

Evaluation of near infrared and nuclear magnetic resonance spectroscopy for rapid quality control of South African extra virgin olive oils

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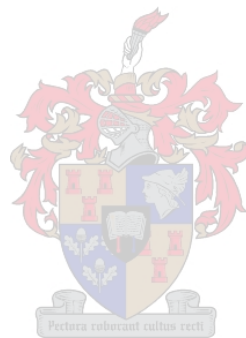
December 2005

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 other university for a degree.

Kerstin Eberle

Date



Abstract

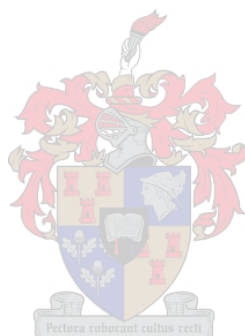
Near infrared (NIR) spectroscopy prediction models were developed for the rapid determination of the peroxide value, free fatty acid content, specific extinction coefficients at 232 and 270 nm, thiobarbituric acid reactive substances value, pigment, total polyphenol, oleic acid, linoleic acid and saturated fatty acid contents, as well as the $\alpha:\beta$ ratio of oleic and linoleic acids in South African extra virgin olive oils. Extra virgin olive oils were obtained from producers in the Western Cape region and were oxidised under accelerated conditions (artificial light and 35°C) for up to 10 weeks, to obtain an adequate range of reference values for the purpose of developing robust NIR prediction models.

NIR spectra were collected in parallel to chemical reference methods on two Fourier transform spectrophotometers, i.e., the Perkin Elmer IdentiCheck™ at different resolutions (64, 32, 16 and 8 cm^{-1}) and path lengths (0.2 and 0.5 mm) in transmittance mode and the Büchi NIRLab N-200 at 8 cm^{-1} resolution and a path length of 0.6 mm in transreflectance mode. Partial least squares prediction models were developed for each respective reference method and the various instrument settings, path lengths and sample holders. These models were statistically compared to evaluate the effect of different scanning conditions on prediction model performance. The comparisons revealed that spectra collected at a low resolution (64 cm^{-1}) resulted in equally accurate prediction models as spectra of higher resolutions (32, 16 and 8 cm^{-1}) and small differences in path length (0.2 and 0.5 mm) caused no significant differences ($p > 0.05$) in model performance. When comparing prediction models developed from transmittance spectra (0.2 mm path length and 64 cm^{-1} resolution) and transreflectance spectra (0.6 mm path length and 8 cm^{-1} resolution) significant differences in performance were observed for only a few reference methods. Good results were obtained for the peroxide value ($SEP = 4.15 \text{ meq O}_2 \text{ kg}^{-1}$, $R^2 = 0.92$), specific extinction coefficient K_{232} ($SEP = 0.94$, $R^2 = 0.94$), linoleic acid ($SEP = 0.83\%$, $R^2 = 0.90$) and saturated fatty acid content ($SEP = 0.91\%$, $R^2 = 0.88$) prediction models.

^{13}C Nuclear magnetic resonance (NMR) spectroscopy was employed to investigate the classification of the olive oils according to oxidation level, cultivar and geographical origin by means of multivariate statistical analyses. Selected resonance intensities and peak heights were used for principal component analysis (PCA) and linear discriminant analysis (LDA). PCA of NMR intensity and peak height data failed to classify olive oils on the basis of differences in oxidation level or geographical origin, under the present experimental conditions. However, principal component score plots showed evidence of grouping on the basis of cultivar differences. LDA was able to successfully classify single cultivar olive oils into their respective cultivar groups, but was unable to correctly classify oils according to geographical origin. PCA showed no obvious differences in performance between NMR intensity and peak height data,

while LDA of the peak intensity data gave better results than for the peak height data for cultivar and geographical origin based classifications.

The implementation of NIR spectroscopy as a quality screening method for South African extra virgin olive oils may provide an economical and efficient alternative to official chemical methods and, thereby, facilitate the realisation of a locally regulated quality control system for extra virgin olive oils. NMR spectroscopy is a valuable technique, both as a reference method for NIR prediction models, and as a means of classifying olive oils according to their cultivar composition.



Uittreksel

Naby infrarooi (NIR) spektroskopie voorspellingsmodelle is ontwikkel vir die vinnige bepaling van die peroksiedwaarde, vry vetsuurinhoud, spesifieke ekstinksiekoëffisiente by 232 en 270 nm, tiobarbituursuur reagerende substansie waarde, pigment, totale polifenole, oleïnsuur, linoleïnsuur en versadigde vetsuurinhoud, asook die $\alpha:\beta$ verhouding van oleïn- en linoleïnsuur. Suid-Afrikaanse ekstra edel olyf olie is verskaf deur produsente in die Wes-Kaap omgewing en geoksideer onder versnelde toestande (kunsmatige lig en 35°C) vir tot 10 weke om 'n geskikte reeks van verwysingswaardes te verkry vir die ontwikkeling van robuuste NIR voorspellingsmodelle.

NIR spektra is in parallel met chemiese verwysingsmetodes op twee verskillende Fourier transformasie spektrofotometers, i.e. die Perkin Elmer IdentiCheck™ by verskillende resolusies (64, 32, 16 en 8 cm⁻¹) en padlengtes (0.2 en 0.5 mm) in transmissie en die Büchi NIRLab N-200 by 'n resolusie van 8 cm⁻¹ en 'n padlengte van 0.6 mm in transfleksie versamel. Parsiële kleinste kwadrate voorspellingsmodelle is ontwikkel vir elk van die verwysingsmetodes en die onderskeie instrument verstellings, padlengtes en monsterhouers is statisties vergelyk om die effek van verskillende NIR metingstoestande op die modelprestasie te beoordeel. Die vergelykings het getoon dat spektra wat by 'n laer resolusie (64 cm⁻¹) versamel is, modelle van gelykstaande akkuraatheid as dié by hoër resolusies (32, 16 en 8 cm⁻¹) gelewer het, en dat klein verskille in padlengte (0.2 en 0.5 mm) geen betekenisvolle verskil ($p > 0.05$) op die modelle se prestasie gehad het nie. Wanneer voorspellingsmodelle van onderskeidelik transmissie (0.2 mm padlengte en 64 cm⁻¹ resolusie) en transfleksie spektra (0.6 mm padlengte en 8 cm⁻¹ resolusie) vergelyk is, het net 'n paar verwysingsmetodes betekenisvolle verskille getoon. Goeie resultate is verkry vir die peroksiedwaarde ($SEP = 4.15$ meq O₂ kg⁻¹, $R^2 = 0.92$), spesifieke ekstinksiekoëffisient K_{232} ($SEP = 0.94$, $R^2 = 0.94$), linoleïnsuur ($SEP = 0.83\%$, $R^2 = 0.90$) en versadigde vetsuurinhoud ($SEP = 0.91\%$, $R^2 = 0.88$) voorspellingsmodelle.

¹³C Kern magnetiese resonansie (KMR) spektroskopie data van dieselfde stel olyfolie monsters is verder benut om die klassifikasie van olyfolie op grond van oksidasievlak, kultivar en geografiese oorsprong, deur middel van meerveranderlike statistiese analise, te ontleed. Spesifieke NMR resonansie intensiteite en piekhoogtes is vir hoofkomponent en lineêre diskriminante analises (LDA) gebruik. Die hoofkomponent analise het, onder die spesifieke omstandighede, getoon dat geen duidelike korrelasie bestaan tussen KMR intensiteits- of piekhoogte data en die oksidasie vlak of geografiese oorsprong van die olyfolies nie, maar hoofkomponent grafieke het groepering van monsters ten opsigte van kultivarverskille getoon. LDA kon enkel kultivar olyfolies suksesvol klassifiseer, maar kon die olies nie ten opsigte van geografiese verskille korrek klassifiseer nie. Hoofkomponent analise het geen opmerkbare verskille tussen KMR intensiteits- en piekhoogte data getoon nie, terwyl LDA van die

piekhoogtes swakker resultate as met intensiteitsdata, vir beide kultivar en geografiese oorsprong klassifikasies, gelewer het.

Die implementering van NIR spektroskopie as 'n kwaliteitskontrolemetode vir Suid-Afrikaanse ekstra edel olyfolies is geskik as 'n ekonomiese alternatief vir amptelike chemiese metodes en kan dus die totstandkoming van 'n plaaslik gereguleerde kwaliteitskontrolestelsel vir ekstra edel olyfolies vereenvoudig. KMR spektroskopie is 'n waardevolle tegniek, beide as 'n verwysingsmetode vir NIR voorspellingsmodelle en as 'n klassifikasiemetode vir die bepaling van olyfolie kultivarsamestelling.



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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.

List of abbreviations

ANN = Artificial neural networks

AOAC = Association of Analytical Chemists

AOCS = American Oil Chemists' Society

AOTF = Acousto-optical tunable filter

CCDs = Charged coupled devices

ECN = Equivalent carbon number

FT-NIR = Fourier transform near infrared

HDLs = High density lipoproteins

InGaAs = Indium gallium arsenide

IOOC = International Olive Oil Council

L = Linoleic acid

LDA = Linear discriminant analysis

LDLs = Low density lipoproteins

LED = Light emitting diode

MLR = Multiple linear regression

MSC = Multiplicative scatter correction

MUFAs = Monounsaturated fatty acids

NMR = Nuclear magnetic resonance

NIR = Near infrared

O = Oleic acid

OSC = Orthogonal signal correction

P = Palmitic acid

PbS = Lead sulphide

PCA = Principal component analysis

PCR = Principal component regression

PLS = Partial least squares regression

PRESS = Predicted residual error sum of squares

PUFAs = Polyunsaturated fatty acids

PV = Peroxide value

R^2 = Coefficient of determination

r = Coefficient of correlation

RMSEP = Root mean square error of prediction

RPD = Ratio of standard error of prediction to standard deviation

S = Saturated fatty acids



SAOGA = South African Olive Growers Association

SA Olive = South African Olive Industry Association

SD = Standard deviation

SD_{Büchi} = Standard deviation of prediction model developed from Büchi spectra

SD_P = Standard deviation of predicted values

SD_{PE} = Standard deviation of prediction model developed from Perkin Elmer spectra

SE = Standard error

SEC = Standard error of calibration

SECV = Standard error of cross-validation

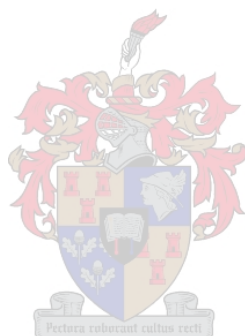
SEL = Standard error of laboratory

SEP = Standard error of prediction

SIMCA = Soft independent modeling of class analogy

SNV = Standard normal variate

TBARS = Thiobarbituric acid reactive substances



List of equations

Reference value statistics

$$\text{Mean}(x) = \sum \frac{x}{n}$$

$$\text{Range}(x) = \text{maximum} - \text{minimum}$$

$$\text{SD}(x) = \sqrt{\frac{\sum x^2 - \frac{\sum x^2}{n}}{n-1}}$$

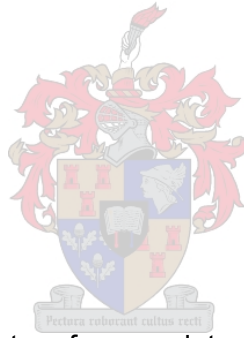
$$\text{SE}(x) = \frac{\text{SD}(x)}{n^2}$$

$$\text{SEL} = \sqrt{\frac{\sum (y_1 - y_2)^2}{2n}}$$

Where x = reference value

n = number of samples

y₁ and y₂ = values of duplicate reference determinations



Validation statistics

$$\text{SD}_P(y) = \sqrt{\frac{\sum y^2 - \frac{\sum y^2}{n}}{n-1}}$$

$$\text{SEP} = \sqrt{\frac{\sum (x-y)^2 - \frac{(\sum (x-y))^2}{n}}{n-1}}$$

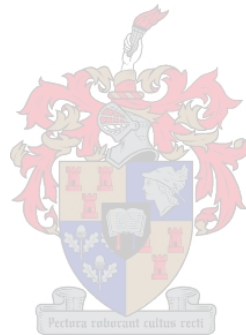
$$r = \frac{\sum(x \times y) - \frac{(\sum x \times \sum y)}{n}}{\sqrt{\left[\sum x^2 - \frac{(\sum x)^2}{n} \right] \times \left[\sum y^2 - \frac{(\sum y)^2}{n} \right]}}$$

$$RPD = \frac{SD(x)}{SEP}$$

Where x = reference value

y = predicted value

n = number of samples



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Chapter 1

INTRODUCTION



Introduction

Olive oil has become a popular product in many countries outside the Mediterranean, especially in areas where the increasing standard of living leads to a concomitant increase in health consciousness (Bianchi, 2002). The South African olive industry is still in its early stages and olive production is increasing steadily, compared to a rather stagnant olive industry in leading olive oil producing countries, like Spain, Italy and Greece. The increasing health consciousness of today's more cosmopolitan South African consumer and growing awareness of the importance of a healthy diet in disease prevention elucidates the increase in demand for olive products in this country.

Olive oil distinguishes itself from other vegetable oils due to its high stability, good taste and aroma and nutritional benefits. The health promoting effects of extra virgin olive oil are no longer just alleged but proven, which sustained the increase in consumption. These beneficial health effects have largely been ascribed to the oil's high oleic acid (18:1) content and its natural antioxidants (Ryan *et al.*, 1998). The monounsaturated fatty acid, oleic acid, lowers total and low-density lipoprotein (LDL) or "bad" cholesterol, does not lower high-density lipoprotein (HDL) or "good" cholesterol, and reduces LDL oxidative power, resulting in a protective effect against coronary heart disease and atherosclerosis (Calabrese, 2002). The major antioxidants found in olive oil are phenolic compounds and tocopherol or vitamin E. These antioxidants play an important biological role in that they inhibit LDL oxidation and scavenge free radicals.

The health-promoting and superior sensory characteristics of olive oil are, however, only prominent in good quality extra virgin olive oil and a higher price is requested for these oils because of the high production costs associated with extra virgin oil extraction (Bianchi, 2002). This aspect tempts many olive oil producers to manipulate low quality oils to meet the requirements of an extra virgin olive oil. Lack in quality control regulations regarding olive oil imports from Mediterranean countries has allowed the marketing of adulterated and over-classified oils on South African supermarket shelves. In October 2001 the first severe case of olive oil adulteration was revealed in three different brands of supposedly Italian olive oil, which was actually of local origin (Cilliers, 2001). This oil contained recycled sunflower oil, coloured with a hazardous green colourant. Clearly, the admixture of expensive extra virgin olive oils with seed oils or lower-grade olive oils is more than a potential problem in South Africa.

To combat this problem requires the provision of certificates of analysis and authentication, uniform labelling regulations and access to laboratories that routinely perform rapid and accurate quality control analyses on olive oils produced in, or imported to South Africa. At present, the quality control of olive oil in South Africa is limited to one accredited laboratory, which analyses oil samples for olive oil producers that are willing to pay the high cost of these analyses. The certificate of analysis issued by such laboratory generally only includes

the measurement of the free fatty acids and the fatty acid composition, which is according to international standards not sufficient to verify the virgin quality of an olive oil (IOOC, 2003). Furthermore, the certificate of analysis is requested, at the most, only once a year by leading olive oil producers in South Africa. It is obvious that a need exists for a more affordable and regular quality control option for the South African olive oil producer. Unfortunately, South Africa has no effective legislation protecting consumers from suspect imports with inaccurate and/or misleading labelling (Anon., 2004). The best protection for the consumer would be the institution of a local quality control system for olive oil, which compels importing companies as well as local producers to have their product analysed on a regular basis and comply with minimum requirements.

Quality criteria, as stipulated by the International Olive Oil Council (IOOC), include the free fatty acid content, peroxide value, UV absorption, moisture content, volatile matter content, insoluble impurities, flash point, trace metals and finally sensory evaluation (IOOC, 2003). The most frequently used, conventional quality control procedures of olive oil involve the determination of the free fatty acid content, peroxide value, UV absorption, halogenated solvents, phenol content, pigment content, Rancimat test and organoleptic evaluation by a panel test (Bianchi, 2002). The free fatty acid content, peroxide value, UV absorption and halogenated solvents are an indication of oil deterioration, the presence of hydroperoxides, drastic thermal and chemical treatment or oxidation and harmful contamination, respectively. The phenol content gives an indication of the antioxidant capacity, whereas the pigment content plays an important role in consumer acceptability, authenticity and also influences antioxidant properties of the oil. The Rancimat test is an accelerated oxidation test that gives an indication of the oils' general stability and resistance to oxidation.

All of the above mentioned quality analysis methods are time-consuming procedures that require the use of large amounts of glassware and potentially hazardous reagents, and are destructive to the sample (Yildiz *et al.*, 2001). The cost of performing these labour-intensive, wet-chemical or spectrophotometric analyses is inevitably high, due to the large expenses on glassware, solvents, waste removal and highly trained laboratory technicians. As a consequence, it has been attempted to upgrade the official methods of fat and oil analysis a number of times over the past years to develop more rapid and cost-effective measuring techniques. The IOOC has not as yet adopted any alternative analysis methods and still only approves the use of the conventional wet-chemical methods of the American Oil Chemists' Society (AOCS, 1996).

Near infrared (NIR) spectroscopy is a common technique in agriculture and in the food industry and first gained popularity in protein, moisture and oil determination in wheat (McClure, 2003). Today, NIR spectroscopy has found wide application in the analysis of fats and oils,

including the quantitative measurement of quality parameters in vegetable oils. The free fatty acid content and peroxide value of several vegetable oils has been successfully determined with NIR spectroscopy (Cho *et al.*, 1998; Ha *et al.*, 1998; Che Man & Moh, 1998; Moh *et al.*, 1999; Li *et al.*, 2000; Yildiz *et al.*, 2001). NIR calibrations for the prediction of the free fatty acid content, peroxide value, specific extinction coefficients at 270, 232 and 225 nm, total polyphenol content, Rancimat test, moisture content and panel test score have been developed for virgin olive oil (Garrido *et al.*, 2000; Garrido-Varo *et al.*, 2004). Good coefficients of determination between NIR spectroscopy and conventional chlorophyll ($R^2 = 0.986$) and carotenoid ($R^2 = 0.970$) determination by UV-Vis spectrophotometry were obtained with virgin olive oil (Jimenez-Marquez, 2003). Subsequently, other groups of researchers developed NIR calibration models for olive oil quality parameters (Conte *et al.*, 2003; L.S. Conte, 2004, Dipartimento di Scienze degli Alimenti, Universita degli Studi di Udine, Udine, Italy, personal communication; Mailer, 2004), including the free fatty acid content, peroxide value, total polyphenol content, Rancimat test and various fatty acid contents.

NIR spectroscopy has several advantages when compared to the conventional methods, including high speed, little sample preparation and only small amounts of sample are required (Yildiz *et al.*, 2001). It is environmentally friendly and extremely economical, since no chemical waste is produced and labour requirements are low due to minimal sample preparation. Modern NIR spectroscopy systems effectively allow one to automate quality control methods by building a user-friendly interface and developing calibrations into the system so that measurement results are given to the user directly after scanning the sample (Li *et al.*, 2000). Therefore, a NIR instrument equipped with appropriate calibration software would be a useful tool for rapid routine quality control analysis of freshly pressed and stored olive oil. The approval of the application of NIR spectroscopy for the measurement of the most important quality control methods in South Africa would facilitate the routine quality control of olive oil and would thus promote the establishment of a reliable quality control system. Major extra virgin olive oil producers in the country could install a NIR instrument at their presses for immediate and regular quality control during production. Small-scale producers could benefit by sending samples to laboratories equipped with a NIR instrument and receiving results quicker and at a lower price compared to laboratories applying conventional methods.

The objective of this research was to evaluate two rapid spectroscopic techniques (NIR and NMR spectroscopy) as routine quality control procedures for use in the South African olive oil industry. NIR prediction models were developed for the determination of the peroxide value, free fatty acid content, specific extinction coefficients K_{232} and K_{270} , pigment and total polyphenol content and thiobarbituric acid reactive substances value. Nuclear magnetic resonance (NMR) spectroscopy was applied to establish the linoleic, oleic and saturated fatty

acid content, as well as the $\alpha:\beta$ ratio of both linoleic and oleic fatty acids in extra virgin olive oil and was further employed as a reference method for the development of NIR prediction models for these parameters. All calibration models were developed from spectra collected on two different NIR spectrophotometers and the effect of distinct instrument settings on performance was investigated. Lastly, NMR spectroscopy combined with multivariate statistics was evaluated as an alternative method for oxidative stability studies on olive oil.

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Chapter 2

LITERATURE REVIEW

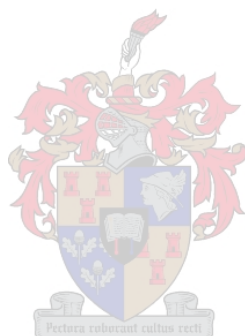


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1. Introduction

Olive cultivation is probably the oldest agricultural practice known to man and dates back to around 3500 B.C. (Anon., 2003a). Although twenty million year old fossil remains of an ancestral form of the olive tree were found in Italy, actual cultivation of the olive tree has its roots in Crete and only reached Italy in the fifth century B.C (Anon., 1997). Over the millennia olive cultivation spread from Greece to Syria, Palestine, Israel, Southern Turkey and Cyprus. During the eighth century the olive culture moved westwards due to Roman influences and reached Southern Italy and France, as well as North Africa.

The olive tree symbolised wealth, glory and peace and its oil was used for medicinal and spiritual purposes besides its obvious function as a food product (Anon., 1997). Olympic winners were rewarded with olive tree branches and winners at the Panathenaic Games received olive oil in amphorae (Fig 1) (Anon., 2003a). The vase itself represented the quality of the already certified olive oil and this is one of the very first examples of product certification in world history.

Whereas the ancient cultures of the Mediterranean basin have already praised their olive oil for millennia for its medicinal and culinary purposes and even certified this product (Anon., 2003a), Southern Africa was only introduced to the “liquid gold” in the early twentieth century (Costa, 1998). Compared to the major olive oil producing countries in the Mediterranean, South Africa is still a newcomer to the industry and although more and more farmers have entered the olive oil business over the past years, the majority of manufacturers comprise small scale production units. Most of South African olive oil is sold locally in selected stores, and since no regulations regarding olive oil quality and authenticity exist as yet in the country, this aspect of quality control is generally not part of the production process.

The following literature review looks at all facets of olive oil and its production that have an impact on the quality and subsequent quality control of this product, such as its extraction, chemical composition and oxidative stability. Finally, an alternative method for olive oil quality control, namely near infrared spectroscopy, is introduced.

2. Olive oil

2.1 The olive oil industry in South Africa

2.1.1 History

The olive tree (*Olea europaea* L.) was first brought to South Africa in the days of Jan van Riebeeck, who described in his diary entry of 18th July 1661 that vines, citrus trees and olive trees were well adapted to the climate of the Western Cape (Anon., 2004a). In the early years of the twentieth century several experimental plantings of olive trees were made (Costa, 1998). From 1925 olive oil cultivars, imported from Italy by Ferdinando Costa, were



Figure 1 Panathenaic amphorae.

grown on a commercial scale in the Paarl valley, where he cultivated olives and in 1936 set up an olive press to produce his first olive oil (Costa, 2004).

The local olive oil production was not satisfying demand in this new industry and soon imported olive oil from the Mediterranean countries threatened to saturate the market and made it difficult for South African oil producers to enter the market (Costa, 1998). In 1956, the South African Olive Growers Association (SAOGA) was established (renamed to South African Olive Industry Association, referred to as SA Olive, in November 2004), whose goal is to support local producers. The South African olive industry grew sluggishly in its early years, however, since the 1970s there has been rapid development and increasing demand.

2.1.2 Olive cultivars

Olive cultivars can generally be divided into three categories, i.e., table olives, which are suitable for pickling when green or black, oil olives which are only used for oil production and dual purpose olives, which are used for both table olive and oil production (Costa, 1998). The most popular table olive cultivars planted in South Africa today are Mission, Kalamata, Manzanilla and Barouni (Fitchet, 2004), whereas the Leccino, Frantoio and Coratina cultivars are most popular for oil production (De Bruyn, 1999). The Mission olive is the most widely cultivated olive in South Africa and constitutes ca. 85% of the trees planted in the Cape (Fitchet, 2004). Black table olives with a good texture and taste can be produced from this cultivar, as well as fruity, good quality oil (Costa, 1998). Frantoio olives have a high oil content ranging between 15 and 22%, which makes this cultivar solely useful for oil extraction (Anon., 2004b). The Leccino cultivar bears very small fruits weighing only 2 to 2.5 g with a good oil yield of 14 to 18%. Coratina olives are medium to large sized fruits (5 to 6 g) that have an oil yield between 17 and 23% and are characteristic for their fruity oil (Anon., 2004c). A relatively novel olive cultivar in South Africa is the semi-dwarf Favolosa or FS17 cultivar, which is cloned from Frantoio and is suitable for high density orchards (Anon., 2004d). This medium-sized olive has an oil content of 20 to 25% and generates high quality oil.

2.1.3 Production

The largest olive oil producing countries in the world include Spain, Italy, Greece, Portugal, Tunisia, Turkey and Syria, of which the European Community countries alone comprise 60% of the total world production (Anon., 2004e). Although South Africa is still a minor contributor to the world production of olives and their oil, the local industry is growing steadily and the demand for olive oil is growing by 20% annually (Anon., 2003a). The Western Cape is the most important olive producing region in South Africa, representing over 120 olive producers, and with the Paarl region containing around 90% of South Africa's estimated 450 000 bearing olive trees (Anon.,

2004f). Nonetheless, several farmers in the Beaufort West and Prince Albert region have successfully planted olive trees and obtain good crops (Costa, 1998). Farmers in the Northern Cape and Free State are also increasingly entering into the olive industry (Snyman & Van den Berg, 2002). Approximately 1500 hectares are under olive cultivation in South Africa, compared to a world total of 10 million hectares, and between 3500 and 4500 tons of olives are produced annually, of which 1500 to 2000 tons are oil olives (Anon., 2004f). At present, the South African production of olive oil is 400 000 litres per annum (Fitchet, 2004). The SA Olive estimates that by the year 2008 local olive oil production will have increased to 1.6 million litres per annum and that 12 000 tons of table olives will be harvested each year (Fitchet, 2004). Statistical figures indicate that in a few years the South African olive market might meet local demands and excess olive products would have to be exported (Jooste, 2001). This, however, only holds true for table olives. South African exporters of olive oil face a world oversupply and cannot be price competitive as a result of subsidies given to European producers (Anon., 2000). In comparison to mechanised picking of olives by major producing countries, South Africa can afford hand-picking due to low labour costs and thus produces high quality table olives. Table olive production in Europe is decreasing and Australia imports up to 7000 tons of olives per annum, thus ideal export markets for South African producers could be Australia, the United States and Asia, as well as Europe (Jooste, 2001).

Olive oil imports to South Africa over the years 1999 to 2002 showed a 75% increase and this growth trend is likely to continue (Field, 2003). This phenomenon can largely be attributed to the globalisation of trade that has generally occurred in recent years due to the liberalisation of World Trade Organisation agreements (Mili & Zuniga, 2002). Extra virgin olive oil imports, mainly coming from Italy and Spain, account for ca. 64% of the local market (Fitchet, 2004).

2.2 Olive oil extraction

The oil content of olive fruits largely varies depending on the cultivar, the ripeness of the olive and environmental conditions and is usually between 12 to 30% oil (on a mass per mass basis) (Costa, 1998). The majority of the oil is found in the mesocarp or fruit tissue of the olive and some oil is present in the kernel (Costa, 1998), which is why olives are generally milled in whole for oil extraction (Anon., 2004a). To obtain high quality oil it is important that processing of the olives occurs within two to three days from harvest (Bianchi, 2002). The ripeness stage at which olives are harvested for oil production varies according to the cultivar and at which stage the oil content of the fruit is at its maximum.

The extraction of olive oil from fresh olives can be accomplished by several processing methods, which include pressing, centrifugation and extraction by non-mechanical means

(Boskou, 2002). The first step in each of these processing methods is to clean the olives from stems, twigs and leaves and in some cases the olives may be washed with water to remove dirt and pesticides (Hoffmann; 1989; Anon., 2004a).

2.2.1 *Crushing*

The oil is released from the olives by crushing, which can be achieved by entering the olives into a stone mill, hammer mill (Fig 2a) or metal-toothed grinder (Anon., 2004a). The stone mill uses stone rollers or wheels that roll in circles on a slab of granite to grind the olives into a paste. The hammer mill has swinging arms that push the olives into the sides of a rotating chamber through centrifugal action (Fig 2b) and the metal-toothed grinder has an electric motor attached to a toothed grinder that pulverizes the olives as they are flung away from the centre of the grinder. The two last mentioned methods are more economic due to continuous and high through put, however, they may affect the organoleptic quality of the final product because the paste is heated up as a result of friction. Hammer crushers have a greater capacity to extract phenolic compounds than the stone mill (Amirante *et al.*, 2002).

There is no distinctive difference in the taste between oil extracted from de-stoned olives and whole olives, however, advantages of de-stoning olives before crushing are a higher phenol level and higher induction times (period of time before exponential phase of oxidation is reached), mainly due to a reduction in the heating effect in the absence of stone fracturing. The acidity and peroxide value of oil extracted from de-stoned mash is also lower, leaving only one disadvantage of de-stoning olives, namely a lower oil yield compared to crushed whole olives.

2.2.2 *Malaxation*

Mixing or malaxation of the olive paste for 20 to 40 minutes increases the percentage of free oil by allowing small oil droplets to combine into bigger ones, which can be separated from the oil-water emulsion into a continuous liquid phase during the oil extraction process (Hoffmann, 1989). The paste is often heated during this process, since heating the olive paste will decrease viscosity and improve water oil separation. This increases yield, but also leads to increased oxidation and enzymatic breakdown of the paste and thus is not recommendable. Excessive increases in mixing temperature (above 30 to 35°C) and time also have a negative effect on the natural antioxidant content of the oil (Amirante *et al.*, 2002). The most common mixer used is a horizontal trough with spiral mixing blades (Fig 2c) (Anon., 2004a).

2.2.3 *Pressing or centrifugation*

The pressing of olives for oil extraction is the oldest method of oil extraction and is still in limited use today (Boskou, 2002). In this procedure the olives are mostly crushed by stone mills to break up the kernel and olive flesh, thus releasing the oil from the cells without the

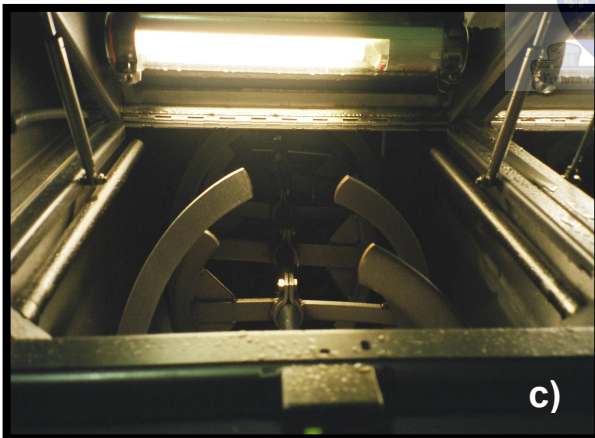
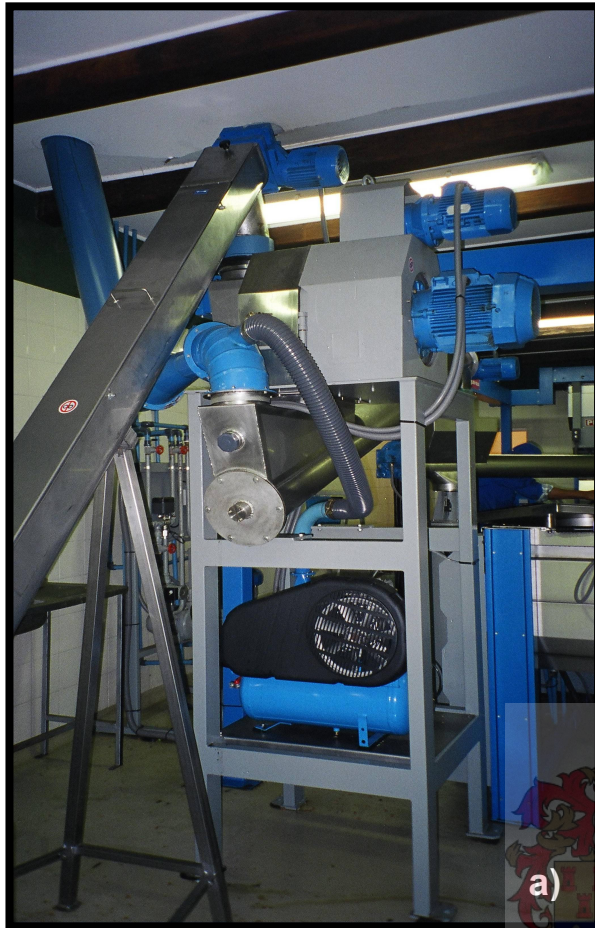


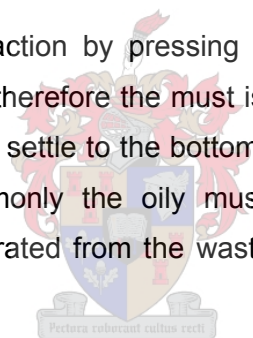
Figure 2 Photos depicting a) a hammer mill, b) the rotating chamber of a hammer mill and c) a horizontal trough malaxer and its spiral mixing blades.

disadvantageous effects of frictional heat. The paste obtained from the mill is then transferred to pressing bags piled one above the other on pressing plates and the oil is drained out of the bags, while the solids remain behind (Hoffmann, 1989; Bianchi, 2002). A more modern oil press is the hydraulic olive press that makes use of a piston to squeeze the paste which has been applied to stacks of filter-like discs (Fig 3a) (Anon., 2004a).

There are three centrifugation methods applied for the extraction of olive oil, namely the centrifugal decanter and dual phase- and triple-phase centrifugation (Anon., 2004a). All centrifugation methods make use of horizontal centrifuges, which separate the olive paste into pomace and must. The centrifugal decanter spins the olive paste in a horizontal drum during which the heavier flesh and pits go to the outside and the water and oil is tapped off from the centre (Fig 3b). During dual-phase and triple-phase centrifugation the olive paste is directly separated into oil and a mixture of water and pomace and thus does not require an additional separation step (Boskou, 2002).

2.2.4 Separation

The turbid oil obtained from extraction by pressing or centrifugation still contains dispersed water, skin and pulp particles and therefore the must is stored in tanks for a two to three month period during which these particles settle to the bottom of the container and can then be tapped off (Bianchi, 2002). More commonly the oily must obtained after pressing or horizontal centrifugation is immediately separated from the wastewater by vertical centrifugation (Fig 3c) (Anon., 2004a).



2.2.5 Extraction by non-mechanical means

Extraction by non-mechanical means is normally applied to the olive paste remaining after the “first press” by pressing or centrifugation.

Percolation extraction involves the dipping of rows of metal discs or plates into the olive paste. The difference in surface tension between the distinct components then leads the oil to coat these plates and it is removed with scrapers in a continuous process (Anon., 2004a). Processing aids such as enzymes, talc, solvents, alkali and steam can be used to increase oil yield by way of extracting more oil from the pomace, however, these methods are not in accordance with the legal definition of virgin olive oil and are not used in better quality oils.

Olive residue oil is obtained by solvent extraction of the pomace with hexane and has a high acidity and bad flavour and thus needs to undergo neutralisation, bleaching and deodorisation before it is fit for consumption.



Figure 3 Photos depicting a) a hydraulic press, b) a centrifugal decanter or horizontal centrifuge and c) a separator or vertical centrifuge for the extraction and separation of extra virgin olive oil.

2.3 Olive oil chemical composition

2.3.1 Fatty acids and triacylglycerols

Olive oil has a unique fatty acid profile compared to other vegetable oils, containing mainly oleic (18:1), linoleic (18:2), palmitoleic (16:1) and stearic acids (18:0) (Ryan *et al.*, 1998). The fatty acid composition of olive oil may vary widely, depending on the maturation stage of the olives, cultivar, oil extraction system and the growing conditions and area (Kailis, 2003; Salvador *et al.*, 2003). It usually is in the ranges of 55 to 85% oleic, 7.5 to 20% linoleic, 0.3 to 3.5% palmitoleic, 0.5 to 5% stearic, 7.5 to 20% palmitic, and 0.0 to 1.5% linolenic acid (Boskou, 2002). The composition of triacylglycerols in olive oil mainly constitutes OOO (40 to 59%), POO (12 to 20%), OOL (12.5 to 20%), POL (5.5 to 7%), and SOO (3 to 7%), where O stands for oleic, P for palmitic, S for stearic and L for linoleic acid.

2.3.2 Polyphenols

Olive oil is mainly distinguished by other vegetable oils through its minor constituents, e.g. polyphenols, which contribute to the oils beneficial role in human nutrition (Boskou, 2002). The polyphenols are a complex mixture of polar compounds and their complete chemical nature has not yet been revealed (Ryan *et al.*, 1998). The most abundant polyphenolic compounds in olive oil are tyrosol and hydroxytyrosol. The total polyphenol content of olive oil can vary considerably with different cultivars, environmental conditions, harvesting times and extraction methods, however, values between 100 and 300 mg kg⁻¹ are considered acceptable for extra virgin olive oils (Ryan *et al.*, 1998; Salvador *et al.*, 2001; Uccella, 2001; Amirante *et al.*, 2002; Salvador *et al.*, 2003). The highest concentration of polyphenols is found when the oil content in the olive fruit reaches a maximum value (Roca & Minguez-Mosquera, 2001). Polyphenols play a role in both the stability and the flavour of the oil. Hydroxytyrosol, tyrosol, caffeic acid and *p*-hydroxybenzoic acid mostly influence the sensory characteristics of olive oil. Hydroxytyrosol is indicative of good quality oils and tyrosol is mainly found in poor quality oils (Kiritsakis, 1998).

2.3.3 Flavour components

Volatile flavour components are formed in olive fruits through an enzyme-catalysed process that takes place on crushing of the olive. On disruption of the plant tissue, lipoxygenase comes into contact with polyunsaturated fatty acids, resulting in the oxidation of the latter and formation of mainly linoleic and linolenic hydroperoxides (Boskou, 2002). These hydroperoxides are decomposed by a specific lyase, generating C₆ aldehydes and C₁₂ or C₉ oxo-acids. The aldehydes are further converted to alcohols by the action of dehydrogenase enzymes or to hexyl esters by transferases. Other pathways that can contribute to the aroma of olive oil include fermentation or conversion of amino acids, enzymatic activities originating from moulds and

oxidative processes (Angerosa *et al.*, 2004). However, these pathways mainly yield off-flavours, whereas the lipoxygenase pathway is prevalent in good quality oil (Fig 4).

Aldehydes, alcohols, esters, hydrocarbons, ketones and furans are the groups of compounds that largely characterise the taste and aroma of olive oil (Kiritsakis, 1998). Hexanal, trans-2-hexenal, 1-hexanol, and 3-methylbutan-1-ol make up the majority of the volatile components in olive oil. The analysis of volatiles by means of capillary gas chromatography reveals the main volatile compounds found in virgin olive oil, many being responsible for its distinct flavour notes (Table 1) (Boskou, 2002).

Flavour compounds reach their highest concentration in the olive during the semi-black stage and decrease with further ripening, due to reduced enzyme activity (Kiritsakis, 1998; Angerosa *et al.*, 2004). On storage of the olive fruits volatile compounds are slowly lost. Flavour components are influenced by a variety of external factors such as the olive cultivar, climate, maturity stage, storage conditions of the fruit and olive processing (Kiritsakis, 1998; Ryan *et al.*, 1998; Angerosa *et al.*, 2004).

2.3.4 Pigments

The two major pigments found in olive oil are chlorophylls and carotenoids. The main chlorophyll found in olive oil is pheophytin *a*, and its concentration may vary between 10 and 30 mg kg⁻¹, depending on factors such as cultivar, climate and pressing methods (Boskou, 2002). The most common carotenoids present are β -carotene and lutein and their content ranges between 1 and 20 mg kg⁻¹ oil. Pheophytin *a* makes up the largest part of the total pigment content (44 to 58%), followed by lutein (18 to 38%) and β -carotene (6 to 17%) (Gandul-Rojas & Minguéz-Mosquera, 1996). During oil production the extraction process results in the transformation of some chlorophyll into pheophytin, when the central Mg²⁺ ion of the porphyrin ring is substituted by H⁺ (Gandul-Rojas *et al.*, 2000). This results in a colour change of the oil from bright green to olive brown. Differences in olive cultivars or stages of fruit ripeness can also be directly correlated to oil colour (Minguéz-Mosquera *et al.*, 1991).

The total chlorophyll and carotenoid contents of olives decrease as they mature. When the olive fruit is still green, the carotenoid fraction degrades more rapidly compared to the chlorophyll fraction, however, as the fruit changes colour, the degradation of chlorophylls is higher than that of carotenoids (Minguéz-Mosquera & Gallardo-Guerrero, 1995). Thus, green olives give oil with a green hue due to the high chlorophyll to carotenoid ratio, whereas ripe olives give yellow oil due to the decrease in the chlorophyll to carotenoid ratio. This ratio of chlorophyll to carotenoid pigments is independent of variety and the different pigment content of olive fruits and is more or less constant within a range of 2.5 to 3.7 mg total chlorophyll per mg total carotenoid (Roca & Minguéz-Mosquera, 2001). The pigment loss caused by oil extraction

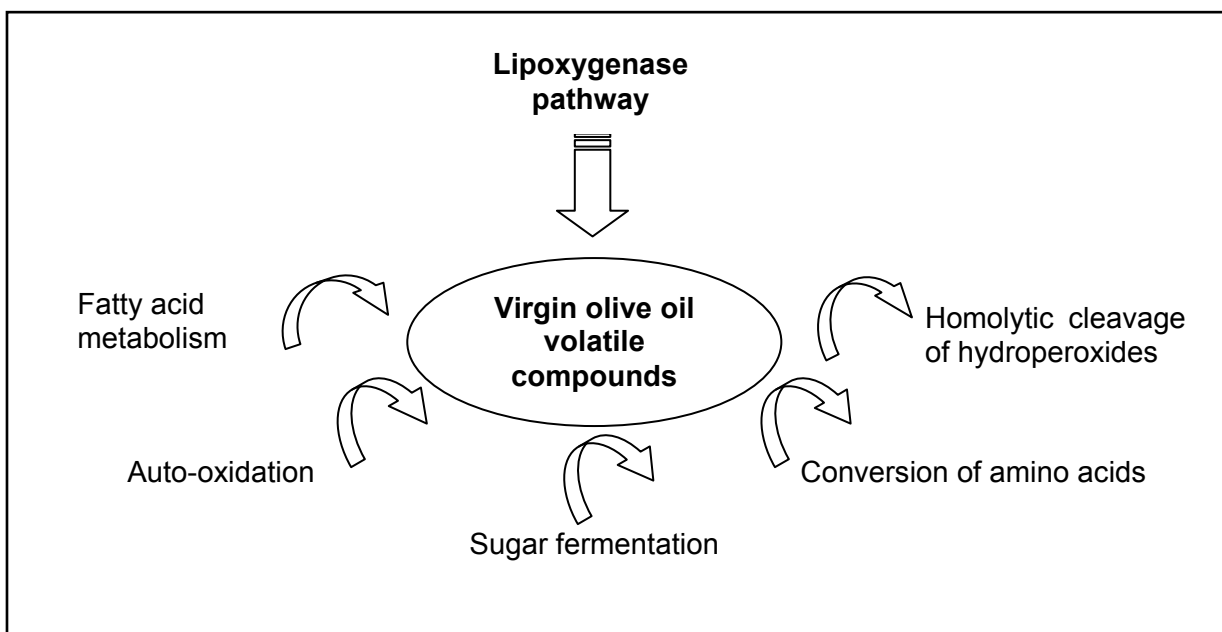


Figure 4 The enzymatic and chemical reactions involved in the production of volatile compounds (Adapted from Angerosa *et al.*, 2004).



Table 1 Several volatile compounds in virgin olive oil related to certain basic positive flavour attributes (Adapted from Morales *et al.*, 1997).

Volatile compound	Flavour note
Ethyl acetate, 3-methylbutanal, ethylfuran, ethyl propanoate, pentan-3-one, 4-methylpentan-2-one, pent-1-en-3-one, hexyl acetate	Sweet
3-Methylbutanal, heptan-2-one, aldehyde C ₆ branched, hexyl acetate, 6-methyl-5-hepten-2-one, nonan-2-one, hexan-1-ol, methyl nonanoate	Fruity
Butyl acetate, hexanal, (E)-2-pentenal, (Z)-2-pentenal, (Z)-3-hexenal, (E)-2-hexenal, (E)-2-hexen-1-ol	Green
Butyl acetate, pentan-1-ol, acetic acid, propanoic acid	Pungent
(E)-2-hexenal	Bitter

is more pronounced for the chlorophyll component and, therefore, the chlorophyll to carotenoid ratio in the oil is always approximately one.

2.3.5 Vitamins

Tocopherols play an important role in the oxidative stability and nutritional qualities of olive oil (Ryan *et al.*, 1998). The tocopherol content of olives varies greatly between distinct cultivars, however, is always highest during the first period of harvesting. The tocopherol content in olive oil fluctuates widely between good and poor quality olive oil and values may range between 5 and 300 mg kg⁻¹. Fluctuations may be attributed to different olive processing procedures and storage of oil, in addition to discrepancies between analytical methods (Ryan *et al.*, 1998; Rastrelli *et al.*, 2002). The tocopherol content of a good quality olive oil should be above 100 mg kg⁻¹ (Boskou, 2002).

The main component of the mixture of tocopherols found in olive oil is α -tocopherol, which constitutes up to 95% of the total. The remaining 5% consist of β -tocopherols and γ -tocopherols. α -Tocopherol, also known as vitamin E, is an effective antioxidant that quenches free radicals and contributes to the oxidative stability of olive oil (Deiana *et al.*, 2002). Its antioxidant activity is at a peak at concentrations of ca. 100 mg kg⁻¹, rather than at higher concentrations (500 to 1000 mg kg⁻¹) and a pro-oxidant effect at different levels of addition has also been reported (Blekas *et al.*, 1995; Mateos *et al.*, 2003). β -Carotene, commonly known as vitamin A, occurs in the olive oil pigment fraction and has beneficial effects on human health by acting as an antioxidant (Boskou, 2002).

2.3.6 Sterols

The sterols present in olive oil can be divided into four classes, i.e., the common sterols (4- α -desmethylsterols), 4- α -methylsterols, 4,4-dimethylsterols (triterpene alcohols) and triterpene dialcohols (Boskou, 2002).

The desmethylsterol component of olive oil makes up the largest fraction (100 to 200 mg 100 g⁻¹) of the total sterol content and contributes ca. 75 to 90% β -sitosterol, 5 to 36% Δ^5 -avenasterol and 3% campesterol to the total sterols (Boskou, 2002). Other desmethylsterols are only present in trace amounts in olive oil. Olive oils' stability towards deterioration at elevated temperatures can be partly attributed to the presence of Δ^7 -avenasterol, which has an ethylidene side chain that impedes oxidative polymerisation in heated oils. 4- α -Methylsterols are commonly found in olive oil in small quantities (20 to 70 mg 100 g⁻¹), since they are intermediate compounds in the biosynthesis of sterols. Triterpene alcohols occur in concentrations ranging from 100 to 150 mg 100 g⁻¹ oil. Triterpene dialcohols in olive oil consists of mainly two compounds, namely erythrodiol and uvaol. Their content differs greatly between olive oil and

residue oil (obtained by solvent extraction of second press) and thus their relative content in the total sterol fraction is used as a means of discriminating between olive oil and residue oil.

2.3.7 Waxes

Olives only contain small amounts of waxes with values usually not exceeding 35 mg 100 g⁻¹ oil (Boskou, 2002). Pressed olive oil has much higher wax contents than residue oil and this difference in wax content is officially used to distinguish between these two oil categories. The waxes are composed of several types of distinct C₃₆ to C₄₆ esters of fatty alcohols with fatty acids.

2.3.8 Metals

Iron and copper can be found in trace amounts in virgin olive oil. These metals typically originate from the soil and fertilizers or through contamination from pressing machinery and storage tanks (Boskou, 2002). Since iron and copper can act as oxidation promoters it is essential to use stainless steel equipment in the production of olive oil.

2.4 Health aspects

The consumption of olive oil as the principal source of fat reflects the eating patterns of Mediterranean countries such as Crete, Greece and Southern Italy about thirty years ago. This Mediterranean diet, more specifically defined as “the dietary patterns found in olive-growing areas of the Mediterranean region more than thirty years ago” (Willett *et al.*, 1995), has been long associated with general good health. The highest life expectancy in the world was found in the Mediterranean region in the early 1960s and the occurrence of coronary heart disease, certain cancers and diet-related chronic diseases was also the lowest in Mediterranean countries. The Mediterranean diet largely comprises plant foods, with olive oil as the principal source of fat and dairy products, fish and poultry consumption in moderate amounts, whereas red meat is consumed in low amounts. Additionally, the diet includes low to moderate amounts of wine and regular physical activity.

The health benefits of olive oil compared to other fats, and in particular animal fats, are ascribed to its high content of monounsaturated fatty acids (MUFAs) and its antioxidant properties. Oleic acid has been proven to prevent thrombosis and is also less likely to cause oxidation of low-density lipoproteins (LDLs) than polyunsaturated fatty acids (PUFAs). The former is thought to increase the risk of coronary heart disease and atherosclerosis (Willett *et al.*, 1995). PUFAs, which are the main components in most other vegetable oils such as soybean, sunflower, safflower and corn oil, lower overall cholesterol levels meaning that both good high-

density lipoproteins (HDLs) and bad LDLs are reduced (Anon., 2004g). On the other hand, MUFAs aid in reducing LDL and increasing HDL levels (Anon., 1999).

Prostaglandins that are formed from arachidonic acid play an important role in the development of tumours and oleic acid has been found to lower their production in the body. This is considered one of the reasons for olive oil to be implicated in lowered risk of breast, digestive tract and prostate cancer. Since olive oil has been consumed in considerable amounts in the Mediterranean region for several thousand years, it is clear that no negative long-term effects are evident (Willett *et al.*, 1995). The increased replacement of animal fats with polyunsaturated fats is, however, a newer dietary trend and thus the long-term effects of its usage as principal fat are unknown.

Antioxidants found in olive oil, such as vitamin E, vitamin A and phenolic compounds, have several beneficial effects on the human body (Anon., 2004g). Oxidative stress arising from an imbalance in free radical production and antioxidants in the body can lead to deterioration of normal cell functions and accelerated ageing. The phenols, some of which besides acting as free radical scavengers also inhibit platelet aggregation, are anti-inflammatory, anti-bacterial and promote nitric acid formation, which is a vasodilator. Lastly, olive oil has a beneficial effect on the muscle tone and activity of the gall bladder and thereby stimulates the digestion of lipids and prevents the development of gallstones.

2.5 Olive oil categories

The International Olive Oil Council (IOOC) classifies olive oil according to distinct trade standards, which define different quality degrees of olive oil and virgin olive oil (IOOC, 2003).

Olive oil is defined as oil obtained exclusively from the fruit of the olive tree (*Olea europaea* L.) and is free of any oil obtained using solvents or re-esterification processes and of any oils of other kinds (IOOC, 2003). **Virgin olive oil**, on the other hand, is defined as the oil extracted from the fruit of the olive tree solely by mechanical or other physical means under conditions that do not lead to alterations in the oil, and which has not undergone any treatment other than washing, decantation, centrifugation and filtration. Oil obtained by treating olive pomace (olive paste after virgin/ first press) with solvents or other physical treatments, excluding re-esterification processes and the mixture with oils of other kinds, is classified as **olive-pomace** or **residue oil**.

Under the three above-mentioned categories the oils are designated to more specific grades, such as extra virgin olive oil, virgin olive oil, ordinary virgin olive oil, lampante virgin olive oil, refined olive oil, olive oil, olive-pomace oil, crude olive-pomace oil and refined olive-pomace oil (IOOC, 2003). Since South African olive oil producers only manufacture virgin olive oils, the

differences between extra virgin olive oil, virgin olive oil and lampante virgin olive oil are of particular interest.

Extra virgin olive oil is a virgin olive oil, which has a free fatty acid content, expressed as oleic acid, of not more than 0.8%. The free acidity of **virgin olive oil** is not allowed to exceed 2%, whereas that of **ordinary virgin olive oil** may not be more than 3.3%. **Lampante virgin olive oil** is considered unfit for consumption as it is and therefore this oil is intended for refining or technical uses not involving human consumption. Its free fatty acid content is thus allowed to exceed 3.3% oleic acid (IOOC, 2003). Table 2 indicates the chemical characteristics of the various oil categories as stipulated by the European Community.

2.6 Oxidative stability

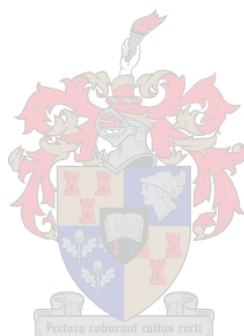
One aspect of a good quality olive oil is the ability to maintain its natural, original chemical composition throughout its lifetime from the mill to the consumer and remain unchanged as long as possible (Bianchi, 2002). The deterioration of olive oil can generally be defined as the chemical processes leading to a change in the original chemical composition and organoleptic characteristics. The transformation products are derived oil compounds, which were either not present or only present in small amounts in the oil enclosed in the fruit tissue (Hoffmann, 1989). They are formed mostly during or after oil production as a result of external influences. Oxidation is the main cause of modifications in the chemical composition of olive oil and can be caused by three principal environmental factors, i.e., light, high temperatures and metal catalysts. The oils' inherent fatty acid composition and antioxidant capacity, as well as the conditions and duration of storage of both the olives and the oil are of great importance.

The condition of the olive fruits used for the extraction of oil has a significant influence on the final product and rotten olives will produce oil of high acidity and generally low quality independent of the extraction process applied (Kiritsakis *et al.*, 1998). The peroxide value of oils obtained from olives stored after harvest increases during storage, compared to oil obtained from freshly harvested olives. Storage conditions and packaging materials influence olive oil quality and different oxidation rates are observed in glass packaging and plastic packaging, as well as during storage in artificial light and direct sunlight (Kiritsakis & Dugan, 1984). Glass packaging gives better protection to oxidation than polyethylene packaging, since the former is less permeable to oxygen. The oxidation of oil proceeds slowly in darkness, faster in artificial light and even faster in sunlight. In darkness, the oxidation process proceeds faster at higher temperatures than at room temperature (Gomez-Alonso *et al.*, 2004).

The initial amount of tocopherol in olive oil is one of the components, which influence oxidative stability (Deiana *et al.*, 2002). Tocopherols and orthodiphenols also are the first compounds to be degraded during oxidation and thus their measurement is a useful means of

Table 2 Chemical characteristics of the different categories of olive oil as specified by the European Community standards (Adapted from Garrido-Varo *et al.*, 2004).

Category	Free fatty acids (%)	Peroxide value (meqO ₂ kg ⁻¹)	K ₂₃₂	K ₂₇₀
Extra virgin olive oil	<1.0	≤20	≤2.50	≤0.20
Virgin olive oil	≤2.0	≤20	≤2.60	≤0.25
Ordinary virgin olive oil	<3.3	≤20	≤2.60	≤0.25
Lampante virgin olive oil	>3.3	>20	≤3.70	>0.25
Olive oil	≤1.5	≤15	≤3.30	≤1.00
Olive-pomace oil	≤1.5	≤15	≤5.30	≤2.00



estimating the average life of olive oil (Gutierrez *et al.*, 2002). Polyphenolic compounds contribute approximately 50% to the oxidative stability of virgin olive oil (Gutierrez *et al.*, 2001), and chlorophylls were found to slightly increase the stability of oil stored in the dark (Gutierrez-Rosales *et al.*, 1992). Therefore, phenolic compounds and pigments are quality parameters, which are strongly related to the oxidative stability (Gutierrez *et al.*, 2002).

2.6.1 Oxidation reactions

Three forms of oxidation affect the quality of olive oil and include auto-oxidation, photo-oxidation and lipoxygenase oxidation (Bianchi, 2002).

2.6.1.1 Auto-oxidation

Auto-oxidation occurs when fatty acids react with molecular oxygen and leads to a complex chain of reactions involving free radical mechanisms (Hoffmann, 1989; Gunstone, 2004). Auto-oxidation is initiated at unsaturated fatty acid chains, which hold so-called active α -methylene groups whose hydrogen atom is easily removed by radical formation or by inducing conditions such as heat, light and catalysts. These α -methylene groups appear due to the attractive force a double bond has on the bonding electrons of the valence electrons, of one of the hydrogen atoms of the methylenic groups next to it. The abstraction of this hydrogen atom by molecular oxygen or other inducing agents is easier when the methylenic group lies between two double bonds. Therefore, polyunsaturated fatty acids such as linoleic and linolenic acid in olive oil are most susceptible to auto-oxidation. Following the cleavage of the hydrogen bond, a free radical is formed on these fatty acid chains (reaction 1). This process is called the initiation step of auto-oxidation and is preceding the propagation reactions. Propagation involves the addition of molecular oxygen to the free radical formed during initiation, resulting in a peroxy free radical (reaction 2), which in turn reacts with a new unsaturated fatty acid (reaction 3). This reaction results in the formation of hydroperoxides and free alkyl radicals, the latter of which react with molecular oxygen in a new cycle. Hydroperoxides are fairly non-volatile, tasteless and odourless compounds that can be further attacked to form secondary oxidation products. Auto-oxidation usually ceases due to a depletion of oxygen in the termination step.



Secondary oxidation products are formed during decomposition of hydroperoxides by radical inducing conditions such as heat, radiation and metal catalysts (Hoffmann, 1989).

Peroxides can be converted into alkoxy radicals by homolysis and this intermediate reaction leads to the formation of several secondary oxidation products (Bianchi, 2002). The reduction of the alkoxy radical gives an alcohol, whereas disproportionation with another radical produces a carbonyl compound, alcohol and a reduced radical. The abstraction of a hydrogen atom from another molecule transforms the alkoxy radical into an alcohol and a new free radical. The aldehydes and carbonyls formed are responsible for the typical off-flavours of rancidity in oil (Hoffmann, 1989).

Another effect of auto-oxidation of an unsaturated fatty acid is the conjugation of double bonds (Hoffmann, 1989). The initial radical formation at the α -methylene carbon atom causes mobility of all the bonding electrons in its two adjacent carbon atoms, leading to a shift of the double bond within this three carbon chain or allylic group. Initial free radical formation often takes place on the methylene group between two double bonds. In this unstable situation, conjugation of the double bonds occurs by shifting of bonding electrons to create a more stable state. Further, the double bond being shifted changes from the *cis* to the *trans* conformation (Gunstone, 2004).

2.6.1.2 Photo-oxidation

Photo-oxidation occurs when radiation energy excites molecular oxygen into a higher energy state by energy transfer from a sensitiser, such as chlorophyll, and singlet oxygen is formed (Bianchi, 2002; Gunstone, 2004). The formation of hydroperoxides does not involve free radical reactions but the action of singlet oxygen, which is produced by the transfer of excitation energy from chromophoric impurities to oxygen (Kiritsakis *et al.*, 1983). The singlet oxygen reacts with fatty acid double bonds more rapidly than triplet oxygen and enters an 'ene' pericyclic reaction forming six regiomer hydroperoxides of linoleates at a rate of 1450 times faster than triplet oxygen. These hydroperoxides can then initiate the free radical mechanism of auto-oxidation (Gunstone, 2004).

2.6.1.3 Lipoxygenase oxidation

During lipoxygenase oxidation molecular oxygen is directly inserted into free fatty acids by lipoxygenase enzymes (Bianchi, 2002). Triglycerides are split into their fatty acids by a lipolytic acyl hydrolase. The free fatty acids formed are then attacked by lipoxygenase, hydroperoxide lyase, alcohol dehydrogenase and alcohol acyltransferase, respectively, resulting in six carbon atom aldehydes, alcohols and esters of alcohols. The complex mixture of volatile compounds produced during this enzymatic pathway in part defines the sensory characteristics of olive oil.

As deterioration of the oil progresses the volatiles present in fresh olive oil are gradually replaced by volatile decomposition products produced during the oxidation reactions. Hexanal is

produced by the breakdown of linoleate-13-OOH, nonanal and 2-decenal from oleate-9-OOH, and 2-heptenal arises from linoleate-12-OOH, resulting in the development of fatty, waxy, painty and oxidised off-flavours, respectively (Morales et al., 1997).

2.6.2 Measuring oxidative stability

Since oxidative rancidity is the major form of deterioration in oil, its acceptability depends on the extent to which this deterioration has occurred and therefore some criterion for assessing the oxidative status of olive oil is necessary (Gray, 1978). Although sensory analysis is one of the most sensitive methods available, it is not practical for routine analysis and lacks in reproducibility. Therefore, a wide range of chemical and physical analysis methods exists, which measure oxidation of lipids at different stages of the oxidation process and with different underlying principles. The chemical methods attempt to improve the reproducibility and sensitivity of evaluating oils, compared to sensory analysis by a tasting panel, by quantifying the oxidative status. The ultimate criterion, in terms of quality control, for the suitability of any analysis method is its agreement with sensory perception of off-flavours and odours (Gray, 1978). However, there is no single ideal method which correlates well with changes in sensory properties of an oxidised oil throughout the entire course of oxidation or which measures all oxidative pathways concurrently.

Each of the methods discussed below gives valuable information about a particular stage in the oxidative deterioration of a lipid, and some are more applicable to certain lipid systems than others. Hence, a choice has to be made, which methods to use for the analysis of a particular lipid and this depends on a number of factors such as the nature of the sample, the type of information required, and the time available. Generally, at least two oxidation parameters are required to evaluate the oxidative stability of olive oil (Ryan *et al.*, 1998).

The measurement of oxidative stability can generally be divided into two categories, i.e., methods measuring the extent of lipid oxidation and susceptibility tests (Gray, 1978). Susceptibility tests or accelerated stability tests measure the stability of a lipid under standardised conditions that accelerate oxidative deterioration and include methods such as the Schaal oven test, active oxygen method and Rancimat method. The induction period is then measured as the time required to reach an endpoint of oxidation corresponding either to a level of detectable rancidity or a sudden change in the rate of oxidation (Frankel, 1993). However, the determination of the end of the induction period requires additional methods that test the oxidative status of the lipid, to give an indication of the point at which rancidity arises. For the purpose of this study, only the different analyses applied to measure the extent of lipid oxidation will be considered. Appendix 1 gives a list of publications, which have considered a variety of oxidation experiments on olive oil.

2.6.2.1 Peroxide value

The peroxide value is the most widely used method to evaluate the presence of primary oxidation products. It is an iodometric method, which measures the presence of hydroperoxides in terms of milli-equivalents of active oxygen in one kilogram of oil (Bianchi, 2002). Hydroperoxides react with iodide ions to liberate iodine from potassium iodide (reaction 4) (Nawar, 1996).



The amount of hydroperoxides in an oil sample is estimated by titration with sodium thiosulfate in the presence of potassium iodide. The liberated iodine is converted to a blue starch-iodine complex by the addition of starch indicator and the volume sodium thiosulfate required to reach a colourless endpoint is used to calculate the amount of oxygen milli-equivalents. The American Oil Chemists' Society (AOCS) has developed an official method for the determination of the peroxide value (AOCS Cd8b-90), which is applicable to all fats and oils (AOCS, 1996). This method is commonly used for the analysis of olive oil, although it is highly empirical and any variation in the procedure may lead to variations in the results (Gray, 1978). Since the peroxide value measures oil oxidation in its early stages, its value for oil under oxidative conditions always passes through a maximum and then declines as hydroperoxides are broken down into secondary oxidation products. Good correlations between peroxide values and sensory panel test scores have been reported for some vegetable oils (Hahm & Min, 1995).

2.6.2.2 Ultraviolet absorption

The ultraviolet absorption method is based on the determination of extinction coefficients at 232 and 270 nm corresponding to the maximum absorbencies of conjugated diene and triene systems, respectively, which are formed during oxidation or refining processes (Ryan *et al.*, 1998). A 1% oil solution in cyclohexane is prepared and the absorbance at 232 and 270 nm, respectively, is measured by a UV-Vis spectrophotometer (IOOC, 2001). The extinction coefficients, K_{232} and K_{270} , are obtained by dividing the absorbance at the respective wavelengths by the product of the concentration of the oil solution in $\text{g } 100 \text{ ml}^{-1}$ and the cuvette path length in cm.

Whereas the conjugated dienes absorbing at 232 nm are formed during initial stages of oxidation, the conjugated trienes, ethylenic diketones, ketodienes and ketodienals absorbing at 270 nm arise in secondary stages of oxidation (White, 1995). The magnitude of change in the UV absorption is not readily related to the degree of oxidation because the effects on the different unsaturated fatty acids vary in type and magnitude (Gray, 1978). However, the

extinction coefficients can be used as relative measurements of oil oxidation and the method is widely applied for determining the degree of rancidity of oil and fat-containing foods. The method also does not depend on chemical reaction or colour development and values for the K_{232} coefficient correlate well with the peroxide value (White, 1995). Similar to the peroxide value, the K_{232} coefficient value does not increase infinitely, but reaches a plateau at a certain concentration, where the breakdown and formation of conjugated dienes cancel each other out.

2.6.2.3 Thiobarbituric acid reactive substances value

The determination of the thiobarbituric acid reactive substances (TBARS) value is not as commonly applied in the evaluation of oxidative stability of olive oil, as the peroxide value and free fatty acids, since this method is not classified as a quality parameter by the IOOC. However, it has been used as a measurement of the oxidative condition of olive oil (Lugasi *et al.*, 1995; Kiritsakis *et al.*, 1983). The TBARS method (AOCS official method Cd19-90) is based on the colour reaction between TBA and secondary oxidation products of polyunsaturated fatty acids (Frankel, 1993; AOCS, 1996). The oil sample is diluted with butan-1-ol and allowed to react with TBA for two hours in a water bath at 95°C. Malondialdehyde or more widely defined, thiobarbituric acid reactive substances (TBARS) are formed from fatty acids with mainly three or more double bonds and react with TBA to produce a red chromagen, whose absorbance can be measured at 530 nm (Bianchi, 2002).

The TBARS value represents a quantification of the degree of oxidation of the linolenic fatty acids in olive oil, which are only found in minor amounts. Nevertheless, good correlation between the TBARS value and the peroxide value are usually obtained (Gray, 1978; Ryan *et al.*, 1998). Alkanals, 2-alkenals and 2,4-alkadienals produce a yellow pigment with TBA that absorbs at 450 nm, while only 2,4-alkadienals and 2-alkenals produce a red pigment that absorbs at 530 nm (Gray, 1978). However, the absorbance at 450 nm can additionally be produced by the reaction of TBA with aldehydes, which have not undergone oxidation. Although both wavelengths are indicative of rancidity, 530 nm is thus taken as the characteristic wavelength of the TBA-malonaldehyde complex. The relative TBARS value varies from product to product and for this reason meaningful results from the TBA test are only obtained when samples of a single product are compared at different stages of oxidation. Once the relationship between TBARS value and change in flavour is established for olive oil, the TBARS value could be used as an index of flavour.

2.6.2.4 Total carbonyl compounds

Carbonyl compounds, such as aldehydes and ketones, are considered the major contributors to the development of oxidised flavours and thus many methods have been proposed, which

determine carbonyl compounds for possible correlation with sensory evaluations (White, 1995). The most widely applied method that measures total carbonyl compounds was developed by Henick *et al.* (1954) and is now adopted as the standard method of the Japanese Oil Chemists' Society. The experimental procedure is based on the formation of 2,4-dinitrophenylhydrazones from the carbonyl compounds in the presence of a trichloroacetic acid catalyst. This reaction results in coloured hydrazones that are measured spectrophotometrically in an alkaline solution. This procedure mainly detects non-volatile carbonyls and although these may be precursors to more volatile, odorous compounds, they make no direct contribution to the off-flavour such as the volatile carbonyls (Gray, 1978).

The measurement of volatile carbonyl compounds requires a distinct method of detection such as gas-liquid chromatography (White, 1995). The total carbonyl value is a useful method for the measurement of the oxidative status of certain oils in that it measures the actual flavour compounds formed during oxidation. However, this method also has many disadvantages, which cause it to be rather unpopular. The solvents used during the procedure must be purified to eliminate interference from existing carbonyls. The biggest problem is the decomposition of hydroperoxides under experimental conditions, which causes additional formation of carbonyls and an overestimation of the carbonyl content.

2.6.2.5 Anisidine value

The anisidine value also relies on the reaction of secondary oxidation products with reagents to give a coloured product that can be measured spectrophotometrically. α - and β -Aldehydes react with *p*-anisidine reagent in an acetic acid solution and the resulting Schiff base compounds formed during the reaction between aldehyde and amino group produce an anil with a yellow chromophoric group, whose absorbance is measured at 350 nm (White, 1995). The anisidine value determination is a fairly quick and simple method that can be performed directly on an oil solution. The absence of heat or strong acids during the reaction ensures that hydroperoxides do not interfere. Nonetheless, the anisidine value is not very sensitive and not well suited for the prediction of oxidised flavours in oils. The procedure mostly detects 2-alkenals, which are not solely responsible for the formation of off-flavours, and is thus more appropriate as a routine method to determine treatment before processing of crude oil rather than as a research tool.

2.6.3 Measuring components that influence oxidative stability

2.6.3.1 Free fatty acids

Free fatty acids are naturally found in olive oil in low concentrations, even in freshly pressed extra virgin olive oil. They are remainders of triglyceride synthesis and their concentrations are usually not lower than 0.2 to 0.3% in extra virgin olive oil (Bianchi, 2002). The measurement of

free fatty acids as a quality index, on the other hand, takes into account the free fatty acids formed during hydrolysis of acylglycerols by lipolytic enzymes, microorganisms or by hydrolytic processes during extraction (Ryan *et al.*, 1998). Examples of extrinsic factors which lead to a high free fatty acid content in an oil are fruit fly infestation of the olive fruit, delays between harvesting and extraction, fungal diseases such as *gloesporium* and *macrophoma*, as well as prolonged contact between oil and vegetation water after extraction (Costa, 2003). The hydrolytic products lead to the formation of undesirable aroma in the oil and thus a low as possible free fatty acid content is sought-after in olive oil of good quality. Free fatty acids have an effect on the stability of the oil due to their pro-oxidant action, which is exerted by the accelerating effect of the carboxylic group on the decomposition of hydroperoxides (Frega *et al.*, 1999). The free fatty acid content can thus be regarded as an index of lipase activity, fruit quality and freshness, as well as storage time and stability of the oil. This, however, only holds true for virgin olive oils, since refined oils have less than or equal amounts of free fatty acids due to their removal during the refining process (Bianchi, 2002).

The most commonly used method for the determination of free fatty acids in olive oil is based on acid-base titration using phenolphthalein as an indicator (Ryan *et al.*, 1998). The oil sample is dissolved in an organic solvent and titrated against an alkaline solution to a phenolphthalein endpoint. The acidity, expressed as percentage oleic acid, is determined according to equation 1. The Association of Official Analytical Chemists (AOAC) method (AOAC 940.28) uses sodium hydroxide as the base and dissolves the oil in pure ethanol (AOAC, 2000). The solvent and titrant may differ between various official methods (AOCS, British or European Community standard), however, all titration methods rely on the same principle of measuring the amount of base necessary to neutralise the free acids present in the sample.

$$\text{FFA}\% = \frac{(\text{volume of alkaline solution} \times \text{molarity of alkaline solution} \times 282)}{10 \times \text{sample mass}} \quad (1)$$

2.6.3.2 Total polyphenols

The determination of individual phenolic substances takes considerable expertise and work and, therefore, the total polyphenol content is normally measured for purposes of oxidative stability investigations (Ryan *et al.*, 1998). The total polyphenol content is determined spectrophotometrically following the reaction of Folin-Ciocalteu reagent with a methanol extract of the oil (Gutfinger, 1981; Morello *et al.*, 2004). A solution of oil in hexane is centrifuged three times with a 60:40 methanol-water solution in order to extract the polar phenolic fraction. A suitable aliquot of the combined extracts is then reacted with Folin-Ciocalteu reagent for two hours, after which the absorbance is read at 725 nm. The total phenol content is estimated by

preparing a standard curve of caffeic acid and the results are expressed as mg caffeic acid per kilogram oil (Gutierrez *et al.*, 2001; Gutierrez *et al.*, 2002). The disadvantages of this fairly simple method are the interference of other compounds, since the Folin reagent is not entirely specific for phenols. The complex nature of the phenolic fraction makes the selection of an absorbing wavelength another difficulty and in general, spectrophotometric measurements tend to overestimate the phenolic content. Nonetheless, the determination of total polyphenols is a widely applied method, since it gives a good indication of the quality of olive oil and can be used to predict its oxidative stability and antioxidant capacity.

2.6.3.3 Pigments

Pigments, in particular chlorophyll, play an essential role in photo-oxidation in that they act as photosensitisers and capture and concentrate light energy. Chlorophylls have a pro-oxidant effect in oils exposed to light, however, in darkness the same compounds seem to protect the oil from oxidation by capturing free radicals (Gutierrez-Rosales *et al.*, 1992). Carotenoids are singlet oxygen quenchers and protect olive oil from photo-oxidation (Boskou, 2002). The chlorophyll and carotenoid content of virgin olive oil is an important consideration in the evaluation of oxidative stability, since a high pigment concentration in olive oil stored in the dark could contribute to increased resistance against oxidation. Carotenoids and chlorophylls in olive oil are easily quantified spectrophotometrically, by measuring the absorbance of oil diluted in cyclohexane at 470 and 670 nm, respectively. The amount of pigment in mg kg⁻¹ oil can then be calculated by use of the specific extinction coefficients in cyclohexane, which are 613 g 100 mL⁻¹ for chlorophyll and 2000 g 100 mL⁻¹ for carotenoids when using a 1 cm path length (Minguez-Mosquera *et al.*, 1991; Marquez, 2003).

2.7 Quality control

The term quality can be defined in many ways, depending on the situation and individual perception. However, in terms of food products it is mostly defined as the degree of excellence and includes factors such as taste, appearance and nutritional content (Potter & Hotchkiss, 1998).

Quality considerations differ from the perspective of the producer and the consumer. The olive grower or processor perceives the oil content of olive fruits as a major quality consideration, whereas the consumer measures the quality of olive oil according to its nutritional and sensory attributes (Costa, 1998). These attributes are again defined by a number of factors during the various stages of olive oil production. The condition of the fruit when harvested, the absence of pesticide residues, the method of harvesting and storing the unprocessed fruits, the time between harvesting and processing, cleanliness of fruit and equipment, the extraction

technique used and the storage conditions of the oil all play a major role in the quality of the bottled product.

Nutritional and sensory characteristics of an olive oil can be related to several chemical measurements and this leads to a set of chemical analyses or quality parameters, which represent a suitable indication of the nutritional and sensory quality of the oil. The oxidative status of edible oil inevitably has a direct effect on the nutritional and sensorial quality and thus most chemical analyses involve the measurement of oxidative stability or compounds having an influence on this stability.

The most important compounds that have an influence on the stability of olive oil are the polyphenols and pigments found in olive oil. The polyphenols in olive oil are of nutritional importance and are of particular interest regarding the oxidative stability of olive oil (Salvador *et al.*, 2001). These natural antioxidants help to heighten the human body's antioxidant defence mechanism (Calabrese, 2002) and in the oil itself contribute to approximately 50% of the stability (Gutierrez *et al.*, 2001). Thus, it can be expected that the polyphenol content of olive oil is correlated with oxidative stability parameters such as the peroxide value and the free fatty acid percentage, as well as sensorial quality (Ryan *et al.*, 1998). Olive oil pigments have implications in auto-oxidation and photo-oxidation processes and their quantity is also directly correlated with the colour of the oil, which plays a significant role in the consumers' association with quality (Minguez-Mosquera *et al.*, 1991; Ryan *et al.*, 1998). Since the pigments decompose during prolonged storage (Roca *et al.*, 2003) and are not present in substantial amounts in processed or heat treated oils, the IOOC has described their presence as a quality criterion.

In terms of virgin olive oil, the most commonly applied chemical analyses for the study of the oxidative stability are the peroxide value, percentage free fatty acids expressed as oleic acid, absorbencies in the ultraviolet region, polyphenol analysis, pigment analysis and stability determined by the Rancimat method (Bianchi, 2002). However, the standards set by the IOOC, the European Commission Regulation 2568/91 and the Codex Alimentarius (1993) require more elaborate analyses (Table 3).

The European commission lists many more specifications regarding minor fatty acid and sterol content (not listed in Table 3). These detailed requirements regarding the composition of olive oil are more of concern when investigating the adulteration of olive oil and are not relevant for general quality control. Quality parameters that are analysed to obtain an indication of the nutritional and sensory qualities should thus not be confused with parameters specifying the genuineness or authenticity of olive oil. According to Ryan *et al.* (1998), genuineness is the state of a food to be natural, without any sophistication or modification of its composition. Of course a certain quality parameter can also be an indication of the oil's authenticity. For instance, the pigment content of an olive oil was used to develop an index of authenticity for

Table 3 The different regulations regarding olive oil characteristics (Adapted from Bianchi, 2002).

	IOOC	Codex Alimentarius (1993)	EU Commission 2568/91
	Extra virgin olive oil	Virgin olive oil	Extra virgin olive oil
Free acidity (% oleic acid)	< 0.8	na.	< 1.0
Peroxide value (meq O₂ kg⁻¹)	< 20	< 20	< 20
Absorbancy in UV	270 nm < 0.22 232 nm < 2.50	na.	270 nm < 0.20 232 nm < 2.40
Moisture and volatile matter (%m/m)	< 0.2	< 0.2	na.
Insoluble impurities (%m m⁻¹)	< 0.1	< 0.1	na.
Trace metals (mg kg⁻¹)	Iron < 3.0 Copper < 0.1	Iron < 5.0 Copper < 0.4	na.
Panel test	Median = 0 for negative attributes	na.	> 6.5
Iodine value	na.	75-94	na.
Refractive index (20°C)	na.	1.4677-1.4705	na.
Saponification value (mg KOH kg⁻¹)	na.	184-196	na.
Unsaponifiables (g kg⁻¹)	na.	< 15	na.
Contaminants (ppm)	na.	Pb < 0.1 As < 0.1 Solvents < 0.2	Halogenated solvents < 0.2
Waxes	na.	na.	< 2.50
Sat. fatty acids in triacylglycerol position 2 (%)	na.	na.	< 1.3
Stigmastadienes (mg kg⁻¹)	na.	na.	< 0.15
Diff. between HPLC and theoretical calculation of ECN42	na.	na.	< 0.2

na. = not applicable, i.e., no specifications by regulators.

virgin olive oil (Gandul-Rojas *et al.*, 2000; Roca *et al.*, 2003). The ratio of chlorophyll to carotenoid was determined to be around one, and the ratio of minor carotenoids to lutein to be around 0.5 for a genuine virgin olive oil.

2.8 Sensory analysis

The sensory attributes of virgin olive oil are formed by the combined effect of many volatile and some non-volatile compounds on our gustatory and olfactory receptors (Bianchi, 2002). In a good quality virgin olive oil the volatile compounds present mainly originate from the lipoxygenase pathway, which occurs naturally on tissue disruption of the olive. These C₆ aldehydes and alcohols and their related esters create the typical green, grassy and unripe fruit odour of fresh virgin olive oil. Phenolic compounds found in the oil are mainly responsible for bitter and pungent sensory attributes. Negative sensory notes are produced during the oxidation of the oil, due to the formation of off-flavour compounds from decomposition of hydroperoxides, leading to rancid odours. Additional negative attributes include fusty, musty, winey, muddy and metallic sensory notes. Fusty relates to the flavour of an olive oil that has been pressed from olives stored in piles and which have undergone anaerobic fermentation (IOOC, 1996). Olives that have been stored in humid conditions and therefore have developed extensive fungal and yeast contamination will result in musty or mouldy oil. Winey oil contains acetic acid, ethyl acetate and ethanol arising from fermentation and muddy odours are perceived in oil that has been left in contact with tank sediment. Metallic flavours can be found in olive oil due to long-lasting contact with metallic surfaces during crushing, mixing, pressing or storage.

The IOOC and the European Community have adopted the above-mentioned positive and negative sensory attributes in their panel test profile sheet (IOOC, 1996a). The profile sheet of the IOOC comprises an unstructured scale, 10 cm long, for seven negative attributes, i.e. fusty, musty, winey/vinegary/acid/sour, muddy, metallic, rancid and others (IOOC, 1996a). There are only three positive attributes, i.e. fruity, bitter and pungent. The median of each positive and negative attribute is calculated and the oil is categorised according to the median of defects and the median of the fruity attribute. The median of the fruity attribute has to be above 0 for extra virgin and virgin olive oils, but is allowed to be 0 for ordinary and lampante olive oils. The median of defects has to be 0 for extra virgin, between 0 and 2.5 for virgin, between 2.5 and 6 for ordinary olive oil and can be above 6 for lampante olive oil (Bianchi, 2002).

The IOOC has besides the profile sheet and statistical evaluation of the results also standardised the presentation of the sample (IOOC, 1987) and training of tasters (IOOC, 1996b). A 15 mL olive oil sample at $28 \pm 2^{\circ}\text{C}$ is to be presented in a dark-coloured, narrow-mouthed tasting glass, covered with a watch glass and randomly coded. These conditions allow

the olive oil odours and flavours to be concentrated and prevent the judgement of the oil according to its colour.

Organoleptic evaluation of oils, based on odour and flavour perception, provides very useful information regarding the consumer acceptance of the product (Frankel, 1993). Although this method is very sensitive, it is highly dependent on the degree of training the taste panel received. Furthermore, the scoring given for a particular olive oil by a panellist may vary greatly from laboratory to laboratory. A number of drawbacks and weak points in the statistical evaluation of the IOOC sensory analysis method have been recognised, yet attempts to adopt other sensory evaluation techniques have failed (Van Bruggen *et al.*, 1995).

Many attempts have been made to find a more objective and standardised method to evaluate the sensory properties of olive oil for quality control purposes. Instrumental methods, such as gas chromatography (GC) and NMR spectroscopy have been applied to gain information on the sensory properties of olive oil (Kiritsakis, 1998; Mannina *et al.*, 1999). The sensory characterisation of virgin olive oil and its relationship with the headspace composition has been investigated (Servili *et al.*, 1995). GC-mass spectrometry was applied for the analysis of the volatile components of olive oil and the relationship between these data and sensory panel test data was evaluated by partial least squares regression analysis. Good prediction results for panel test scores were also obtained by coupling high resolution gas chromatography with artificial neural network processing (Angerosa *et al.*, 1996). H^1 nuclear magnetic resonance spectroscopy allows the detection and quantification of several flavour components in virgin olive oil, including phenols, alcohols and aldehydes (Sacchi *et al.*, 1997), by relating particular areas of resonances to flavour compounds (Mannina & Segre, 2002). The presence of specific flavour compounds can then be translated into sensory panel test scores (Mannina *et al.*, 2003).

The prediction of sensory properties by near infrared spectroscopy has been used for tenderness, toughness and juiciness measurements of beef (Hildrum *et al.*, 1995; Lui *et al.*, 2003) and the prediction of sensory properties of espresso (Esteban-Diez *et al.*, 2004). Relationships between sensory analysis and near infrared spectroscopy were also developed for apples (Mehinagic *et al.*, 2003) and white wine (Cozzolino *et al.*, 2005). To date there is only one study reporting the prediction of olive oil sensory panel test scores by near infrared spectroscopy (Garrido-Varo *et al.*, 2004).

2.9 Adulteration of olive oil

The adulteration of olive oil is a serious problem in the modern olive oil industry and is not only a crisis in major olive producing countries, but is even affecting South African olive oil suppliers and consumers (McKenzie & Koch, 2004). The increasing consumer interest due to the outstanding nutritional and sensory properties and the economic value of olive oil compared to

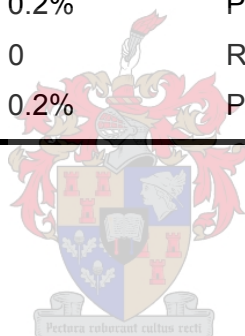
other vegetable oils makes this product prone to adulteration by cheaper oils. Olive oil adulteration can be divided into two types, namely the blending of virgin olive oils with inferior quality olive oils such as refined olive oil or pomace oil and, secondly, the addition of other vegetable oils to olive oil (Dourtoglou *et al.*, 2003). The former mainly occurs in major olive oil producing countries such as Greece, Italy and Spain. The addition of other vegetable oils (e.g. sunflower, canola and rapeseed) is mostly a problem in countries importing olive oil (e.g. USA and Canada), and where seed oils are locally produced and less expensive (Li-Chan, 1994).

The fatty acid composition of olive oil is the most important indicator of adulteration by other oils and is mostly determined by gas chromatography. Since the IOOC and other official bodies have established specific limits with regard to the percentage of distinct fatty acids in genuine olive oil, this method can be applied to distinguish pure from adulterated oils (Christopoulou *et al.*, 2004). The maximum limits of fatty acids in olive oils and olive pomace oils are as follows: Oleic 55.0 to 83.0%, stearic 0.5 to 5.0%, palmitic 7.5 to 20.0%, palmitoleic 0.3 to 3.5%, linoleic 3.5 to 21.0%, myristic $\leq 0.05\%$, linolenic $\leq 1.0\%$, arachidic $\leq 0.6\%$, eicosenoic $\leq 0.4\%$, behenic $\leq 0.2\%$ and lignoceric $\leq 0.2\%$ (IOOC, 2003). Any fatty acid present in amounts exceeding the above-mentioned limits will be indicative of adulteration with seed oil (Table 4). However, the fatty acid composition is not in all cases successful in detecting fraud (Christopoulou *et al.*, 2004).

The fatty acid determination by means of gas chromatography cannot be applied for the reliable uncovering of adulteration of olive oil with sunflower, cotton, corn, sesame, hazelnut, almond and safflower oils. Gas chromatography involves methyl ester formation from triacylglycerols and thus no information can be gained on the triacylglycerol composition. Therefore, useful information for the revealing of seed oil adulteration is lost. High performance liquid chromatography (HPLC) allows the determination of triglyceride composition and in olive oil the largest triglyceride peaks are represented by LOO, LOP, OOO, POO, POP and SOO (O = oleic, L = linoleic, P = palmitic, S = stearic) (Bianchi, 2002). Many commercially important seed oils have high concentrations of LLL, LLO and LLP, but mostly low contents of LOO and LOP. These trends in triglyceride composition can be exploited for the detection of adulterants in olive oil. The trilinolein (LLL) content and the Equivalent Carbon Number 42 (ECN42) are the parameters most effective in detecting seed oil adulteration in even small quantities (Christopoulou *et al.*, 2004). The ECN42 is calculated by subtracting the experimental ECN42, determined by HPLC, from the theoretical ECN42, obtained by a special computer programme based on GC determination of fatty acid composition. The ECN42 was adopted by the IOOC as a purity criteria and it specifies an ECN42 below 0.2 for all edible virgin olive oils (IOOC, 2003). This limit is effective in exposing the adulteration of olive oil with sunflower, soybean, cotton,

Table 4 Presence of fatty acids used for the detection of seed oils (Adapted from Bianchi, 2002).

Fatty acid present above limit	% fatty acid	Adulterant
Lauric acid	> 0	Oil babassu, coconut, palm kernel
Myristic acid	> 0.05%	Oil babassu, coconut, palm kernel
Palmitic acid	> 20%	Palm oil
Stearic acid	> 5%	Peanut, soyabean, sunflower
Oleic acid	< 55%	Babassu, coconut, cottonseed, grapeseed, maize, palm kernel, safflower
Linoleic acid	> 21%	Peanut, cottonseed, grapeseed, maize, safflower, sesame, soyabean, sunflower
Linolenic acid	> 0.9%	Rapeseed, soyabean
Arachidic acid	> 0.6%	Peanut
Eicosenoic acid	> 0.4%	Rapeseed
Behenic acid	> 0.2%	Peanut, sunflower
Erucic acid	> 0	Rapeseed, safflower
Lignoceric acid	> 0.2%	Peanut, rapeseed



maize, walnut, sesame, safflower, canola and rapeseed oil (Christopoulou *et al.*, 2004). However, mixtures of olive oil with hazelnut, almond, peanut or mustard oils cannot be detected at concentrations lower than 5% by the ECN42. In fact, the presence of below 5% hazelnut or almond oil in olive oil cannot be identified by both LLL content and ECN42 values and thus creates the greatest problems.

Several sterols found in the minor components fraction of olive oil are characteristic for it and their absence or presence of other sterols can be used to distinguish genuine olive oil (Bianchi, 2002). The total sterol content of virgin, refined and ordinary olive oil has to be equal or larger than 1000 mg kg⁻¹. A concentration of cholesterol above 0.5%, brassicasterol above 0.2% and campesterol and stigmasterol above 4% may be indicative of various seed oil admixtures. The two triterpenes, erythrodiol and uvaol, making part of the sterol fraction are an effective tool in detecting solvent-extracted olive residue oils (crude or refined olive pomace oil) in higher grade olive oils. Virgin olive oils have a maximum triterpene content of 4.5% of the total sterol fraction, whereas olive residue oils always contain more than 4.5% of erythrodiol and uvaol.

2.9.1 Nuclear magnetic resonance spectroscopy in the detection of adulteration

All the above-mentioned methods of detecting olive oil adulteration involve very sophisticated and time-consuming analysis methods and therefore much effort has been put into the development of more rapid and economic detection methods. ¹H and ¹³C nuclear magnetic resonance (NMR) spectroscopy has raised particular interest in the authentication of olive oil, since this method can identify the fatty acids and their position on the glycerol backbone, gives information on the degree of saturation, and can quantify minor olive oil components (Mannina & Segre, 2002; Hidalgo & Zamora, 2003). The carbonyl region of an olive oil ¹³C NMR spectrum shows two groups of resonances, which hold information on the 1,3-glycerol (α -position) and 2-glycerol position (β -position) fatty acid chains (Vlahov, 1999). Although the individual fatty acid chains cannot be differentiated, it is possible to distinguish between saturated chains and unsaturated chains and it was established that unsaturated fatty acids are almost exclusively found in the β -position of the triglycerol, whereas saturated fatty acids are attached at the α -positions (Mannina *et al.*, 1999; Mavromoustakos *et al.*, 1997). This is the only instrumental means by which the positional distribution of fatty acids can be determined and is much faster than enzymatic methods previously applied. An α : β ratio of below 2:1 of oleic or linoleic acid implies the preference of these unsaturated fatty acids for the β -position and can therefore be employed as an indicator of olive oil adulteration (McKenzie & Koch, 2004). The fact that less than 1.5% of saturated fatty acids in olive oil are found in the β -position of the triglyceride also allows the detection of esterified olive oils (Sacchi *et al.*, 1997). In these oils the neutralisation

process causes the abundant number of free fatty acids to randomly distribute on glycerol and thus the percentage of saturated fatty acids on the β -position of the glycerol increases to around 15. Oleic acid and linoleic acid concentrations can be determined by four characteristic peaks present in the olefinic region of the ^{13}C NMR spectrum and these allow the detection of olive oil adulterated with seed oils (Mavromoustakos *et al.*, 2000). ^{31}P NMR spectroscopy combined with multivariate analysis methods allows the detection of adulteration of extra virgin olive oil with as little as 5% lampante and refined olive oil (Fragaki *et al.*, 2005). As with chromatography, ^1H NMR can also be used to analyse minor components of olive oil, such as sterols, squalene, cycloartenol, chlorophyll and phenols, which can be indicators of quality and authenticity (Vlahov, 1999; Guillen & Ruiz, 2001; Mannina *et al.*, 2003). Appendix 3 tabulates publications concerning the analysis of olive oil by NMR spectroscopy.

2.9.2 Infrared and near infrared spectroscopy in the detection of adulteration

Another spectroscopic method, which has been used in the authentication of olive oil is near infrared and infrared spectroscopy. Near infrared spectroscopy combined with discriminant analysis (Bewig *et al.*, 1994) and principal component analysis (Sato, 1994) has been successfully applied to differentiate between types of vegetable oils. The ability of near infrared spectroscopy to discriminate between virgin olive oils of different geographical origin has been confirmed (Bertran *et al.*, 2000). The use of infrared spectroscopy in the detection of refined olive oil and hazelnut oil adulteration of extra virgin olive oil has been studied (Lai *et al.*, 1995). Fourier transform total attenuated reflectance infrared spectroscopy has been applied for the detection of olive oil adulteration with sunflower oil down to a 2% level of contamination (Tay *et al.*, 2002). The detection and quantification of sunflower oil, corn oil and olive residue oil in virgin olive oil was achieved by near infrared spectroscopy (Wesley *et al.*, 1995), where adulteration levels of only 1% sunflower oil could be detected (Downey *et al.*, 2002). Recently, quantitative models predicting olive oil adulterants within an error range of less than 1% were developed (Christy *et al.*, 2004). This shows that near infrared spectroscopy is a powerful tool for the rapid detection and quantification of olive oil adulteration and has potential for application in rapid quality control procedures.

3. Near Infrared Spectroscopy

3.1 History

The near infrared (NIR) region of the electromagnetic spectrum was discovered as early as March 1800 by William Herschel, who presented his work titled "Experiments on the refrangibility of the invisible rays of the sun" in the *Philosophical Transactions of the Royal Society* (**90**, 284-292) (Workman, 2004). Herschel proved that there exists light radiation beyond the visible

spectrum by studying the heating effects of the different colours in the visible region (Davies, 1998; McClure, 2003; Pasquini, 2003). He observed that the heating effect of the sun rays extended beyond the red visible light and thus came to the conclusion that invisible radiation was present. However, his discovery did not find application until 1881, when Abney and Festing photographically recorded the NIR spectra of organic liquids between 700 and 1200 nm, from which they ascertained that hydrogen bonds play an important role in near infrared absorption (Osborne *et al.*, 1993). In the early 1900s, spectra of pure compounds were measured by Coblentz (Pasquini, 2003) between 800 and 2800 nm and he was first to characterise C-H bond absorbance bands (Osborne *et al.*, 1993). Later, between 1922 and 1929 Ellis confirmed this work by studying more functional groups of organic compounds and in 1928 Brackett identified NIR absorbance bands of CH₃, CH₂ and CH groups (McClure, 2003). More studies on assigning organic functional groups to absorption bands were published in the 1930s by Wulf and Liddel, who investigated aliphatic and aromatic amines, phenols and alcohols in the 1500 nm region. The absorption characteristics of water in the NIR region were first considered by Collins (1925), who assigned water bands at five distinct wavelengths and studied their shift with changes in temperature. Between 1935 and 1940 Ellis, Kinsey and Fox expanded on his work.

The development of the photoelectric detector for the NIR region around 1945 made it possible to record NIR spectra and the first instrument of this kind was the Cary 14 (Osborne *et al.*, 1993). This NIR spectrophotometer was obtainable in 1954 and initiated increasing research in the field of NIR spectroscopy. Kaye, Willis, Goddu, Wheeler and Whetsel were the researchers that performed qualitative and quantitative studies of various functional groups with instruments such as the Cary 14 and Beckman DK-2, which formed a strong basis for modern NIR spectroscopy (McClure, 2003). A major contribution to NIR spectroscopy becoming an accepted analytical technique was research conducted in the field of agriculture by K. Norris of the United States Agricultural Research Service (ARS) in the early 1960s (Barton, 2002). Around 1965 Norris modified a Cary 14 spectrophotometer, previously used for transmission NIR spectroscopy, to be able to record diffuse reflectance spectra (McClure, 2003). This instrument was used to measure the moisture, protein and oil content in wheat by applying multiple linear regression calibrations and was the first agricultural application of NIR reflectance spectroscopy. It was not long until other agricultural products, such as tobacco and food products, were analysed with NIR reflectance spectroscopy. In 1975, NIR spectroscopy was accepted by the Canadian Grain Commission as an official protein testing method (Osborne *et al.*, 1993). Later, Norris developed practical applications for NIR transmittance spectroscopy, such as the analysis of intact cereal grains and this research lead to the development of a series of NIR transmittance instruments. As on-line instruments emerged, technically advanced

transmittance and reflectance instruments were built and new calibration methods were developed, NIR spectroscopy boomed and extended into pharmaceutical, industrial, cosmetic and food processing applications (Osborne *et al.*, 1993).

3.2 Instrumentation

NIR spectrophotometers can be classified according to their wavelength selection technology into filter and scanning instruments (Wetzel, 2001). Filter NIR instruments only radiate from a small number of discrete wavelengths in the NIR region. Examples of instruments applying discrete wavelengths are filter based, light emitting diode (LED) based and acousto-optical tunable filter (AOTF) based instruments (Fig 5) (Pasquini, 2003). These systems are employed to allow the channelling of variably broad wavelength bands. LED and AOTF spectrophotometers do not include moving parts in their filter system, but rely on emission of a narrow band of radiation (Osborne *et al.*, 1993) and applying different radio frequencies across a crystal, respectively, to achieve wavelength selection (McClure, 2001). Discrete wavelength instruments are mostly only of application for analytes absorbing in a specific segment of the NIR spectral region (Blanco & Villarroya, 2002). The advantage of this equipment is its lower cost compared to whole spectrum instruments, as well as its suitability for use in portable NIR scanners.

Dispersive instruments based on diffraction gratings and interferometric (Fourier transform) instruments are the most common whole spectrum or scanning NIR instruments (Fig 5) (Pasquini, 2003). Grating dispersive instruments split the light radiation spatially by means of an optical interference such as grating monochromators, whereas Fourier transform (FT) instruments make use of interferometers and Fourier transformation to recapture intensities of single wavelengths (Osborne *et al.*, 1993; Gunasekaran & Irudayaraj, 2001). An interferometer consists of a beam splitter that splits the incoming radiation and allows one half to be reflected from a moving mirror and one half from a fixed mirror, after which they are reunited before reaching the sample and detector (Wetzel, 2001). The interference data collected at different mirror positions is then subjected to Fourier transformation. FT-NIR instruments display good resolution and signal to noise ratios and are a particularly good choice for research purposes, where broad application and calibration transfer are important.

Most NIR spectroscopy instruments are equipped with semiconductor detectors, which can either be indium gallium arsenide (InGaAs) or lead sulphide (PbS) detectors (Stchur *et al.*, 2002). Although InGaAs detectors give a faster response (Wetzel, 2001) and higher signal to noise ratio when compared to PbS detectors, the latter is often preferred due to its considerably lower cost (Osborne *et al.*, 1993; Stchur *et al.*, 2002). Multi-channel detectors such as charged coupled devices (CCDs) can be implemented by positioning several detection units in rows or

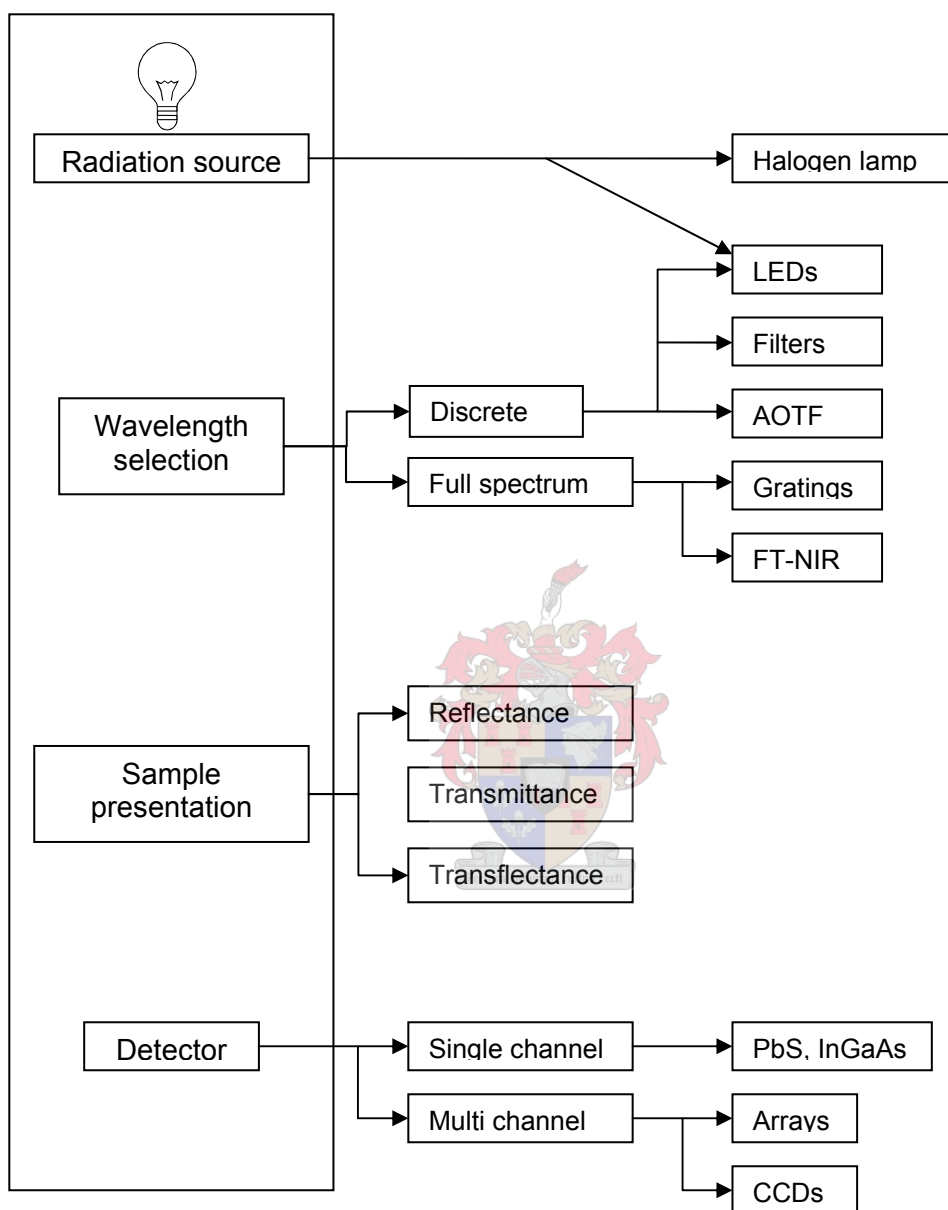


Figure 5 Basic components of a near infrared instrument (AOTF = acousto-optical tunable filter, CCDs = charged coupled devices, FT-NIR = Fourier transform near infrared, InGaAs = indium gallium arsenide, LEDs = light emitting diode, PbS = lead sulphide) (Adapted from Blanco & Villarroya, 2002).

planes, respectively (Fig 5) (Blanco & Villarroya, 2002). The radiation source for most NIR instruments is either a tungsten coil or a halogen lamp (McClure, 2001; Pasquini, 2003).

3.2.1 Sample presentation modes

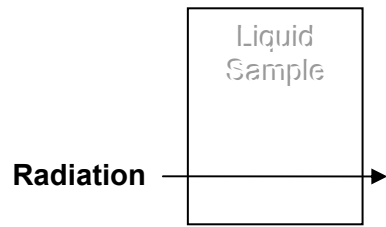
Near infrared absorption (780 to 2500 nm) by substances is up to 1000 times lower than the absorption resulting from fundamental bands in the mid infrared region (2500 to 10 000 nm) (Pasquini, 2003). This allows intact samples to be used for analysis without prior dilution or disruption of tissues and has resulted in several different sample presentation modes for NIR measurements. The most commonly applied measurement modes applied are transmittance, transreflectance, and diffuse reflectance (Fig 6). Transmittance NIR spectra are obtained when measuring liquids in quartz cuvettes with an optical path length usually ranging between 0.2 and 50 mm and the radiation penetrates the sample without obstruction (Fig 6a) (Pasquini, 2003). Radiation that is transmitted through a sample, however, reflected by an object such as a mirror or metal plate, and is transmitted back through the sample is known as transreflectance (Fig 6b). In this mode the radiation beam passed twice through the sample, thereby doubling the optical path length. Unique for NIR spectroscopy is the ability to measure solid samples, which occurs by diffuse reflectance of the radiation from the solid matter (Fig 6c). However, scattering and absorbance of some radiation by the solid may contribute to inaccuracies.

3.3 Theory

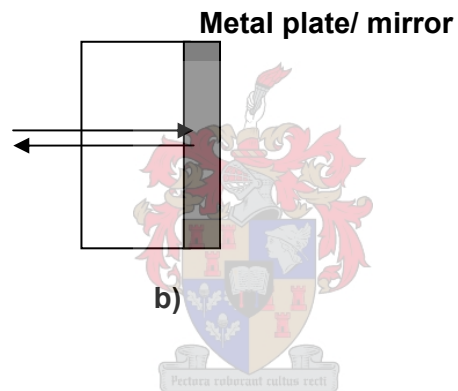
Near infrared spectroscopy is a type of vibrational spectroscopy, which exploits photon energy in the wavelength region from 780 to 2500 nm (Miller, 2001; Pasquini, 2003). Near infrared radiation can be applied in the field of spectroscopy due to phenomena related to the absorption and emission of energy by molecules. Electromagnetic radiation can be pictured as a flow of photons with energy proportional to the frequency of the radiation (Osborne *et al.*, 1993). The atoms in molecules are bound together by chemical bonds, which can act like springs and result in vibrations of the bonds. Since, each pair of atoms in a molecule has distinctive bond length, strength and direction, each bond vibrates at unique frequencies (Murray, 2004).

3.3.1 The harmonic model

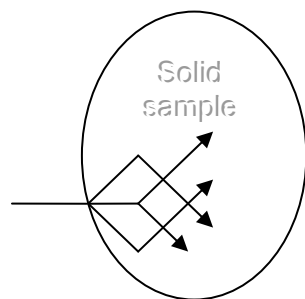
From these basic findings Hooke's Law can be derived, which states that "for a simple harmonic oscillator the frequency of a vibration (ν) is equal to the reciprocal of 2π times the speed of light times the square root of the force constant (5×10^5 dynes cm^{-1}) times the sum of the two masses divided by their product" (Barton, 2002; Pasquini, 2003). The quantum mechanical model for a harmonic oscillator was proposed by Planck in 1900. This model states that the energy of a vibrating bond is discontinuous and the absorption or emission of single



a)



b)



c)

Figure 6 Three different possibilities of sample presentation: a) Transmittance, b) transreflectance and c) diffuse reflectance.

bundles of energy can lead to a change in vibrational energy states (Osborne *et al.*, 1993). The quantum theory therefore only allows the vibrational transition to occur at frequency changes of one ($\Delta v = \pm 1$). This change in energy can be induced by electromagnetic radiation of exactly the same frequency as the vibrating bond, in which case the interaction between radiation and vibration relies on the presence of a dipole moment across the bond. This principle of harmonic oscillation explains the fundamental absorbencies for diatomic molecules in the infrared region, however, NIR absorbencies are produced from vibrational transitions of 2, 3 or even 4 times the fundamental frequency (overtones), and are thus theoretically not possible according to Hooke's law and the quantum theory (Miller, 2001; Barton, 2002).

3.3.2 The anharmonic model

The Franck-Codon principle explains the formation of NIR absorbance bands at inaccurate multiples of the fundamental frequencies (Barton, 2002). This theory states that atoms are most of the time found in vibrational states, which have a low kinetic energy and photons are therefore most likely absorbed by slow moving nuclei. The excitation caused by the absorption of the photon is not instantly transferred to the nuclei and thus slow movement of the nuclei will continue immediately after absorption. It is further assumed that transitions occur mostly when nuclear configurations are the same in the vibrational energy states and when nuclei have small kinetic energies. These small deviations result in anharmonicity, which in turn leads to overtone and combination bands that are not precise multiples of the fundamental frequency.

Anharmonic bond vibration can further be described by the fact that bonds having a dipole moment are more easily expanded than compressed, due to coulombic repulsion and dissociation between the two atoms (Miller, 2001; Murray, 2004). Coulombic repulsion causes a more rapid increase in potential energy than predicted by the harmonic model, whereas dissociation causes the potential energy to level off (Osborne *et al.*, 1993; Pasquini, 2003). Thus, anharmonic oscillation only differs from harmonic oscillation in that the oscillation frequency decreases steadily with increasing multiples of the fundamental frequency. Energy transitions can therefore occur over more than one vibrational state and these create first, second and higher overtones in the near infrared region (Blanco & Villarroya, 2002). First and second overtone bands give the most valuable information, however, combination and difference bands may also be formed by the interaction of different vibrations (Osborne *et al.*, 1993). These have frequencies that originated from the sum or difference of multiples of their fundamental frequency.

3.3.1 Chemical assignments

Hydrogen containing functional groups are the most anharmonic and cause overtone bands due to stretching vibrations and combination bands of stretching and bending vibration modes (Murray, 2004). Organic compounds are largely composed of hydrocarbon groups, making the near infrared region ideal for the investigation of food components. The prevalent near infrared spectral assignments are the methyl CH stretching vibrations, methylene CH stretching vibrations, aromatic CH stretching vibrations and OH stretching vibrations (Workman, 2004). Minor bands that are of importance include those created by vibrations from methoxy CH, carbonyl, NH from amides and amines, as well as NH from amine salts.

Organic CH first overtone bands have been assigned by studying different alkanes in the wavelength region from 1690 to 1770 nm and five bands (1693, 1710, 1724, 1757 and 1770 nm) in the region were identified (Osborne *et al.*, 1993). CH₂ groups in fatty acids display first overtones at 1740 and 1770 nm and =CH first overtone at 1680 nm. The most important chemical assignments regarding oil analysis that were made in the NIR region are shown in Table 6.

3.4 Chemometrics

The most recent advances in NIR spectroscopy have been in the field of chemometrics, which involves the application of mathematical procedures for processing, evaluating and interpreting large amounts of data, such as NIR spectral data (Stchur *et al.*, 2002). Chemometrics are used to find statistical correlations between the spectral data and the constituent of the sample to be measured and are indispensable for the development of qualitative or quantitative calibrations.

3.4.2 Quantitative calibrations

Before any chemometric calculation can be applied, a well-planned calibration experiment has to be performed during which two sets of data have to be collected. Firstly, a reference data set has to be constructed by analysing a sample population for the component of interest, according to the traditional chemical or physical method (Osborne *et al.*, 1993). Secondly, the corresponding spectral data of the same sample population have to be collected. The reference data should be representative of the samples to be analysed in future by the NIR instrument (Osborne *et al.*, 1993), and must include varying concentrations of the component that are distributed evenly between extreme values (Wiedemann *et al.*, 1998; Pasquini, 2003). The minimum and maximum values of concentrations of the component should be around 5 times the reproducibility of the reference method to give a reliable calibration. Therefore, it is important to be aware of the repeatability and reproducibility of the reference method, since the NIR prediction model will give results in terms of the reference method used for calibration

Table 6 Absorption bands of typical vegetable oil functional groups in the near infrared region
(Adapted from Christy *et al.*, 2004; Garrido-Varo *et al.*, 2004).

Wavelength (nm)	Functional group	Assignment
1168	CH ₃ -	C-H str. 2 nd ovt.
1211	-CH ₂ -	C-H str. 2 nd ovt.
1391	CH ₃ -	2C-H str. + C-H def.
1414	-CH ₂ -	2C-H str. + C-H def.
1664	<i>Cis</i> R ₁ CH=CHR ₂ CH ₃ -	<i>Cis</i> CH
1723	-CH ₂ -	C-H 1 st ovt.
1761	-CH ₂ -	C-H 1 st ovt.
1901	C=O	2 nd ovt. C=O str.
1931	C=O	2 nd ovt. C=O str. (ester)
2124	-COOR	C-H and C=O str.
2145	-HC=CH-	=C-H and C=C str.
2176	-HC=CH-	CH asymmetric str. and C=C str.
2304	CH ₂	CH asymmetric str. and CH def.
2344	CH ₂	CH asymmetric str. and CH def.
2445	CH ₂ , C=O	CH asymmetric str. and C=O str.
2480	CH ₃ , C=O	CH asymmetric str. and C=O str.

str. = Stretch

ovt. = Overtone

def. = Scissoring



(Williams, 2001). Other important aspects are the number of samples employed for building the model (Wiedemann *et al.*, 1998), which should be a minimum of 50 to 100 for agricultural products (Pasquini, 2003), and factors influencing NIR measurements, such as temperature and moisture content of samples (Williams, 2001). The aim of chemometric processing, in the form of multivariate analysis methods, is then to deduce an algorithm from these data, which is able to predict the constituent measured by the reference method from spectral data in future samples (Osborne *et al.*, 1993). Many different multivariate analysis methods exist for both qualitative and quantitative applications (Fig 7), however, only the most important methods for quantitative calibrations are mentioned in detail here.

3.4.3 Multivariate analysis methods for quantitative applications

3.4.3.1 Multiple linear regression

Multiple linear regression (MLR) analysis is based on the selection of a few wavelengths that are used to find a linear combination that minimises the error in calculating the constituent of interest from spectral data (Chaminade *et al.*, 1998). The selection of wavelengths for the multiple regression is usually based on the smallest residual sum of squares (Beebe & Kowalski, 1987), however, a choice based only on the residual sum of squares cannot be made, since it will always decrease as wavelengths are added to the regression equation (Osborne *et al.*, 1993). The standard error of prediction (*SEP*) tends not to decrease indefinitely with the addition of more wavelengths, but equations having the smallest *SEP* generally include too many wavelengths. This tends to lead to over-fitting of the data and small *SEP* and coefficient of correlation (*r*) values do not necessarily indicate an accurate calibration (Williams, 2001). Alternatively, the t-test may be used as a criterion by rejecting all equations that do not have significance at 5 percent. Since all these selection methods have their drawbacks, most applications of multiple linear regressions apply stepwise selection procedures, which include forward step-up, forward stepwise and backward stepwise regression (Osborne *et al.*, 1993).

Forward step-up regression selects the wavelength with the highest coefficient of correlation first and all subsequent wavelength selections depend on the primary wavelength (Williams, 2001). Forward stepwise regression starts with the wavelength giving the best one-term equation and then adds one wavelength at a time, each selected on the basis of the smallest residual sum of squares (Osborne *et al.*, 1993). Backward stepwise regression may be applied in cases where it is possible to include all wavelengths in the first step, followed by stepwise removal of wavelengths, which results in the smallest increase of residual sum of squares.

The MLR calibration method was suitable for selecting wavelengths from the spectra of filter NIR instruments, however, the selection of just two or three wavelengths from the hundreds

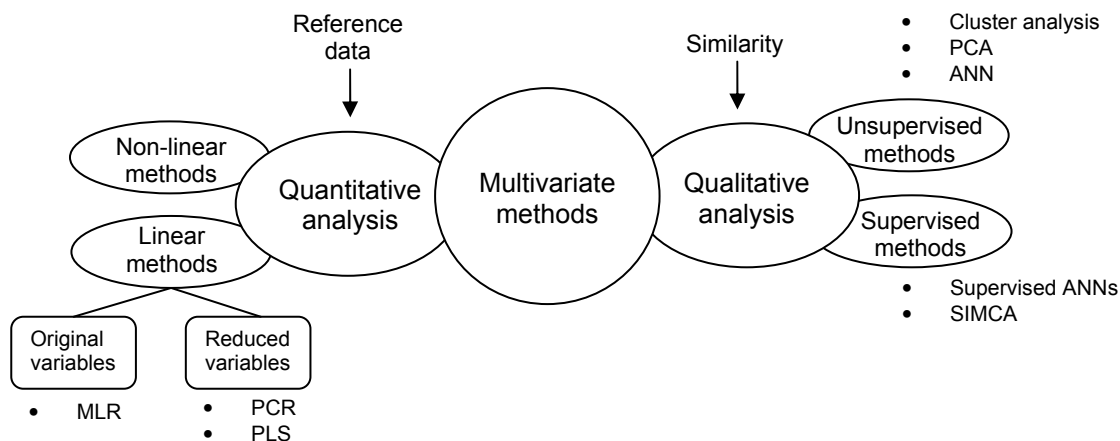


Figure 7 Diagram classifying the various multivariate analysis methods (ANN = artificial neural networks, MLR = multiple linear regression, PCA = principal component analysis, PCR = principal component regression, PLS = partial least squares regression) (Adapted from Blanco & Villarroya, 2002).



of measuring wavelengths of a dispersive NIR instrument can be very difficult (Osborne *et al.*, 1993). MLR is sensitive to noise and only efficient if no interferences and colinearities between properties exist at the selected wavelengths (Chaminade *et al.*, 1998). Therefore, MLR is less popular for monochromators and calibration methods that use all the available wavelengths of the spectrum have been developed.

3.4.3.2 Full spectrum regression methods

Multiple linear regression was the only calibration method available until 1977, after which it was mostly replaced or combined with Fourier transform, and a few years later principal component regression (PCR) and partial least squares regression (PLS) were introduced (McClure, 2003). These full-spectrum methods both have the aim of compressing data without losing valuable information and of avoiding over-fitting (Osborne *et al.*, 1993). PCR and PLS underlie the same principle of building principal component factors from the original spectra (principal component analysis).

The principal component analysis (PCA) of spectral data results in a much smaller number of variables or principal components, which represent the majority of the variation between the measured samples as linear combinations of the spectral data (Osborne *et al.*, 1993). Assuming that absorbencies of the sample are measured at 700 wavelengths, then the spectral data can be seen as a cloud of points, each representing one sample, in a 700-dimensional space. To reduce the dimensions each point can be projected onto a smaller dimensional space, eventually resulting in a two-dimensional plot that has a new x-coordinate or factor, which represents the linear combination of all spectral measurements. This linear combination is the sum of all the products of each wavelength (λ) and corresponding loading (w) and gives the score (z) of each sample on the factor (equation 2).

$$z = w_1\lambda_1 + w_2\lambda_2 + \dots + w_n\lambda_n \quad (2)$$

The first principal component describes most of the spectral variations and each consecutive factor contains less information (Beebe & Kowalski, 1987). Once the spectral data has been reduced to the most important factors, the factors and loadings can be used to build a multiple linear regression. This combination of PCA and MLR is referred to as PCR (Stchur *et al.*, 2002).

PLS regression was developed in the late 1970s and its advantage over PCR is the calculation of factors from both spectral and reference value information (Beebe & Kowalski, 1987; Osborne *et al.*, 1993). Compared to PCR, PLS factors are chosen according to the most relevant variation in NIR data and thus the first factor does not only include the biggest spectral

variations but also variations due to high correlation with reference data (Martens & Naes, 2001).

3.4.1 Qualitative calibrations

Qualitative applications of NIR spectroscopy include the identification or classification of samples into groups of distinct characteristics and can be achieved by two different methods, namely supervised and unsupervised methods (Fig 7) (Blanco & Villarroya, 2002; Pasquini, 2003). In supervised qualitative calibrations the classes or identity of the samples is known, whereas in unsupervised methods the number of possible classes in the training sample set is unknown. In both methods it is important to include as much variation within the training sample set as possible to ensure successful classification of future samples (Pasquini, 2003). Qualitative multivariate analysis methods, also known as pattern recognition methods, are used to recognise similarities and regularities in the NIR data. The most commonly applied methods are principal component analysis, cluster analysis, artificial neural networks (ANN) and soft independent modelling of class analogy (SIMCA) (Fig 7) (Blanco & Villarroya, 2002; Stchur *et al.*, 2002).

3.4.4 Pre-treatment of spectra

Spectral pre-treatments have the purpose of reducing spectral information that is unrelated to the constituent for whom the calibration is developed (Blanco & Villarroya, 2002), such as radiation scattering in solid samples or other spectrum baseline affecting trends (Pasquini, 2003). A wide variety of pre-treatment options are available in modern software packages, but the more frequently applied pre-treatments include derivatives, multiplicative scatter correction and normalisation.

3.4.4.1 Derivatives

The derivative spectra are calculated by taking the difference between absorbance ($\log 1/R$) measurements at gaps of about 10 nm (Osborne *et al.*, 1993). In the same manner, second derivative calibrations can be made by calculating the difference between two neighbouring first derivative measurements. Higher derivatives can be derived, however, for NIR spectroscopy applications these are seldom useful (Osborne *et al.*, 1993), since the signal to noise ratio decreases with each addition of a derivative treatment to the spectral data (Stchur *et al.*, 2002). Overlapping peaks in an original spectrum can be observed as distinct downward peaks in a second derivative spectrum and offset or sloping baselines become zero (Ozaki *et al.*, 2001). Derivative treatments are thus especially valuable for reducing particle size effects (Osborne *et*

al., 1993), packing variations or path length differences (Wiedemann *et al.*, 1998) and may also accentuate spectral differences between compounds (Chaminade *et al.*, 1998).

3.4.4.2 Scatter correction

During multiplicative scatter correction (MSC) a linear relationship between each spectrum and the mean spectrum of the calibration set is determined (Chaminade *et al.*, 1998). The offset of this regression line is then subtracted from the absorbance values of each spectrum and the difference is divided by the slope of the regression line. This pre-treatment reduces light scattering effects, as well as particle size effects, packing variations or path length differences (Wiedemann *et al.*, 1998).

Standard normal variate (SNV) transformation is a different type of scatter correction, which involves the division of the difference between the absorbance and the average absorbance by the standard deviation of the absorbance (Chaminade *et al.*, 1998). It is used to normalise spectra that have been affected by light scattering.

3.4.4.3 Normalisation

Normalisation of spectra can be achieved with four different algorithms (Chaminade *et al.*, 1998). The absorbance (transmittance, transreflectance or reflectance) at each wavelength can be divided by the average absorbance, the maximum absorbance, the sum of the total absorbance or each spectrum can be scaled between zero and one. The main purpose of this pre-treatment is to eliminate offset deviations in spectra.

3.4.5 Evaluation of calibration efficiency

The efficiency of a calibration is evaluated by using the calibration to analyse the validation sample set (Williams, 2001). For this purpose the standard error of calibration (*SEC*) and prediction (*SEP*), the bias and the coefficient of determination (R^2) between NIR measurements and reference data is calculated. The R^2 shows the degree to which the NIR data agree with the reference data, and the bias expresses the mean difference between NIR and reference data. The *SEC* and *SEP* equal the standard deviation of the differences between NIR data and reference or predicted values, respectively. The *SEC* should always be higher than the standard error of the reference method (*SE*) (Stchur *et al.*, 2002). If this is not the case the calibration model can be considered over-fitted.

Generally, the most effective calibration is the one with the highest R^2 and lowest *SEP* (Williams, 2001). More emphasis should, however, be laid on the *SEP* value, since the predictive ability measured by the R^2 is dependent on the range of the reference values. Thus, a reference value range far beyond that of interest for future measurement could give a very high

coefficient of determination, although the *SEP* is larger than it would be for a smaller reference sample range (Osborne *et al.*, 1993). Another term that can be used for the evaluation of the *SEP* with regard to the standard deviation of the predicted values (SD_p) is the *RPD* (ratio of *SEP* to SD_p) (Williams, 2001). *SEP* values should be considerably lower than SD_p values, resulting in a *RPD* of at least 3. The *SEP* value, which gives an indication of the predictive ability of the model, should also be as close as possible to the standard error of laboratory (*SEL*) between duplicate reference measurements. A graph of residuals, which is drawn by plotting the reference values minus the predicted values versus the predicted values, can be used to examine the calibration plot for nonlinearity and possible outliers.

For any regression algorithm the best number of variables to be included in the final model must be determined (Pasquini, 2003). This involves the selection of wavelengths in MLR (see 3.4.3.1) and factors in the full spectrum calibration methods. The smallest possible number of factors should be chosen, however, in cases where the component of interest occurs in only small concentrations in the sample, more principal components may be acceptable (Wiedemann *et al.*, 1998). To choose the minimum number of factors without excluding valuable information, the predicted residual error sum of squares (*PRESS*) may be calculated and plotted against the factors (Stchur *et al.*, 2002). The number of factors, which include the majority of the *PRESS* will incorporate most of the sample variance. Scores can give an indication of sample homogeneity and cluster formation, whereas loadings show how variables are weighted in the principal component space. Residuals describe the amount of information that is not included by the principal components (Chaminade *et al.*, 1998).

Since with PLS regression the performance of the calibration set will increase continuously as more factors are added, over-fitting can become a problem (Osborne *et al.*, 1993). Having a separate validation set and choosing the number of factors according to the best performance of this validation set can solve this. Alternatively, cross validation may be applied, which involves leaving one sample out and then performing a calibration with the remaining samples. A prediction is made for the sample left out and this procedure is repeated for each sample. Finally, an average squared prediction error is calculated, which will have a minimum value for the optimum number of factors. This procedure may also be applied to PCR.

3.5 Near infrared spectroscopy of vegetable oils

In 1956, NIR chemical assignments for several chemical groups characteristic of lipids were determined by investigating the spectra of synthetic fatty acids (Holman & Edmondson, 1956). The earliest application of near infrared analysis of vegetable oils was published in 1991 (Sato *et al.*, 1991). The fatty acid composition of butter fat, pig milk fat, soybean oil and palm oil was determined by gas chromatography and the NIR transmittance spectra between 1600 and 2300

nm were measured to build a qualitative calibration. The aim was to collect a library of NIR spectra of various fats and oils and then identify unknown samples by comparing their spectra to those in the library. Other qualitative near infrared studies applied discriminant (Bewig *et al.*, 1994) and principal component (Sato, 1994) analysis to distinguish between different vegetable oils. Principal component analysis was also used to detect and quantify adulterants in virgin olive oil (Wesley *et al.*, 1995). Studies on the relationship between near infrared second derivative spectra and oxidation of oil in terms of hydroperoxides revealed that the absorption peak at 2084 nm is highly correlated to hydroperoxides and is suitable for determining the lipid oxidation in edible oils (Takamura *et al.*, 1995).

From the late 1990s onwards advances in calibration methods and computer programs made it possible to develop quantitative calibrations for different constituents in vegetable oils that are an indication of oxidative status. Multiple linear regression has been applied for the construction of peroxide value and free fatty acid content prediction models for soybean oil (Cho *et al.*, 1998). By selecting two wavelengths corresponding to hydroperoxide bands (2080 and 2020 nm) and three wavelengths for the free fatty acid calibration equation (2008, 1442 and 1752 nm), good coefficients of determination and standard errors of prediction (*SEP*) of 9.67 meq kg⁻¹ and 0.137 mg g⁻¹, respectively, were achieved. The prediction of peroxide values and free fatty acid values by near infrared spectroscopy was also performed on sesame oil (Ha *et al.*, 1998). A multiple linear regression model was developed for the determination of the free fatty acid content in palm oil (Che Man & Moh, 1998). The wavelength selection was based on the presence of carboxylic acid overtone bands in the wavelength region 1850 to 2050 nm and the selection of three wavelengths (1882, 2010 and 2040 nm) resulted in the smallest root mean square error of prediction (*RMSEP*), i.e. 0.077, 0.041 and 0.059% for crude palm oil, refined bleached deodorised palm olein and refined bleached deodorised palm oil, respectively.

In 1999 a partial least squares calibration was performed on crude palm oil NIR spectra to develop a prediction model for the peroxide value as determined by the official American Oil Chemists' Society (AOCS) method (Moh *et al.*, 1999). The wavelength region between 1350 and 1480 nm was selected and for a reference value range of 2.17 to 10.28 meq kg⁻¹, a coefficient of determination (*R*²) of 0.996 and *SEP* of 0.170 meq kg⁻¹ was obtained. Using a reference value range of 0 to 10 meq kg⁻¹, produced by spiking hydroperoxide free canola oil with triphenylphosphine and triphenylphosphine oxide solutions, a partial least squares calibration was constructed and validation of the calibration with olive oil and sunflower oil gave *R*²s of 0.94 and 0.98, respectively (Li *et al.*, 2000).

NIR instruments have also been calibrated for other conventional oil analyses methods, except peroxide value and free fatty acid content. Several chemical properties, i.e., the free fatty acid content (Standard error of cross-validation (*SECV*) = 0.06%, *R*² = 0.98), peroxide value

($SECV = 1.55 \text{ meq kg}^{-1}$, $R^2 = 0.66$), specific extinction coefficients K_{270} ($SECV = 0.02$, $R^2 = 0.77$), K_{232} ($SECV = 0.07$, $R^2 = 0.64$) and K_{225} ($SECV = 0.02$, $R^2 = 0.92$), total polyphenol content ($SECV = 43.2 \text{ mg kg}^{-1}$, $R^2 = 0.90$), oxidative stability as determined by the Rancimat ($SECV = 9.10 \text{ h}$, $R^2 = 0.94$) and moisture content ($SECV = 0.04\%$, $R^2 = 0.76$) of virgin olive oil were predicted by NIR (Garrido *et al.*, 2000). This study also included the first attempt to predict the sensory panel test score of olive oil by NIR and resulted in satisfactory results ($SECV = 0.50$, $R^2 = 0.88$). Partial least squares regression models with first derivative pre-treatment gave good results for the peroxide value ($SEP = 0.760 \text{ meq kg}^{-1}$, $R^2 = 0.994$), conjugated diene value ($SEP = 0.021\%$, $R^2 = 0.857$) and anisidine value ($SEP = 0.920$, $R^2 = 0.901$) of soybean oil (Yildiz *et al.*, 2001). The oleic acid, linoleic and linolenic acid contents in olive oil were quantified using partial least squares regression models with orthogonal signal correction (OSC) as spectral treatment (Bertran *et al.*, 2001). Selected wavelength regions were used to include overtones and combination bands of CH and C=CH bonds (1100 to 1280, 1660 to 1780 and 2000 to 2240 nm). OSC was found to decrease the number of PLS factors, however, did not increase predictive ability. Prediction models for the total carotenoid and chlorophyll content in virgin olive oil have been developed for visible-NIR transmittance spectroscopy and a SEP of 0.66 ($R^2 = 0.970$) and 0.96 mg kg^{-1} ($R^2 = 0.986$) was achieved for carotenoids and chlorophylls, respectively (Jimenez-Marquez, 2003). NIR reflectance prediction models for the determination of the free fatty acid percentage, peroxide value and oleic acid content of Italian virgin olive oils were developed in 2003 (Conte *et al.*, 2003). This research was expanded in 2004 and NIR transmittance calibrations were built for other virgin olive oil quality criteria, such as UV absorption, Rancimat test and tocopherol, polyphenol and linoleic acid contents (L.S. Conte, 2004, Dipartimento di Scienze degli Alimenti, Universita degli Studi di Udine, Udine, Italy, personal communication). NIR spectroscopy was evaluated as a quality tool for Australian olive oils by developing PLS prediction models for the free fatty acid content ($SECV = 0.07\%$, $R^2 = 0.97$), peroxide value ($SECV = 1.34 \text{ meq kg}^{-1}$, $R^2 = 0.92$), total polyphenol ($SECV = 58.67 \text{ mg kg}^{-1}$, $R^2 = 0.89$) and chlorophyll ($SECV = 0.51 \text{ mg kg}^{-1}$, $R^2 = 0.98$) content, Rancimat test ($SECV = 0.97 \text{ h}$, $R^2 = 0.88$) and various fatty acid contents (Mailer, 2004). A summary of the current publications on the analysis of vegetable oils with NIR spectroscopy is tabulated in Appendix 2.

4. Conclusions

Olive oil is a food product known to man for already several thousand years and therefore it has been studied extensively in many aspects. The fatty acid composition, flavour profile and minor component content of olive oil has been characterised through comprehensive research and this gave insight to the beneficial effects of regular olive oil consumption on human health. Scientific evidence revealing the effectiveness of regular olive oil consumption in preventing

cardiovascular diseases, as well as certain cancers, has initiated a new interest in olive oil by the modern health-conscious community and has led to the extension of the industry beyond Mediterranean countries. South Africa with its climate and environmental conditions suitable for olive cultivation is one of the countries that entered the industry.

The high demand for olive oil together with its high economic value, compared to other vegetable oils, has made this product prone to adulteration and thus increasingly strict quality control measures are becoming essential. The IOOC, Codex Alimentarius and European Commission have standardised quality control procedures and have specified the different olive oil categories. However, since these quality control procedures involve complex chemical procedures, many attempts have been made to facilitate olive oil quality control by means of instrumental methods. Although several studies have looked at near infrared spectroscopy as an alternative quality control tool for other vegetable oils, the application of NIR spectroscopy to olive oil quality control is a relatively new development. Recent olive oil literature is mainly aimed at the detection and quantification of olive oil adulteration with alternative methods such as nuclear magnetic resonance spectroscopy and near infrared spectroscopy.

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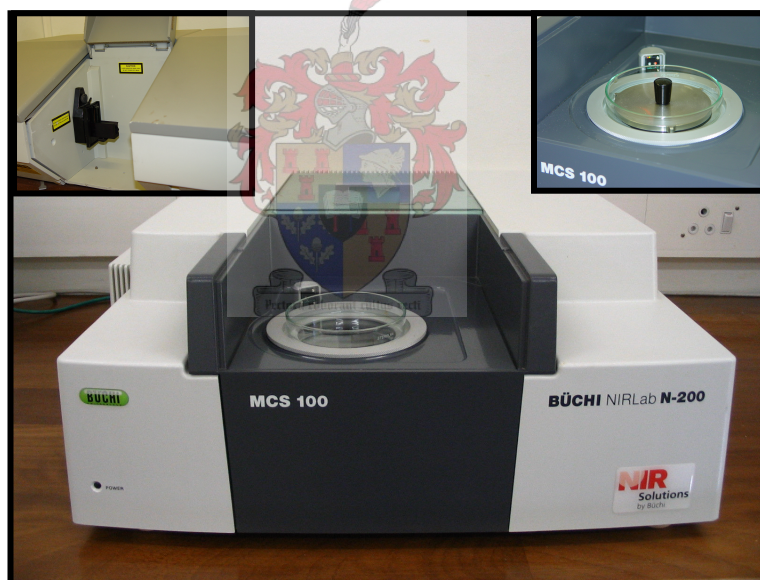
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Chapter 3

COMPARISON OF FT-NIR SPECTROSCOPY PARTIAL LEAST SQUARE PREDICTION MODELS FOR SOUTH AFRICAN EXTRA VIRGIN OLIVE OIL USING SPECTRA COLLECTED ON TWO SPECTROPHOTOMETERS AT DIFFERENT RESOLUTIONS AND PATH LENGTHS



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Comparison of FT–NIR spectroscopy partial least square regression models for South African extra virgin olive oil using spectra collected on two spectrophotometers at different resolutions and path lengths

Abstract

Near infrared (NIR) partial least square (PLS) regression models for the determination of several extra virgin olive oil quality parameters (peroxide value, free fatty acid content, specific extinction coefficients K_{232} and K_{270} , linoleic, oleic and saturated acid content, α : β ratios of linoleic and oleic acid, pigment and total polyphenol content, thiobarbituric acid reactive substances value) were developed from spectra collected on two different Fourier transform near infrared (FT-NIR) spectrophotometers using different sample holders, path lengths and resolutions. Spectra were recorded on the Perkin Elmer IdentiCheck™ spectrophotometer (9091-4000 cm^{-1}) in transmittance mode at resolutions of 64, 32, 16 and 8 cm^{-1} at each of two path lengths (0.2 and 0.5 mm) and on the Büchi NIRLab N-200 instrument (10 224-4000 cm^{-1}) in transreflectance mode at a fixed resolution of 8 cm^{-1} and a path length of 0.6 mm. The PLS regression models, for each respective parameter, were statistically compared to evaluate the effect of different sample scanning conditions on model performance. The comparison of the regression models developed from Perkin Elmer spectra revealed that spectra recorded at the lowest resolution of 64 cm^{-1} produced equally accurate models when compared to higher resolution spectra and that the two path lengths resulted in no significant differences. Comparisons between PLS models developed from Büchi and Perkin Elmer spectra, respectively, showed significant differences for only a few parameters. In general, reliable prediction results were obtained for the peroxide value ($SEP = 4.15 \text{ meq O}_2 \text{ kg}^{-1}$, $R^2 = 0.87$), K_{232} ($SEP = 0.94$, $R^2 = 0.94$), linoleic acid ($SEP = 0.83\%$, $R^2 = 0.90$) and saturated acid content ($SEP = 0.91\%$, $R^2 = 0.88$) PLS regression models.

Introduction

The superior nutritional and sensory characteristics of extra virgin olive oil are the basis for the high economic value and popularity of olive oils with health-conscious consumers. Therefore, the most important quality parameters for this product relate to the measurement of oxidative deterioration, which has a direct effect on nutritional and sensorial quality. Some of the most commonly performed chemical analyses for quality control of extra virgin olive oil are the peroxide value, free fatty acid content (expressed as % oleic acid), specific extinction coefficients at 232 and 270 nm, polyphenol content and pigment analysis (Bianchi, 2002). The thiobarbituric acid reactive substances value, although seldom used in official olive oil quality control procedures, can be useful for oxidative stability studies, since it can be directly related to changes in flavour (Gray, 1978).

The adulteration of extra virgin olive oil with low-cost vegetable oils has developed into an increasing problem in many countries and the detection of adulterated olive oils is thus becoming an essential part of quality control procedures (Christy *et al.*, 2004). The fatty acid composition of olive oil is the most obvious indicator of adulteration by foreign oils and can be determined by either gas chromatography (GC) or nuclear magnetic resonance (NMR) spectroscopy. The disadvantage of NMR spectroscopy is that it can only measure the major fatty acids in olive oil (oleic, linoleic and saturated acids), compared to GC which allows quantification of all present fatty acids. However, NMR spectroscopy is able to identify the position of linoleic (C18:2) or oleic (C18:1) fatty acids on the triacylglycerol backbone and the distribution of these fatty acids on the α and β positions is an effective parameter to detect adulterated olive oils (McKenzie & Koch, 2004). Unsaturated fatty acids in olive oils are predominantly situated at the β position of the glycerol backbone and thus the α : β ratio of oleic or linoleic acid should be lower than 2:1 (2 x α positions, 1 x β position) to indicate the preference of these unsaturated fatty acids for the β position.

All of the above mentioned analysis methods are time-consuming procedures that require the use of large amounts of glassware and potentially hazardous reagents. The cost of performing these labour-intensive, wet-chemical or spectrophotometric analyses is inevitably high, due to the large expenses on glassware, solvents, waste removal and highly trained laboratory technicians. Near infrared (NIR) spectroscopy has several advantages when compared to the conventional methods, including high speed, no sample preparation, only small amounts of sample are required and it is environmentally friendly, as no chemical waste is produced (Yildiz *et al.*, 2001).

NIR spectroscopy has found wide application in the analysis of fats and oils, including the quantitative measurement of quality parameters in vegetable oils (Takamura *et al.*, 1995; Che Man & Moh, 1998; Cho *et al.*, 1998; Ha *et al.*, 1998; Moh *et al.*, 1999; Li *et al.*, 2000; Yildiz *et al.*, 2001). The free fatty acid content and peroxide value of several vegetable oils has been successfully determined with NIR spectroscopy. NIR prediction models were developed for virgin olive oil for the determination of the free fatty acid content, peroxide value, total polyphenol content, oxidative stability as determined by the Rancimat (Garrido *et al.*, 2000; Conte *et al.*, 2003, L.S. Conte, 2004, Dipartimento di Scienze degli Alimenti, Universita degli Studi di Udine, Udine, Italy; Mailer, 2004), oleic and linoleic acid content (Conte *et al.*, 2003, L.S. Conte, 2004, Dipartimento di Scienze degli Alimenti, Universita degli Studi di Udine, Udine, Italy; Mailer, 2004) and specific extinction coefficients K_{270} , K_{232} (L.S. Conte, 2004, Dipartimento di Scienze degli Alimenti, Universita degli Studi di Udine, Udine, Italy) and K_{225} (Garrido *et al.*, 2000). Prediction models were also developed for the moisture content (Garrido *et al.*, 2000),

tocopherol content (L.S. Conte, 2004, Dipartimento di Scienze degli Alimenti, Università degli Studi di Udine, Udine, Italy) and various fatty acids (Mailer, 2004). Good correlation between NIR spectroscopy and the conventional chlorophyll and carotenoid determinations by UV-Vis spectrophotometry were obtained with virgin olive oil (Jiménez-Marquez, 2003). In all cases it was concluded that NIR spectroscopy might well be an economical and efficient alternative to chemical analyses.

What has not been taken into consideration before is the effect, which different instruments, scanning settings and sample presentation modes might have on the performance of regression models developed from identical sample sets. Some FT-NIR spectrophotometers offer the possibility to use different resolutions, which arise from differing distances the mirror of an interferometer moves. The larger the distance the mirror moves, the higher the resolution of the generated spectrum and more detail is revealed. A higher resolution results in longer scanning times and also decreases the signal to noise ratio and therefore an optimum resolution, which reveals all spectral detail without weakening the signal to noise ratio, needs to be determined. Besides resolution, both the sample presentation mode and path length have an effect on the spectral data due to physical influences and intensities of absorption.

The objective of this study was to statistically compare the performance of PLS regression models, for each of 13 respective quality parameters, developed from extra virgin olive oil spectra recorded on two respective Fourier transform near infrared (FT-NIR) instruments at different resolutions and path lengths, to determine the most appropriate instrument settings and sample presentation modes. Several FT-NIR spectroscopy PLS regression models, which could serve as a complete quality control tool for South African extra virgin olive oil producers, were developed. These regression models included peroxide value, free fatty acid content, UV absorption characteristics, linoleic, oleic and saturated fatty acids content, as well as the $\alpha:\beta$ ratio of both linoleic and oleic fatty acids, pigment content, total polyphenol content and thiobarbituric acid reactive substances value.

Materials and methods

Samples

Fourteen South African extra virgin olive oils (seasons 2003 and 2004), pressed from different olive cultivars (Mission, Frantoio, Coratina and Leccino) and originating from 5 regions in the Western Cape province (Stellenbosch, Somerset West, Paarl, Worcester and Riebeek Kasteel), were exposed to accelerated oxidation conditions to obtain a sample set that included an adequate range of oxidation levels for the development of PLS regression models. Plastic Petri-dishes were filled with 15 mL of extra virgin olive oil and stored uncovered in an illuminated incubation room at 35°C. The olive oil samples (pure cultivars and blends) were analysed when

they arrived from the producer, as well as weekly for up to five weeks of oxidation in the incubation room. Excluding the peroxide value, the degree of oxidation obtained after five weeks was too low to develop adequate prediction models. An additional set of 9 extra virgin olive oils (seasons 2003 and 2004), pressed from different olive cultivars (Mission, Frantoio and Coratina), was obtained from 4 regions (Paarl, Somerset West, Riebeek Kasteel and Robertson) and stored as before. The oil samples were again analysed when they arrived from the producer and to extent the range of reference values, every second week up to 10 weeks of oxidation. As the peroxide values needed extension at the lower range, these measurements were performed twice weekly for three and a half weeks. Development of turbidity in some oil samples resulted in overestimation of spectrophotometric absorbance and these samples were not included in the respective model developments.

Chemical analyses

Peroxide value

The peroxide value (PV) was determined according to the American Oil Chemists' Society (AOCS) official method (AOCS Cd 8b-90) (AOCS, 1996). The following adjustments were made to this method. Sample weights were reduced to 0.5 g and 10 mL glacial acetic acid (BDH, Wadeville, South Africa): isooctane (Merck, Darmstadt, Germany) (3:2) was added to the sample in a 100 mL Erlenmeyer flask. Then 0.5 mL of saturated potassium iodide (Saarchem, Merck Chemicals (Pty) Ltd, Wadeville, South Africa) solution was added and after one minute the reaction was stopped by adding 10 mL distilled water.

Free fatty acid content

The free fatty acid (FFA) content, expressed as % oleic acid, was determined according to the Association of Official Analytical Chemists (AOAC) official method (AOAC 940.28) (Horwitz, 2000).

Specific extinction coefficients at 232 nm and 270 nm

The two parameters K_{232} and K_{270} were determined according to the recommended method of the International Olive Oil Council (IOOC) (COI/T.20/Doc.19) (IOOC, 2001).

Pigment content

Chlorophyll and carotenoid pigments were quantified by spectrophotometric measurement at 670 and 470 nm, respectively (Beckman Coulter DU530 Life Science UV-Vis spectrophotometer) (Jiménez-Marquez, 2003). Three gram oil samples were diluted with hexane (HPLC grade *n*-hexane) (Saarchem, Merck Chemicals (Pty) Ltd, Wadeville, South

Africa) in 10 mL volumetric flasks and the absorbance of the solution was measured in glass cuvettes (Hellma) with a path length of 10 mm.

Total polyphenol content

The total polyphenol (TP) content (mg kg^{-1}) was determined by means of the Folin-Ciocalteu method (Capannesi *et al.*, 2000; Gutierrez *et al.*, 2002). Prior to reaction with the Folin-Ciocalteu reagent (Merck, Darmstadt, Germany) 10 g oil were diluted with hexane (Kimix, Chemicals & Laboratory Suppliers, Cape Town, South Africa) in a 25 mL volumetric flask and the water-soluble polyphenol fraction was extracted by means of centrifugation (Beckman Coulter Tj-25 centrifuge) with a methanol (Saarchem, Merck Chemicals (Pty) Ltd, Wadeville, South Africa): water mixture (6:4). Each 25 mL sample together with 10 mL of methanol-water was transferred to a 50 mL polyflor centrifuge tube and centrifuged three times for five minutes at 4425 rpm. After each centrifugation the methanol fraction was separated from the oil fraction and replaced by a fresh methanol: water solution. A small amount (0.5 mL) of the combined extracts of each sample was then mixed with Folin reagent and 7.5% sodium carbonate (Saarchem, Merck Chemicals (Pty) Ltd, Wadeville, South Africa) solution (1:5:4), respectively, and was allowed to react for two hours at 30°C. The absorbance of the combined extract solutions and caffeic acid (Sigma, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) standards (0.01, 0.02, 0.04, 0.06 and 0.08 mg mL^{-1} in distilled H_2O) was measured at 725 nm (Beckman Coulter DU530 Life Science UV-Vis spectrophotometer). The caffeic acid standard curve was used to estimate the total polyphenol content in the olive oil samples.

Thiobarbituric acid reactive substances value

The thiobarbituric acid reactive substances (TBARS) value was determined according to the AOCS official method (AOCS Cd 19-90) (AOCS, 1996).

Spectroscopic analysis

Nuclear magnetic resonance spectroscopy

Fourier-transform nuclear magnetic resonance (NMR) ^{13}C spectra were recorded on a Varian^{Unity} Inova 600 MHz (14.09 Tesla) spectrometer operating at 150 MHz for ^{13}C to determine the oleic (C18:1), linoleic (C18:2) and saturated fatty acids contents and α : β oleic and α : β linoleic ratios (McKenzie & Koch, 2004). Sample solutions in 5 mm NMR tubes consisted of 40% (w/w) olive oil in deuterated chloroform (CDCl_3) (Aldrich, Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The spectral window ranged from 200 to -5 ppm. A 90° pulse was used to collect the ^{13}C NMR spectra with a pulse delay of 45 seconds. Inverse-gated proton decoupling was applied. To optimise resolution Gaussian multiplication was applied to the free induction decay

(FID) using a line broadening of -0.092 and Gaussian enhancement of 1.088. Zero-filling to 524k was employed before the FID was Fourier-transformed and, finally, the peaks were integrated by deconvolution analysis. From the carbonyl region at around 172 ppm the percentage of saturated fatty acids was determined, whereas the olefinic region around 130 ppm was used to measure the oleic and linoleic acid content and $\alpha:\beta$ ratios. By NMR it is possible to distinguish between fatty acids attached to the α or β position of the glycerol backbone as the same carbon on a fatty acid chain will resonate at different chemical shifts depending on whether it is attached to the α or β position. As NMR has the advantage that the triacylglycerides themselves can be analysed without the cleavage of the fatty acids from the glycerol backbone, the $\alpha:\beta$ ratio information can be gained directly from the NMR spectrum and calculated in the same way as the linoleic and oleic acids.

Fourier transform near infrared spectroscopy

Near infrared spectra of the extra virgin olive oil samples were recorded immediately after the chemical reference methods on two respective spectrophotometers, i.e., a Perkin Elmer (PE) IdentiCheck™ FT-NIR and a Büchi NIRLab N-200 FT-NIR instrument.

Transmittance spectra were recorded on the PE IdentiCheck™ spectrophotometer from 9091-4000 cm^{-1} (1100-2500 nm) at 2 nm intervals, resulting in 701 data points. Spectra were measured at four different resolutions (64, 32, 16 and 8 cm^{-1}) at each of two path lengths (0.2 and 0.5 mm) in quartz Suprasil cuvettes (Hellma). A background spectrum of air was taken before each sample and the quartz cuvettes were cleaned with acetone before each sample.

The Büchi NIRLab N-200 FT-NIR spectrophotometer was used to record transmittance spectra from 10 224-4000 cm^{-1} (978-2500 nm) at 4 cm^{-1} intervals, resulting in 1557 data points, at a fixed resolution of 8 cm^{-1} . Five milliliters of oil were placed into a glass Petri dish and covered with a transmittance cover having three 0.3 mm high projections on its lower surface. The beam passed through the oil from below, reflected from the cover and passed through the oil layer a second time resulting in a total path length of 0.6 mm. The transmittance cover was cleaned with warm Extran MA 03 phosphate-free soap (Merck Chemicals (Pty) Ltd, Wadeville, South Africa) water and acetone between each oil sample.

Data analysis

Spectral characterisation was performed on the raw data (no pre-treatment) and the FT-NIR spectra ($n = 104$) pre-treated with multiplicative scatter correction (MSC) were investigated in terms of spectral differences due to oxidation status by means of principal component analysis (PCA) using Büchi NIRCal (version 4.21) software. Score plots of principal components (PCs) 1

and 2 were constructed for both instruments. To improve visual clarity of the PC plots, one spectrally different outlier was deleted from the PE sample set.

Partial least square (PLS) regression models were developed using Büchi NIRCal (version 4.21) software. Regression models were developed for each parameter and spectral data recorded on the Büchi NIR-Lab N-200 (13 regression models) as well as for each parameter and spectra recorded at the different resolutions and path lengths on the PE IdentiCheck™ (8 regression models for each of the 13 parameters). To allow comparison between regression models for each respective parameter, identical validation sets were chosen for each parameter by selecting every third sample from a list of ascending reference values. The remaining two-thirds of the sample set were placed in the calibration set. A PLS regression was fitted to all calibrations, with PLS factors differing between individual calibrations. The regression models were developed and compared without pre-treatment and without any outliers and wavelength regions removed. The paired comparison test was based on the statistical principles suggested by Snedecor and Cochran (1989) and the comparison of two competing calibrations performed according to Fearn's criteria (Fearn, 1996). The Excel sheet of Fearn's criteria version 2.0 was modified to give *p-values* instead of confidence levels, which indicates more precisely whether calibration models differ significantly or not.

Comparisons were made between PE regression models that were developed from spectra measured at equal resolutions but with different path lengths (e.g. 8 cm⁻¹ and 0.5 mm versus 8 cm⁻¹ and 0.2 mm), as well as spectra measured with equal path lengths but at different resolutions (e.g. 0.5 mm and 8 cm⁻¹ versus 0.5 mm and 64 cm⁻¹). The combination of path length and resolution, which resulted statistically in the best performing model, for each parameter, according to the paired comparison tests, was optimised by the application of appropriate pre-treatment(s) (multiplicative scatter correction (MSC) full, normalisation by closure (NCL) and between 0 and 1 (N0-1), 1st derivative 4 points (DB1) and Savitzky-Golay 9 points (DG1), smoothing average 9 points (A9), Savitzky-Golay 9 points (S9) or standard normal variate (SNV)). When no significant differences were observed between different resolutions, decisions were based on time-efficiency, i.e., lowest resolution. The Büchi regression models of each parameter were also optimised by the application of appropriate pre-treatments for comparison with the best performing, optimised PE models.

Paired comparison (Fearn, 1996) tests were subsequently performed between the optimised Büchi and PE PLS regression models. Validation and calibration sets, for each respective parameter, were kept the same as before and no outliers or wavelength regions were removed. Regression model accuracy and precision was evaluated according to lowest standard errors of prediction (*SEP*) and highest coefficient of determination (*R*²) values and a *SEP* as close as possible to the standard error of laboratory (*SEL*).

Results and discussion

NIR spectra

Spectroscopic characterisation

The FT-NIR spectra of an extra virgin olive oil at low (week 0), medium (week 6) and high (week 10) degree of oxidation, as recorded on both the PE and Büchi spectrophotometers, are shown in Figure 1. Olive oil spectra are characterised by strong absorption at 8333 (1200 nm) (CH_2 second overtone), 5767 and 5666 cm^{-1} (1734 and 1765 nm) (CH_2 first overtones), as well as at 4329 and 4264 cm^{-1} (2310 and 2345 nm) (CH_2 stretch bend combinations) (Osborne *et al.*, 1993). The absorption across the whole spectrum increases slightly as the oil becomes oxidised, however, the increase in oxidation is more evidently seen at the absorption bands between 5000 and 4545 cm^{-1} (2000 and 2200 nm) which are ascribed to unsaturated fatty acid moieties (Osborne *et al.*, 1993). An increase in absorption in this region is correlated with an increase in unsaturation (Garrido-Varo *et al.*, 2004), probably caused by the formation of unsaturated hydroperoxides during oxidation (Gunstone & Norris, 1983; Garrido-Varo *et al.*, 2004).

The Büchi spectra (Fig 1a) show considerably higher absorbance values and more defined (CH_2 first overtone and CH_2 stretch bend combination) oil peaks, when compared to the PE spectra (Fig 1b, 64 cm^{-1} resolution, 0.2 mm path length), which can be attributed to the longer path length (0.6 mm) and higher resolution (8 cm^{-1}) of the Büchi spectra. Figure 2 shows the differences in spectral appearance of PE spectra at the 4 different resolutions and indicates that particularly the absorbencies at the CH_2 stretch bend combinations in the region between 4329 and 4264 cm^{-1} (2310 and 2345 nm) are affected by the variation in resolutions.

Principal component analysis

Differences in the spectra as obtained by the two FT-NIR instruments can also be seen in the PCA score plots (Fig 3). The PCA score plot (PC1 versus PC2) of the MSC pre-treated spectra collected on the Büchi spectrophotometer (Fig 3a) illustrates the separation of spectra into clusters of olive oil samples oxidised for 0-3 weeks and 4-10 weeks, respectively. PC1 and PC2 account for 67% and 21% of the spectral variation, respectively, after variation due to physical influences had been removed by MSC pre-treatment. The two clusters formed due to differing degrees of oxidation are separated along the PC2 axis. A small group of spectra has also split from each respective main cluster along the PC1 axis. This deviation from the main

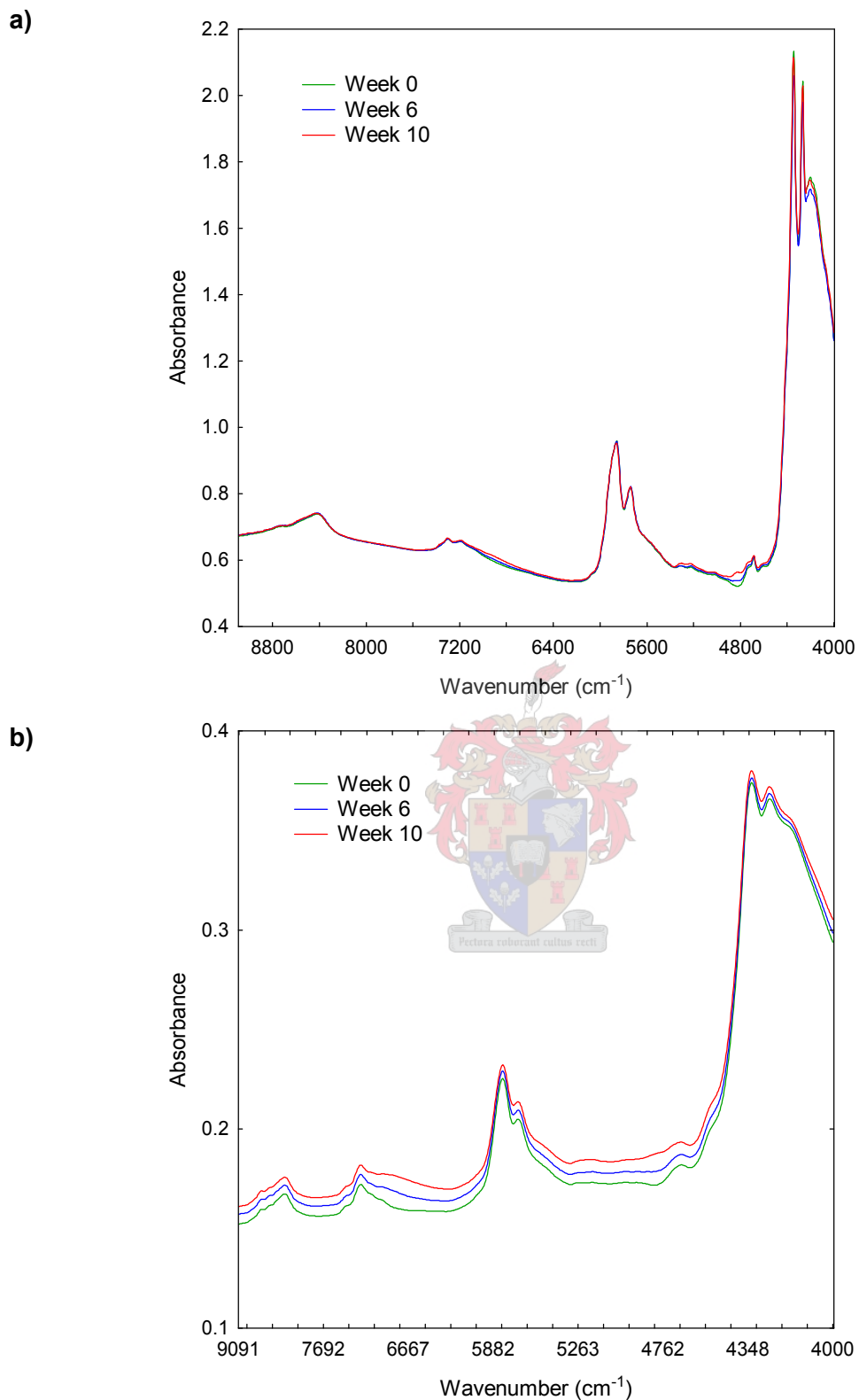


Figure 1 Spectra of an extra virgin olive oil at low (week 0), medium (week 6) and high (week 10) degree of oxidation, as recorded on the a) Büchi NIRLab 200 spectrophotometer with a path length of 0.6 mm and resolution of 8 cm^{-1} and the b) Perkin Elmer IdentiCheck™ spectrophotometer with a path length of 0.2 mm and resolution of 64 cm^{-1} .

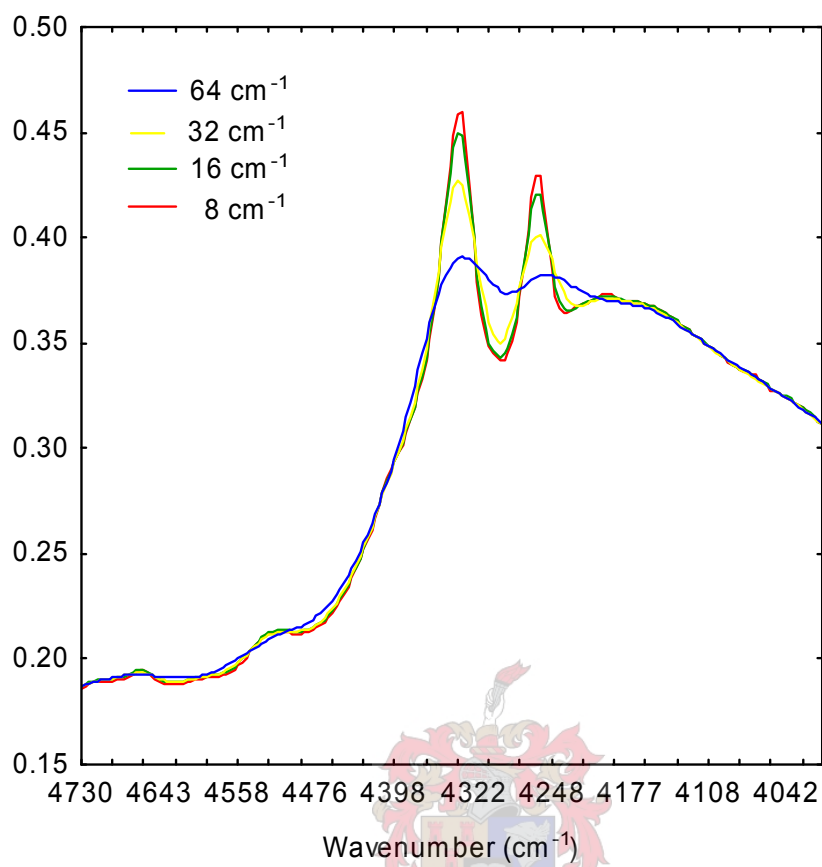
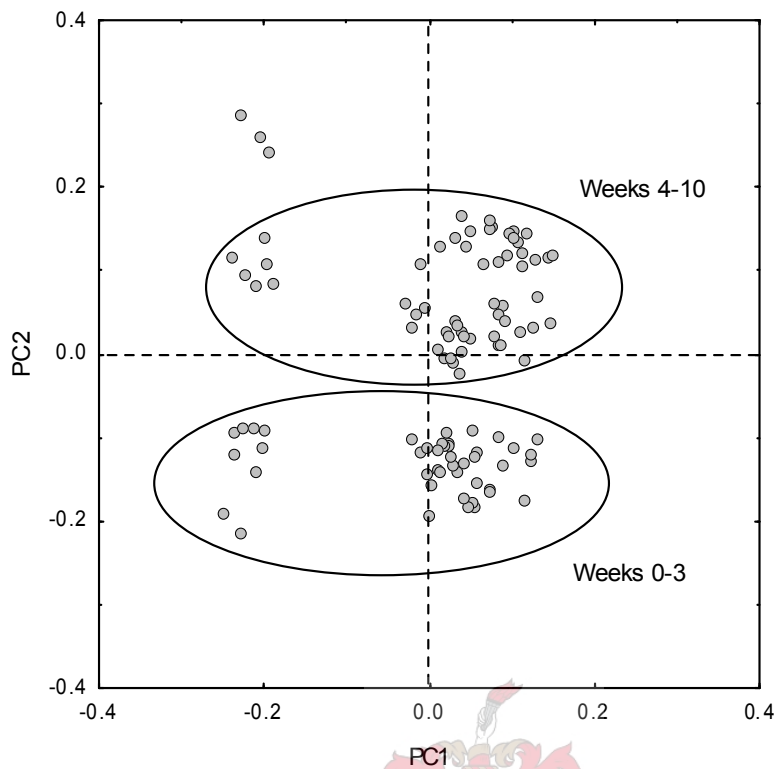


Figure 2 Spectra (4730-4000 cm^{-1}) of an extra virgin olive oil measured on the Perkin Elmer IdentiCheck™ at different resolutions (64, 32, 16 and 8 cm^{-1}) showing the difference in spectral appearance.

a)



b)

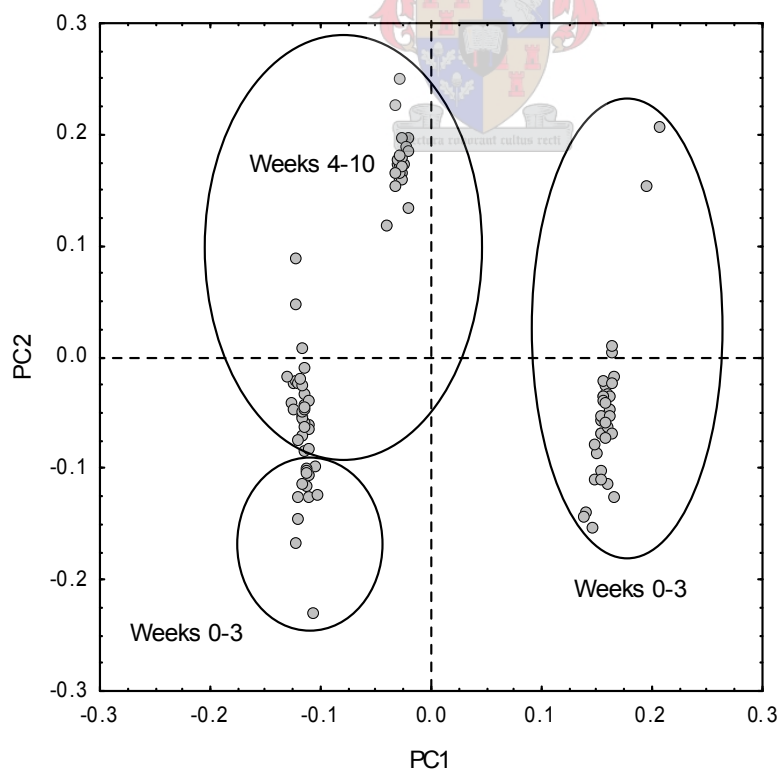


Figure 3 PCA score plots (PC1 vs PC2) of a) Büchi (8 cm^{-1} resolution, 0.6 mm path length) and b) Perkin Elmer spectra (64 cm^{-1} resolution, 0.2 mm path length).

clusters, as well as the three outliers found outside the clusters, may have occurred due to experimental variation during scanning, since no common feature could be observed within this group of samples.

The PCA score plot (PC1 versus PC2) of the spectra recorded on the PE spectrophotometer (Fig 3b) reveals that these spectra separated along the PC1 axis rather than the PC2 axis, even though a group of samples oxidised for 0-3 weeks was only distinguished from the 4-10 weeks samples along the PC2 axis. PC1 accounts for the largest percentage of spectral variation (69%), followed by PC2 (18%).

PCA of the Büchi spectra without any pre-treatment (results not shown) to remove physical influences, showed that PC1 accounted for 96% (67% after MSC pre-treatment) of the spectral variation, compared to 73% (69% after MSC pre-treatment) of PC1 for the PE spectra. This indicates that more scattering, due to physical influences (e.g. bottom of glass Petri-dish), was present in the Büchi spectra when compared to the PE spectra. It has been reported (Garrido-Varo *et al.*, 2004) that besides inevitable environmental and sample preparation-related variations, the use of a transmittance sample holder adds additional discrepancies. It was observed that differences in absorbance levels occurred due to possible variation in path length and loss of reflectance surface, when using identical or distinct sample holders over a period of time, and this could have also been the cause of spectral variation in the Büchi spectra.

Reference data

The descriptive statistics of the sample sets, for each respective parameter, used for PLS regression model development are summarised in Table 1. Minimum and maximum values were always placed in the calibration set and thus it was ensured that the standard deviation of the calibration set was larger than that of the validation set. The *SEL* was determined for duplicate determinations and gives an indication of the precision that was obtained under the specific experimental conditions. No duplicate analyses were performed with the NMR spectrometer, therefore no *SEL*'s were obtained for the fatty acid composition parameters.

Comparison of calibration models using Fearn's criteria

The *p-values* for the paired comparisons between PLS regression models developed from PE spectra recorded at different resolutions with quartz cuvettes of 0.2 and 0.5 mm path length, are listed in Tables 2 and 3, respectively. No significant differences ($p > 0.05$) in standard deviations were observed between models developed from 64, 32, and 16 cm^{-1} resolution spectra, respectively, for each of the 0.2 mm (Table 2) and 0.5 mm (Table 3) path lengths. Significant differences ($p < 0.05$) between models developed from spectra collected at the same path length, but at different resolutions were determined between 64 cm^{-1} and 8 cm^{-1} . Spectra with a

Table 1 Summary of descriptive statistics for the reference values.

Parameter	Calibration set				Validation set				
	<i>n</i>	Range	Mean	<i>SD</i> ^e	<i>n</i>	Range	Mean	<i>SD</i>	<i>SEL</i> ^f
PV^a (meqO ₂ kg ⁻¹)	90	2.18 - 74.02	20.51	15.40	44	2.47 - 60.62	20.16	14.62	1.41
FFA^b content (% oleic acid)	72	0.25 - 4.52	0.49	0.51	35	0.26 - 1.5	0.46	0.22	0.032
K₂₃₂ extinction coefficient	70	1.67 - 20.36	4.76	3.68	34	1.83 - 18.83	4.61	3.40	0.42
K₂₇₀ extinction coefficient	70	0.10 - 2.03	0.28	0.32	34	0.10 - 1.38	0.26	0.23	0.048
Linoleic acid (%)	70	0.00 - 15.68	10.90	2.95	34	6.62 -15.48	11.09	2.33	na
Oleic acid (%)	70	58.90 - 77.90	71.75	2.75	34	67.19 - 76.21	71.85	2.21	na ^g
Saturated acids (%)	70	10.73 - 41.09	17.36	3.98	34	11.35 - 20.77	17.04	2.49	na
α:β ratio linoleic acid	68	0.90 - 1.40	1.13	0.11	33	0.92 - 1.30	1.13	0.10	na
α:β ratio oleic acid	70	1.20 - 1.84	1.58	0.11	34	1.40 - 1.82	1.58	0.10	na
Chlorophyll (mg kg ⁻¹)	65	0.082 - 25.23	6.19	5.48	32	0.33 - 25.04	6.17	5.32	0.47
Carotenoids (mg kg ⁻¹)	64	0.12 - 13.13	3.13	2.15	32	0.13 - 8.35	3.18	1.95	0.23
TP^c content (mg kg ⁻¹)	67	44.49 - 738.76	201.87	130.74	31	51.62 - 442.81	187.44	101.55	9.24
TBARS^d value	69	0.0089 - 0.0386	0.0175	0.0046	34	0.0100 - 0.0370	0.0177	0.0049	0.00132

^aPV = peroxide value

^bFFA = free fatty acids

^cTP = total polyphenol

^dTBARS = thiobarbituric acid reactive substances

^e*SD* = standard deviation

^f*SEL* = standard error of laboratory = $\sqrt{[\sum(y_1 - y_2)^2] [2n]^{-1}}$

n = number of samples

^gna = not applicable as only single NMR analyses were performed

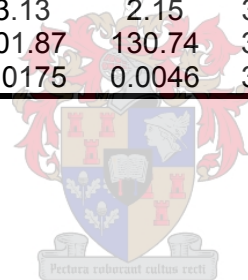


Table 2 *P-values* for paired comparison between PLS regression models developed from Perkin Elmer spectra measured at different resolutions with a path length of 0.2 mm.

Parameter	Resolution (cm ⁻¹)	32	16	8
Peroxide value	64	0.29	0.37	0.36
Free fatty acid content	64	0.82	0.98	0.00 (SD ₆₄ ^a < SD ₈ ^b)
K ₂₃₂	64	0.70	0.28	0.78
K ₂₇₀	64	0.76	0.48	0.84
Linoleic acid	64	0.24	0.92	0.39
Oleic acid	64	0.43	0.78	0.76
Saturated acids	64	0.23	0.31	0.94
α:β ratio linoleic acid	64	0.94	0.62	0.15
α:β ratio oleic acid	64	0.33	0.28	0.17
Chlorophyll	64	0.11	0.46	0.64
Carotenoids	64	0.11	0.40	0.67
Total polyphenol content	64	0.63	0.26	0.00 (SD ₆₄ < SD ₈)
TBARS value	64	0.06	0.48	0.34

^aSD₆₄ = standard deviation at 64 cm⁻¹ resolution.

^bSD₈ = standard deviation at 8 cm⁻¹ resolution

Table 3 *P-values* for paired comparison between regression models developed from Perkin Elmer spectra measured at different resolutions with a path length of 0.5 mm.

Parameter	Resolution (cm ⁻¹)	32	16	8
Peroxide value	64	0.66	0.65	0.28
Free fatty acid content	64	0.39	0.69	0.07
K ₂₃₂	64	0.79	0.37	0.99
K ₂₇₀	64	1.00	0.39	0.65
Linoleic acid	64	0.90	0.87	0.42
Oleic acid	64	0.40	0.89	0.03 (SD ₆₄ ^a < SD ₈ ^b)
Saturated acids	64	0.62	0.37	0.70
α:β ratio linoleic acid	64	0.76	0.17	0.96
α:β ratio oleic acid	64	0.32	0.09	0.15
Chlorophyll	64	0.32	0.44	0.58
Carotenoids	64	0.73	0.97	0.43
Total polyphenol content	64	0.34	0.91	0.33
TBARS value	64	0.75	0.22	0.99

^aSD₆₄ = standard deviation at 64 cm⁻¹ resolution.

^bSD₈ = standard deviation at 8 cm⁻¹ resolution

resolution of 64 cm^{-1} gave significantly better models for the FFA (0.2 mm path length) (Table 2), TP (0.2 mm path length) (Table 2) and oleic acid (0.5 mm path length) (Table 3) contents, indicating that the lower signal to noise ratio with high resolutions might be responsible for a decrease in regression model performance. Due to shorter scanning times than at higher resolutions, 64 cm^{-1} resolution was used for further model comparisons. The comparisons between regression models developed from spectra measured at equal resolutions, but different path lengths revealed no significant differences ($p > 0.05$) in model performance (Table 4). There was a tendency for the 0.2 mm path length to give better calibration performance with the chemical parameters, whereas the 0.5 mm path length tended to give better performance with the NMR spectroscopic parameters (Table 5). These tendencies were not conclusive and, therefore, the most time-efficient set-up for equally accurate PE calibration models would be using either a 0.2 or 0.5 mm path length quartz cuvette and 64 cm^{-1} resolution.

For the purpose of this study, the combination of a 64 cm^{-1} resolution with the 0.2 mm path length quartz cuvette was chosen as optimal. Subsequently, the 0.2 mm and 64 cm^{-1} PE PLS regression models were compared to those developed from Büchi spectra (Table 6). Significant differences ($p < 0.05$) were observed for three models, i.e., the FFA content, TBARS value and oleic acid content calibrations, which performed better when developed from PE spectra. The better performance of certain regression models developed from PE spectra could be due to these being recorded from oil samples contained in quartz cuvettes with a strictly controlled path length. The Büchi sample holder, in comparison, allowed for much more variation to influence each single measurement, due to unevenness of the bottom of the glass Petri-dish, resulting in the path length not always to be exactly 0.6 mm. Moreover, 10 similar but not identical Petri-dishes were used, which could have added to the variation. Transmittance could thus be the preferred mode of sample presentation for homogenous products, such as olive oil, instead of transfectance, which could be more appropriate for less homogenous samples. Conversely, the Büchi sample holder is easier to handle and clean and can be replaced quicker and at a lower price if broken, compared to quartz cuvettes.

Lastly, a paired comparison was conducted between the best performing PLS regression models for each parameter and instrument, after optimising the respective models by applying pre-treatments. As with the PLS regression models developed from raw spectra (no pre-treatment), the PE models were significantly better ($p < 0.05$) for certain parameters, i.e., PV and K_{232} (Table 7). The Büchi spectra produced a significantly better ($p < 0.05$) regression model for the $\alpha:\beta$ ratio of oleic acid. These differences in performance compared to the PLS models developed from raw spectra (no pre-treatment) confirm that the application of pre-treatments has a significant effect on the final performance of the models and that regression models for each respective parameter may require different types of pre-treatments for the best

Table 4 *P-values* for paired comparison tests comparing regression model performance between PE spectra measured at equal resolutions and two different path lengths.

Parameter	Path length (mm)	0.2			
		Resolution (cm ⁻¹)	64	32	16
Peroxide value	0.5	64	0.54		
		32		0.47	
		16			0.61
Free fatty acid content	0.5	64	0.24		
		32		0.80	
		16			0.33
K ₂₃₂	0.5	64	0.43		
		32		0.52	
		16			0.45
K ₂₇₀	0.5	64	0.84		
		32		1.00	
		16			0.66
Linoleic acid	0.5	64	0.53		
		32		0.10	
		16			0.70
Oleic acid	0.5	64	0.42		
		32		0.38	
		16			0.68
Saturated acids	0.5	64	0.76		
		32		0.20	
		16			0.48
α : β ratio linoleic acid	0.5	64	0.92		
		32		0.74	
		16			0.39
α : β ratio oleic acid	0.5	64	0.06		
		32		0.06	
		16			0.64
Chlorophyll	0.5	64	0.55		
		32		0.59	
		16			0.99
Carotenoids	0.5	64	0.41		
		32		0.12	
		16			0.42
Total polyphenol content	0.5	64	0.07		
		32		0.14	
		16			0.13
TBARS value	0.5	64	0.33		
		32		0.82	
		16			0.23

Table 5 Best Perkin Elmer calibrations according to paired comparison tests (selection of best calibration was based on the smallest standard deviation, since there was no significant difference between 0.2 mm and 0.5 mm calibrations).

Parameter	Path length (mm)	Resolution (cm ⁻¹)
Peroxide value	0.2	64
Free fatty acid content	0.2	64
K ₂₃₂	0.2	64
K ₂₇₀	0.2	64
Linoleic acid	0.2	64
Oleic acid	0.5	64
Saturated acids	0.5	64
α:β ratio linoleic acid	0.5	64
α:β ratio oleic acid	0.5	64
Chlorophyll	0.5	64
Carotenoids	0.2	64
Total polyphenol content	0.5	64
TBARS value	0.5	64

Table 6 Paired comparison between best Perkin Elmer (0.2 mm path length, 64cm⁻¹ resolution) and Büchi PLS regression models without pre-treatment.

Parameter	p-value
Peroxide value	0.33
Free fatty acid content	0.02 (SD _{PE} ^a < SD _{Büchi} ^b)
K ₂₃₂	0.83
K ₂₇₀	0.06
Linoleic acid	0.29
Oleic acid	0.04 (SD _{PE} < SD _{Büchi})
Saturated acids	0.66
α:β ratio linoleic acid	0.07
α:β ratio oleic acid	0.11
Chlorophyll	0.95
Carotenoids	0.78
Total polyphenol content	0.97
TBARS value	0.03 (SD _{PE} < SD _{Büchi})

^aSD_{PE} = standard deviation for Perkin Elmer.

^bSD_{Büchi} = standard deviation for Büchi.

Table 7 Prediction results for the optimised PLS regression models obtained with the Büchi and PE spectra.

Parameter	NIR	Pre-treatment	PLS factors	SEP	R ²	Bias [#]	RPD ^l	p-value
Peroxide value	Büchi	MSC ^a	8	5.28	0.87	0.38	2.77	0.01
	PE	N0-1 ^b	6	4.15	0.92	0.31	3.52	(SD _{PE} ^j < SD _{Büchi} ^k)
Free fatty acid content	Büchi	SNV ^c	8	0.15	0.58	0.014	1.47	0.25
	PE	MSC	7	0.12	0.69	-0.0041	1.83	
K₂₃₂	Büchi	MSC	4	1.33	0.87	-0.10	2.56	0.00
	PE	None	6	0.94	0.94	-0.0064	3.62	(SD _{PE} < SD _{Büchi})
K₂₇₀	Büchi	MSC	3	0.13	0.71	-0.0084	1.77	0.07
	PE	MSC	6	0.094	0.87	-0.020	2.45	
Linoleic acid	Büchi	S9 ^d -DB1 ^e	8	0.83	0.88	0.14	2.81	0.93
	PE	DB1-MSC	8	0.83	0.90	0.071	2.81	
Oleic acid	Büchi	A9 ^f -DB1	8	1.47	0.56	0.12	1.50	0.76
	PE	None	8	1.53	0.53	-0.24	1.44	
Saturated acids	Büchi	DB1-MSC	6	1.09	0.81	-0.18	2.28	0.30
	PE	DB1-NCL ^g	8	0.91	0.88	0.12	2.74	
α:β ratio linoleic acid	Büchi	A9-DB1	6	0.092	0.14	0.0022	1.09	0.22
α:β ratio oleic acid	PE	none	6	0.083	0.29	0.0097	1.20	
Chlorophyll	Büchi	DB1-MSC	8	0.040	0.85	0.0035	2.50	0.03
	PE	NCL	8	0.056	0.71	0.0074	1.79	(SD _{Büchi} < SD _{PE})
Carotenoids	Büchi	DB1-NCL	3	4.42	0.31	-0.019	1.20	0.09
	PE	MSC-DG1 ^h	8	3.58	0.56	-0.12	1.49	
Total polyphenol content	Büchi	MSC	3	1.35	0.52	-0.11	1.44	0.12
	PE	N0-1	5	1.14	0.66	0.030	1.71	
TBARS value	Büchi	MSC	2	82.10	0.34	-4.81	1.24	0.21
	PE	MSC	2	89.66	0.21	-10.27	1.13	
TBARS value	Büchi	N0-1	3	0.0042	0.24	0.00043	1.17	0.13
	PE	NCL-DB1	5	0.0038	0.40	0.00037	1.29	

[#]by a paired t-test at 95% confidence level, all biases were not significantly different

^aMSC = multiplicative scatter correction

^eDB1 = 1st derivative 4 points

^bN0-1 = normalisation between 0 and 1

^fA9 = smoothing average 9 points

^cSNV = standard normal variate

^gNCL = normalisation by closure

^dS9 = smoothing Savitzky-Golay 9 points

^hDG1 = 1st derivative Savitzky-Golay 9 points

possible outcome. Scatter correction (MSC and SNV) was the most effective pre-treatment for the majority of the Büchi regression models, whereas for the PE regression normalisation (by closure or between 0 and 1) and MSC worked equally well. Regarding the better performance of the PV and K_{232} optimised PE models, one might suggest similar reasons as for the untreated PLS models, i.e., the presence of less external variation, leading to reduced scattering when using quartz cuvettes compared to Petri-dishes. The majority of the comparisons revealed, however, that these differences in the spectra do not have a significant effect on the regression model performance and therefore it cannot be said with certainty that one instrument should be preferred to the other.

Evaluation of regression models

The optimised PE and Büchi (pre-treatments applied) PLS regression model prediction results are shown in Table 7. It should be kept in mind that the prediction results include extremely low and high reference values and therefore the calibrations could be improved further by removal of these samples if analyses of future samples are not expected to have such extreme values. However, to be able to compare calibrations it was essential to use identical calibration and validation sets for each respective parameter.

All standard errors of calibration (*SEC*) were larger than the standard errors of the respective reference method, indicating that the calibrations were not over-fitted (Stchur *et al.*, 2002) (results not shown). *SEL* values for the chemical properties were considerably lower than *SEP* values obtained for the corresponding calibration models, indicating the need to further improve models before they could be implemented in olive oil quality control. The $\alpha:\beta$ ratio models could be improved by extending the sample set with adulterated olive oil samples, which will include $\alpha:\beta$ ratio values above 2. According to the *RPD* values ($SD_{\text{validation}} \text{SEP}^{-1}$), the models for the PV, specific extinction coefficient K_{232} , linoleic acid and saturated fatty acids contents would be applicable for quality control screening. The TBARS value, TP content and $\alpha:\beta$ ratio linoleic acid models performed poorly with both instruments and it is questionable, whether these parameters will be applicable for NIR regression models. For the majority of regressions the PE models had higher R^2 values and lower *SEP*'s, however, this slightly better performance only resulted in significantly lower ($p < 0.05$) standard deviations in two cases, i.e., PV and K_{232} . The prediction results of the models developed in this study were not as successful as those reported recently by Mailer (2004). This can be explained by the fact that the regression models were not fully optimized as the samples in the calibration and validation set had to kept identical to allow comparisons between the two sample presentation modes.

The validation plots of Büchi and PE regression models for all the quality parameters are shown in Figures 4-16. The PV, FFA content and K_{232} are important quality parameters for

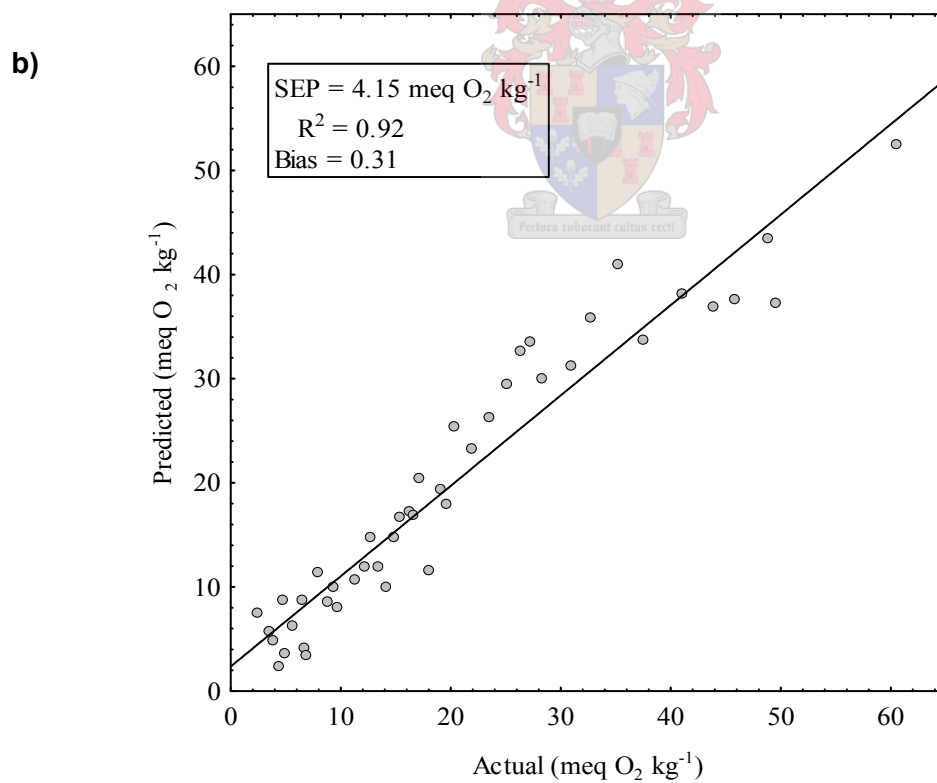
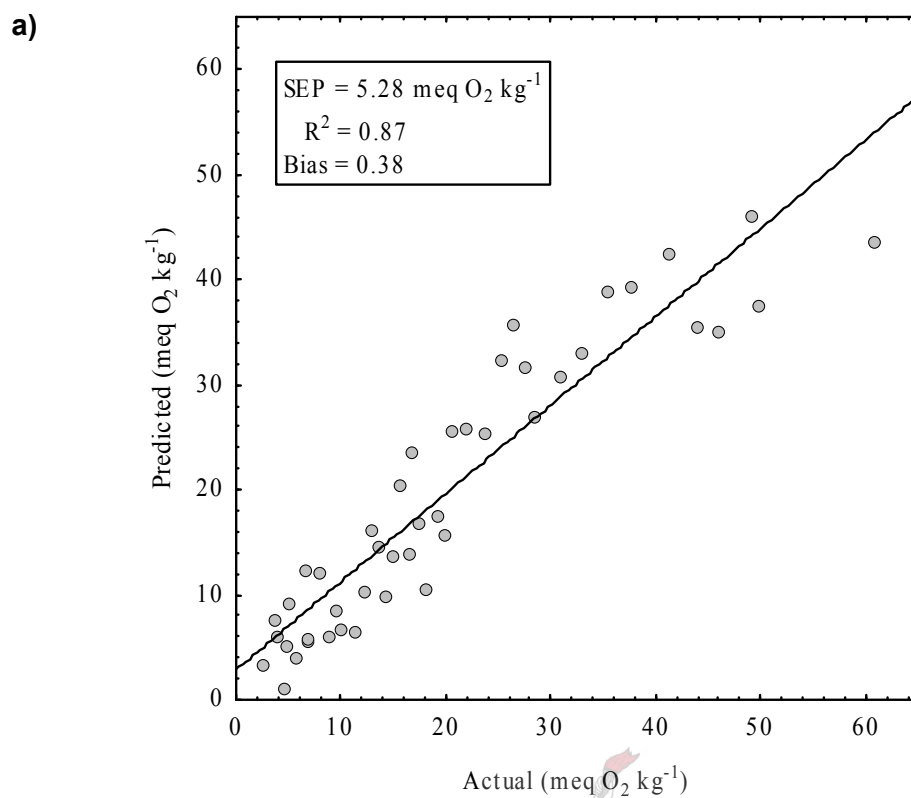


Figure 4 Validation plot of the optimised peroxide value PLS regression model developed from a) Büchi spectra (MSC) and b) Perkin Elmer spectra (normalisation, MSC, 64 cm⁻¹ resolution, 0.2 mm path length).

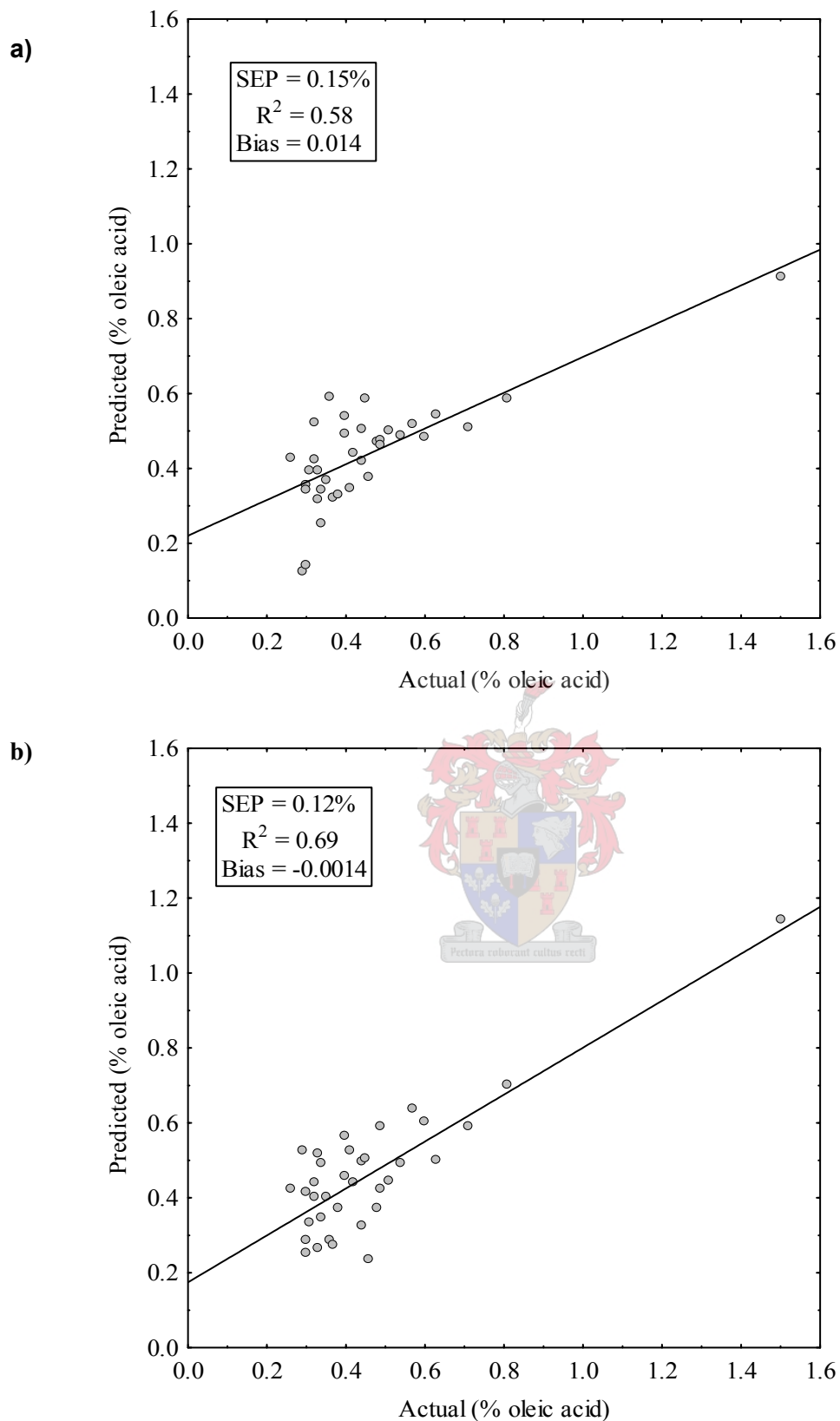


Figure 5 Validation plot of the optimised free fatty acid PLS regression model developed from a) Büchi spectra (MSC) and b) Perkin Elmer spectra (normalisation, MSC, 64 cm^{-1} resolution, 0.2 mm path length).

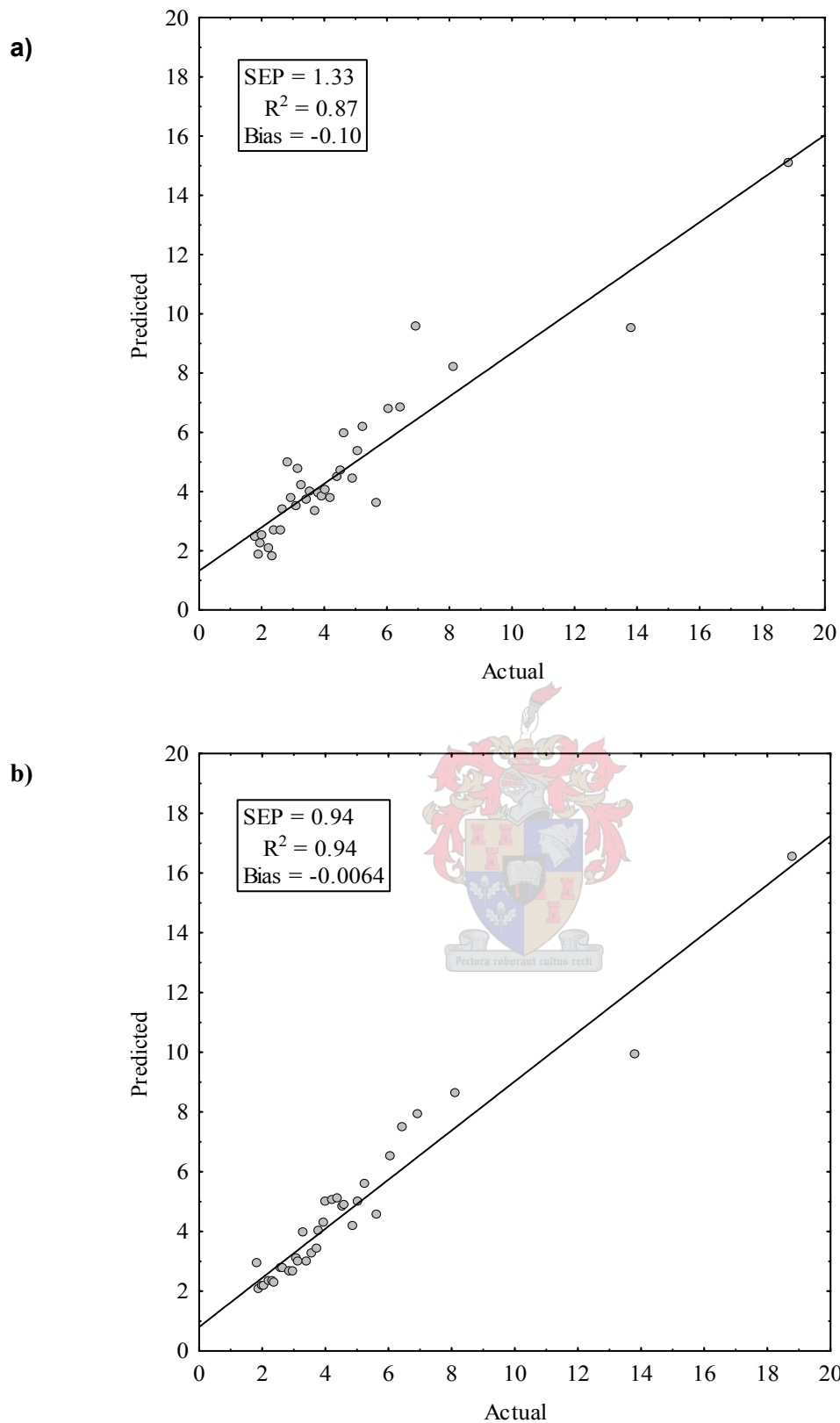


Figure 6 Validation plot of the optimised specific extinction coefficient K_{232} PLS regression model developed from a) Büchi spectra (MSC) and b) Perkin Elmer spectra (normalisation, MSC, 64 cm^{-1} resolution, 0.2 mm path length).

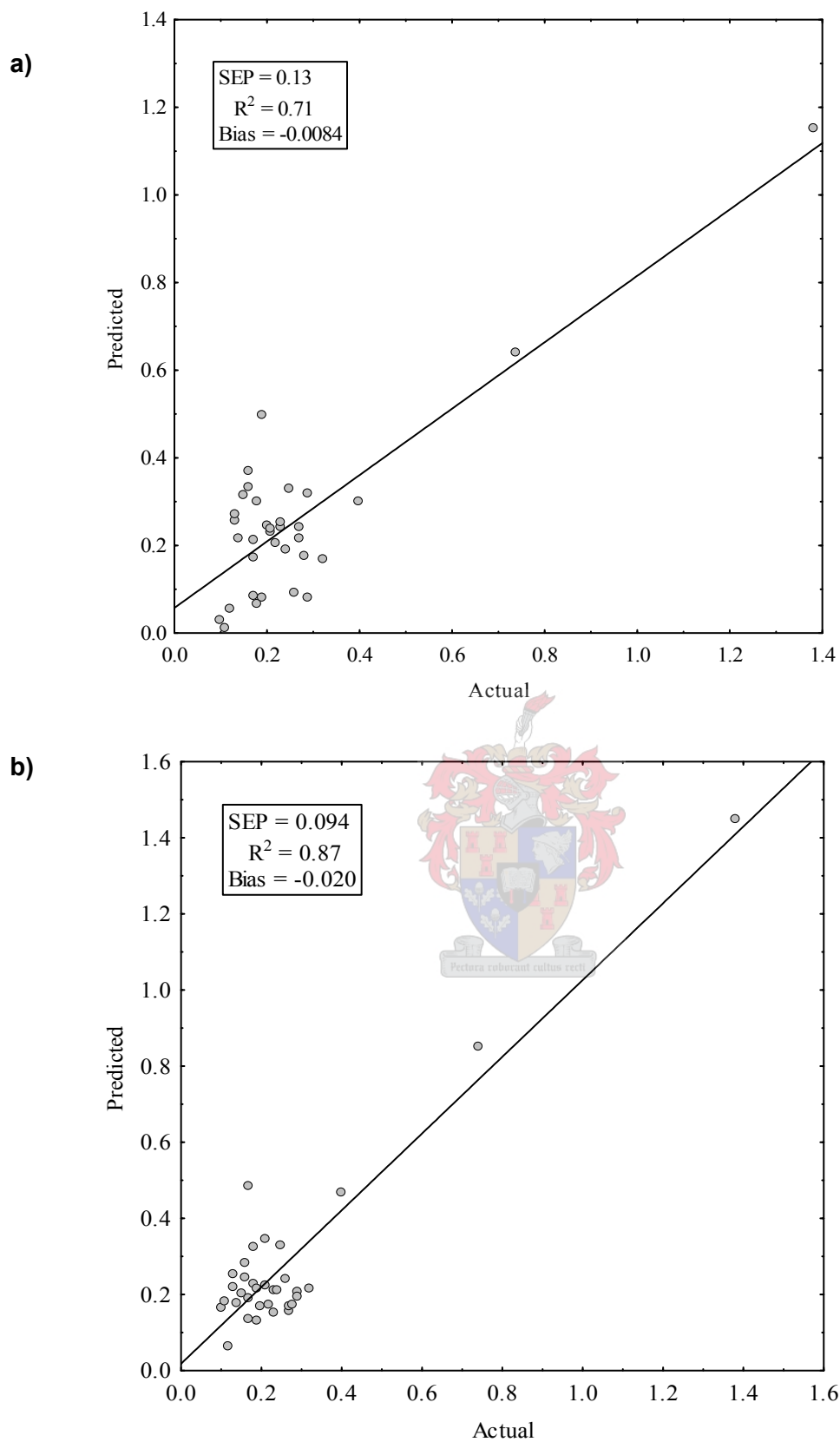


Figure 7 Validation plot of the optimised specific extinction coefficient K_{270} PLS regression model developed from a) Büchi spectra (MSC) and b) Perkin Elmer spectra (MSC, 64 cm^{-1} resolution, 0.2 mm path length).

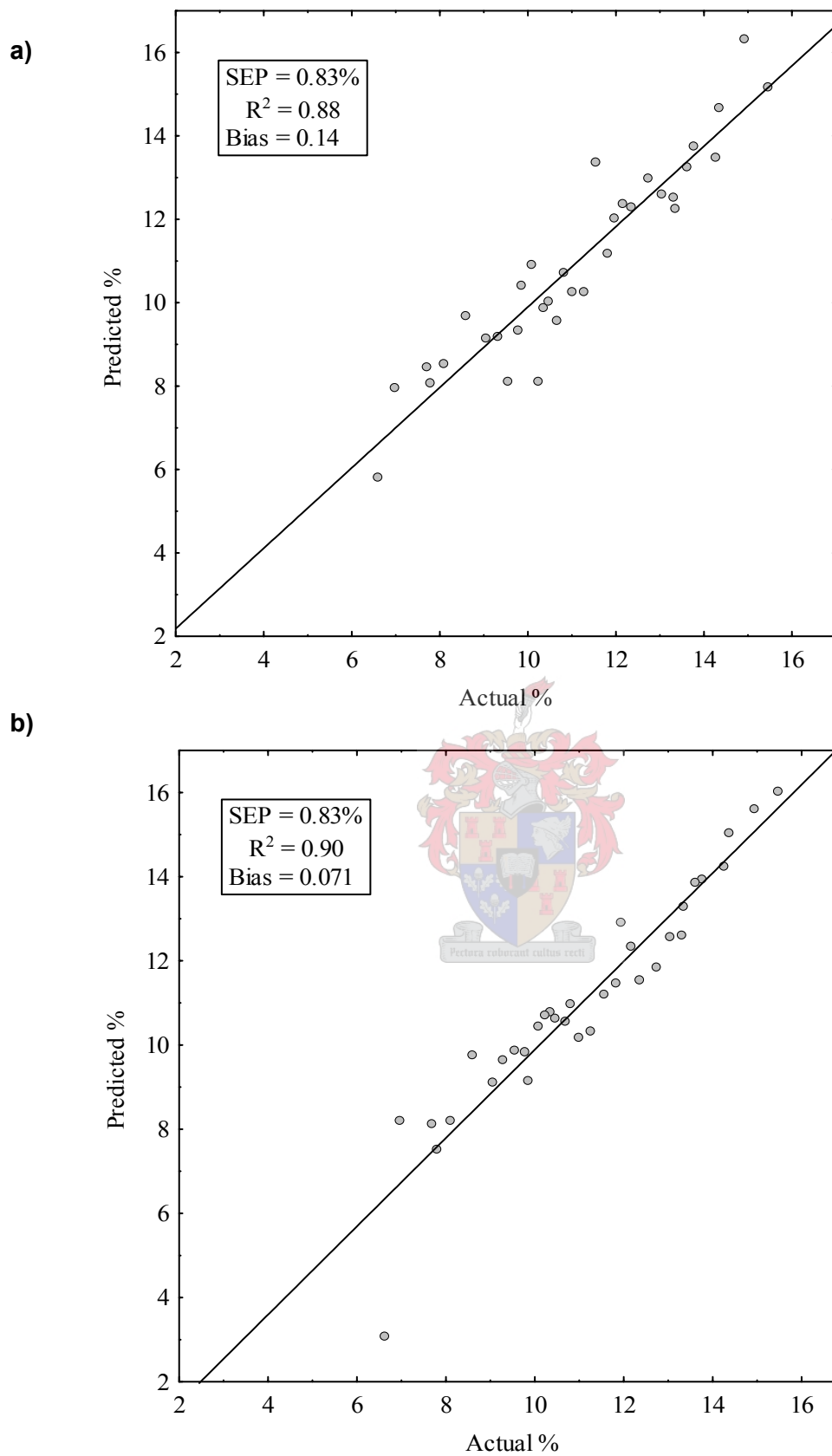


Figure 8 Validation plot of the optimised linoleic acid content PLS regression model developed from a) Büchi spectra (MSC) and b) Perkin Elmer spectra (normalisation, MSC, 64 cm^{-1} resolution, 0.2 mm path length).

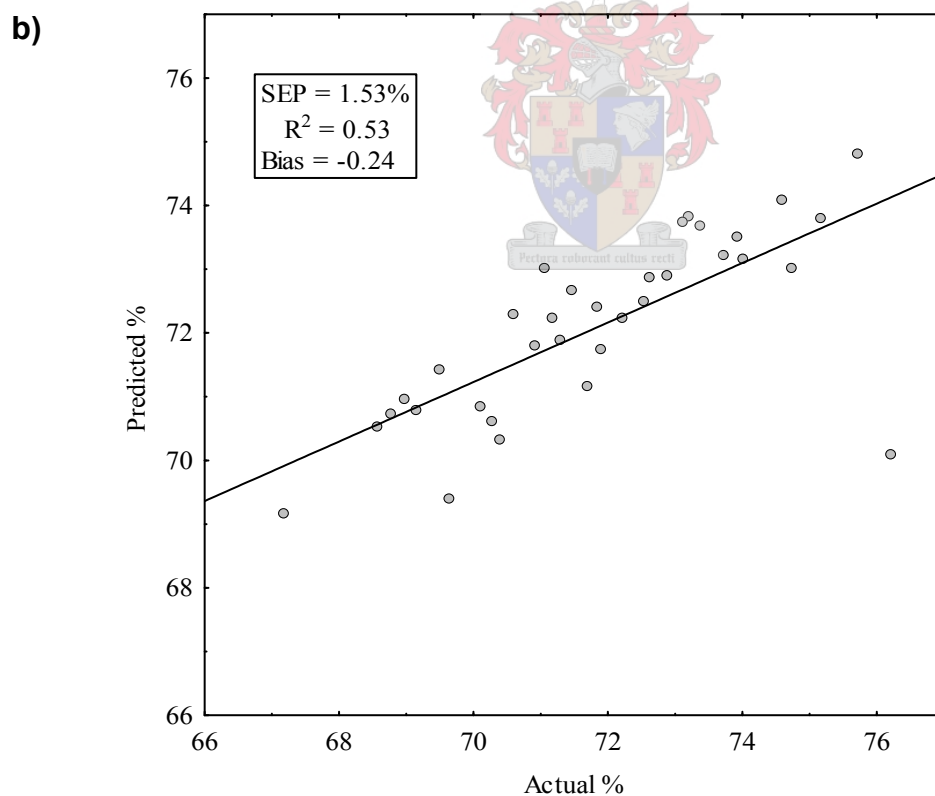
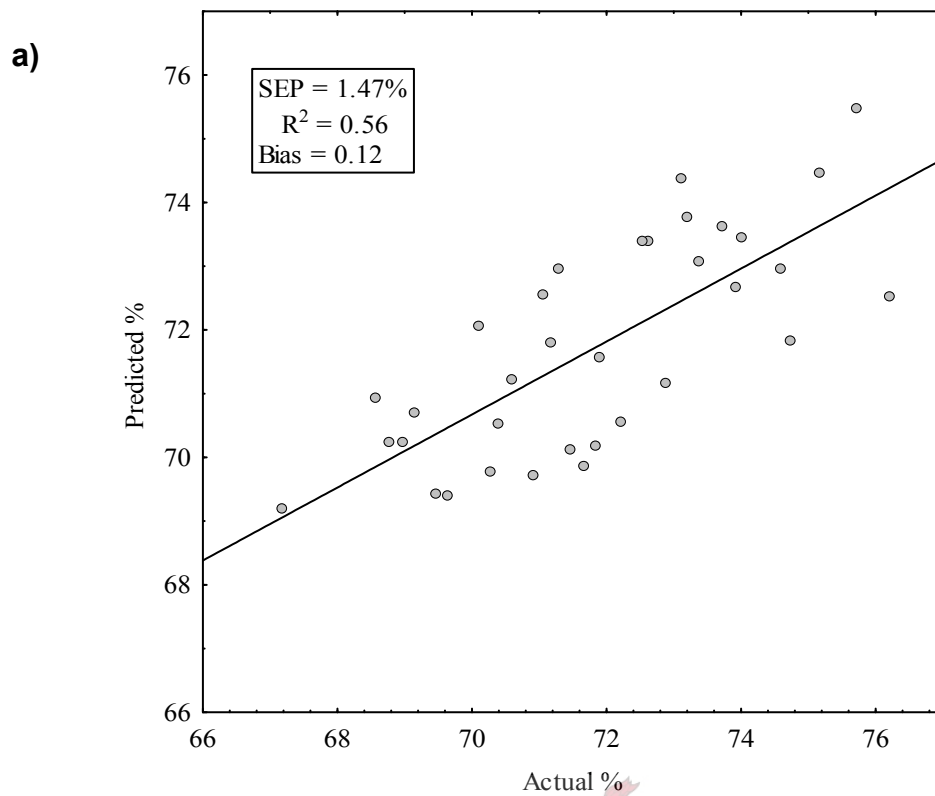


Figure 9 Validation plot of the optimised oleic acid content PLS regression model developed from a) Büchi spectra (Smoothing, 1st derivative) and b) Perkin Elmer spectra (No pre-treatment, 64 cm^{-1} resolution, 0.2 mm path length).

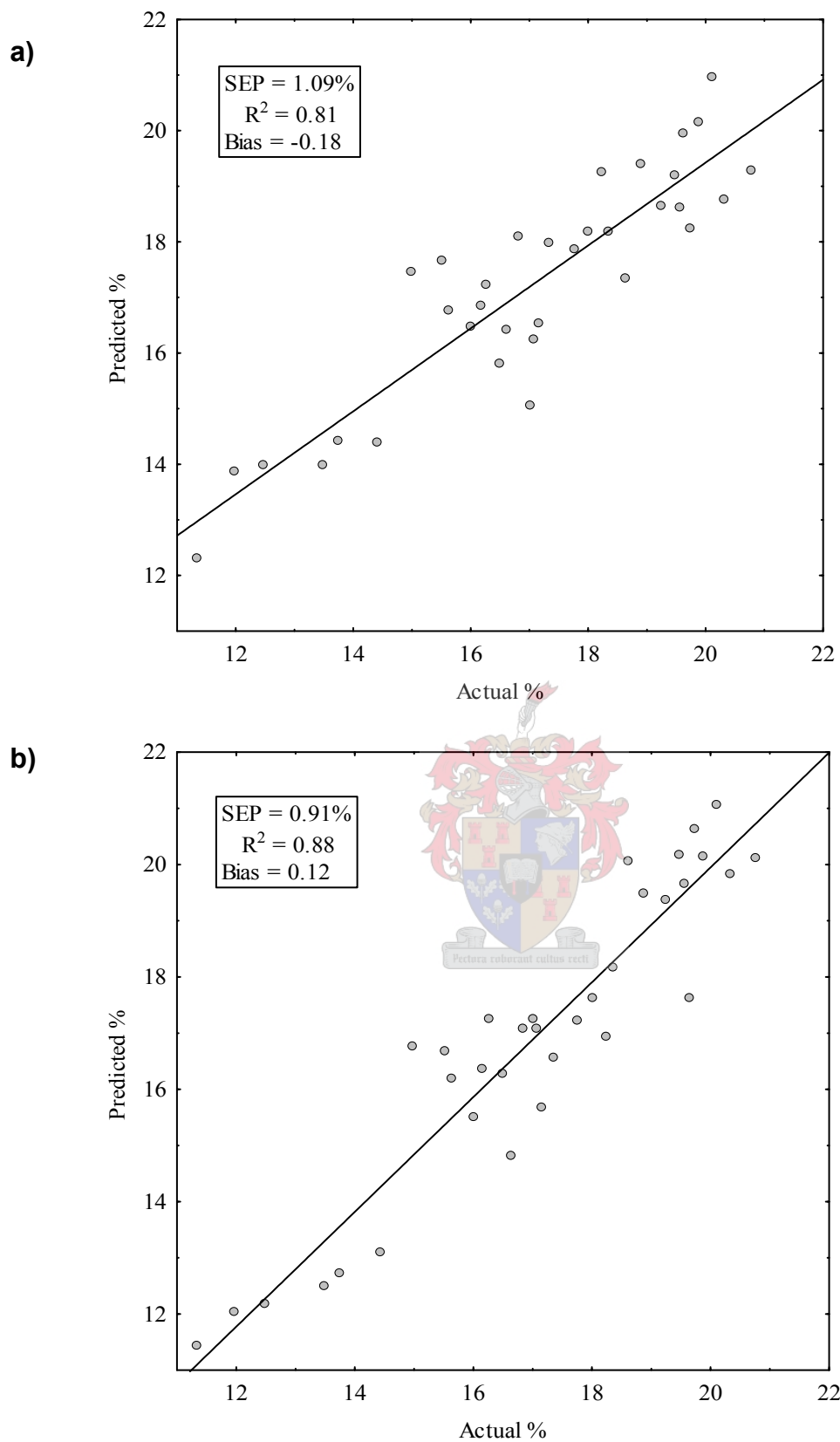


Figure 10 Validation plot of the optimised saturated acid content PLS regression model developed from a) Büchi spectra (1st derivative, MSC) and b) Perkin Elmer spectra (1st derivative, normalisation, 64 cm⁻¹ resolution, 0.2 mm path length).

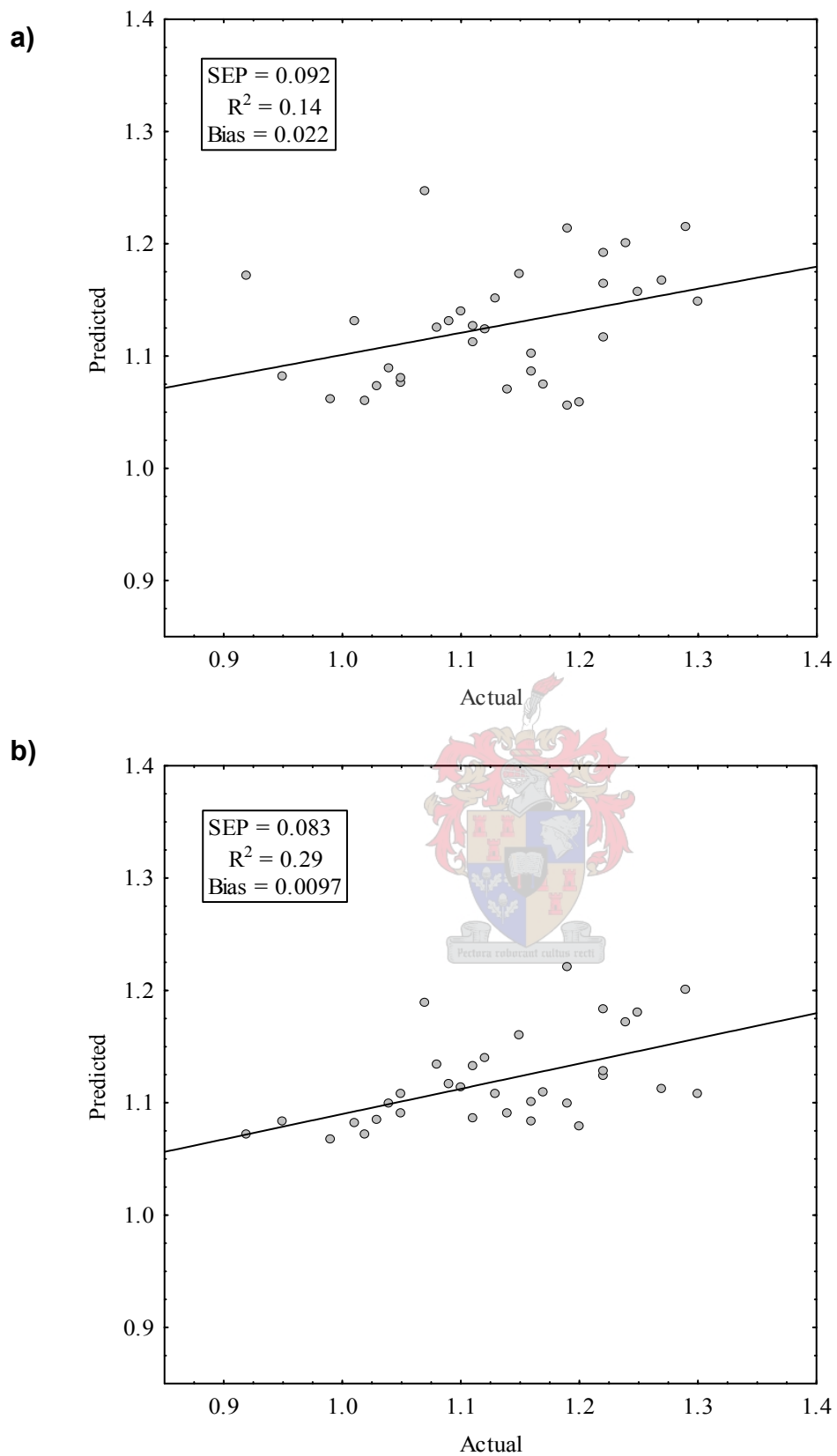


Figure 11 Validation plot of the optimised $\alpha:\beta$ ratio linoleic acid PLS regression model developed from a) Büchi spectra (Smoothing, 1st derivative) and b) Perkin Elmer spectra (No pre-treatment, 64 cm^{-1} resolution, 0.2 mm path length).

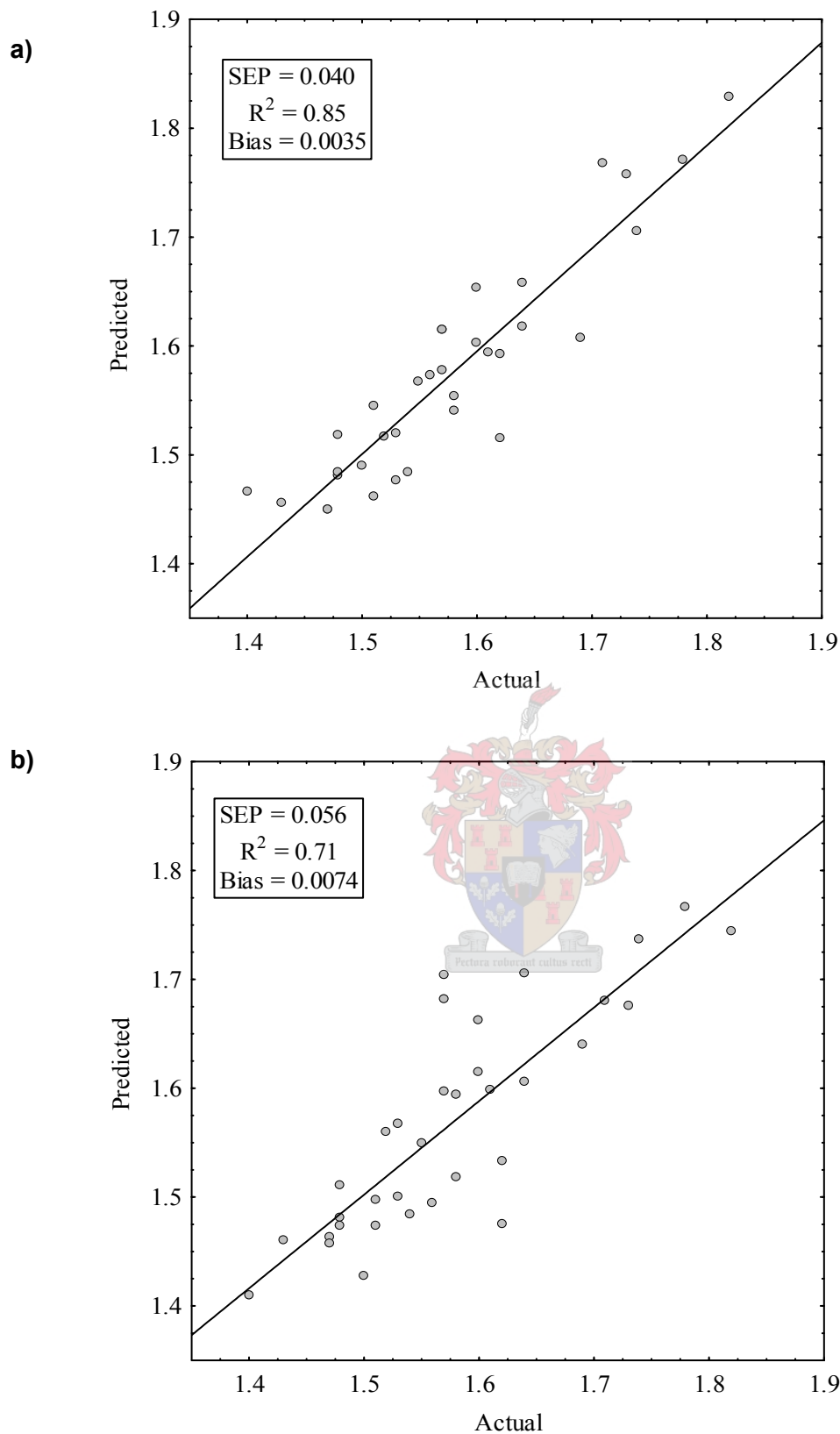


Figure 12 Validation plot of the optimised $\alpha:\beta$ ratio oleic acid PLS regression model developed from a) Büchi spectra (1st derivative, MSC) and b) Perkin Elmer spectra (normalisation, 64 cm^{-1} resolution, 0.2 mm path length).

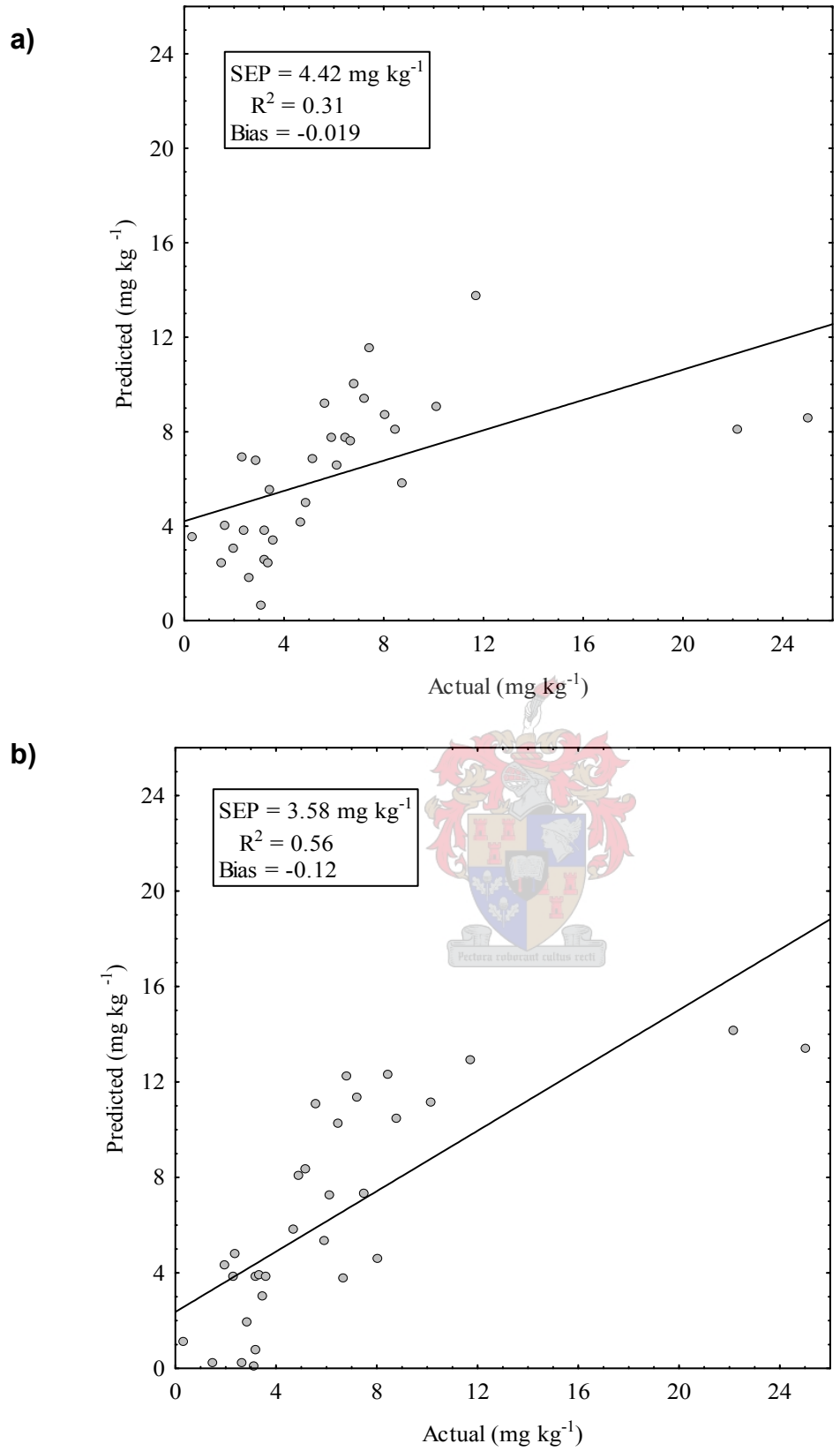


Figure 13 Validation plot of the optimised chlorophyll content PLS regression model developed from a) Büchi spectra (1st derivative, normalisation) and b) Perkin Elmer spectra (MSC, 1st derivative, 64 cm⁻¹ resolution, 0.2 mm path length).

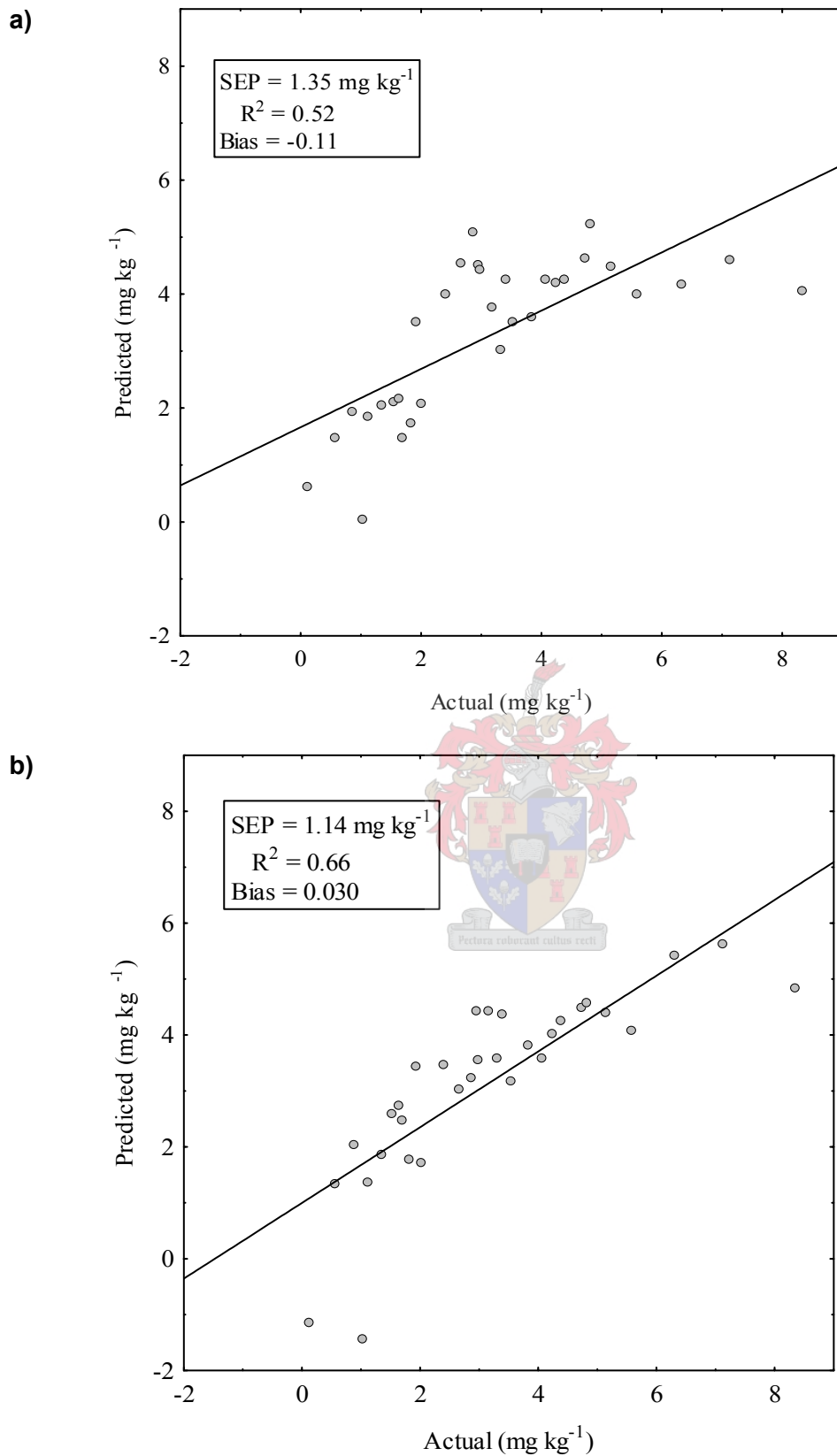


Figure 14 Validation plot of the optimised carotenoid content PLS regression model developed from a) Büchi spectra (MSC) and b) Perkin Elmer spectra (normalisation, MSC, 64 cm⁻¹ resolution, 0.2 mm path length).

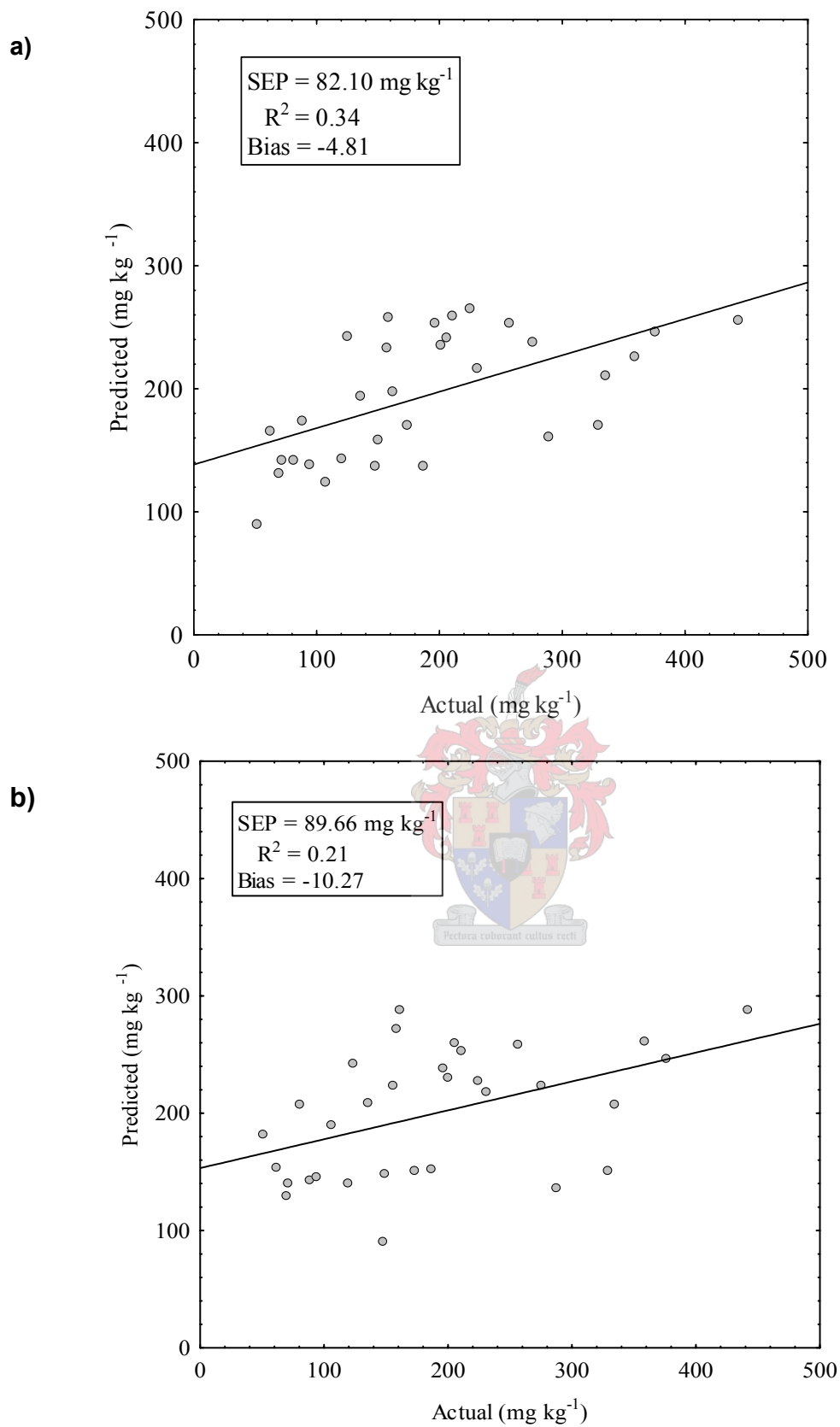


Figure 15 Validation plot of the optimised total polyphenol content PLS regression model developed from a) Büchi spectra (MSC) and b) Perkin Elmer spectra (MSC, 64 cm⁻¹ resolution, 0.2 mm path length).

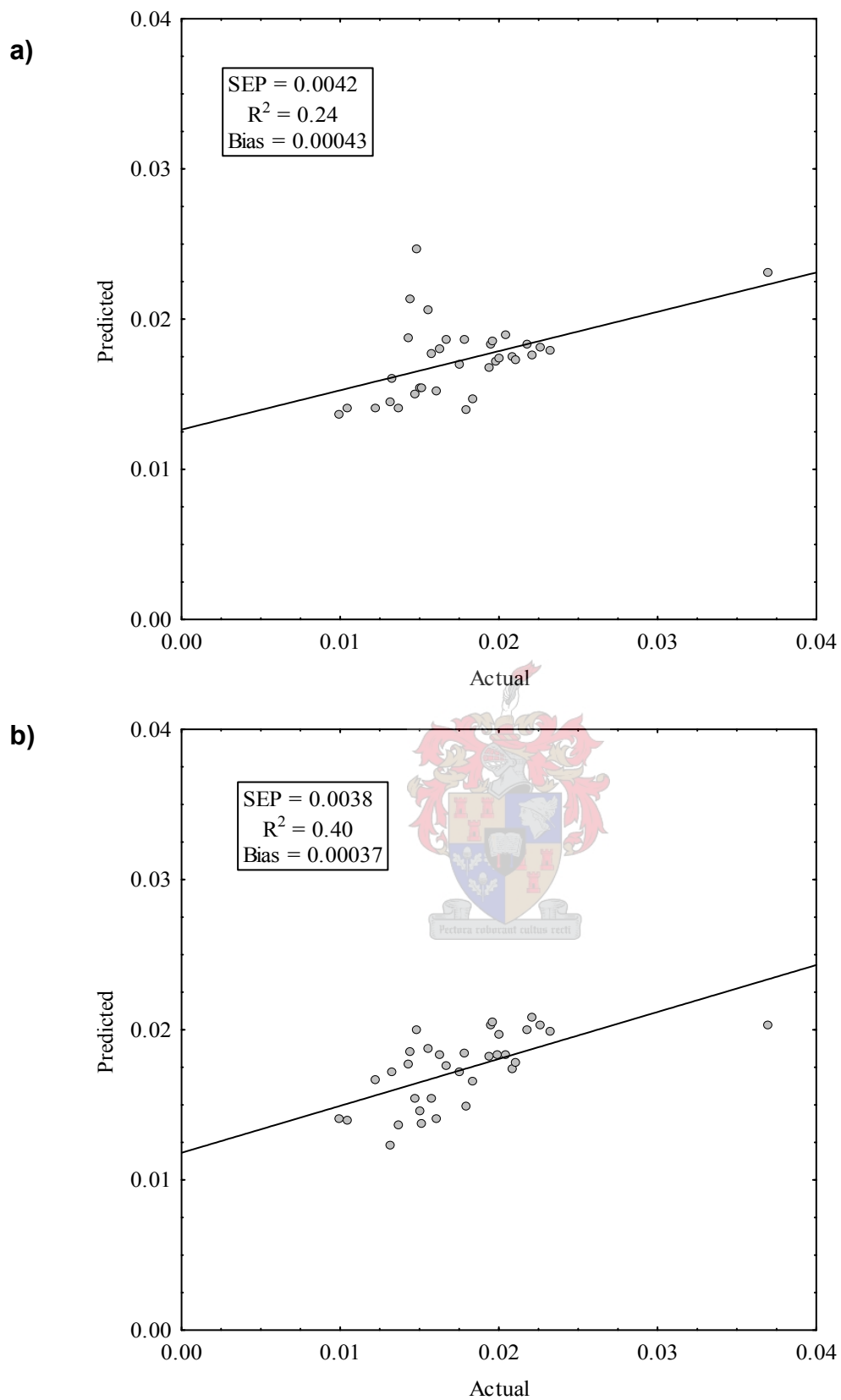


Figure 16 Validation plot of the optimised thiobarbituric acid reactive substances value PLS regression model developed from a) Büchi spectra (normalisation) and b) Perkin Elmer spectra (normalisation, 1st derivative, 64 cm^{-1} resolution, 0.2 mm path length).

extra virgin olive oil and have defined limits. The IOOC requires a PV below 20 meq O₂ kg⁻¹, a FFA content below 0.8% oleic acid and K₂₃₂ below 2.50 (K₂₇₀ < 0.22) (IOOC, 2003). The linoleic (3.5-21%) and oleic fatty acid (55-83%) content and their α:β ratios (< 2) are useful indicators of olive oil adulteration with other vegetable oils, as is the saturated fatty acid content (7.5-20%). Although the pigment content of olive oil is not a characteristic regulated by law, the chlorophyll and carotenoid content of virgin olive oil is an important consideration in the evaluation of oxidative stability, since a high pigment concentration in olive oil stored in the dark could contribute to increased resistance against oxidation (Boskou, 2002). Similarly, polyphenolic compounds occurring in virgin olive oil have, due to their anti-oxidant properties, a positive influence on the oxidative stability of the oil and human-related health benefits.

Conclusions

The comparison of PLS regression models developed from spectra recorded on a PE FT-NIR spectrophotometer, at different resolutions and path lengths, demonstrated that lower resolutions (64 cm⁻¹) and small differences in path length (0.2 and 0.5 mm) did not have a significant effect on regression model performance. A tendency for spectra recorded on a Büchi FT-NIR spectrophotometer to produce slightly weaker PLS regression models for particular parameters might suggest that transmittance spectra are more appropriate for olive oil applications than reflectance spectra. However, there is no conclusive evidence that PE spectra will produce better olive oil regression models and for application purposes the most user-friendly and time-efficient instrument, instrument setting and sample presentation mode could be used. Since this study was mainly concerned with the comparisons of olive oil FT-NIR regression models in terms of resolution and path length, improvement of some regression models is necessary before they can be applied as a tool for South African extra virgin olive oil quality control.

Acknowledgements

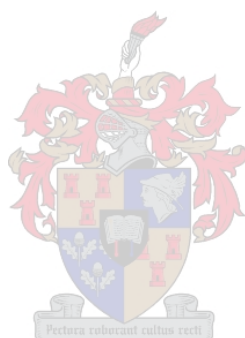
We acknowledge Büchi, Flawil, Switzerland for the loan of the Büchi NIRLab N-200 spectrophotometer and NIRCal (version 4.21) software and Dr Martin Kidd, Centre for Statistical Consultation, Stellenbosch University, South Africa for help with statistical analyses performed. We thank the olive oil producers in the Western Cape region, South Africa for supplying extra virgin olive oil samples.

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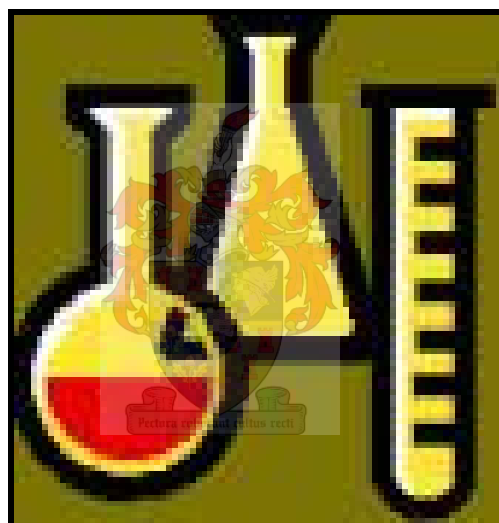
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Chapter 4

METHODOLOGY OF REFERENCE DATA



Methodology of reference data

Introduction

The determination of the accuracy of chemical reference methods is a critical point in assuring the development of valid near infrared (NIR) spectroscopy prediction models and it has been stressed for long that the more accurate the reference determinations, the more accurate the NIR predictions (Coates, 2002). Therefore, it is essential that reference methods are optimised and their accuracy maximised, before they are applied on a sample set that is going to be used to develop a NIR prediction model.

All analytical test methods used for the development of NIR prediction models during this study were optimised before final analysis of the olive oil samples. During this trial period official methods of analysis were adapted, if necessary, and unofficial methods published by previous researchers were evaluated and described in detail. The repeatability and the variance, between duplicate analyses of each reference method, were determined after 10 repetitive measurements of the same sample. These 10 repetitive measurements included sample preparation of each individual sample and thus represent the repeatability of the entire test procedure. Repeatability tests were performed twice on two different days (Repetition 1 and 2) on the same oil sample for each reference method, and a box and whisker plot of the non-outlier range and median was drawn. The standard deviation and 5 and 95 percentile boundaries were calculated, to determine a maximum acceptable difference between duplicate values. Since, repetition 1 and 2 were not carried out immediately after another and the analysts' accuracy improved with time, repetition 2 represents the best repeatability results, which is why only the latter are tabulated. Repeatability in this context means "the value below which the absolute difference between two single independent test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time, may be expected to lie with a probability of 95%" (IOOC, 2001).

Peroxide value

The peroxide value was measured according to the official method of the American Oil Chemists' Society (AOCS), method Cd 8b-90 (AOCS, 1996). This method determines peroxides produced during oil oxidation by quantifying the amount of iodine liberated from potassium iodide due to its oxidation by oxidising substances, such as hydroperoxides (Nawar, 1996). The milliequivalent oxygen per kilogram ($\text{meq O}_2 \text{ kg}^{-1}$) of oil is estimated by converting the liberated iodine into a blue iodine-starch complex, which is titrated against sodium thiosulfate to a colourless endpoint. This iodometric method is highly empirical and any variation in the test

conditions may lead to unreliable results (AOCS, 1996). It also tends to give erratic results when the sample has a peroxide value above 70 meq kg⁻¹.

The problem that was encountered during the initial trial of the peroxide value determination was the failure to obtain a blue starch-iodine complex on addition of the starch indicator solution. Since soluble starch (Kimix, Chemicals & Laboratory Suppliers, Cape Town, South Africa), which is not recommended by the AOCS method, was used to prepare a 0.5% starch solution for the first titrations, it was assumed that the starch was unsuitable for use in this experiment. However, after testing several different starch preparations, including potato starch (BDH, Laboratory Supplies, Poole, England), starch prepared from instant potato mash powder (Bromo Foods (Pty) Ltd, Cadbury), as well as soluble starch obtained from another laboratory, still no blue starch-iodine complex was formed. The formation of a strong blue colour with all starch preparations during the titration carried out to standardise the titrant, sodium thiosulfate, lead to the conclusion that another empirical error must have caused the problem. It was eventually found that the failure to form a blue colour on addition of the starch indicator was only due to the sample solution containing the potassium iodide already being titrated to the endpoint. This lead to the neutralisation of the iodine and, thus, no iodine was left to form a coloured complex once the starch indicator was added. The AOCS method requires the titration of the sample solution until the yellow iodine colour has almost disappeared, before adding 0.5 mL of starch indicator. During this study it was found that only when a light yellow colour was still clearly visible in the solution, the starch indicator formed the blue colour complex, and care must be taken not to over-titrate the sample solution in this first step of the titration. This is a crucial point in the experiment, since it depends on the sensitivity of the starch solution and the degree of oxidation in the sample and is also subjective to each analyst.

An additional difficulty faced in connection with the colour of the starch-iodine complex was the addition of sodium lauryl sulfate (Saarchem, Merck Chemicals (Pty) Ltd, Wadeville, South Africa), which caused the starch-iodine complex to develop a brown colour instead of a black/blue colour. Consequently, it was decided to leave out the addition of 0.5 mL of a 10% sodium lauryl sulfate solution, since it does not play a crucial role during the reaction and alleviated the problem of the brown colour. The AOCS official method was further modified in the following aspects. Firstly, the sample weight was reduced to 0.5 g and 10 mL of glacial acetic acid (BDH, Wadeville, South Africa)-isooctane (Merck, Darmstadt, Germany) solution was added to the sample in a 100 mL Erlenmeyer flask immediately before titration of each sample. When adding the glacial acetic acid-isooctane solution simultaneously to all samples to be analysed, and thereby allowing the last samples to stand considerably longer than the first one, an unacceptable degree of variation was generated. After reaction with 0.5 mL saturated potassium iodide (Saarchem, Merck Chemicals (Pty) Ltd, Wadeville, South Africa) solution, 10

mL of distilled water were added and the sample solution was titrated with 0.02 M sodium thiosulfate (Saarchem, Merck Chemicals (Pty) Ltd, Wadeville, South Africa). A 1% starch indicator solution was added after vaguely lightening the yellow iodine colour by adding a few drops of sodium thiosulfate solution. The 0.02 M sodium thiosulfate solution was standardised with 0.01- 0.02 g of potassium dichromate (Kimix, Chemicals & Laboratory Suppliers, Cape Town, South Africa).

For the peroxide value the standard deviation was 0.49 meq O₂ kg⁻¹ and the 5 and 95 percentile boundaries were 5.08 and 6.61 meq O₂ kg⁻¹, respectively, for repetition 2 (Table 1). Therefore, peroxide values measured under similar conditions would typically deviate 0.49 meq O₂ kg⁻¹ from the mean and duplicate values would show a repeatability of 1.53 meq O₂ kg⁻¹, which represents the difference between the 95 and 5 percentile boundaries (Fig 1). When the peroxide value of duplicate determinations differed more than 1.53, the measurement was repeated. However, in the case of peroxide values that considerably exceed the range of values used for this statistical evaluation it might not always be appropriate. The peroxide value range for the two different repetitions differs noticeably, since measurements were not taken on the same day and repetition 1 and 2 should therefore be regarded as two different oils. This holds true for all reference methods.

Free fatty acid content

The free fatty acid (FFA) content, expressed as percentage oleic acid, was determined according to the AOAC official method 940.28 (AOAC, 2000), which is equivalent to the AOCS official method Ca-5a (AOCS, 1996). This titration method relies on the principle of measuring the amount of base required to neutralise the free fatty acids in the oil sample. The acid-base titration is accomplished by diluting the sample in ethanol and titrating with an alkaline solution to a phenolphthalein endpoint (Ryan *et al.*, 1998). The free fatty acid content in olive oil is then calculated according to equation 1.

$$\text{FFA (\% oleic acid)} = \frac{V \times M \times 282}{10 \times m} \quad (1)$$

where V = volume NaOH solution

M = molarity of NaOH solution

m = mass of oil sample

Ethanol (96%) (Saarchem, Merck Chemicals (Pty) Ltd, Wadeville, South Africa), neutralised by adding 0.3 mL phenolphthalein (Merck, Darmstadt, Germany) per 100 mL of alcohol and sufficient 0.1 M sodium hydroxide (Saarchem, Merck Chemicals (Pty) Ltd, Wadeville, South

Table 1 Summary of the descriptive statistics of the repeatability tests during repetition 2 for each chemical reference method.

Reference method	<i>n</i>	Mean	<i>SD</i>	<i>SE</i>	95% PB	5% PB	Repeatability
Peroxide value (meq O₂ kg⁻¹)	10	5.67	0.49	0.16	6.61	5.08	1.53
FFA content (% oleic acid)	10	0.40	0.028	0.0087	0.46	0.36	0.10
K₂₃₂	10	2.65	0.11	0.034	2.88	2.50	0.38
K₂₇₀	10	0.41	0.045	0.014	0.49	0.34	0.15
Chlorophyll content (mg kg⁻¹)	10	9.20	0.068	0.022	9.32	9.08	0.24
Carotenoid content (mg kg⁻¹)	10	6.45	0.044	0.014	6.51	6.37	0.14
Total polyphenol content (mg kg⁻¹)	10	183.01	6.57	2.08	189.65	172.48	17.17
TBARS value	10	0.0220	0.000479	0.000152	0.0226	0.0213	0.0013

SD = standard deviation

SE = standard error

PB = percentile boundaries

Repeatability = 95% PB – 5% PB

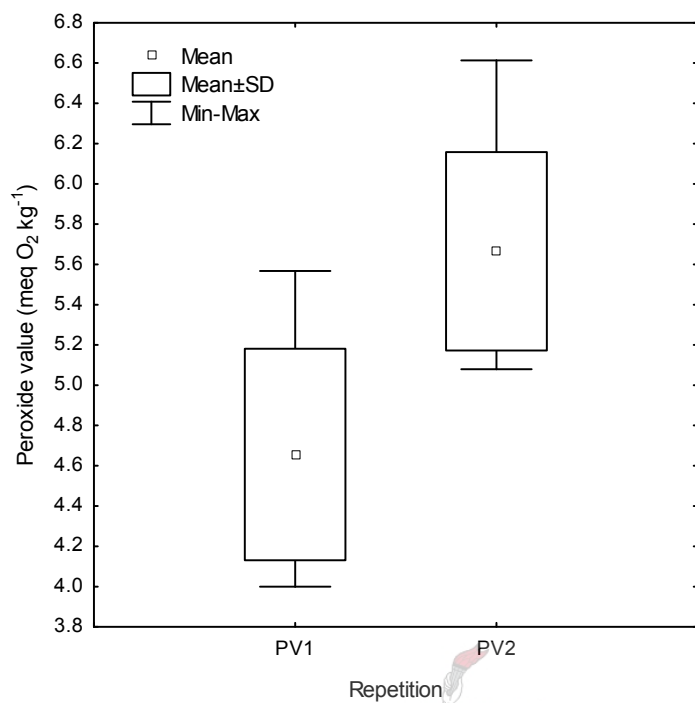
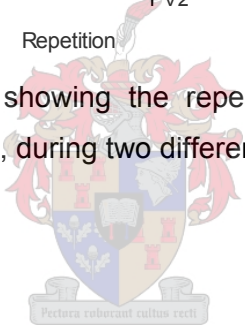


Figure 1 Box and whisker plot showing the repeatability of 10 repetitive peroxide value measurements on the same sample, during two different repetitions (PV1 and PV2).



Africa) to produce a faint pink colour, was used for the dilution of the oil. Since this neutralisation is subject to variation between different analysts, it is important that the testing of one group of samples is always done by the same analyst and that he/she always neutralises the alcohol to the same extent. If the ethanol is not completely neutralised, the free fatty acid content of the sample will be overestimated.

The official method was again changed in a few aspects to better suit the measurement of the free fatty acid content of extra virgin olive oil under the particular conditions. The sample mass was reduced to 1 g, a 100 mL Erlenmeyer flask was used and 20 mL neutralised ethanol and 3 drops of phenolphthalein solution (1% in ethanol) were added. The volume of ethanol was increased to 20 mL, as the addition of only 10 mL neutralised ethanol to the oil sample resulted in a cloudy solution on titration. The sample solution was then gently heated over a Bunsen burner, to completely dissolve the oil, and was titrated against 0.1 M sodium hydroxide solution to a light pink phenolphthalein endpoint persisting for at least one minute.

The sodium hydroxide solution was standardised according to the potassium hydrogen phthalate official method 936.16 (AOAC, 2000). Approximately 0.0002 g of potassium phthalate (BDH, Laboratory Supplies, Poole, England) was weighed off into a 250 ml Erlenmeyer flask. Distilled water (50 mL) and three drops of phenolphthalein were added, after which the solution was titrated to the phenolphthalein endpoint (pH 8.6) with the sodium hydroxide solution. A blank titration was performed with 50 mL distilled water and three drops of phenolphthalein, during which the water was titrated to a colour matching that of the pH 8.6 potassium phthalate solution. The volume sodium hydroxide solution used in the blank titration was subtracted from that used in the first titration and the molarity of the sodium hydroxide solution was calculated (AOAC, 2000).

The repeatability tests (Fig 2) for the free fatty acid content determination indicated that duplicate values with a maximum difference of 0.10% could be achieved and that the standard deviation of values from the mean was typically 0.028% (Table 1).

Specific extinction coefficients, K_{232} and K_{270}

The spectrophotometric investigation of the extra virgin olive oil samples in the ultraviolet region was accomplished by measuring the absorbance of a 1% oil solution in *n*-hexane (HPLC grade) (Saarchem, Merck Chemicals (Pty) Ltd, Wadeville, South Africa) at wavelengths of 232 nm and 270 nm (Beckman Coulter DU530 Life Science UV-Vis spectrophotometer). The absorption at each of these wavelengths gives an indication of the degree of conjugated diene and triene systems, respectively, which are formed as a result of oxidation or refining processes (Ryan *et al.*, 1998). The absorptions are expressed as specific extinction, *E*, more commonly referred to as the specific extinction coefficient *K*, which equals the absorbance divided by the product of

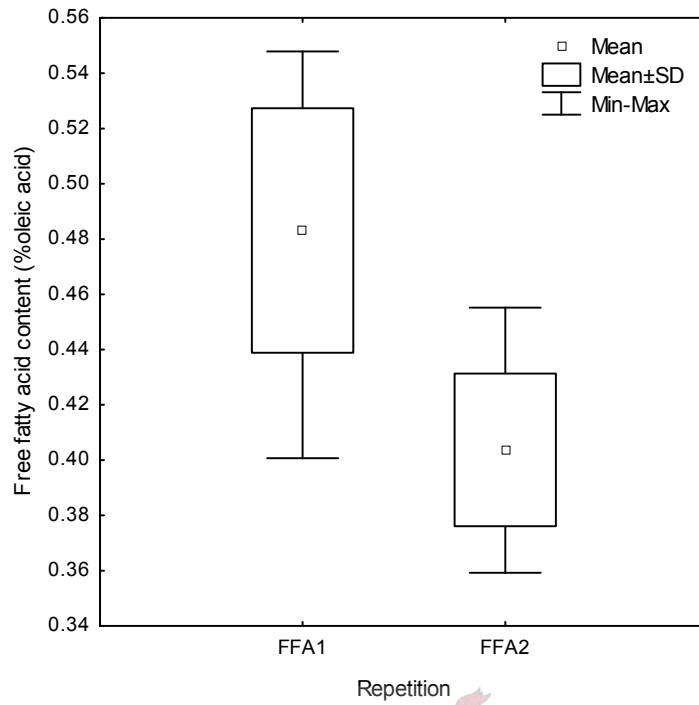
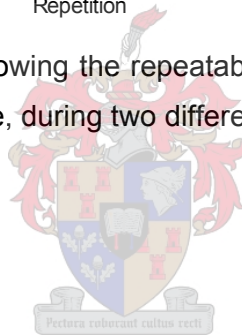


Figure 2 Box and whisker plot showing the repeatability of 10 repetitive free fatty acid content measurements on the same sample, during two different repetitions.



the concentration of the oil solution ($\text{g } 100 \text{ mL}^{-1}$) and the cuvette path length (cm). The procedure described by the International Olive Oil Council (IOOC) was used as a reference for this method (IOOC, 2001).

This method is very straightforward and the only problem encountered with the procedure itself, was the requirement that all absorbance values must fall within the range of 0.1 to 0.8. With the suggested 1% solution of extra virgin oil, the absorbance fell outside this specified range and even with reduced sample masses absorbencies would either be outside the range at 232 nm or at 270 nm. After several trials it was established that a sample mass of 0.030 to 0.035 g, diluted to 10 mL with *n*-hexane, resulted in absorbencies closest to the specified range of 0.1 to 0.8 for the oil used during repeatability tests. However, absorbance values at both 232 and 270 nm were not consistently within the range and often values above 0.8 or below 0.1 were obtained. The optimal concentration of the sample solution, giving absorbencies between 0.1 and 0.8 for both wavelengths, may thus vary for each type of oil and even between different qualities of one type of oil, which would make it necessary to test different sample concentrations for distinct oils. Since this is very unpractical, the sample concentration was kept at 0.030 to 0.035 $\text{g } 10 \text{ mL}^{-1}$ throughout the extinction coefficient measurements for the NIR prediction models. When measuring the absorbencies, it is essential that only matched sets or pairs of quartz cuvettes are used to exclude preventable variation in measurements. The cuvettes should be thoroughly rinsed with first acetone, then hexane and finally the sample solution, to avoid measurement inaccuracy through mixing of different samples.

The repeatability of the K_{232} measurements was found to be 0.38 for repetition 2 (Fig 3). The standard deviation from the mean of 0.11 was below the maximum limit of 0.39 set by the AOCS official method for conjugated dienes (K_{232}). The repeatability of the K_{270} measurements during repetition 2 was 0.15 and the standard deviation 0.045 (Fig 4). Although this is a big improvement when compared to the analysis at repetition 1, these values are still higher than those determined by the IOOC, which may be attributed to the fact that it was difficult to obtain absorbance values between 0.1 and 0.8 at both 232 and 270 nm.

Chlorophyll and carotenoid content

Carotenoids and chlorophylls in olive oil are easily quantified spectrophotometrically by measuring the absorbance of oil diluted in *n*-hexane at 470 and 670 nm, respectively. Since the quantification of the pigments in olive oil is not a quality criterium set by the IOOC, Codex Alimentarius or the European Union, there is no official method for the determination of the pigment content in olive oil. Several researchers have investigated the pigment content in virgin

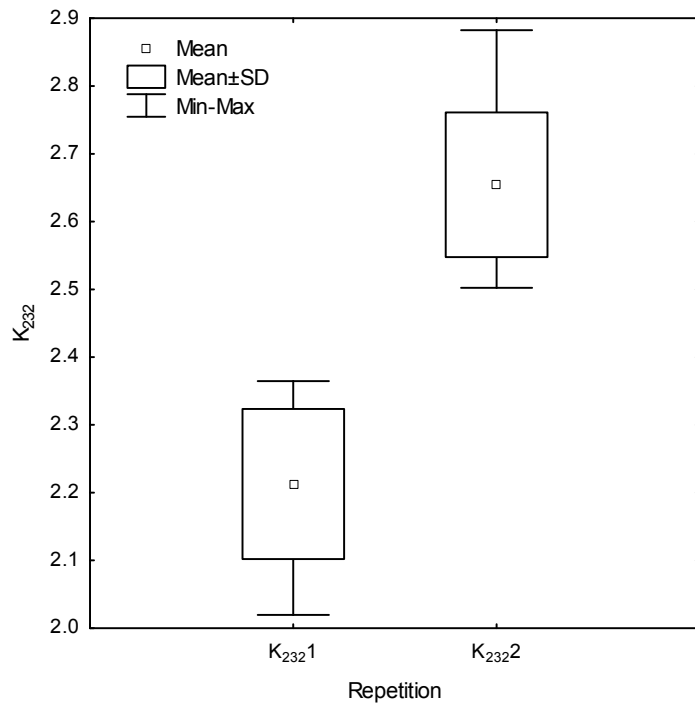


Figure 3 Box and whisker plot showing the repeatability of 10 repetitive K_{232} measurements on the same sample, during two different repetitions (K_{2321} and K_{2322}).

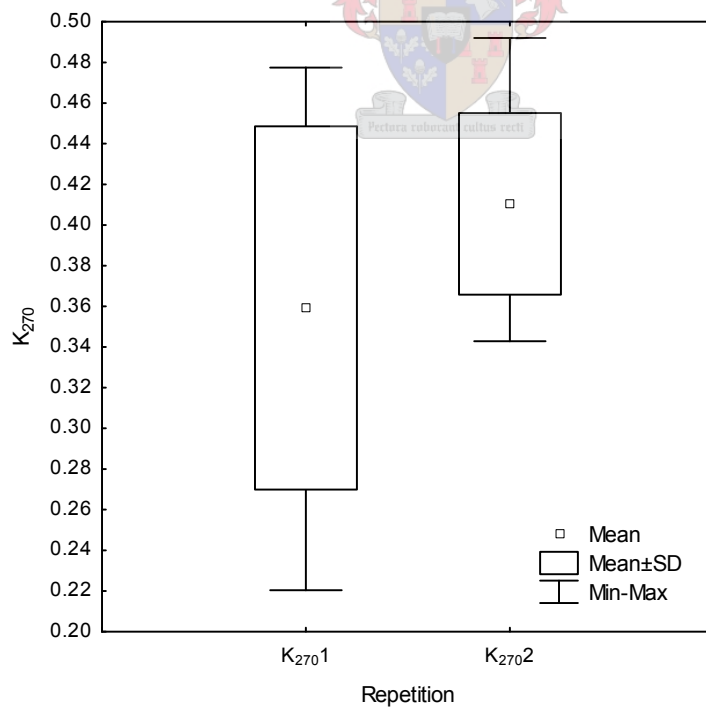


Figure 4 Box and whisker plot showing the repeatability of 10 repetitive K_{270} measurements on the same sample, during two different repetitions (K_{2701} and K_{2702}).

olive oils by spectrophotometry and this method seems to be a reliable method to estimate the total chlorophyll (chlorophyll and pheophytin a) and carotenoid (lutein and β -carotene) content (Minguez-Mosquera *et al.*, 1991; Gutierrez *et al.*, 2001; Salvador *et al.*, 2001; Jimenez-Marquez, 2003).

In this study the method applied by Jimenez-Marquez (2003) was taken as reference. Three grams of olive oil were weighed into a 10 mL volumetric flask and dissolved and made up to volume with *n*-hexane (HPLC grade) (Saarchem, Merck Chemicals (Pty) Ltd, Wadeville, South Africa). The sample solutions' absorbencies at 470 nm (carotenoids) and 670 nm (chlorophylls) were then measured in a 1 cm path length glass cuvette (Beckman Coulter DU530 Life Science UV-Vis spectrophotometer). The use of glass cuvettes instead of disposable plastic cuvettes was necessary due to the incompatibility of the particular plastic with *n*-hexane. This incompatibility with the plastic caused the cuvettes to turn opaque after several minutes of containing the sample solution.

The pigment content was calculated from the absorbencies by use of the extinction coefficients in cyclohexane, which were given in the bibliography as 613 for chlorophyll and 2000 for carotenoids (Jimenez-Marquez, 2003). It is important to note that these extinction coefficients have units of $\text{g } 100 \text{ mL}^{-1} \text{ cm}^{-1}$ instead of M cm^{-1} (molar extinction coefficient, ϵ) and consequently, the concentration of the pigments (*c*) in the formula $A_\lambda = \epsilon c L$, must be given as $\text{g } 100 \text{ mL}^{-1}$ as well (Anon., 2004). By determining the dilution of the sample of olive oil the concentration of the pigments in mg kg^{-1} oil can be calculated (equation 2).

$$A = \epsilon \times c \times L = \left(\frac{1}{M \times cm} \right) \times M \times cm \quad (2)$$

$$A = E \times c \times L = \left(\frac{1}{\text{g}100\text{mL}^{-1} \times cm} \right) \times \text{g}100\text{mL}^{-1} \times cm$$

$$c = \frac{A}{E}$$

where A = absorbance

E = extinction coefficient

c = pigment concentration

L = cuvette path length, 1 cm

$$\begin{aligned} \text{Example: carotenoid content} &= \frac{0.379}{2000\text{g}100\text{mL}^{-1}} \\ &= 1.895 \times 10^{-4} \text{g}100\text{mL}^{-1} \end{aligned}$$

$$\begin{aligned}
&= 1.895 \times 10^{-4} \frac{\text{g}}{\frac{(3.00 \text{g} \times 10)}{100 \text{mL}}} \\
&= 6.31 \times 10^{-6} \text{gg}^{-1} \text{oil} \\
&= 6.31 \text{mgkg}^{-1}
\end{aligned}$$

The box and whisker plots for the chlorophyll (Fig 5) and carotenoid (Fig 6) content repeatability test results show very similar standard deviations from the mean for repetition 1 and 2. A repeatability of 0.24 and 0.14 mg kg⁻¹ for the chlorophyll and carotenoid content, respectively, was achieved for repetition 2 (Table 1).

Total polyphenol content determination using the Folin-Ciocalteu reagent

Several publications were consulted to develop a suitable method for the determination of the total polyphenol content in olive oil and in particular the extraction procedure (Gutfinger, 1981; Capannesi *et al.*, 2000; Mosca *et al.*, 2000; Gutierrez *et al.*, 2002). Although all cited methods for the colorimetric determination of polyphenols with the Folin-Ciocalteu reagent are very similar, many different procedures for the extraction of the polyphenols from the oil have been applied. The extraction of polyphenols during this research was performed by diluting a 10 g olive oil sample with hexane (reagent grade) (Kimix, Chemicals & Laboratory Suppliers, Cape Town, South Africa) up to a volume of 25 mL and then centrifuging this oil solution with 10 mL of a methanol (Saarchem, Merck Chemicals (Pty) Ltd, Wadeville, South Africa): water (6:4) solution (Beckman Coulter Tj-25). The centrifuge tubes were thoroughly shaken by hand before centrifugation. After centrifugation at 3000 g (4425 rpm) for five minutes the methanol extract was removed with an Eppendorf pipette and 10 mL of fresh methanol: water solution was added to the same oil-hexane mixture, after which centrifugation was repeated. In this way three separate methanol extracts of the same sample were collected and their polyphenol content determined, and it was thereby established that a considerable amount of polyphenols is still extracted from the oil during the third centrifugation. Thus, all oil samples were centrifuged three times with 10 mL methanol: water solution and the extracts combined before analysis.

A big problem experienced during the extraction of the polyphenols from the oil, was the centrifuge tubes to be used. Initially, 50 ml polypropylene screw cap tubes were used, however, as the polypropylene was found to be incompatible with hexane it was decided to rather use polyethylene tubes. Polyethylene tubes were only available with snap-caps and these leaked during centrifugation, due to the nature of the sample mixture, according to the supplier. Finally, polyflor centrifuge tubes with screw caps were obtained, which although leaking when they were

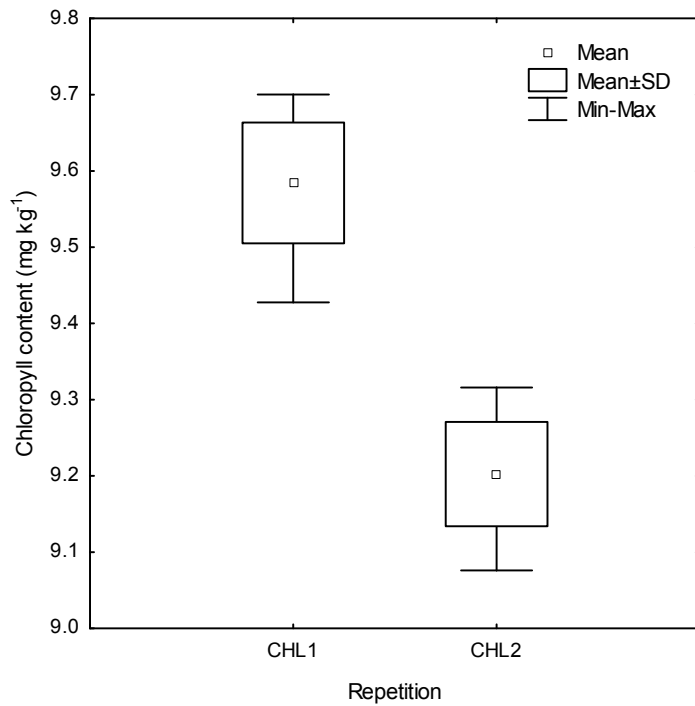


Figure 5 Box and whisker plot showing the repeatability of 10 repetitive chlorophyll content measurements on the same sample, during two different repetitions (CHL1 and CHL2).

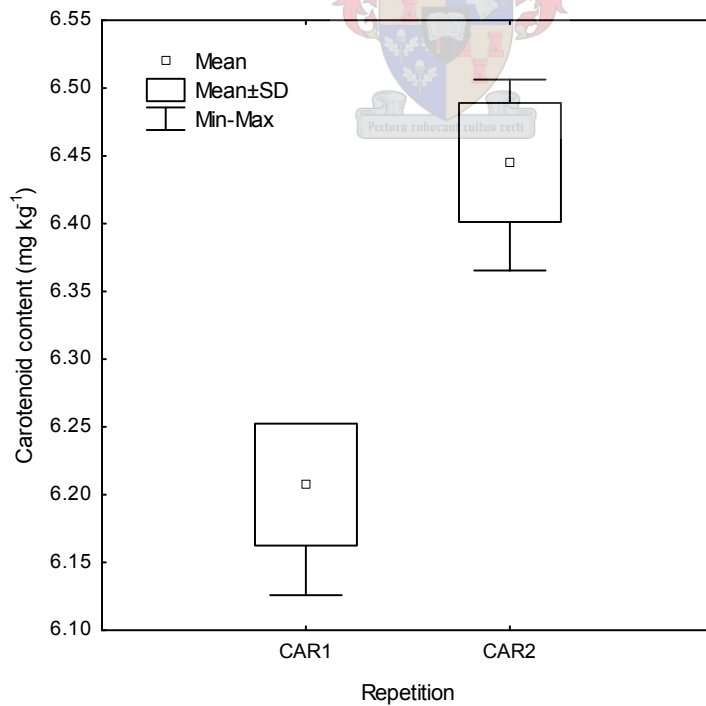


Figure 6 Box and whisker plot showing the repeatability of 10 repetitive carotenoid content measurements on the same sample, during two different repetitions (CAR1 and CAR2).

vortexed, did not leak when the tubes were shaken by hand and not during centrifugation.

From the combined methanol extracts of three centrifugations, 0.5 mL were placed into a test tube followed by 2.5 mL Folin reagent (10%) (Merck, Darmstadt, Germany) and 2 mL sodium carbonate solution (7.5%) (Saarchem, Merck Chemicals (Pty) Ltd, Wadeville, South Africa). The test tubes were vortexed and incubated at 30°C for two hours, after which the absorbance at 725 nm was measured (Beckman Coulter DU530 Life Science UV-Vis spectrophotometer). The absorbance of a standard caffeic acid (Sigma, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) dilution range (0.01, 0.02, 0.04, 0.06 and 0.08 mg mL⁻¹ distilled H₂O) was also measured and the concentration of polyphenols in the oil samples, expressed as mg caffeic acid kg⁻¹, could thus be estimated from the developed standard curve.

The repeatability for the total polyphenol content method was 78.71 mg kg⁻¹ for repetition 1, at which the mean value was 230.52 mg kg⁻¹ (Fig 7). However, for a mean value of 183.01 mg kg⁻¹ during repetition 2 the standard deviation was considerably lower and the repeatability was 17.17 mg kg⁻¹ (Table 1).

Thiobarbituric acid reactive substance value

The 2-thiobarbituric acid reactive substance (TBARS) value was measured according to the direct method of the American Oil Chemists' Society, method Cd 19-90 (AOCS, 1996). The TBARS value is defined as "the increase of absorbance measured at 530 nm due to the reaction of the equivalent of 1 mg of sample per 1 mL volume with 2-thiobarbituric acid". Secondary oxidation products, also known as thiobarbituric acid reactive substances, of polyunsaturated fatty acids react with thiobarbituric acid in a condensation reaction, which results in the formation of a red chromagen that can be detected by absorbance measurement at 530 nm (Bianchi, 2002). This direct method is a rather uncomplicated means of determining the oxidation of the polyunsaturated fatty acids linoleic (C18:2) and linolenic (C18:3) acid in olive oil.

In preparation for the TBARS value analysis, the thiobarbituric acid (TBA) reagent was always prepared the day before the analysis was performed, by dissolving thiobarbituric acid (Merck, Darmstadt, Germany) in 1-butanol (Saarchem, Merck Chemicals (Pty) Ltd, Wadeville, South Africa) with the aid of a magnetic stirrer. For each new set of analyses fresh reagent was prepared to prevent the incorporation of variation in results due to the use of TBA reagent in an unreliable condition. After several trials it was found that for extra virgin olive oil a sample weight of 50 to 200 mg gives a too low sample concentration when diluted with 1-butanol, as a comparatively lower TBARS value is expected than in other vegetable oils. Sample masses between 0.7 and 0.8 g were thus diluted with 1-butanol in 25 mL volumetric flasks to obtain an absorbance reading in the range of 0.2 to 0.8. Instead of a reaction mixture of 5 mL sample solution and 5 mL TBA reagent, these volumes were reduced to 2 mL each, to reduce the

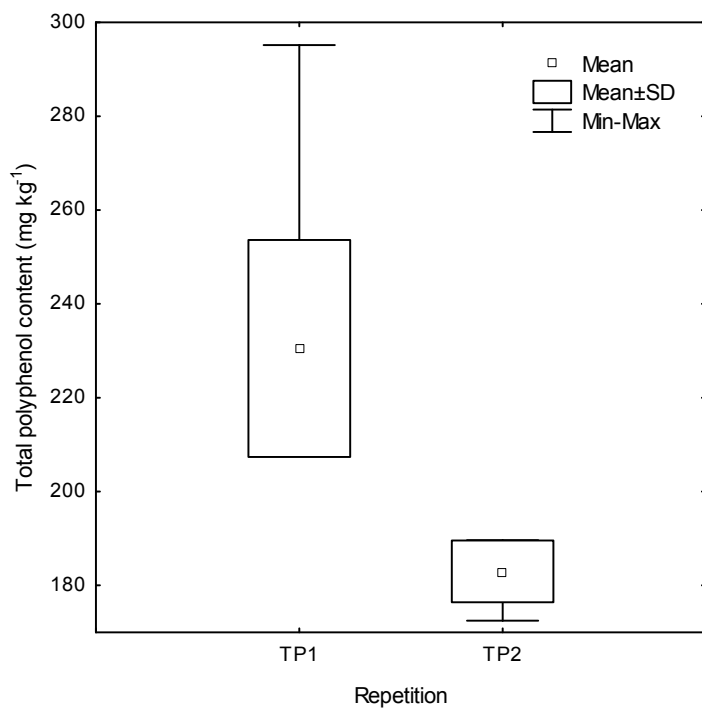


Figure 7 Box and whisker plot showing the repeatability of 10 repetitive total polyphenol content measurements on the same sample, during two different repetitions (TP1 and TP2).




consumption of solvent and thiobarbituric acid. Particular care was taken that after reaction in the water bath (95°C) all test tubes were cooled down to the same degree, by placing the test tube holder in a cold water bath (cold tap water) for a minimum of ten minutes, as large variations in temperature of the samples may result in inconsistent absorbance measurements by the spectrophotometer (Beckman Coulter DU530 Life Science UV-Vis spectrophotometer).

Figure 8 shows that the repeatability for repetition 2 is significantly better than that during repetition 1, indicating a lower standard deviation and smaller range of the percentile boundaries. Duplicate values of the TBARS value should, under the specific conditions, not differ by more than 0.0013 with a 95% probability (Table 1).

Conclusion

Optimisation of the above-mentioned olive oil test methods, to suit the specific conditions and available resources of this study, resulted in an acceptable degree of accuracy. These reference methods were thus employed during NIR prediction model development. Good standard deviations for each reference method were maintained during the actual experiment discussed in Chapter 3, considering the wide range of test values measured.

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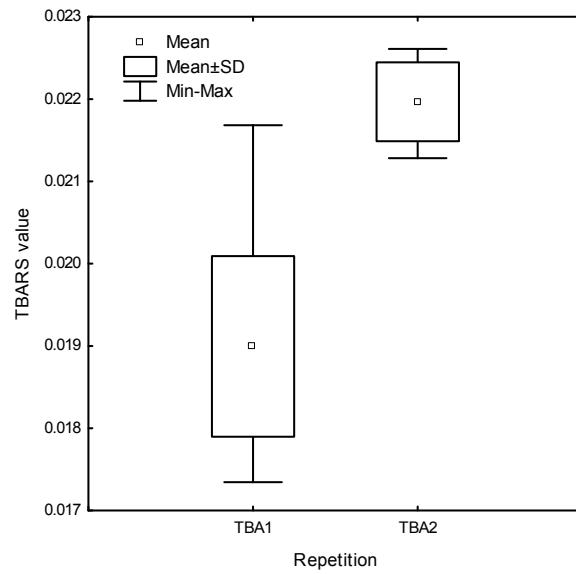
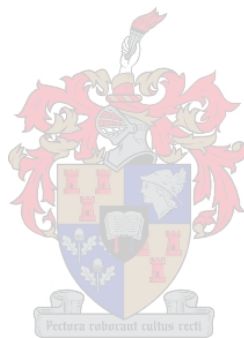


Figure 8 Box and whisker plot showing the repeatability of 10 repetitive TBARS value measurements on the same sample, during two different repetitions (TBA1 and TBA2).



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Chapter 5

CLASSIFICATION OF SOUTH AFRICAN EXTRA VIRGIN OLIVE OILS OF DIFFERENT OXIDATION LEVELS, CULTIVARS AND GEOGRAPHICAL ORIGIN BY ^{13}C NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY AND MULTIVARIATE STATISTICAL ANALYSES



Classification of South African extra virgin olive oils of different oxidation levels, cultivars and geographical origin by ^{13}C nuclear magnetic resonance spectroscopy and multivariate statistical analyses

Abstract

Principal component and linear discriminant analyses of ^{13}C NMR intensity and peak height data, to classify South African extra virgin olive oils according to different oxidation levels, cultivars and geographical regions, were investigated. Principal component analysis (PCA) on NMR intensity and peak height data was unable to classify the olive oils according to their degree of oxidation and geographical origin under the present conditions, but demonstrated a grouping of olive oils on the basis of cultivar differences. Linear discriminant analysis (LDA) of the intensity data was able to correctly classify single cultivar olive oils into their respective cultivar groups, however, was unsuccessful in classifying the olive oils according to geographical origin. PCA showed no obvious differences in performance between intensity and peak height data, however, LDA of the intensity data gave better results than for the peak height data for cultivar and geographical origin based classifications.

Introduction

The basic principles of nuclear magnetic resonance (NMR) spectroscopy rely on the fact that most elements have at least one isotope, which is magnetic and when such a magnetic nucleus is placed in a strong magnetic field, it orientates itself in either the same direction or opposite direction as the field (Hore, 2000). These two directions or states have different energy levels and the difference in energy is unique for each nucleus and depends on the strength of the magnetic field. This difference in energy can be measured by exposing the nucleus to electromagnetic radiation, causing the nucleus to change from a lower energy level to an adjacent higher energy level (Friebolin, 2005). The radiation required to induce this nuclear magnetic radiation (NMR) transition is called the radiofrequency field. Following the electromagnetic pulse, the nuclei return to their state of equilibrium and a weak signal of resonance frequency can be recorded, giving information on a molecule's structure, its conformation or molecular motion (Anon., 1995; Hore, 2000; Friebolin, 2005).

A plot of resonance frequency against intensity of the particular nuclei in different chemical environments creates a NMR spectrum (McKenzie & Koch, 2004). Since the frequency of the pulse has to be in resonance with the spin system of the nucleus, to make energy transfer possible, the radiofrequency for any given isotope in different chemical environments results in distinct chemically shifted spectral lines (Anon., 1995). The most

commonly used nuclei in NMR spectroscopy are ^1H and ^{13}C , however ^{19}F , ^{31}P and ^{15}N can also be applied.

Food products and other organic compounds are mainly composed of carbon and proton atoms and thus ^1H and ^{13}C high-resolution NMR spectroscopy is the most suitable method for structure elucidation in organic compounds (McKenzie & Koch, 2004). Protons have a high natural abundance of 99.98%, which makes the acquisition of ^1H NMR spectra faster compared to ^{13}C spectra. ^{13}C has a natural abundance of only 1.11% and this increases the signal averaging time significantly. ^{13}C NMR is therefore comparatively insensitive, leading to a low signal-to-noise ratio of the spectra (Robien, 2003). Nevertheless, ^{13}C NMR spectroscopy has become increasingly popular due to the introduction of Fourier transformation, which leads to much shorter acquisition times and improved sensitivity.

The ^{13}C resonances of olive oil can be grouped into four different regions, namely the carbonyl region (172-174 ppm), the olefinic carbon region (124-134 ppm), the glycerol carbon region (60-72 ppm) and the aliphatic carbon region (10-35 ppm) (Vlahov, 1999). The carbonyl region of an olive oil ^{13}C NMR spectrum shows two groups of resonances, which hold information on the α - and β -position of fatty acid chains on the glycerol backbone. Although the individual fatty acid chains cannot be differentiated, it is possible to distinguish between saturated chains and unsaturated chains and it was established that unsaturated fatty acids are almost exclusively found in the β -position of the triglycerol, whereas saturated fatty acids are attached at the α -position (Mannina *et al.*, 1999; McKenzie & Koch, 2004). The olefinic region has the widest frequency range on the ^{13}C spectrum and contains information on the carbons in unsaturated fatty chains, which are mainly oleic (C18:1) and linoleic (C18:2) acid in olive oil (Vlahov, 1999). These two fatty acids are present in olive oil at concentrations of 55-83% and 3.5-20%, respectively, and their quantity plays an important role in the detection of olive oil adulteration. Although it is not possible to make a complete fatty acid compositional analysis as is done with gas chromatography (GC), it has been confirmed that ^{13}C NMR can quantify major fatty acids with the same accuracy as GC (Mavromoustakos *et al.*, 1997; McKenzie & Koch, 2004). The glycerol region can be of use in determining the diacylglycerol concentration in olive oils, which is a direct indicator of lipolytic action and can be correlated to the quality of the raw material used for olive oil pressing (Sacchi *et al.*, 1997). The aliphatic region of the ^{13}C NMR spectrum consists of ^{13}C resonances on saturated and unsaturated fatty acid chains (Vlahov, 1999). Several minor components of olive oil, such as sterols, can be detected by investigating the ^{13}C NMR spectrum, however, these are not of relevance for the classification of olive oils according to oxidative status, cultivar and geographical origin.

The information on the fatty acid profile of olive oils that can be extracted from a ^{13}C NMR spectrum has found application in the detection and quantification of virgin olive oil

adulteration with other seed oils (Sacchi *et al.*, 1997; Mavromoustakos *et al.*, 2000; Zamora *et al.*, 2001; Zamora *et al.*, 2002; Hidalgo & Zamora, 2003; McKenzie & Koch, 2004). Furthermore, this information combined with multivariate statistical analyses, such as principal component analysis (PCA) and linear discriminant analysis (LDA), has been employed to discriminate between olive oils of different geographical origin and distinct cultivars (Shaw *et al.*, 1997; Vlahov *et al.*, 1999; Sacco *et al.*, 2000; Vlahov *et al.*, 2001; Mannina *et al.*, 2003; Vlahov *et al.*, 2003). PCA makes the representation of a data set of intercorrelated variables, in this case NMR intensities or peak heights, easier and instead of dividing the variables into dependent and independent, converts the original variables into new uncorrelated principal components, which can present information on underlying relationships among a set of samples (Afifi & Clark, 1996). Discriminant analysis, on the other hand, is used to classify samples into one of two or more distinct groups based on a known property, such as cultivar, and could, e.g., be used as a model to predict the cultivar from which a particular olive oil was pressed. Chemical determinations of oil stability (Rancimat, fatty acid and triacylglycerol composition, phenols, tocopherol) have been successfully correlated to ^{13}C NMR spectroscopy data (Hidalgo *et al.*, 2002), however, there is no sighting in the literature of any attempt to apply multivariate statistical analyses to ^{13}C NMR data to discriminate between olive oils of different oxidation levels.

Multivariate statistical models are usually built from the intensities (peak area obtained by integration) of NMR peaks, however, since the process of integration is rather intricate and time-consuming, it was investigated whether the heights of selected peaks could have equally strong discriminant power when employed during PCA and LDA. The main objective of this study was to evaluate the feasibility of PCA and LDA of ^{13}C NMR spectral data (intensities and peak heights) to distinguish between South African extra virgin olive oils of different oxidation levels, cultivars and geographical origins. Since, the South African market is flooded with olive oil imports from the Mediterranean and has experienced local incidents of fraud (Cilliers, 2001), the lack of a locally regulated quality control system is becoming a serious problem. The application of NMR spectroscopy combined with multivariate statistical models, as a quality control tool for the South Africa olive oil industry, would facilitate origin based authentication of olive oils and could be used to endorse the Proudly South African campaign.

During this research, we have shown that for PCA of NMR data the peak heights provide equally precise results when compared to intensity data. We have proven that ^{13}C NMR spectroscopy is not suitable for the classification of multivarietal extra virgin olive oils according to oxidation level differences and that geographical origin based classifications by LDA are only successful when evaluating olive oils pressed from the same single cultivar. The cultivar an olive oil is pressed from has the most significant influence on its major fatty acid composition and

this is illustrated by the successful classification of single cultivar olive oils into their respective groups of cultivars by LDA.

Materials and methods

Sample oxidation

Fourteen South African extra virgin olive oils (seasons 2003 and 2004), pressed from different olive cultivars (Mission, Frantoio, Coratina and Leccino) and originating from 5 regions in the Western Cape (Stellenbosch, Somerset West, Paarl, Worcester and Riebeek Kasteel), were exposed to accelerated oxidation conditions to obtain a sample set that included a range of oxidation levels. Plastic Petri-dishes were filled with 15 mL of extra virgin olive oil and stored uncovered in an illuminated incubation room at 35°C. The olive oil samples (pure cultivars and blends) were analysed by NMR spectroscopy when they arrived from the producer, as well as weekly for up to five weeks of oxidation in the incubation room. The peroxide value of the olive oil samples was measured at the same time intervals. As it was found that the degree of oxidation obtained after five weeks was still relatively low an additional set of 9 extra virgin olive oils (seasons 2003 and 2004), pressed from different olive cultivars (Mission, Frantoio, Coratina), was obtained from 4 regions (Paarl, Somerset West, Riebeek Kasteel and Robertson) and stored as before. The oil samples (pure cultivars and blends) were again analysed by NMR spectroscopy, however, to extend the oxidation level of the oils, every second week up to 10 weeks of oxidation. As not all the olive oil samples were available in sufficient volumes to complete oxidation for 5 or 10 weeks, respectively, a total of 104 South African extra virgin olive oil samples were analysed and included during data analysis.

Nuclear magnetic resonance spectroscopy

Fourier-transform nuclear magnetic resonance (NMR) ^{13}C spectra were recorded on a Varian^{Unity} Inova 600 MHz (14.09 Tesla) spectrometer operating at 150 (McKenzie & Koch, 2004). Sample solutions in 5 mm NMR tubes consisted of 40% (w/w) olive oil in deuterated chloroform (CDCl_3) (Aldrich, Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The spectral window ranged from 200 to -5 ppm. A 90° pulse was used to collect the ^{13}C NMR spectra with a pulse delay of 45 seconds. Inverse-gated proton decoupling was applied. To optimise resolution Gaussian multiplication was applied to the free induction decay (FID) using a line broadening of -0.092 and Gaussian enhancement of 1.088. Zero-filling to 524k was employed before the FID was Fourier transformed and, finally, the peaks in the carbonyl and olefinic region of the spectrum were integrated by deconvolution analysis.

Peroxide value

The peroxide value was determined according to the American Oil Chemists' Society (AOCS) official method (AOCS Cd 8b-90) (AOCS, 1996). The following adjustments were made to this method. Sample weights were reduced to 0.5 g and 10 mL glacial acetic acid: isooctane (3:2) was added to the sample in a 100 mL Erlenmeyer flask. Half a milliliter of saturated potassium iodide solution was added and after one minute the reaction was stopped by adding 10 mL distilled water.

Data analysis

The intensities (peak areas) of the C9 and 10 α and β oleyl peaks, and the C9, 10, 12 and 13 α and β linoleyl peaks in the olefinic region of the NMR spectrum, as well as the saturated acid, oleic and linoleic α and oleic and linoleic β carbonyl intensities in the carbonyl region (Table 1) were obtained by means of integration (MestRe-C version 9.1). The oleic and linoleic peaks in the carbonyl region were integrated as a group due to them overlapping. The peak heights of 21 peaks, referenced to deuterated chloroform at 77 ppm, and covering the whole range of the NMR spectrum (Table 2) were also recorded (MestRe-C version 9.1) and assigned according to chemical shifts reported in literature (Shaw *et al.*, 1997; Vlahov *et al.*, 2001; Zamora *et al.*, 2001). Additionally, to the oleyl and linoleyl peaks in the carbonyl and olefinic regions, the resonances of ^{13}C atoms on saturated, oleic, linoleic or linolenic fatty acid chains in the aliphatic region of the NMR spectrum were included, as well as the resonances of ^{13}C at the α and β positions on the glycerol molecule.

Principal component analysis (STATISTICA version 7) was employed to reveal any relationship between NMR intensity or peak height data and the oxidation level, geographical origin or cultivar of the olive oil samples, by means of examining the principal component score plots. Linear discriminant analysis (STATISTICA version 7), using leave-one-out cross-validation, was performed on the NMR intensity and peak height data to built models for the classification of the olive oil samples according to geographical region and cultivar.

Results and discussion

The ^{13}C NMR spectrum of a fresh extra virgin olive oil is shown in Figure 1, indicating the olefinic, carbonyl, glycerol carbon and aliphatic regions of the spectrum. The carbonyl and olefinic regions are the most important regions when evaluating olive oil, as these reveal information on the position of saturated and unsaturated fatty acids on the glycerol backbone and can be used to quantify major fatty acids (i.e., oleic, linoleic and saturated). The unsaturated fatty acids are of particular importance during oxidation reactions, since they are

Table 1 The chemical shifts and assignments of the 15 selected ^{13}C NMR peaks in the carbonyl and olefinic regions, for which the intensities were used for principal component analysis and linear discriminant analysis.

Peak no.	Chemical shift	Assignment	Spectral region
1	172.59	S1	Carbonyl
2	172.56	O+L1 α	
3	172.20	O+L1 β	
4	129.82	L13 β	Olefinic
5	129.81	L13 α	
6	129.70	O10 β	
7	129.68	O10 α	
8	129.63	L9 α	
9	129.61	L9 β	
10	129.41	O9 α	
11	129.38	O9 β	
12	127.90	L10 β	
13	127.88	L10 α	
14	127.72	L12 α	
15	127.71	L12 β	

S = saturated

O = oleic

L = linoleic

1, 13, 10 *etc.* = carbon position on fatty acid chain

α = outer fatty acid chain positions on the triacylglycerol molecule

β = inner fatty acid chain position on the triacylglycerol molecule



Table 2 The chemical shifts and assignments of 21 ¹³C NMR peaks, for which the peak heights were used during principal component analysis and linear discriminant analysis.

Peak no.	Chemical shift (ppm)	Assignment	Spectral region
1	172.56	O1 α	Carbonyl
2	172.20	O1 β	
3	129.70	O10 β	Olefinic
4	129.68	O10 α	
5	129.41	O9 α	
6	129.38	O9 β	
7	127.90	L10 β	
8	127.88	L10 α	
9	127.72	L12 α	
10	127.71	L12 β	
11	68.83	Glycerol C β	Glycerol
12	61.84	Glycerol C α	
13	33.92	O/L2 β	Aliphatic
14	33.76	S/O/L2 α	
15	31.73	S/O/L16	
16	29.13	O/L/Ln4, O/L6	
17	27.03	O/L/Ln8, O11, L14	
18	25.45	L/Ln11, Ln14	
19	24.66	S/O/L3	
20	22.47	S/O/L17	
21	13.83	S/O/L18	

S = saturated

O = oleic

L = linoleic

Ln = linolenic

1, 10, 9 *etc.* = carbon position on fatty acid chain

α = outer fatty acid chain positions on the triacylglycerol molecule

β = inner fatty acid chain position on the triacylglycerol molecule

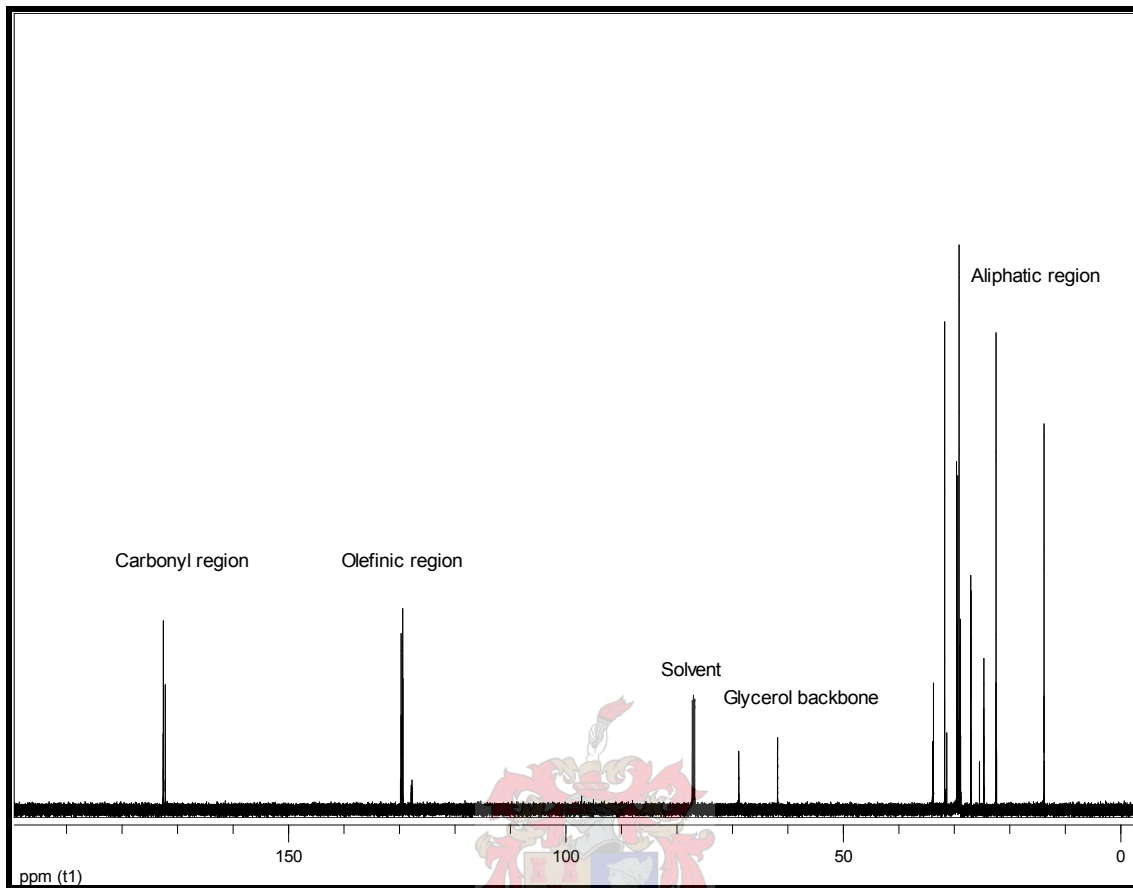


Figure 1 ^{13}C NMR spectrum of a fresh extra virgin olive oil, indicating its distinct resonance regions.



most prone to oxidation (Gray, 1978). The conjugation of double bonds is a common occurrence during oxidation and these shifts in double bond systems in unsaturated fatty acids are expected to cause changes in the intensities of the respective ^{13}C resonances. Therefore, the intensities of 15 ^{13}C NMR peaks in the carbonyl and olefinic region (Table 1), which represent several ^{13}C resonances on oleic and linoleic acid chains (with the exception of peak 1), were determined.

Figure 2 illustrates the score plot of principal component (PC) 2 versus PC3 for the 15 peak intensities with samples labeled according to oxidation week, i.e., the time in weeks, which the oil was stored under accelerated oxidation conditions. The peroxide values differed notably between olive oils oxidised for the respective number of weeks and thus the time of oxidation was directly proportional to the oxidation level of the oils. Four extremely oxidised samples that were separated from the main group of samples are not shown on the score plot to improve visibility of the remaining samples. No clustering of olive oil samples of similar oxidation level occurred, as was also the case when plotting PC1 versus PC2, and the various weeks of oxidation were evenly spread on the PCA score plot. Similar results were obtained with the score plot of PC1 versus PC2 for the peak height data (Fig. 3). Twenty samples, which appeared as outliers on the score plot due to extremely low peak heights, are not shown on the score plot to improve visibility of the remaining samples. The PCA score plots were subsequently investigated for any other type of grouping such as geographical origin and cultivar.

Figure 4 depicts the same peak intensity PCA score plot as Figure 2 (PC2 vs PC3) with samples labeled according to geographical origin. Although no distinct grouping is evident, it can be noticed that samples originating from the same geographical area tend to cluster together, irrespective of their degree of oxidation. For the peak height data the same trend could be observed as samples originating from the same geographical region again tended to form clusters (Fig. 5). Even though PCA did not show well-defined groups of geographical region, it seems that differences in olive oil fatty acid composition due to distinct geographical origins are predominant over the slight changes in the fatty acid structure occurring due to oxidation. Another factor, which could have prevented the classification of oils according to oxidation level, is the fact that the sample set (0 weeks oxidation) contained freshly pressed olive oils and oils pressed during the previous season. Thus, variation in degree of oxidation within each particular group of oxidation level (week) was also included.

Since, no correlation was noted between NMR data and the degree of oxidation of olive oils, even when expressed as peroxide values (results not shown), LDA was only employed to investigate the classification of the oils according to geographical origin and cultivars. LDA of the NMR intensity and peak height data reflected the trends seen during PCA in that it failed to

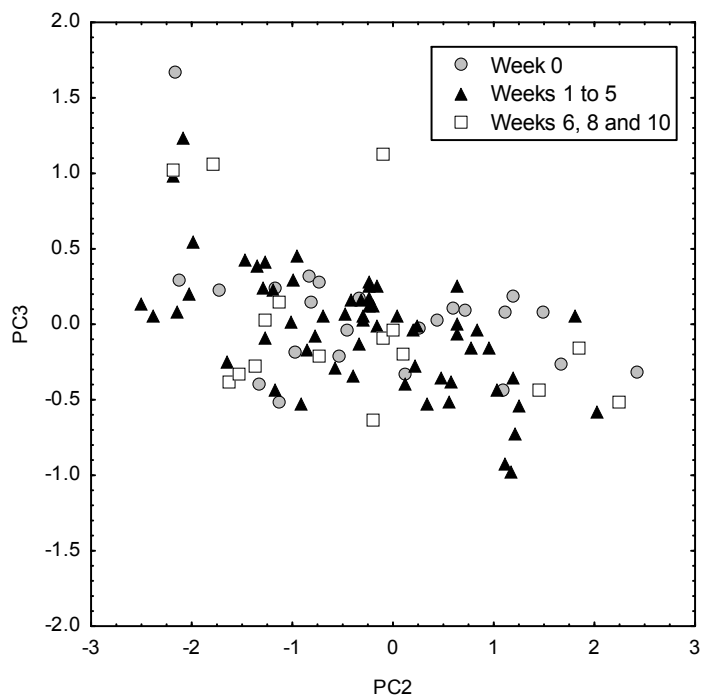


Figure 2 PCA score plot of PC2 versus PC3 for the 15 peak intensities, indicating the sample oxidation level from weeks 0 to 10.

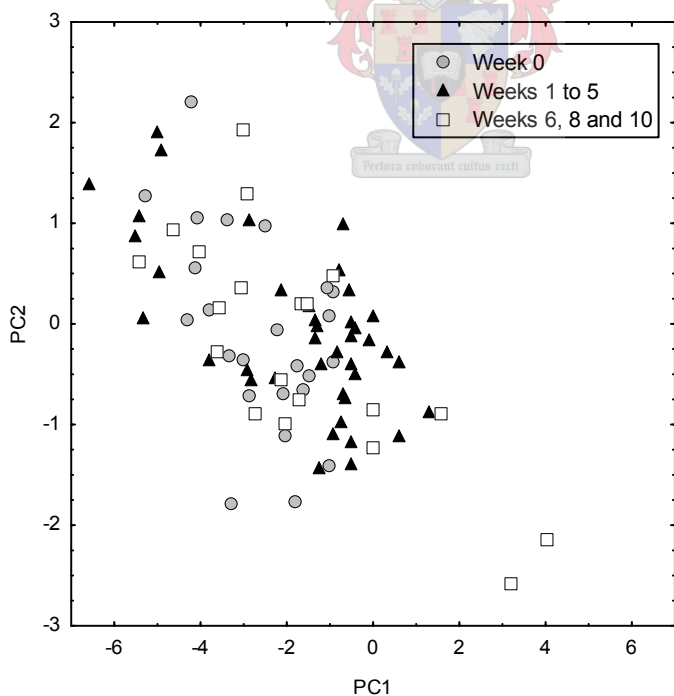


Figure 3 PCA score plot of PC1 versus PC2 for the 21 peak heights, indicating the sample oxidation level from weeks 0 to 10.

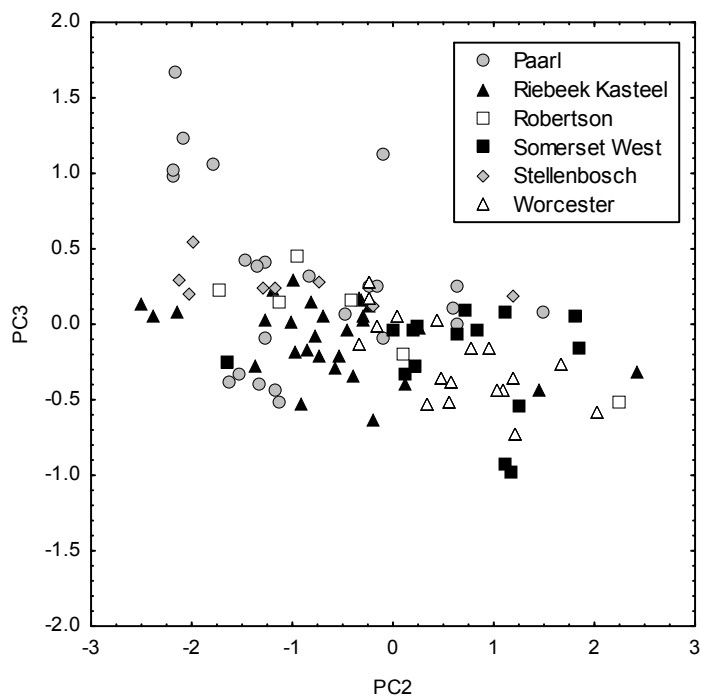


Figure 4 PCA score plot of PC2 versus PC3 for the 15 peak intensities, indicating the geographical origin of each sample.

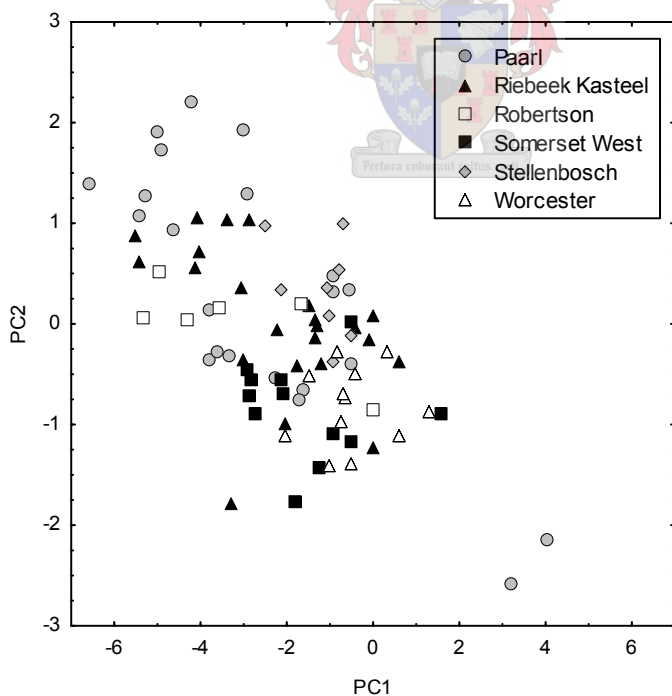


Figure 5 PCA score plot of PC1 versus PC2 for the 21 peak heights, indicating the geographical origin of each sample.

consistently predict the geographical origin of the olive oil samples. Figure 6 displays the results of the LDA on the intensity data. Each histogram represents one region in the Western Cape and indicates the percentage of samples from that particular region, which were misclassified and correctly classified. It can be observed that only for the Worcester (61%) and Stellenbosch (63%) regions, more than half of the samples were classified correctly, whereas for Riebeeek Kasteel (37%) and Robertson (33%) only a third of the sample set was correctly classified and for Somerset West (19%) and Paarl (13%) even less. The LDA of NMR peak heights revealed similar results (Fig. 7). The samples from the Worcester (67%) and Stellenbosch (63%) areas were again most correctly classified. These trends are not as apparent when observing the sample distribution on the PCA score plots (Figs. 4 and 5), which indicates that LDA is a more powerful classification method than PCA.

The classification of blended olive oils (multivarietal) according to geographical origin by multivariate statistical analysis of ^{13}C NMR data is difficult (Vlahov *et al.*, 2001) and clear grouping of distinct geographical areas has as yet only been achieved with olive oils pressed from a single olive cultivar (monovarietal) (Vlahov *et al.*, 1999; Mannina *et al.*, 2003; Vlahov *et al.*, 2003). The presence of different monovarietal and blended olive oils in the sample set of this study could thus be the reason for not achieving evident classification of samples originating from different geographical areas. It is, however, unlikely that extra virgin olive oils produced in different regions of South Africa will ever only represent monovarietal olive oils and the results obtained in this study are therefore more realistic in terms of applicability.

When only the monovarietal olive oils were used for PCA of both the NMR intensity (Fig 8) and peak height data (Fig 9), the clustering of oil samples from the same olive cultivar became very clear, but the grouping according to oxidation level or geographical area was again not evident at all (results not shown). A closer look at the cultivar composition of the blended and single cultivar olive oils included in the sample set indicated that cultivar had a considerable influence on the classification of olive oils according to the geographical area. On the PCA score plot of the intensity data of all samples (results not shown), blended olive oils containing the cultivar Mission were mainly situated in the left lower quadrant of the plot as in Figure 8, whereas blended oils containing the Frantoio cultivar were mainly situated in the upper half of the plot as can be related to in Figure 8. This strong influence of the cultivar on the olive oil fatty acid composition has also been found to obscure geographical classification in other olive oil NMR studies (Vlahov *et al.*, 2003). The PCA score plots, therefore, represent a combination of the effect of geographical origin and cultivar, which overpowers the factors contributing to oxidation level. As PCA most clearly showed a separation of samples according to cultivar, when only single cultivar olive oils were included in the analysis, LDA was applied to this monovarietal sample set to classify the respective olive oil samples. For the NMR intensity data

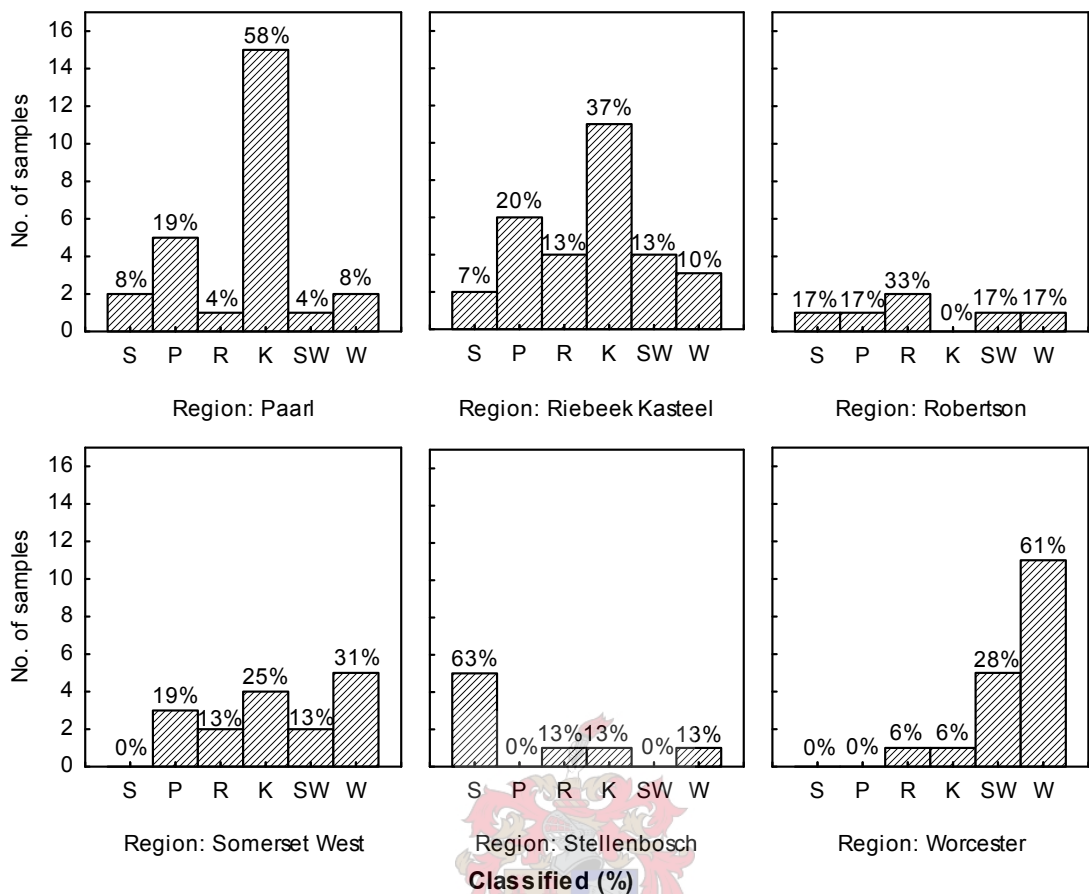


Figure 6 LDA results of the NMR peak intensities for the geographical origin classification showing the percentages of samples classified correctly and incorrectly for each region (S = Stellenbosch, P = Paarl, R = Robertson, K = Riebeeck Kasteel, SW = Somerset West, W = Worcester).

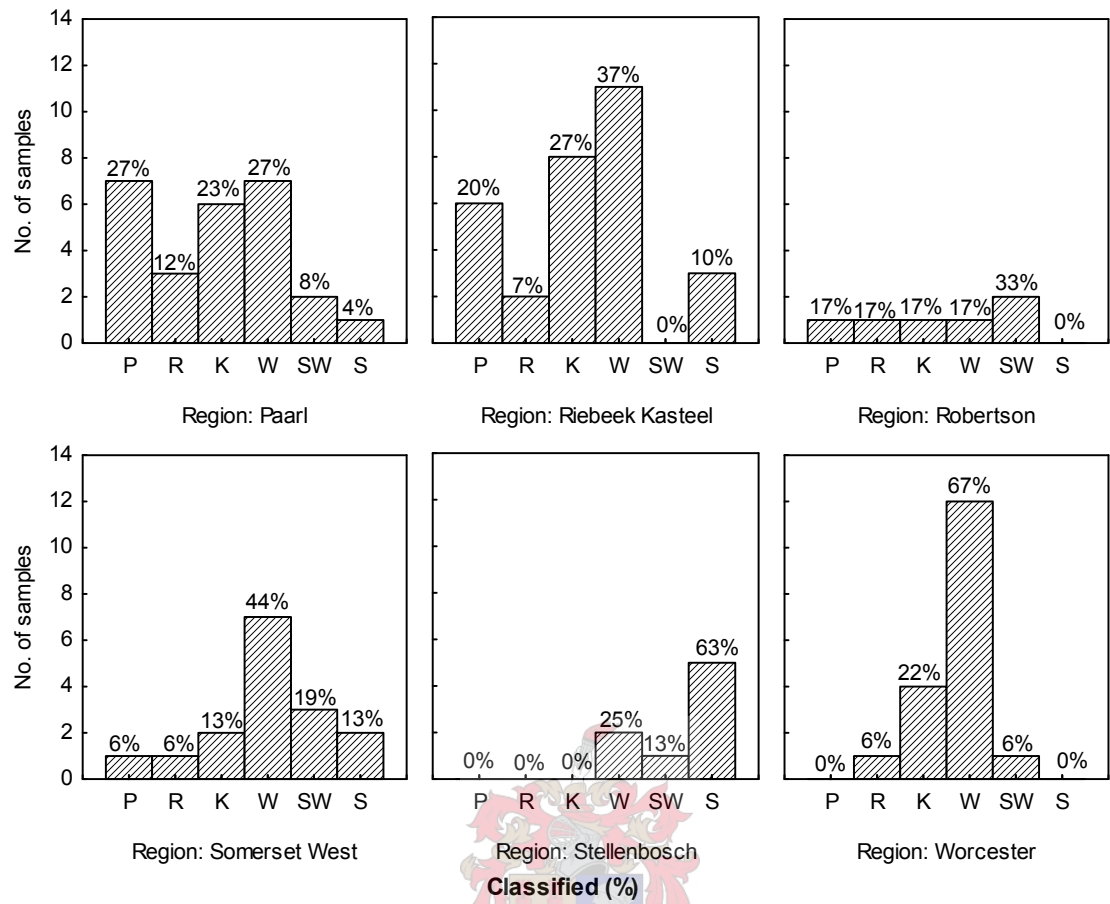


Figure 7 LDA results of the NMR peak heights for the geographical origin classification showing the percentages of samples classified correctly and incorrectly for each region (P = Paarl, R = Robertson, K = Riebeeck Kasteel, W = Worcester, S = Stellenbosch, SW = Somerset West).

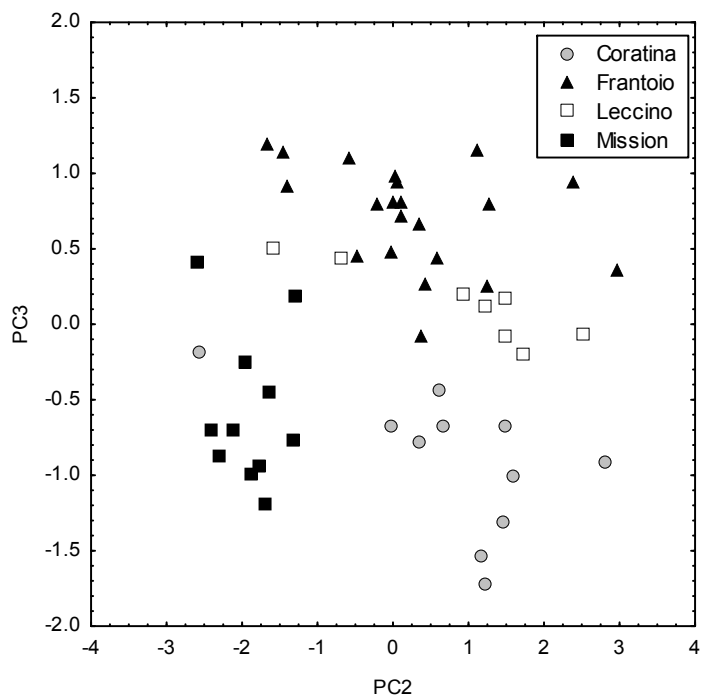


Figure 8 PCA score plot of PC2 versus PC3 for the peak intensities of the monovarietal olive oil samples only, showing the groups of cultivars.

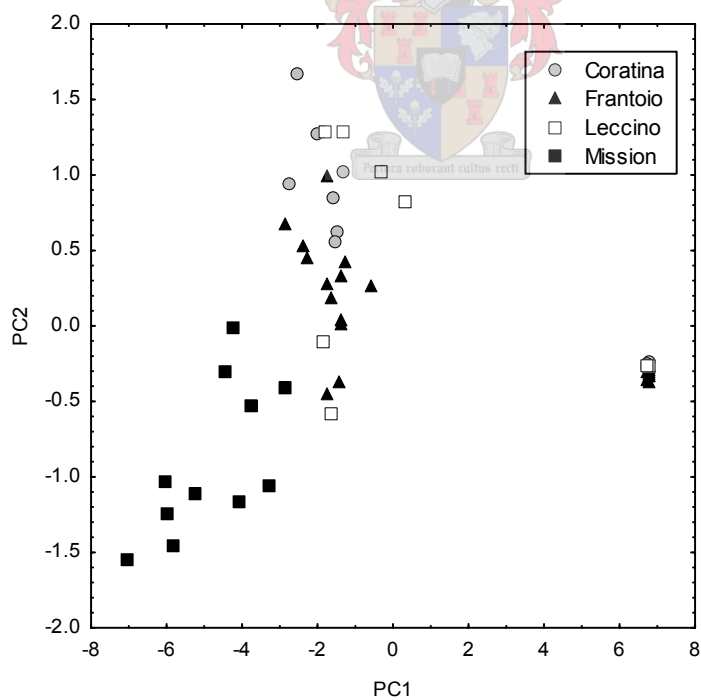


Figure 9 PCA score plot of PC1 versus PC2 for the peak heights of the monovarietal olive oil samples only, showing the groups of cultivars.

all olive oil samples pressed from Coratina and the majority of those pressed from Mission (82%) and Frantoio (81%) were classified correctly (Fig. 10). Only 63% of the Leccino samples were classified correctly. These results agree with those obtained during PCA, since Coratina and Mission samples formed well-defined clusters on the PCA score plot (Fig. 8) and the Leccino samples overlapped with the Frantoio samples. For the cultivar classification by LDA, differences in performance between intensity and peak height data became more apparent, as only the Mission (91%) and Frantoio (81%) samples could successfully be classified with peak height data (Fig. 11). Leccino and Coratina samples considerably overlapped on the PCA score plot (Fig. 9) and, correspondingly, these two cultivars could not be classified correctly. When comparing the results obtained with LDA to the actual fatty acid composition of the single cultivar olive oil samples, as determined by NMR, it can be observed that the oil samples pressed from Mission, which were successfully classified during LDA, had a higher linoleic and lower saturated fatty acid content than the other cultivars. Less consistent differences were observed for the other cultivars, as well as for oils of the same cultivar but different geographical region.

Conclusions

The classification of olive oils according to their oxidation level using NMR data was unsuccessful, due to the strong influence of olive cultivar and geographical region on the fatty acid composition of the olive oil samples. The sample set used in this study contained a large range of South African extra virgin olive oils, consisting of pure cultivars and blends of different oxidation levels, which made it difficult to classify samples according to minor changes in specific NMR intensities or peak heights. The study could be confirmed with a sample set consisting of olive oils of a single cultivar and geographical area to cancel the effect of these innate influences. Accelerated oxidation could be commenced with samples that were all pressed during the same season and are equally fresh, to prevent variation within distinct oxidation level groups. However, these circumstances would not represent conditions encountered when testing different olive oils marketed in South Africa and such statistical model would probably not be applicable in the industry.

^1H NMR spectroscopy should be considered as an alternative method for discrimination of olive oils of different oxidation levels by PCA. Although ^1H NMR spectroscopy does not allow the determination of the acyl position in the triglyceride molecule, it allows measurement of glycerol, saturated and unsaturated protons (Hidalgo & Zamora, 2003), diglycerides, hydroperoxides and minor compounds, such as volatile compounds (Mannina & Segre, 2002), which show more marked changes during oxidation.

The PCA of peak intensities and peak heights showed no convincing differences in performance and we can therefore deduce that the heights of NMR peaks, instead of their

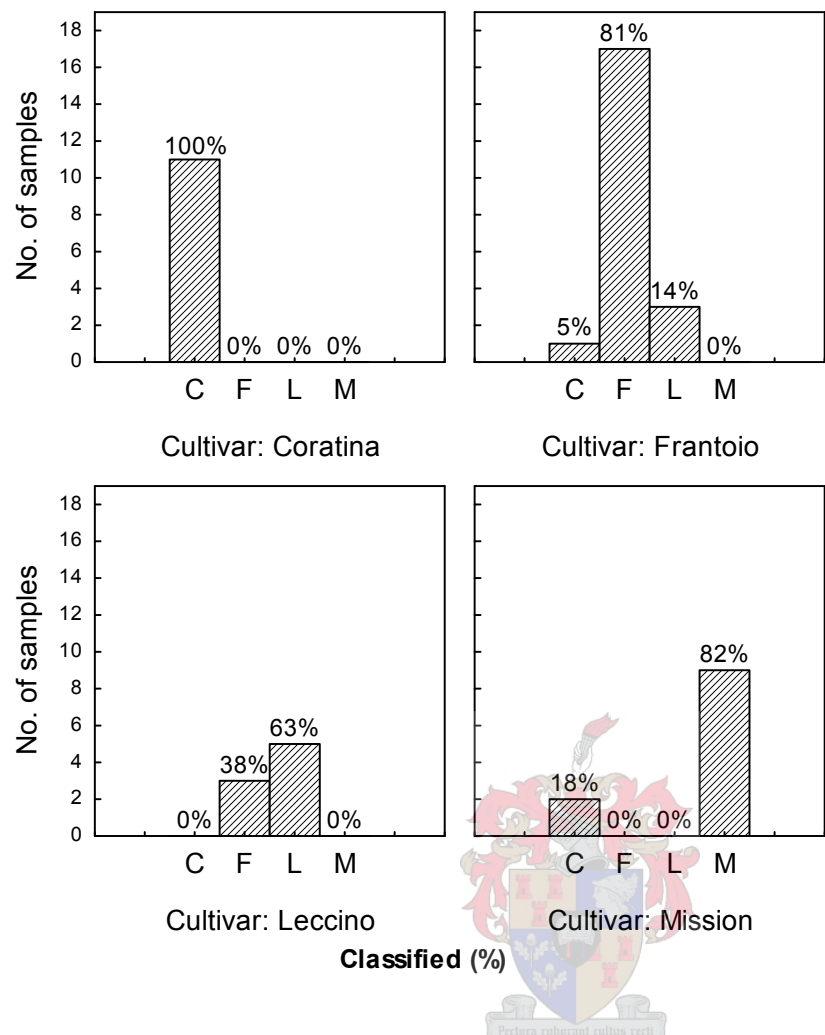


Figure 10 LDA results of the NMR peak intensities for the cultivar classification showing the percentages of samples classified correctly and incorrectly for each cultivar group (C = Coratina, F = Frantoio, L = Leccino, M = Mission).

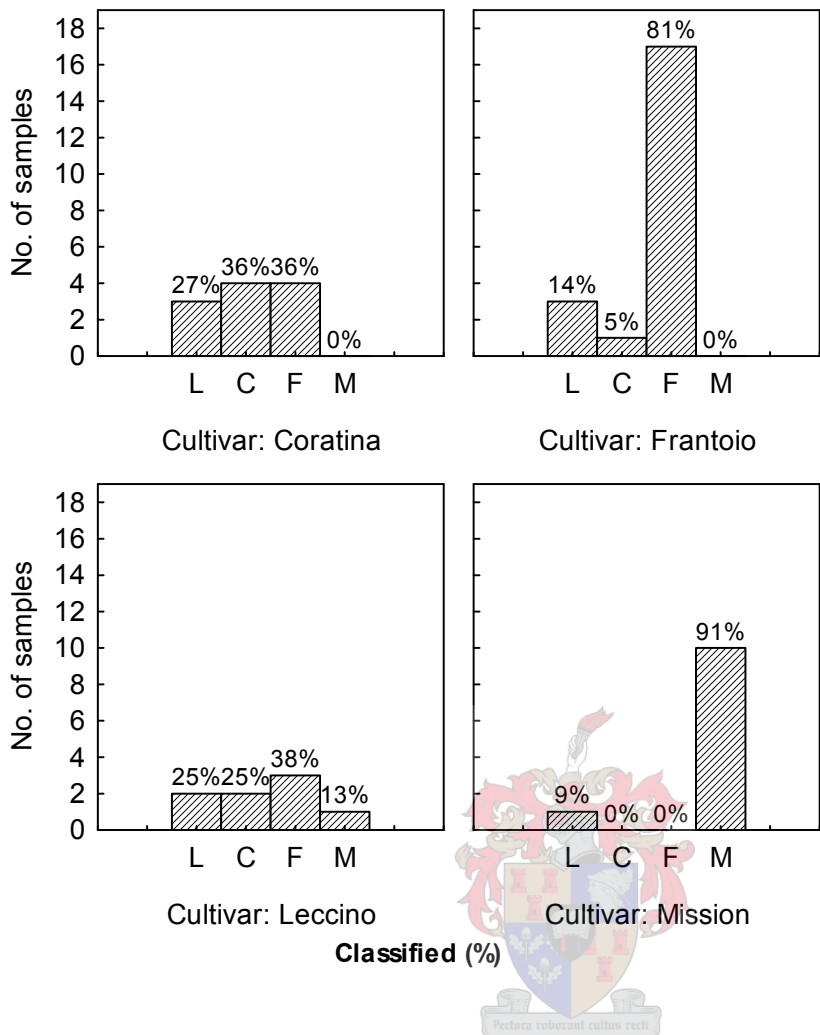


Figure 11 LDA results of the NMR peak heights for the cultivar classification showing the percentages of samples classified correctly and incorrectly for each cultivar group (C = Coratina, F = Frantoio, L = Leccino, M = Mission).

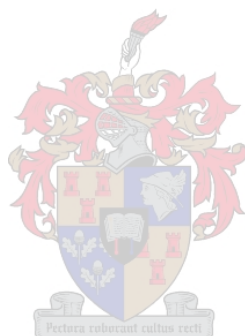
intensities may equally well be used for PCA to observe trends in a data set. However, for statistical models, such as LDA, NMR intensities give better results.

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Chapter 6

GENERAL DISCUSSION AND CONCLUSIONS



General Discussion and Conclusions

The South African olive industry is a relatively young, developing industry that is experiencing steady growth in both olive production and demand, and is expected to continue expanding in the years to come (Anon., 2004). All olive oil pressed in South Africa is of extra virgin quality and local producers have achieved international awards and export orders, confirming the superior quality of local olive oils. Nevertheless, almost 70% of the local consumption of olive oil is imported from mainly Spain, Italy and Greece. A great percentage of these imports consists of extra virgin olive oils from previous seasons, which when arriving on South African supermarket shelves have too often lost their extra virgin quality. As no regulating body exists in South Africa, yet, that would be able to verify the quality of imported, as well as locally produced oils, this study investigated the practicability of an affordable olive oil quality control technique by means of near infrared (NIR) spectroscopy.

The application of NIR spectroscopy to the analysis of olive oils has to date mainly concentrated on the detection of adulteration and the classification of olive oils according to their geographical origin or cultivar by qualitative NIR spectroscopy, and only few studies have been published on quantitative NIR applications (Garrido *et al.*, 2000; Bertran *et al.*, 2001; Jimenez-Marquez, 2003; Conte *et al.*, 2003; Garrido-Varo *et al.*, 2004). During this study, NIR spectroscopy prediction models were developed for olive oil quality criteria, including the official parameters peroxide value ($R^2 = 0.92$, $SEP = 4.15$ meq O₂ kg⁻¹), free fatty acid content ($R^2 = 0.69$, $SEP = 0.12\%$) and specific extinction coefficients K₂₃₂ ($R^2 = 0.94$, $SEP = 0.94$) and K₂₇₀ ($R^2 = 0.87$, $SEP = 0.094$), as well as the unofficial parameters chlorophyll ($R^2 = 0.56$, $SEP = 3.58$ mg kg⁻¹), carotenoid ($R^2 = 0.66$, $SEP = 1.14$ mg kg⁻¹) and total polyphenol content ($R^2 = 0.34$, $SEP = 82.10$ mg kg⁻¹) and thiobarbituric acid reactive substance value ($R^2 = 0.40$, $SEP = 0.0038$). Except for the thiobarbituric acid value, prediction models have been previously developed for these quality criteria for Italian and Spanish olive oils (Garrido *et al.*, 2000; Marquez, 2003; Conte *et al.*, 2003; Garrido-Varo *et al.*, 2004). The free fatty acid, total polyphenol, chlorophyll and carotenoid content prediction models developed for South African olive oil performed weaker than those developed for Spanish olive oils, whereas the peroxide value and specific extinction coefficient prediction models for South African olive oils showed better coefficients of determination when compared to those determined by Garrido *et al.* (2000). NIR prediction models were also developed for the determination of the linoleic ($R^2 = 0.90$, $SEP = 0.83\%$), oleic ($R^2 = 0.56$, $SEP = 1.47\%$) and saturated fatty acid contents ($R^2 = 0.88$, $SEP = 0.91\%$), as well as the positional distribution of linoleic ($R^2 = 0.29$, $SEP = 0.083$) and oleic acids ($R^2 = 0.85$, $SEP = 0.040$) on the triacylglycerol, expressed as the $\alpha:\beta$ ratios, with the application of ¹³C NMR spectroscopy as reference method. The application of ¹³C NMR spectroscopy as a reference method for the development of NIR prediction models was the first time investigated during this

study and prediction models for the saturated fatty acid content, as well as for the $\alpha:\beta$ ratios of oleic and linoleic acid have not been developed previously by other researchers. Except for the linoleic acid $\alpha:\beta$ ratio prediction model, acceptable model performance was achieved, although better coefficients of determination and *SEPs* were attained for the oleic and linoleic acid content by other researchers (L.S. Conte, Dipartimento di Scienze degli Alimenti, Università degli Studi di Udine, Udine, Italy, personal communication).

The weaker performance of some of the models developed during this study, compared to previous studies, indicates that reference methods might have to be optimised further, but also shows that there is room for improvement of these prediction models. It should be kept in mind that the aim of this research was, additionally, to compare prediction models developed from spectra collected on two spectrophotometers (Perkin Elmer IdentiCheck™ and Büchi NIRLab N-200), at different resolutions (64, 32, 16 and 8 cm^{-1}), path lengths (0.2, 0.5 and 0.6 mm) and sample presentation modes (transmittance and transreflectance). The performance of the respective prediction models was, therefore, compromised by the fact that calibration and validation sets had to be identical for the various models of the same parameter, to allow relevant statistical comparisons. Thus, with further research specifically aimed at developing NIR prediction models for the implementation in South African olive oil quality control, better model efficiency should be achieved.

The comparison of regression models developed from spectra collected on two spectrophotometers under diverse measuring conditions revealed that within one instrument (Perkin Elmer), lower resolutions (64 instead of 8 cm^{-1}) did not compromise the quality of NIR prediction models and small differences in path length (0.5 and 0.2 mm) did not show significant differences in model performance. In general, transmittance spectra (Perkin Elmer) collected at a resolution of 64 cm^{-1} and path length of 0.2 mm produced slightly better prediction models than transreflectance spectra (Büchi) collected at a resolution of 8 cm^{-1} and a path length of 0.6 mm, indicating that transmittance spectra might be more applicable for olive oil. However, since these differences in performance were not significant ($p > 0.05$) there is no definite evidence that either of the instruments produced better prediction models. Hence, during the development of NIR prediction models for application in olive oil quality control, the most user-friendly and time-efficient instrument settings could be chosen.

Statistical analysis of the ^{13}C NMR intensity and peak height data revealed that there was no correlation between the NMR resonances under investigation and the oxidation level of the olive oils. However, principal component analysis score plots showed limited grouping of the olive oil samples (various blends and single cultivars) according to geographical origin and single cultivar olive oils could be clearly grouped according to their respective cultivars used for pressing the oil. Linear discriminant analysis was unsuccessful in classifying olive oils into

groups of similar geographical origin, but could effectively classify single cultivar olive oils into their distinct cultivar groups. The results obtained during statistical analyses of the NMR data of the olive oils indicated that minor differences in intensity values due to oxidation related changes in fatty acid structure were covered up by the more evident differences in fatty acid composition due to cultivar and geographical origin. The classification of blended olive oils according to geographical origin by multivariate statistical analysis of ^{13}C NMR data is difficult (Vlahov *et al.*, 2001) and clear grouping of distinct geographical areas has as yet only been achieved with olive oils pressed from a single olive cultivar (Vlahov *et al.*, 1999; Mannina *et al.*, 2003; Vlahov *et al.*, 2003). The sample set of olive oils during this study consisted of different single cultivar oils and blended oils of varying degrees of oxidation, which explains why these could not be classified effectively into groups of geographical regions. On the other hand, the composition of this sample set is a realistic representation of South African olive oils that would have to be classified by the model in future and thus the applicability of such classification model is doubtful. The classification of single cultivar olive oils by linear discriminant analysis and ^{13}C NMR spectroscopy showed promising results and if stricter regulations regarding the labeling of olive oils in South Africa should arise, NMR spectroscopy could be a useful tool in identifying olive oil cultivar compositions.

NMR intensity data gave considerably better results than NMR peak heights during linear discriminant analysis, although, during principal component analysis no evident differences in results could be noted between intensity and peak height data. This observation points out that for the preliminary analysis of NMR data by principal component analysis, such as to identify trends in a data set, peak heights may well be sufficient for analysis. The heights of NMR peaks can more easily and rapidly be determined, by the use of the appropriate software accompanying the NMR spectrometer, than the peak intensities. A lot of unfruitful effort can thus be avoided by initially investigating possible trends in the data by using the selected NMR peak heights, before completing more detailed analyses of the data by using NMR intensities in combination with statistical models, such as linear discriminant analysis.

From the results obtained during this study of NIR and NMR spectroscopy of South African extra virgin olive oils, it is evident that NIR spectroscopy is more successful than NMR spectroscopy in detecting chemical changes that occur as olive oil becomes oxidised. On the other hand, NMR spectroscopy was able to classify olive oils according to differences in the fatty acid composition, e.g. due to cultivar differences, which could not be achieved as effectively with NIR spectral data. It was established that NMR spectroscopy can productively be applied as a reference method for the development of NIR prediction models, such as for the rapid determination of major fatty acids in olive oil and the $\alpha:\beta$ ratios of oleic and linoleic acids. With NMR spectroscopy as reference method, NIR prediction models can therefore be employed to

rapidly gain information on, not only chemical parameters of olive oil, but the fatty acid composition as well. Since, NIR spectroscopy is a faster, more affordable analytical technique that requires less sample preparation and expertise than NMR spectroscopy, the former would be the preferred method to confirm the quality and authenticity of South African olive oils. It can thus be concluded that with further research and improvement of the current NIR prediction models for the rapid quality control of South African extra virgin olive oil and with appropriate support from the industry and government, NIR spectroscopy could be implemented as a quality control tool.

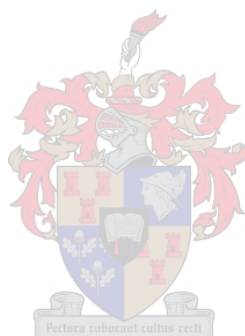
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APPENDICES



Appendix 1 Summary of research published on oxidation experiments on olive oil.

Analysis Method	Limit EVOO	Sample Oxidation	Time to Limit	Author(s)
Free fatty acids (% oleic acid)	< 0.8	<ul style="list-style-type: none"> Vials at 36°C, with and without fluorescent light, sampling every 24 h for 1 week then at day 14, 28, 56 and 90 		Gutierrez-Rosales <i>et al.</i> , 1992
		<ul style="list-style-type: none"> FFA range prepared by spiking oil with 0.15% enzyme, incubator 60°C, sample taken at 15 min intervals 		Che Man & Moh, 1998
		<ul style="list-style-type: none"> Oil from olives stored at 5/ 7.5°C in air/ 2% O₂/ 5% CO₂/ 2% O₂ + 5% CO₂ for 40 days 		Kiritsakis <i>et al.</i> , 1998
		<ul style="list-style-type: none"> Rancimat (Air at 10 L/ h and 100°C), Sampling at 8 ox. times 		Gutierrez <i>et al.</i> , 2002
		<ul style="list-style-type: none"> Clear 1L glass bottles at 30°C + light/ 2°C in dark for 6 months 	Did not reach limit	Gutierrez & Fernandez, 2002
K₂₃₂ and K₂₇₀	270 nm < 0.22 232 nm < 2.50	<ul style="list-style-type: none"> Oven test at 65 and 100°C in Petri dishes, room temp. and 50°C in clear jar, loosely capped 		Kiritsakis <i>et al.</i> , 1983
		<ul style="list-style-type: none"> Vials at 36°C, with and without fluorescent light, sampling every 24 h for 1 week then at day 14, 28, 56 and 90 		Gutierrez-Rosales <i>et al.</i> , 1992
		<ul style="list-style-type: none"> Transparent, open glass bottles (3 cm Ø), 40°C in dark 		Blekas <i>et al.</i> , 1995
		<ul style="list-style-type: none"> Oil from olives stored at 5/ 7.5°C in air/ 2% O₂/ 5% CO₂/ 2% O₂ + 5% CO₂ for 40 days 		Kiritsakis <i>et al.</i> , 1998
		<ul style="list-style-type: none"> Rancimat (Air at 10 L/ h and 100°C), Sampling at 8 ox. times 		Gutierrez <i>et al.</i> , 2002
		<ul style="list-style-type: none"> Clear 1L glass bottles at 30°C + light/ 2°C in dark for 6 months 	K ₂₃₂ limit not exceeded, K ₂₇₀ limit exceeded at ca. 50-60 days for oil at 30°C	Gutierrez & Fernandez, 2002

Analysis Method	Limit EVOO	Sample Oxidation	Time to Limit	Author(s)
TBARS value	Na.	<ul style="list-style-type: none"> Oven test at 65 and 100°C in Petri dishes, room temp. and 50°C in clear jar, loosely capped 		Kiritsakis <i>et al.</i> , 1983
Total polyphenols	> 100 mg/ L	<ul style="list-style-type: none"> Oil from olives stored at 5/ 7.5°C in air/ 2% O₂/ 5% CO₂/ 2% O₂ + 5% CO₂ for 40 days Rancimat (Air at 10 L/ h and 100°C), Sampling at 8 ox. times Oil stored in amber glass bottles in darkness for 12 months 	Below limit for late harvesting period oils	Kiritsakis <i>et al.</i> , 1998 Gutierrez <i>et al.</i> , 2002 Morello <i>et al.</i> , 2004
Chlorophyll/ carotenoid content	Na.	<ul style="list-style-type: none"> Vials at 36°C, with and without fluorescent light, sampling every 24 h for 1 week then at day 14, 28, 56 and 90 Oil from olives stored at 5/ 7.5°C in air/ 2% O₂/ 5% CO₂/ 2% O₂ + 5% CO₂ for 40 days Rancimat (Air at 10 L/ h and 100°C), Sampling at 8 ox. times Oil stored in amber glass bottles in darkness for 12 months 		Gutierrez-Rosales <i>et al.</i> , 1992 Kiritsakis <i>et al.</i> , 1998 Gutierrez <i>et al.</i> , 2002 Morello <i>et al.</i> , 2004
Sensory analysis	> 6.5	<ul style="list-style-type: none"> Rancimat (Air at 10 L/ h and 100°C), Sampling at 8 ox. times Clear 1L glass bottles at 30°C + light/ 2°C in dark for 6 months 	2°C oil stayed above limit, 30°C oil lost extra virgin quality at 49-56 days	Gutierrez <i>et al.</i> , 2002 Gutierrez & Fernandez, 2002

Analysis Method	Limit EVOO	Sample Oxidation	Time to Limit	Author(s)
Peroxide value	< 20 meq O ₂ kg ⁻¹	<ul style="list-style-type: none"> Oven test at 65 and 100°C in Petri dishes, room temp. and 50°C in clear jar, loosely capped 		Kiritsakis <i>et al.</i> , 1983
		<ul style="list-style-type: none"> Oils contained in glass and polyethylene bottles stored in light and dark, 5 cm headspace and no headspace, respectively 	In light: ca. 1 month In dark: ca. 5 month	Kiritsakis, 1984
		<ul style="list-style-type: none"> Vials at 36°C, with and without fluorescent light, sampling every 24 h for 1 week then at day 14, 28, 56 and 90 		Gutierrez-Rosales <i>et al.</i> , 1992
		<ul style="list-style-type: none"> Transparent, open glass bottles (3 cm Ø), 40°C in dark 		Blekas <i>et al.</i> , 1995
		<ul style="list-style-type: none"> 10 g oil in 50 ml screw capped Erlenmeyer flask oxidized in dark in shaker oven at 60°C 		Satue <i>et al.</i> , 1995
		<ul style="list-style-type: none"> Oil from olives stored at 5/ 7.5°C in air/ 2% O₂/ 5% CO₂/ 2% O₂ + 5% CO₂ for 40 days AND oil samples heated at 63°C 		Kiritsakis <i>et al.</i> , 1998
		<ul style="list-style-type: none"> Storage at room temp. in the dark in screw-capped glass bottles for 7 months AND oxidation at 37/75°C in water bath in bulk phase/ thin-layer up to 144 h 		Deiana <i>et al.</i> , 2002
		<ul style="list-style-type: none"> Clear 1L glass bottles at 30°C + light/ 2°C in dark for 6 months 	Max. PV did not exceed limit	Gutierrez & Fernandez, 2002
		<ul style="list-style-type: none"> Rancimat (Air at 10 L/ h and 100°C), Sampling at 8 ox. times 		Gutierrez <i>et al.</i> , 2002
		<ul style="list-style-type: none"> Oils in transparent glass bottles (no headspace) stored in dark at room temperature 	Above limit after 24 months	Psomiadou & Tsimidou, 2002
		<ul style="list-style-type: none"> Stored in clear/ dark, closed glass bottles, filled/ half-empty at room temp. in diffuse light for 12 months, samples taken every 2 months 	Half-empty clear bottle – 10 months Half-empty dark bottle – 12 months	Rastrelli <i>et al.</i> , 2002

EVOO = Extra virgin olive oil
Na. = Not applicable

Appendix 2 Current knowledge on quantitative near infrared spectroscopic measurements of vegetable oils.

Parameter	Application	Reference method	RMSEP/ SEP/ SECV	R ²	Author(s)
Peroxide value	Canola, olive and safflower (purified), soybean and cotton seed (not purified)	AOAC official method	2084 nm: Canola SE = 8.5 meq kg ⁻¹ Olive SE = 12.7 meq kg ⁻¹ Safflower SE = 19.2 meq kg ⁻¹ Soybean SE = 0.83 meq kg ⁻¹ Cotton seed SE = 1.37 meq kg ⁻¹	2084 nm: Canola R ² = 0.99 Olive R ² = 0.99 Safflower R ² = 0.99 Soybean R ² = 0.99 Cotton seed R ² = 0.98	Takamura <i>et al.</i> , 1995
	Soybean oil		SEP = 9.67 meq kg ⁻¹ oil	R ² = 0.99	Cho <i>et al.</i> , 1998
	Sesame oil		SEP = 1.48 meq kg ⁻¹	R ² = 0.54	Ha <i>et al.</i> , 1998
	Palm oil	AOCS Official Method Cd 8-53	SEP = 0.170 meq kg ⁻¹	R ² = 0.99	Moh <i>et al.</i> , 1999
	Canola oil (olive and sunflower for validation)	AOCS Official Method Cd 8-53	Olive SD = 0.50 meq kg ⁻¹ Sunflower SD = 0.38 meq kg ⁻¹	Olive R ² = 0.94 Sunflower R ² = 0.98	Li <i>et al.</i> , 2000
	Soybean oil	AOCS Official Method Cd 8-53	SEP = 1.640 meq kg ⁻¹ (1 mm) SEP = 0.760 meq kg ⁻¹ (2 mm)	R ² = 0.98 (1 mm) R ² = 0.99 (2 mm)	Yildiz <i>et al.</i> , 2001
	Olive oil	IUPAC method 2-501	SECV = 1.34 meq kg ⁻¹	R ² = 0.92	Mailer, 2004
	Olive oil	EEC official method	SECV = 1.55 meq kg ⁻¹	R ² = 0.66	Garrido <i>et al.</i> , 2000
Free fatty acids	Palm oil	AOCS titration method, FFA expressed as % palmitic acid	RMSEP = 0.077% crude palm oil RMSEP = 0.041% RBD palm oleic RMSEP = 0.059% RBD palm oil	R ² = 0.99 crude palm oil R ² = 0.94 RBD palm olein R ² = 0.94 RBD palm oil	Che Man & Moh, 1998
	Soybean oil		SEP = 0.137 mg KOH g ⁻¹ oil	R ² = 0.99	Cho <i>et al.</i> , 1998
	Sesame oil		SEP = 0.51 mg KOH g ⁻¹	R ² = 0.98	Ha <i>et al.</i> , 1998
	Olive oil	EEC official method	SECV = 0.06%	R ² = 0.98	Garrido <i>et al.</i> , 2000
	Olive oil	EEC official method	Na.	R ² = 0.66	L.S. Conte, personal communication.
	Olive oil	AOCS Aa 6-38	SECV = 0.07%	R ² = 0.97	Mailer, 2004

Parameter	Application	Reference method	RMSEP/ SEP/ SECV	R ²	Author(s)
Anisidine value	Soybean oil	AOCS Official Method Cd 18-90	SEP = 0.590 (1mm) SEP = 0.920 (2mm)	R ² = 0.78 (1 mm) R ² = 0.90 (2 mm)	Yildiz <i>et al.</i> , 2001
Fatty acid composition	Soybean oil, palm oil	Gas chromatography	x	x	Sato <i>et al.</i> , 1991
	Sesame oil	Gas chromatography	Palmitic SEP = 0.33% Stearic SEP = 0.14% Oleic SEP = 0.52% Linoleic SEP = 1.04%	Palmitic R ² = 0.90 Stearic R ² = 0.78 Oleic R ² = 0.93 Linoleic R ² = 0.92	Ha <i>et al.</i> , 1998
	Olive oil	Gas liquid chromatography	Oleic acid RMSEP = 0.78% Linolenic acid RMSEP = 0.10% Linoleic acid RMSEP = 0.31%	Na.	Bertran <i>et al.</i> , 2001
	Olive oil	Gas chromatography	Oleic acid SECV = 0.47% Linoleic acid SECV = 0.23% Linolenic acid SECV = 0.06% Stearic acid SECV = 0.26% Palmitoleic acid SECV = 0.12% Pamitic acid SECV = 0.48%	Oleic acid R ² = 0.99 Linoleic acid R ² = 1.0 Linolenic acid R ² = 0.85 Stearic acid R ² = 0.86 Palmitoleic acid R ² = 0.87 Pamitic acid R ² = 0.91	Mailer, 2004
	Olive oil	EEC official methods	Oleic SEP = 0.383% Linoleic SEP = 0.108%	Oleic acid R ² = 0.88 Linoleic acid R ² = 0.99	L.S. Conte, personal communication.
K₂₃₂ and K₂₇₀	Soybean oil	CD value: AOCS Official Method Ti 1a-64	SEP (K ₂₃₂) = 0.021% (1 mm) SEP (K ₂₃₂) = 0.021% (2 mm)	R ² (K ₂₃₂) = 0.91 (1 mm) R ² (K ₂₃₂) = 0.86 (2 mm)	Yildiz <i>et al.</i> , 2001
	Olive oil	Na.	SECV (K ₂₃₂) = 0.07 SECV (K ₂₇₀) = 0.02	R ² (K ₂₃₂) = 0.64 R ² (K ₂₇₀) = 0.77	Garrido <i>et al.</i> , 2000
K₂₂₅	Olive oil	Na.	SECV = 0.02	R ² = 0.92	Garrido <i>et al.</i> , 2000
Total polyphenols	Olive oil	Cortesi method	SEP = 25.215 mg kg ⁻¹	R ² = 0.90	L.S. Conte, personal communication.
	Olive oil	Na.	SECV = 43.2 mg kg ⁻¹	R ² = 0.90	Garrido <i>et al.</i> , 2000
	Olive oil	Folin Ciocalteu	SECV = 58.67 mg kg ⁻¹	R ² = 0.89	Mailer, 2004

Parameter	Application	Reference method	RMSEP/ SEP/ SECV	R ²	Author(s)
Rancimat test	Olive oil	Rancimat (110°C, air flow rate 20L h ⁻¹)	SEP = 2.991 h	R ² = 0.86	L.S. Conte, personal communication.
	Olive oil	Rancimat	SECV = 9.10 h	R ² = 0.94	Garrido <i>et al.</i> , 2000
	Olive oil	Rancimat (130°C, air flow rate 20 L h ⁻¹)	SECV = 0.97 h	R ² = 0.88	Mailer, 2004
Tocopherol content	Olive oil	Dieffenbacher method	SEP = 49.424 mg kg ⁻¹	R ² = 0.62	L.S. Conte, personal communication.
Pigment content	Olive oil	UV-Vis spectrophotometry	Carotenoids SEP = 0.66 mg kg ⁻¹ Chlorophylls SEP = 0.96 mg kg ⁻¹	Carotenoids R ² = 0.97 Chlorophylls R ² = 0.98	Jimenez-Marquez, 2003
	Olive oil	AOCS method Ch 4-91	Chlorophyll SECV = 0.51 mg kg ⁻¹	R ² = 0.98	Mailer, 2004
Moisture	Olive oil	Na.	SECV = 0.04%	R ² = 0.76	Garrido <i>et al.</i> , 2000
Panel test score	Olive oil	Na.	SECV = 0.50	R ² = 0.88	Garrido <i>et al.</i> , 2000

RMSEP = Root mean square error of prediction

SD = Standard deviation

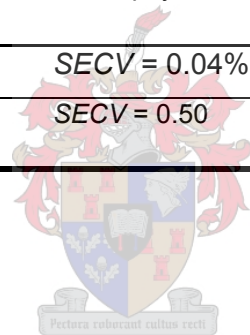
SE = Standard error

SEP = Standard error of prediction

SECV = Standard error of cross-validation

R² = Coefficient of determination

Na. = Not available



Appendix 3 Summary of publications on the analysis of olive oil with NMR spectroscopy.

Title	Journal, Vol., No., Pg.	Authors
A high field ^1H nuclear magnetic resonance study of the minor components in virgin olive oils	<i>J. Am. Oil Chem. Soc.</i> , 73 (6), 747-758	Sacchi <i>et al.</i> , 1996
Discrimination of the variety and region of origin of extra virgin olive oils using ^{13}C NMR and multivariate calibration with variable reduction	<i>Analytica Chimica Acta</i> , 348 , 357-374	Shaw <i>et al.</i> , 1997
^1H and ^{13}C NMR of virgin olive oil	<i>Magnetic resonance in Chemistry</i> , 35 , 125-132	Sacchi <i>et al.</i> , 1997
^{13}C NMR analysis of the triacylglycerol composition of Greek extra virgin olive oils	<i>Magnetic resonance in Chemistry</i> , 35 , 3-7	Mavromoustakos <i>et al.</i> , 1997
Rapid determination of iodine value by ^1H nuclear magnetic resonance spectroscopy	<i>J. Am. Oil Chem. Soc.</i> , 75 (1), 15-19	Miyake <i>et al.</i> , 1998
Characterization of Italian extra virgin olive oils using ^1H NMR spectroscopy	<i>J. Agric. Food Chem.</i> , 46 , 3947-3951	Sacchi <i>et al.</i> , 1998
Application of NMR to the study of olive oils	<i>Progress in Nuclear Magnetic Resonance Spectroscopy</i> , 35 (4), 341-357	Vlahov, 1999
Use of ^{13}C nuclear magnetic resonance distortionless enhancement by polarization transfer pulse sequence and multivariate analysis to discriminate olive oil cultivars	<i>J. Am. Oil Chem. Soc.</i> , 76 (10), 1223-1231	Vlahov <i>et al.</i> , 1999
A novel analytical method to detect adulteration of virgin olive oil by other oils	<i>J. Am. Oil Chem. Soc.</i> , 77 (4), 405-411	Mavromoustakos <i>et al.</i> , 2000
Characterization of Italian olive oils based on analytical and nuclear magnetic resonance determinations	<i>J. Am. Oil Chem. Soc.</i> , 77 (6), 619-625	Sacco <i>et al.</i> , 2000
High resolution ^1H nuclear magnetic resonance in the study of edible oils and fats	<i>Trends in Food Sc. & Tech.</i> , 12 , 328-228	Guillen & Ruiz, 2001

P.D.O. (Protected designation of origin): Geographical characterization of Tuscan extra virgin olive oils using high field ^1H NMR spectroscopy	<i>Ital. J. Food Sci.</i> , 1 (13), 53-63	Mannina <i>et al.</i> , 2001
Quantitative ^{13}C NMR method using the DEPT pulse sequence for the determination of the geographical origin (DOP) of olive oils	<i>Magnetic Resonance in Chemistry</i> , 39 , 689-695	Vlahov <i>et al.</i> , 2001
Use of high resolution ^{13}C nuclear magnetic resonance spectroscopy for the screening of virgin olive oils	<i>J. Am. Oil Chem. Soc.</i> , 78 (1), 89-94	Zamora <i>et al.</i> , 2001
Oil stability prediction by ^{13}C nuclear magnetic resonance spectroscopy	<i>J. Agric. Food Chem.</i> , 50 , 5825-5831	Hidalgo <i>et al.</i> , 2002
High resolution nuclear magnetic resonance: from chemical structure to food authenticity	<i>Grasas y Aceites</i> , 53 (1), 22-33	Mannina & Segre, 2002
Classification of vegetable oils by high resolution ^{13}C NMR spectroscopy using chromatographically obtained oil fractions	<i>J. Am. Oil Chem. Soc.</i> , 79 (3), 267-272	Zamora <i>et al.</i> , 2002
Edible oil analysis by high-resolution nuclear magnetic resonance spectroscopy: recent advances and future perspectives	<i>Trends in Food Sci. & Tech.</i> , 14 , 499-506	Hidalgo & Zamora, 2003
Olive oil as seen by NMR and chemometrics	<i>Spectroscopy Europe</i> , 15 (3), 6-14	Mannina <i>et al.</i> , 2003
Study of the cultivar-composition relationship in Sicilian olive oils by GC, NMR and statistical methods	<i>J. Agric. Food Chem.</i> , 51 , 120-127	Mannina <i>et al.</i> , 2003
Determination of geographical origin of olive oils using ^{13}C nuclear magnetic resonance spectroscopy. I – Classification of olive oils of the Puglia region with denomination of protected origin	<i>J. Agric. Food Chem.</i> , 51 , 5612-5615	Vlahov <i>et al.</i> , 2003
Rapid major component analysis and authentication of South African extra virgin olive oils by means of ^{13}C nuclear magnetic resonance spectroscopy	<i>South African Journal of Science</i> , 100 , 349-354	McKenzie & Koch, 2004
Detection of extra virgin olive oil adulteration with lampante olive oil and refined olive oil using nuclear magnetic resonance spectroscopy and multivariate statistical analysis	<i>J. Agric. Food Chem.</i> , 53 (8), 2810-2816	Fragaki <i>et al.</i> , 2005

