyphenols would provide for an important contribution to the dietary TAC as proposed by Nijveldt et al. (200) and Nardini et al.(142)

The first phase of the research evaluated self-reported dietary flavonoid intake through a questionnaire by comparison to a superior dietary assessment method and to biomarkers in the rooibos intervention trial. FFQs have, however, not been deemed the optimal dietary assessment method for use in studies with small numbers of subjects, where accurate absolute intakes are required, and in clinical work when exact intakes are required.(18) This all relates to it not being advisable for assessing group means in intervention trials.(634) It also appears that in intensive studies where multiple dietary recalls or records are practical, both these methods are better than a FFQ for assessing the dietary intake.(86,389) Despite these concerns, the application of FFQs to clinical settings were listed among the future dietary assessment research directions listed by Willett.(30) Considering participant burden and limited budgets, multiple recalls or records are not considered practical options for assessing the dietary intake in many studies.(86) Self-administered FFQs offer a convenient means for dietary assessment as they involve a low participant burden and undemanding data analysis in comparison to other assessment methods.(413) Because of the low cost, the modest demand on subjects and the documented validity for assessing long-term diet, Willett (30) considered the questionnaire approach as probably useful for clinical research. In the rooibos intervention trial, the comprehensive FFQ developed and its resultant abbreviated FFQ were administered and applied in an intervention setting.

A study by Schaefer et al.,(351) aimed at absolute validation of the FFQ method against a known diet, did not provide support for using FFQs in clinical trials.(376) This study in which subjects were cycled through three different dietary phases in addition to their usual diets for six week periods, found that the FFQ lacked efficacy in assessing macronutrient intakes, as 'blurring' of the dietary intakes in the FFQ applications occurred across the three phases of the study.(351) The study was based on the conviction that FFQs could capture dietary behaviour in the short term.(376) However, the advocates of the FFQ approach, like Willett,(634) denote that the efficacy of FFQ responses greatly depends on the participants' accumulated mental image, or knowledge, of their customary diet shaped over the longer term past, which would normally be the case when FFQs are used in epidemiological studies. FFQs are therefore not sensitive to fluctuations in dietary intake over a short period.(376) According to Drewnowski et al.,(376) this viewpoint admits that FFQs have less to do with memory for what was consumed, but more with a mental image developed about the habitual diet. The validation of the FFQ in the study of Schaefer et al. (351)

occurred in a situation where the participants were provided with standard diets for a period of time that differed from their usual consumption pattern. According to Drewnowski et al.,(376) this represented an unnatural situation, which was not the case in the rooibos intervention trial.

The findings of the rooibos intervention did not support a 'blurring' of the dietary intakes across the trial periods, as the mean daily total flavonoid intakes as assessed by the dietary records and that of the FFQ were closely related in each of the trial periods, which reflected the changed dietary conditions of the trial periods. Although the FFQ cannot perform the same as dietary records when assessing the absolute dietary intake of a subject, it could be of clinical use.(347) This is because it performs fairly well in ranking subjects by levels of dietary intake, as found in this and other studies. It would thus distinguish subjects in their intake of the dietary component of interest in clinical feeding trials. The outcome of the research demonstrates the effectiveness of the FFQ to obtain dietary data in intervention settings to draw conclusions regarding the influence of the dietary component, which is total flavonoids on the disease state, CHD. This supports its use in clinical trials to assess group intakes, unless a correct absolute value of the dietary component is required. In such instances, the use of the FFQ should possibly complement rather than substitute the use of another superior dietary assessment method as information on the intakes of other dietary components may also be necessary, which cannot be provided by this developed FFQ.

Buzzard and Sievert (55) had proposed that measures to assess dietary change be developed and evaluated. Dietary information derived from baseline or cross-sectional data is inadequate to assess the performance of dietary assessment methods to measure change in dietary behaviour.(50) A brief FFQ might be a suitable tool to assess dietary change in total flavonoid intakes, as the developed abbreviated FFQ was responsive to the total flavonoid intake changes within the rooibos intervention trial and even responsive to the total flavonoid intake within the washout and control trial periods on the exclusion of flavonoid-rich seasonal foods from its food list. The administration of the abbreviated FFQ in the further participant group had a cross-sectional design, which did not allow for determining its responsiveness in this phase of the research. It remains to be confirmed whether the abbreviated FFQ is suitable to detect consumption changes over time. This may inherently be a limitation of the second phase of the research. A concern in an intervention setting is whether an intervention caused a change in dietary intake other than that targeted by the intervention,(41) which with the use of the abbreviated FFQ will have to be overcome by using a complementary dietary assessment method, like dietary records, as was used in the rooibos intervention trial.

Cade et al. (17) in their FFQ development and validation review reported that less than half of all FFQs being validated were tested for reproducibility, although the reproducibility evaluation of a

FFQ is not considered as critical, but still an omission if not done.(445) To adequately assess reproducibility, sufficient time must be allowed between the two administrations of the questionnaire to avoid subjects remembering their responses, which may be a concern in the second phase of the research due to the short time interval of two weeks between the questionnaire administrations. Although a longer time interval would be preferable, the administration and evaluation of the abbreviated FFQ in the second phase of the research was linked to the design of the questionnaire for use in the rooibos intervention trial. The open-ended format of the questionnaire frequency responses may not have supported a natural frequency response remembrance for the participants in the repeat administration.

10.5   RECOMMENDATIONS

All measures advance over time. In the case of the developed abbreviated FFQ, the correlations for the technical requirements of an assessment tool are satisfactory and do not seem to require further improvement. An improved absolute intake estimate and an ability to categorize the subjects, however, could benefit from further investigation. FFQs are precoded with a limited number of dietary items in the food list. By providing the frequency of consumption and a portion size of the consumed items, a rough estimate of the participant dietary intake can be obtained.(86) It is likely that modification or addition of dietary items and possible format changes to minimize the effect of some subject errors, could improve the estimates. The extensive dietary data collected in the two phases of the research provided a good resource with which to primarily address the food list of the abbreviated FFQ to accomplish the above. Further research on this FFQ should consider whether the questionnaire would still perform equally well if seasonal fruits were integrated within the food list. These fruits, which were excluded from the food list, were items that could contribute to and discriminate in the total flavonoid intake. Both the food list compilation and confirmation analyses would be required to address this issue. The chance, however, is that inclusion of these fruits in the food list could exaggerate the total flavonoid intake overestimation on extending the current food list through their inclusions, as they were among the top foods contributing to the absolute daily total flavonoid intake.

In the confirmation of the food list, the opportunity arose to further refine the questionnaire considering the slight overestimation in the absolute intake, which is most likely proportional to the overestimation occurring prominently at higher levels of intake. This could possibly be attributed to the overestimation of the tea consumption at its higher levels of intake. Further investigation is needed so that the FFQ could fully reflect a more truthful tea intake that would be related to the portion size consumed or to the consumption frequency. Tea contributed greatly to the daily total flavonoid intake in this study. Therefore, a better representation of the actual quantity consumed is important for assessing the absolute intake in the FFQ. Multiple portion sizes may be an option as

applied in other studies.(24) More relevant to this questionnaire, the participants could provide their frequency response by indicating their daily intake as an *intake range* in the questionnaire. It may be a viable option to ignore the required indication of the number of times per day, and only answering the number of times per week or per two weeks which would take into account the likelihood that the same amount of tea was not consumed on those tea drinking days over the two-week time frame.

Additional qualitative information on the variation in the tea preparation, for specifically biochemical exposure comparisons, may have an added advantage, as the preparation technique may influence the flavonoid content on consumption. The tea type and amount consumed, along with the preparation method of brewing time and beverage temperature, influences the concentration of flavonoids in tea.(571) To allow for better exposure and possible effect indications applying biochemical comparisons, consideration could be given to obtain more focused qualitative tea consumption information as applied by Hakim et al.(31,571) They designed the Arizona Tea Questionnaire to serve as a supplement to the traditional Arizona FFQ, which asks information on the variation in the tea preparation like tea leaf concentration, brewing time, strength and beverage temperature. The qualitative information obtained from this questionnaire can thus be used complementary to the quantitative tea estimate, which is obtained from a FFQ.(31) Lack of such qualitative information may impact epidemiological investigations of the relationship between tea consumption and disease risk.(571)

The findings of the research, in addition, highlighted the importance of beverage consumption in the form of tea, followed by fruit juice, in contributing to flavonoid intakes. It also seems that the intake of beverages possibly do not only contribute greatly to a South African total flavonoid intake, but also to the dietary TAC.(635) Emphasis should thus be placed on consumption of beverages, alongside that of foods, in dietary intake assessments and in dietary intake guidelines and recommendations, especially when the dietary contribution of antioxidants to human health promotion and CVD prevention is considered. The intake of even small amounts of dietary flavonoids was reported as important in circumstances where the intake of other dietary antioxidant sources is low.(11) The tea drinking habit may be a convenient dietary means to increase total flavonoid and antioxidant intake, without increasing energy and sugar intakes.(299)

With reference to the seasonal fruit, it could be argued that the exclusion of these dietary items from the food list of the abbreviated FFQ that contributed to the absolute intake and the between-person variation in intake deters the questionnaire of providing a true estimation of the total flavonoid intake. With the data collection not spanning over the usual long-term dietary intake and thus all the seasons, the devised food list included only those dietary items captured in the time

span of the research. Development of a well-defined food list for an abbreviated FFQ should innately span all seasons of the year to incorporate the most eminent dietary items in the food list. Within the South African context, it may additionally relate to other flavonoid-rich fruits, like grapes and even stone fruits, like apricots and plums, which are only available in summer. Grapes are available from November through to April peaking in December to February, apricots in November and December and plums from November through to March peaking in January and February (544). These were not considered for inclusion in the developed abbreviated FFQ food list. However, when these highly seasonal fruits are available, they may be consumed in the place of other flavonoid-rich fruits, possibly not influencing the total flavonoid intake to such an extent as it might influence the subclass intakes.

The dietary item exclusion on seasonal grounds draws attention to administration time of the abbreviated FFQ that becomes an important aspect to consider. It is known that in addition to individual preferences other factors, like season and climate, alter fruit and vegetable intake.(52) Different kinds of fruit are available in different seasons of the year.(441) What is important for such estimates concerning FFQs, is that current seasonal fruit intake influences the report of the past intake providing evidence of seasonal bias.(636) In this research the percentage contribution of black tea and rooibos, as the two main contributory items to the total flavonoid intake, reduced in the further participant group administration, which took place in late October to early November representing early summer, compared to the rooibos intervention trial which was conducted mainly in the winter season (July to August). The contribution of beer, raw onions and white wine increased and contributed as discriminating items. The effect of season is the most plausible reason for these findings as these dietary items are usually consumed in warmer weather conditions.

These results set the stage for a seasonal influence beyond possibly that of fruit and vegetables alone, but also on beverage choices and consumption. To be assured that any observed changes did not reflect seasonal changes in consumption patterns, total flavonoid intake estimates should be assessed in the same season when either some plant foods are in season with plentiful supply or when out of season, like citrus, berries and summer stone fruit.

Reproducibility studies will, therefore, have to be performed during similar seasons and not include different seasons because of seasonal variations not only in vegetable and fruit intake, but possibly even in beverage consumption. This highlights a limitation in the use of the abbreviated FFQ in that data on the daily total flavonoid intake should not be collected during the main seasons for citrus fruit, guava and strawberry intakes. These seasonal items could be included in the food list and the resulting performance of the questionnaire evaluated on such food list inclusions. In the

case of interest in flavonoid subclass intakes, seasonal fruit availability of citrus fruits, guavas and strawberries, will greatly impact the intake of the flavonoid flavanone and anthocyanidin subclasses, respectively, caused by within-person variability from season to season. The inclusion of even other flavonoid-rich fruits, which fell outside the seasonal time frame of this research, in the abbreviated FFQ food list, will require its resulting performance to be evaluated on such food list inclusions.

The above scenario may be particularly relevant in intervention studies focused on such dietary items, such as citrus fruits or grapes, as the research did find that due to the exclusion of the documented seasonal fruits from its food list in the rooibos intervention trial, the abbreviated FFQ did not perform well in the washout and control trial periods. As a result, the total flavonoid intake estimates from the dietary records captured the intake of these fruits, whereas the abbreviated FFQ could not account for their contributions to the total flavonoid intake, particularly on exclusion of all teas as major contributors to the total flavonoid intake.

In the application of the abbreviated FFQ to assess dietary change in total flavonoid intake, the above need to be considered in its application. The questionnaire would be able to assess dietary change where the change is related to foundation total flavonoid intakes or when citrus fruit, guava, strawberries and summer fruits are not in full season. In particular with intervention work related to the different types of tea, there are larger contributions of the single teas. Although the abbreviated FFQ might be sensitive enough in such applications, it may possibly not be so when the intervention diet incorporates flavonoid-rich dietary items not included in the food list. Or, if more complex, some changes in intake are likely to be missed because the questionnaire does not include a comprehensive list of all foods that contain flavonoids, which is a problem all short questionnaires encounter when assessing a limited dietary scope.(434) The groundwork has been set yet further work may be required to explore whether the abbreviated questionnaire can assess dietary change in total flavonoid intake when the dietary change or the intervention diet is more complex. The abbreviated questionnaire application within the rooibos washout and control periods where the required flavonoid intake was restricted, did not perform as soundly. Although the comprehensive FFQ deals with many dietary items and is more suited for this purpose, it places a greater burden on the subjects in terms of the time required for it to be completed.

Even though FFQs provide a limited number of dietary items in the food lists and do not allow recipes or specific ingredients to be imputed for analysis,(351) the vegetable category in the questionnaire, considering vegetable intake as ingredient in mixed or composite dishes, provided for this. Although the comprehensive FFQ food list incorporated a spectrum of vegetables in mixed dishes as a food category to account for vegetables found in composite food mixtures, only the

inclusion of onion was retained in the abbreviated FFQ food list. As for the determined herb amounts added to South African recipe formulations, the same process should be applied to mixed dish recipe formulations in published South African cookbooks containing onions as ingredient, to obtain more standardized amounts on its ingredient use so that these amounts could be used for the small, medium and large portion amounts and not the currently used gram estimates.

The two-week time frame of the questionnaire, as considered for the rooibos intervention trial, may need attention. The argument that diet for a one-month period in a year as time frame in some FFQs may not represent long-term habitual diet and may not be relevant to disease risk,(428) also applies to a total flavonoid intake estimate over two weeks. Long-term diet, rather than the recent intake, is the relevant exposure when, for instance studying CVD.(257) Chronic diseases emerge over a number of years and are the outcome of several pathogenic processes. Protective effects like the reduction, inhibition or reversal of these processes may necessitate extended periods of exposure to bioactive compounds.(3) Assessment of the usual dietary intake of individuals and groups within a population is fundamental to the study of diet and their relationship to disease.(394) The FFQ is typically designed to assess usual intake without the need for repeated assessments.(380) However, in the use of this developed questionnaire, repeated assessments would be required to obtain a reflection of the usual total flavonoid intake.

In epidemiological studies, additional dietary records or recalls would probably increase the validity, although the increased costs might prove too expensive.(470) However, in the use of this abbreviated FFQ, a balance could be found between more details and the least possible burden to the participants (494) to ensure a more representative total flavonoid intake on repeat administration. Repeat intake assessments seem to be a necessity for assessing total flavonoid intake when considering the seasonal influence. De Vries et al.,(74) calculated that the within-person coefficient of variation of 63% indicates that the number of days required to assess individual intake, within 20% of the actual intake 95% of the time, is 38 days. They concluded that the results of one or three-day dietary records is possibly insufficient to represent habitual average daily flavonoid intake, particularly where individual subclass flavonoid intakes are concerned.(74) Repeated plasma flavonoid measurements in time are needed to obtain estimations that represent long-term intake.(194) Shahar et al.,(637) who found that significant seasonal changes occur in dietary intake, recommended that a FFQ should be administered at least twice a year to capture the seasonal quantitative differences between the summer and winter dietary intakes, which favours recurring administration. Wolfe et al. (419) also suggested that multiple brief assessments should be undertaken when trying to average typical consumption over more than one season.

Although the assessment of the total flavonoid intake is possible through this abbreviated FFQ, which can be used as a quick method to assess the intake of those adult South Africans following a diet similar to a Western diet, its generalized use and the generalization of its findings must be cautioned. Although the research sample, both the internal rooibos intervention trial sample and external further participant group sample, were to some extent diverse in terms of gender, age and educational backgrounds, there was an overall predominance of females, younger aged and higher educated subjects of whom most had executive/professional occupations. The study sample was not as diverse in race/ethnicity representation, as only white and coloured adults participated. The highest rates for IHD observed among the South African population groups, with the exception of the Indian population group, are for the white and coloured population groups, with a low rate observed in the black population group.(638) Those adults who agreed to take part were possibly not representative of the general South African adult white and coloured population at risk for CHD, as they were volunteers and possibly more interested in their personal health and highly motivated participants, in addition to being largely representative of the Western Cape Province of SA. This may raise some justifiable concerns about the generalizability of the use of the abbreviated FFQ and its findings within the population group of South African adults at risk for CHD.

Further work needs to be undertaken to obtain a broader participant representation to fully understand the potential broader application of the abbreviated FFQ. Although the findings of reasonable validity and reproducibility are positive, it is possible that the findings could differ from that obtained from a broader and more diverse participant representation. Further testing would have to include representation of more men, other racial/ethnic groups and a larger range of socio-economic characteristics pertaining to lower literacy subjects or subjects of a lower socioeconomic status and, in particular, also across other geographical regions to extend the sample diversity to confirm and expand the FFQ use and its findings.

Validity and reproducibility estimates are not a measure of the questionnaire by itself, but the outcome of the interaction of the questionnaire and the particular participant group to whom it was administered.(639) Dietary intake varies among diverse populations,(86) as persons from different ethnic and socioeconomic groups and from different geographical areas are likely to differ by the dietary items commonly consumed, the recipes and the methods of preparation and possibly in typical portion sizes.(409,639) As a result, the major food sources of specific nutrients, food group choices and nutrient intake may differ.(400) It may call for the creation of a FFQ with a somewhat dissimilar food list that requires evaluation, as the food list would need to be sensitive to the dietary behaviour of the included groups.(400) There is a greater consumption of fruit and vegetables among people with higher income and education,(5) which would have manipulated the current abbreviated FFQ food list. Edible wild greens would probably have been incorporated in the food

list on a greater racial/ethnic representation of blacks. Edible wild greens are a potentially important source of flavonols in the Greek diet, as they are habitually eaten as part of the traditional Greek diet and have a far higher content of flavonols in comparison to the usual fresh vegetables, fruits and beverages typically consumed in Europe.(640) The low occurrence rate of IHD among the South African black population is though on the increase in the urbanized sector (638) with dietary intakes related to a Western diet.(25) Even the consumption of tea, which is the major contributor to the daily total flavonoid intake and a major source of the between-person variation in the intake, reflect local preferences and traditions as to the types consumed and the drinking habit, which can cause considerable variation in intake between populations.(31,571)

Participant, demographic characteristics and other attributes, like motivation, nutrition knowledge and ability to complete the questionnaire, are known to affect self-reports.(86,379) This may also necessitate further evaluation of the FFQ even without a dissimilar food list since participant factors (379,405) and the underlying between-person variation in nutrient intake affects the performance of any dietary assessment method (405) in addition to the questionnaire properties, such as layout and data editing procedures.(379) Further evaluative investigations are always necessary and useful to fully establish the relevance and performance of a questionnaire in other groups or populations,(405,409,482) as a FFQ valid for one population may be invalid when applied to another.(510) It should be kept in mind that questionnaires are culture-specific and that the assessment of intake in a culturally different group might require the development and validation of a different measure.(405) The currently developed abbreviated FFQ may thus require modifications for wider administration.

Further research is needed between total flavonoid intake and/or flavonoid-rich dietary sources and health promotion and disease prevention, particularly CHD. A next step to expand the use of the abbreviated FFQ and further its performance evaluation in its current format, or a revised version, could be in health intervention research among South African adults at risk for CHD. This would be relevant in particular to rooibos herbal tea consumption and in addition to more detailed dietary assessment measures, like dietary records. Such research would provide biochemical measurements that could be investigated as exposure and/or effect biomarkers of total flavonoid intake that, on the one hand, could reduce or on the other hand, strengthen the dependence on reference methods in such dietary assessment, but would overall add to expand the dietary biomarker research in terms of polyphenols.

As people eat various plant foods from various environmental surroundings, the mean flavonoid intake may be the most appropriate intake to estimate.(248) Although this was computed in the study, use of the USDA flavonoid database in the absence of a South African database could

possibly have underestimated the flavonoid intake assessment, as the flavonoid contents of plants grown in SA may be higher than that grown elsewhere, due to sunlight exposure during cultivation.(166)   The flavonoid intake level of South African adults may be higher than the assessed estimations in this research. The compilation of a South African-based regional and/or national food composition database may be a future endeavor to undertake. Food composition data is essential for dietary intake assessment, which requires the development and the continued updating and expansion of the food composition database in the interest of diet-health related research.(55)

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ADDENDUM A:

Tables for Chapter 4

ADDENDUM A

TABLES RELATED TO CHAPTER 4

Table 4.1: Mean daily total flavonoid intake, daily intake range estimates of some summed flavonoid subclasses in several countries based on national representative samples and non-representative subsamples

COUNTRY	Population	Dietary intake assessment method[a]	Flavonoids determined[b]	Summed mean daily flavonoid intake (mg/day)[b,c]	Prime sources of flavonoid intake (if noted)
DENMARK (337)	Danish Household Consumption Survey	Dietary history	1 Flavone 2 Flavonols 3 Flavanones	23–46	
FINLAND (11,262,256 respectively)	Finnish Mobile Clinic Health Examination Survey, 5 133 adults, 30-69 years	Dietary history	2 Flavones 3 Flavonols	3.4	Apples, onions (64%); Other: Fruits, berries, sweetened juices, jams (mainly berry) and vegetables
	Finnish Mobile Clinic Health Examination Survey, 10 054 adults	Dietary history	2 Flavones 4 Flavonols 3 Flavanones	24.2±26.7[d]	Oranges, apples, grapefruit, onions, white cabbage, berries, juices (95%)
	Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study, 25 372 male (M)[e] smokers, 50-69 years	Self-administered modified dietary history questionnaire	2 Flavones 3 Flavonols	9.9 (Median=8.0)	
FRANCE (South-Western) (190)	Paquid study, 1 367 elderly subjects	Three-day dietary records and questionnaire	2 Flavones 3 Flavonols	14.4 from dietary records 17.6 from more detailed questionnaire (more detailed vegetable information)	Fruits (35.2%), vegetables (19.1%), wine (16.9%), tea (16.0%)
GERMANY (338)	Plasma biomarker study, 48 female (F)[e] students, 22-36 years	Seven-day dietary records	2 Flavonols 2 Flavanones	Flavonols: quercetin 17.9±9.5, kaempferol 4.7±4.8 Flavonones: naringenin 12.1±15.6, hesperetin 17.4±14.3	Flavonols: Green and black tea (40%), onions (25%), apples (11%) Flavanones: Exclusively citrus fruits
JAPAN (Northern) (309)	Volunteers, 115 F, 29-78 years	Three-day dietary records	1 Flavone 4 Flavonols 2 Isoflavones	Flavone and flavonols: 16.7±9.2 Isoflavones: 47.2±23.6	Onions (45.9%), apples (7.2%), green tea (5.4%)

(Table 4.1 continued on next page)

COUNTRY	Population	Dietary intake assessment method[a]	Flavonoids determined[b]	Summed mean daily flavonoid intake (mg/day)[b,c]	Prime sources of flavonoid intake (if noted)
SOUTH WALES (165)	Caerphilly study, 1 900 M (1979), 2 168 M (1983), 45-59 years	Food frequency questionnaire (FFQ)[f]	3 Flavonols	First survey FFQ (n=1 900 men) 26.3±12.5; Weighed dietary records (n=665 men) 26.2±16.0; Second survey FFQ (n=2 168 men) 26.0±13.2	Black tea (82%), onions (10%)
THE NETHER-LANDS (45,258,111, 174,339 respectively)	Zutphen Elderly Study, 1985-1990 , 805 M, 65-84 years	Dietary history	2 Flavones 3 Flavonols	Baseline: 25.9±14.5 1990: 26.6±13.2 Mean=26	Black tea (61%), onions (13%), apples (10%)
	Zutphen study cohort, 552 elderly M (followed up for stroke incidence)	Dietary history	2 Flavones 3 Flavonols	Baseline: 19.8±9.2 in stroke cases 22.2±9.1 in non-cases	Black tea (70%), apples (10%)
	Dutch National Food Consumption Survey, 4 112 adults, 19+ years	Two-day dietary records	2 Flavones 3 Flavonols	23	Tea (48%), onions (29%), apples (7%)
	Dutch National Food Consumption Survey, 6 200 subjects, 1-97 years	Two-day dietary records	6 Catechins	50±56	Tea main source Children: Chocolates second (20%) followed by apples/pears Adults: Chocolate and apples/pears equally important Elderly: Apples/Pears
	MORGEN study (three Dutch cities), 13 651 adults, 20-59 years	FFQ	Flavones Flavonols Catechins	58±46	Overall: Tea, apples Catechins: Tea (72%), apples (12%), chocolate (4%) Flavonols and flavones: Tea (47%), apples (14%), onions (14%)
UNITED KINGDOM, London, Greenwich (340)	Population based case-control study, 607 asthma cases, 864 controls, 16-50 years	FFQ	2 Flavones 3 Flavonols 6 Catechins	Median interquartile intakes: Flavones 0.25 Flavonols 33.2 Catechins 81.2	

COUNTRY	Population	Dietary intake assessment method[a]	Flavonoids determined[b]	Summed mean daily flavonoid intake (mg/day)[b,c]	Prime sources of flavonoid intake (if noted)
UNITED STATES (US) (254,153,232, 259,250 respectively)	US health professionals, 34 789 M, 40-75 years	FFQ	2 Flavones 3 Flavonols	20.1	Tea (25%), onions (25%), apples (10%), broccoli (7%)
	US health professionals, 37 886 M, 78 884 F, 40-75 years	FFQ	2 Flavones 3 Flavonols	20–22	Tea (M 26%; F 35%), onions (M 24%; F 23%), apples (M 10%; F 8%), broccoli (M 7%; F 8%), red wine (M 0.3%; F 0.2%); Remainder variety of fruits, vegetables and juices
	US health professionals (Women's Health Study), 38 445 F, 45-89 years	FFQ	2 Flavones 3 Flavonols	24.6±18.5	Tea (31.2%), onions (23.2%), broccoli (7.8%), apples (7.7%)
	US registered nurses (Nurses' Health Study), 66 360 F, 30-55 years	FFQ	2 Flavones 3 Flavonols	21.2±16	Six major foods (83.4%): Tea (35%), onions (23.3%), apples (9.2%), broccoli (8.3%), tomatoes (5.9%), grape products (raisins, grapes, red wine) (1.7%)
	Iowa Women's Health Study, 34 492 post menopausal F, 55-69 years	FFQ	2 Flavones 3 Flavonols	13.9	Tea (36%), apples (17%), broccoli (9%)
SEVEN COUNTRIES STUDY (253)	16 cohorts, 12 763 M, 40-59 years	Seven-day dietary record (also one-day and four-days)	2 Flavones 3 Flavonols	2.6 West Finland to 68.2 Ushibuku, Japan	Main sources differed in cohorts: Tea: Japan (>80%), Zutphen (60%); Red wine: Italian cohorts (40%); Vegetables and fruits (mainly onions, apples): US, Finland, former Yugoslavia, Greece
DIFFERENT COUNTRIES (N=14) (74)	17 international subjects	Dietary records (one- and three-day) FFQ (8 Dutch subjects)	2 Flavones 3 Flavonols	International subjects: 27.6±19.5 Dutch subjects: three-day dietary records 34.1±31.2; FFQ: 41.9±23.7	Tea (37%), onions (26%), vegetables (14%), fruits (22%), red wine (1%)

[a] The food frequency questionnaires used as dietary assessment method in these epidemiological studies were originally developed to assess the total diet and did not have a prior flavonoid-specific intake hypothesis in the compilation.

[b] The flavonoid intake estimates were primarily computed from unpublished and published Dutch food composition content values obtained through food analysis in The Netherlands with input of additional values from published content values for other foods, among others American foods obtained through food analysis in the United States *(Table 4.1 continued on next page)*

[c] Mean daily intake refers to only a few summed flavonoid compounds (mostly two flavones and three flavonols)

[d] Mean±standard deviation

[e] M= Male(s); F= Female(s)

[f] FFQ= Food frequency questionnaire

Table 4.2: **Mean and median daily total flavonoid intake estimates of the major flavonoid subclasses in several countries based on national representative samples and non-representative subsamples**

COUNTRY	Population	Dietary intake assessment method[a]	Flavonoids determined[b]	Mean daily total flavonoid intake (mg/day)[b, c]	Prime sources of flavonoid intake (if noted)
AUSTRALIA (89,164 respectively)	National Nutrition Survey, 13 858 adults, 18+ years	24-hour recall	5 major subclasses	454	Tea (76%), oranges, other citrus fruit, grapes, wine, apples, leaf and stalk vegetables
	24 healthy young Australian women	Twelve-day dietary records	2 Flavones 4 Flavonols 3 Flavanones 6 Catechins	128±19.9[d]	Catechins (59%) with tea (green, black), apples (with/without skin), red wine, dark chocolate, cocoa
FINLAND (Eastern) (169)	Kuopio Ischaemic Heart Disease Risk Factor Study, 1 380 middle-aged men	Four-day dietary records	5 major subclasses	129 Energy-adjusted: 128.5±206.7 Not energy adjusted: 128.5±207.7	Flavanols (84%) with black tea most likely the prime source
GREECE (261,137 respectively)	Hospital-based coronary heart disease (CHD)[e] control study, 329 patients, 570 controls	FFQ[f]	6 major subclasses	Median quintile (Q)[g] intakes of cases and controls: (lowest) Q1, 69.4 Q2, 110 Q3, 148.4 Q4, 185.0 Q5, 235.8 (highest)	Flavanones (35.5 mg/day) followed by flavanols (20.8 mg/day from wine and tea); Minimal isoflavones (median intake of highest quintile: <1 mg/day)
	Weekly menu representative of traditional Greek plant-based diet	Weekly menu	6 major subclasses	118.6	Oranges (32%), red wine (23%), apples (18%), parsley (6%), onion (5%)
ITALY (341,263 respectively)	Large scale multicentric (6 Italian areas) case-control study of breast cancer, 2 569 women, 2 588 controls	FFQ	6 major subclasses	Median intake of control subjects: Flavanones 33.7 Flavanols 36.4 Flavonols 18.6 Flavones 0.5 Anthocyanidins 10.4 Isoflavones 21.7	Flavanones: Oranges, other citrus fruits Flavanols: Red wine, grapes, other fruits Flavonols: Various common vegetables and fruits Flavones: Aromatic herbs Anthocyanidins: Onions and garlic Isoflavones: Pulses
	Case-control study of acute myocardial infarction (Milan), 760 patients, 682 controls	FFQ	6 major subclasses	Control subjects: 134.0	Flavanols 55.6 mg/day; Flavanones 38.2 mg/day; Flavonols 20.6 mg/day; Anthocyanidins 19.2 mg/day Flavones 0.44 mg/day; Isoflavones 25.5 ug/day

(Table 4.2 continued on next page)

COUNTRY	Population	Dietary intake assessment method[a]	Flavonoids determined[b]	Mean daily total flavonoid intake (mg/day)[b, c]	Prime sources of flavonoid intake (if noted)
UNITED STATES (US) (248,105, 342 respectively)	Iowa Women's Health Study, 34 849 post menopausal women, 55-69 years	FFQ	6 major subclasses	Median Q intakes: (lowest) Q1, 95.8 Q2, 167.5 Q3, 238.9 Q4, 336.5 Q5, 603.3 (highest)	Tea (26%), apples and pears (17%), bran added to food (9%), beans or lentils (9%), peaches, oranges, orange juice (all 5%), strawberries and grapefruit (both 4%), other fruit juices (3%), chocolate (2%), blueberries (1%)
	National Health and Nutrition Examination Survey (NHANES) 1999-2002, 8 809 adults, 19+ years	24-hour dietary recall	6 major subclasses	189.7±11.2	Tea 157 mg/day, citrus fruit juice 8 mg/day, wine 4 mg/day, citrus fruits 3 mg/day
	NHANES 1999-2002, 8 809 adults, 19+ years	24-hour dietary recall	6 major subclasses	189.7±11.2	Flavanols (83.5%) Tea consumers (21.3% drinking tea daily) 697.9 mg/day Tea non-consumers 32.6 mg/day

[a] The food frequency questionnaires used as dietary assessment method in these epidemiological studies were originally developed to assess the total diet and did not have a prior flavonoid-specific intake hypothesis in the compilation.

[b] The flavonoid intake estimates were primarily computed from the United States Department of Agriculture database integrated with published values for other foods

[c] Flavonols, flavones, flavanones, flavanols and anthocyanidins forming the five major flavonoid subclasses with isoflavones added to form the sixth major flavonoid subclass

[d] Mean±standard deviation

[e] CHD= Coronary heart disease

[f] FFQ= Food frequency questionnaire

[g] Q= Quintile

Table 4.3: **Dietary assessment methods, description, advantages and disadvantages of the dietary history, 24-hour recall and dietary record**

Dietary assessment method and description	Advantages	Disadvantages
Dietary history (Retrospective): (6,52,344,354) Extensive detailed interview (1-2 hours) designed to assess overall usual, long-term or customary diet covering a wide range of items and data on food preparation, most frequently from a 24-hour recall and cross-check with a frequency food list followed by a three-day dietary record (the latter often not used).	Used as gold standard against which other methods are validated; Reasonably valid against other dietary assessment methods and good reliability on different occasions.	Intensive interview by skilled interviewers (trained dietician/ nutritionist); Require much training on the part of the interviewer; Dependent on quality of the interview; Expensive and time consuming for wide application; Relies on subject memory; High subject burden.
24-hour recall (Retrospective): (6,52,344,354) Designed to quantitatively assess current nutrient intake; Subjects asked to recall and describe exact intake of all foods and beverages and the quantity the preceding day / previous 24-hours; Require trained interviewer (dietician/nutritionist) to make appropriate enquiries about type of items consumed and possible omissions (snacks), and assess portion amounts; Interviewer generally use food models and household measures to help subject quantify actual amount of foods and beverages consumed. Another technique to increase reproducibility of the 24-hour recall is referred to as the multiple pass 24-hour recall. The multiple pass technique increases retrieval of the requested information by allowing the subject to review the food and beverage intake of the previous day several times.(14,343)	Administered by persons with less training; Personal contact; Open-ended; Does not alter food intake pattern; Only requires short-term memory (memory of recent intake more precise); Quantities estimated rather accurately; Simple, quick (relatively brief requiring about 20 to 30 minutes or so); Relatively low interviewer and subject burden (compared to dietary records); Inexpensive/Cost-effective; No subject literacy and limited numeracy skills required – suitable for low-income and low-literacy populations; Used across broad populations of different ethnicities; Widely used assessment in clinical dietetic practice; If recall is unannounced, the diet is not changed (although behaviour change could not be excluded, subject memory might be improved by notification that details of yesterday's dietary intake would be asked); Valid for assessing average intake of groups or populations fairly well, but not for individuals.	Well trained interviewer (dietician / nutritionist) skilled and familiar in identification of available foods and in preparation practices generally used in specific region / ethnic group; Accurate quantitative information on recent intake, but do not represent usual intake as it provides no information on day-to-day variation in diet (single day's intake not representative of usual diet for individuals); Problems with subject memory and description of consumed amounts (food models, containers, photographs, etc. may assist) that may provide an under- or overestimate of intake; Conceptualization skills required; Multiple recalls required to achieve greater day-to-day representativeness.
Food/Dietary record/ Food inventory/diary (Prospective): (6,52,343, 344,354) Detailed subject recording of all dietary items actually consumed with measurement of the portion sizes by either weighing using scales (weighed method), measuring or estimation using household measures/utensils (cups, tablespoons) (estimated method) in conjunction with food models, collection of pictures, etc.;	Weighed method often used as gold standard for validating other dietary assessment methods (seven-day weighed records often considered most accurate and practical method of assessing intake under free-living conditions when neither a dietary history nor observation is possible); Reasonably valid (with the advent of biomarkers, the dietary record is known to have weaknesses for validation); Open-ended; Little reliance on memory and omission of consumed items might be minimal;	Subjects trained to record consumption (description of foods, cooking methods and amounts consumed); How to weigh or estimate portion sizes with reference to food photos, models, systems like the PETRA scales [which record the weight directly from the scales on to a magnetic tape contained within the machine], etc.); Requires subjects to be literate (and numerate); Expensive and time-consuming; High subject burden; High degree of subject co-operation;

(Table 4.3 continued on next page)

Dietary assessment method and description	Advantages	Disadvantages
Food/Dietary record/ Food inventory/diary (Prospective) *(cont.)* Recording of all foods and beverages done immediately before/at the time of consumption and estimating leftover amounts; Typically obtained for three to seven days (need not be consecutive days, but must include representation of the weekdays); Completed records checked by dietician/nutritionist for completeness, missing entries and legibility.	Accurate to reflect current intake, but may not reflect usual intake; For greater representativeness day-to-day variation considered by obtaining multiple records (average of more, seven days more representative of usual intake than a single day).	Compliance concern (writing down everything soon gets tiresome); Motivated subjects; Small and unrepresentative subject sample; Accuracy of recording deteriorates after first few days; Recording errors if not done as items are consumed; Likely to influence habitual diet and/or induce behaviour change due to participation (reduce intake or consume more monotonous diet to ease recording); Training effect of repetition; Skilled reviewer can make records more accurate, clarify items and amounts and add omitted items or amounts.

ADDENDUM B:

Ethical Approval

Cape Peninsula
University of Technology

13 May 2009
CPUT/HW-REC 2009/H006

**P.O. Box 1906 ● Bellville 7535 South Africa ●Tel: +27 21 442 6162 ● Fax +27 21 447 2963
Symphony Road Bellville 7535**

OFFICE OF THE CHAIRPERSON:
HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HW-REC)

At a meeting of the Health and Wellness Sciences-REC on 8 May 2009 the addendum to a previously approved study entitled: Modulation of Blood Oxidative Stress Markers and DNA Damage by Rooibos in Volunteers at risk for Coronary Heart Disease', was approved.

This certificate should be viewed with the certificate:
28 May 2007

TITLE:
Effects of rooibos consumption on plasma markers of oxidative stress and DNA strand breaks in peripheral leukocytes in volunteers at risk for coronary heart disease

PRINCIPAL INVESTIGATOR:
Dr J Marnewick

Comment:
The informed consent for the original study included the clause that blood samples could be stored for future analysis.

The ethical standard of anonymity will be upheld in this follow-up study.

Research activities are restricted to those detailed in the un-dated short addendum to the original proposal, submitted in April 2009.

Approval will not extend beyond 31 December 2009.

Prof PENELOPE ENGEL-HILLS
CHAIR: HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE

e-mail: *engelhillsp@cput.ac.za*

07 October 2009   **MAILED**

Ms I Venter
Department of Human Nutrition
3rd Floor, Clinical building
Stellenbosch University
Tygerberg campus
7505

Dear Ms Venter

"Development and evaluation of an abbreviated food frequency questionaire to assess daily total flavonoid intake using a rooibos intervention study model."

ETHICS REFERENCE NO: N09/08/223

RE : FINAL APPROVAL

At a meeting that was held on 16 September 2009 the Health Research Ethics Committee considered your application for the registration and approval of the abovementioned project. The Committee referred the project back to you awaiting further information that was required.

This information was supplied and the project was finally approved on 6 October 2009 for a period of one year from this date.

Notwithstanding this approval, the Committee can request that work on this project be halted temporarily in anticipation of more information that they might deem necessary to make their final decision.

Please quote the above-mentioned project number in ALL future correspondence.

Please note that a progress report obtainable on www.sun.ac.za/rds/ should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly and subjected to an external audit.
Translations of the consent document in the languages applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239
The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Yours faithfully

MRS MERTRUDE DAVIDS
RESEARCH DEVELOPMENT AND SUPPORT
Tel: 021 938 9207 / E-mail: mertrude@sun.ac.za
Fax: 021 931 3352

ADDENDUM C:

Abbreviated Food Frequency Questionnaire

ABBREVIATED FOOD FREQUENCY QUESTIONNAIRE:
AVERAGE DAILY TOTAL FLAVONOID INTAKE

Dear participant

Thank you for participating in this research project. The aim of this questionnaire is to determine your flavonoid intake. The consumption of flavonoids is important, as studies have indicated that the intake of flavonoid-containing foods and beverages is associated with a reduced risk for coronary heart and other chronic diseases. Consuming flavonoid-containing foods and beverages regularly could therefore be beneficial to our health. Foods, such as fruit, vegetables, legumes, nuts and chocolate, and beverages, such as fruit juices, cocoa drinks, tea and wine, as well as herbs and spices contain flavonoids. The intakes of only some flavonoid-containing items of those foods and beverages mentioned above are asked about in this questionnaire.

Please check your allocated participant code in the blocks above.
(Please do not write your name on the questionnaire)

The questionnaire should take about 10 minutes of your time to complete.

Cape Peninsula
University of Technology

FACULTY OF APPLIED SCIENCES
DEPARTMENT OF AGRICULTURAL AND FOOD SCIENCES
PROGRAMME: CONSUMER SCIENCE: FOOD AND NUTRITION

IN COLLABORATION WITH THE SOUTH AFRICAN HERBAL RESEARCH GROUP, CAPE PENINSULA UNIVERSITY OF TECHNOLOGY
AND THE DIVISION OF HUMAN NUTRITION, STELLENBOSCH UNIVERSITY

Date: July 2009
Final

INSTRUCTIONS FOR COMPLETION

This questionnaire is about your consumption of flavonoid-containing foods and beverages during the **past 2 weeks**. The flavonoid-containing foods and beverages are grouped in sections in the questionnaire as fruit juices (A), fresh fruit (B), dried fruit (C), vegetables (D), alcoholic beverages (E), other (non-alcoholic) beverages (F), chocolate (G) and lastly herbs added to food (H). These section headings are indicated as highlighted areas that include guidelines on which foods/beverages, and their additions, you need to consider when completing the section and which to ignore.

Please follow the instructions below to complete the questionnaire as accurately as possible as flavonoids typically occur in small amounts in foods/beverages. For the flavonoid-containing foods/beverages you consumed over the **past 2 weeks**:

1. If the **food/beverage** (indicated in the first column) was **consumed,** tick it in the corresponding block in the second column.

Example: If in Section F (Other beverages, non-alcoholic - tea) you consumed black tea and rooibos in the past 2 weeks you should tick black tea and rooibos in the second column as indicated below:

Food/Beverage and description	Tick if consumed	Reference amount (medium serving)	Your amount consumed?			How often did you consume it?			Office use		
			Small	Medium	Large	Times per day	Days per week OR	Days per 2 weeks	Food code	Reviewed	Captured
F: OTHER BEVERAGES, NON-ALCOHOLIC - TEA											
*** Ignore additions such as milk, sugar, sweeteners honey and/or lemon juice added to tea.**											
Black/English tea, plain or flavoured	√	1c = 1 small mug	½c	1c	Large mug				14365		
Black/English ice tea, plain or flavoured		1c = 1 small mug	½c	1c	Large mug				99341		
Green tea, plain or flavoured		1c = 1 small mug	½c	1c	Large mug				99070		
Green ice tea, plain or flavoured		1c = 1 small mug	½c	1c	Large mug				99343		
Rooibos, plain or flavoured	√	1c = 1 small mug	½c	1c	Large mug						
Rooibos ice tea, plain or flavoured		1c = 1 small mug	½c	1c	Large mug						

2. Indicate the **amount of the foods/beverages** you consumed as a small, medium or large amount by clearly circling the appropriate amount consumed column. A small amount is about one-half (½) the medium amount and a large amount about one-and-a-half (1½) times the medium amount or more. The "c" in the amounts consumed columns indicates cup, with a half (½) cup as 125ml.

NOTE: Additional amount descriptions are provided in the reference amount column (third column) to guide the medium amount estimation so that the amounts consumed can be easier estimated and more accurately. Use the sketches provided to guide the portion sizes of some of the fruits and vegetables you may have consumed.

Example: If you consume a cup of black tea at a time (every day and three times during the day) and a cup of rooibos at a time (every second Sunday, twice during the day) you should circle these beverage amounts consumed in the appropriate amount consumed columns as indicated below:

Food/Beverage and description	Tick if consumed	Reference amount (medium serving)	Your amount consumed?			How often did you consume it?			Office use		
			Small	Medium	Large	Times per day	Days per week OR	Days per 2 weeks	Food code	Reviewed	Captured
F: OTHER BEVERAGES, NON-ALCOHOLIC - TEA											
*** Ignore additions such as milk, sugar, sweeteners, honey and/or lemon juice added to tea.**											
Black/English tea, plain or flavoured	√	1c = 1 small mug	½c	(1c)	Large mug				14365		
Black/English ice tea, plain or flavoured		1c = 1 small mug	½c	1c	Large mug				99341		
Green tea, plain or flavoured		1c = 1 small mug	½c	1c	Large mug				99070		
Green ice tea, plain or flavoured		1c = 1 small mug	½c	1c	Large mug				99343		
Rooibos, plain or flavoured	√	1c = 1 small mug	½c	(1c)	Large mug						
Rooibos ice tea, plain or flavoured		1c = 1 small mug	½c	1c	Large mug						

3. Also indicate **how often in the past 2 weeks** you consumed these foods/beverages. First indicate how many times per day you consumed the food/beverage. Then indicate how many days you consumed the food/beverage as the number of days per week OR per 2 weeks. *PLEASE NOTE*: For all the items consumed you should complete the per day column AND the per week column OR the per 2 weeks column.

Example: As you consume black tea three times daily you should indicate the number 3 in the per day column and the number 7 in the per week column (see indication below). For the rooibos consumed you should indicate the number 2 in the per day column as you drink it twice per day and the number 1 in the per 2 weeks column as you drink rooibos every second Sunday (see indication below).

Food/Beverage and description	Tick if consumed	Reference amount (medium serving)	Your amount consumed?			How often did you consume it?			Office use		
			Small	Medium	Large	Times per day	Days per week OR	Days per 2 weeks	Food code	Reviewed	Captured
F: OTHER BEVERAGES, NON-ALCOHOLIC - TEA											
*** Ignore additions such as milk, sugar, sweeteners, honey and/or lemon juice added to tea.**											
Black/English tea, plain or flavoured	√	1c = 1 small mug	½c	(1c)	Large mug	3	7		14365		
Black/English ice tea, plain or flavoured		1c = 1 small mug	½c	1c	Large mug				99341		
Green tea, plain or flavoured		1c = 1 small mug	½c	1c	Large mug				99070		
Green ice tea, plain or flavoured		1c = 1 small mug	½c	1c	Large mug				99343		
Rooibos, plain or flavoured	√	1c = 1 small mug	½c	(1c)	Large mug	2		1			
Rooibos ice tea, plain or flavoured		1c = 1 small mug	½c	1c	Large mug						

4. *NOTE:* All listed food and beverage **items not consumed** are left blank.

Food/Beverage and description	Tick if consumed	Reference amount (medium serving)	Your amount consumed?			How often did you consume it?			Office use		
			Small	Medium	Large	Times per day	Days per week OR	Days per 2 weeks	Food code	Reviewed	Captured
A: FRUIT JUICES * Only indicate 100% pure juices and blends consumed. * Do not indicate fruit juice and yoghurt blends, dairy fruit blends and nectars containing only a percentage fruit juice (mostly 40% to 50%). * Ignore whether the beverages consumed were sweetened or unsweetened.											
Apple, including apple and pear, mango and apple			¼c	½c	¾c				9D16CB		
Berry and mixed berry			¼c	½c	¾c				9D16CB/89D07/9911Q		
Orange and orange juice blends, including mango and orange, peach and orange and breakfast punch			¼c	½c	¾c				9D16CB/92D6R		
Tropical and tropical fruit blends, including granadilla, kiwi, mango, paw paw, pineapple			¼c	½c	¾c				9D16CB/92D6R		
B: FRESH FRUIT * Use the sketches as guide to indicate the fruit size consumed, e.g. look at the sketches and if you consumed a small apple circle F1.											
Apple, fresh with peel		See sketches, p.1	F1	F2	F3				9060		
Apple, fresh without peel		See sketches, p.1	F1	F2	F3				9064		
Banana		See sketches, p.2	F6	F7	F8				9040		
Pear, fresh with skin		See sketches, p.1	F28	F29	F30				9252		
Pear, fresh without skin		See sketches, p.1	F28	F29	F30				9902S		
C: DRIED FRUIT * Ignore very small amounts of dried fruit included in products such as breakfast cereals and bars.											
Pear, halves		2 halves = ¼c	1	2	3						
D: VEGETABLES * The vegetables are indicated as either raw or cooked that includes all cooking methods (i.e. boiling, steaming, frying, baking and roasting). * Ignore whether the vegetables consumed were prepared with or without additions such as margarine, butter, oil, sugar and mayonnaise or salad dressing. * The reference amount (medium serving) of vegetables consumed as a mixed dish ingredient is indicated as the household measure 1 H DSP that indicates a heaped (H) dessertspoon (DSP). In this case a small amount is about 1 heaped (H) teaspoon (TSP) and a large amount about 1 heaped (H) tablespoon (TBS) or more.											
Onions, cooked as side dish or main soup/salad/dish ingredient			¼c	½c	¾c				11283		
Onions, cooked as ingredient in mixed dishes (stews, bredies, quiches, pastas, etc.), pizzas, soups, burgers, sandwiches, salads			1 H TSP	1 H DSP	1 H TBS				11283		
E: ALCOHOLIC BEVERAGES											
Apple cider		Can = 340ml / Long Tom = 500ml	½ can	1 can	Long Tom				99083		
Wine, red		1 red wine glass = 175ml	½ x 175ml	175ml	2 x 175ml				14090		
F: OTHER BEVERAGES * Ignore additions such as milk, sugar, sweeteners, honey and/or lemon juice added to tea.											
Black/English tea, plain or flavoured		1c = 1 small mug	½c	1c	Large mug				14355		
Black/English ice tea, plain or flavoured		1c = 1 small mug	½c	1c	Large mug				9934		
Green tea, plain or flavoured		1c = 1 small mug	½c	1c	Large mug				99070		
Green ice tea, plain or flavoured		1c = 1 small mug	½c	1c	Large mug				9934S		
Rooibos, plain or flavoured		1c = 1 small mug	½c	1c	Large mug						
Rooibos ice tea, plain or flavoured		1c = 1 small mug	½c	1c	Large mug						
G: CHOCOLATE * Ignore chocolate coated bars, sweets and confectionary, other chocolate coatings, assorted centres and white chocolate.											
Milk chocolate, with/without additions such as nuts, fruit flavours, etc.		40 g = 6 blocks	3 blocks	6 blocks	9 blocks				19120		
Dark chocolate with/without additions such as nuts, fruit flavours, etc.		40 g = 6 blocks	3 blocks	6 blocks	9 blocks				9982		
H: HERBS ADDED TO FOOD * The medium amount indicated as reference ("mildly flavoured") equals the amount that is usually included in a recipe or as garnish. * A small amount indicates that a smaller amount than the usual recipe amount was used ("lightly flavoured"). * A large amount indicates that more than the usual recipe amount was used ("strongly flavoured").				Added flavour							
Parsley - popular as garnish and used in cooking with meat, fish, chicken, eggs and vegetables		Dried	Light	Mild	Strong				2D29EI		

Thank you for your time.
Please take a moment to check whether you have ticked all the foods/beverages you consumed in the past 2 weeks. Please also ensure that you have circled the amounts of these foods/beverages consumed and indicated how often you consumed these foods/beverages per day as well as either per week OR per 2 weeks.