

**USE OF NEAR INFRARED SPECTROSCOPY (NIRS) AND  
SPECTROPHOTOMETRIC METHODS IN QUALITY CONTROL OF GREEN  
ROOIBOS (*ASPALATHUS LINEARIS*) AND HONEYBUSH (*CYCLOPIA  
GENISTOIDES*)**

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Thesis presented in partial fulfilment for the degree of

**MASTER OF SCIENCE IN FOOD SCIENCE**



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## DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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## ABSTRACT

Near infrared spectroscopy (NIRS) and UV/Vis spectrophotometric methods were investigated as rapid and/or more economical methods to quantify the major monomeric phenolic compounds, soluble solid content (SSC), total polyphenol content (TPC) and total antioxidant activity (TAA) of green rooibos and *Cyclopia genistoides*. Principal component analysis (PCA) and linear discriminant analysis (LDA) of near infrared spectral data were applied to distinguish between green rooibos and *C. genistoides* and to classify green rooibos according to cultivation area and *C. genistoides* according to type and harvest date.

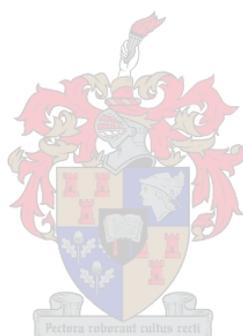
NIRS was able to predict the aspalathin (SEP = 0.45 g.100 g<sup>-1</sup>; r = 0.92) and the dihydrochalcone contents (SEP = 0.49 g.100 g<sup>-1</sup>; r = 0.93) of green rooibos plant material. NIRS can be used for screening of the nothofagin content (SEP = 0.10 g.100 g<sup>-1</sup>; r = 0.84) of green rooibos and the mangiferin (SEP = 0.46 g.100 g<sup>-1</sup>; r = 0.86) and hesperidin (SEP = 0.38 g.100 g<sup>-1</sup>; r = 0.85) contents of green *C. genistoides* plant material. Poor correlations were found between the predicted and the measured values for the aspalathin content of green rooibos water extracts, mangiferin and hesperidin contents of green *C. genistoides* water extracts and TPC, SSC and TAA of green rooibos and *C. genistoides* water extracts.

The aspalathin content of green rooibos water extracts determined by UV spectroscopy correlated with the aspalathin content quantified using HPLC (r = 0.97), but a slope of 1.90 was obtained ( $y = 1.9037x + 14.2828$ ), indicating that UV spectroscopy overestimated the aspalathin content. A similar correlation was found between the aspalathin content determined by UV spectroscopy and the dihydrochalcone content quantified using HPLC (r = 0.97;  $y = 1.7084x + 14.6903$ ). The TPC of water extracts of green rooibos can be determined using UV spectroscopy (r = 0.99;  $y = 1.0829x - 0.6498$ ) and aspalathin as standard. The mangiferin content of green *C. genistoides* methanol extracts can be determined using the aluminium chloride colorimetric method (r = 0.90;  $y = 1.2304x - 5.949$ ).

A good correlation (r = 0.97) existed between the TPC and the aspalathin content of green rooibos water extracts, but not between the TPC and the mangiferin content (r = 0.22) of green *C. genistoides* water extracts. TAA of green rooibos water extracts correlated with its aspalathin content (r = 0.96), but a moderate correlation was found between the TAA of green *C. genistoides* water extracts and its mangiferin content (r = 0.75). The TAA correlated with the TPC (r = 0.99) in the case of green rooibos water extracts, but not in the case of green *C. genistoides* (r = 0.27).

NIRS together with PCA or LDA was able to distinguish between green rooibos and *C. genistoides* plant material and to classify green *C. genistoides* according to type. Although PCA could not classify green rooibos plant material according to cultivation area and *C. genistoides* plant

material according to harvesting date, LDA was able to classify between 67 and 100% of the green rooibos samples and between 55 and 100% of the *C. genistoides* samples correctly.



## UITTREKSEL

Naby infrarooi spektroskopie (NIRS) en UV/Vis spektrofotometriese metodes is ondersoek as vinnige en/of meer ekonomiese metodes om die belangrikste monomeriese fenoliese verbindings, oplosbare vastestofinhoud (OVI), totale polifenolinhoud (TPI) en totale antioksidant aktiwiteit (TAA) van groen rooibos en *Cyclopia genistoides* te kwantifiseer. Hoofkomponent analise (HA) en lineêre diskriminant analise (LDA) van naby infrarooi spektroskopiese data is toegepas om tussen groen rooibos en *C. genistoides* te onderskei en om groen rooibos te klassifiseer op grond van verbouingsarea en *C. genistoides* op grond van tipe en oesdatum.

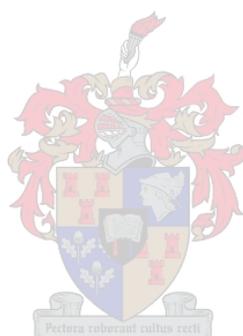
Dit was moontlik om die aspalatien- (SEP = 0.45 g.100 g<sup>-1</sup>; r = 0.92) en dihidrogalkooninhoud (SEP = 0.49 g.100 g<sup>-1</sup>; r = 0.93) van groen rooibos plantmateriaal met behulp van NIRS te voorspel. NIRS kan gebruik word om die notofagieninhoud (SEP = 0.10 g.100 g<sup>-1</sup>; r = 0.84) van groen rooibos, en die mangiferien- (SEP = 0.46 g.100 g<sup>-1</sup>; r = 0.86) en hesperidieninhoud (SEP = 0.38 g.100 g<sup>-1</sup>; r = 0.85) van groen *C. genistoides* plantmateriaal rofweg te bepaal. Swak korrelasies is gevind tussen die NIRS voorspelde en die bepaalde waardes vir die aspalatieninhoud van groen rooibos waterekstrakte, mangiferien- en hesperidieninhoud van *C. genistoides* waterekstrakte en die TPI, OVI en TAA van groen rooibos and *C. genistoides* waterekstrakte.

Die aspalatieninhoud van groen rooibos waterekstrakte, soos bepaal met behulp van UV spektroskopie, het met die aspalatieninhoud wat gekwantifiseer is deur HPLC (r = 0.97) gekorreleer, maar 'n helling van 1.90 ( $y = 1.9037x + 14.2828$ ) is verkry wat toon dat UV spektroskopie die aspalatieninhoud oorskat het. 'n Soortgelyke korrelasie is gevind tussen die aspalatieninhoud wat bepaal is met behulp van UV spektroskopie en die dihidrogalkooninhoud soos gekwantifiseer met HPLC (r = 0.97;  $y = 1.7084x + 14.6903$ ). The TPI van rooibos waterekstrakte kan bepaal word met behulp van UV spektroskopie (r = 0.99;  $y = 1.0829x - 0.6498$ ) en aspalatien as standaard. Die mangiferieninhoud van groen *C. genistoides* metanolekstrakte kan bepaal word deur die aluminiumchloried kolorimetriese metode (r = 0.90;  $y = 1.2304x - 5.949$ ) te gebruik.

'n Goeie korrelasie (r = 0.97) is tussen die TPI en die aspalatieninhoud van groen rooibos waterekstrakte verkry, maar nie tussen die TPI en die mangiferieninhoud van groen *C. genistoides* waterekstrakte nie (r = 0.22). TAA van groen rooibos waterekstrakte het met die aspalatieninhoud (r = 0.96) gekorreleer, maar 'n effens swakker korrelasie is gevind tussen die TAA van groen *C. genistoides* waterekstrakte en die mangiferieninhoud (r = 0.75). Die TAA het met die TPI (r = 0.99) in die geval van groen rooibos waterekstrakte gekorreleer, maar nie in die geval van groen *C. genistoides* nie (r = 0.27).

Dit was moontlik om te onderskei tussen groen rooibos en *C. genistoides* plantmateriaal en om *C. genistoides* plantmateriaal te klassifiseer op grond van tipe deur gebruik te maak van HA en

LDA. Alhoewel dit nie moontlik was om rooibos en *C. genistoides* te klassifiseer op grond van verbouingsarea en oesdatum met HA nie, was dit moontlik om tussen 67 en 100% van die groen rooibos monsters van die verskillende verbouingsareas en tussen 55 en 100% van die *C. genistoides* monsters wat op die verskillende datums geoes is, korrek te klassifiseer met behulp van LDA.



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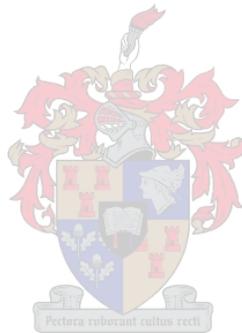
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**ABBREVIATIONS**

ABTS	2,2'-azinobis(3-ethyl-benzothiazoline-6-sulphonate)
AlCl <sub>3</sub>	Aluminium chloride
AOTF	Acousto-optical tunable filter
ANN	Artificial neural network
CCD	Charge coupled device
DAC	4-dimethylaminocinnamaldehyde
DAD	Diode array detector
DMSO	Dimethyl sulfoxide
DPPH	$\alpha,\alpha$ -diphenyl- $\beta$ -picrylhydrazyl
DT	De-trending
EDTA	Ethylenediaminetetra-acetic acid di-sodium salt dehydrate
EtOH	Ethanol
FC	Folin-Ciocalteu
FRAP	Ferric reducing antioxidant power
FT	Fourier transform
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
InGaAs	Indium gallium arsenide
IR	Infrared
LDA	Linear discriminant analysis
LED	Light-emitting diodes
M	Molar
MeOH	Methanol
MLR	Multiple linear regression
MSC	Multiplicative scatter correction
NIRS	Near infrared spectroscopy
nm	Nanometer
OSC	Orthogonal signal correction
PC	Principal component
PCA	Principal component analysis
PbS	Lead sulphide
PbSe	Lead selenide
PLS	Partial least square
r	Coefficient of correlation

RP	Reversed phase
RPD	Ratio of standard error of performance to standard deviation
SD	Standard deviation
SECV	Standard error of cross validation
SEL	Standard error of laboratory
SEP	Standard error of prediction
SIMCA	Soft independent modeling of class analogy
SNV	Standard normal variate
SSC	Soluble solid content
TAA	Total antioxidant activity
TEAC	Trolox equivalent antioxidant concentration
TeO <sub>2</sub>	Tellurium dioxide
TLC	Thin-layer chromatography
TPC	Total polyphenol content
UV	Ultraviolet
UV/Vis	Ultraviolet-visible
v/v	Volume per volume
m/v	Mass per volume



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Language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

# CHAPTER 1



Photograph supplied by Rooibos Ltd,

## Introduction

## CHAPTER 1

### INTRODUCTION

The growing interest in natural plant products by the nutraceutical and cosmetic industry resulted in a large number of polyphenol-enriched extracts on the market (Andersen *et al.*, 2005; Wright, 2005). Due to their high antioxidant activity (Von Gadow *et al.*, 1997; Richards, 2002; Schulz *et al.*, 2003; Joubert *et al.*, 2004), two indigenous plants, rooibos (*Aspalathus linearis*) (Coetzee *et al.*, 1953) and honeybush (*Cyclopia spp.*) (Kies, 1951) are increasingly being used by the South African industry for the development of new products.

The major monomeric polyphenols in green (unoxidised) rooibos are the dihydrochalcones, aspalathin and nothofagin (Joubert, 1996). In the plant material, the content of these compounds vary from 4.45-11.9 and 0.34-1.33 g.100 g<sup>-1</sup> for aspalathin and nothofagin, respectively (Schulz *et al.*, 2003). Aspalathin contributes 22-57% of the total antioxidant activity (TAA) of green rooibos extracts (Schulz *et al.*, 2003).

Mangiferin and hesperidin are the major monomeric polyphenols of honeybush (Joubert *et al.*, 2003). Of several species investigated, *C. genistoides* had the highest mangiferin content (Joubert *et al.*, 2003), making it an obvious choice for preparation of mangiferin-enriched extracts. Values for mangiferin content in plant material ranged between 4.93 and 6.37 g.100 g<sup>-1</sup>, while the hesperidin content varied between 2.23 and 5.21 g.100 g<sup>-1</sup> for the *C. genistoides* plant material. The Overberg type had a higher mangiferin, whereas the West Coast type had a higher hesperidin content (Joubert *et al.*, 2003). The contribution of mangiferin to the TAA of aqueous extracts prepared from *C. genistoides* was found to be ca. 22% (Richards, 2002). The contribution of hesperidin was substantially lower (>1%), not only due to the lower concentration, but also because it is less potent than mangiferin in the 2,2'-azinobis(3-ethyl-benzothiazoline-6-sulphonate) (ABTS) assay (Richards, 2002). Its anti-inflammatory properties (Guardia *et al.*, 2001; Rotelli *et al.*, 2003) add to its value in nutraceutical products.

For production of extracts high in aspalathin and mangiferin content, their concentration in the plant material is a deciding factor. Minimum levels, i.e., 4% aspalathin and mangiferin in rooibos and honeybush, respectively, are required by one European company in order to obtain extracts with the required levels of these compounds in the final dried extract (B. Weinreich, Raps and Co., Germany, personal communication, 2005). The higher the aspalathin or mangiferin content the more valuable the plant material. Rapid analysis or screening of the raw plant material at an early stage of processing is a prerequisite to identify plant material with sufficient quantities of these phenolic compounds to ensure extracts with the desired phenolic content.

Marker compounds or pharmacologically active components are being used for evaluation of the quality and authenticity of herbal medicines, for the identification/verification of the herb or herbal medicines and for evaluating the quantitative herbal composition of a herbal product (Liang *et al.*, 2004). The phenolic composition of herbal plant material differs considerably among the plants, species and even the same plant extracts (He, 2000). The age of the plant, harvest season, locality, climate, drying process and storage conditions are factors contributing to this variation and these factors have made quality control of herbal material a challenge for the food and phytopharmaceutical industries (He, 2000; Laasonen *et al.*, 2002). Currently, no regulatory quality standards or requirements pertaining to composition or compound exist for rooibos or honeybush tea (Anon., 2000).

Product specifications for rooibos and honeybush extracts produced by local extract manufacturers comprise total polyphenol content (TPC) and TAA, as determined with the Folin-Ciocalteu (Singleton & Rossi, 1965) and ABTS assays (Re *et al.*, 1999), respectively (E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, personal communication, 2004). HPLC is used by a German manufacturer for the analysis of aspalathin- and mangiferin-enriched extracts from green rooibos and honeybush, respectively, to ensure that the extracts contain the specified quantity of these compounds. No such quality specifications exist for rooibos and honeybush extracts.

Although high performance liquid chromatography (HPLC) methods have high selectivity and are accurate, it is time-consuming, requiring the use of environmentally unfriendly chemicals and trained laboratory personnel (Huie, 2002; Laasonen, 2003). Another major disadvantage of HPLC is its high cost due to expensive equipment and solvents (Harborne, 1998). Rapid and/or more economical methods are required by industry for quality control purposes. Colorimetric methods with higher selectivity than the Folin-Ciocalteu method can be used for the determination of specific classes of phenolic compounds. Ultraviolet (UV) spectra of xanthenes undergo characteristic bathochromic shifts with aluminium chloride ( $\text{AlCl}_3$ ) depending on the number and position of the hydroxyl substituents (Harborne, 1998). Mangiferin contains a keto group, as well as a dihydroxyl group on the benzene ring, which could form aluminum chloride complexes. The high mangiferin content, compared to the other phenolic compounds in *C. genistoides* (Joubert *et al.*, 2003; Van der Merwe, 2004) suggests the possible use of this method for determination of mangiferin with minor interference of other phenolic compounds, e.g. hesperidin. Chang *et al.* (2002) showed that hesperetin, the aglycone of hesperidin and a flavanone, lacking the orthodihydroxyl groups on the A- or B-ring, has very low reactivity towards  $\text{AlCl}_3$ .

Near infrared spectroscopy (NIRS) is a rapid, easy-to-use and non-destructive analytical technique (Day & Fearn, 1982). It requires minimum or no sample preparation (Stark *et al.*, 1986) and can be carried out in-line (Singh Sahni *et al.*, 2004) or on-line (Osborne *et al.*, 1993; Hoyer, 1997). Quantitative and qualitative NIRS analyses have been used successfully in many

applications in the food (Osborne, 1991; Kawano *et al.*, 1992; Rannou & Downey, 1997; Blanco *et al.*, 1998; Büning-Pfaue *et al.*, 1998; Schulz *et al.*, 1998; Lister *et al.*, 2000; Cozzolino & Murray, 2004; Cozzolino *et al.*, 2004) and non-food (Grunewald *et al.*, 1998; Cleve *et al.*, 2000; Demattê *et al.*, 2004; Lima *et al.*, 2004) industries. NIRS has become a useful tool for analysis of herbal plants e.g. to determine the echinacosides in *Enchinacea* roots (Schulz *et al.*, 2002), harpagoside in devil's claw (*Harpagophytum procumbens*) (Joubert *et al.*, 2005) and sennosides A and B in *Senna* (*Cassia senna* L.) leaves (Pudelko-Körner *et al.*, 1996). Furthermore, NIRS has been used for quantification of quality parameters of green (Luypaert *et al.*, 2003; Schulz, 2004; Zhang *et al.*, 2004) and black teas (Hall *et al.*, 1988). It has also been used to predict the aspalathin content in rooibos tea, but on a sample set with limited seasonal variation (Schulz *et al.*, 2003). Other applications of NIRS include identification (Laasonen *et al.*, 2002), as well as classification of herbal or medicinal plant species (Lister *et al.*, 2000; Laasonen *et al.*, 2002), cultivation area (Woo *et al.*, 2002), geographical origin (Woo *et al.*, 1999), and processing methods, e.g. the discrimination between unfermented and fermented rooibos (Schulz *et al.*, 2003). It has also been used for discrimination of black teas of different quality (Osborne & Fearn, 1988) and for authentication of different foods and food ingredients (Downey, 1996; Downey, 1998; Laasonen *et al.*, 2002).

The analytical technique used to ensure quality of herbal medicinal products should also be able to discriminate between related species and/or potential adulterants (Anon., 2001). The ability of NIRS to discriminate between plant species is of interest, not only from a research point of view, but also to the industry for authentication purposes, especially when it is difficult to identify plant material visually when it is e.g. pulverised. A light, green colour is normally associated with green rooibos and indicative of good retention of aspalathin and thus a product of higher value (E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, personal communication, 2004). Green *C. genistoides* has a bright, green colour and is less susceptible to discolouration. Adulteration with green *C. genistoides* is therefore likely to enhance the colour of green rooibos. If HPLC is not employed, adulteration of green rooibos with green *C. genistoides* may go undetected. NIRS offers the potential to replace HPLC, once calibrated, to differentiate between the two teas and detect adulteration in a more rapid and economical manner.

The objectives of this study were to:

- develop NIRS calibration models for the prediction of aspalathin, nothofagin and dihydrochalcone contents of dried, green rooibos plant material and the mangiferin and hesperidin contents of dried, green *C. genistoides* plant material;
- develop NIRS calibration models for the prediction of aspalathin content of green rooibos water extracts, mangiferin and hesperidin contents of green *C. genistoides* water extracts and TPC and soluble solid content and TAA of both green rooibos and *C. genistoides* water extracts;

- investigate the potential of UV spectroscopy and an aluminium chloride colorimetric method to determine the aspalathin content of green rooibos water extracts and mangiferin content of green *C. genistoides* water and methanol extracts, respectively; and
- differentiate between dried, green rooibos and *C. genistoides* and to classify dried, green rooibos according to cultivation area and dried, green *C. genistoides* according to type and harvest date.

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## CHAPTER 2



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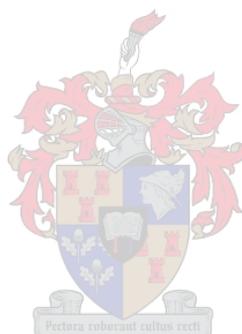
## Literature review

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## CHAPTER 2

### LITERATURE REVIEW

#### 1. INTRODUCTION

The growing interest in natural plant products by the nutraceutical and cosmetic industries during the past number of years resulted in a large number of polyphenol-enriched extracts on the market (Andersen *et al.*, 2005; Wright, 2005). Rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia spp.*) are two indigenous South Africa herbal plants containing high quantities of the polyphenolic compounds, aspalathin and mangiferin, respectively, contributing to their antioxidant activity (Von Gadow *et al.*, 1997a; Richards, 2002; Schulz *et al.*, 2003a; Joubert *et al.*, 2004).

This chapter gives an overview of rooibos and honeybush and their respective polyphenolic compositions. Procedures that can be used in quality control and standardisation of these herbal plants, i.e. near infrared (NIR) and UV/Vis spectrophotometric methods as opposed to high performance liquid chromatography (HPLC) analysis are discussed. Since near infrared spectroscopy (NIRS) is not as yet applied extensively by the herbal industry in South Africa, special attention was given to NIRS in this review. The principle of NIRS, development of calibration methods (sample set, reference method, chemometrics) and the required statistics for validation of the prediction models are therefore discussed.



#### 2. ROOIBOS

##### 2.1. Background

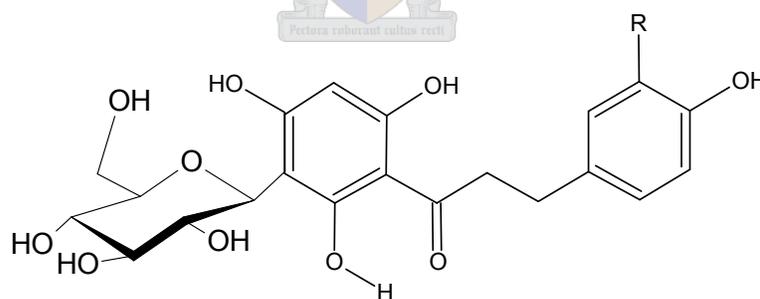
Rooibos tea is the common name for the plant, *Aspalathus linearis* spp. *linearis*, and its infusion. *Aspalathus linearis* is one of 278 species of the genus *Aspalathus* (Dahlgren, 1968), an indigenous leguminous scrub endemic to the North Western Cape fynbos area of South Africa (Coetzee *et al.*, 1953; Cheney & Scholtz, 1963). The consumption of rooibos tea by the Hottentots was first reported in 1772 by Carl Thunberg, marketing began in 1902 (Morton, 1983) and commercial cultivation commenced in 1930 (Anon., 1967). After being mostly consumed locally, exports increased significantly since 1993, with more than 60% of total sales exported by 2003. Local sales for 2003 were approximately 4200 tons and export sales about 6400 tons. The total sales increased with 150% from 1993 and the exports with 742% (Genis, 2005). The absence of caffeine and its low tannin content (Blommaert & Steenkamp, 1978) compared to black tea, make it a popular herbal tea, both locally and internationally. Rooibos tea is mainly exported to Germany, but other markets include Japan, the Netherlands, Poland, the United

Kingdom, the United States of America, Malaysia, Australia, Zimbabwe, Belgium, China, Korea, Bulgaria, Singapore, Finland, Austria, Canada, France, Italy, New Zealand, Switzerland, Taiwan, UAE, Sweden and Sri Lanka (D. de Villiers, Cape Natural Tea Products, South Africa, personal communication, 2004).

The leaves and stems are harvested and processed during the dry summer months, from January to March. Processing entails shredding, fermentation (oxidation) for the development of the characteristic red-brown colour and sweet flavour, followed by drying (Cheney & Scholtz, 1963), sieving and steam pasteurisation before bulk packaging (Joubert, 1994). As oxidation results in the degradation of the dihydrochalcones, aspalathin and nothofagin (Joubert, 1996), as well as a decrease in antioxidant activity (Von Gadow *et al.*, 1997b; Standley *et al.*, 2001), unfermented (unoxidised or green) rooibos is preferred for the preparation of aspalathin-enriched extracts for the nutraceutical, cosmetic and functional food industries (Schulz *et al.*, 2003a; Joubert *et al.*, 2004). Bramati *et al.* (2003) reported a 2-fold higher total antioxidant activity (TAA) value for green rooibos.

## 2.2. Phenolic composition

The phenolic composition of rooibos is unique in that it contains aspalathin (Figure 1), a rare C-C dihydrochalcone glycoside (Koeppen, 1970). It is the primary monomeric flavonoid in green rooibos (Koeppen & Roux, 1966) comprising between 4.45 and 11.9 g.100 g<sup>-1</sup> of the plant material (Schulz *et al.*, 2003a). Approximately 35 to 68% of the total polyphenol content (TPC) of water extracts of green rooibos is explained by its aspalathin content (Schulz *et al.*, 2003a).



**Figure 1** Molecular structure of aspalathin (R=OH) and nothofagin (R=H) (Joubert, 1996).

Schulz *et al.* (2003a) found a good linear relationship between the TAA and the aspalathin content of the water extracts, with aspalathin contributing 22-57% of the TAA. Nothofagin (Figure 1), similar in structure to aspalathin except for the hydroxylation pattern of the B-ring (Joubert, 1996), is present in substantially lower quantities, but still in higher quantities than the other flavonoids (Joubert, 1996). They comprise the flavonol aglycone quercetin (Snyckers & Salemi, 1974) and its glycosides, isoquercitrin and rutin (Koeppen *et al.*, 1962), the flavone

aglycones, luteolin (Snyckers & Salemi, 1974) and chrysoeriol (Rabe *et al.*, 1994), and the flavone glycosides, orientin and iso-orientin (Koeppen *et al.*, 1962; Koeppen & Roux, 1965) and their 4'-deoxy analogues, isovitexin and vitexin (Rabe *et al.*, 1994). Low levels of three condensed tannins have been identified, i.e. (+)-catechin, procyanidin B3 and a profistininidin triflavonoid (Ferreira *et al.*, 1995). The phenolic acids present in fermented rooibos are the phenolic carboxylic acids, *p*-hydroxybenzoic acid, protocatechuic acid and vanillic acid, and the hydroxycinnamic acids, *o*-coumaric acid, caffeic acid and ferulic acid (Rabe *et al.*, 1994). Rooibos tannin, isolated from green rooibos, is a heterogenous proanthocyanidin type polymer, with (+)-catechin and (-)-epicatechin as chain-extending units (Ferreira *et al.*, 1995; Marais *et al.*, 1998). Antioxidant activity of varying potency has been demonstrated for these phenolic compounds, with aspalathin one of the most potent (Von Gadow *et al.*, 1997a; Joubert *et al.*, 2004; Snijman *et al.*, 2004).

### 3. HONEYBUSH

#### 3.1. Background

*Cyclopia*, an indigenous leguminous scrub endemic to the Cape fynbos region of South Africa (Kies, 1951; Schutte, 1995), was traditionally used for its medicinal properties (Watt & Breyer-Brandwijk, 1932), but later also as a herbal infusion called honeybush tea, known for its characteristic pleasant sweet honey-like flavour (Du Toit *et al.*, 1998). Honeybush is low in tannins (Terblanche, 1982) and contains no or traces of caffeine (Greenish, 1881). Until the 1990's, honeybush tea was only consumed locally to a limited extent. Research on antioxidants, cultivation and health properties catalysed growth of the industry and market development. Sales have shown a significant increase from 50 tons sold in 1999 to 250 tons sold in 2005. Between 80-90% of honeybush tea produced is exported to Europe, United Kingdom, United States of America and Japan (E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, personal communication, 2005).

There are approximately 24 *Cyclopia* species (Van Wyk, 1997) which grow in different areas of the fynbos biome and differ in appearance (Kies, 1951; Schutte, 1995). Honeybush tea is manufactured from mainly three species, namely *C. intermedia* ("bergtee"), *C. subternata* ("vleitee") and *C. genistoides* ("heuningtee") of which *C. subternata* and *C. genistoides* are cultivated (E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, personal communication, 2004).

Similar to rooibos, both the leaves and stems of the plant are used for processing (Du Toit *et al.*, 1998). Previously the plants were harvested during flowering as it was believed to be responsible for the sweet flavour, however, harvesting currently takes place during summer and early autumn (E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, personal

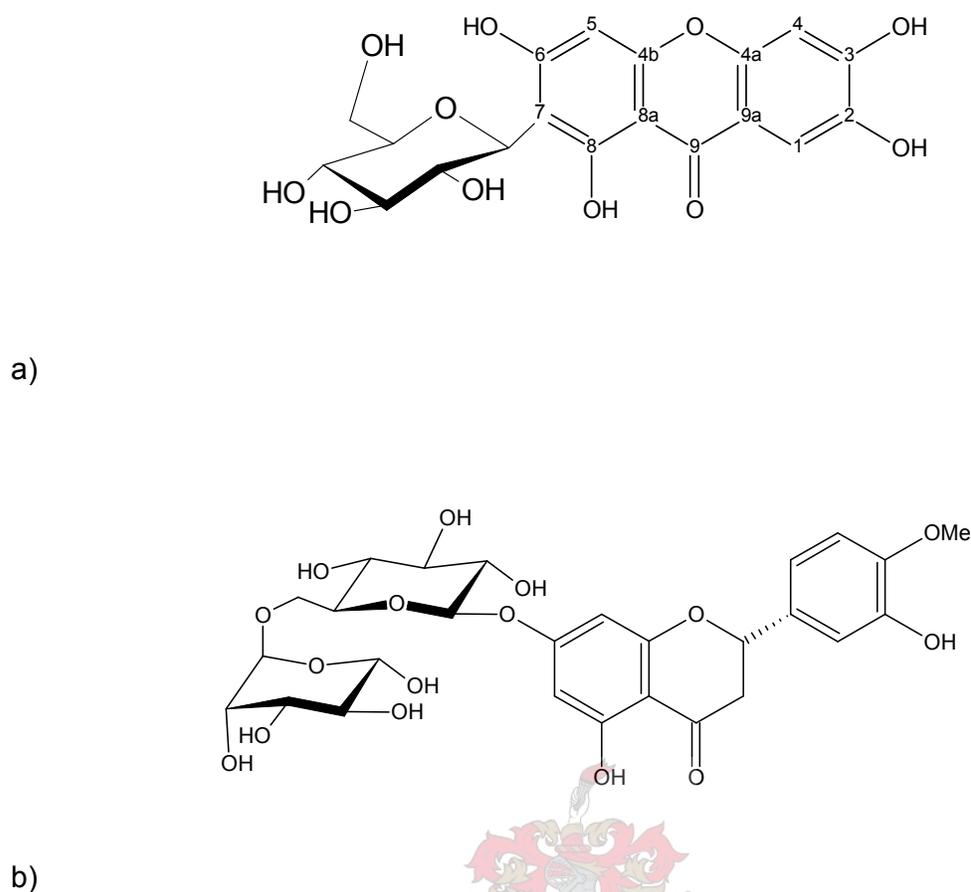
communication, 2004). The shoots are shredded, fermented at elevated temperatures (Du Toit & Joubert, 1999), and sun- or oven-dried. Although fermentation is necessary for the development of an acceptable flavour it reduces the TPC (Du Toit & Joubert, 1998a; Hubbe, 2000; Richards, 2002), including the mangiferin content, as well as the antioxidant potency of honeybush tea (Hubbe, 2000; Richards, 2002). This contributed to the development of green honeybush (De Beer & Joubert, 2002), as an alternative product for either the herbal tea market or agro-processing industry (E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, personal communication, 2004).

### 3.2. Phenolic composition

The phenolic composition, both qualitative and quantitative, differs among *Cyclopia* species (De Nysschen *et al.*, 1996; Ferreira *et al.*, 1998; Joubert *et al.*, 2003; Kamara *et al.*, 2003; Kamara *et al.*, 2004), as well as types within species (Joubert *et al.*, 2003). The first investigation of fermented *C. intermedia* identified the flavanones naringenin, eriodictyol, hesperetin and hesperidin, the flavone luteolin, the isoflavones formononetin, afrormosin, calycosin, pseudobaptigen and fujikinetin, the coumestans medicagol, flemichapparin and sophoracoumestan B, the xanthones mangiferin and isomangiferin and the phenolic acid *p*-coumaric acid (Ferreira *et al.*, 1998). In a follow-up study on minor compounds, four flavanone glucosides, five kaempferol glycosides, the isoflavone, wistin, a diglycosylated isoflavone, two flavones, tyrosol and a methoxy analogue, and a diglycosylated benzaldehyde were isolated (Kamara *et al.*, 2003).

*Cyclopia subternata* possesses a different phenolic profile. Apart from mangiferin, hesperidin, luteolin and *p*-coumaric acid, unfermented *C. subternata* contains the flavanones narirutin and eriocitrin, the flavones 5-deoxyluteolin and scolymoside, the isoflavone orobol, the flavonol C-6-glucosylkaempferol, a glycosylated flavan, and the flavanol epigallocatechin gallate. Other compounds include 4-glucosyltyrosol and shikimic acid (Kamara *et al.*, 2004). No coumestans have been isolated.

The major phenolic compounds identified in unfermented *C. genistoides* are mangiferin and isomangiferin (Brandt, 2004). Based on ultraviolet-diode array detector (UV-DAD) identification, eriodictyol, eriocitrin, hesperidin, hesperetin and luteolin were shown to be present (Van der Merwe, 2004). According to De Nysschen *et al.* (1996) the major phenolic compounds in the leaves of 22 unfermented *Cyclopia* species were mangiferin, hesperetin and isosakuranetin. HPLC analysis of *C. intermedia*, *C. sessiliflora*, *C. maculata* (Genadendal type) and *C. genistoides* showed mangiferin (Figure 2a), isomangiferin and hesperidin (Figure 2b) to be the major compounds (Joubert *et al.*, 2003). *Cyclopia genistoides* and *C. intermedia* had the highest mangiferin and hesperidin contents, respectively (Joubert *et al.*, 2003). According to Richards (2002) the soluble solids of water extracts of *C. genistoides* had a significantly higher xanthone content than *C. intermedia* and *C. subternata*. The total polyphenols of



**Figure 2** Molecular structures of a) mangiferin and b) hesperidin present in *Cyclopia genistoides* (Brand, 2004; Van der Merwe, 2004; Sukpondma *et al.*, 2005).

*C. genistoides*, *C. intermedia* and *C. subternata* comprise 86, 20 and 12% of xanthones, respectively. The total polyphenol and total flavonoid contents of the water soluble solids of *C. genistoides* are higher than that of *C. intermedia* (Du Toit & Joubert, 1998a). According to Richards (2002) *C. genistoides* and *C. subternata* had the highest total water soluble solid content but the TPC of *C. subternata* was the highest of the four *Cyclopia* species, *C. genistoides*, *C. intermedia*, *C. sessiliflora* and *C. subternata* (Richards, 2002).

## 4. QUALITY CONTROL AND AUTHENTICATION

### 4.1. Quality control and authentication

Quality control is an important aspect in the food, nutraceutical and pharmaceutical industry. According to Wilkinson (1998) the following aspects need to be taken into account when assessing the quality of herbal products for nutraceutical development: Macroscopic and

microscopic properties; its botanical name and family; its geographical source; the method of drying and/or extraction (including solvents used); and consistent analytical data (e.g. TLC, HPLC). Marker or pharmacologically active components are used for standardisation and quality control procedures of herbal products (He, 2000; Liang *et al.*, 2004; Ong, 2004). Examples of marker compounds are hyperoside in St. John's Wort (Rager *et al.*, 2002), harpagoside in devil's claw (Guillerault *et al.*, 1994), echinacoside in *Enchinacea* (Perry *et al.*, 2001) and flavonoids in *Ginko biloba* (Deng & Zito, 2003). Phytochemicals (which include phenolic compounds, saponins, alkaloids and other classes of natural products in botanical extracts) in herbal plant material differ significantly among species and even the same plant extracts (He, 2000). The harvest season, geographical area, climate, drying process, age and storage conditions are all factors contributing to this variation and have made quality control of herbal material a challenge for the food and phytopharmaceutical industries (He, 2000; Laasonen *et al.*, 2002).

Authentication and adulteration of products are other aspects of quality control that have become more important for manufacturers, because of the increasing competitiveness in the food industry. It is important that the product conforms to the description provided by the producer or processor and that no adulterants were added (Downey, 1995; Downey, 1996; Downey, 1998). According to The European Agency for the Evaluation of Medical Products, the identification of herbal drugs is one of the first tests to be applied in ensuring quality, safety, and efficacy of herbal medicinal products. Identification testing should be able to discriminate between related species and/or potential adulterants (Anon., 2001).

#### **4.2. Quality evaluation of rooibos and honeybush**

Current standards and requirements for export of green rooibos and honeybush (Anon., 2000) do not address any of the requirements for the evaluation of herbal products discussed in the previous section. Local extract manufacturers make use of TPC and TAA, as determined with the Folin-Ciocalteu (FC) and ABTS assays, respectively, to standardise their products (E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, personal communication, 2004). A German manufacturer of an aspalathin-enriched extract from green rooibos use HPLC analysis to ensure that the product contains the specified quantity of aspalathin. No such quality parameters exist for the plant material. Tables 1 and 2 give a summary of analytical methods used in studies on rooibos and honeybush to quantify phenolic compounds and determine the antioxidant activity of extracts.

**Table 1** Conventional analytical methods used for determining polyphenol composition and antioxidant activity of fermented and unfermented rooibos tea and polyphenol-enriched extracts.

Parameter	Method	Reference
Total polyphenol content	Folin-Ciocalteu method <sup>a</sup>	Joubert, 1988; Joubert, 1996; Winterton, 1999; Marnewick <i>et al.</i> , 2000; Bramati <i>et al.</i> , 2003; Marnewick <i>et al.</i> , 2003; Schulz <i>et al.</i> , 2003a; Joubert <i>et al.</i> , 2004; Van der Merwe, 2004
Dihydrochalcones		
Aspalathin content	HPLC <sup>b</sup>	Joubert, 1996; Bramati <i>et al.</i> , 2002; Bramati <i>et al.</i> , 2003; Schulz <i>et al.</i> , 2003a; Joubert <i>et al.</i> , 2004, Van der Merwe, 2004
Nothofagin content	HPLC	Joubert, 1996; Schulz <i>et al.</i> , 2003a; Van der Merwe, 2004
Flavanols	DAC colorimetrically <sup>c</sup>	Van der Merwe, 2004
Flavonols + Flavones	Spectrophotometrically <sup>d</sup>	Van der Merwe, 2004
Flavonols		
Isoquercitrin/hyperoside	HPLC	Bramati <i>et al.</i> , 2002; Bramati <i>et al.</i> , 2003
Isoquercitrin/rutin	HPLC	Van der Merwe, 2004
Rutin	HPLC	Bramati <i>et al.</i> , 2002; Bramati <i>et al.</i> , 2003
Quercetin	HPLC	Bramati <i>et al.</i> , 2002; Bramati <i>et al.</i> , 2003; Van der Merwe, 2004
Flavones		
Chrysoeriol	HPLC	Bramati <i>et al.</i> , 2002; Bramati <i>et al.</i> , 2003
Iso-orientin	HPLC	Bramati <i>et al.</i> , 2002; Bramati <i>et al.</i> , 2003; Van der Merwe, 2004
Luteolin	HPLC	Bramati <i>et al.</i> , 2002; Bramati <i>et al.</i> , 2003; Van der Merwe, 2004
Orientin	HPLC	Bramati <i>et al.</i> , 2002; Bramati <i>et al.</i> , 2003; Van der Merwe, 2004
Vitexin	HPLC	Bramati <i>et al.</i> , 2002; Bramati <i>et al.</i> , 2003; Van der Merwe, 2004
Isovitexin	HPLC	Bramati <i>et al.</i> , 2002; Bramati <i>et al.</i> , 2003; Van der Merwe, 2004

Table 1 (continued)

Parameter	Method	Reference
Antioxidant activity	ABTS <sup>e</sup>	Bramati <i>et al.</i> , 2003; Schulz <i>et al.</i> , 2003a
	DPPH <sup>f</sup>	Von Gadow <i>et al.</i> , 1997a,b; Winterton, 1999; Joubert <i>et al.</i> , 2000; Standley <i>et al.</i> , 2001; Joubert <i>et al.</i> , 2004
	Superoxide anion radical scavenging <sup>g</sup>	Winterton, 1999; Joubert <i>et al.</i> , 2000; Standley <i>et al.</i> , 2001; Joubert <i>et al.</i> , 2004
	$\beta$ -Carotene bleaching <sup>h</sup>	Von Gadow <i>et al.</i> , 1997a,b,c
	Rancimat lipid peroxidation <sup>i</sup>	Von Gadow <i>et al.</i> , 1997a
	Linoleic acid-phosphate buffer emulsion <sup>j</sup>	Winterton, 1999
	Methyl linoleate micellar system <sup>k</sup>	Winterton, 1999
	Sunflower oil-in-water emulsion	Winterton, 1999

<sup>a</sup> Singleton & Rossi, 1965

<sup>b</sup> Reversed-phase high-performance liquid chromatography

<sup>c</sup> McMurrugh & McDowell, 1978

<sup>d</sup> Mazza *et al.*, 1999

<sup>e</sup> Radical cation 2,2'-azinobis(3-ethyl-benzothiazoline-6-sulphonate) decolourisation assay (Re *et al.*, 1999)

<sup>f</sup>  $\alpha,\alpha$ -diphenyl- $\beta$ -picrylhydrazyl (DPPH) radical scavenging method (Brand-Williams *et al.*, 1995)

<sup>g</sup> Robak & Gryglewski, 1988

<sup>h</sup> Pratt, 1980

<sup>i</sup> Ho *et al.*, 1992

<sup>j</sup> Lingnert *et al.*, 1979

<sup>k</sup> Miyake & Shibamoto, 1997

**Table 2** Conventional analytical methods used for determining polyphenol composition and antioxidant activity of honeybush tea and polyphenol-enriched extracts.

Parameter	Method	Reference
Total polyphenol content	Folin-Ciocalteu assay colorimetric method <sup>a</sup>	Du Toit & Joubert, 1998a,b; Du Toit & Joubert, 1999; Marnewick <i>et al.</i> , 2000; Richards, 2002; Marnewick <i>et al.</i> , 2003
Xanthones (M+I) <sup>b</sup>	HPLC <sup>c</sup>	Richards, 2002
Mangiferin (M)	HPLC	De Nysschen <i>et al.</i> , 1996; Joubert <i>et al.</i> , 2003; Van der Merwe, 2004
Isomangiferin (I)	HPLC	Joubert <i>et al.</i> , 2003
Flavanols	DAC colometrically <sup>d</sup>	Richards, 2002, Van der Merwe, 2004
Flavonols + Flavones	Spectrophotometrically <sup>e</sup>	Richards, 2002, Van der Merwe, 2004
Luteolin	HPLC	Van der Merwe, 2004
Flavanones		
Hesperidin	HPLC	Joubert <i>et al.</i> , 2003; Richards, 2002; Van der Merwe, 2004
Hesperetin	HPLC	Van der Merwe, 2004
Eriocitrin	HPLC	Van der Merwe, 2004
Eriodictyol	HPLC	Van der Merwe, 2004
Narirutin	HPLC	Van der Merwe, 2004

Table 2 (continued)

Quality parameter	Method	Reference
Antioxidant activity	ABTS <sup>f</sup>	Richards, 2002
	Superoxide anion radical scavenging <sup>g</sup>	Hubbe & Joubert, 2000a
	FRAP <sup>h</sup>	Richards, 2002
	DPPH <sup>i</sup>	Hubbe & Joubert, 2000b

<sup>a</sup> Singleton & Rossi, 1965

<sup>b</sup> Mangiferin and isomangiferin co-eluted

<sup>c</sup> Reversed-phase high-performance liquid chromatography

<sup>d</sup> McMurrrough & McDowell, 1978

<sup>e</sup> Mazza *et al.*, 1999

<sup>f</sup> Radical cation 2,2'-azinobis(3-ethyl-benzothiazoline-6-sulphonate) decolourisation assay (Re *et al.*, 1999)

<sup>g</sup> Robak & Gryglewski, 1988

<sup>h</sup> Ferric reducing antioxidant power assay (Benzie & Strain, 1996)

<sup>i</sup>  $\alpha,\alpha$ -diphenyl- $\beta$ -picrylhydrazyl (DPPH) radical scavenging method (Brand-Williams *et al.*, 1995)



## 5. PROCEDURES FOR ROUTINE QUANTIFICATION OF PHENOLIC COMPOSITION

### 5.1. Extraction methods and conditions with special reference to rooibos and honeybush

The choice of extraction method and conditions is of great importance as it is essential to extract the desired chemical components from the plant material in adequate quantities (Huie, 2002). Pressurised fluid extraction (also known as accelerated solvent extraction) and supercritical fluid extraction (Da Costa *et al.*, 1999; Huie, 2002; Escribano-Bailón & Santos Buelga, 2003; Chen *et al.*, 2004; Bergeron *et al.*, 2005) have been investigated as alternatives to conventional solid-liquid extraction to obtain optimum extraction of active compounds or groups of compounds from the plant material of interest. Bergeron *et al.* (2005) showed that these methods did not give superior extraction of total flavonoids from *Scutellaria lateriflora* compared to standard hot water or 70% ethanol extraction.

The diversity and complexity of the chemical composition of plant material make it difficult to predict optimum extraction conditions. Solubility of phenolic compounds in the extraction solvent has a major effect on the quantities extracted. Frequently used solvent systems comprise hot water extraction (Bergeron *et al.*, 2005; Ivanova *et al.*, 2005) or organic solvents such as methanol, ethanol, acetonitrile or mixtures of these solvents with water, depending on the polarity of the compounds (Lin *et al.*, 1996; Ferrari *et al.*, 2000; Moure *et al.*, 2001; Lee *et al.*, 2002; Jayasinghe *et al.*, 2003; Ong, 2004; Bergeron *et al.*, 2005; Gálvez *et al.*, 2005; Lin & Giusti, 2005).

Aspalathin in rooibos is readily soluble in water and other polar solvents, but insoluble in non-polar solvents (Koeppen & Roux, 1966). Methanol extracts of rooibos has slightly higher aspalathin content than aqueous extracts (Bramati *et al.*, 2002). Extraction of aspalathin with a 30% acetonitrile-water mixture is used by a manufacturer of green rooibos extracts for quantification purposes of the aspalathin content of the plant material before processing (E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, personal communication, 2004). Jaganyi and Wheeler (2003) determined the first-order rate constant for extraction of aspalathin from fermented rooibos (1.00-1.40 mm fraction), which is *ca.* 5 times less than that for extraction of flavanols from Japanese green tea.

Mangiferin, a glucoside, is the only water-soluble xanthone (Harborne, 1998). Improved extraction efficiency is obtained, using solvents of lower polarity (Markham, 1975). Methanol is usually used to extract mangiferin (Ferrari *et al.*, 2000). For the production of Vimang®, a mangiferin extract from mango stem bark, methanol is used for initial extraction (Núñez-Sellés *et al.*, 2002). Lin *et al.* (1996) and Tang *et al.* (2004) used 70% methanol-water and 80% methanol-water mixtures, respectively, to extract mangiferin from traditional Chinese medicines.

Hesperidin, the rutoside of hesperetin, is not readily soluble in water (Harborne, 1998). Calabrò *et al.* (2004) investigated several extraction conditions to determine the most suitable

method for extraction of flavonoids, which included hesperidin. Ether or hexane was used to extract the sample under controlled temperatures (25 or 40°C) for a fixed extraction time (20 or 40 min) whereafter the residue was extracted with ethyl acetate, methanol or acetonitrile with the same temperature and time conditions. It was found that hesperidin was best extracted with ether and ethyl acetate at 40°C for 20 min. Sonication of powdered Daflon® tablets with methanol:DMSO (1:1) at 30°C for 30 min was found to be a very efficient system for the complete extraction of hesperidin in these tablets (Kanaze *et al.*, 2003). Other factors that may affect the quantity of the component extracted are the mass ratio (solvent-to-solid ratio), temperature of the solvent, contact time and physical form of the plant material. Pinelo *et al.* (2005) found a linear relationship between solvent-to-solid ratio and solid yields despite of the solvent used in a study done on grape pomace. They also demonstrated the enhancing effect of increasing temperature on the solubility of the solute and the diffusion coefficient for grape pomace. High temperature increases the efficiency of extraction of polyphenols, since heat causes cell walls to be permeable, it increases solubility and the diffusion coefficients of the compounds to be extracted and decreases the viscosity of subsequent separating processes such as filtering (Escribano-Bailón & Santos-Buelga, 2003). Joubert (1988, 1990) has shown that extraction of total polyphenols from fermented rooibos is improved by increasing the temperature of the water, the contact time and the solvent-to-solid ratio from 5:1 to 10:1. Flavonoid type compounds are responsible for the largest part of the total polyphenol extracted (Joubert, 1990). Increasing extraction time (up to 30 min) resulted in an increase in the antioxidant activity of rooibos aqueous extracts (Von Gadow *et al.*, 1997c). Particle size reduction eases the extraction process and allows for greater yields (Escribano-Bailón & Santos-Buelga, 2003), i.e. the polyphenol content and the antioxidant activity (Moure *et al.*, 2001).

A summary of extraction conditions used in green rooibos and honeybush studies are shown in Tables 3 and 4. Conventional solid-liquid extraction was used. In some cases ultrasonication was applied to enhance extraction. Water has been predominantly used, since the focus of these studies was the relevance of water to a “cup of tea”. Compounds of interest were aspalathin in rooibos tea (Joubert, 1996) and mangiferin (Van der Merwe, 2004), mangiferin + isomangiferin (Richards, 2002) and hesperidin (Richards, 2002; Van der Merwe, 2004) in honeybush tea. In cases where quantification of the flavonoid content of the plant material was of interest, water was used to extract aspalathin in rooibos (Schulz *et al.*, 2003) and methanol to extract mangiferin (De Nysschen *et al.*, 1996; Joubert *et al.*, 2003) and hesperidin (Joubert *et al.*, 2003) in honeybush tea. In the case of water extractions high temperatures was used for 5-15 min. For methanol extraction it was left at room temperature for 4 h or sonicated for 30 min.

**Table 3** Extraction conditions used and the quantitative data for aspalathin and nothofagin content of green rooibos.

Compound	Plant material	Extraction solvent	Plant:solvent ratio	Extraction method	Extraction time	Quantity (g.100 g <sup>-1</sup> plant material) <sup>a</sup>	Reference
Aspalathin	Leaves + stems	Water	20 g:250 ml	Freshly boiled water, followed by steeping on a steam bath <sup>b</sup>	15 min	1.47	Joubert, 1996
	Leaves	Water	0.5 g:50 ml	100°C	15 min	4.45-11.90	Schulz <i>et al.</i> , 2003a
	Stems	Water	0.5 g:50 ml	100°C	15 min	7.89	Schulz <i>et al.</i> , 2003a
	Leaves + stems	Water	0.5 g:50 ml	100°C	15 min	4.52-9.34	Schulz <i>et al.</i> , 2003a
	Leaves + stems	Water	1 g:60 ml	Freshly boiled water, followed by steeping	10 min	4.99	Bramati <i>et al.</i> , 2003
Nothofagin	Leaves + stems	Water		Freshly boiled water, followed by steeping on a steam bath <sup>b</sup>	15 min	0.43	Joubert, 1996
	Leaves	Water	0.5 g:50 ml	100°C	15 min	0.68-1.33	Schulz <i>et al.</i> , 2003a
	Stems	Water	0.5 g:50 ml	100°C	15 min	1.25	Schulz <i>et al.</i> , 2003a
	Leaves + stems	Water	0.5 g:50 ml	100°C	15 min	0.34-1.03	Schulz <i>et al.</i> , 2003a

<sup>a</sup> Recalculated from published data.

<sup>b</sup> Aqueous extracts was followed by liquid-liquid extraction, under reflux for 6 h with ethyl acetate.

**Table 4** Extraction conditions used and the quantitative data for mangiferin, isomangiferin, xanthone and hesperidin content of green honeybush.

<i>Cyclopia</i> species	Compound	Plant material	Plant:solvent ratio	Extraction solvent	Extraction method	Extraction time	Quantity (g.100 g <sup>-1</sup> plant material) <sup>a</sup>	Reference
<i>C. intermedia</i>	Mangiferin (M)	Leaves+stems	0.25 g:30 ml	Methanol	Sonication	30 min	1.69	Joubert <i>et al.</i> , 2003
		Leaves		Methanol	Room temperature	4 h	Not quantified	De Nysschen <i>et al.</i> , 1996
		Leaves+stems	100 g:1000 ml	Deionised water	Steeping	5 min	0.68	Van der Merwe, 2004
	Isomangiferin (I)	Leaves+stems	0.25 g:30 ml	Methanol	Sonication	30 min	0.22	Joubert <i>et al.</i> , 2003
		Xanthone (M+I) <sup>b</sup>	Leaves+stems	100 g:1000 ml	Deionised water	Steeping	5 min	0.78
	Hesperidin	Leaves+stems	0.25 g:30 ml	Methanol	Sonication	30 min	1.76	Joubert <i>et al.</i> , 2003
		Leaves+stems	100 g:1000 ml	Deionised water	Steeping	5 min	0.35	Richards, 2002
		Leaves+stems	100 g:1000 ml	Deionised water	Steeping	5 min	0.31	Van der Merwe, 2004
		Leaves+stems	100 g:1000 ml	Deionised water	Steeping	5 min	0.31	Van der Merwe, 2004
<i>C. genistoides</i>	Mangiferin	Leaves+stems	0.25 g:30 ml	Methanol	Sonication	30 min	3.61	Joubert <i>et al.</i> , 2003
		Leaves		Methanol	Room temp	4 h	Not quantified	De Nysschen <i>et al.</i> , 1996
		Leaves+stems	100 g:1000 ml	Deionised water	Steeping	5 min	3.89	Van der Merwe, 2004
	Isomangiferin	Leaves+stems	0.25 g:30 ml	Methanol	Sonication	30 min	0.54	Joubert <i>et al.</i> , 2003
	Xanthone (M+I)	Leaves+stems	100 g:1000 ml	Deionised water	Steeping	5 min	1.71	Richards, 2002
	Hesperidin	Leaves+stems	0.25 g:30 ml	Methanol	Sonication	30 min	1.50	Joubert <i>et al.</i> , 2003
		Leaves+stems	100 g:1000 ml	Deionised water	Steeping	5 min	0.15	Richards, 2002
		Leaves+stems	100 g:1000 ml	Deionised water	Steeping	5 min	0.36	Van der Merwe, 2004
		Leaves+stems	100 g:1000 ml	Deionised water	Steeping	5 min	0.36	Van der Merwe, 2004
<i>C. subternata</i>	Mangiferin	Leaf		Methanol	Room temperature	4 h	Not quantified	De Nysschen <i>et al.</i> , 1996
		Leaves+stems	100 g:1000 ml	Deionised water	Steeping	5 min	0.44	Van der Merwe, 2004
	Xanthone (M+I)	Leaves+stems	100 g:1000 ml	Deionised water	Steeping	5 min	1.69	Richards, 2002
	Hesperidin	Leaves+stems	100 g:1000 ml	Deionised water	Steeping	5 min	0.16	Van der Merwe, 2004
		Leaves+stems	100 g:1000 ml	Deionised water	Steeping	5 min	0.48	Richards, 2002

<sup>a</sup> Recalculated from published data.

<sup>b</sup> Mangiferin and isomangiferin co-eluted.

## 5.2. Quantification

Quantification of phenolic compounds is either based on determination of a class of compounds such as flavonoids, a sub-class such as flavanones, or individual compounds such as hesperidin (Harborne, 1998). Spectrophotometric and colorimetric methods are relatively cheap compared to methods such as HPLC and although it is not as accurate it might be convenient for routine analysis.

### 5.2.1. Ultraviolet-visible spectroscopy

UV/Vis radiation forms part of the electromagnetic radiation range between 180-780 nm. Colourless compounds absorb in the UV range between 200-400 nm and coloured compounds in the visible range between 400-780 nm. The use of UV/Vis spectroscopy for identification of unknown compounds is related to the relative complexity of the spectrum and the general position of the wavelength maxima. Spectral measurements in two or three other solvents and comparison with literature data aid identification (Harborne, 1998).

The UV spectra for flavanones in methanol are all alike and can be attributed to the fact that they have little or no conjugation between the rings of the molecule. A major absorption peak is found in the range between 270 and 295 nm. Flavones and flavonols are readily distinguished from flavanones by their UV spectra. Their spectra show two absorption peaks in the region 250-400 nm, i.e. 310-370 nm (Band I) and 250-295 nm (Band II). Dihydrochalcone spectra (MeOH) give similar information than that obtained from the flavanones. Dihydrochalcones also have no conjugation between the rings of the molecule, and the introduction of free hydroxyls on the B-ring therefore has no effect on the UV spectrum (Santos-Buelga *et al.*, 2003). Xanthenes have absorption maximums between 230-245, 250-265, 305-330 and 340-400 nm (Harborne, 1998). The UV ( $\lambda$  max) spectral data of aspalathin and other major compounds of rooibos in ethanol are listed in Table 5. The UV/Vis ( $\lambda$  max) spectral data of mangiferin in different solutions are summarised in Table 6.

The absorbance maxima of a certain compound can be used to estimate the concentration of compounds of similar structure. Aspalathin has for example a very similar molecular structure than nothofagin. Quantification is usually done in terms of the major compound present. Due to the diverse chemical composition of plant extracts, other compounds could absorb at the same wavelength, leading to overestimation of results. The compounds also do not have the same molar absorptivity, aggravating overestimation or resulting in underestimation.

### 5.2.2. Colorimetric methods

In colorimetric methods, compounds are reacted with chemicals to form colour complexes which exhibit a selective light absorptive capacity that can be measured in the visible range with a spectrophotometer. This measurement will then determine the concentration of the

**Table 5** UV ( $\lambda$  max) spectral data of the major phenolic compounds present in rooibos in ethanol.

Compound	UV ( $\lambda$ max) (nm)	References
Aspalathin	290	Koepen & Roux, 1966
Nothofagin	n.a. <sup>a</sup>	-
Isoorientin	255, 268, 350	Harborne, 1998
Orientin	255, 268, 350	Harborne, 1998
Vitexin	269, 336	Harborne, 1998
Rutin	259, 266sh <sup>b</sup> , 363	Harborne, 1998
Isovitexin	269, 336	Harborne, 1998
Luteolin	255, 268, 350	Harborne, 1998
Quercetin	255, 374	Harborne, 1998
Chrysoeriol	252, 269, 350	Harborne, 1998

<sup>a</sup> not available<sup>b</sup> peak with shoulder**Table 6** UV/Vis ( $\lambda$  max) spectral data of mangiferin in different solutions.

Solution	UV ( $\lambda$ max) (nm)	References
EtOH	242, 258	Harborne, 1998
95% EtOH	240, 258, 316, 364	Harborne, 1998
MeOH	237, 254, 268, 312, 364	Núñez Sellés <i>et al.</i> , 2002
MeOH + AlCl <sub>3</sub>	235, 268 321sh <sup>a</sup> , 352, 392	Núñez Sellés <i>et al.</i> , 2002
MeOH + AlCl <sub>3</sub> + HCl	226, 258, 276sh, 316, 330, 395	Núñez Sellés <i>et al.</i> , 2002
MeOH + KOH	235, 268, 298, 341	Núñez Sellés <i>et al.</i> , 2002
MeOH + NaOAc	237, 263, 301sh	Núñez Sellés <i>et al.</i> , 2002

<sup>a</sup> peak with shoulder

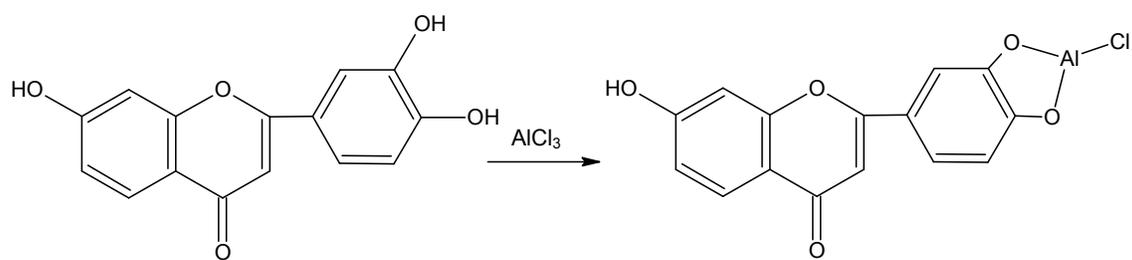
compound responsible for the colour (Boltz & Mellon, 1966). Different compounds or groups of flavonoids will react differently with different chemicals due to their molecular structure.

The TPC of rooibos and honeybush extracts, as a quality parameter, is routinely determined using the FC colorimetric method (Joubert, 1988; Joubert, 1996; Du Toit & Joubert, 1998a; Bramati *et al.*, 2003; Schulz *et al.*, 2003; Joubert *et al.*, 2004). The original method has undergone several adaptations, but adherence to the original method is recommended (Prior *et al.*, 2005). The FC method is an electron transfer based assay that gives reducing capacity (Prior *et al.*, 2005). The method, based on the reducing power of phenolic hydroxyl groups, detects all phenols with varying sensitivities (Singleton & Rossi, 1965). It has found application in the determination of TPC of a wide variety of plant extracts and wine (Zheng & Wang, 2001; Liu *et al.*, 2002; Borbalán *et al.*, 2003; Costin *et al.*, 2003; Baydar *et al.*, 2004; Ivanova *et al.*, 2005). Readily oxidised substances such as ascorbic acid, sulfite, sulfur dioxide and a high sugar content cause interference (Singleton *et al.*, 1999).

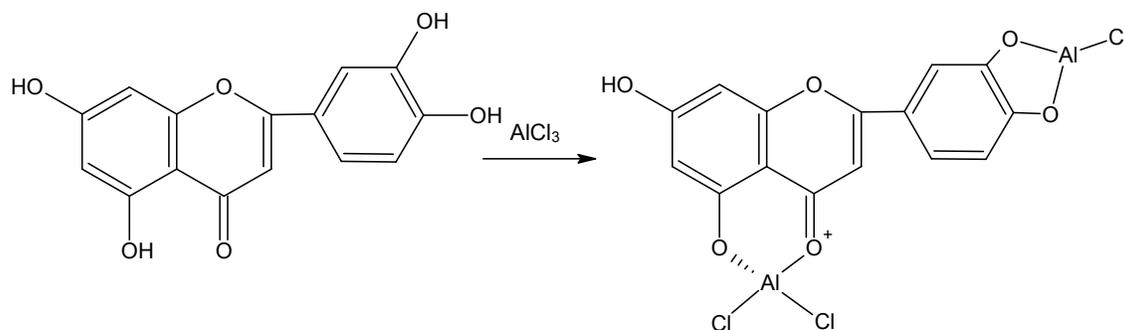
For the determination of certain classes of phenolic compounds more specific colorimetric methods are used. Proanthocyanidins release coloured anthocyanidins when heated in butanol-HCl mixtures (Porter's reagent) (Porter *et al.*, 1986), flavanols react with acidified vanillin (Goldstein & Swain, 1963, Sarkar & Howarth, 1976) or 4-dimethylaminocinnamaldehyde (DAC) (McMurrrough & McDowell, 1978; Treutter, 1989; Treutter *et al.*, 1994) to give coloured products, flavones and flavonols form yellow complexes with  $\text{AlCl}_3$  (Jurd, 1962; Mabry *et al.*, 1970; Chang *et al.*, 2002; Kosalec *et al.*, 2004) and pH-shift with subsequent change in colour is used for the determination of anthocyanins (Ribéreau-Gayon, 1972).

The principle of the  $\text{AlCl}_3$  colorimetric method is that  $\text{AlCl}_3$  forms acid stable yellow complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols (Jurd, 1962; Mabry *et al.*, 1970; Chang *et al.*, 2002). If flavones and flavonols lack a free C-5 or C-3 hydroxyl group no complexes will form with addition of the  $\text{AlCl}_3$  solution (Jurd, 1962). Aluminium chloride can also form acid labile complexes with the orthodihydroxyl groups on the A- or B-ring of flavonoids (Chang *et al.*, 2002). Complex formation results in significant bathochromic shifts of Bands I and II (Mabry *et al.*, 1970). An illustration of  $\text{AlCl}_3$  complex formations of flavones and flavonols is depicted in Figure 3. Flavanones, having a C-5 hydroxyl group, which forms complexes with  $\text{AlCl}_3$ , give a low insignificant absorbance at 415 nm (Chang *et al.*, 2002).

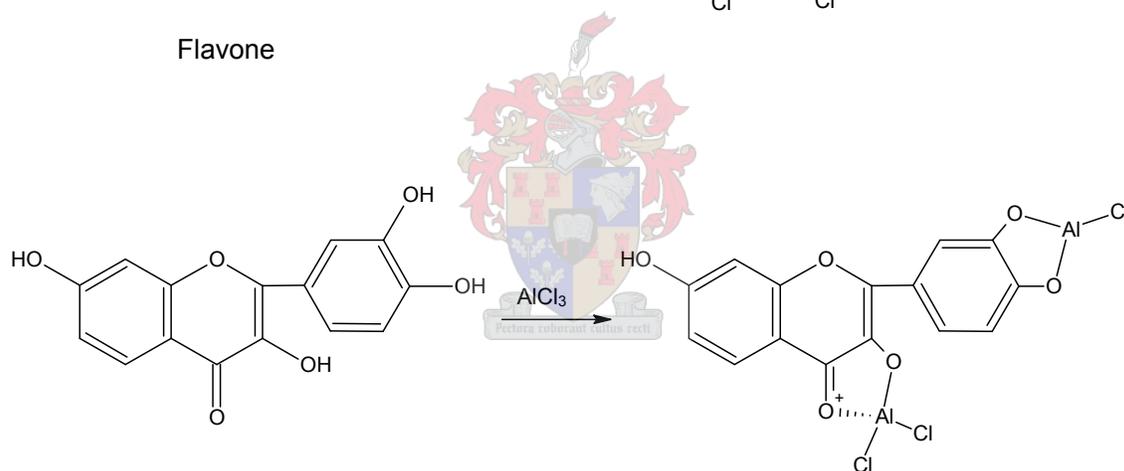
The molecular structure of mangiferin, and particularly the keto group adjacent to a hydroxyl group, lends itself to complexation with  $\text{AlCl}_3$ . The spectra of xanthenes undergo characteristic bathochromic shifts with  $\text{AlCl}_3$  and differ according to the number and position of the hydroxyl substituents (Harborne, 1998). The high quantity of mangiferin compared to the other phenolic compounds in *C. genistoides* (Joubert *et al.*, 2003; Van der Merwe, 2004)



Flavone



Flavone



Flavonol

**Figure 3** Illustration of aluminum chloride complexes of flavones and flavonols (adopted from Mabry *et al.*, 1970).

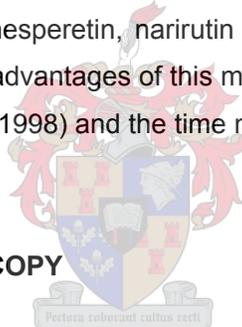
suggests the possible use of this method for determination of mangiferin with minor interference of other phenolic compounds.

A major advantage of these methods is their adaptivity for the use of microtiter plates. This enables large numbers of samples to be measured in one run. Microtiter plates have been

used to measure the antioxidant activity of individual flavonoids in apple juice (Van der Sluis *et al.*, 2000).

### 5.2.3. High-performance liquid chromatography (HPLC)

HPLC has been used for the determination of flavonoids since the 1970's and has become a popular method for separation, screening, chemotaxonomical surveys or quantitative analysis of plants, food products (Santos-Buelga *et al.*, 2003) and herbal medicines (Liang *et al.*, 2004). Merken and Beecher (2000) give an overview of a wide range of HPLC applications for the quantification of individual flavonoids within subclasses present in food. HPLC has been used to quantify isoquercitrin (Bramati *et al.*, 2003; Schulz *et al.*, 2003), iso-orientin, orientin, rutin (Bramati *et al.*, 2002; Bramati *et al.*, 2003; Schulz *et al.*, 2003), isoquercitrin + hyperoside, luteolin, quercetin, vitexin, isovitexin, chrysoeriol (Bramati *et al.*, 2002; Bramati *et al.*, 2003), aspalathin (Joubert, 1996; Bramati *et al.*, 2002; Bramati *et al.*, 2003; Schulz *et al.*, 2003) and nothofagin (Joubert, 1996; Schulz *et al.*, 2003) in rooibos, and xanthone (Richards, 2002), mangiferin (De Nysschen *et al.*, 1996; Joubert *et al.*, 2003; Richards, 2002; Van der Merwe, 2004), isomangiferin (Joubert *et al.*, 2003), hesperidin (Joubert *et al.*, 2003; Richards, 2003; Van der Merwe, 2004), eriocitrin, hesperetin, narirutin and luteolin (Van der Merwe, 2004) in honeybush (Tables 1 & 2). The disadvantages of this method are its high cost due to expensive equipment and solvents (Harborne, 1998) and the time needed for analysis.



## 6. NEAR INFRARED SPECTROSCOPY

### 6.1. Background

Near infrared spectroscopy (NIRS) dates back to the early 1800's when Fredrick William Herschel, a professional musician and astronomer (Herschel, 1800; Davies, 1991), discovered the first non-visible region in the absorption spectrum (Stark *et al.*, 1986). From 1800 to the 1950's it was a dormant period for NIRS and only a small number of articles were reported. It was with the work of Karl Norris, the father of near infrared (NIR), and co-workers (Butler, 1983) later in the 1950's when its potential was recognised (Day & Fearn, 1982). Today NIRS has become the alternative quality control method in the food industry because of its advantages over other analytical techniques. It is a fast, easy-to-use and a non-destructive analytical technique (Day & Fearn, 1982) and can be carried out on-line (Osborne *et al.*, 1993a; Hoyer, 1997) or in-line (Singh Sahni *et al.*, 2004). Minimal or no sample preparation or pretreatment is necessary, which contributes to it being a rapid technique (Day & Fearn, 1982; Osborne, 2000). It can record spectra for liquids, slurries and solids (Williams & Stevenson, 1990; Blanco & Villarroya, 2002). One of the major advantages of NIRS is that it allows several constituents to be measured at the same time (Osborne, 2000). The NIR instrumentation is often rugged and

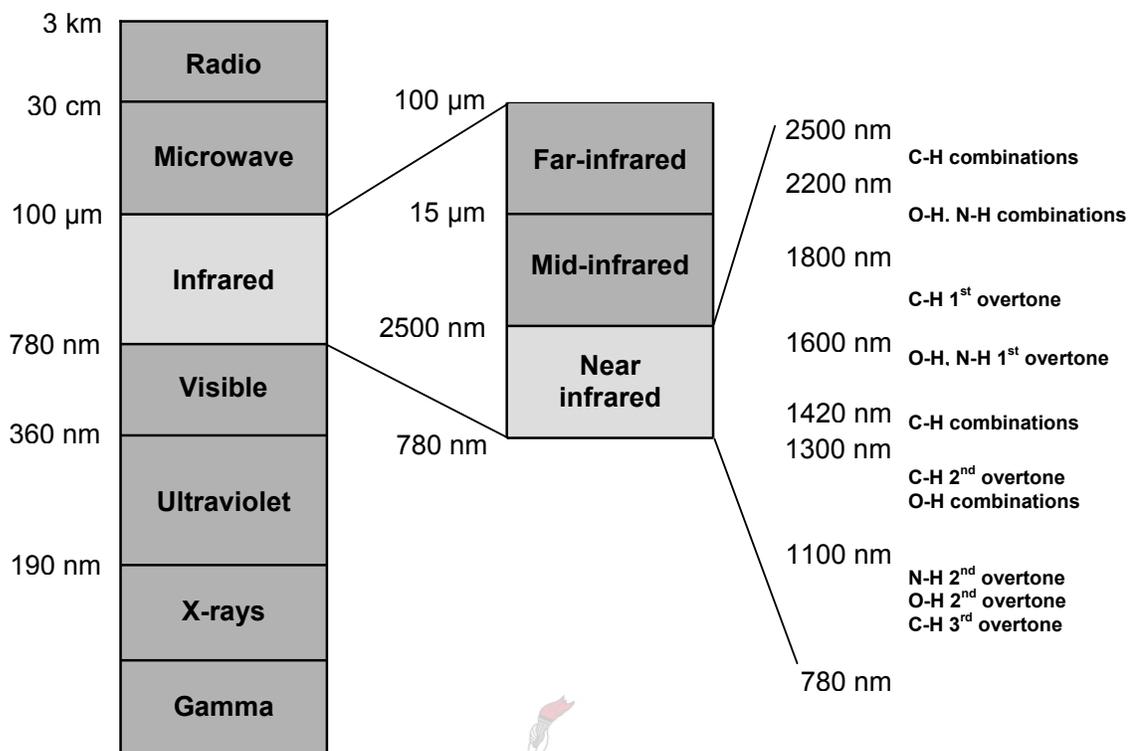
can be used inside or outside laboratory environments (Stark *et al.*, 1986). Portable NIR spectrophotometers such as hand-held instruments and equipment that can be carried in a backpack or mounted on a vehicle are available (Blanco & Villarroya, 2002). The only prerequisite of NIRS application for quantitative purposes is to develop a separate reliable calibration model for every commodity, constituent or quality measurement (Williams, 2001).

## 6.2. Principles of NIRS

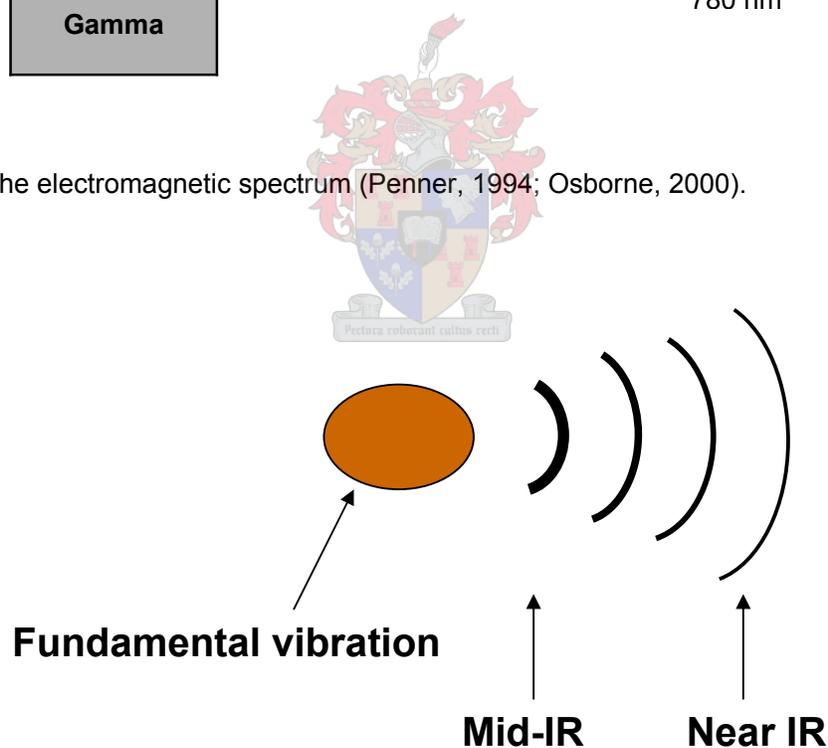
Spectroscopy involves the production, measurement and interpretation of spectra arising from the interaction of electromagnetic radiation with matter (Penner, 1994). The IR region forms that part of the electromagnetic spectrum in the wavelength range between 780-100 000 nm and is divided into near IR, mid-IR and far-IR sub-regions (Penner, 1994; Osborne, 2000). The NIR region covers the part of the wavelength range spanning from 780-2500 nm (Figure 4) (Osborne, 2000) corresponding to a wavenumber range of 4000-12 800  $\text{cm}^{-1}$  (Davies, 1998). In the NIR wavelength range absorption of light is primarily due to overtones and combinations of fundamental vibrations occurring in the mid-infrared spectrum and will be one to three orders of magnitude weaker than those in the mid-infrared spectrum (Figure 5) (Stark *et al.*, 1986). These spectra in the NIR region are rich in physical and chemical information of molecules and may give valuable information about the composition of a food product (Katsumoto *et al.*, 2001).

Electromagnetic radiation may be considered as a wave with the properties of simple harmonic motion and may be defined in terms of the frequency of vibrations or the wavelength (Osborne, 2000). The harmonic oscillator model can be used to describe the vibration of molecules (Osborne *et al.*, 1993a). Molecules consist of atoms combined by covalent bonds sharing electrons between them. Atoms oscillate through their bonds in a molecule like two balls attached to a spring. The vibration frequency, defined as the number of oscillations the wave will make at a given point per second (Penner, 1994), and unique to a bond, is dependent upon the atomic masses ( $m_1$  and  $m_2$ ) and the force constant (strength) of the interatomic bond (Whetsel, 1968). When the fundamental frequency of a specific vibration,  $\nu$ , is equal to the frequency of the radiation, and when there is a vibrational change in the dipole moment, there will be a net transfer of energy in the form of discrete packets, called quanta, from the radiation to the molecule. This causes the radiation to be absorbed, which excites a vibrational change in the molecule (Osborne, 2000; Laasonen, 2003). The radiation energy should therefore be high enough to produce vibrational changes in the molecules (Blanco *et al.*, 1998). Using Hooke's law, defined as an atom shifting from its equilibrium position with strength proportional to the shift, the vibrational frequency ( $f$ ) of a diatomic molecule can be determined (Blanco *et al.*, 1998):

$$f = \frac{1}{2\pi c} \sqrt{\frac{k}{\mu}}$$



**Figure 4** The electromagnetic spectrum (Penner, 1994; Osborne, 2000).



**Figure 5** Overtones and combinations of a fundamental vibration occurring in the mid-infrared spectrum.

where  $c$  is the speed of light,  $k$  the force constant of the bond between the two atoms and  $\mu$  the reduced mass,

$$\mu = \frac{m_1 m_2}{m_1 + m_2}$$

.....2

where  $m_1$  and  $m_2$  are the atomic masses. The variation of the potential energy with bond distance is a parabola centered about the equilibrium distance with evenly spaced vibrational energy levels (Blanco *et al.*, 1998). The energy of the different, evenly spaced levels can be calculated from (Blanco & Villarroya, 2002; Laasonen, 2003):

$$E_{\text{vib}} = \left( v + \frac{1}{2} \right) \frac{h}{2\pi} \sqrt{\frac{k}{\mu}}$$

.....3

Since the selection rule for harmonic oscillator transitions is  $\Delta v = \pm 1$  and energy levels are equally spaced, the energy difference between two consecutive levels will always be  $E_{(v+1)} - E_v = f$ , which is called the “fundamental frequency” of the band (Whetsel, 1968; Blanco *et al.*, 1998).

Vibration of actual chemical bonds cannot be explained using the harmonic oscillator model but rather using the model of an anharmonic oscillator by which energy levels are not evenly spaced (Whetsel, 1968). The anharmonic oscillator behaves like the harmonic oscillator but with the energy difference decreasing as  $v$  increases:

$$\Delta E_{\text{vib}} = h\nu [1 - (2v + \Delta v + 1)\gamma]$$

.....4

where  $\gamma$  is the anharmonicity factor. These changes from 0 to 2, 0 to 3 are known as overtones (first and second overtone correspondingly) (Whetsel, 1968; Osborne *et al.*, 1993a; Blanco & Villarroya, 2002). Because of the decrease in energy change with the increase in  $v$ , the first overtone band will be an order of magnitude less intense than a fundamental band, the second less intense than the first and so forth (Whetsel, 1968). Overtones appear between 780-2000 nm, depending on the overtone order and the bond nature and strength. The frequencies correspond to approximate multiples of the fundamental vibration (Osborne *et al.*, 1993a; Blanco & Villarroya, 2002).

Two or more vibrational modes can also interact to cause simultaneous energy changes (Osborne, 2000; Blanco & Villarroya, 2002), such as in the case of polyatomic molecules (Blanco & Villarroya, 2002). These absorption bands are called combination bands (Osborne, 2000; Blanco & Villarroya, 2002). The frequencies will then be the sum of the multiples of each of the interacting frequency, for instance in the case of a triatomic molecule, there will be 3 first

overtones, 3 second overtones, etc. (Osborne, 2000). NIR combination bands appear between 1900 and 2500 nm (Blanco & Villarroya, 2002). Any sample will absorb at a certain wavelength, depending on the properties of the chemical entities present. NIRS can thus be used to determine certain functional groups present in a sample (Gunasekaran & Irudayaraj, 2001).

### **6.3. Instrumentation**

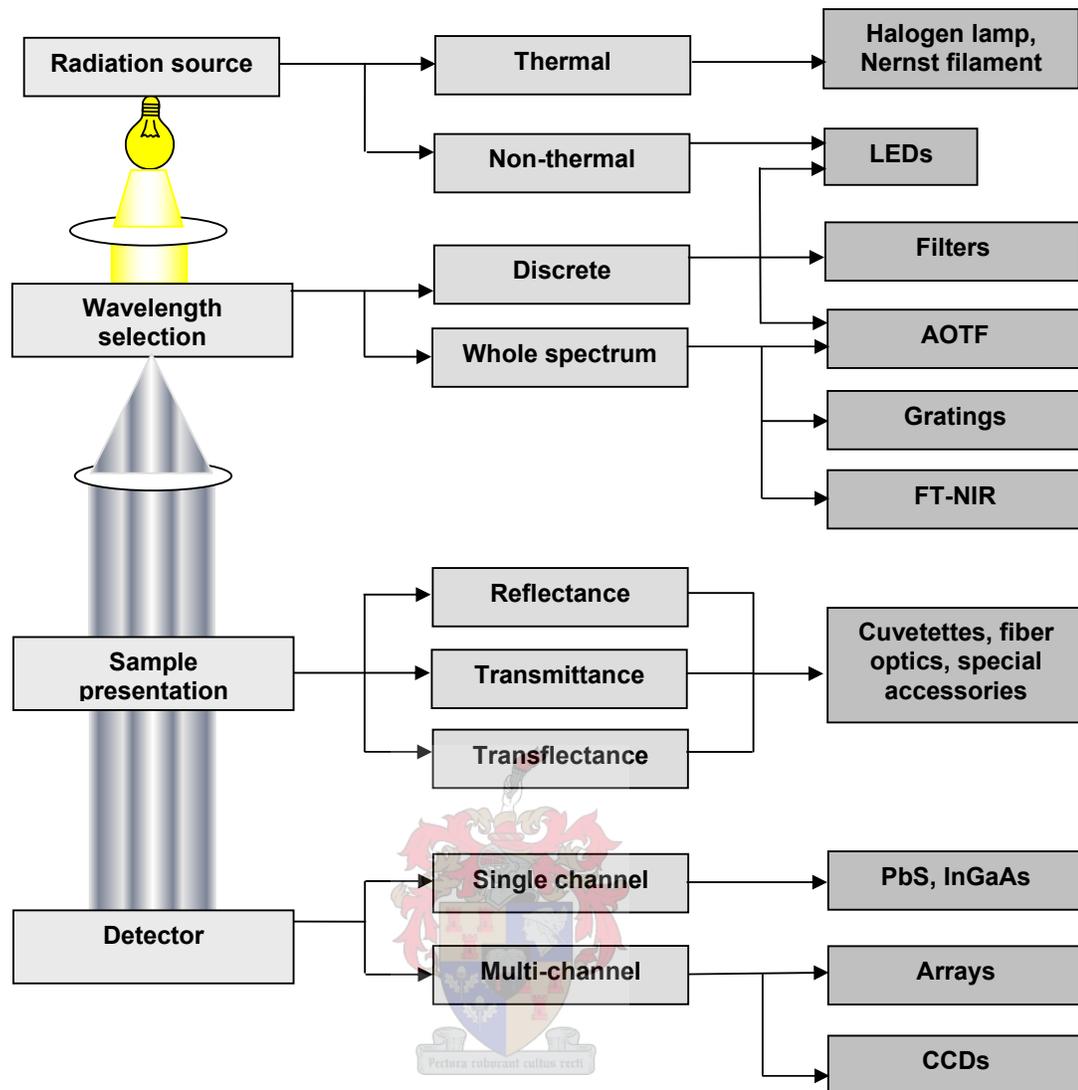
NIRS equipment can include a variety of devices (Figure 6) depending on the characteristics of the sample and analytical conditions and needs, which makes the technique very flexible (Blanco & Villarroya, 2002). It is therefore important to make the correct choice of instrument for the specific application (Osborne, 2000). All NIR instruments possess the same essential building blocks: A radiation source, wavelength selectors, sample presentation modes and a detector (Blanco & Villarroya, 2002).

#### **6.3.1. Radiation source**

NIRS radiation sources can be thermal or non-thermal. The thermal sources consist of a radiant filament producing thermal radiation, for example the Nernst filament, which is a heated ceramic filament containing rare-earth oxides, or quartz-halogen lamps, and produces radiation spanning from a narrow range to a large range of frequencies in the NIR region (Osborne *et al.*, 1993a). Tungsten-halogen (thermal) lamps are usually employed as a radiation source and should meet the criteria as described in McClure (2001). These lamps give a longer life span and are more stable because of the cleaning action of the halogen (McClure, 2001). The non-thermal sources consist of discharge lamps, light-emitting diodes, laser diodes or lasers and emit much narrower bands of radiation than the thermal sources. Non-thermal sources are more efficient compared to thermal sources, because most of the energy consumed appears as emitted radiation over a narrow range of wavelengths, and they can be electronically adjusted, simplifying the design of the instrument and reducing the power consumption (Osborne *et al.*, 1993a).

#### **6.3.2. Wavelength selectors**

NIR spectrophotometers can be distinguished on the basis of wavelength selection, i.e. discrete wavelength and whole spectrum. A discrete wavelength spectrophotometer irradiates a sample with only a few wavelengths selected using light source filters or light-emitting diodes (LEDs) (Blanco & Villarroya, 2002). Filter instruments are designed for specific applications in specific spectral regions. Usually between six and twenty interference filters are chosen to obtain the wavelengths that will be absorbed by certain constituents in the samples for a specific application (Penner, 1994). These are the easiest to use and least expensive instruments (Osborne, 2000).



**Figure 6** Principle features of NIRS equipment (LEDs = Light-emitting diodes; AOTF = Acousto-optical tunable filter; FT-NIR = Fourier transform near infrared; PbS = Lead sulphide; InGaAs = Indium gallium arsenide; CCDs = Charge coupled devices) (adapted from Blanco & Villarroya (2002)).

The whole-spectrum NIR instruments usually include a diffraction grating, although they may be of the Fourier transform near infrared (FT-NIR) type. These instruments are much more flexible than discrete wavelength instruments and can be used in a wider variety of measurements (Blanco & Villarroya, 2002).

The principle of grating monochromator instruments is to spread out radiation according to wavelength (McClure, 2001). It can be used in the transmittance and the reflectance mode, although the latter is simpler and much more efficient (Osborne *et al.*, 1993a). It is normally used for research purposes or when a wide range of different applications is required (Osborne, 2000).

Acousto-optical tunable filters (AOTFs), also known as dispersive monochromator wavelength-selection devices (Osborne, 2000), choose wavelengths by using radio-frequency signals to change the refractive index of a crystal, usually tellurium dioxide (TeO<sub>2</sub>) (Blanco & Villarroya, 2002). AOTFs do not include any moving parts, ensuring more reliable, reproducible wavelength scans than those provided by other devices. It is also very stable which makes AOTFs especially suitable for equipment exposed to destructive conditions, as in production plants (Osborne, 2000; Blanco & Villarroya, 2002).

The FT-NIR such as the Michelson interferometer splits light into two beams and then recombine them after a path difference has been introduced to create the conditions for optical interference (Osborne *et al.*, 1993a). Simultaneous measures of all wavelengths allow the light to be imaged onto one detector making it a multiplexing instrument. Although frequencies could be measured successfully it can be photometrically poor for weak absorption features. To use it for all its advantages a sufficient number of wavelengths should be measured (Osborne *et al.*, 1993a).

### **6.3.3. Sample presentation modes**

The different sample presentations modes make it possible to apply NIR to a wide range of food analyses. There are techniques for all types of liquids, slurries, powdered or solid samples (Osborne, 2000). Radiation interacting with a sample may be absorbed, transmitted or reflected. According to the law of conservation of energy the total radiant power incident on the sample must equal the sum of the radiation power absorbed, transmitted and reflected (Osborne *et al.*, 1993a).

#### **6.3.3.1. Diffuse transmittance mode**

In the transmittance measurements reflection is eliminated so that the proportion of radiation attenuated by the sample may be measured as transmittance (Osborne, 2000). Proportionality exists between transmittance, the concentration of the absorbing species and the path length (Beer's law). Since the path length for the specific substance may be fixed by means of a static or flow-through sample cuvette or a pair of fiber-optic probes, the absorbance is linearly related

to the concentration, and a calibration can be developed using samples of known concentrations. Beer's law can only be used for clear transparent liquid samples when there is no light scattering, because scattering changes the path length through which the radiation passes and the amount of scattering differs from sample to sample. When a sample like liquid whole milk is used the fat globules in the milk will scatter the light which makes Beer's law invalid. This is then known as diffuse transmittance (Coventry, 1988; Osborne, 2000) (Figure 7a). The wavelength region normally used for diffuse transmittance measurements is 700-1100 nm. Diffuse transmittance measurements are usually used to analyse liquid samples but can also be used to measure solid samples such as meat, cheese and whole grain (Penner, 1994).

#### **6.3.3.2. Diffuse reflectance mode**

Diffuse reflectance measurements (Figure 7b) are used to analyse solid or granular samples (Penner, 1994). With diffuse reflectance measurements the amount of scattering for these samples that transmits through 1 cm is so small that the path length for the wavelength region between 1100-2500 nm becomes large and most of the radiation is reflected (Osborne, 2000).

#### **6.3.3.3. Transflectance mode**

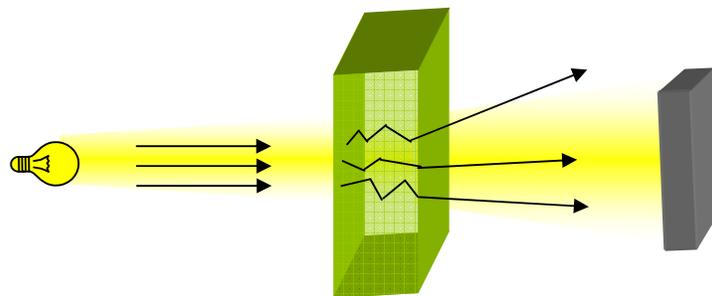
Diffuse reflectance and transmittance modes can be combined to form a transflectance mode (Figure 7c). It can be used to measure turbid liquids by placing the sample between the quartz window (radiation source) and the diffuse reflecting material such as a transflectance cover. The radiation is transmitted through the sample, reflected from the transflectance cover and then transmitted back through the sample. Some radiation will be scattered as it transmits through the sample. The light that is backscattered will then also transmit back with the light reflected from the transflectance cover (Stark *et al.*, 1986).

#### **6.3.3.4. Interactance mode**

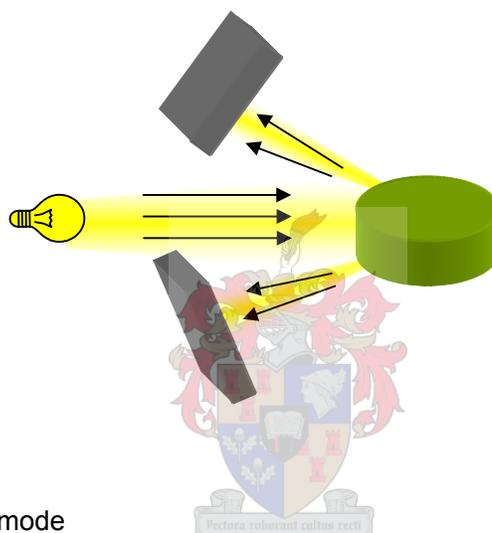
Transmittance and reflectance can also be combined to form an interactance mode that will illuminate and detect at laterally separated points on the sample's surface. A fiber-optic probe is normally used. It is very useful for the analysis of large samples such as whole fruit (Osborne, 2000).

#### **6.3.4. Detectors**

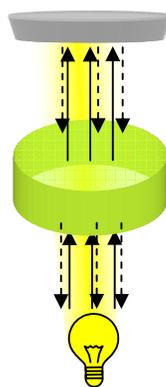
IR detectors can be differentiated according to their spectral response, their speed of response and the minimum amount of radiant power they can detect (McClure, 2001). There are two broad categories of IR detectors according to operation principles, namely thermal detectors and photon detectors of which the latter are used in NIR applications (Osborne *et al.*, 1993a). For photon detectors it is necessary for the incident radiation to be strong enough to liberate charge carriers from either the crystal lattice (intrinsic detectors) or from impurities intentionally



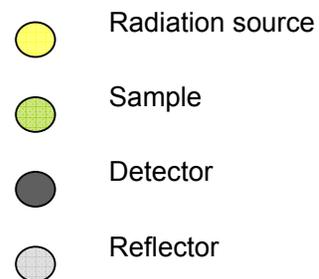
a) Diffuse transmittance mode



b) Diffuse reflectance mode



c) Transflectance mode



**Figure 7** Sample presentation modes (Osborne, 2000).

added to the host crystal (extrinsic detectors) (McClure, 2001).

The detection devices most widely used for NIRS analysis can be divided into two groups, i.e. single and multichannel detectors. Single channel detectors include lead-salt semiconductors, lead sulphide (PbS) used for the range 1100-2500 nm, lead selenide (PbSe) used for 2500-3500 nm, epitaxially-grown indium gallium arsenide (InGaAs) used for 800-1700 nm and silicon detectors used for 400-1100 nm (Osborne *et al.*, 1993a; Blanco & Villarroya, 2002).

Multi-channel detectors comprise diode arrays, where several detection elements are arranged in rows, or charged coupled devices (CCDs), where several detection elements are arranged in planes. Multi-channel detectors can for this reason record many wavelengths at once. This type of detector has given rise to NIR-imaging spectroscopy where spectra are recorded by using cameras that can determine composition at different points in space and record the shape and size of the object. Making measurements at different wavelengths provides a three-dimensional image that is a function of the spatial composition of the sample and the irradiation wavelength used (Blanco & Villarroya, 2002).

#### **6.4. Calibration development**

Calibration is included in all scientific activities (Geladi, 2002) and provides the basic functions of analytical chemistry, i.e. separation, identification and quantification (Osborne, 2000). The aim of calibration is to develop a model that correlates the NIR spectra to the values obtained by the independent reference methods (Beebe & Kowalski, 1987). Calibration is therefore the relationship between the  $\log(1/R)$  or absorbance values for the amount of the component obtained from the set of samples determined by an NIR instrument and the values for the amount of the component determined by the reference method and is expressed as an approximation involving some form of regression equation (Hruschka, 2001). The calibration can then be used for a new set of samples to determine the amount of a component in that sample.

##### **6.4.1. Calibration data set**

In the development of a calibration set it is crucial to have a sufficient number of samples (Honigs *et al.*, 1984). The samples should include all possible sources of variation that may occur at any stage and time of the existence of that product. Some sources of variation include the growing location, season, chemical composition, physical texture, bulk density after grinding, sample type (including diversity), colour, texture, storage conditions, temperature, humidity of work area, processing conditions, stage of maturity of material being analysed and presence of foreign materials (Williams, 2001). The variation should be as large as possible in all directions. Failure to include all sources of variation may lead to outliers in later analyses (Næs & Isaksson, 1994).

For many materials, sample preparation may be time-consuming and is very important for both reference and NIR reflectance testing and can account for 60-70% of the overall error of the testing. This regularly involves grinding to reduce the size of a sample, but also includes the preparation of an uniform slurry or other forms of preparations that makes direct testing or testing in a sample cell possible (Williams & Stevenson, 1990). The same sample ground to different particle sizes will result in substantially different spectra concluding that particle size can be a function of wavelength (Grant *et al.*, 1987; Osborne, 2000).

Water is the most important compound of all material in the agricultural and food industries which may affect NIRS calibration development (Williams, 2001). Strong absorbance by water in the 1450 and 1940 nm areas dominate the spectra of high-moisture foods. In cases where NIRS wavelength selection is affected by the water absorbance bands, reference analyses must also take moisture content into consideration (Williams & Stevenson, 1990). Where the moisture content of the sample changes during processing, it is important to take that into account to ensure that the right NIRS prediction values are correlated to the correct reference measurements (Williams, 2001).

#### **6.4.2. Reference method**

Reference data are necessary during calibration because the NIR instrument reports data in terms of the reference data. The reference methods are those that have been used before the introduction of NIRS and will always be more accurate than NIRS. Reference methods include for example the Kjeldahl test in measuring the protein content, the Dumas procedure which measures the nitrogen present (Williams, 2001) and HPLC which measures compounds such as aspalathin in rooibos tea (Schulz *et al.*, 2003). Accuracy and reproducibility of reference methods are important. Reference data must be attached to the correct NIRS spectral data (Williams, 2001).

#### **6.4.3. Chemometrics**

Large amounts of spectral data are usually obtained from NIR instruments and yield useful analytical information (Osborne *et al.*, 1993a; Blanco & Villarroya, 2002). Spectral data also contain noise, uncertainties, variabilities, interactions, non-linearities and unrecognised features. Chemometrics are required to extract as much relevant information from it as possible (Wold, 1995).

##### **6.4.3.1. Pretreatments for NIR spectra**

Pretreatments of spectral data are usually required to reduce the noise, correct baseline variations, enhanced resolution and/or to normalise the data (Katsumoto *et al.*, 2001; Delwiche & Reeves, 2004).

The moving-average and Savitzky-Golay are the most common smoothing methods used to reduce high-frequency noise (Savitzky & Golay, 1964). High-frequency noise is associated with the detector and electronic circuits (Katsumoto *et al.*, 2001), whereas low-frequency noise can be associated with instrument drift during the scanning measurements due to sources like a poor interferometer in an FT-NIR instrument or poor detector electronics (Savitzky & Golay, 1964; Eriksson *et al.*, 1999). Other noise reduction methods include wavelets, which remove high- and low-frequency noise as well as localised noise caused by an occurrence like scattering, eigenvector reconstruction and artificial neural networks (ANN) (Katsumoto *et al.*, 2001).

Baseline correction methods comprise derivatives (Griffiths, 1987; Griffiths *et al.*, 1987) and multiplicative scatter correction (MSC) (Geladi *et al.*, 1985), which are the most popular methods used, as well as standard normal variate (SNV), de-trending (DT) (Barnes *et al.*, 1989) and orthogonal signal correction (OSC) (Sjöblom *et al.*, 1998; Wold *et al.*, 1998). Derivatives reduce most of the particle size effects (Osborne *et al.*, 1993a). Apart from baseline correction it has also been used for resolution enhancement, deconvolution of background (Osborne, 2000) and to reduce scatter effects (Isaksson & Næs, 1988). The one disadvantage of the derivative methods is that the signal-to-noise ratio gets worse each time a spectrum is differentiated (Katsumoto *et al.*, 2001).

MSC was developed with the aim of correcting light scattering variations in reflectance spectroscopy. MSC corrects spectra according to a simple linear univariate fit to a standard spectrum, estimated by least squares regression using the standard spectrum (Katsumoto *et al.*, 2001). The MSC algorithm proceeds by computing the mean spectrum of the sample set and rotating each sample spectrum so that a regression line drawn through it aligns with a regression line drawn through the mean spectrum (Osborne, 2000).

Besides derivative methods, difference spectra, mean centering and Fourier self-deconvolution are used to enhance the resolution (Katsumoto *et al.*, 2001). These methods are important in the separation of overlapping bands and explaining the existence of hidden bands (Noda, 1993; Ozaki & Noda, 2000).

Centering and normalisation methods include mean centering, vectors normalised to constant Euclidean norm and mean normalisation (Katsumoto *et al.*, 2001).

#### **6.4.3.2. Reduction of variables**

Due to the vast amount of spectral information, the large number of samples required to build classification and calibration models, and the high correlation in spectra there is a need for variable-reduction methods that allow the dimensions of the original data to be reduced to a few uncorrelated variables containing only relevant information from the samples (Blanco & Villarroya, 2002). Principal component analysis (PCA) is such a method which searches for directions of maximum variability in sample groupings and uses them as new axes called

principal components which then contain the relevant information in a reduced number of variables and can be used as new variables, instead of the original data, in following calculations (Cowe & McNicol, 1985; Blanco & Villarroya, 2002). This reconstruction of the spectra and examination of the individual components give more detail than the original data obtained through an examination of the original spectra (Cowe *et al.*, 1990). By using principal components it is possible to identify which chemical structures influence the spectral variation. PCA provides a simple procedure for regression modeling. No reference to any set of analytical values is necessary to examine spectral variation and by looking at the shapes of the components it is possible to see in which areas in the spectra the absorbance bands can be detected (Cowe & McNicol, 1985). PCA is therefore a method of data description and compression (Devaux *et al.*, 1988).

#### **6.4.3.3. Multivariate calibration methods**

A variety of multivariate analysis techniques can be used to extract the analytical information contained in NIR spectra. These methods are used to develop models, which are capable to predict the properties of unknown samples. There are mainly two groups of multivariate analysis methods dividing calibration into two parts, namely qualitative and quantitative analysis.

##### *Qualitative analysis*

Qualitative analyses can be useful to screen samples during early evaluation of processing stages. Data reduction, e.g. PCA is usually required before analysis. In some instances this step alone is sufficient to classify groups (Downey, 1996).

Qualitative multivariate analysis is collectively known as pattern-recognition methods (Osborne, 2000). It is possible to compare spectra and look for similarities or differences (Wüst & Rudzik, 2003). Usually a spectral library of known substances is built (which defines the mean and standard deviation of each sample type in multidimensional space) and then the spectrum of an unknown sample is compared to the spectral library to see if it belongs to it or not (Wüst & Rudzik, 2003). Other discriminant or classification methods include soft independent modeling of class analogy (SIMCA), artificial neural networks (ANNs), *K*-nearest neighbours analysis (*K*-NN), linear discriminant analysis (LDA), multiple discriminant analysis (MDA), factorial discriminant analysis (FDA) and canonical variates analysis (CVA) (Downey, 1996).

##### *Quantitative analysis*

Quantitative analysis is used to develop models to predict some properties of unknown samples or the quantities present in the sample (Blanco & Villarroya, 2002). The quantitative analysis

methods most often used are multiple linear regression (MLR), principal component regression (PCR) and partial least squares (PLS) regression (Beebe & Kowalski, 1987).

MLR, the simplest multivariate-regression method, can be defined as minimising the sum of the squares of the deviations of the predicted values from the true values (Beebe & Kowalski, 1987). MLR usually uses less than five spectral wavelengths (Blanco & Villarroya, 2002) and is therefore more often used with filter instruments and occasionally with instruments recording the whole spectra (Blanco *et al.*, 1998). It presumes concentration to be a function of absorbance, which involves the knowledge of the concentrations of the target analytes as well as all other components contributing to the overall signal (Blanco & Villarroya, 2002). This method will therefore be an useful choice when there is a linear relationship between the analytical signal and the concentration, when spectral noise is low and when the target components do not interact with other sample components (Blanco *et al.*, 1998).

PCR and PLS regression are the regression methods more often used in NIRS and can be used in specific spectral regions or the whole spectrum allowing more information to be included in the calibration model (Blanco & Villarroya, 2002). Several components in a sample can be determined at the same time (Blanco *et al.*, 1998). PCR uses the principal components provided by PCA to perform regression on the sample property to be predicted, while PLS finds the directions of greatest variability by considering both spectral and measured-property information, with the new axes called PLS components or PLS factors (Blanco & Villarroya, 2002). PCR and PLS are factor-based modeling procedures. When redefining the variables it is usually the aim to use the smallest number of factors that will describe the original variables best. The first principal component is the linear combination that has the smallest squared errors when used to estimate the original variables and will therefore best describe the variation in the samples. The second principal component will be less descriptive and each of the following factors will describe less of the information in the model-building phase (Beebe & Kowalski, 1987).

There are situations where there is no linear relationship between the spectral data and measured property as a result of instrumental factors or the physico-chemical nature of sample. Non-linear calibration methods, like the artificial neural networks (ANNs) which are iterative computational algorithms that allow experimental variables to be fitted to a specific response, can be used in such cases (Blanco *et al.*, 2000; Blanco & Villarroya, 2002).

#### **6.4.4. Validation**

When calibration is finished, it is important to determine the prediction ability of the calibration equation for a different sample set or for a whole population of samples. Such quantity is usually unknown and cannot be measured directly. Therefore, an estimate has to be calculated from a limited set of samples (Næs & Isaksson, 1991). The accuracy of the calibration is therefore verified by analysis of a validation sample set. Two methods normally used include

independent validation which uses an independent data set apart from the calibration data set (Esbensen, 2000) and cross validation which uses the calibration data set only. The latter delete one sample at a time and perform the calibration on the rest of the samples (Næs & Isaksson, 1991).

#### **6.4.5. Statistical evaluation**

“Statistics is the art of drawing conclusions from data, and making decisions, in the presence of variability” (Wold, 1995). This is the final step in calibration development and is needed for interpretation of the gathered data and evaluation of the efficiency and accuracy of the calibration model.

The statistics most often used are: The standard error of prediction (SEP) or the standard error of cross-validation (SECV); the bias; the coefficient of correlation ( $r$ ) and the ratio of standard error of performance to standard deviation (RPD) (Osborne *et al.*, 1993a; Williams, 2001) (Table 7). The SEP measures how well the calibration is going to perform in future analysis (Fearn, 2002) and should be as close as possible to the standard error of laboratory (SEL). It can be used to evaluate the accuracy of a calibration by indicating the variability in deviations of  $x$  (reference data) from  $y$  (NIR data). The bias gives the average amount by which the results differ. Together the SEP and the bias indicate and evaluate the overall accuracy of the prediction model. The SEP and bias should be as low as possible with a slope close to 1.0 (Williams, 2001). When cross-validation is done the accuracy of the calibration can be indicated using the SECV. Another statistic that enables the evaluation of an SEP in terms of the SD of the reference data is the RPD, which gives an indication of the efficiency of the calibration model (Williams & Sobering, 1993, Williams, 2001). The guidelines for the interpretation of the RPD value are given in Table 8. The recommended RPD value for the use of NIRS in quality control is 5.0 or more. For screening purposes a value more than 3.1 would be sufficient. The coefficient of correlation ( $r$ ) shows how good the NIR predicted data correlate with the reference data over the range of composition. The correlation coefficient should be high ( $>0.95$ ) to indicate a good correlation, while with a low value ( $\leq 0.80$ ) it is not possible to obtain consistently high accuracy by NIRS analysis. Table 9 gives guidelines for interpretation of  $r$ . For quality control analysis it is necessary to have a  $r$  value above 0.95 but for screening purposes a  $r$  value of 0.81 or more would be adequate.

#### **6.5. Applications of NIRS analysis**

NIRS can be applied in different areas in the food industry. These include analysis of raw material to ensure that the end product will meet the specifications, analysis of products at critical stages during processing to monitor the composition, analysis of finished products to confirm authentication, analysis of by-products to determine future sales and analysis of effluents and other wastes for pollution control (Williams & Stevenson, 1990).

**Table 7** Equations for statistical calculations.

Statistic	Equation	Recommendations
<b>SD<sup>a</sup></b>	$\sqrt{\sum y^2 - \frac{(\sum y)^2}{n}}$	
<b>SEL<sup>b</sup></b>	$\sqrt{\frac{\sum (y_1 - y_2)^2}{2n}}$	As small as possible
<b>SEP<sup>c</sup> / SECV<sup>d</sup></b>	$\sqrt{\frac{\sum_{i=1}^n (y_i - \hat{y}_i - BIAS)^2}{n-1}}$	As small as possible or close as possible to SEL value
<b>V-BIAS<sup>e</sup></b>	$\frac{1}{n} \sum_{i=1}^n (y_i - \hat{y}_i)$	As close to zero as possible
<b>R<sup>f</sup></b>	$\frac{\sum_{i=1}^{n_s} (\hat{y}_i - y_i)^2}{\sqrt{\sum_{i=1}^{n_s} (y_i - \hat{y}_i)^2}}$	See Table 8
<b>RPD<sup>g</sup></b>	$\frac{SD_{\hat{y}}}{SEP} \text{ or } \frac{SD_{\hat{y}}}{SECV}$	See Table 9

<sup>a</sup> Standard deviation<sup>b</sup> Standard error of laboratory<sup>c</sup> Standard error of prediction<sup>d</sup> Standard error of cross validation<sup>e</sup> Bias of the validation set<sup>f</sup> Coefficient of correlation<sup>g</sup> Ratio of standard error of performance to standard deviation $y$  = reference value $\hat{y}$  = predicted value $y_i$  = reference value for the  $i^{\text{th}}$  sample $\hat{y}_i$  = NIR predicted values for the  $i^{\text{th}}$  sample $y_1$  and  $y_2$  = duplicate reference values $n$  = number of samples $t$  = number of terms in the model

**Table 8** Guidelines for interpretation of RPD (Williams, 2001).

RPD value	Classification	Application
0.0-2.3	Very poor	Not recommended
2.4-3.0	Poor	Very rough screening
3.1-4.9	Fair	Screening
5.0-6.4	Good	Quality control
6.5-6.8	Very good	Process control
8.1+	Excellent	Any application

**Table 9** Guidelines for interpretation of r (Williams, 2001).

r value	Interpretation
Up to 0.5	Cannot use in NIRS calibration
0.51-0.70	Poor correlation. Investigation is necessarily
0.71-0.80	Can be used for rough screening. More than 50% of variance in y (NIR data) accounted for by x (reference data)
0.81-0.90	Can be used for screening and some approximate calibrations
0.91-0.95	Can be used in most applications but with caution. More research is necessarily
0.96-0.98	Can be used in most applications, including quality assurance
0.99+	Can be used in any applications

Application of NIRS in the food industry was reported as early as 1938 in gelatine studies (Ellis & Bath, 1938). It was originally limited to grain analysis but today both qualitative and quantitative applications are widely used in many fields including dairy (Wüst & Rudzik, 2003; Blazquez *et al.*, 2004), meat and fish (Wold & Isaksson, 1997; Cozzolino & Murray, 2004; Realini *et al.*, 2004;), fruit and vegetables (Kawano *et al.*, 1992; Goula & Adamopoulos, 2003; Xing *et al.*, 2003; Walsh *et al.*, 2004), cereal and cereal products (Osborne, 1991; Wilson *et al.*, 1991; Windham *et al.*, 1993; Bao *et al.*, 2001), fats and oils (Man & Moh, 1998; Schulz *et al.*, 1998; Schulz *et al.*, 2003b), spreads and condiments (Iizuka & Aishima, 1999), tea (Grant *et al.*, 1987; Hall *et al.*, 1988; Osborne & Fearn, 1988; Schulz *et al.*, 1999; Luypaert *et al.*, 2003; Schulz, 2004; Zhang *et al.*, 2004), coffee (Pizarro *et al.*, 2004), confectionery (Tarkosová & Copíková, 2000), honey (García-Alvarez *et al.*, 2000); wine and grapes (Baumgarten, 1987; Chauchard *et al.*, 2004; Cozzolino *et al.*, 2004), distillation industry (Gomez-Cordoves & Bartolome, 1993), feed (Murray & Hall, 1983), forage (Park *et al.*, 1998; Stuth *et al.*, 2003), manure (Reeves, 2001), pharmaceuticals (Blanco *et al.*, 1998), diagnostic medicine (Hock *et al.*, 1997; Suto *et al.*, 2004), cosmetics (Grunewald *et al.*, 1998), tobacco (Hana *et al.*, 1997), soils (Demattê *et al.*, 2004), textiles (Cleve *et al.*, 2000), environmental studies (Iwamoto *et al.*, 1995; Büning-Pfaue *et al.*, 1998; Lister *et al.*, 2000), and fossil and other fuels (Lima *et al.*, 2004).

NIRS qualitative analysis has been used for authentication of meat (Downey & Beauchêne, 1997), tea (Budínová *et al.*, 1998), coffee (Downey *et al.*, 1994; Downey & BouSSION, 1996; Downey *et al.*, 1997), orange and other juices (Scotter *et al.*, 1992; Shilton *et al.*, 1998), rice (Osborne *et al.*, 1993b), oils (Bewig *et al.*, 1994; Sato, 1994) and wheat (Downey *et al.*, 1986; Downey, 1996; Downey, 1998). Quantitative analysis has also been used for authentication of food products. Applications include adulteration of milk with non-milk fat (Sato, 1994), milk fat with foreign fat (Sato *et al.*, 1990), Arabica with Robusta coffee (Downey & Spengler, 1996) and extra virgin olive oils with sunflower oil (Downey *et al.*, 2002).

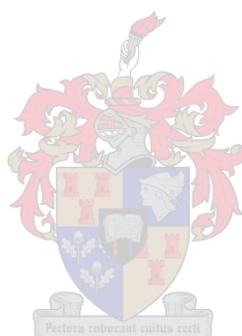
A major emphasis is placed on non-destructive quality evaluation methods for agricultural and biological materials that are useful in evaluating composition analysis (Gunasekaran & Irudayaraj, 2001). NIRS has become a useful tool for the analysis of plant species (Ren & Chen, 1999; Fuzzati, 2004) and herbal products (Laasonen, 2003). It has been used for quantification of quality parameters in green and black tea (Table 10). It has also been used to predict the aspalathin content in rooibos tea (Schulz *et al.*, 2003). There are numerous applications of the use of NIRS for discriminant analysis, including identification (Laasonen *et al.*, 2002) as well as classification of plant species (Lister *et al.*, 2000; Laasonen *et al.*, 2002), cultivation area (Woo *et al.*, 2002) and processing methods (Schulz *et al.*, 2003a) (Table 11). NIRS has also been used for discrimination of black teas of differing qualities (Osborne & Fearn, 1988).

**Table 10** NIRS quantitative applications in analysis of tea and medicinal plants.

Sample	Parameter	References
Green rooibos tea	aspalathin	Schulz <i>et al.</i> , 2003a
Green tea	total antioxidant capacity	Zhang <i>et al.</i> , 2004
	caffeine content, epigallocatechin gallate, epicatechin, total antioxidant capacity	Luypaert <i>et al.</i> , 2003
	total polyphenol content, epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate, caffeine, gallic acid, theobromine	Schulz <i>et al.</i> , 1999
	total nitrogen content	Ikegaya <i>et al.</i> , 1985
	caffeine content	Ikegaya <i>et al.</i> , 1987
Black tea	quality, theaflavin content, moisture	Hall <i>et al.</i> , 1988
	caffeine content	Schmidt <i>et al.</i> , 1998
American ginseng ( <i>Panax quinquefolium</i> ) root powder	Ginsenosides	Ren & Chen, 1999
Ginseng ( <i>Panax ginseng</i> C.A. Meyer) roots	ginsenosides Rg1, Rb1, Re	Corti <i>et al.</i> , 1990; Lin <i>et al.</i> , 2000
Asan ginseng ( <i>Panax ginseng</i> ) roots	moisture content	Ren & Chen, 1997
<i>Enchinacea</i> roots	echinacoside	Schulz <i>et al.</i> , 2002
Devil's claw ( <i>Harpagophytum procumbens</i> ) root	harpagoside, 8- $\rho$ -coumaroyl harpagide, moisture	Joubert <i>et al.</i> , 2005
Senna ( <i>Cassia senna</i> L.) leaves	sennosides A and B	Pudelko-Körner <i>et al.</i> , 1996
Primrose ( <i>Primula veris</i> L.) ethanolic extract of the flowers	3',4',5'-trimethoxyflavone	Schulz & Albroscheit, 1988
St. John's Wort extracts	hyperforin, I3,II8-biapigenin	Rager <i>et al.</i> , 2002

**Table 11** NIRS qualitative applications for tea and medicinal plants.

Types of application	Description of the application	References
Discrimination	unfermented and fermented roibos	Schulz <i>et al.</i> , 2003
Discrimination	black teas of differing quality	Osborne & Fearn, 1988
Classification	cultivation area of ginseng	Woo <i>et al.</i> , 2002
Classification	<i>Gliricidia</i> spp. provenances	Lister <i>et al.</i> , 2000
Discrimination	herbal medicines according to geographical origin	Woo <i>et al.</i> , 1999
Identification	<i>Echinacea purpurea</i> dried roots	Laasonen <i>et al.</i> , 2002
Detection	adulteration of <i>Echinacea purpurea</i> with <i>Enchinacea angustifolia</i> , <i>Enchinacea pallida</i> or <i>Parthenium integrifolium</i>	Laasonen <i>et al.</i> , 2002

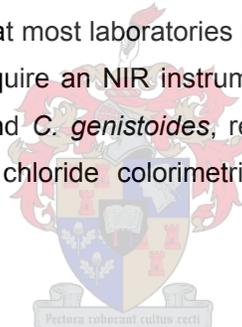


## 7. CONCLUSION

High quantities of aspalathin in rooibos and mangiferin and hesperidin in *C. genistoides* make the development of extracts of these plants for the nutraceutical and cosmetic industry viable. Furthermore, a lot of emphasis is placed on the importance of standardisation of marker or active compounds in nutraceutical products to ensure quality. Alternative methods to HPLC are required for routine screening of plant material for these phenolic compounds, as well as their quantification in the final product. Ideally, these methods should be both rapid and cheaper than HPLC.

NIRS showed good potential due to its fast analysis time and low running cost. NIRS applications for quantification of a range of phenolic compounds and antioxidant activity in herbal or medicinal plants indicate its possible use to predict aspalathin and nothofagin in rooibos, mangiferin and hesperidin in honeybush tea, as well as total polyphenols, antioxidant activity and soluble solids in both plants. NIRS has also been used in different classification analysis of herbal or medical plant material to authenticate plant material.

UV/Vis spectrophotometry and colorimetry have the potential to be used as alternative cheaper methods to NIRS, given that most laboratories possess spectrophotometers and not all laboratories have the capital to acquire an NIR instrument. The high quantities of aspalathin and mangiferin in green rooibos and *C. genistoides*, respectively, make the use of ultraviolet spectroscopy and the aluminium chloride colorimetric method to estimate the respective compounds, possible.



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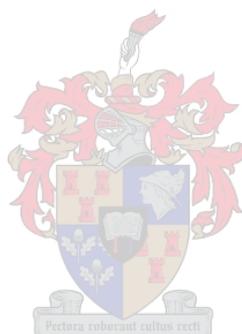
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## CHAPTER 3



**Quantification of the major phenolic compounds, soluble solid content and total antioxidant activity of green rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia genistoides*) by means of near infrared spectroscopy**

## CHAPTER 3

**QUANTIFICATION OF THE MAJOR PHENOLIC COMPOUNDS, SOLUBLE SOLID  
CONTENT AND TOTAL ANTIOXIDANT ACTIVITY OF GREEN ROOIBOS  
(*ASPALATHUS LINEARIS*) AND HONEYBUSH (*CYCLOPIA GENISTOIDES*) BY  
MEANS OF NEAR INFRARED SPECTROSCOPY**

**Abstract**

Near infrared spectroscopy (NIRS) was used to develop calibration models to predict aspalathin, nothofagin and dihydrochalcone contents of dried, green rooibos, and mangiferin and hesperidin contents of dried, green *Cyclopia genistoides* plant material, as well as of their respective water extracts. Calibration models were also developed to predict the total polyphenol content (TPC), soluble solid content (SSC) and total antioxidant activity (TAA) of green rooibos and *C. genistoides* water extracts. NIRS can effectively predict the aspalathin content of dried, green rooibos with a standard error of prediction (SEP) and correlation coefficient ( $r$ ) of  $0.45 \text{ g}\cdot 100 \text{ g}^{-1}$  and 0.92, respectively, and the dihydrochalcone content of rooibos with an SEP of  $0.49 \text{ g}\cdot 100 \text{ g}^{-1}$  and  $r$  of 0.93. Extending the aspalathin content range of the sample set with samples manipulated with a rooibos extract powder containing a high concentration of aspalathin ( $15.95 \text{ g}\cdot 100 \text{ g}^{-1}$ ) and nothofagin ( $1.94 \text{ g}\cdot 100 \text{ g}^{-1}$ ), slightly less accurate models were obtained for the aspalathin (SEP =  $0.53 \text{ g}\cdot 100 \text{ g}^{-1}$ ;  $r = 0.93$ ) and dihydrochalcone (SEP =  $0.57 \text{ g}\cdot 100 \text{ g}^{-1}$ ;  $r = 0.94$ ) contents. The NIRS calibration model developed for nothofagin content (SEP =  $0.10 \text{ g}\cdot 100 \text{ g}^{-1}$ ;  $r = 0.84$ ) (extended range: SEP =  $0.10 \text{ g}\cdot 100 \text{ g}^{-1}$ ;  $r = 0.88$ ) of dried, green rooibos, as well as the calibration models developed for mangiferin (SEP =  $0.46 \text{ g}\cdot 100 \text{ g}^{-1}$ ;  $r = 0.86$ ) and hesperidin (SEP =  $0.38 \text{ g}\cdot 100 \text{ g}^{-1}$ ;  $r = 0.85$ ) contents of dried, green *C. genistoides* can be used for screening purposes. The current transmittance NIRS calibration models developed for TPC, SSC and the TAA of rooibos and *C. genistoides* water extracts did not give acceptable results.

**Introduction**

The antioxidant properties of green (unoxidised) rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia spp.*) have led to an increasing interest in the development of polyphenol-enriched extracts for the nutraceutical and cosmetic industries. Aspalathin, a potent antioxidant (Von Gadow *et al.*, 1997; Schulz *et al.*, 2003; Joubert *et al.*, 2004) and unique to rooibos (Koeppen & Roux, 1966), comprises between  $4.45$  and  $11.9 \text{ g}\cdot 100 \text{ g}^{-1}$  of the plant material and is the main contributor to the total polyphenol content (TPC) (ca. 35-68%) and total antioxidant activity (TAA) (22-57%) of green rooibos water extracts (Schulz *et al.*, 2003). Nothofagin, another structurally related dihydrochalcone, is present in lower quantities ( $0.34$ - $1.33 \text{ g}\cdot 100 \text{ g}^{-1}$ ) in the plant material (Schulz *et al.*, 2003), but still in substantially higher quantities than the other

flavonoids (Joubert, 1996; Bramati *et al.*, 2003; Schulz *et al.*, 2003). Nothofagin has comparable radical scavenging activity to aspalathin (Snijman *et al.*, 2004) and the higher the nothofagin and particularly the aspalathin content, the more valuable the plant material.

Mangiferin and hesperidin are the two major phenolic compounds present in green *C. genistoides*. The mangiferin content varies between 4.93 and 6.37 g.100 g<sup>-1</sup>, while hesperidin, present in lower quantities, varies between 2.23 and 5.21 g.100 g<sup>-1</sup> in the plant material (Joubert *et al.*, 2003). The contribution of mangiferin to the TAA of *C. genistoides* water extracts is ca. 22%, while that of hesperidin is substantially lower (<1%), not only due to its lower concentration, but also because it is less potent as radical scavenger in the ABTS assay than mangiferin (Richards, 2002). However, the anti-inflammatory properties of hesperidin (Guardia *et al.*, 2001; Rotelli *et al.*, 2003) add to its value in nutraceutical products.

High quantities of these phenolic compounds in the plant material are required for the manufacturing of enriched extracts for the nutraceutical and cosmetic industries. Rapid analysis or screening of raw plant material at an early stage of processing is therefore a prerequisite to identify plant material with sufficient quantities of these compounds to ensure that extracts meet specifications. Minimum levels, i.e. 4 g.100 g<sup>-1</sup> aspalathin and mangiferin in dried, green rooibos and honeybush plant material, respectively, have been set by a major extract manufacturer in order to obtain extracts with levels of 15 g.100 g<sup>-1</sup> and higher of these compounds (B. Weinreich, Raps and Co., Germany, personal communication, 2005).

Standardisation of minimum levels of individual compounds is necessary to ensure consistency and efficacy of herbal extracts (He, 2000; Huie, 2002). Quantification of marker or pharmacologically active compounds in herbal products has been used to evaluate the quality of herbal medicines (Liang *et al.*, 2004), i.e. hyperoside in St. John's Wort (Rager *et al.*, 2002), harpagoside in devil's claw (*Harpagophytum procumbens*) (Guillerault *et al.*, 1994), enchinacoside in *Enchinacea* (Perry *et al.*, 2001) and flavonoids in *Ginko biloba* (Deng & Zito, 2003).

No quality standards or requirements relating to the composition or active compounds exist for green rooibos or honeybush (Anon., 2000). TPC and TAA, as determined with the Folin-Ciocalteu and ABTS assays, respectively, are used by local extract manufacturers to standardise products (E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, personal communication, 2004). A German manufacturer of green rooibos extracts standardises its product on the basis of aspalathin content as determined by HPLC (B. Weinreich, Raps and Co., Germany, personal communication, 2005). Due to the cost involved, local manufacturers of rooibos extracts do not use HPLC. Although HPLC is an accurate method to quantify individual compounds, it is time-consuming, labour intensive and requires large quantities of reagents (Huie, 2002; Laasonen, 2003) and expensive equipment (Harborne, 1998). This clearly emphasises the need for a rapid and more economical technique for quality control.

Near infrared spectroscopy (NIRS) is not only a rapid, easy-to-use and non-destructive analytical technique (Day & Fearn, 1982), but also has low running costs due to minimum sample preparation and furthermore no chemicals are required. NIRS analyses have been used successfully to develop calibration models to predict compounds in herbal and medicinal plant species, i.e. echinacosides in *Enchinacea* roots (Schulz *et al.*, 2002), harpagoside in devil's claw (*H. procumbens*) root (Joubert *et al.*, 2005) and sennosides A and B in *Senna* (*Cassia senna* L.) leaves (Pudelko-Körner *et al.*, 1996). It has also been used for the quantification of total polyphenol, epicatechin, epicatechin gallate, epigallocatechin gallate, caffeine, theogalline, gallic acid, theobromine and epigallocatechin contents (Schulz *et al.*, 1999; Luyypaert *et al.*, 2003; Schulz, 2004) and TAA of green tea (Luyypaert *et al.*, 2003; Zhang *et al.*, 2004), as well as theaflavin, moisture (Hall *et al.*, 1988) and caffeine contents (Schmidt *et al.*, 1998) of black tea. The aspalathin content of rooibos tea has been quantified using NIRS, but on a sample set with limited seasonal variation (Schulz *et al.*, 2003). This merits further improvement of the robustness of the calibration model by including plant material with more variation. There is no evidence of NIRS being used to predict the nothofagin and dihydrocalcone contents of dried, green rooibos, mangiferin and hesperidin contents of dried, green *C. genistoides*, or the TPC, SSC and TAA of either green rooibos or *C. genistoides* water extracts.

The objectives of this study were to:

- develop NIRS calibration models for the prediction of aspalathin, nothofagin and dihydrocalcone contents of dried, green rooibos plant material and the mangiferin and hesperidin contents of dried, green *C. genistoides* plant material; and
- develop NIRS calibration models for the prediction of aspalathin content of green rooibos water extracts, mangiferin and hesperidin contents of green *C. genistoides* water extracts and TPC, SSC and TAA of both green rooibos and *C. genistoides* water extracts.

## Materials and methods

### Chemicals

Purified aspalathin (Batch A/02) was isolated by Petra Snijman at the PROMEC (Programme on Mycotoxins and Experimental Carcinogenesis) Unit of the Medical Research Council (MRC, Bellville, South Africa). Mangiferin, hesperidin (97%), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), dimethyl sulfoxide (DMSO) (99.5%), ascorbic acid, potassium persulphate, gallic acid, acetonitrile R Chromasolv® (Riedel-de Haën) for liquid chromatography and glacial acetic acid (min. 99.8%) (Riedel-de Haën) were obtained from Sigma-Aldrich (Cape Town, South Africa). ABTS (2,2'-azinobis(3-ethyl-benzothiazoline-6-sulphonate)) was obtained from Roche (Johannesburg, South Africa). Folin-Ciocalteu's phenol reagent, methanol and sodium carbonate were purchased from Merck Chemicals (Pty) Ltd (Cape Town, South Africa). Ethanol (99%) was obtained from Illovo (Cape Town, South Africa). Deionised water refers to

water treated with a Modulab Water Purification System (Separations, Cape Town, South Africa). For HPLC analysis, deionised water was purified by means of a Milli-Q 185 Académic Plus water purification system (Microsep (Pty) Ltd, Bellville, South Africa).

#### *Green rooibos plant material*

A large selection of dried, green rooibos plant material ( $n = 340$ ) harvested during different years (1984, 1999, 2002 and 2004) and from different production areas (Citrusdal and Clanwilliam, South Africa) was obtained from the sample collection of ARC Infruitec-Nietvoorbij. Fresh plant material was dried at 40°C to ca. 8-10% moisture content and ground with a Retsch mill (1 mm sieve). Samples harvested during 1984 and 2004 included young growth, older plant material and flowers which provided variation that could be obtained during the growth cycle. Included in the sample set were samples consisting of only toppings (i.e. the first harvest of a newly-established plantation) and samples consisting of either leaves or stems. The aspalathin and nothofagin contents of nine samples were increased by adding varying amounts of a high aspalathin (15.95 g.100 g<sup>-1</sup>) and nothofagin (1.94 g.100 g<sup>-1</sup>) content rooibos extract powder to extend the range of the aspalathin and nothofagin contents.

#### *Green C. genistoides plant material*

The dried, green *C. genistoides* plant material ( $n = 240$ ) was obtained from ARC Infruitec-Nietvoorbij (harvested during 2001, 2003, 2004 and 2005). The fresh plant material was dried at 40°C to ca. 8-10% moisture content and ground with a Retsch mill (1 mm sieve). Samples harvested during 2001, 2003 and 2004 represented two types, i.e. West Coast and Overberg and both types were harvested in the Pearly Beach area. Harvesting took place approximately every 5-6 weeks from 28/03/01 to 11/07/01 and every 6-8 weeks from 31/03/2003 to 26/01/2004 to include variation that could occur during the growth cycle. Some plants were also re-harvested on 26/01/2004 to obtain young regrowth. The 2005 samples represented the West Coast type harvested at Reins Farm, Albertinia. Included were also samples ( $n = 10$ ) comprising of either leaves or stems which extended the range of the mangiferin and hesperidin contents.

#### *Preparation of green rooibos acetonitrile-water extracts*

Acetonitrile-water extracts of green rooibos were prepared in duplicate by extracting ca. 0.5 g ground, dried plant material with 15 mL acetonitrile and 30 mL boiling deionised water. The samples were heated on a steam bath for 15 min, cooled to room temperature and filled to volume (100 mL) with 1% (w/v) ascorbic acid solution. The extracts were filtered through Whatman No. 4 filter paper, followed by 0.45 µm Millipore Millex-HV hydrophilic PVDF syringe filters (25 mm diameter) (Microsep (Pty) Ltd, Bellville, South Africa) directly into HPLC auto sampler vials for immediate HPLC analysis.

#### *Preparation of green rooibos water extracts*

Water extracts of 102 green rooibos samples that covered a range of 0.15-10.59% aspalathin content (based on aspalathin content in acetonitrile-water extracts) were prepared by extracting ca. 0.5 g ground, dried plant material with 45 mL boiling deionised water. The samples were heated on a steam bath for 10 min, cooled to room temperature and filled to volume (100 mL) with deionised water. After filtration through Whatman No. 4 filter paper, aliquots of each extract were stored at -18°C until analysed (HPLC, TPC, TAA and NIRS).

#### *Preparation of green C. genistoides methanol extracts*

Methanol extracts of green *C. genistoides* were prepared in duplicate by extracting ca. 0.5 g ground, dried plant material with 50 mL methanol (100%). The samples were heated in a water bath at 64°C for 30 min, cooled to room temperature and filled to volume (100 mL) with methanol. The extracts were filtered through Whatman No. 4 filter paper, followed by 0.45 µm Millipore Millex-HV hydrophilic PVDF syringe filters (25 mm diameter) (Microsep (Pty) Ltd, Bellville, South Africa) directly into HPLC auto sampler vials for immediate HPLC analysis.

#### *Preparation of green C. genistoides water extracts*

Water extracts of green *C. genistoides* were prepared by extracting ca. 2 g ground, dried plant material with 150 mL boiling deionised water for 2 min with continuous stirring (magnetic stirrer) and filtered through Whatman No. 4 filter paper. Aliquots of each extract were stored at -18°C until analysed (HPLC, TPC, TAA and NIRS).

#### *Soluble solid content of green rooibos and C. genistoides water extracts*

The SSC of the water extracts was determined gravimetrically in duplicate by evaporating 15 and 20 mL rooibos and *C. genistoides* water extracts, respectively, in nickel moisture dishes on a steam bath until dry. It was subsequently dried at 100°C for 60 min and results were expressed as mg.100 mL<sup>-1</sup> extract.

#### *Quantification of aspalathin, nothofagin, dihydrochalcone, mangiferin and hesperidin by HPLC*

The green rooibos water-acetonitrile and *C. genistoides* methanol extracts were immediately analysed upon preparation. The water extracts of rooibos and *C. genistoides*, upon defrosting, were diluted with a 1% (m/v) ascorbic acid solution (rooibos: 2000 µL + 400 µL; honeybush: 3000 µL + 300 µL). The extracts were filtered through 0.45 µm Millipore Millex-HV hydrophilic PVDF syringe filters (25 mm diameter) (Microsep (Pty) Ltd, Bellville, South Africa) directly into HPLC auto sampler vials. Quantification of aspalathin and nothofagin in the green rooibos water-acetonitrile and water extracts and mangiferin and hesperidin in the green *C. genistoides* methanol and water extracts were conducted in duplicate by reversed-phase HPLC, using a LaChrom (Merck/Hitachi) HPLC system (Merck, Cape Town, South Africa) which comprised of

an L7000 interface, L7400 UV detector, L7100 pump and L7200 autosampler. An L7450 DAD detector was used to confirm peak identify. Separation was performed on a Phenomenex Synergy MAX-RP C12 80 Å column (TMS endcapping, 4 µm, 150 x 4.5 mm ID) (Separations, Johannesburg, South Africa) at 30°C, using the solvent gradient program of Schulz *et al.* (2003) for aspalathin and nothofagin and that of Joubert *et al.* (2003) for the mangiferin and hesperidin determinations. The solvents used for separation were 2% acetic acid and acetonitrile at a flow rate of 1 mL.min<sup>-1</sup>. A Phenomenex Degasser Model DG-4400 (Separations, Cape Town, South Africa) was used to degas solvents in-line.

Measurements were made at 288 nm for aspalathin and nothofagin and at 320 nm and 288 nm for mangiferin and hesperidin, respectively. Ten µL of the samples were injected for the analysis. Standard dilution series of aspalathin (concentration range 0.08-4.0 µg injected; R<sup>2</sup> = 1), mangiferin (concentration range 0.01-6.16 µg injected; R<sup>2</sup> = 1) and hesperidin (concentration range 0.01-4.77 µg injected; R<sup>2</sup> = 1) were analysed weekly. An absorbance ratio in terms of aspalathin (Joubert, 2004; unpublished data) was used to calculate nothofagin. The dihydrochalcone content was calculated as the sum of the aspalathin and nothofagin contents. Peak area integration was done with the LaChrom Multisystem Software D700. Results were expressed on an as-is basis (plant material) and slight differences in moisture content were not taken into account.

#### *Total polyphenol content of green rooibos and C. genistoides water extracts*

The TPC of green rooibos and *C. genistoides* water extracts was determined in duplicate using a 20:1 scaled-down version (final volume 5 mL) of the Folin-Ciocalteu method of Singleton and Rossi (1965). Gallic acid was used to prepare a calibration curve (concentration range 10.16-101.61 µg.mL<sup>-1</sup>; R<sup>2</sup> = 1) and the TPC of the samples was expressed as mg gallic acid equivalents (GAE).100 mL<sup>-1</sup> extract. The extracts were diluted to obtain absorbance within the range of the calibration curve. Measurements were done in 1 cm path length disposable cuvettes (Plastibrand PS-cuvette, Sigma-Aldrich, Cape Town, South Africa) at 765 nm using a GBC UV/Vis 911A spectrophotometer (Wirsam, Cape Town, South Africa).

#### *Total antioxidant activity of green rooibos and C. genistoides water extracts*

TAA of the green rooibos and *C. genistoides* water extracts was determined using the ABTS decolorisation assay of Re *et al.* (1999). The ABTS radical cation (ABTS<sup>•+</sup>) was generated by reacting 7 mmol ABTS water solution with 2.45 mmol potassium persulfate overnight, followed by dilution with ethanol to an absorbance of 0.70 (± 0.02) at 734 nm. The extracts were diluted with deionised water to give 40-60% inhibition of the ABTS<sup>•+</sup> solution after 4 min at 37°C. The TAA of the samples was calculated from a calibration curve prepared with Trolox (concentration range 0.003-0.02 µmol.mL<sup>-1</sup>; R<sup>2</sup> = 1). Measurements were done in 1 cm path length disposable

cuvettes (Plastibrand PS-cuvette, Sigma-Aldrich, Cape Town, South Africa) at 734 nm using a GBC UV/Vis 911A spectrophotometer (Wirsam, Cape Town, South Africa).

#### *Near infrared spectroscopy measurements*

##### Dried, green rooibos and *C. genistoides* plant material

A Büchi NIRLab N-200 Fourier transform near infrared (FT-NIR) spectrophotometer with NIRLabWare (version 3.0) near infrared (NIR) measurement software was used to perform the NIRS measurements in diffuse reflectance mode. The ground, dried plant material was presented to the instrument in rotating glass petri-dishes and the NIR spectra collected from 1000-2500 nm at a resolution of 12 cm<sup>-1</sup> resulting in 1557 data points.

##### Green rooibos and *C. genistoides* water extracts

Using the Perkin Elmer IdentiCheck™ FT-NIR spectrophotometer, Spectrum IdentiCheck NIR measurement software (version 2.0) and a 0.2 mm quartz cuvette, spectra of water extracts of green rooibos and *C. genistoides* were collected in diffuse transmittance mode from 1100-2500 nm with 2 nm intervals at a resolution of 16 cm<sup>-1</sup> resulting in 701 data points.

##### Purified aspalathin, mangiferin and hesperidin

A Perkin Elmer IdentiCheck™ FT-NIR spectrophotometer with Spectrum IdentiCheck NIR measurement software (version 2.0) was used to perform the NIRS measurements. The purified aspalathin, mangiferin and hesperidin powders were presented to the instrument in 4 mL Chromocol glass vials and the NIRS spectra were collected in diffuse reflectance mode from 1100-2500 nm with 2 nm intervals at a resolution of 16 cm<sup>-1</sup> resulting in 701 data points. For comparative purposes one sample of dried, green rooibos and *C. genistoides* each was analysed.

#### *Spectral characterisation*

Spectral characterisation was performed on the raw spectra (no pre-treatment). MSC pre-treated FT-NIR spectra were investigated by means of principal component analysis (PCA) using Büchi NIRCal (version 4.21) software. Loading plots of principal components (PCs) 1-4 were constructed and used to describe the physical and chemical influences, causing spectral variation.

#### *NIRS calibration model development*

Büchi NIRCal (version 4.21) was used for calibration model development. The pretreatments, multivariate calibration methods and number of samples in the calibration and validation sets used for each respective compound are summarised in Table 1. The calibration models were validated by means of independent validation and no outliers were removed.

**Table 1** Summary of pretreatments, multivariate calibration methods and number of samples in the calibration and validation sets used during calibration model development of the respective compounds in dried, green rooibos and *C. genistoides*.

Compound	Pretreatment(s)	Multivariate calibration method	Calibration set (n)	Validation set (n)
<b><u>Green rooibos plant material</u></b>				
Aspalathin, nothofagin and dihydrochalcone contents	Db1 <sup>a</sup>	PLS <sup>b</sup>	220	111
Aspalathin, nothofagin and dihydrochalcone contents (extended aspalathin and nothofagin contents)	n01 <sup>c</sup> , db1	PLS	227	113
<b><u>Green <i>C. genistoides</i> plant material</u></b>				
Mangiferin content	MSC <sup>d</sup>	PLS	160	80
Hesperidin content	Db1	PLS	160	80
<b><u>Green rooibos water extracts</u></b>				
Aspalathin and total polyphenol contents	No pre-treatment	PLS	69	33
Soluble solid content	MSC	PLS	69	33
Total antioxidant activity	No pre-treatment	PLS	69	32
<b><u>Green <i>C. genistoides</i> water extracts</u></b>				
Mangiferin content	No pre-treatment	PLS	85	42
Hesperidin content	Db1	PCR <sup>e</sup>	85	42
Total polyphenol content	No pre-treatment	PCR	85	42
Soluble solid content	Db1	PLS	85	42
Total antioxidant activity	No pre-treatment	PLS	75	36

<sup>a</sup> First derivative

<sup>b</sup> Partial least squares regression

<sup>c</sup> Normalisation (between 0 and 1)

<sup>d</sup> Multiplicative scatter correction

<sup>e</sup> Principal component regression

The accuracy of the calibration models was expressed by means of the standard error of prediction (SEP) (Chapter 2, p. 44), the correlation coefficient ( $r$ ) and the ratio of SEP to standard deviation of the validation set (RPD) (Chapter 2, p. 44), which is an indication of the efficiency of a calibration. The goal of model development is to obtain a calibration model with a low SEP, a high  $r$ , preferably above 0.91 and a RPD higher than 5. The SEP should also be as close as possible to the standard error of laboratory (SEL) (Chapter 2, p. 44).

## Results and discussion

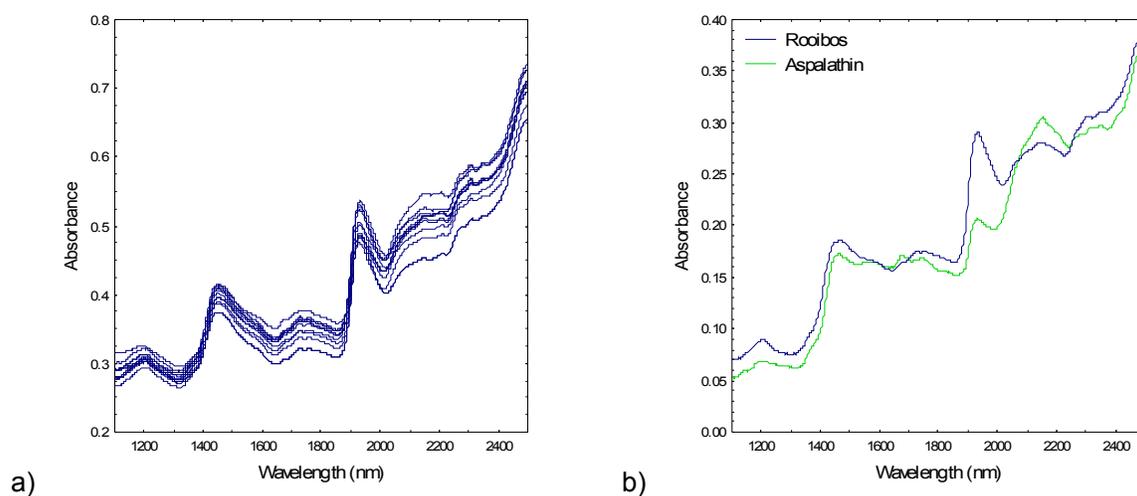
### *Spectral characterisation*

Typical raw (no-pretreatment) NIR spectra of ground, dried, green rooibos and *C. genistoides* plant material as well as that of purified aspalathin, mangiferin and hesperidin are shown in Figures 1 and 2. It is, to our knowledge, the first time that NIR spectra are presented for these pure compounds.

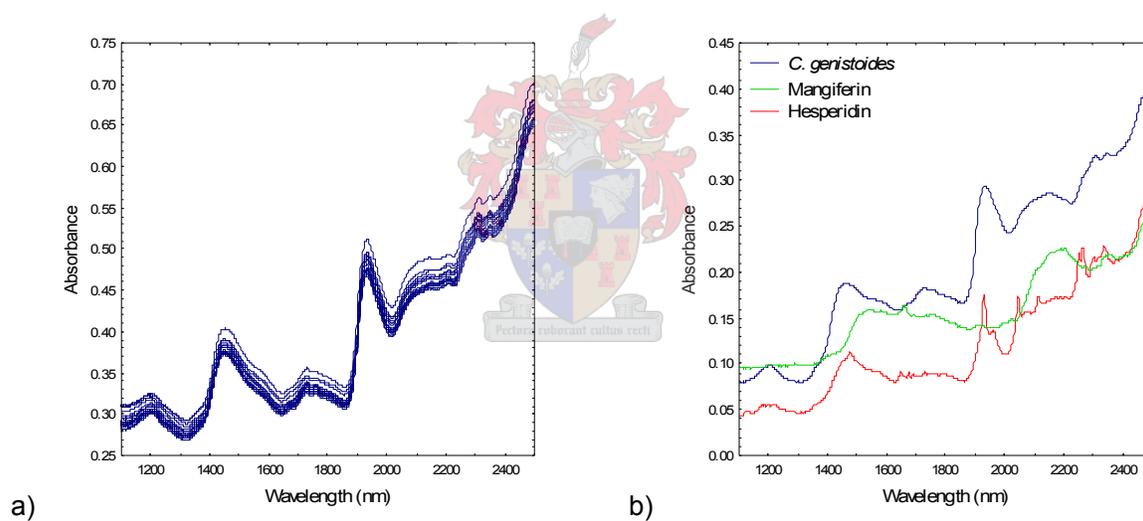
The spectra of both the green rooibos and *C. genistoides* plant material showed broad absorption bands at 1450 nm (O-H stretch first overtone, associated with water) and 1940 nm (O-H stretch and O-H deformation, associated with water) (Osborne *et al.*, 1993). A broad band is also observed at 2100 nm (2 x O-H deformation and 2 x C-O stretch) in both rooibos and *C. genistoides*. These bonds, (O-H and C-O) are abundant in aspalathin, nothofagin, mangiferin and hesperidin (Figure 3). This broad absorption band could, however, also be due to C-H stretch and C=O stretch bond vibrations which would normally occur at approximately 2200 nm. The C-H and C=O bonds are found in the structures of aspalathin, mangiferin and hesperidin. The C=O stretch second overtone would have been expected to overlap with the O-H stretch first overtone bond at 1940 nm (Osborne *et al.*, 1993).

The NIRS spectrum of the purified aspalathin shows absorptions at similar wavelengths than the spectra of the green rooibos plant material. The absorption at 2100 nm is particularly pronounced (2 x O-H deformation and 2 x C-O stretch). The mangiferin spectrum shows a broad band at *ca.* 2150 (2 x O-H deformation and 2 x C-O stretch as well as C=O stretch second overtone) which is also present in the *C. genistoides* spectrum but at a slightly shifted position towards 2100 nm. The hesperidin spectrum shows distinct absorption bands at 1450 (O-H stretch O-H deformation, associated with water), 1940 (O-H stretch O-H deformation, associated with water), 2050 (O-H stretch and O-H deformation), 2100 (2 x O-H deformation and 2 x C-O stretch) and 2250 nm (O-H stretch and O-H deformation), which are all bonds abundant in the hesperidin structure.

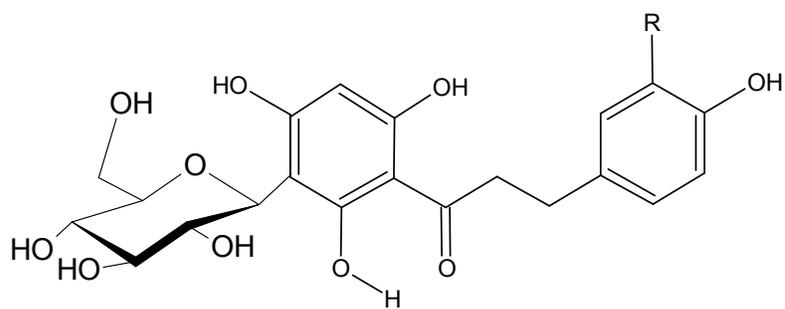
After exclusion of particle size variation by means of multiplicative scatter correction (MSC), the largest remaining contributor to spectral variation in the plant material was moisture content. The loading plots of the first two principle components (PCs) for both rooibos and *C. genistoides* plant material (Figure 4) show strong absorption at *ca.* 1940 nm (O-H stretch and O-H deformation). This wavelength region corresponds most strongly to the absorption of water



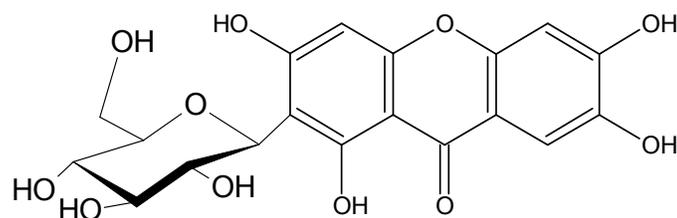
**Figure 1** a) Typical NIR spectra of ground, dried, green rooibos plant material and b) NIR spectra of purified aspalathin powder and a ground, dried, green rooibos plant material sample.



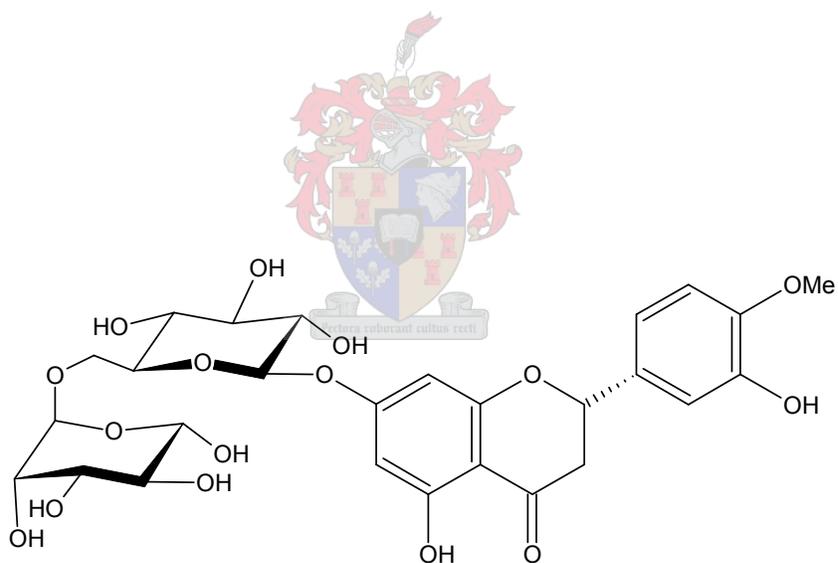
**Figure 2** a) Typical NIR spectra of ground, dried, green *C. genistoides* plant material and b) NIR spectra of mangiferin and hesperidin powder and a ground, dried, green *C. genistoides* plant material sample.



a)

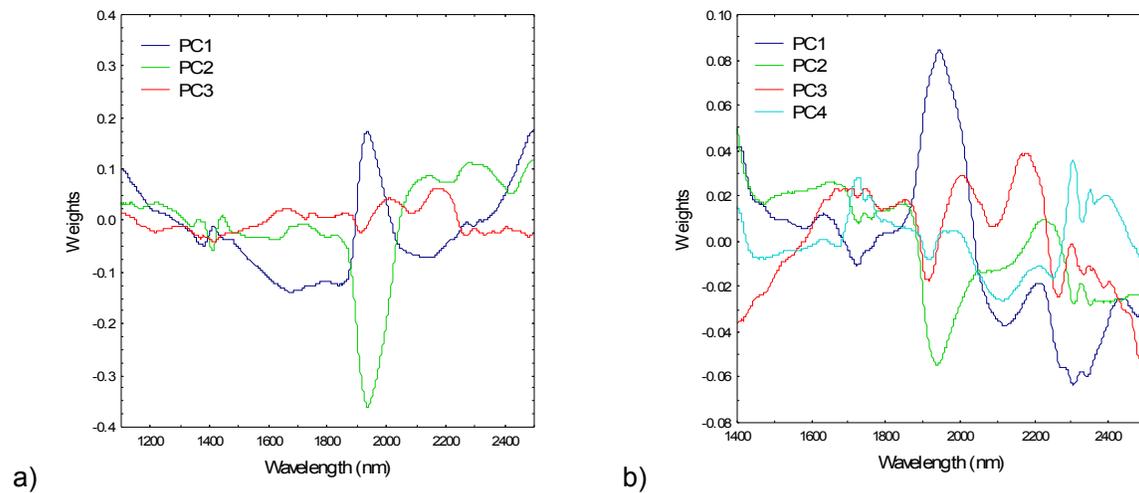


b)

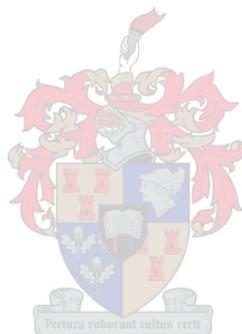


c)

**Figure 3** Molecular structures of a) aspalathin (R=OH) and nothofagin (R=H) present in rooibos (Joubert, 1996), and b) mangiferin and c) hesperidin present in *Cyclopia genistoides* (Brandt, 2004; Van der Merwe, 2004).



**Figure 4** Loading plots of a) the first three principal components of the MSC pretreated green rooibos plant material spectra and b) the first four principal components of the MSC pretreated green *C. genistoides* plant material spectra.



(or sample moisture) (Osborne *et al.*, 1993). The relative contributions of each PC to the explained variation are, as expected, large for the first few factors, with 96% of the variation explained by the first 3 factors (PC1 = 48%, PC2 = 45% and PC3 = 3%) for rooibos and 95% of the variation explained by the first 4 factors for *C. genistoides* (PC1 = 72%, PC2 = 15%, PC3 = 6% and PC4 = 2%).

PC1 and PC2 of the rooibos plant material show, apart from the largest weight at 1940 nm (O-H stretch and O-H deformation), weights at 1410 nm (O-H stretch first overtone) which is associated with the most abundant structural group of aspalathin and nothofagin, namely O-H (Figure 3). PC3, which accounted for 3% of the variation, shows absorption at ca. 2200 nm (C-H stretch and C=O stretch) which may be associated with the C=O group and C-H groups in both aspalathin and nothofagin.

PC1 and PC2 of the *C. genistoides* plant material shows, apart from the largest weight at 1940 nm, absorption at 2280 nm which could be related to the O-H stretch and C-C stretch, as well as the C-H stretch and C-H deformation bond vibrations present in mangiferin and hesperidin. PC3 has absorption bands at ca. 1910 nm (O-H stretch first overtone), 2000 (2 x O-H deformation and C-O deformation), 2200 (C-H stretch and C=O stretch), 2280 (C-H stretch and C-H deformation) and 2360 nm (O-H deformation second overtone), which are all related to the most abundant structural groups present in mangiferin and hesperidin (Figure 3). PC4 shows similar weights to that of PC3.

#### *NIRS calibration development*

#### NIRS calibration models for aspalathin, nothofagin and dihydrochalcone contents of dried, green rooibos plant material and the mangiferin and hesperidin contents of dried, green *C. genistoides* plant material

A summary of the reference data for aspalathin, nothofagin and dihydrochalcone contents of the dried, green rooibos plant material and mangiferin and hesperidin contents of the dried, green *C. genistoides* plant material is given in Table 2. Typical HPLC chromatograms of a green rooibos acetonitrile-water extract and a *C. genistoides* methanol extract are depicted in Figure 5. The distribution of the aspalathin, nothofagin and dihydrochalcone contents are depicted in Figure 6 and that of the mangiferin and hesperidin contents in Figure 7. A Gaussian distribution was noticed for all of the compounds due to the difficulty to obtain an adequate number of samples with low and high contents of the active compounds. It is likely that this type of distribution will produce more accurate predictions near the mean and less accurate predictions for samples near the extremes of the range (Williams, 2001).

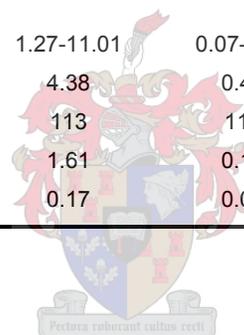
**Table 2** Summary of the reference data for aspalathin, nothofagin and dihydrochalcone contents of dried, green rooibos plant material and mangiferin and hesperidin contents of dried, green *C. genistoides* plant material.

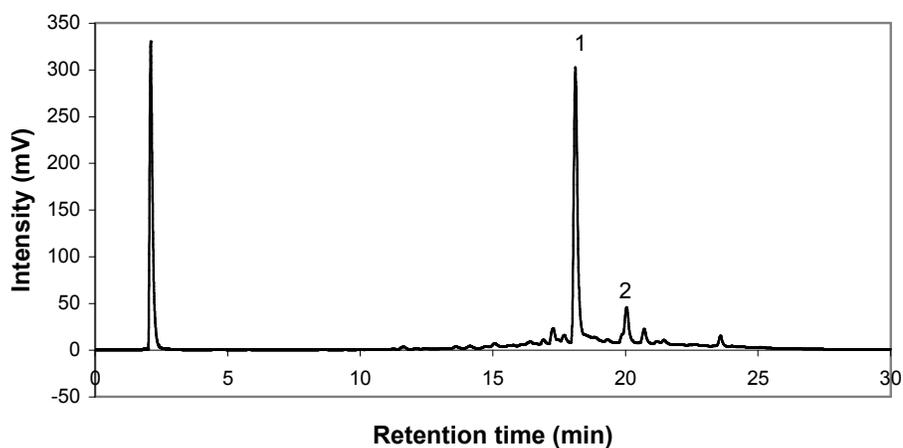
	Aspalathin		Dihydrochalcone		Nothofagin		Mangiferin	Hesperidin
	1	2 <sup>a</sup>	1	2 <sup>a</sup>	1	2 <sup>a</sup>		
<b>n</b>	331	340	331	340	331	340	240	240
<b>Calibration set</b>								
<b>Range (g.100 g<sup>-1</sup>)</b>	0.60-8.61	0.60-10.59	0.66-9.61	0.66-11.83	0.07-1.09	0.07-1.24	0.70-7.21	0.60-4.80
<b>Mean (g.100 g<sup>-1</sup>)</b>	3.77	3.91	4.21	4.37	0.45	0.46	3.64	2.04
<b>n</b>	220	227	220	227	220	227	160	160
<b>Validation set</b>								
<b>Range (g.100 g<sup>-1</sup>)</b>	0.85-7.36	1.13-9.79	0.92-8.21	1.27-11.01	0.07-0.85	0.10-1.21	1.17-7.18	0.70-3.81
<b>Mean (g.100 g<sup>-1</sup>)</b>	3.78	3.93	4.22	4.38	0.44	0.46	3.68	1.98
<b>n</b>	111	113	111	113	111	113	80	80
<b>SD<sup>b</sup></b>	1.18	1.43	1.33	1.61	0.18	0.21	0.90	0.72
<b>SEL<sup>c</sup></b>	0.15	0.15	0.17	0.17	0.02	0.03	0.08	0.03

<sup>a</sup> Including 9 samples with increased aspalathin and nothofagin contents.

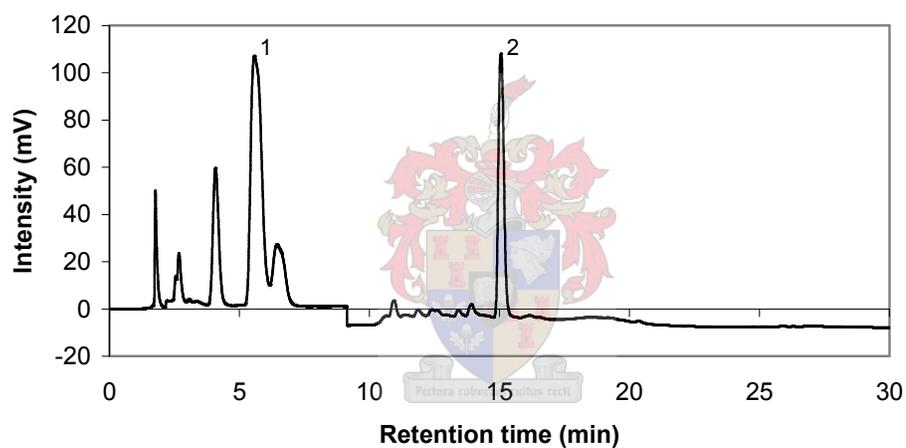
<sup>b</sup> Standard deviation of reference data of validation set.

<sup>c</sup> Standard error of laboratory of reference data of complete data set.



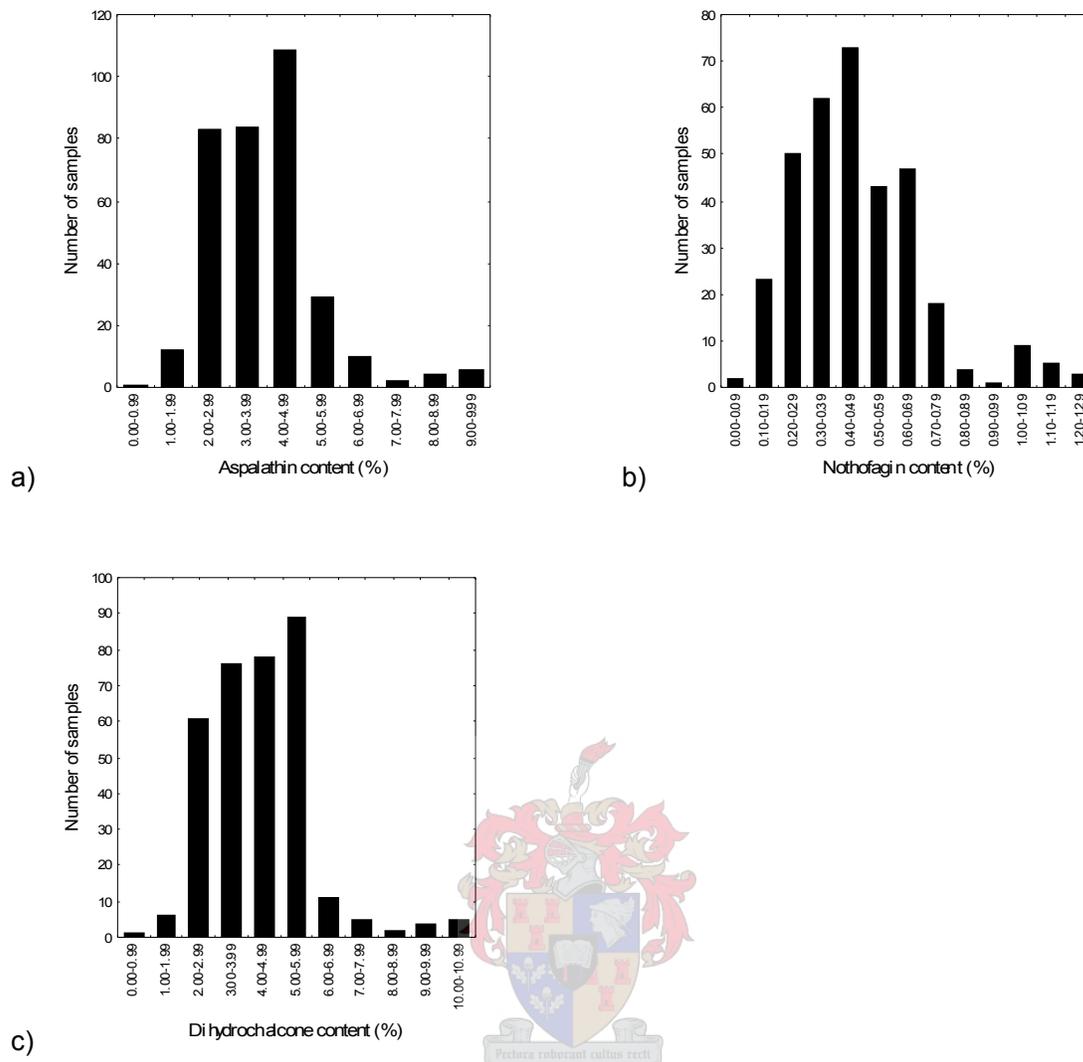


a)

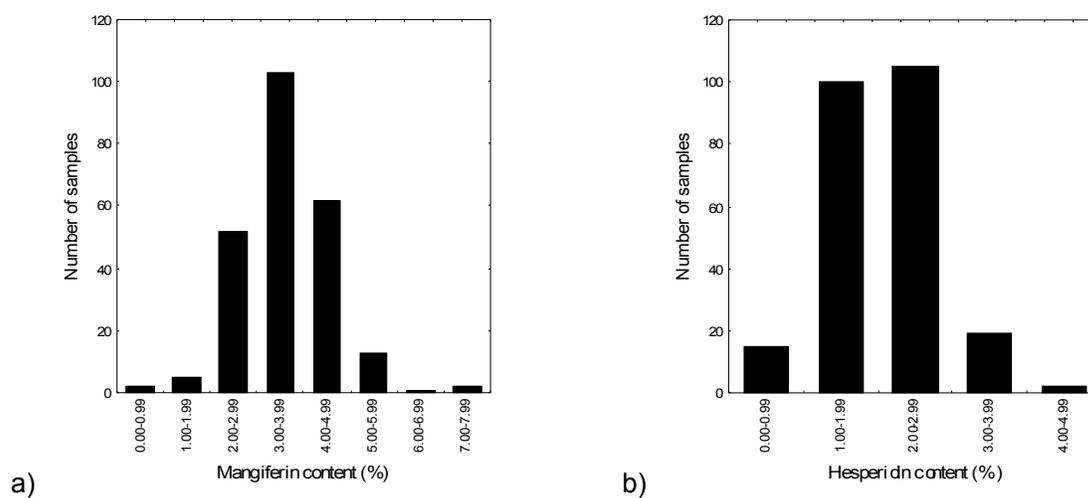


b)

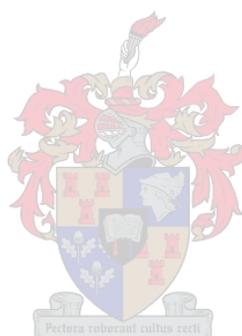
**Figure 5** HPLC chromatogram of a green rooibos acetonitrile-water extract a) with absorbance of aspalathin (1) and nothofagin (2) at 288 nm and the HPLC chromatogram of a green *C. genistoides* methanol extract b) with absorbance of mangiferin (1) and hesperidin (2) at 320 and 280 nm, respectively. The change-over from 320 to 280 nm occurred at 9.1 min.



**Figure 6** Histograms of the distribution of a) aspalathin, b) nothofagin and c) dihydrochalcone contents of the dried, green rooibos plant material samples (calibration and validation sets combined, including samples with increased aspalathin and nothofagin contents).



**Figure 7** Histogram of the distribution of a) mangiferin and b) hesperidin contents of the ground, dried, green *C. genistoides* plant material samples (calibration and validation sets combined).



#### Aspalathin and dihydrochalcone contents of dried, green rooibos plant material

The aspalathin and the dihydrochalcone contents of dried, green rooibos plant material could be successfully predicted by NIRS analysis. The SEP for the aspalathin and dihydrochalcone contents calibrations was determined as 0.45 g.100 g<sup>-1</sup> and 0.49 g.100 g<sup>-1</sup>, respectively (Table 3). These values did not compare as well with their SEL values of 0.15 g.100 g<sup>-1</sup> and 0.17 g.100 g<sup>-1</sup> (Table 2), but the NIRS models were not expected to be as sensitive as the HPLC reference method. A small bias of 0.01% and good correlation coefficients (*r*) of 0.92 and 0.93 were, respectively, observed (Table 3 and Figures 8a and 9a). The respective RPD values of 2.62 and 2.71 indicated that the accuracy of the calibration models is adequate for screening purposes during quality control (Williams, 2001). The calibration model for aspalathin content showed improvement on that obtained by Schulz *et al.* (2003) (range = ca. 4-10 g.100 g<sup>-1</sup>; SECV = 0.65 g.100 g<sup>-1</sup>; *r* = 0.87) who used a smaller sample set with limited seasonal variation.

After having extended the aspalathin and nothofagin contents range by including samples that were manipulated by adding dried rooibos extract powder with a high (15.95 g.100 g<sup>-1</sup>) aspalathin and nothofagin (1.94 g.100 g<sup>-1</sup>) content (Table 2), similar results were found for the aspalathin (SEP 0.53 g.100 g<sup>-1</sup>; *r* = 0.93; RPD = 2.70) (Table 3 and Figure 8b), as well as the dihydrochalcone content calibrations (SEP 0.57 g.100 g<sup>-1</sup>, *r* = 0.94; RPD = 2.89) (Table 3 and Figure 9b). Although the accuracies of these calibrations were slightly lower, the increased RPD value suggested more robust calibration models.

#### Nothofagin content of dried, green rooibos plant material

Slightly less accurate results were obtained for the calibration model developed for the nothofagin content (SEP = 0.10 g.100 g<sup>-1</sup>; *r* = 0.84; RPD = 2.10) of dried, green rooibos (Table 3, Figure 10a). Although the accuracy was similar after inclusion of the 9 samples with higher aspalathin and nothofagin contents the efficiency did decrease to some extent (SEP = 0.10 g.100 g<sup>-1</sup>; *r* = 0.88; RPD = 1.80) (Table 3, Figure 10b). The nothofagin calibration models can be used for screening and some approximate calibrations (Williams, 2001). Nothofagin is less abundant in green rooibos compared to aspalathin which could have affected the accuracy of the calibration.

#### Mangiferin and hesperidin contents of dried, green *C. genistoides* plant material

The validation results of the mangiferin calibration model (SEP = 0.46 g.100 g<sup>-1</sup>; *r* = 0.86; RPD = 1.96) indicated that it can be used for screening and approximate predictions (Table 3, Figure 11a). The hesperidin calibration model gave similar results (SEP = 0.38 g.100 g<sup>-1</sup>; *r* = 0.85; RPD = 1.90) (Table 3, Figure 11b).

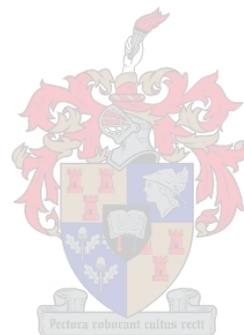
The slightly less accurate results for the prediction of mangiferin and hesperidin might be due to the solubility of these compounds. Mangiferin is slightly less soluble in methanol than in water, while hesperidin is readily soluble in methanol compared to not being readily soluble in

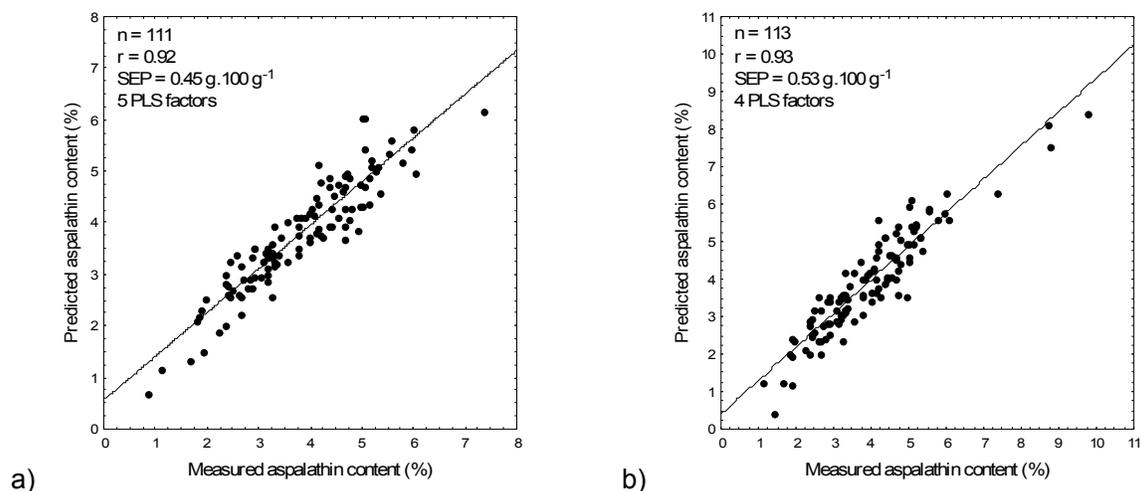
**Table 3** Summary of the NIRS validation results for the prediction of aspalathin, nothofagin and dihydrochalcone contents of dried, green rooibos and mangiferin and hesperidin contents of dried, green *C. genistoides* plant material.

	Aspalathin		Dihydrochalcone		Nothofagin		Mangiferin	Hesperidin
	1	2 <sup>a</sup>	1	2 <sup>a</sup>	1	2 <sup>a</sup>		
<b>SEP (g.100 g<sup>-1</sup>)</b>	0.45	0.53	0.49	0.57	0.10	0.10	0.46	0.38
<b>r</b>	0.92	0.93	0.93	0.94	0.84	0.88	0.86	0.85
<b>Bias</b>	0.01	0.01	0.01	-0.002	-0.01	-0.01	-0.04	0.02
<b>PLS factors<sup>b</sup></b>	5	4	5	4	5	4	4	6
<b>RPD</b>	2.62	2.70	2.71	2.89	1.8	2.10	1.96	1.90

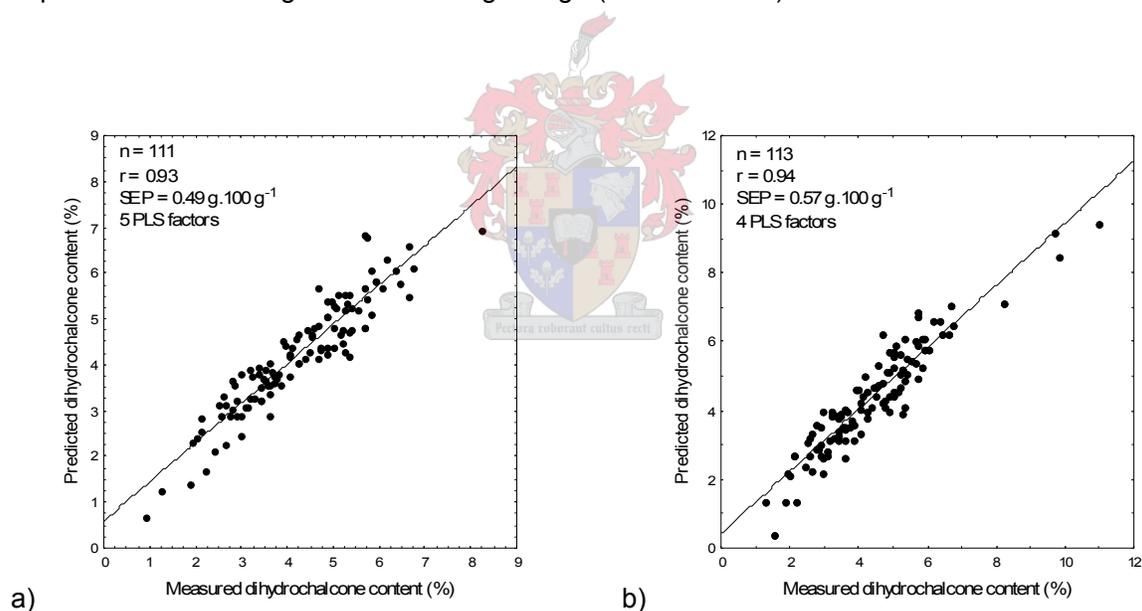
<sup>a</sup> Including 9 samples with increased aspalathin and nothofagin contents.

<sup>b</sup> Number of PLS factors used.

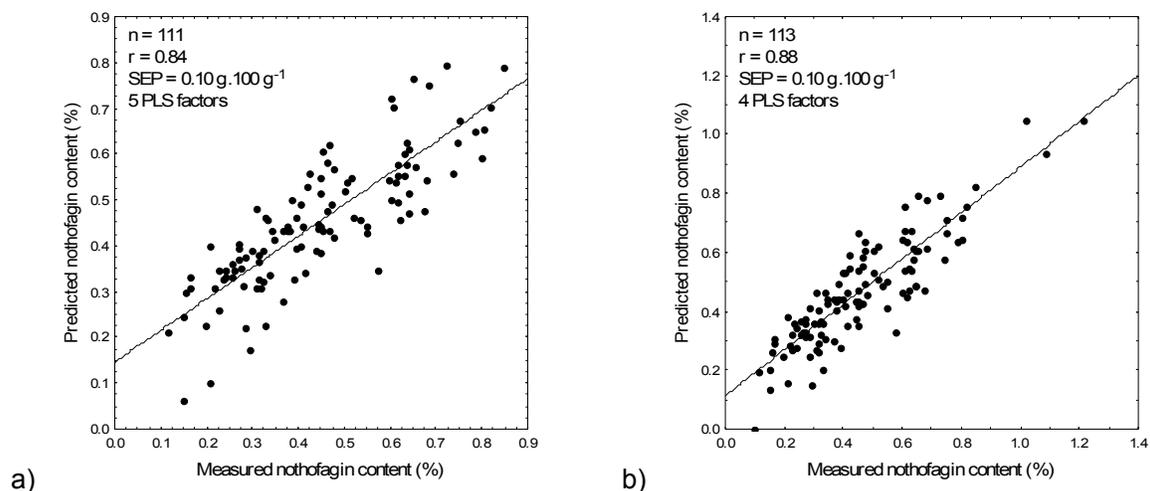




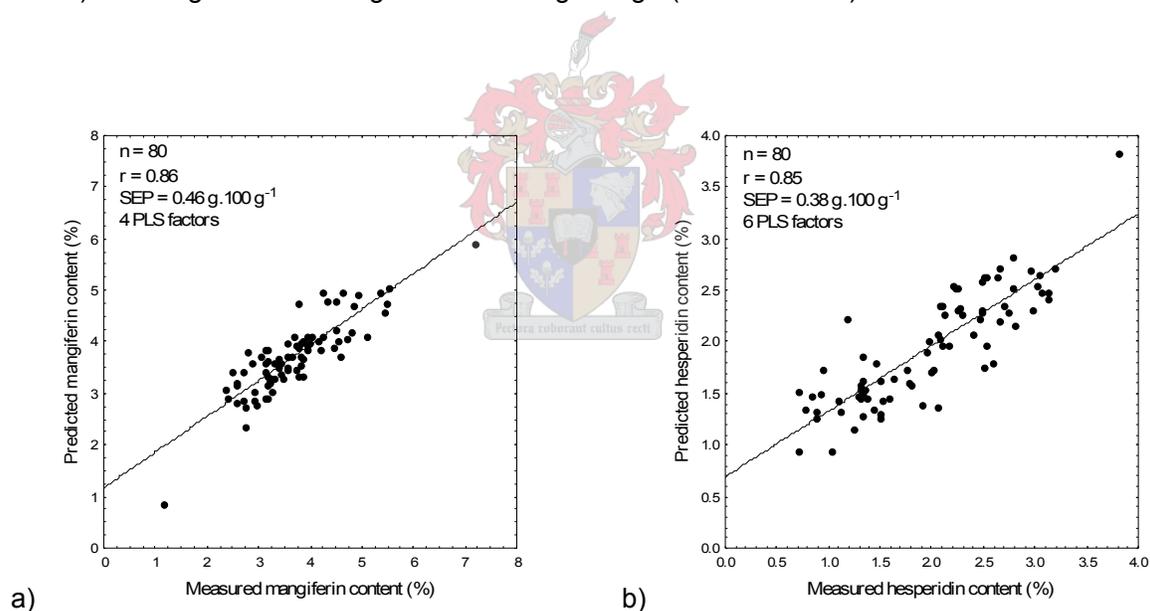
**Figure 8** Validation plots of the predicted aspalathin values versus the measured (HPLC) aspalathin values for calibration models for green rooibos plant material with different aspalathin content ranges: a) aspalathin content range =  $0.60\text{-}8.61 \text{ g} \cdot 100 \text{ g}^{-1}$  (calibration set) and b) aspalathin content range =  $0.60\text{-}10.59 \text{ g} \cdot 100 \text{ g}^{-1}$  (calibration set).



**Figure 9** Validation plots of the predicted dihydrochalcone values versus the measured (HPLC) dihydrochalcone values for calibration models for green rooibos plant material with different dihydrochalcone content ranges: a) dihydrochalcone content range =  $0.66\text{-}9.61 \text{ g} \cdot 100 \text{ g}^{-1}$  (calibration set) and b) dihydrochalcone content range =  $0.66\text{-}11.83 \text{ g} \cdot 100 \text{ g}^{-1}$  (calibration set).



**Figure 10** Validation plots of the predicted nothofagin values versus the measured (HPLC) nothofagin values for calibration models for green rooibos plant material with different nothofagin content ranges: a) nothofagin content range =  $0.07\text{-}1.09 \text{ g} \cdot 100 \text{ g}^{-1}$  (calibration set) and b) nothofagin content range =  $0.07\text{-}1.24 \text{ g} \cdot 100 \text{ g}^{-1}$  (calibration set).



**Figure 11** Validation plots of a) the predicted mangiferin values versus the measured (HPLC) mangiferin values and b) the predicted hesperidin values versus the measured (HPLC) hesperidin values for the calibration models for green *C. genistoides* plant material.

water. The use of methanol extractions for HPLC analysis of mangiferin and hesperidin could thus have led to underestimation of mangiferin. Preparation of *C. genistoides* extracts with different solvents for optimum extraction of the individual compounds, during reference analysis might improve these calibration models.

#### Residual variance plots

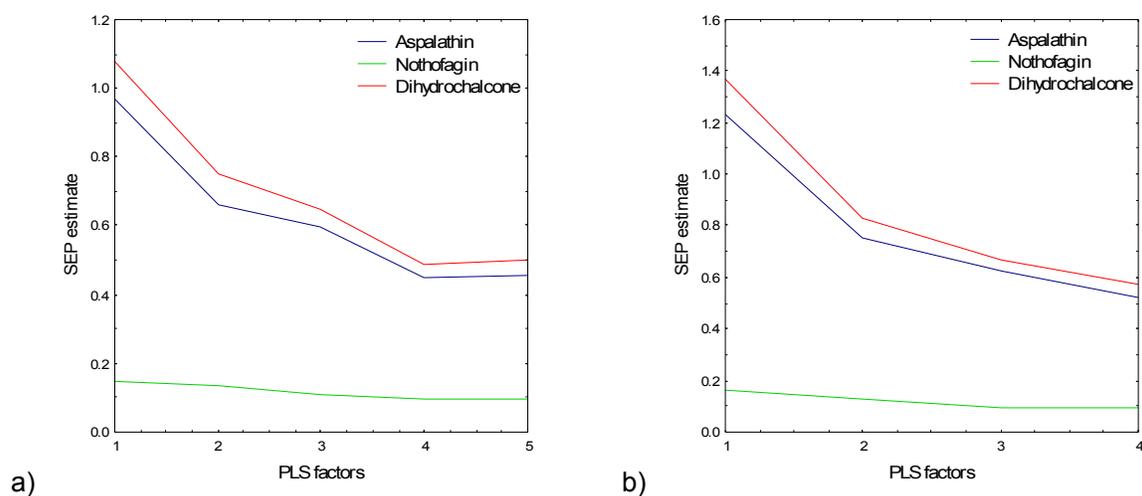
The residual variance plots showing the number of PLS factors used for the development of the calibration models for the prediction of aspalathin, nothofagin and dihydrochalcone contents are depicted in Figure 12 and those for the prediction of mangiferin and hesperidin contents in Figure 13. The number of PLS factors used was chosen according to the lowest estimated SEP value before it flattens or increase again. The number of factors should be chosen with caution to prevent over-fitting, but also to include the necessarily factors to improve the accuracy of the calibration model.

#### NIRS calibration models for green rooibos and *C. genistoides* water extracts

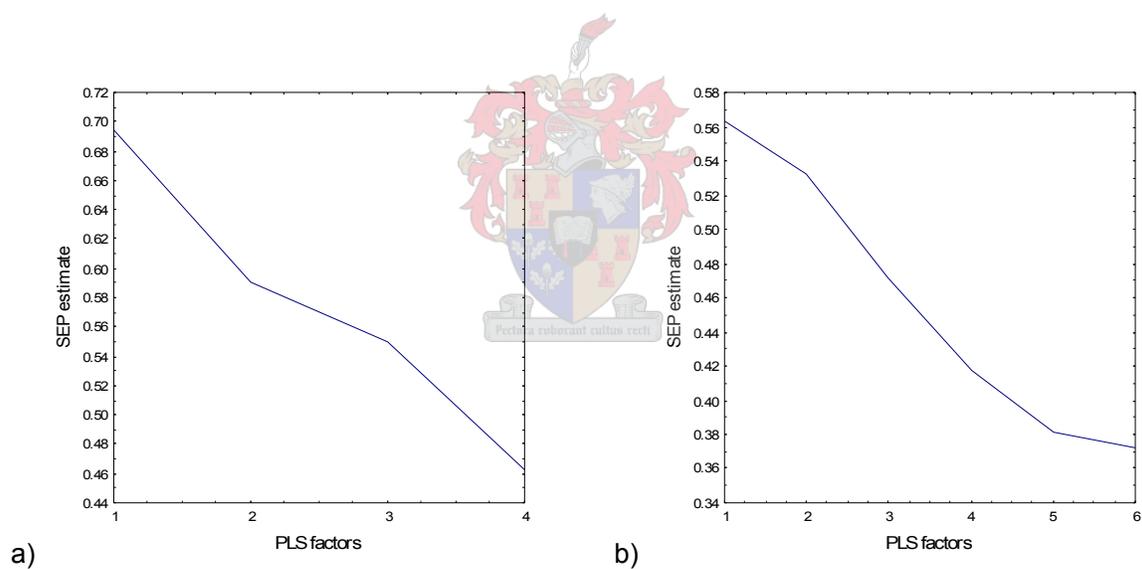
A summary of the reference data for aspalathin, total polyphenol and soluble solid contents and the TAA of the green rooibos water extracts is given in Table 4. Reasonable correlations, but poor accuracy were found for the predictions of the aspalathin (SEP = 8.33 mg.100 mL<sup>-1</sup>; r = 0.72; RPD = 1.44), total polyphenol (SEP = 17.49 mg.100 mL<sup>-1</sup>, r = 0.61, RPD = 1.24) and soluble solid contents (SEP = 44.97 mg.100 mL<sup>-1</sup>; r = 0.46; RPD = 1.20) and TAA (SEP = 112.63 μmol.100 mL<sup>-1</sup>; r = 0.68; RPD = 1.24) of green rooibos water extracts (Table 5, Figure 14).

A summary of the reference data for mangiferin, hesperidin, total polyphenol and soluble solid contents and the TAA of the green *C. genistoides* water extracts is given in Table 6. Poor correlations were found for the prediction of mangiferin (SEP = 12.70 mg.100 mL<sup>-1</sup>; r = 0.48; RPD = 1.29), hesperidin (SEP = 2.50 mg.100 mL<sup>-1</sup>; r = 0.15; RPD = 1.01), total polyphenol (SEP = 18.94 mg.100 mL<sup>-1</sup>; r = 0.31; RPD = 1.03) and soluble solid contents (SEP = 0.06 mg.100 mL<sup>-1</sup>; r = 0.22; RPD = 1.67) and TAA (SEP = 110.50 μmol.100 mL<sup>-1</sup>; r = 0.59; RPD = 1.23) for the green *C. genistoides* water extracts (Table 7, Figure 15).

The reason for these poor calibrations might be due to the low concentrations of the compounds, and soluble solids present, and the low TAA of the water extracts. Solubility of compounds such as hesperidin, which is not readily soluble in water, could also have contributed to the poor results obtained in the case of prediction of the hesperidin content due to under-estimation of the reference values. Because of the poor solubility of hesperidin in water, precipitation upon cooling to room temperature could have resulted in the extracts being turbid. The diffuse transreflectance mode was initially considered, since this measurement is based on light that moves from the radiation source, transmit through the sample, reflect from



**Figure 12** Residual variance plots of the estimated SEP values versus the number of PLS factors used for the development of calibration models for aspalathin, nothofagin and the dihydrochalcone contents of the two sample sets: a)  $n = 331$  and b)  $n = 340$ .



**Figure 13** Residual variance plots of the estimated SEP values versus the number of PLS factors used for the development of calibration models for: a) mangiferin and b) hesperidin.

**Table 4** Summary of the reference data for the aspalathin, total polyphenol and soluble solid contents and total antioxidant activity of green rooibos water extracts.

	Aspalathin <sup>a</sup>	Total polyphenol <sup>a</sup>	Soluble solid <sup>a</sup>	Total antioxidant activity <sup>b</sup>
<b>n</b>	102	102	102	101
<b>Calibration set</b>				
<b>Range</b>	0.31-50.94	8.42-102.14	51.33-269.67	48.23-634.99
<b>Mean</b>	21.28	51.23	146.24	328.38
<b>n</b>	69	69	69	69
<b>Validation set</b>				
<b>Range</b>	1.05-49.88	9.26-94.55	55.00-250.33	55.47-610.56
<b>Mean</b>	21.62	50.14	143.47	325.19
<b>n</b>	33	33	33	32
<b>SD<sup>c</sup></b>	12.02	21.65	53.98	139.96
<b>SEL<sup>d</sup></b>	0.19	0.42	0.01	2.19

<sup>a</sup> Expressed as mg.100 mL<sup>-1</sup> water extract.

<sup>b</sup> Expressed as  $\mu$ mol Trolox equivalents.100 mL<sup>-1</sup> water extract.

<sup>c</sup> Standard deviation of reference data of validation sample set.

<sup>d</sup> Standard error of laboratory of reference data of complete data set.

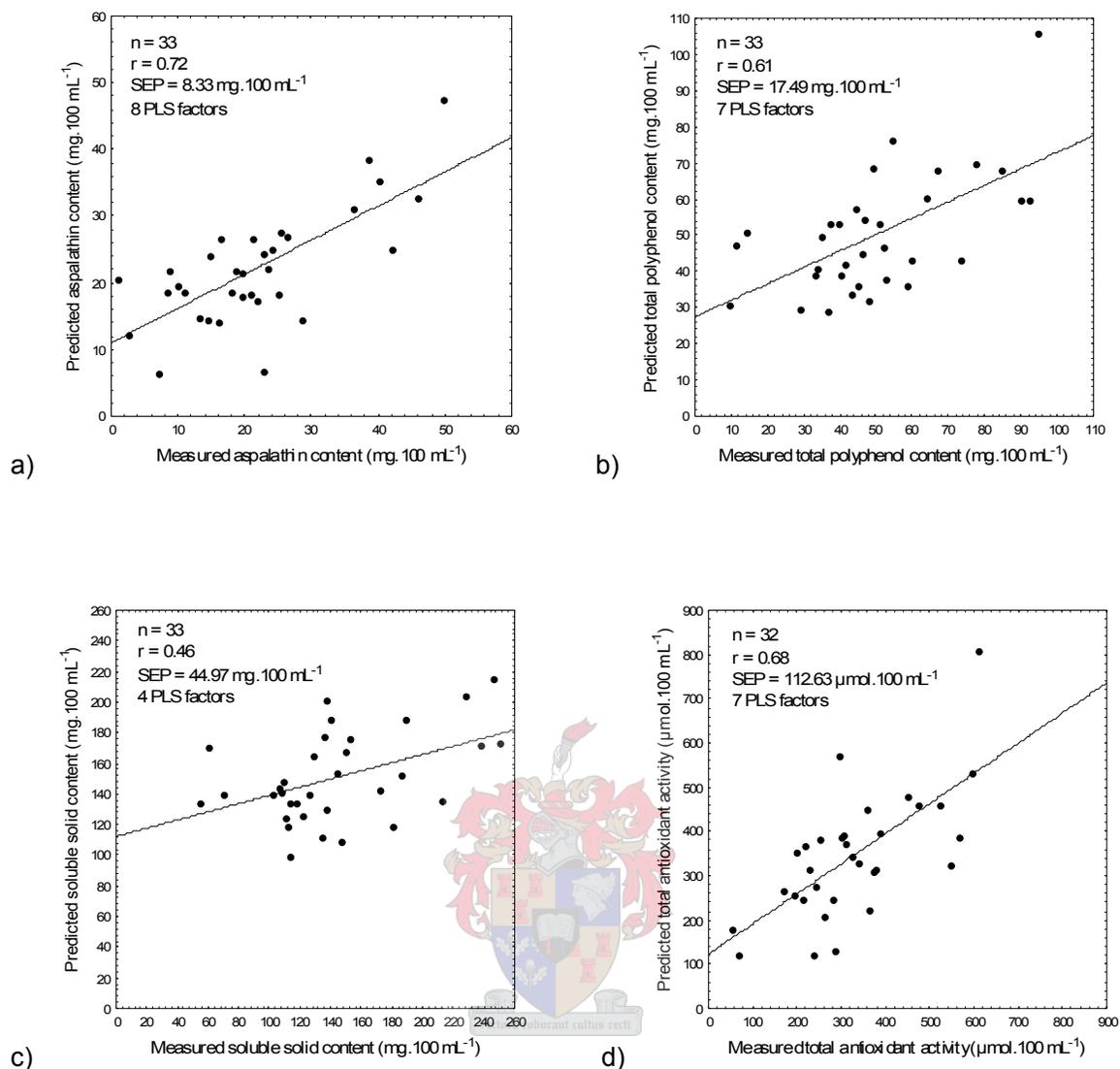
**Table 5** Summary of the NIRS validation results for aspalathin, total polyphenol, soluble solid contents and total antioxidant activity of the green rooibos water extracts.

	Aspalathin <sup>a</sup>	Total polyphenol <sup>a</sup>	Soluble solid <sup>a</sup>	Total antioxidant activity <sup>b</sup>
<b>SEP</b>	8.33	17.49	44.97	112.63
<b>r</b>	0.72	0.61	0.46	0.68
<b>Bias</b>	-0.51	-0.15	-7.41	-19.07
<b>PLS factors<sup>c</sup></b>	8	7	4	7
<b>RPD</b>	1.44	1.24	1.20	1.24

<sup>a</sup> Expressed as mg.100 mL<sup>-1</sup> water extract.

<sup>b</sup> Expressed as  $\mu$ mol Trolox equivalents.100 mL<sup>-1</sup> water extract.

<sup>c</sup> Number of PLS factors used.



**Figure 14** Validation plots of a) the predicted aspalathin values versus the measured (HPLC) aspalathin values, b) the predicted TPC values versus the measured (Folin-Ciocalteu assay) TPC values, c) the predicted SSC values versus the measured SSC values and d) the predicted TAA values versus the measured (ABTS assay) TAA values for the calibration models for green rooibos water extracts.

**Table 6** Summary of the reference data for mangiferin, hesperidin, total polyphenol and soluble solid contents and total antioxidant activity of the green *C. genistoides* water extracts.

	Mangiferin <sup>a</sup>	Hesperidin <sup>a</sup>	Total polyphenol <sup>a</sup>	Soluble solid <sup>a</sup>	Total antioxidant activity <sup>b</sup>
<b>n</b>	127	127	127	127	111
<b>Calibration set</b>					
<b>Range</b>	14.39-98.38	5.90-20.24	75.64-177.13	0.27-0.59	507.43-1250.42
<b>Mean</b>	46.73	10.98	132.14	0.41	820.02
<b>n</b>	85	85	85	85	75
<b>Validation set</b>					
<b>Range</b>	15.65-81.50	6.67-17.56	96.97-175.43	0.29-0.55	562.95-1160.58
<b>Mean</b>	43.29	11.05	132.78	0.41	811.59
<b>n</b>	42	42	42	42	36
<b>SD<sup>c</sup></b>	16.43	2.53	19.42	0.10	136.34
<b>SEL<sup>d</sup></b>	1.13	0.50	1.79	0.01	17.96

<sup>a</sup> Expressed as mg.100 mL<sup>-1</sup> water extract.

<sup>b</sup> Expressed as μmol Trolox equivalents.100 mL<sup>-1</sup> water extract.

<sup>c</sup> Standard deviation of reference data of validation sample set.

<sup>d</sup> Standard error of laboratory of reference data of complete data set.

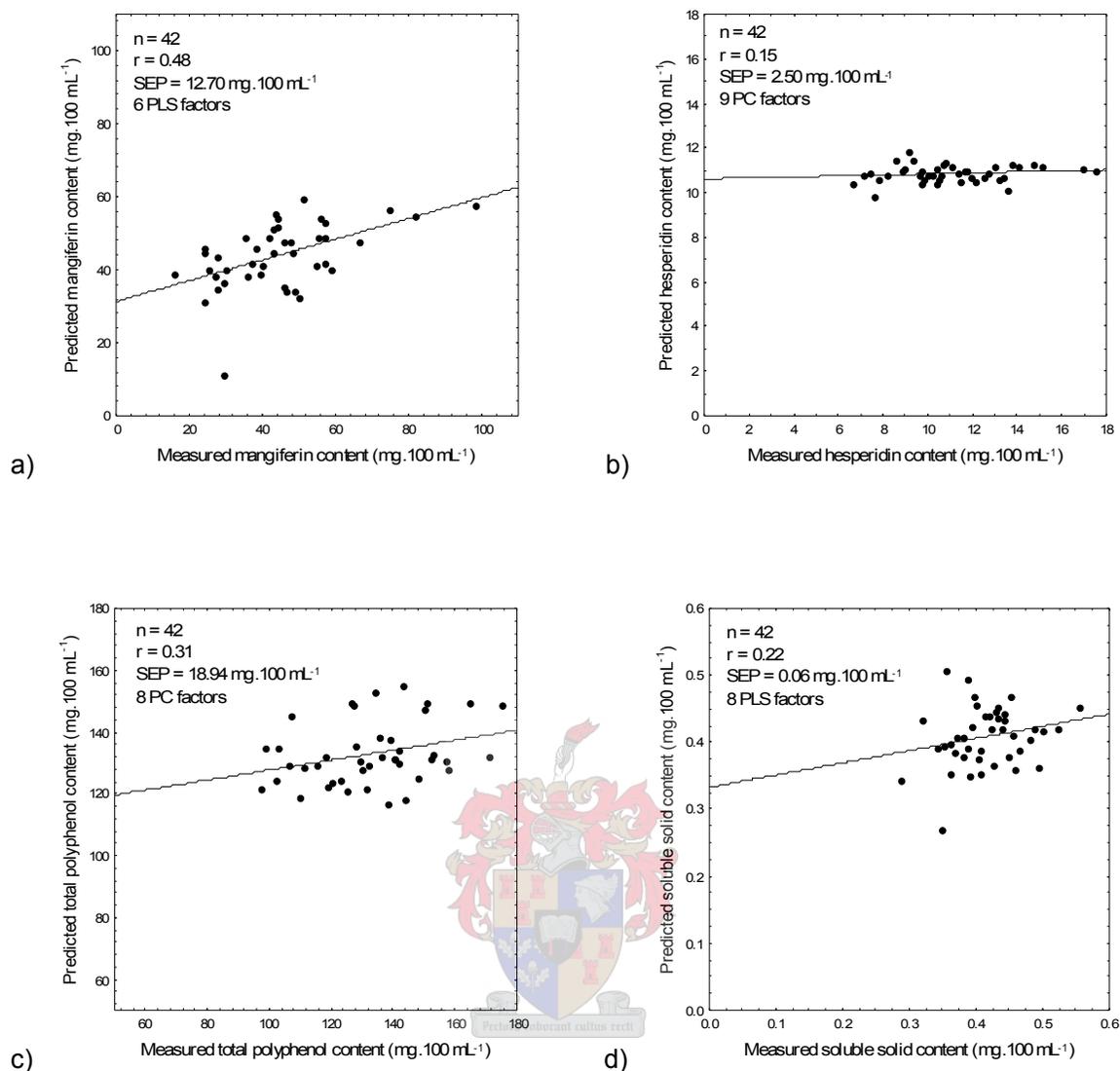
**Table 7** Summary of the NIRS validation results for mangiferin, hesperidin, total polyphenol and soluble solid contents and total antioxidant activity of green *C. genistoides* water extracts.

	Mangiferin <sup>a</sup>	Hesperidin <sup>a</sup>	Total polyphenol <sup>a</sup>	Soluble solids <sup>a</sup>	TAA <sup>b</sup>
<b>SEP</b>	12.70	2.50	18.94	0.06	110.50
<b>r</b>	0.48	0.15	0.31	0.22	0.59
<b>Bias</b>	-0.38	0.18	-0.21	0.01	-4.58
<b>PLS factors<sup>c</sup></b>	6	9	8	8	4
<b>RPD</b>	1.29	1.01	1.03	1.67	1.23

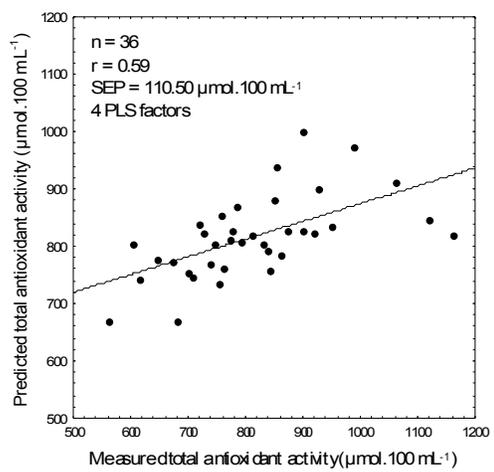
<sup>a</sup> Expressed as mg.100 mL<sup>-1</sup> water extract.

<sup>b</sup> Expressed as μmol Trolox equivalents.100 mL<sup>-1</sup> water extract.

<sup>c</sup> Number of PLS factors or principal components used.

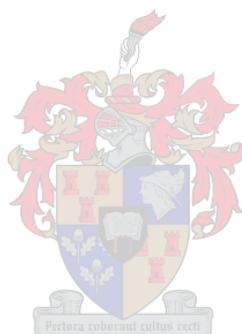


**Figure 15** Validation plots of a) the predicted mangiferin values versus the measured (HPLC) mangiferin values, b) the predicted hesperidin values versus the measured (HPLC) hesperidin values, c) the predicted TPC values versus the measured (Folin-Ciocalteu assay) TPC values, d) the predicted SSC values versus the measured SSC values and e) the predicted TAA values versus the measured (ABTS assay) TAA values for the calibration models for green *C. genistoides* water extracts.



f)

Figure 15 Continued/...



the transmittance cover, and transmit back through the sample (Stark *et al.*, 1986). However, due to the uneven surface of the petri-dishes the path length was not consistent, and the transmittance mode and quartz cuvettes were used. The diffuse transmittance mode should be investigated in future studies using an appropriate sample holder.

## Conclusions

Near infrared spectroscopy showed promise as a rapid method to quantify the aspalathin and dihydrochalcone contents in green rooibos plant material, since an accuracy of *ca.* 0.5 g.100 g<sup>-1</sup> would be acceptable for the prediction of these compounds by the industry. It could be used to standardise green rooibos plant material at an early stage of processing. Nothofagin, mangiferin and hesperidin NIRS calibration models could be used for screening purposes. Nothofagin is present in much smaller quantities than aspalathin which could have contributed to the slightly less accurate calibration models. The lower accuracy of the mangiferin and hesperidin calibration models could be due to the difference in solubility of these compounds. Preparation of different extracts using different solvents for optimum extraction of each compound might improve the accuracy of the reference data and therefore the calibration models. All the calibration models developed for the green rooibos and *C. genistoides* plant material could be improved by adding more samples to give a more even distribution of the aspalathin, nothofagin, dihydrochalcone, mangiferin and hesperidin contents. NIRS did not give acceptable results for the prediction of the different compounds, total polyphenol content, soluble solid content and total antioxidant activity of green rooibos and *C. genistoides* water extracts. The main reason might be the low concentrations present in the water extracts. The low hesperidin content present due to its poor solubility in water led to underestimation of hesperidin content which contributed to the poor calibration model.

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## CHAPTER 4



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**Determination of aspalathin content of green rooibos  
(*Aspalathus linearis*) by UV spectroscopy and  
mangiferin content of honeybush (*Cyclopia  
genistoides*) with an aluminium chloride colorimetric  
method**

## CHAPTER 4

**DETERMINATION OF ASPALATHIN CONTENT OF GREEN ROOIBOS  
(*ASPALATHUS LINEARIS*) BY UV SPECTROSCOPY AND MANGIFERIN CONTENT  
OF HONEYBUSH (*CYCLOPIA GENISTOIDES*) WITH AN ALUMINIUM CHLORIDE  
COLORIMETRIC METHOD**

**Abstract**

The potential of UV spectroscopy and the aluminium chloride colorimetric method to determine aspalathin in green rooibos and mangiferin in green honeybush (*C. genistoides*), respectively, was investigated. The aspalathin content of water extracts determined using UV spectroscopy correlated ( $r = 0.97$ ) with the aspalathin content as quantified using HPLC ( $y = 1.9037x + 14.2828$ ), but a slope of 1.90 was obtained, indicating that UV spectroscopy overestimated the aspalathin content. The aspalathin content determined by UV spectroscopy gave a similar correlation ( $r = 0.97$ ;  $y = 1.7084x + 14.6903$ ) with the dihydrochalcone content as quantified using HPLC. The total polyphenol content (TPC) of water extracts of rooibos tea can be determined using UV spectroscopy and aspalathin as standard ( $r = 0.99$ ;  $y = 1.0829x - 0.6498$ ). Both the TPC ( $r = 0.97$ ) and total antioxidant activity (TAA) ( $r = 0.96$ ) of rooibos water extracts correlated with the aspalathin (HPLC) content. A good correlation of TAA with TPC ( $r = 0.99$ ) was thus obtained.

Aluminium chloride resulted in characteristic bathochromic and hyperchromic shifts in the spectrum of mangiferin, but it had little effect on the spectrum of hesperidin due to the absence of the orthodihydroxyl groups on the A and B rings. A correlation coefficient ( $r$ ) of 0.78 was found for the mangiferin content in water extracts determined using an aluminum chloride colorimetric method and mangiferin content quantified using HPLC. The correlation ( $r = 0.90$ ) was improved when using methanol for extract preparation. The mangiferin content of the water extracts did not correlate with its TPC ( $r = 0.22$ ). A moderate correlation was found between the TAA and the mangiferin content ( $r = 0.75$ ). A poor correlation was obtained between the TAA and TPC of the water extracts of *C. genistoides* ( $r = 0.27$ ).

**Introduction**

A large number of polyphenol-enriched extracts is on the market due to the growing interest of the nutraceutical and cosmetic industries in natural plant products (Andersen *et al.*, 2005; Wright, 2005). High quantities of the dihydrochalcone, aspalathin, and the xanthone, mangiferin, in green rooibos and honeybush (*Cyclopia genistoides*), respectively, make both these herbal plants generally good sources of these compounds. However, since their concentration in the plant material can vary considerably, screening of plant material before the manufacture of enriched extracts is essential to ensure that the final product contains the

minimum required levels. Rapid analytical methods are required for quality control of the raw material, as well as the final product.

Near infrared spectroscopy (NIRS) has been investigated as a rapid, non-destructive technique to determine aspalathin and mangiferin content in green rooibos and *C. genistoides*, respectively (Chapter 3). Although the running cost of NIRS is low, extract manufacturers consider the initial capital cost prohibitive. Since UV/Vis spectrophotometers are available in most quality control laboratories, spectrophotometric assays could potentially offer the quality control analyst an alternative. Depending on the properties of the compound, UV/Vis spectrophotometry or colorimetry can be used for quantification of some compounds, especially if they are present in high quantities relative to other compounds. In the case of green rooibos, this is true for aspalathin, comprising between 4.45 and 11.9 g.100 g<sup>-1</sup> of the plant material (Schulz *et al.*, 2003), while nothofagin comprises 1.33 g.100 g<sup>-1</sup> and the other flavonoids ca. 0.92 g.100 g<sup>-1</sup> (Bramati *et al.*, 2003).

Colorimetric methods are widely used to determine specific classes of phenolic compounds. One such method is the aluminium chloride (AlCl<sub>3</sub>) method, which is relatively specific for flavones and flavonols (Jurd, 1962; Mabry *et al.*, 1970; Chang *et al.*, 2002). Aluminium chloride forms a complex with the C-4 keto group and either the C-3 or C-5 hydroxyl group, as well as the orthodihydroxyl groups on the A- or B-ring of flavonoids (Chang *et al.*, 2002). These structural requirements suggest that mangiferin with a conjugated carboxyl chromophore and C-1 hydroxyl group, as well as the C-6 and C-7 hydroxyl groups could also form complexes with AlCl<sub>3</sub> (Figure 1). Spectra of xanthenes undergo characteristic bathochromic shifts with AlCl<sub>3</sub> depending on the number and position of the hydroxyl substituents (Harborne, 1998). Hesperidin, a flavanone and another major phenolic compound of *C. genistoides*, comprising between 2.23 and 5.21 g.100 g<sup>-1</sup> of the plant material (Joubert *et al.*, 2003), lacks the orthodihydroxyl groups on the A- or B-ring. It would therefore have very low reactivity towards AlCl<sub>3</sub> as confirmed for its aglycone, hesperitin (Mabry *et al.*, 1970; Chang *et al.*, 2002). The high quantities of mangiferin (4.93-6.37 g.100 g<sup>-1</sup>) present in green *C. genistoides* plant material (Van der Merwe, 2004) further support the possible use of this method for determination of mangiferin content with minor interference of other phenolic compounds.

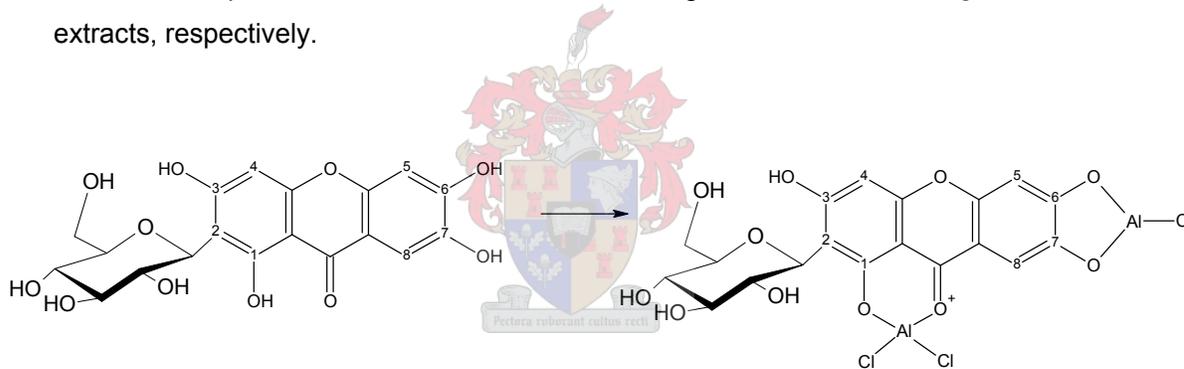
Both aspalathin and mangiferin are extracted for their antioxidant properties. It is therefore of interest to determine whether the aspalathin and mangiferin contents of extracts correlate with their total antioxidant activity (TAA), a quality parameter employed by local extract manufacturers. Using two sets of samples, Schulz *et al.* (2003) obtained conflicting results for the correlation of TAA of rooibos water extracts with their aspalathin content. This aspect was therefore revisited in the present study.

Another quality parameter used by local extract manufacturers, in the absence of quantification of aspalathin and mangiferin, is the total polyphenol content (TPC) of extracts.

Research on a variety of plant extracts has shown different results for the relationship between the TPC and the TAA, ranging from a good correlation (Cai *et al.*, 2004; Gorinstein *et al.*, 2004; Soong & Barlow, 2004) to no relationship (Bocco *et al.*, 1998; Heinonen *et al.*, 1998; Kähkönen *et al.*, 1999). Since the basic mechanism of the Folin-Ciocalteu method is an oxidation-reduction reaction, it can be considered as another TAA method (Prior *et al.*, 2005), suggesting that either method should suffice in quality control if a good correlation is obtained.

The objectives of this study were to investigate:

- the potential of UV spectroscopy to determine the aspalathin content of green rooibos tea water extracts;
- the potential of the aluminium chloride colorimetric method to determine the mangiferin content of green *C. genistoides* water and methanol extracts;
- the contribution of aspalathin and mangiferin to the TPC of the green rooibos and *C. genistoides* water extracts, respectively;
- the relationship between the aspalathin and mangiferin contents and the TAA of green rooibos and *C. genistoides* water extracts, respectively; and
- the relationship between the TAA and the TPC of green rooibos and *C. genistoides* water extracts, respectively.



**Figure 1** Potential complexation of mangiferin with  $\text{AlCl}_3$ .

## Materials and methods

### Chemicals

Aspalathin (Batch A/02, purity  $\geq 95\%$ ) was isolated by Petra Snijman at the PROMEC (Programme on Mycotoxins and Experimental Carcinogenesis) Unit of the Medical Research Council (MRC, Bellville, South Africa). Tannin, isolated from the n-butanol fraction of green rooibos water extract, was supplied by Prof. D. Ferreira, Department of Chemistry, University of the Free State, Bloemfontein (presently at University of Mississippi, USA). Mangiferin, hesperidin (97%), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), dimethyl sulfoxide (DMSO) (99.5%), ascorbic acid, potassium persulphate, gallic acid, aluminium chloride (anhydrous, sublimed), acetonitrile R Chromasolv® (Riedel-de Haën) for liquid chromatography and glacial acetic acid (min. 99.8%; Riedel-de Haën) were obtained from Sigma-Aldrich (Cape

Town, South Africa). Folin-Ciocalteu's phenol reagent and sodium carbonate, potassium acetate, methanol and EDTA (ethylenediaminetetra-acetic acid di-sodium salt) were purchased from Merck Chemicals (Pty) Ltd (Cape Town, South Africa). ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate)) was obtained from Roche (Johannesburg, South Africa). Ethanol (99%) was obtained from Illovo (Cape Town, South Africa). Deionised water was prepared with a Modulab Water Purification System (Separations, Cape Town, South Africa). For HPLC eluent preparation, deionised water was further purified by means of a Milli-Q 185 Académic Plus water purification system (Microsep (Pty) Ltd, Bellville, South Africa).

#### *Plant material*

The rooibos plant material (n = 73) used in the present study were selected based on their aspalathin content as determined on acetonitrile-water extracts (Chapter 3). The samples covered the aspalathin range between 0.15-10.59%. The *C. genistoides* samples collected from the sample set described in Chapter 3 were used for the preparation of the methanol extracts (n = 226), but only a selection of the samples harvested during 2003 were used for the preparation of the water extracts (n = 126).

#### *Preparation of extracts*

Water extracts were prepared from green rooibos and *C. genistoides*, while methanol extracts were prepared from green *C. genistoides* only. The extraction procedures were as described in Chapter 3.

#### *Quantification of the aspalathin, dihydrochalcone and mangiferin contents of extracts by HPLC*

The same procedures were followed as described in Chapter 3. The dihydrochalcone content was calculated as the sum of the aspalathin and nothofagin contents.

#### *Total polyphenol content of water extracts*

The Folin-Ciocalteu colorimetric method was used to determine the total polyphenol content as described in Chapter 3.

#### *Total antioxidant activity of water extracts*

The ABTS decolorisation assay was used to determine total antioxidant activity as described in Chapter 3.

#### *UV/Vis spectra of aspalathin*

Aspalathin (2.30 mg) was dissolved in 1 mL DMSO whereafter it was diluted with deionised water to give a final concentration of 48.81  $\mu\text{M}$ . The UV/Vis spectrum of aspalathin was recorded between 200 and 400 nm in UV disposable cuvettes (1 cm path length; Plastibrand

UV-cuvette, Sigma-Aldrich, Cape Town, South Africa) with a UNIKON 923 Double Beam UV/Vis Spectrophotometer (Analytical & Diagnostic Products, South Africa), using the same solvent composition as reference.

#### *Quantification of aspalathin in water extracts of green rooibos using UV spectroscopy*

A Beckman DU-65 spectrophotometer (Beckman, Cape Town, South Africa) was used to perform spectrophotometric measurements of the green rooibos water extracts at a wavelength of 288 nm in UV disposable cuvettes (1 cm path length; Plastibrand UV-cuvette, Sigma-Aldrich, Cape Town, South Africa). Purified aspalathin (Batch A/02) was used to prepare a calibration curve (concentration range 3.18-31.84  $\mu\text{g}\cdot\text{mL}^{-1}$ ;  $R^2 = 1$ ). The extracts were diluted with deionised water to obtain absorbances within the range 0.2-0.8. The aspalathin content value of the extracts was expressed as  $\text{mg}\cdot 100\text{ mL}^{-1}$  extract.

#### *Measurement of UV absorbance of green rooibos tannin*

The absorbance of tannin, dissolved in water (concentration range = 21.6-324  $\mu\text{g}\cdot\text{mL}^{-1}$ ), at 288 nm was measured in UV disposable cuvettes (1 cm path length; Plastibrand UV-cuvette, Sigma-Aldrich, Cape Town, South Africa) using a Beckman DU-65 spectrophotometer (Beckman, Cape Town, South Africa).

#### *UV/Vis spectra of mangiferin and hesperidin in the presence of $\text{AlCl}_3$ , potassium acetate and $\text{AlCl}_3 + \text{potassium acetate}$*

The aluminum chloride colorimetric method modified from the procedure reported by Chang *et al.* (2002) was used. Disposable glass tubes and plastic caps were pretreated with EDTA, to eliminate metal contamination, and thoroughly rinsed with deionised water before drying and use. Mangiferin (20.24 mg) and hesperidin (10.72 mg) were each dissolved in 1 mL DMSO whereafter it was diluted with 80% ethanol to give final concentrations of 191.69  $\mu\text{M}$  and 70.23  $\mu\text{M}$ , respectively. The different reaction mixtures prepared are summarised in Table 1. The reference samples were similarly prepared, except that 0.5 mL DMSO-ethanol mixture instead of mangiferin and hesperidin was added. The reaction mixture was vortexed, incubated at 30°C in a temperature controlled laboratory oven for an hour and the absorbance measurement (200-500 nm) performed in UV disposable cuvettes (1 cm path length; Plastibrand UV-cuvette, Sigma-Aldrich, Cape Town, South Africa) with a UNIKON 923 Double Beam UV/Vis Spectrophotometer (Analytical & Diagnostic Products, South Africa).

#### *Aluminum chloride colorimetric measurement of mangiferin in water and methanol extracts of green *C. genistoides**

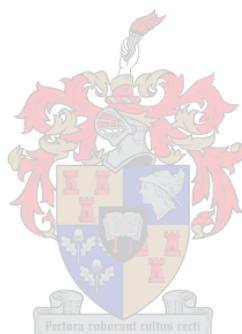
Disposable glass tubes and plastic caps were pretreated with EDTA as described previously.

**Table 1** Summary of the reaction mixtures for the UV/Vis spectra of mangiferin and hesperidin in the presence of  $\text{AlCl}_3$ , potassium acetate and  $\text{AlCl}_3$  + potassium acetate.

Reaction mixture designation	EtOH	Deionised water	Mangiferin/hesperidin	Potassium acetate <sup>a</sup>	$\text{AlCl}_3$ <sup>b</sup>
Water	1.5	3.1	0.5		
Potassium acetate	1.5	0.1	0.5	3	
$\text{AlCl}_3$	1.5	3	0.5		0.1
$\text{AlCl}_3$ + potassium acetate	1.5		0.5	3	0.1

<sup>a</sup> 0.03 M potassium acetate was prepared by dissolving 1.472 g.500 mL<sup>-1</sup> deionised water.

<sup>b</sup> 10% (m/v)  $\text{AlCl}_3$  solution was prepared by dissolving 1 g.10 mL<sup>-1</sup> deionised water.



Mangiferin (20 mg dissolved in 10 mL DMSO) was diluted with 80% ethanol to prepare a standard series (concentration range 20.14-200.70  $\mu\text{g}\cdot\text{mL}^{-1}$ ;  $R^2 = 1$ ) for preparation of a calibration curve. The standard solutions and samples (0.5 mL) were mixed with 1.5 mL ethanol, 0.1 mL 10%  $\text{AlCl}_3$  ( $m/v$ ) and 3 mL 0.03 M potassium acetate. The standard and sample blanks were similarly prepared, but the  $\text{AlCl}_3$  solution was replaced with water. The reaction mixture was vortexed, incubated at 30°C in a temperature controlled laboratory oven for an hour and the absorbance measured in disposable plastic cuvettes (1 cm path length; Plastibrand PS-cuvette, Sigma-Aldrich, Cape Town, South Africa) with a GBC UV/Vis 911A spectrophotometer (Wirsam, Cape Town, South Africa) at 410 nm. The tea extract (2 mL) was diluted to 10 mL with the extraction solvent (water or methanol) before being added to the reaction mixture. The mangiferin content value of the extracts was expressed as  $\text{mg}\cdot 100\text{ mL}^{-1}$  extract.

#### *Statistical analysis*

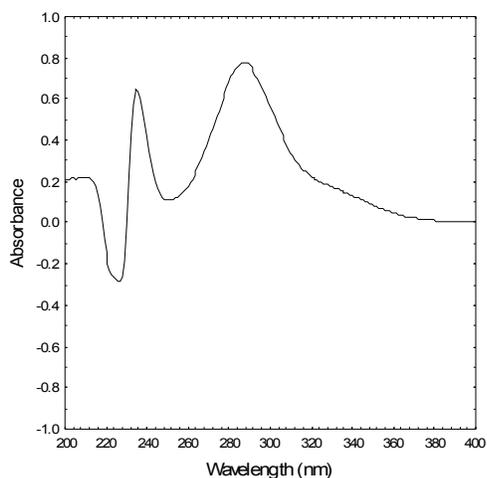
Correlations were performed with Microsoft® Office Excel 2003 (Part of Microsoft Office Professional Edition 2003).

### **Results and discussion**

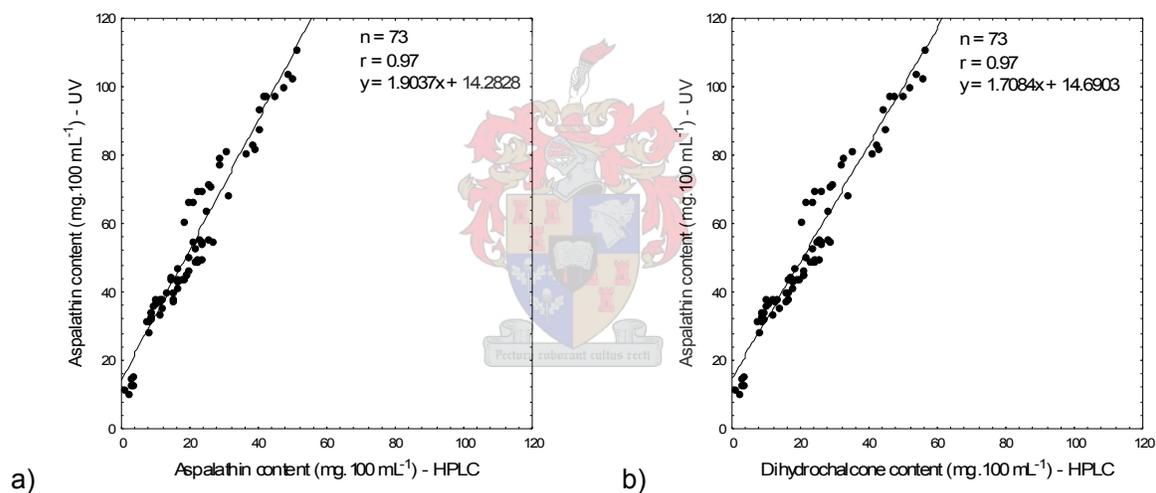
#### *UV spectrophotometric measurement of aspalathin content of green rooibos water extracts*

The UV/Vis spectrum of aspalathin is depicted in Figure 2. The  $\lambda_{\text{max}}$  (nm) with mainly water as solvent was 284 nm. The  $\lambda_{\text{max}}$  (nm) will depend on solvent composition, i.e. 290 nm in ethanol (Koeppen & Roux, 1966) and 288 nm in an acetonitrile-2% acetic acid-water mixture (Schulz *et al.*, 2003). Good correlation was obtained between the aspalathin content estimated with UV spectroscopy and that determined using HPLC ( $r = 0.97$ ) (Figure 3a). The y-intercept of 14.28 indicated background absorbance that could not be attributed to aspalathin, yet it was relatively consistent, based on the good correlation obtained. A slope of 1.90 was obtained, indicating that UV spectroscopy overestimated the aspalathin content. This could partly be explained by the absorbance of nothofagin, another dihydrochalcone, which could comprise as much as 1.33% of the plant material whereas aspalathin could comprise between 4.45 and 11.9% (Schulz *et al.*, 2003).

The contribution of nothofagin was taken into account by calculating the dihydrochalcone content. The same correlation coefficient was found between the aspalathin content of rooibos extract estimated with UV spectroscopy and the dihydrochalcone content determined using HPLC ( $r = 0.97$ ) (Figure 3b). The slope changed only slightly from 1.90 to 1.71. The overestimation indicated that other compounds absorbing at 288 nm, are present in relative high quantities. Flavonoids such as iso-orientin, orientin, vitexin, rutin, isovitexin, luteolin, quercetin and chrysoeriol would also absorb at 288 nm. However, they are not present in high quantities in the plant material, comprising in total less than 1% (Bramati *et al.*, 2003). Rooibos tannin (40



**Figure 2** UV/Vis spectrum of aspalathin in water between 200 and 400 nm.



**Figure 3** Correlation of UV spectrophotometric estimated aspalathin content ( $\text{mg.100 mL}^{-1}$ ) with a) HPLC measured aspalathin content ( $\text{mg.100 mL}^{-1}$ ) and b) the dihydrochalcone content ( $\text{mg.100 mL}^{-1}$ ) of green rooibos water extracts.

$\mu\text{g}\cdot\text{mL}^{-1}$ ), responsible for the light red-brown colour in rooibos extracts, gave an absorbance of 0.208 at 288 nm ( $y = 0.0055x - 0.0117$ ) compared to 1.479 of aspalathin ( $40 \mu\text{g}\cdot\text{mL}^{-1}$ ) and could therefore make a substantial contribution to the absorbance at 288 nm.

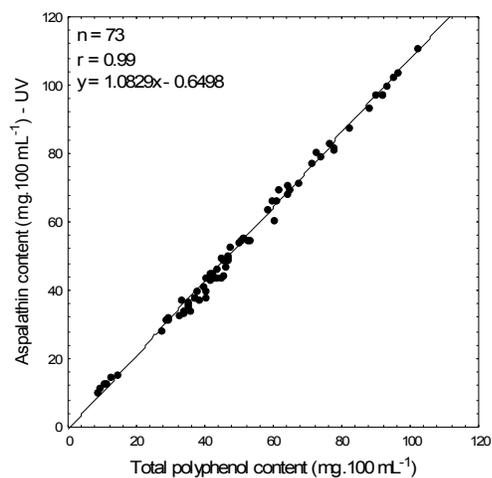
A good correlation was found between the aspalathin content estimated by UV spectroscopy and the TPC determined with the Folin-Ciocalteu method ( $r = 0.99$ ) (Figure 4). The slope and y-intercept were 1.08 and 0.65, respectively. This indicates that the TPC of water extracts of rooibos can be determined using UV spectroscopy, instead of the Folin-Ciocalteu method. No special reagents would therefore be required. However, pure aspalathin is expensive and not easily available. The use of another more economical and easily available phenolic compound as standard, or the use of the extinction coefficient of aspalathin should be investigated.

#### *Aluminum chloride colorimetric estimation of mangiferin content of green C. genistoides water extracts*

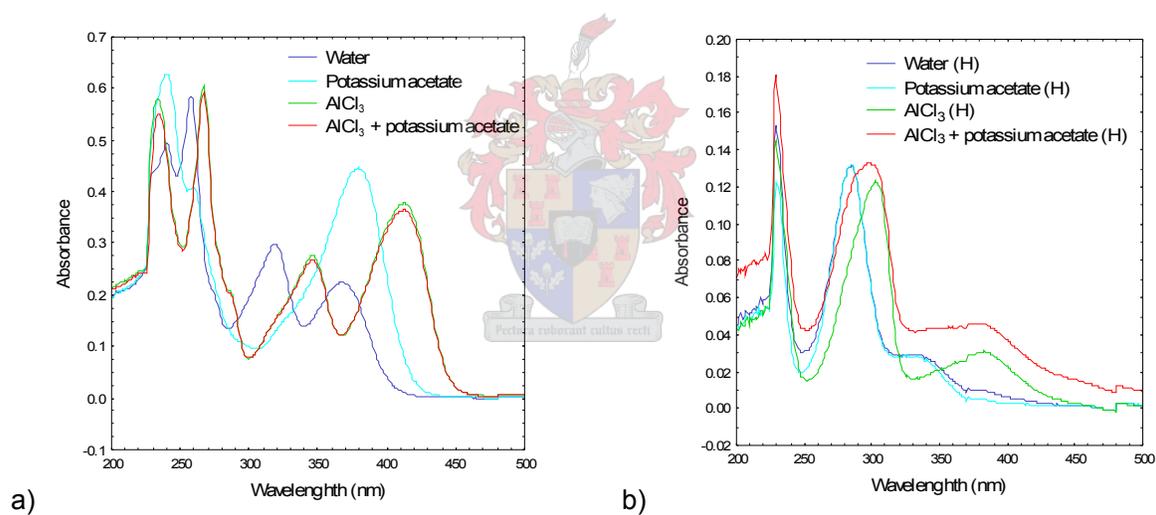
Figure 5a shows the respective effects of potassium acetate,  $\text{AlCl}_3$  and  $\text{AlCl}_3$  + potassium acetate on the UV/Vis spectrum of mangiferin. The  $\lambda_{\text{max}}$  (nm) data are summarised in Table 2. Complex formation of mangiferin with  $\text{AlCl}_3$  resulted in considerable bathochromic shifts of Band I (27 nm) and Band II (43 nm), as well as hyperchromic shifts of Bands I and II. At 410 nm a substantial increase (ca. 0.4) in absorbance was thus obtained. Complexation with potassium acetate resulted in a bathochromic shift of 61 nm and one large peak at 383 nm. Complex formation of mangiferin with  $\text{AlCl}_3$  + potassium acetate gave a similar effect to  $\text{AlCl}_3$  alone, which indicated that potassium acetate did not have an effect on the complex formation between mangiferin and  $\text{AlCl}_3$ . The absorbance of hesperidin in the presence of  $\text{AlCl}_3$  resulted in a small bathochromic shift (17 nm), as well as a hyperchromic shift of Band II at 410 nm, indicating its low reactivity. This is in the same order as the bathochromic shift of 23 nm for band II for a methanolic solution of hesperidin in the presence of  $\text{AlCl}_3/\text{HCl}$  (Mabry *et al.*, 1970). Addition of potassium acetate alone had no effect.  $\text{AlCl}_3$  together with potassium acetate also slightly increased the absorbance at 410 nm.

According to Brandt (2004) isomangiferin, another xanthone, is also present in green *C. genistoides*. However, no isomangiferin was found as determined by HPLC in this study. Other compounds present in *C. genistoides* which contain a C-4 keto and either a C-3 or C-5 hydroxyl group or C-6 and C-7 hydroxyl groups, are eriodictyol and luteolin, which give strong absorbances (Chang *et al.*, 2002). They are both present in very low quantities (Van der Merwe, 2004) and would therefore only make a small contribution to the increase in absorbance.

Only a moderate correlation coefficient ( $r = 0.77$ ) was found between the mangiferin content in water extracts determined using the aluminum chloride colorimetric method and mangiferin content quantified using HPLC (Figure 6). The slope of 0.99 confirmed that



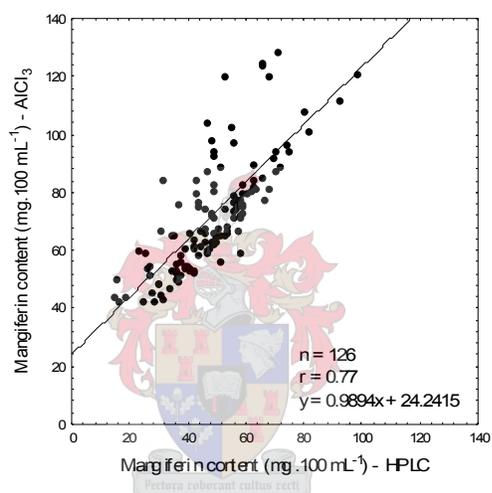
**Figure 4** Correlation of UV spectrophotometric estimated aspalathin content ( $\text{mg.100 mL}^{-1}$ ) with total polyphenol content ( $\text{mg.100 mL}^{-1}$ ) (Folin-Ciocalteu assay) of green rooibos water extracts.



**Figure 5** UV/Vis spectra of mangiferin a) and hesperidin b) in water, potassium acetate,  $\text{AlCl}_3$  and  $\text{AlCl}_3$  + potassium acetate solutions, respectively.

**Table 2**  $\lambda_{\max}$  of mangiferin and hesperidin in water, potassium acetate,  $\text{AlCl}_3$  and  $\text{AlCl}_3$  + potassium acetate.

Reaction mixture designation	$\lambda_{\max}$ (nm)		
	Mangiferin		Hesperidin
	Band I	Band II	Band II
Water	322	372	290
Potassium acetate	383		290
$\text{AlCl}_3$	349	415	307
$\text{AlCl}_3$ + potassium acetate	350	417	300



**Figure 6** Correlation of mangiferin content (mg.100 mL<sup>-1</sup>) of green *C. genistoides* water extracts determined using the aluminum chloride colorimetric method with mangiferin content (mg.100 mL<sup>-1</sup>) quantified using HPLC.

mangiferin is largely responsible for the absorbance. The y-intercept of 24.24, indicating absorbance at 410 nm by other compounds not compensated for, could possibly be explained by the presence of proanthocyanidins in *C. genistoides*. These type of compounds will absorb at between 400 and 600 nm (Hathway & Seakins, 1957).

The methanol extracts gave a good correlation ( $r = 0.90$ ) between the mangiferin content determined using the  $\text{AlCl}_3$  colorimetric method and mangiferin content quantified using HPLC (Figure 7). The slope increased to 1.23, compared to the 0.99 for the water extracts. The overestimation could have been due to compounds such as hesperidin not readily soluble in water, but readily soluble in methanol. Although it has a low reactivity towards  $\text{AlCl}_3$  it might still make a small contribution when it is present in higher quantities such as in the case of methanol extracts. The y-intercept in this case was reduced to 5.95 indicating that methanol was more selective in extraction of compounds that could contribute to background absorbance.

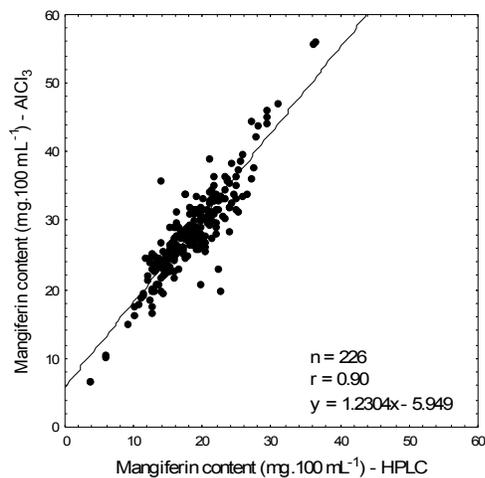
#### *Contribution of aspalathin and mangiferin to the total polyphenol content of extracts*

In the present study, using a large number of samples ( $n = 73$ ), it was found that a good correlation exists between the TPC and the aspalathin content quantified by HPLC ( $r = 0.97$ ) (Figure 8). This is contrary to what was found by Schulz *et al.* (2003), using only a small sample set ( $n = 20$ ;  $r = 0.09$ ). In the present study the variation in the aspalathin content accounted for 93% of the variation in the TPC ( $R^2 = 0.93$ ). Aspalathin is the major compound in green rooibos. Between 35 and 68% of the TPC of green rooibos comprise of aspalathin (Schulz *et al.*, 2003).

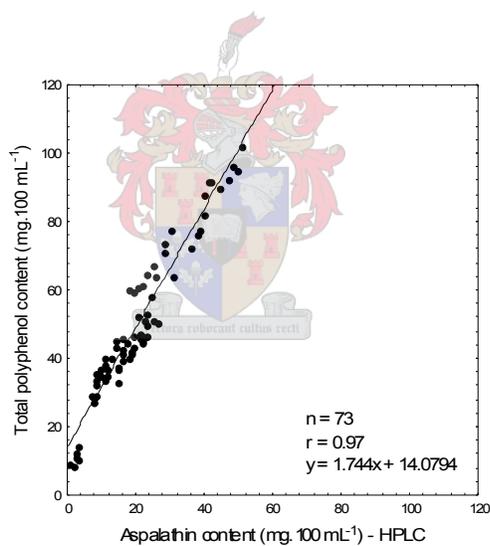
A poor correlation was found between the TPC and the mangiferin content ( $r = 0.22$ ) (Figure 9). The variation in the mangiferin content only accounted for 5% of the variation in the TPC ( $R^2 = 0.05$ ). Hesperidin ( $4.65\text{-}20.24 \text{ mg}\cdot 100 \text{ mL}^{-1}$ ) was present in relatively high quantities but did not vary to the same extent as mangiferin ( $14.39\text{-}98.38 \text{ mg}\cdot 100 \text{ mL}^{-1}$ ). Since it also reacts with the Folin-Ciocalteu reagent, it would contribute to the poor correlation of mangiferin with TPC. The ratio of mangiferin to gallic acid and hesperidin to gallic acid required to give the same colour reaction with the Folin-Ciocalteu reagent is 0.47:1 and 0.98:1 (mass basis), respectively (Richards, 2002). Other phenolic compounds that would contribute to the TPC value were not determined.

#### *Contribution of aspalathin and mangiferin to the total antioxidant activity of extracts*

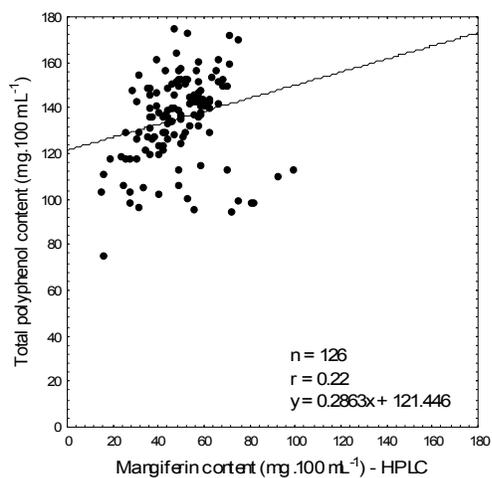
A good correlation was found for the TAA with the aspalathin content quantified by HPLC ( $r = 0.96$ ) (Figure 10) confirming the results found for commercial green rooibos by Schulz *et al.* (2003) ( $n = 15$ ,  $r = 0.90$ ;  $y = 104.73x + 118.54$ ). Schulz *et al.* (2003), however, found contradictory results for experimental green rooibos samples ( $n = 20$ ;  $r = 0.39$ ). In both cases small sample sizes were used. Aspalathin is the major compound in green rooibos and a potent antioxidant (Snijman *et al.*, 2004). The apparent contribution of aspalathin to the TAA of green



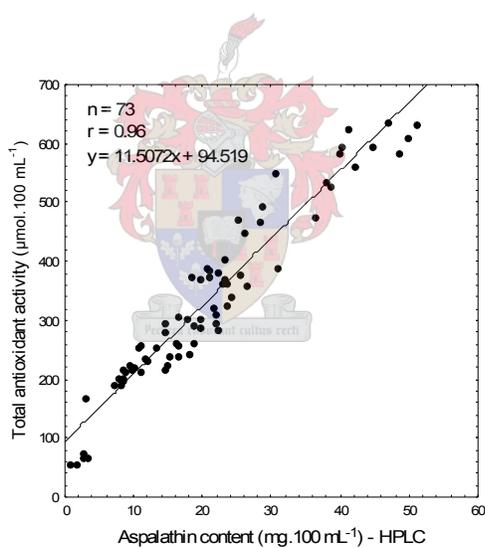
**Figure 7** Correlation of mangiferin content (mg.100 mL<sup>-1</sup>) of green *C. genistoides* methanol extracts determined using the aluminum chloride colorimetric method with mangiferin content (mg.100 mL<sup>-1</sup>) quantified using HPLC.



**Figure 8** Correlation of the total polyphenol content (mg.100 mL<sup>-1</sup>) (Folin-Ciocalteu assay) with aspalathin content (mg.100 mL<sup>-1</sup>) of green rooibos water extracts quantified using HPLC.



**Figure 9** Correlation of the total polyphenol content (mg.100 mL<sup>-1</sup>) (Folin-Ciocalteu assay) with mangiferin content (mg.100 mL<sup>-1</sup>) of green *C. genistoides* water extracts quantified using HPLC.



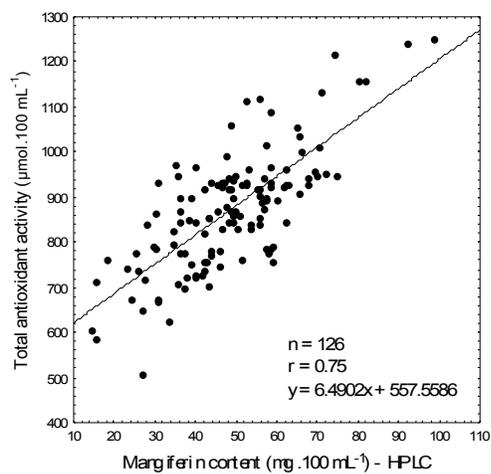
**Figure 10** Correlation of the total antioxidant activity (μmol.100 mL<sup>-1</sup>) (ABTS assay) with the aspalathin content (mg.100 mL<sup>-1</sup>) of green rooibos water extracts quantified using HPLC.

rooibos was 22-57% (Schulz *et al.*, 2003). Other monomeric compounds make a small contribution in terms of quantity (*ca.* 0.92%) (Bramati *et al.*, 2003) and have either similar or less antioxidant potency than aspalathin in the ABTS assay (Snijman *et al.*, 2004).

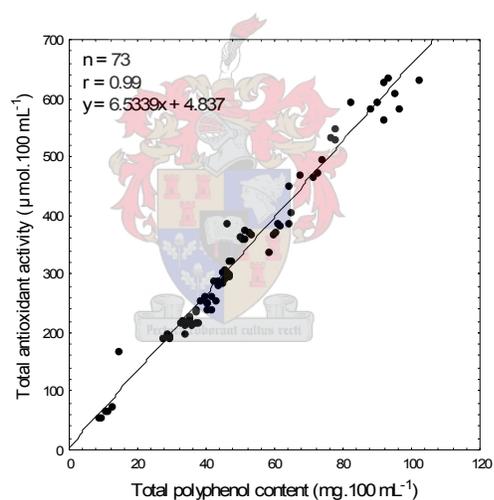
The TAA of the green *C. genistoides* water extracts correlated moderately with the mangiferin content ( $r = 0.75$ ) (Figure 11). Two samples, with almost the same TAA value, e.g. 711.33 and 704.36  $\mu\text{mol}\cdot 100\text{ mL}^{-1}$  (first extract was prepared from old regrowth, while the latter extract was prepared from young regrowth) had substantially different mangiferin contents of 15.27 and 43.20  $\text{mg}\cdot 100\text{ mL}^{-1}$ , respectively. This indicated that the contribution of other compounds to TAA is significant. Mangiferin displays a moderate antioxidant activity in the ABTS assay (Richards, 2002), but it is highly reactive relative to hesperidin. According to Richards (2002) concentrations of 0.618 and 4.128  $\mu\text{mol}$  for mangiferin and hesperidin, respectively, are required to give the same activity as 1  $\mu\text{mol}$  Trolox. Mangiferin contributed 22% of the ABTS radical scavenging activity of *C. genistoides* water extracts compared to <1% by hesperidin (Richards, 2002). This does not take any synergistic effects, if any, into account.

#### *Relationship between the TAA and the total polyphenol content of extracts*

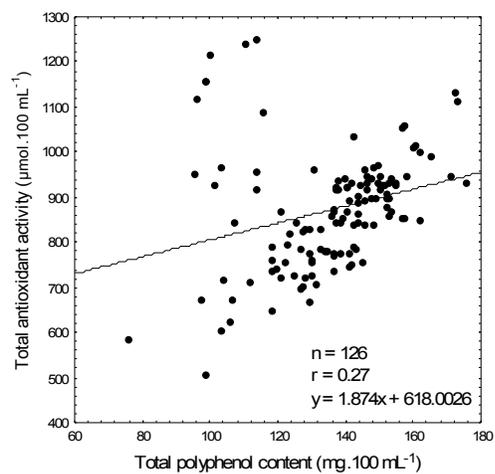
The TAA of the rooibos water extracts gave a good correlation with the TPC ( $r = 0.99$ ) (Figure 12), which is in agreement with results obtained for other plant material (Cai *et al.*, 2004; Gorinstein *et al.*, 2004; Soong & Barlow, 2004). This high correlation is due to the fact that aspalathin is the major compound comprising the largest part of the TPC and that it is a potent antioxidant. Since the basic mechanism of the Folin-Ciocalteu method is an oxidation/reduction reaction it can be considered as another antioxidant capacity method (Huang *et al.*, 2005; Prior *et al.*, 2005), which can thus also be used to estimate the TAA. On the other hand, the TAA gave a poor correlation with the TPC ( $r = 0.27$ ) (Figure 13) of *C. genistoides* water extracts. Since the relative reactivity of mangiferin and hesperidin towards the ABTS radical cation and the Folin-Ciocalteu reagent differs, it would contribute to the poor correlation between TAA and TPC. The relative high quantities of hesperidin present in the samples used in this study thus contributed substantially to the TPC but not to the same extent to the TAA. The poor correlation obtained did not agree with the results of Richards (2002) who found a good correlation between the TAA and the TPC ( $r = 0.98$ ) of *Cyclopia* water extracts using the combined data of samples of different species, i.e. *C. intermedia*, *C. subternata*, *C. sessiliflora* and *C. genistoides*. Not only was a limited sample set ( $n = 24$ ) (Richards, 2002) used, but different species have different phenolic profiles (Ferreira *et al.*, 1998; Kamara *et al.*, 2003; Kamara *et al.*, 2004). The mangiferin and hesperidin contents of these species differ among each other (Joubert *et al.*, 2003; Van der Merwe, 2004). Mangiferin contributes to a high TAA and TPC compared to hesperidin contributing to a low TAA and relative high TPC.



**Figure 11** Correlation of the total antioxidant activity ( $\mu\text{mol}\cdot 100\text{ mL}^{-1}$ ) (ABTS assay) with the mangiferin content ( $\text{mg}\cdot 100\text{ mL}^{-1}$ ) of green *C. genistoides* water extracts quantified using HPLC.



**Figure 12** Correlation of the total antioxidant activity ( $\mu\text{mol}\cdot 100\text{ mL}^{-1}$ ) (ABTS assay) with the total polyphenol content ( $\text{mg}\cdot 100\text{ mL}^{-1}$ ) (Folin-Ciocalteu assay) of green rooibos water extracts.



**Figure 13** Correlation of the total antioxidant activity ( $\mu\text{mol.100 mL}^{-1}$ ) (ABTS assay) with the total polyphenol content ( $\text{mg.100 mL}^{-1}$ ) (Folin-Ciocalteu assay) of green *C. genistoides* water extracts.



## Conclusions

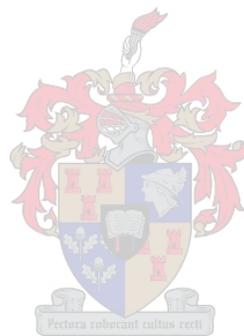
UV spectroscopy overestimated the aspalathin content compared to that determined using HPLC, but it showed good potential to estimate the total polyphenol content of extracts. The good correlation between the total antioxidant activity and the TPC of green rooibos suggests the use of either method in quality control. The aluminium chloride colorimetric method can be used to estimate the mangiferin content in *C. genistoides* methanol extracts, although it overestimated the mangiferin content. The mangiferin content of extracts could not be used as an indication of their TAA and the TPC, as poor correlations with the mangiferin content were obtained.

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## CHAPTER 5



**Differentiation between green rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia genistoides*) and classification of green rooibos cultivation areas and *C. genistoides* types and harvest dates using principal component and linear discriminant analyses**

## CHAPTER 5

# DIFFERENTIATION BETWEEN GREEN ROOIBOS (*ASPALATHUS LINEARIS*) AND HONEYBUSH (*CYCLOPIA GENISTOIDES*) AND CLASSIFICATION OF GREEN ROOIBOS CULTIVATION AREAS AND *C. GENISTOIDES* TYPES AND HARVEST DATES USING PRINCIPAL COMPONENT AND LINEAR DISCRIMINANT ANALYSES

### Abstract

Principal component analysis (PCA) and linear discriminant analysis (LDA) of near infrared spectral data were applied to differentiate between dried, green rooibos and *C. genistoides* and to classify dried, green rooibos according to cultivation area and dried, green *Cyclopia genistoides* according to type and harvest date.

It was possible to differentiate clearly between dried, green rooibos and *C. genistoides* plant material samples using PCA and LDA. Adding samples to increase the variation of the plant material resulted in some overlapping of clusters using PCA, but LDA correctly classify both green rooibos (98.45%) and *C. genistoides* (96.15%). PCA was unable to classify clearly between green rooibos samples from different cultivation areas and *C. genistoides* samples harvested at regular intervals during the course of a year. Samples from the same cultivation area and samples harvested on the same day, however, had a tendency to group together. LDA was able to correctly classify between 50 and 100% of the samples according to the respective cultivation areas and harvest dates. PCA of green *C. genistoides* types (West Coast en Overberg, harvested during 2001) showed two, clearly separated, clusters which was confirmed by 100% correct classification by LDA. Adding green *C. genistoides* samples of both types, harvested during a different season, i.e. 2003, resulted in partial overlapping of the clusters. Excellent classification results were, however, still obtained with LDA for both the West Coast (98.46%) and Overberg (98.36%) types.

### Introduction

Authentication of raw materials and final products is important to guarantee that a food, herbal or pharmaceutical product conforms to the specification provided by the producer or manufacturer and that no adulterants were added (Downey, 1995, 1996, 1998). According to The European Agency for the Evaluation of Medical Products, identification of herbal drugs is one of the first tests to be applied in ensuring quality, safety and efficacy of herbal medicinal products. The test used should be able to differentiate between related species and/or potential adulterants (Anon., 2001).

For development of phenolic-enriched plant extracts for the nutraceutical industry, plant material should contain sufficient quantities of the phenolic compounds to ensure extracts with

high quantities of these compounds. The phenolic composition of plant extracts differs considerably among species and even between extracts prepared from the same plant (He, 2000). Some factors contributing to this variation include the geographical origin or cultivation area, climate, harvest season, age of the plant, drying process and storage conditions, which make effective quality control of herbal plant material a challenge for the food and phytopharmaceutical industries (He, 2000; Laasonen *et al.*, 2002).

Rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.), two herbal plants indigenous to South Africa, are known for their beneficial health and functional properties due to their antioxidant activity. Aspalathin, the major compound present in and unique to rooibos, contributes 22-57% of the total antioxidant activity (TAA) of green rooibos extracts (Schulz *et al.*, 2003) whereas mangiferin, the major compound in *C. genistoides*, contributes 22% of the TAA of green *Cyclopia* extracts (Richards, 2002). A light green colour is normally associated with dried, green rooibos and indicative of good retention of aspalathin and thus a product of higher value. Dried, green *C. genistoides* has a brighter green colour and is less susceptible to discolouration. Adulteration of green rooibos with green *C. genistoides*, which has lower antioxidant activity compared to rooibos, is therefore likely to enhance the colour of dried, green rooibos with subsequent perception of good aspalathin retention. If HPLC is not employed to verify the phenolic profile, adulteration of green rooibos with *C. genistoides* may go undetected.

The mangiferin and hesperidin contents differ between plant material of the two *C. genistoides* types, West Coast (mangiferin: 4.93 g.100 g<sup>-1</sup> and hesperidin: 5.21 g.100 g<sup>-1</sup>) and Overberg (mangiferin: 6.37 g.100 g<sup>-1</sup> and hesperidin: 2.23 g.100 g<sup>-1</sup>) (Joubert *et al.*, 2003). Additionally the harvest date of *C. genistoides* affects the mangiferin and hesperidin contents of the plant material (Joubert *et al.*, 2003).

Near infrared spectroscopy (NIRS) is a rapid analytical technique that has been used for quantitative (reviewed by Schulz, 2004), as well as qualitative analysis of plant material. Qualitative NIRS applications include identification (Laasonen *et al.*, 2002) and classification of plant species (Lister *et al.*, 2000; Laasonen *et al.*, 2002), cultivation area (Woo *et al.*, 2002), geographical origin (Woo *et al.*, 1999) and processing methods, e.g. between unfermented and fermented rooibos (Schulz *et al.*, 2003). NIRS has also been used for the discrimination of black teas of different qualities (Osborne & Fearn, 1988). Principal component analysis (PCA) has been applied to NIRS spectral data to discriminate between different vegetable oils (Sato, 1994) and between different commercially-produced wheat flours (Chiba *et al.*, 1995). There is no evidence of NIRS being used to distinguish between dried, green rooibos and *C. genistoides* or to classify between dried, green rooibos of different cultivation areas, different *C. genistoides* types and *C. genistoides* samples harvested at different dates.

The objectives of this study were to:

- differentiate between dried, green rooibos and *C. genistoides*;

- classify dried, green rooibos according to cultivation area (Nieuwoudtville, Gifberg, Nardouwsberg, Agter Pakhuis, Clanwilliam and Citrusdal);
- classify dried, green *C. genistoides* according to type (West Coast and Overberg); and
- classify dried, green *C. genistoides* according to harvest date (31/03/2003, 15/05/2003, 20/06/2003, 11/08/2003, 15/09/2003, 04/11/2003, 10/12/2003 and 26/01/2004).

## **Materials and methods**

### *Green rooibos plant material*

Dried, green rooibos samples (n = 299), harvested during 1984, 1999 and 2004 were obtained from the sample collection of ARC Infruitec-Nietvoorbij. The fresh plant material was dried at 40°C to ca. 8-10% moisture content and ground with a Retsch mill (1 mm sieve). Samples harvested during 1984 and 2004 at two different cultivation areas, Citrusdal and Clanwilliam, included young growth, older plant material and flowers which provided likely variation that could occur during a growth cycle. Included in the sample set were also samples (n = 10) consisting of only toppings (i.e. the first harvest of a newly-established plantation) and samples (n = 20) consisting of either leaves or stems. Samples harvested during 1999 were from different cultivation areas, i.e. Nieuwoudtville, Gifberg, Nardouwsberg, Agter Pakhuis, Clanwilliam and Citrusdal.

### *Green C. genistoides plant material*

Dried, green *C. genistoides* samples (n = 240) were obtained from ARC Infruitec-Nietvoorbij (harvested during 2001, 2003, 2004 and 2005). The fresh plant material was dried at 40°C to ca. 8-10% moisture content and ground with a Retsch mill (1 mm sieve). Samples harvested during 2001, 2003 and 2004 represented two types, i.e. West Coast and Overberg, and were harvested in the Pearly Beach area. Harvesting took place approximately every 5-6 weeks from 28/03/2001 to 11/07/2001 and every 6-8 weeks from 31/03/2003 to 26/01/2004 to include variation that could occur during a growth cycle. Some of the previously harvested plants were re-harvested on 26/01/2004 to obtain samples comprising of young regrowth. The samples harvested during 2005 were the West Coast type, originating from Reins Farm, Albertinia. Included in the sample set were also samples (n = 10) comprising of either leaves or stems.

### *Near infrared spectroscopy measurements of dried green rooibos and C. genistoides plant material*

A Büchi NIRLab N-200 Fourier transform near infrared (FT-NIR) spectrophotometer, with NIRLabWare (version 3.0) near infrared (NIR) measurement software, was used to perform the NIRS measurements in diffuse reflectance mode. The ground, dried plant material was presented to the instrument in rotating glass petri-dishes and the NIR spectra collected from 1000-2500 nm at a resolution of 12 cm<sup>-1</sup> resulting in 1557 data points.

### *Moisture content determination*

Twenty rooibos and 20 *C. genistoides* samples were selected to evaluate the possible effect of moisture content on the classification of NIR spectra of the dried, green rooibos and *C. genistoides* samples. The moisture content was determined, in duplicate, by calculating the moisture loss after the samples (ca. 5 g) were dried for 16 h in a vacuum oven. NIR spectral data of these samples were recorded as-is (before moisture loss) and at 0% moisture (after drying in vacuum oven) as described earlier.

### *Classification using principal component analysis and linear discriminant analysis*

Principal component analysis (NIRCal version 4.21) was applied to the spectral data of the samples of the respective classification groups as described in Table 1 after pretreatment of the spectra (Table 2). Linear discriminant analysis (LDA) (STATISTICA, version 7), using leave-one-out cross validation, was performed on the first five principal components to develop models for the classification between green rooibos and *C. genistoides* and for the classification of green rooibos cultivation areas and *C. genistoides* types and harvest dates. No outliers were removed. PCA was applied to the NIR spectra, pretreated with multiplicative scatter correction (MSC) and first derivative, of the 40 selected green rooibos and *C. genistoides* samples before and after moisture loss to evaluate the possible effect of moisture content on classification.

## **Results and discussion**

### *Differentiation between green rooibos and green C. genistoides*

The PCA score plot of principal component 1 (PC1) versus PC2 (Figure 1) showed complete separation of the green rooibos (1999) and *C. genistoides* (2001) plant material samples (Table 1, classification group 1). Similar results were found for the LDA analysis of both rooibos and *C. genistoides* with 100% of the samples correctly classified (Figure 2).

The effect of moisture content on the classification of the green rooibos and *C. genistoides* samples was evaluated by applying PCA to the NIR spectral data of 20 rooibos and 20 *C. genistoides* samples to confirm that the classification between samples were not purely due to differences in moisture content. There were little difference in the moisture content of the green rooibos (range: 7.02-8.84%; mean±SD: 7.77±0.62%) and *C. genistoides* (range: 7.29-8.66%; mean±SD: 7.95±0.32%) samples. The NIR spectra of the dried, green rooibos and *C. genistoides* samples collected before and after moisture loss are depicted in Figure 3, respectively, and the corresponding MSC and first derivative pretreated spectra in Figure 4, respectively. The main differences in absorbancies between the green rooibos and *C. genistoides* spectra, before and after moisture loss, were observed in the spectral regions between 1600 and 1690, 1720 and 1730, and 2100 and 2300 nm (Figures 3 & 4). None of these absorption areas correspond to absorption due to water or moisture content. From Figure 3 it is clear that the main difference between the spectra of the dried, green rooibos and

**Table 1** Description of the different classification groups and classes within groups to which PCA and LDA was applied.

Classification group	Class 1	Class 2	Class 3	Class 4	Class 5	Class 6	Class 7	Class 8
1 Green rooibos vs. green <i>C. genistoides</i> (n = 115)	Green rooibos harvested in 1999	Green <i>C. genistoides</i> harvested in 2001						
2 Green rooibos vs. green <i>C. genistoides</i> (n = 539) (over seasons)	Green rooibos harvested during 1984, 1999 and 2004	Green <i>C. genistoides</i> harvested during 2001, 2003 and 2005						
3 Green rooibos (n = 75) from different cultivation areas (within the same harvest season)	Harvested during 1999 at Nieuwoudtville	Harvested during 1999 at Gifberg	Harvested during 1999 at Nardouwsberg	Harvested during 1999 at Agter Pakhuis	Harvested during 1999 at Clanwilliam	Harvested during 1999 at Citrusdal		
4 Types of green <i>C. genistoides</i> (n = 40) (within one season)	West Coast type harvested during 2001	Overberg type harvested during 2001						
5 Types of green <i>C. genistoides</i> (n = 228) (over seasons)	West Coast type harvested during 2001 and 2003	Overberg type harvested during 2001 and 2003						
6 Harvest dates of green <i>C. genistoides</i> (n = 90)	Harvested 31/03/2003	Harvested 15/05/2003	Harvested 20/06/2003	Harvested 11/08/2003	Harvested 15/09/2003	Harvested 04/11/2003	Harvested 10/12/2003	Harvested 26/01/2004

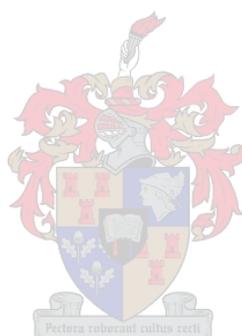
**Table 2** Summary of the pretreatments applied to the respective groups of samples and the principal components (PCs) used.

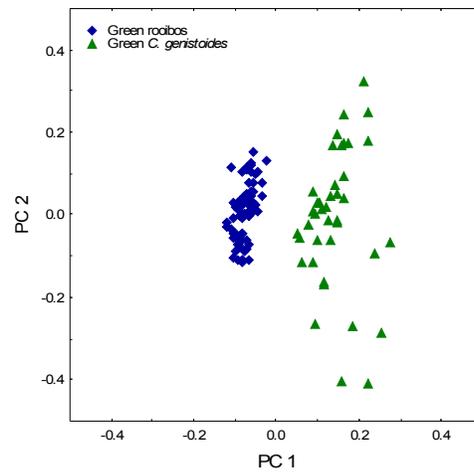
Classification group	Pretreatment	Principal components (PCs)
1	n01 <sup>a</sup> , db1 <sup>b</sup>	PCs 1 & 2
2	n01, db1	PCs 1 & 2
3	MSC <sup>c</sup> , db1	PCs 1 & 2
4	MSC, db1	PCs 1 & 3
5	n01, db1	PCs 1 & 2
6	MSC, db1	PCs 1 & 2

<sup>a</sup> Normalisation (between 0 and 1)

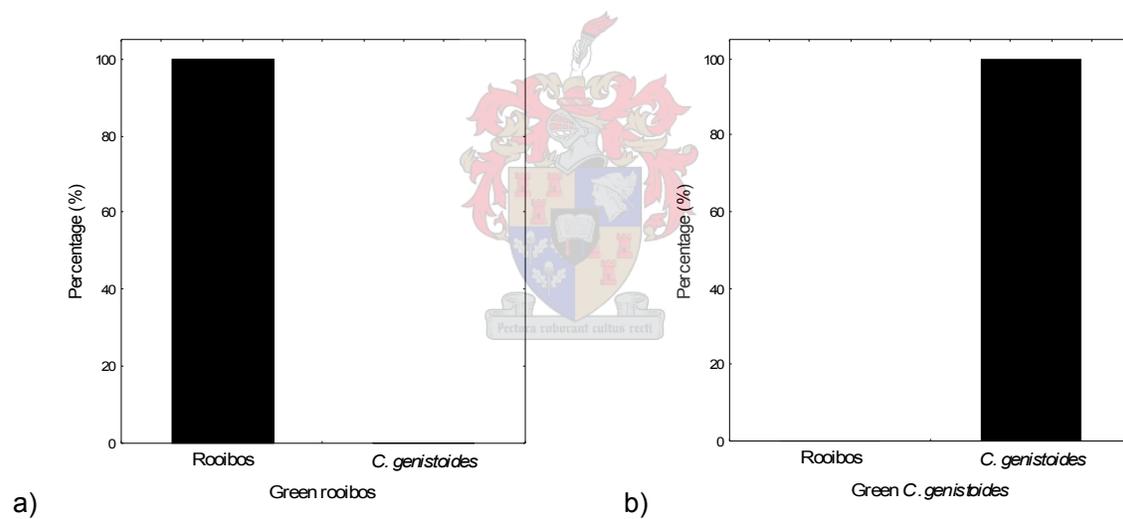
<sup>b</sup> First derivative

<sup>c</sup> Multiplicative scatter correction

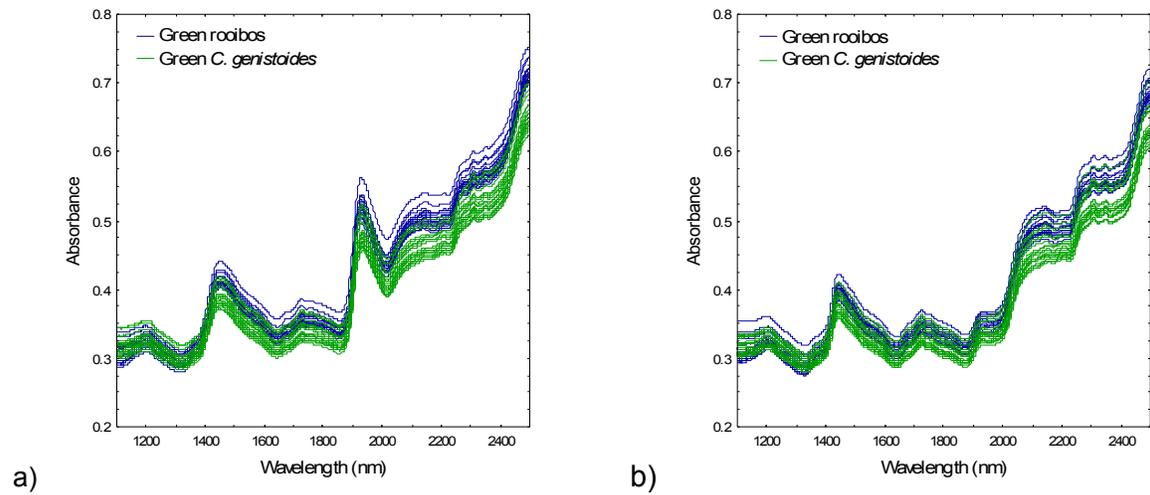




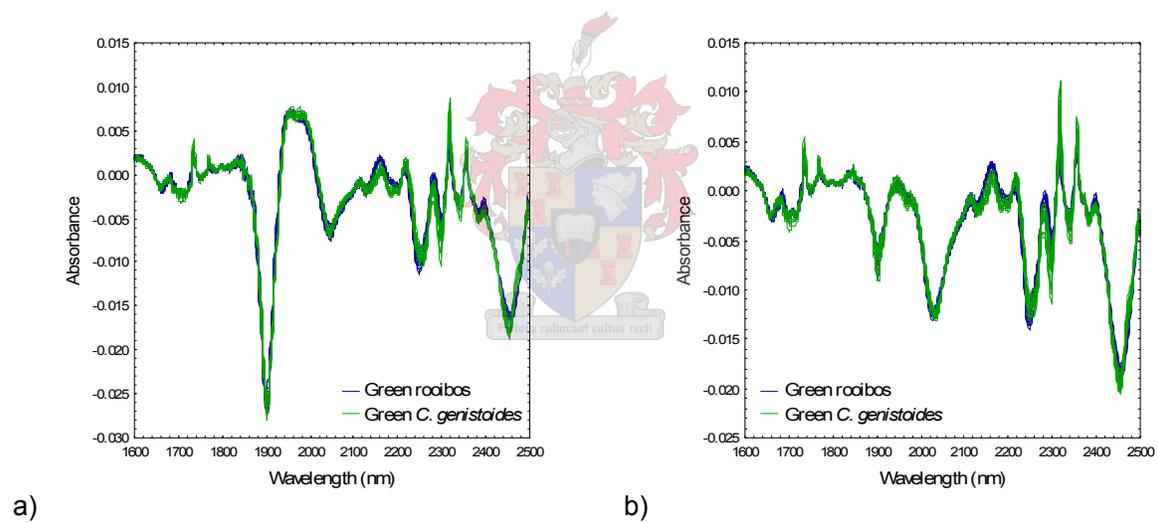
**Figure 1** PCA score plot (PC1 vs PC2) to differentiate between dried, green rooibos (harvested during 1999) and *C. genistoides* (harvested during 2001).



**Figure 2** LDA results for the classification of green rooibos (harvested during 1999) and *C. genistoides* (harvested during 2001) showing 100% correct classification of a) green rooibos and b) green *C. genistoides*.



**Figure 3** Spectra of dried, green rooibos and *C. genistoides* plant material a) before and b) after moisture loss.



**Figure 4** MSC and first derivative pretreated spectra of dried, green rooibos and *C. genistoides* plant material a) before and b) after moisture loss.

*C. genistoides* samples is due to physical differences, e.g. particle size. Differences are observed between the green rooibos and *C. genistoides* spectra (before and after moisture loss) (Figures 3 & 4) at ca. 1680 and 2230 nm, which are the two reference wavelengths used to predict the difference in hardness, i.e. particle size of wheat (AACC, 2000; Williams & Sobering, 1986; Norris *et al.*, 1989). This indicates that green rooibos and *C. genistoides* ground differently when using the same mill and sieve size. The occurrence of thick stems in *C. genistoides* samples could have contributed to the coarseness of the ground plant material. MSC, however, did not completely remove the effect of different particle sizes indicating also chemical differences (Figure 5). It is, therefore, clear that the green rooibos and *C. genistoides* samples did not classify due to differences in moisture content, but rather due to physical and chemical differences between the samples.

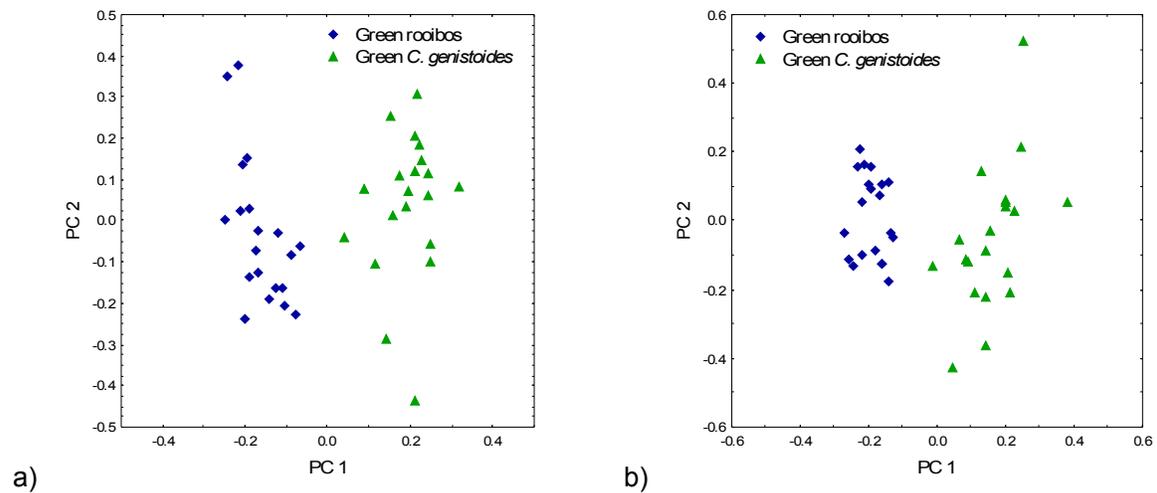
After expanding the sample set and the variation by adding more green rooibos (harvested during 1984 and 2004) and *C. genistoides* (harvested during 2003 and 2005) samples (Table 1, classification group 2), some overlap of clusters occurred (Figure 6). Using LDA 98.45% of the green rooibos and 96.15% of the *C. genistoides* samples were correctly classified (Figure 7).

#### *Classification of green rooibos cultivation areas*

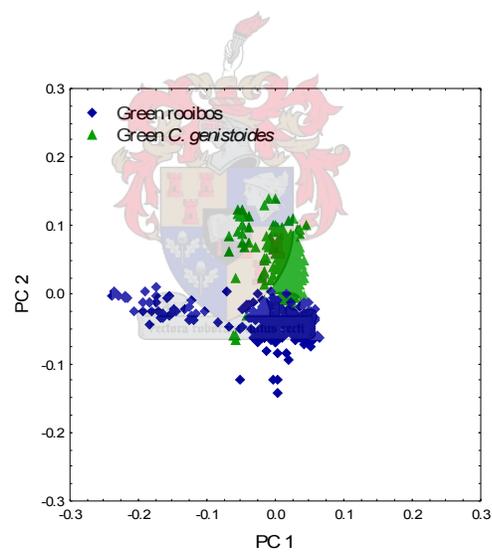
Dried, green rooibos samples from different cultivation areas (Table 1, classification group 3) showed no distinct clusters when plotting PC1 versus PC2. There was, however, a tendency for samples from the same cultivation area to group together (Figure 8). Using LDA correct classifications were obtained for green rooibos samples from Nieuwoudtville (80%), Gifberg (88%), Nardouwsberg (67%), Agter Pakhuis (100%), Clanwilliam (76%) and Citrusdal (85%) (Figure 9). Agter Pakhuis separated clearly from the other cultivation areas based on PC2 (Figure 8). As the samples from Agter Pakhuis had considerably lower aspalathin contents compared to the other samples it is likely that this could have contributed to the clear classification of these samples.

#### *Classification of green C. genistoides types*

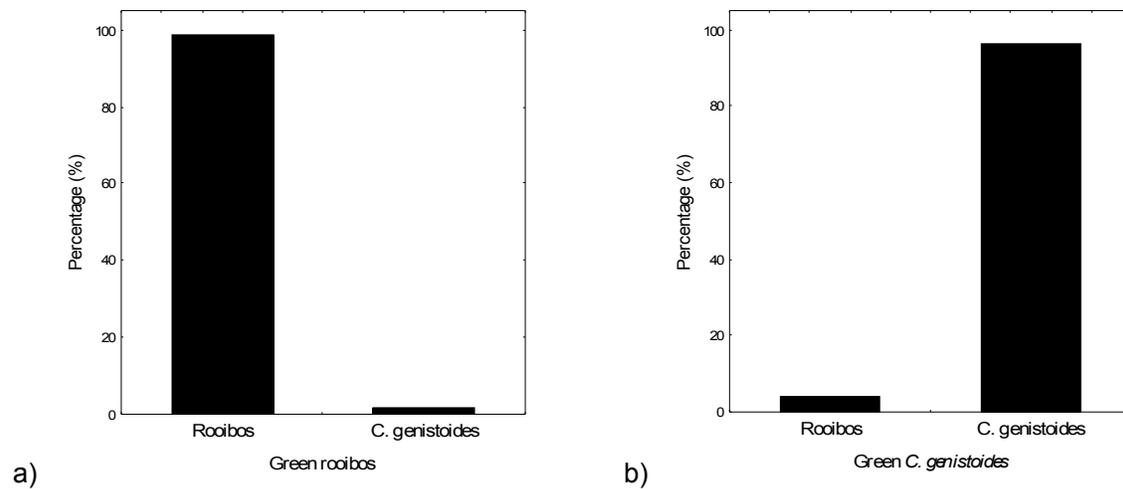
The PCA score plot of PC1 versus PC3 (Figure 10) revealed distinct clustering of the samples of the two *C. genistoides* types, West Coast and Overberg, harvested during 2001 (Table 1, classification group 4). The clear classification of both *C. genistoides* types was confirmed by the 100% correct classification of samples of both types using LDA (Figure 11). The successful classification of these two types could quite likely be due to the differences in their mangiferin and hesperidin contents as the contents of these two compounds differ considerably between the West Coast and Overberg types (Joubert *et al.*, 2003). By adding samples of both types, harvested during a different season, i.e. 2003 (Table 1, classification group 5), some overlap arose between



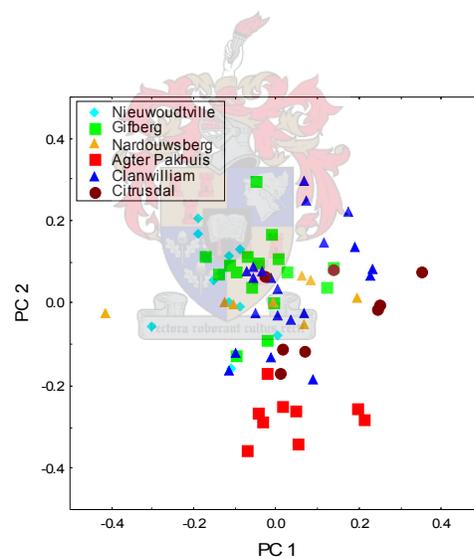
**Figure 5** PCA score plots (PC1 vs PC2) of the MSC and first derivative pretreated spectra showing clear classification between green rooibos and *C. genistoides* plant material samples a) before and b) after moisture loss.



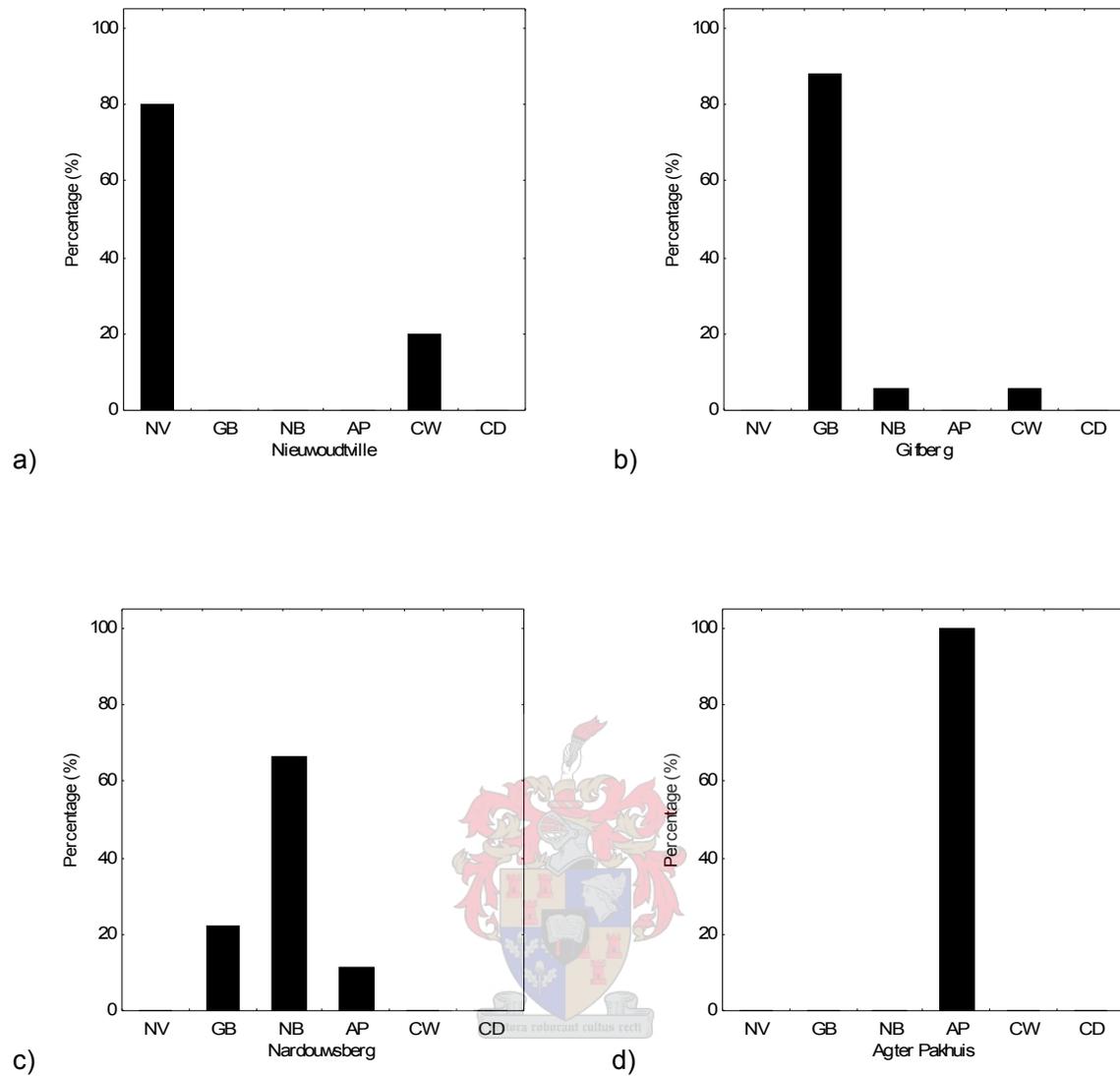
**Figure 6** PCA score plot (PC1 vs PC2) to differentiate between green rooibos (harvested during 1984, 1999 and 2004) and *C. genistoides* (harvested during 2001, 2003 and 2005).



**Figure 7** LDA results for the classification of green rooibos (harvested during 1984, 1999 and 2004) and *C. genistoides* (harvested during 2001, 2003 and 2005) showing the percentage of samples classified correctly and incorrectly for a) green rooibos and b) green *C. genistoides*.



**Figure 8** PCA score plot (PC1 vs PC2) for the classification of green rooibos cultivation areas (Nieuwoudtville, Gifberg, Nardouwsberg, Agter Pakhuis, Clanwilliam and Citrusdal).



**Figure 9** LDA results for the classification of green rooibos cultivation areas showing the percentage of samples classified correctly and incorrectly for each area: a) Nieuwoudville (NV), b) Gifberg (GB), c) Nardouwsberg (NB), d) Agter Pakhuis (AP), e) Clanwilliam (CW) and f) Citrusdal CD).

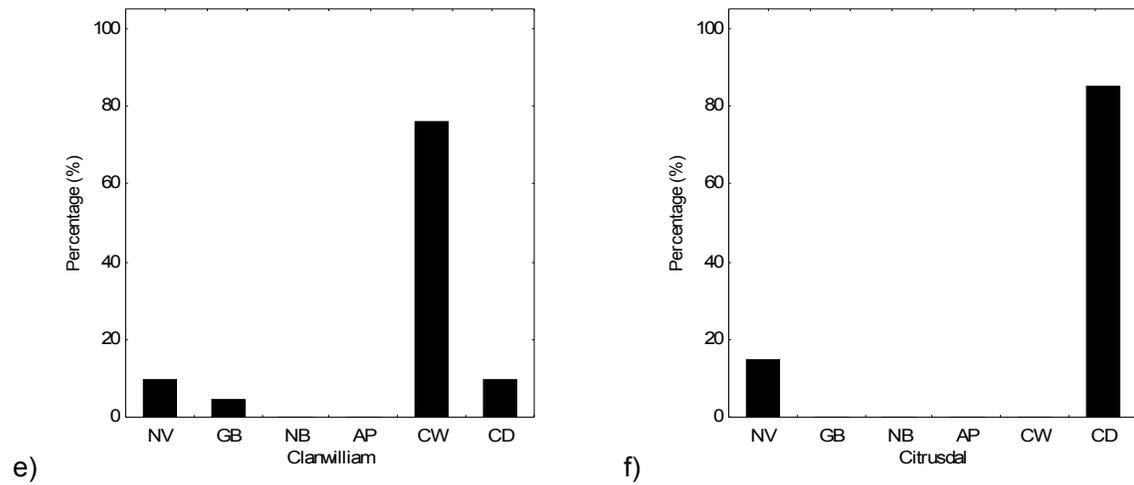


Figure 9 continued/...

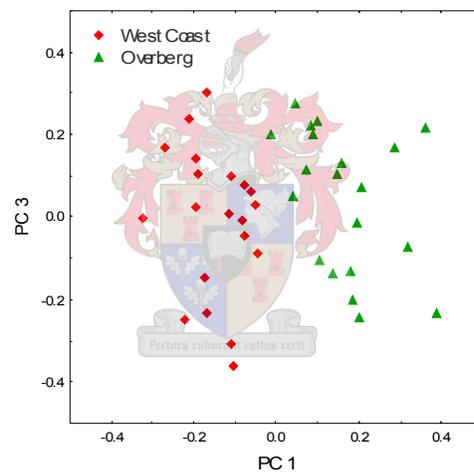
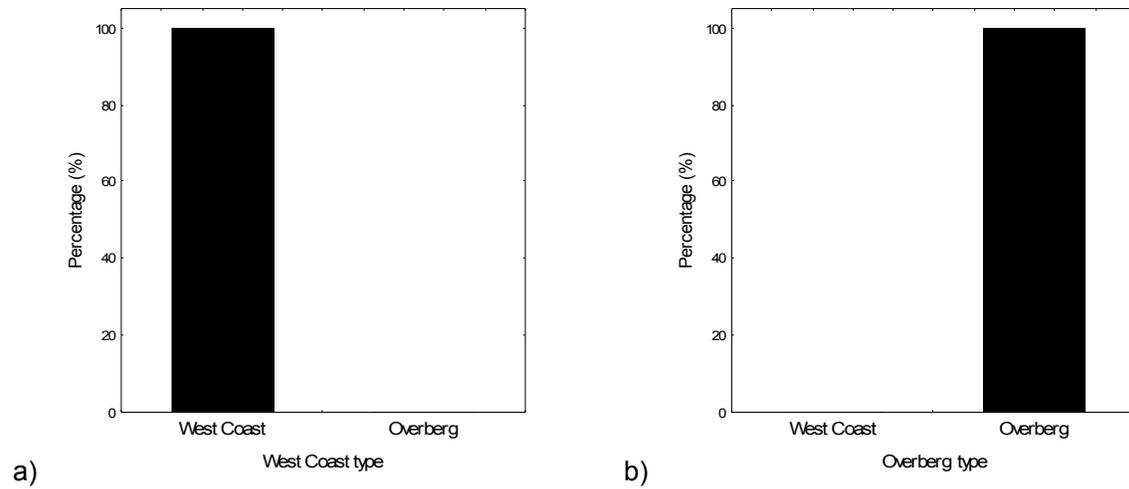
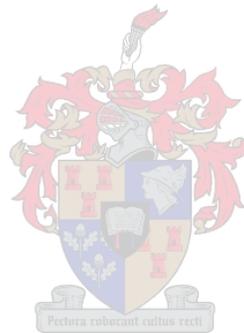


Figure 10 PCA score plot (PC1 vs PC3) of the classification between samples of green *C. genistoides* types (West Coast and Overberg) harvested during 2001.



**Figure 11** LDA results for the classification of green *C. genistoides* types (n = 40; harvested during 2001) showing 100% correct classification for both the a) West Coast and b) Overberg types.



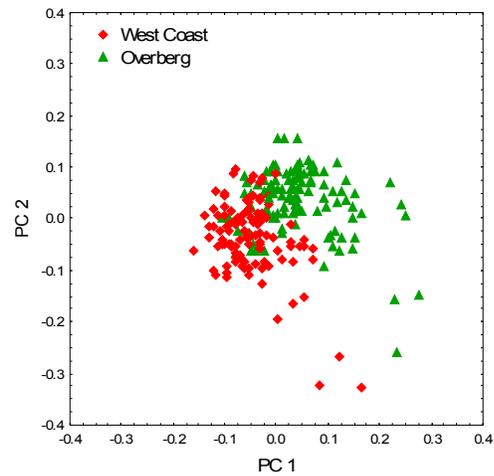
the clusters (Figure 12). LDA, however, still gave good results, with 98.46% of the samples of the West Coast type and 98.36% of the samples of the Overberg type (Figure 13) correctly classified.

#### *Classification of C. genistoides harvest dates*

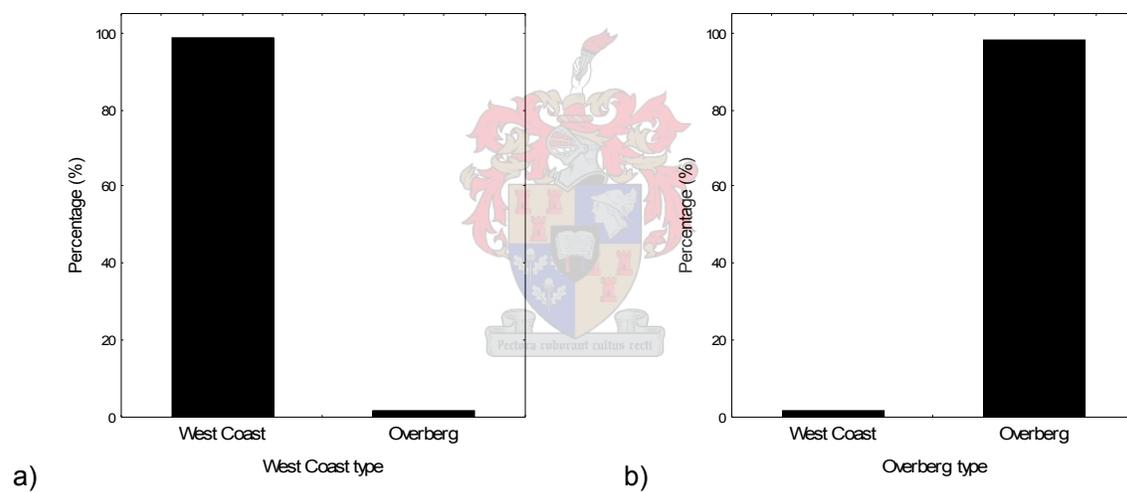
No distinct clusters were observed when plotting PC1 versus PC2 of the green *C. genistoides* (West Coast type only) samples harvested at different dates (Table 1, classification group 6). There was, however, a tendency for samples of the same harvest date to group together (Figure 14). The LDA results are depicted in Figure 15 and it was found that between 55 and 100% of the samples were correctly classified into their respective harvest date classes, i.e. 31/03/2003 (100%), 15/05/2003 (75%), 20/06/2003 (67%), 11/08/2003 (75%), 15/09/2003 (82%), 04/11/2003 (73%), 10/12/2003 (91%) and 26/01/2004 (55%). Classification between the samples could have been due to the different leaf:stem ratios of the samples as the plants have a lower leaf:stem ratio towards the end of summer and after flowering. The mangiferin and hesperidin contents of the samples showed little variation over the entire harvest period which was contradictory to the results found by Joubert *et al.* (2003). In the latter case the samples were harvested only from March to July.

#### **Conclusion**

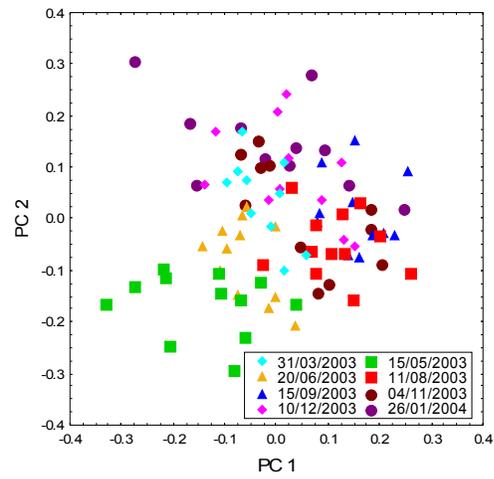
Applying PCA and LDA analyses to NIRS spectral data it was possible to differentiate between dried, green rooibos and *C. genistoides* samples and to classify between the two *C. genistoides* types (West Coast and Overberg). PCA could not clearly classify between green rooibos cultivation areas, nor between *C. genistoides* harvest dates. LDA, however, successfully classified between 55 and 100% of the dried, green rooibos and *C. genistoides* samples according to cultivation area and harvest date, respectively.



**Figure 12** PCA score plot (PC1 vs PC2) of the classification between samples of green *C. genistoides* types (West Coast and Overberg) harvested during 2001 and 2003 combined.

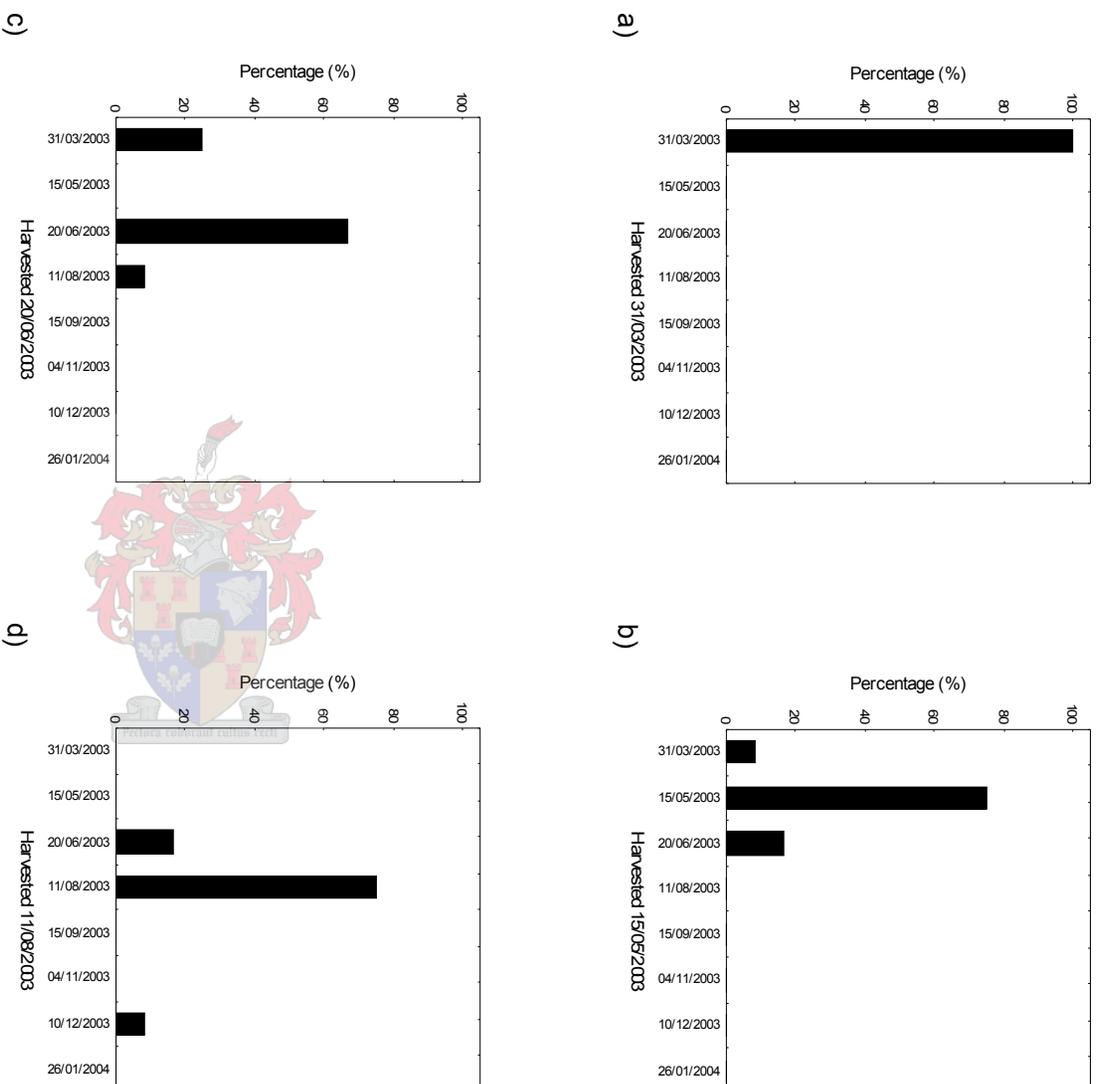


**Figure 13** LDA results for the classification of green *C. genistoides* types (n = 228; harvested during 2001 and 2003) showing the percentage of samples classified correctly and incorrectly for the a) West Coast and b) Overberg types.



**Figure 14** PCA score plot (PC1 vs PC2) to differentiate between dried, green *C. genistoides* (West Coast type only) samples harvested at eight different dates.





**Figure 15** LDA results for the classification of green *C. genistoides* harvest dates showing the percentage of samples classified correctly and incorrectly for each of the eight harvest dates.

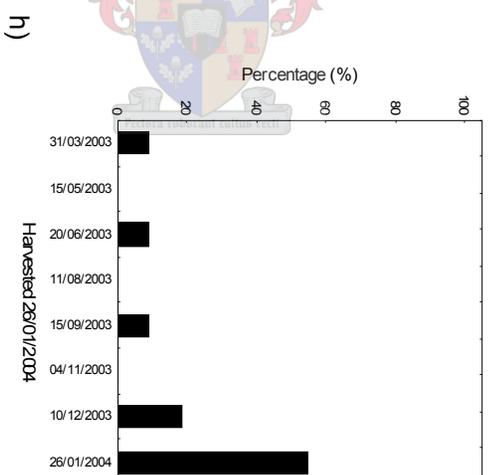
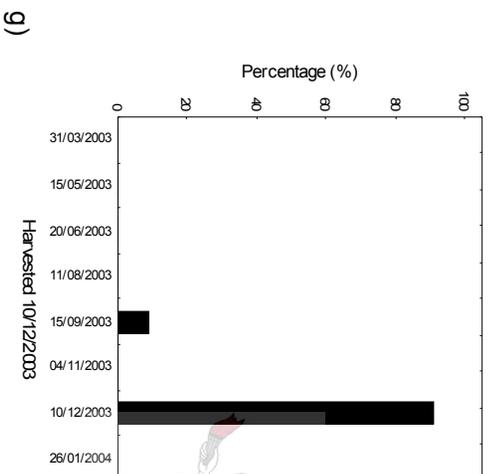
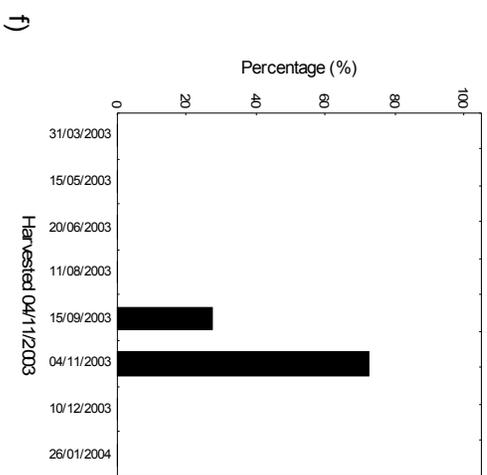
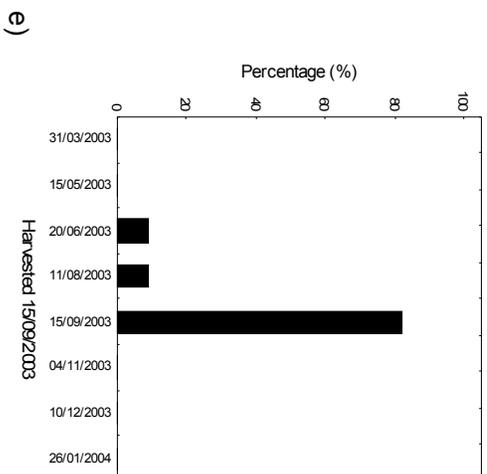
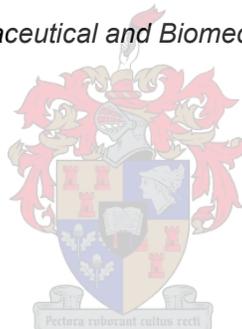


Figure 15 continued/...

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## CHAPTER 6



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## General discussion and conclusion

## CHAPTER 6

### GENERAL DISCUSSION AND CONCLUSION

Green rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.) are two herbal plants containing valuable flavonoids, i.e. aspalathin and mangiferin, respectively, which are major contributors to their antioxidant activity and subsequent potential for the development of polyphenol-enriched extracts for the food, cosmetic and nutraceutical industry. Plant material should contain high quantities of these compounds to make extraction economically feasible. Screening of plant material at an early stage of processing is, therefore, required to ensure extracts with sufficient quantities of aspalathin and mangiferin. During the present study near infrared spectroscopy (NIRS), UV/Vis spectrometry and colorimetry were investigated to identify rapid and/or economical methods to quantify the major phenolic compounds, total polyphenol content (TPC), soluble solid content (SSC) and total antioxidant activity (TAA) of green rooibos and *C. genistoides*. Since the mangiferin and hesperidin contents of green *C. genistoides* vary between the two types, i.e. West Coast and Overberg, as well as with harvest date (Joubert *et al.*, 2003) principal component analysis (PCA) and linear discriminant analysis (LDA) were applied to the near infrared (NIR) spectra, to classify samples according to type and harvest date. The application of NIRS to differentiate between green rooibos and *C. genistoides* was also investigated, as well as to classify green rooibos according to cultivation area.

In order to develop NIRS calibration models for the quantification and qualification of green rooibos and *C. genistoides*, a large number of samples was selected to include different cultivation areas (in the case of rooibos), types (in the case of *C. genistoides*), plants of different ages, plants harvested during different seasons and plants consisting of different leaf:stem ratios. NIRS calibration models for aspalathin, dihydrochalcone and nothofagin were developed using a sample set that included nine samples of which the aspalathin and nothofagin contents were increased by adding varying amounts of a rooibos extract powder, containing 15.95 g.100 g<sup>-1</sup> aspalathin and 1.94 g.100 g<sup>-1</sup> nothofagin. Although a large range of samples was used for both rooibos and *C. genistoides* it was still impossible within the scope of the study to obtain enough samples with low and high aspalathin, dihydrochalcone, nothofagin, mangiferin and hesperidin contents and a Gaussian distribution of the content of all these compounds was found.

Using NIRS it was possible to predict the aspalathin and dihydrochalcone contents of dried, green rooibos plant material with an accuracy of 0.45 and 0.49 g.100 g<sup>-1</sup>, respectively, which would be acceptable by industry for quantification of these compounds. The aspalathin and dihydrochalcone NIRS calibration models on the sample set with the extended aspalathin and nothofagin contents range gave similar results, but with a slightly lower accuracy of 0.53 and 0.57 g.100 g<sup>-1</sup>, respectively. The reason for this might be that the high aspalathin and

nothofagin content rooibos extract powder was only added to nine samples extending the range at the higher concentration, or it might be that the rooibos extract powder that was added had a different texture, since it was a extract powder with a very fine particle size.

Less accurate results were found for the prediction of the nothofagin content of dried, green rooibos and mangiferin and hesperidin contents of dried, green *C. genistoides*. These NIRS calibration models can be used for screening purposes and will still be of great benefit to identify plant material with sufficient quantities of these compounds. The reason for the less accurate results found for the mangiferin and hesperidin calibration models can be that underestimated reference values were used. Mangiferin is slightly less soluble in methanol than water while hesperidin is mostly insoluble in water and readily soluble in methanol. Extraction solvents and conditions used in this study were based on other studies and methods used by the industry. Other solvents and combinations of solvents could be investigated to improve extraction of mangiferin and hesperidin. Similar action could be take to improve extraction of aspalathin and nothofagin.

Since rooibos and honeybush are largely consumed in the form of an infusion (cup of tea), and because local extract manufacturers make use of TPC and TAA to standardise their products (water extracts), development of NIRS calibration models for the prediction of the major phenolic compounds, TPC, SSC and TAA of green rooibos and *C. genistoides* water extracts were also investigated. It was not possible to predict the different phenolic compounds, TPC, SSC and TAA of green rooibos and *C. genistoides* water extracts accurately, due to low concentrations present in the water extracts. Hesperidin, which is not readily soluble in water, could also have contributed to poor prediction of hesperidin in the water extracts. In future studies the prediction of TPC and TAA using NIRS could be investigated determining the TPC and TAA of green rooibos and *C. genistoides* extracts prepared with other solvents. Application of NIRS on the final product, i.e. the dried extract, would also be a possibility, and should be investigated as it would give the quality control analysts a means of rapidly evaluating both the raw material and final product.

UV spectroscopy and an aluminium chloride colorimetric method were investigated as alternative methods to NIRS for the estimation of aspalathin and mangiferin contents, respectively. Although a good correlation existed between the aspalathin content determined using UV spectroscopy and HPLC, aspalathin was overestimated by UV spectroscopy due to other phenolic compounds and tannins present which also absorb at 288 nm (wavelength where aspalathin was measured). Using a conversion factor to compensate for the overestimation, UV spectroscopy can still be used to screen the samples for aspalathin content in routine analysis where a large number of samples needs to be measured and the laboratory do not have a NIRS instrument. Good correlation between the aspalathin content determined using UV spectroscopy (absorbance at 288 nm), and TPC as determined by the Folin-Ciocalteu method, indicated that UV spectroscopy can replace the Folin-Ciocalteu method to determine the TPC

in green rooibos water extracts using aspalathin as standard. However, aspalathin is expensive and an alternative standard that is readily available and more economical to use should be obtained. The relationship between aspalathin and the alternative standard should, however, be established for estimation of the aspalathin content. Since a good correlation existed between the TPC and TAA of the rooibos water extracts, UV spectroscopy could be used to estimate the TAA of green rooibos water extracts indirectly. Good correlations existed between the aspalathin content quantified using HPLC and the TPC and TAA, respectively. This also indicates that aspalathin is largely responsible for the TPC and TAA of rooibos water extracts.

A good correlation was found between the mangiferin content of green *C. genistoides* water extracts determined with the aluminium chloride colorimetric method and HPLC, indicating that the aluminium chloride colorimetric method can be used as an alternative to NIRS for rapid screening of extracts. Although overestimation occurred, this method still gave good results compared to the mangiferin NIRS calibration model.

A moderate correlation was found between the mangiferin content of green *C. genistoides* water extracts and its TAA. Mangiferin is the main contributor to TAA, due to its relative potency and high concentration present in the extracts. No correlation was found between the mangiferin content and TPC, and TPC and TAA of green *C. genistoides* water extracts due to the contribution of hesperidin (which was present in relative high quantities in the extracts) to the TPC, while demonstrating low potency in the TAA assay.

With the application of PCA and LDA analyses on NIR spectral data, NIRS could classify the two *C. genistoides* types (West Coast and Overberg types) and differentiate between green rooibos and *C. genistoides* accurately. The West Coast and Overberg types seemed to separate due to their mangiferin and hesperidin contents, as the Overberg type had, on average, a higher mangiferin content and substantially lower hesperidin content than the West Coast type. The separation of the green rooibos and *C. genistoides* is most likely due to the difference in their phenolic composition. The original spectra of the green rooibos and *C. genistoides* samples showed baseline shifts at 1680 and 2230 nm, which are the wavelengths used to predict difference in hardness, i.e. particle size in wheat, indicating that difference in particle size could be one of the reasons for this classification. The rooibos and *C. genistoides* samples tend to grind differently, although the same mill was used. The presence of thick stems in *C. genistoides* samples could also contribute to these differences in physical texture. MSC was applied to remove particle size variation but did not completely removed the effect of particle size, which indicated that chemical differences also contributed to the classification of green rooibos and *C. genistoides*. Although no separate clusters were found for the different cultivation areas of green rooibos and harvest dates of green *C. genistoides* using PCA, LDA indicated that differences existed between samples harvested at different cultivation areas (in the case of rooibos) and samples harvested at different dates during the course of a year (in the case of *C. genistoides*).

NIRS has the ability to be used in quantitative and qualitative analysis of green rooibos and *C. genistoides*. The only prerequisite is that calibration models are to be developed for each new compound or parameter and this could be expensive and time-consuming, but this is usually a once-off expense. The calibrations, however, need to be validated and updated at regular intervals. The running cost of NIRS spectrophotometers are low, it is a rapid method, no solvents are required, nor is highly trained laboratory personnel required and in most cases no or little sample preparation, such as in the present study where the only preparation of the plant material was drying and milling. UV spectroscopy and the aluminium chloride colorimetric method can be used as alternative screening methods to NIRS since most laboratories have a UV/Vis spectrophotometer. However, the running cost will be more than that of NIRS since solvents and trained laboratory personnel are required. Furthermore, these methods are more time-consuming than NIRS. In the case of large quantities of samples the use of automated and microtiter systems will reduce analysis time and smaller amounts of solvents would be used.

Future studies could focus on the use of NIRS in different stages in the processing of green rooibos and *C. genistoides* extracts, including the final product. The use of NIRS to classify *Cyclopia* species, i.e. *C. genistoides*, *C. intermedia* and *C. subternata* used in the industry, could also be investigated as the phenolic profiles of these species differ. The use of NIRS for the quantification of green rooibos adulteration could also be investigated by preparing different mixtures of green rooibos and *C. genistoides*.

The application of NIRS for prediction of compounds in other plant material to expand its use in the agro-processing and nutraceutical industries would be beneficial since a single instrument can be used for the evaluation of a range of products. Future studies could also investigate the development of NIRS calibration models for the quantification of the aspalathin, nothofagin and dihydrochalcone contents of fresh green rooibos and the mangiferin and hesperidin contents of fresh *C. genistoides* plant material to enable prediction of the content of these components in the plants still in the field. This would enable optimisation in harvest time and aid the plant breeder in search of plants suitable for the development of plants with a high concentration of the sought-after compound.

## References

- Joubert, E., Otto, F., Grüner, S. & Weinreich, B. (2003). Reversed-phase HPLC determination of mangiferin, isomangiferin and hesperidin in *Cyclopia* and the effect of harvesting date on the phenolic composition of *C. genistoides*. *European Food Research and Technology*, **216**, 270-273.