OPTIMISATION OF RETENTION OF MANGIFERIN IN *CYCLOPIA SUBTERNATA* DURING PREPARATION FOR DRYING AND STORAGE OF GREEN HONEYBUSH AND DEVELOPMENT OF NIR SPECTROSCOPY CALIBRATION MODELS FOR RAPID QUANTIFICATION OF MANGIFERIN AND XANTHONE CONTENTS

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Thesis presented in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE IN FOOD SCIENCE

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March 2008

DECLARATION

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

25/02/08

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ABSTRACT

Extraction efficiency of soluble solids (SS), total polyphenols (TP) and xanthones ($AlCl_3$ assay) from dried, green *Cyclopia subternata*, as affected by mass-solvent ratio, extraction time and solvents, was investigated. In addition the effect of solvent composition on extraction of mangiferin and hesperidin was determined. Extraction of 5 g plant material as opposed to 0.5 and 1 g resulted in lower recoveries of SS, TP and xanthones ($P<0.05$). Extraction of SS and TP increased during the initial 20 min of contact time, where after it remained constant ($P>0.05$). Water, 33% acetonitrile, ethanol (50, 80 and 100%), methanol (50 and 100%) and 70% acetone were investigated as extraction solvents. Extraction for 30 min with 33% acetonitrile on a steam bath or 50% ethanol at 64°C on a water bath proved to be the most effective for extraction of SS, TP and xanthones, while 33% acetonitrile was most effective in extracting hesperidin from *C. subternata*. However, 70% acetone was most effective in extracting mangiferin. A poor correlation ($r = 0.54$) was observed for the total antioxidant activity (TAA) of *C. subternata*, as determined for water extracts and with the mangiferin content determined by HPLC. A moderate correlation ($r = 0.85$) was, however, obtained for TAA and TP content.

The mangiferin content of green *C. subternata* can be determined using the aluminium chloride ($AlCl_3$) colorimetric method. A moderate correlation ($r = 0.87$) was found for the xanthone content of the plant material determined using the $AlCl_3$ colorimetric method and mangiferin content quantified by HPLC ($y = 1.2x + 0.54$) following extraction with hot water. For extraction using 33% acetonitrile a weaker correlation ($r = 0.74$; $y = 1.3x + 0.87$) was found between the xanthone and mangiferin contents. The xanthone content (determined by $AlCl_3$) of the plant material as extracted by the two solvents, correlated well ($r = 0.91$). Good correlations were also obtained, when comparing extractions with water and 33% acetonitrile, for determination of the SS (0.94) and mangiferin contents ($r = 0.97$) of the plant material.

Near infrared (NIR) spectroscopy was investigated as a rapid and more economical method for prediction of mangiferin and xanthone contents of dried, green *C. subternata* plant material. NIR spectroscopy calibration models can be used for screening purposes for the mangiferin and (SEP = 0.21 g.100 g^{-1}; $r = 0.82$) and xanthone (SEP = 0.27 g.100 g^{-1}; $r = 0.81$) contents.

The effect of various pre-drying treatments and storage temperatures on the colour, soluble SS, TP, mangiferin and hesperidin contents of green *C. subternata* was investigated. By steaming green *C. subternata* directly after maceration, its colour retention can be improved. Good stability was shown for mangiferin and hesperidin during manufacture and storage of *C. subternata.*

UITTREKSEL

Doeltreffendheid van ekstraksie van oplosbare vastestowwe (OVS), totale polifenole (TP) en xantone ($AlCl_3$ bepaling) van groen, *Cyclopia subternata*, soos geaffekteer deur die massa-oplosmiddel verhouding, ekstraksietyd en oplosmiddels, is ondersoek. Bykomend is die effek van samestelling van oplosmiddel op die ekstaksie van mangiferien en hesperidien bepaal. Ekstraksie van 0.5 and 1 g plantmateriaal het laer OVS, TP en xantoon ($P<0.05$) opbrengste gelewer as dié van 5 g. Ekstraksie van OVS en TP het toegeneem na 20 min kontak tyd, waarna dit konstant gebly het ($P>0.05$). Water, 33% asetonitriel, etanol (50, 80 and 100%), metanol (50 and 100%) en 70% asetoon is ondersoek as ekstraksie oplosmiddels. Ekstraksie vir 30 min met 33% asetonitriel op 'n stoombad of met 50% etanol by 64°C op 'n waterbad was mees effektief vir ekstraksie van OVS, TP en xantone, terwyl 33% asetonitriel mees effektief was vir ekstraksie van hesperidien. In teenstelling was 70% asetoon mees effektief vir die ekstraksie van mangiferien. 'n Swak korrelasie ($r = 0.54$) tussen totale antioksidant aktiwiteit (TAA) en mangiferieninhoud, gekwantifiseer met behulp van HPLC, is waargeneem vir *C. subternata* waterekstrakte. 'n Matige korrelasie ($r = 0.85$) is egter tussen TAA en TP-inhoud waargeneem.

Die mangiferieninhoud van groen *C. subternata* kan bepaal word met behulp van die aluminiumchloried ($AlCl_3$) kolorimetriese metode. 'n Matige korrelasie ($r = 0.87$) vir warm water ekstrakte is waargeneem tussen die xantooninhoud van die plant material ($AlCl_3$ metode) en mangiferieninhoud, gekwantifiseer deur HPLC ($y = 1.2x + 0.54$). Ekstraksie met 33% asetonitriel het 'n swakker korrelasie ($r = 0.74$; $y = 1.3x + 0.87$) getoon tussen xantoon- en mangiferieninhoud. Na ekstraksie met onderskeidelik water en 33% asetonitriel, is 'n goeie korrelasie ($r = 0.91$) vir die xantooninhoud (bepaal deaur $AlCl_3$) van die plantmateriaal tussen die twee oplosmiddels gevind. 'n Goeie korrelasies is ook waargeneem vir die OVS-inhoud ($r = 0.94$) en die mangiferieninhoud ($r = 0.97$) van die plant material tussen laasgenoemde oplosmiddels.

Naby infrarooi (NIR) spektroskopie is ondersoek as 'n vinnige en meer ekonomiese metode om die mangiferien- en xantooninhoud van droë, groen *C. subternata* plant material te voorspel. NIR spektroskopie kalibrasie modelle kan gebruik word om die mangiferien (SEP = 0.21 g.100 g^{-1}; $r = 0.82$) en xantooninhoud (SEP = 0.27 g.100 g^{-1}; $r = 0.81$) rofweg te bepaal.

Die effek van verskillende behandelings voor droging en opbergtemperature op die kleur, OVS, TP, mangiferien- en hesperidieninhoud van groen *C. subternata* is ondersoek. Deur die groen *C. subternata* te stoom direk na dit gekerf is, kan die behoud van kleur verbeter word. Goeie stabiliteit is waargeneem vir mangiferien en hesperidien tydens prosessering en opberging van *C. subternata*.

ACKNOWLEDGEMENTS

I recognise the following persons and institutions for their contribution to the successful completion of this thesis:

Dr Marena Manley, my study leader, for her patience, guidance, positive criticism and continuous support throughout the writing of this thesis;

Dr Lizette Joubert, my co-study leader, for her valuable suggestions, guidance and interest shown during this study;

Dr Martin Kidd for statististical analysis performed and unlimited patience;

ARC Infruitec-Nietvoorbij (especially the friendly staff) for the use of their facilities;

National Research Foundation (NRF) for funding and student bursary (FA 2004050300006);

THRIP (Technology and Human Resources for Industry Programme) for funding;

Raps Foundation, Germany for funding;

Buchi, Flawil, Switzerland for the loan of the NIRLab N-200 and NIRCal 4.21 software;

Marilize Joubert of ARC Infruitec-Nietvoorbij for her assistance during the harvesting of honeybush plant material;

Mariza Botha and Christie Malherbe for assisting me on the HPLC, Dalene de Beer who was always willing to help in times of crisis situations and for her advice. George Dico for his technical assistance.

My mother, for her prayers, unconditional love, support, and ongoing encouragement.

Today is the day to complete what you worked towards yesterday and a wonderful opportunity to achieve what you dream to accomplish tomorrow.

Katrina Costa

ABBREVIATIONS

ABTS	2,2'-Azinobis(3-ethyl-benzothiazoline-6-sulphonate)
ACN	Acetonitrile
ACT	Acetone
AFB1	Aflatoxin B1
AH	Antioxidants
Anon	Anonymous
ANOVA	One-way analysis of variance
ARC	Agricultural Research Council
$AlCl_3$	Aluminium chloride
a_w	Water activity
BHA	Butylated hydroxyanisole
BHT	Bytylated hydroxytoluene
ca.	circa (about)
cm	Centimetre
DAD	Diode array detector
DM	Dry matter
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	α,α-Diphenyl-β-picrylhydrazyl
EC	(-)-Epicatechin
ECG	(-)-Epigallocatechin
EDTA	Ethylenediaminetetra-acetic acid di-sodium salt dehydrate
e.g.	*exempli gratia* (for example)
etc.	*et cetera* (and so forth)
et al.	*et alibi* (and elsewhere)
EtOH	Ethanol
FC	Folin-Ciocalteau
FDA	Food and Drug Administration
FRAP	Ferric reducing antioxidant power
FT	Fourier transform
GAP	Good Agricultural Practice

GLP	Good Laboratory Practice
GMP	Good Manufacturing Practice
GRAS	Generally Recognised As Safe
HCl	Hydrochloric acid
Hd	Hesperidin
HPLC	High performance liquid chromatography
i.e.	*id est* (that is)
ILSI	The International Life Sciences Institute
LC-MS	Liquid chromatography mass spectroscopy
LDL	Low-density lipoprotein
M	Molar
MAE	Microwave-assisted extraction
MeOH	Methanol
Mg	Mangiferin
min	Minutes
mL	Millilitre
mm	Millimetre
MSPD	Matrix solid phase dispersion
m/v	Mass per volume
n	Number of samples
NIR	Near infrared
nm	Nanometer
OTC	Over-the-counter
PCA	Principal component analysis
PDE	Permitted daily exposure
PEF	Pulsed electric field
PFE	Pressurised fluid extraction
PLE	Pressurised liquid extraction
PLS	Partial least square
ppm	Parts per million
PPO	Polyphenol oxidase
r	Coefficient of correlation
R^2	Coefficient of determination

RPD	Ratio of standard error of prediction to standard deviation
$SCCO_2$	Supercritical carbon dioxide extraction
SD	Standard deviation
SECV	Standard error of cross-validation
SEL	Standard error of laboratory
SEP	Standard error of prediction
SFE	Supercritical fluid extraction
spp.	Species
SPE	Solid-phase extraction
SPME	Solid-phase micro extraction
SOP	Standard operating procedures
stm	Steam bath
SS	Soluble solids
TAA	Total antioxidant activity
TGA	Therapeutic Goods Administration
TLC	Thin layer chromatography
TP	Total polyphenol
µg	Microgram
µL	Microlitre
µm	Micrometre
USA	United States of America
UV	Ultraviolet
UV/Vis	Ultraviolet-visible
v/v	Volume per volume
wb	Water bath
Xn	Xanthone

CONTENTS

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

CHAPTER 1
INTRODUCTION

Honeybush tea, prepared from *Cyclopia* species which is endemic to the Cape fynbos biome, is a traditional South African beverage (Du Toit *et al*., 1998). It is still a relatively new entrant on the global tea market, but the popularity of the tea is growing and it could be an alternative herbal beverage on the international market. It is mostly consumed in its fermented (oxidised) form, but small quantities of unfermented (green) honeybush tea are also sold. Health issues, which have become increasingly important among consumers, have resulted in a thriving market for natural products with medicinal value (Pettigrew, 1997). The demand for green honeybush is in line with the positive consumer perception of ordinary green tea (*Camellia sinensis*). Green tea is one of the world's most popular beverages due to its reported health benefits i.e., antimutagenic, antioxidant, anticarcinogenic and anti-arteriosclerotic properties (Wang *et al*., 1989).

The pharmacological effects of green tea are believed to be due to the flavonoids which have been identified as being important for overall health and the prevention of degenerative diseases such as cancer (Anon., 2006). Although the phenolic composition of honeybush tea (Ferreira *et al*., 1998; Kamara *et al.,* 2003; Kamara *et al.,* 2004) differs significantly from that of green tea (Balentine *et al.*, 1997), it has been found to contain flavonoids with significant antioxidant properties (Hubbe & Joubert, 2000a; Hubbe & Joubert, 2000b; Richards, 2002). Honeybush has a very low tannin content (Terblanche, 1982) and contains no caffeine (Greenish, 1881), making it a healthy alternative to other beverages. Although the genus *Cyclopia* (Fabaceae) consists of about 24 species, the commercial supply of honeybush is mostly obtained from *C. subternata, C. intermedia* and *C. genistoides* (Anon., 2007).

Herbal teas have become increasingly popular as health beverages and/or nutraceuticals (Wang *et al*., 2000) and this has contributed to strengthen the honeybush tea industry's existence since its formalisation in the 1990's. During 1997 *ca*. 30 tons of honeybush plant material was processed for production of tea. However, by the year 2000 production had reached about 160 tons and in 2005 it exceeded 350 tons with export comprising the major share of the industry (E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, personal communication, 2006).

Extracts, rich in polyphenols, are prepared from natural plant products and are used as antioxidants in the food, nutraceutical and cosmetic industries (Pettigrew, 1997; Andersen *et al*., 2005). The inhibition of mouse skin tumour formation by a *Cyclopia* extract (Marnewick *et al*., 2005) underlines

its potential application by the pharmaceutical industry. Up to now, however, only in vivo investigation using the species *C. intermedia* has been undertaken. A need exists to search for, isolate and characterise new sources of polyphenols, which could impede unfavourable ageing processes in the human body (Gramza & Korczak, 2005). The focus has recently shifted to xanthones, with sources such as *Garcinia mangostana* (mangosteen) (Anon., 2005) and *Garcinia indica* (Tamil-Selvi *et al*., 2003). Phenolic constituents have also been isolated from mango peel (Berardini *et al*., 2005) and stem bark (Núñez-Sellés *et al*., 2002) with the xanthone glycoside, mangiferin, being the predominant component. A patented process for the extraction of polyphenols from mango stem bark (*Mangifera indica* L.) has been used to produce the product, Vimang® (Núñez-Sellés *et al*., 2002). Xanthones have been found to possess antifungal, antimicrobial, antioxidant and cytotoxic activities (Jung *et al*., 2006).

The main polyphenols identified in *Cyclopia* spp. are mangiferin (a xanthone glycoside) and hesperidin (a flavanone glycoside) (De Nysschen *et al*., 1996; Ferreira *et al*., 1998; Kamara *et al*., 2004). *Cyclopia* spp. contain a complex mixture of phenolic compounds and qualitative analysis performed on fermented *C. intermedia* (Ferreira *et al*., 1998; Kamara *et al*., 2003) and unfermented *C. subternata* (Brand, 2002; Kamara *et al*., 2004) has revealed that phenolic profiles isolated from these two species differ greatly. A recent study showed unfermented *C. genistoides* to be a good source of mangiferin (Joubert *et al*., 2003; Botha, 2005), supporting its use for the preparation of mangiferin-enriched extracts. Its aqueous extract contains *ca*. 10.04 g.100 g^{-1} mangiferin. Unfermented *C. subternata* contains a substantially lower mangiferin content (*ca*. 1.69 g.100 g^{-1}). However, its aqueous soluble solids exhibit the highest total polyphenol (TP) content. Furthermore, its antioxidant activity determined with the 2,2'-azinobis(3-ethyl-benzothiazoline-6-sulphonate) ABTS and FRAP assays was comparable to that of *C. genistoides* (Richards, 2002), suggesting its potential use as antioxidant extract.

The hesperidin content of aqueous extracts of *C. subternata* (*ca*. 0.4 g.100 g^{-1}) is almost half of that of *C. genistoides* (*ca*. 0.92 g.100 g^{-1}) (Van der Merwe, 2005). Although the contribution of hesperidin to the total antioxidant activity (TAA) in the ABTS assay (Richards, 2002) of aqueous extracts of *Cyclopia* spp. is lower than mangiferin, based on concentration and activity, its anti-inflammatory properties (Guardia *et al*., 2001; Rotelli *et al*., 2003), however, makes it a valuable addition to potential nutraceutical products produced *C. subternata.* The renewability of this source of mangiferin and hesperidin through cultivation further supports its use.

Honeybush possesses antimutagenic (Marnewick *et al*., 2000; Richards, 2002; Van der Merwe, 2005) and antioxidant activities (Hubbe, 2000; Richards, 2002). Unfermented *C. subternata* has a significantly ($p<0.05$) higher protection against aflatoxin B_1 (AFB_1) induced mutagenesis (base pair

substitution) than fermented *C. subternata,* as well as unfermented *C. genistoides* (Van der Merwe *et al.*, 2006). The difference between the fermented and unfermented *C. subternata* can be partially attributed to the higher total polyphenol content in the unfermented species.

In South Africa, extraction of phenolic compounds from honeybush is currently undertaken using water as solvent. Consequently this can be related to the composition of an ordinary cup of tea. Whereas mangiferin has good solubility in water (Núñez-Sellés *et al*., 2002), hesperidin is poorly soluble in water (Escribano-Báilon & Santos-Buelga, 2003), emphasing the need to optimise extraction conditions for good extraction of both compounds.

For the preparation of polyphenol-enriched extracts, extracts should be produced from plant material containing high levels of polyphenols. Traditionally processed honeybush (fermented) is not a good source of mangiferin, hesperidin and total polyphenols as major losses occur during processing (Richards, 2002). Enhanced antioxidant and antimutagenic potencies are obtained from green honeybush extracts (Richards, 2002; Van der Merwe *et al*., 2006) indicating that it is the preferred material to be used for preparation of nutraceutical extracts. Manufacturing processes should thus be such to minimise the chemical changes in the plant material. During production of unfermented honeybush, browning of leaves due to oxidative changes takes place, if not inhibited timely, and would result in a reduction in the total polyphenol content. The enzymatic browning of leaves is due to oxidative changes which take place and result in a reduction in the mangiferin content (E. Joubert, ARC, Infruitec-Nietvoorbij, Stellenbosch, South Africa, personal communication, 2005). It is, however, not known to what extent the maceration of leaves, delays in drying and drying conditions of the unfermented plant material affect TP, mangiferin and hesperidin contents and needs further investigation.

Generally, the phenolic composition and contents of plant material differ among the individual plants, species and even extracts of the same plant (He, 2000). In the case of honeybush tea, plants used by industry are not from the same geno- and phenotypes. Therefore large variation in quantitative composition could be expected. Furthermore, factors such as the age of the plant, the leaves (plucking position), harvesting time, locality, climate, drying process and storage conditions all contribute to variation (Fernández *et al*., 2002). This indicates the need for efficient quality control of the plant material used in the food and phytopharmaceutical industries (He, 2000; Laasonen *et al*., 2002; Franke, *et al*., 2004). Howard *et al.* (2002) found spinach, collected during different growing seasons, to have different levels of flavonoid content as well as antioxidant capacities, while genotypes also appeared to play an important role in affecting phenolic metabolism and antioxidant capacity. Du Pont *et al*. (2000) observed differences in the flavonoid glycoside content in eight lettuce varieties. It is well known that

many food antioxidants can be significantly reduced (Du Pont *et al*., 2000; Franke *et al*., 2004) as a consequence of sterilisation, pasteurisation, as well as prolonged storage. However, some processing methods and storage conditions also showed retention of flavonoids or even development of antioxidants with novel properties (Nicoli *et al*., 1997; Manzocco *et al*., 1998).

There are currently no regulatory quality standards for the composition of honeybush tea (Anon., 2000). The ubiquitous presence of mangiferin and hesperidin in *Cyclopia* spp. (De Nyschen *et al*., 1996) and their high concentration relative to other monomeric polyphenols (Van der Merwe, 2005) suggest their use as marker compounds for quality control purposes, either of the plant material or of extracts. The product specifications for extracts comprise currently TP content and TAA as determined by the Folin-Ciocalteau (Singleton & Rossi, 1965) and ABTS assays (Re *et al*., 1999), respectively (E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, personal communication, 2005).

A rapid, easy-to-use method of analysis could be beneficial for screening of the dried plant material at an early stage of processing. This is necessary to ensure minimum requirements in terms of the content of phenolic compounds and confirmation of their retention in the final extracts. NIR spectroscopy, which is also non-destructive, could be such a method (Day & Fearn, 1982; Zhang *et al.*, 2004). Other advantages include little or no sample preparation and no production of chemical wastes (Starke *et al*., 1986).

Qualitative and quantitative NIR spectroscopy analyses have been applied with great success by the food (Osborne, 1991; Kawano *et al*., 1992; Rannou & Downey, 1997; Blanco *et al*., 1998; Büning-Pfaue *et al*., 1998; Karoui *et al*., 2006; Haiduc *et al*., 2007) and also non-food (Grunewald *et al*., 1998; Cleve *et al*., 2000; Dematthê *et al*., 2004; Lima *et al*., 2004; Ludwig *et al*., 2006) industries. It has also become a very useful tool for analysis of herbal plants e.g., the quantification of glycyrrhizic acid in licorice (*Glycyrrhizia uralensis* Fisch) (Wang *et al*., 2007), enchinacosides in Echinacea roots (Schulz *et al*., 2002) and harpagoside in devil's claw (*Harpagophytum procumbens*) root (Joubert *et al*., 2005). The ability of NIR spectroscopy to predict the total antioxidant activity (Zhang *et al*., 2004) and total polyphenol content in green tea was also demonstrated (Zhang *et al.*, 2004; Chen *et al*., 2006a; Chen *et al*., 2006b). NIR spectroscopy can effectively predict the aspalathin and dihydrochalcone contents in dried, green rooibos plant material, but did not give results of acceptable accuracy for the prediction of the different compounds, TP content, SS content and TAA of its water extracts (Manley *et al*., 2006). The prediction of mangiferin and hesperidin contents in dried green *C. genistoides* material was shown to be adequate for screening purposes and can thus be used for quality control (Joubert *et al*., 2006). Robustness of the calibration models was ensured by including two types of *C. genistoides*, samples representative of seasonal variation, as well as samples consisting of only stems or leaves. To date, no

such NIR spectroscopy calibration models exist for the prediction of mangiferin and hesperidin contents in *C. subternata* plant material.

NIR spectroscopy has been applied to identify and classify plant species (Laasonen *et al*., 2002; Zhao *et al*., 2006; Chen *et al*., 2007), cultivation areas (Woo *et al*., 2002) as well as geographical origins (Woo *et al*., 1999; Wang *et al*., 2007). The ideal would be if the analytical technique used to evaluate the quality of herbal products could also discriminate between related species and to detect possible adulteration (Anon., 2001). This would be valuable for the industry for authentication purposes, especially when it is difficult to identify the plant material visually e.g., once it has been pulverised. The ability of NIR spectroscopy to classify between dried, green rooibos and dried, green *C. genistoides,* as well as between different *C. genistoides* types has been demonstrated (Botha, 2005).

Another relatively straightforward method, found to be applicable for quantification of mangiferin in *C. genistoides* (Joubert *et al*., 2007)*,* due to its high mangiferin content, is the colorimetric aluminium chloride method (Chang *et al*., 2002). A good correlation was obtained between the mangiferin content estimated by aluminium chloride and HPLC methods, respectively, for methanol extracts (Joubert *et al*., 2007). With the mangiferin content in *C. subternata* being substantially lower, and its different qualitative phenolic composition compared to that of *C. genistoides*, the investigation into the possibility of using the aluminium chloride method for mangiferin in *C. subternata* is deemed necessary.

The objectives of this study were therefore to:

- optimise extraction of mangiferin and hesperidin from C. subternata to enable accurate quantification using the reference method, i.e. HPLC;
- investigate the effect of different pre-drying treatments for optimal retention of mangiferin and hesperidin contents and colour (green) during drying;
- investigate the effect of long term storage on retention of mangiferin, hesperidin and colour (green);
- investigate the potential of the aluminium chloride colorimetric method to quantify the mangiferin content of *C. subternata*; and
- develop NIR spectroscopy calibration models for the quantification of mangiferin and hesperidin in dried, ground *C. subternata*.

References

Andersen, M.L., Lauridsen, R.K. & Skibsted, L.H. (2005). Power of phenolic compounds. *Functional Foods and Nutraceuticals*, **March 2005**, 44-48.

Anonymous (2000). Agricultural Product Standards Act, Act no. 119 of 1990, Regulation no. 1177 of 24 November 2000.

Anonymous (2001). CPMP/QWP/2820/00 (EMEA/CVMP/81/00). Note for guidance on specifications: Test procedures and acceptance criteria for herbal drugs, herbal drug preparations and herbal medicinal products.

Anonymous (2005). Antioxidant directory. *Nutraceuticals World*, **9** (3), 58-66.

Anonymous (2006). U.S. National Institutes of Health. National Cancer Institute. [WWW document]. URL: http://www.cancer.gov/prevention/agents/Tea_polyphenols.html 8 July 2006.

Anonymous (2007). ARC Honeybush Research Programme: "From the wild to commercialisation". [WWW document]. URL: http://www.arc.agric.za/home.asp?pid=4054 8 October 2007.

Balentine, D.A., Wiseman, S.A. & Bouwens, L.C.M. (1997). The chemistry of tea flavonoids. *Critical Reviews in Food Science and Nutrition*, **37**, 693-704.

Berardini, N., Knödler, M., Schieber, A. & Carle, R. (2005). Utilization of mango peels as a source of pectin and polyphenolics. *Innovative Food Science and Emerging Technologies*, **6**, 442-452.

Blanco, M., Coello, J., Iturriaga, H, Maspoch, S. & De la Pezuela, C. (1998). Near-infrared spectroscopy in the pharmaceutical industry. *Analyst*, **124**, 135-150.

Botha, M. (2005). Use of near infrared spectroscopy (NIRS) and spectrophometric methods in quality control green rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia genistoides*). MSc in Food Science Thesis, Stellenbosch University, South Africa.

Brand, D.L. (2002). Structure and synthesis of polyphenols from *Cyclopia subternata.* MSc Thesis, University of the Free State, South Africa.

Büning-Pfaue, H., Hartmann, R., Harder, J., Kehraus, S. & Urban, C. (1998). NIR-spectrometric analysis of food. Methodical development and achievable performance values. *Fresenius Journal of Analytical Chemistry*, **360**, 832-835.

Chang, C.C., Yang, M.H., Wen, H.M. & Chern, J.C. (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *Journal of Food and Drug Analysis*, **10**, 178-182.

Chen, Q., Zhao, J., Huang, X., Zhang, H. & Liu, M. (2006a). Simultaneous determination of total polyphenols and caffeine contents of green tea by near-infrared reflectance spectroscopy. *Microchemical Journal*, **83**, 42-47.

Chen, Q., Zhao, J., Huang, X., Zhang, H. & Wang, X. (2006b). Feasibility study on qualitative and quantitative analysis in tea by near-infrared reflectance spectroscopy with multivariate calibration. *Analytica Chemica Acta*, **572**, 77-84.

Cleve, E., Bach, E. & Schollmeyer, E. (2000). Using chemometric methods and NIR spectrophotometry in the textile industry. *Analytica Chimica Acta*, **420**, 163-167.

Day, M.S. & Fearn, F.R.B. (1982). Near infra-red reflectance as an analytical technique, Part 1. History and development. *Laboratory Practice*, **31**, 328-330.

Demattê, J.A.M., Campos, R.C., Alves, M.C., Fiorio, P.R. & Nanni, M.R. (2004). Visible-NIR reflectance: A new approach on soil evaluation. *Geodema*, **121**, 95-112.

De Nysschen, A.M., Van Wyk, B-E., Van Heerden, F.R. & Schutte, A.L. (1996). The major phenolic compounds in the leaves of *Cyclopia* species (honeybush tea). *Biochemical Systematics and Ecology*, **24**, 243-246.

Du Pont, M.S., Mondin, Z., Williamsen, G. & Price, K.R. (2000). Effect of variety, processing and storage on the flavonoid content and composition of lettuce and endive. *Journal of Agricultural and Food Chemistry*, **48**, 3957-3964.

Du Toit, J., Joubert, E. & Britz, T.J. (1998). Honeybush tea - a rediscovered indigenous South African herbal tea. *Journal of Sustainable Agriculture*, **12**, 67-84.

Escribano-Bailón, M.T. & Santos-Buelga, C. (2003). Polyphenol extraction from foods. In: Methods in Polyphenol Analysis (edited by C. Santos-Buelga & G. Williamson). Pp. 1-16. The Royal Society of Chemistry, Cambridge.

Ferreira, D., Kamara, B.I., Brandt, E.V. & Joubert, E. (1998). Phenolic compounds from *Cyclopia intermedia* (honeybush tea). *Journal of Agricultural and Food Chemistry*, **46**, 3406-3410.

Fernández, P.L., Pablo F., Martin, J. M., & Gonzalez, A.G., (2002). Study of catechin and xanthine tea profiles as geographical tracers. *Journal of Agricultural and Food Chemistry*. **50**, 1833-1839.

Franke, A.A., Custer, L. J., Arakaki, C. & Murphy, S.P. (2004). Vitamin C and flavonoid levels of fruits and vegetables consumed in Hawaii. *Journal of Food Composition and Analysis,* **17**, 1-35.

Gramza, A. & Korczak, J. (2005). Tea constituents (*Camellia sinensis* L.) as antioxidants in lipid systems. *Trends in Food Science and Technology,* **16**, 351-358.

Greenish, H.G. (1881). Cape tea. *The Pharmaceutical Journal and Transactions*, **3rd series** (550), 549-551.

Grunewald, H., Kurowski, C., Timm, D., Grummisch, U. & Meyhack, U. (1998). Rapid non-destructive raw material identification in the cosmetic industry with near-infrared spectroscopy. *Journal of Near Infrared Spectroscopy*, **6**, 215-222.

Guardia, T., Rotelli, A.E., Juarez, A.O. & Pelzer, L.E. (2001). Anti-inflammatory properties of plant flavonoids. Effects of rutin, quercetin and hesperidin on adjuvant arthritis in rats. *Farmaco*, **56**, 683-687.

Haiduc, A.M., van Duynhoven, J.P.M, Heussen, P., Reska, A.A. & Reiffers-Magnani, C. (2007). Multivariate modelling of the microstructural quality of food emulsions based on NMR. *Food Research International*, **40**, (4), 425-434.

He, X.G. (2000). On-line identification of phytochemical constituents in botanical extracts by combined high-performance liquid chromatographic-diode array detection-mass spectrometric techniques. *Journal of Chromatography* A, **880**, 203-232.

Howard, L.R., Padjaitan, N., Morelock, T. & Gil., M.I. (2002). Antioxidant capacity and phenolic content of spinach as affected by genetics and growing season. *Journal of Agricultural and Food Chemistry,* **50**, 5891-5896.

Hubbe, M.E. (2000). Evaluation of antioxidant and free radical scavenging activities of honeybush tea (*Cyclopia)*. MSc in Food Science Thesis, Stellenbosch University, South Africa.

Hubbe, M.E. & Joubert, E. (2000a). In vitro superoxide anion radical scavenging ability of honeybush tea (*Cyclopia*). In: *Dietary Anticarcinogens and Antimutagens, Chemical and Biological Aspects* (edited by I.T. Johnson & G.R. Fenwick). Pp. 242-244. Cambridge: Royal Society of Chemistry.

Hubbe, M.E. & Joubert, E. (2000b). Hydrogen-donating ability of honeybush tea polyphenols as a measure of antioxidant activity: In: *Polyphenols Communications 2000*, Vol 2 (edited by S. Martens, D. Treutter & G. Forkman). Pp. 361-362. Technische Universität München, Freising-Wiehenstephan.

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

Joubert, E., Manley, M., Gray, B.R. & Schulz, H. (2005). Rapid measurement and evaluation of the effect of drying conditions on harpagoside content in *Harpagophytum procumbens* (devil's claw) root. *Journal of Agricultural and Food Chemistry*, **53**, 3493-3502.

Joubert, E., Manley, M. & Botha, M. (2006). Use of NIRS for the quantification of mangiferin and hesperidin contents from dried green honeybush (*Cyclopia genistoides*) plant material. *Journal of Agricultural and Food Chemistry*, **54**, 5279-5283.

Joubert, E., Manley, M. & Botha, M. (2007). Evaluation of spectrophotometric methods for screening of green rooibos (*Aspalathus linearis*) and green honeybush (*Cyclopia genistoides*) extracts for high levels of bio-active compounds. *Phytochemical Analysis* (http://dx.doi.org/10.1002/pca.1033)

Jung, H-A., Su, B-N., Keller, W.J., Metha R.G. & Kinghorn, A.D. (2006) Antioxidant xanthones from pericap of *Garcinia mangostana* (mangosteen). *Journal of Agricultural and Food Chemistry,* **54**, 2077-2082.

Kamara, B.I., Brandt, E.V., Ferreira, D. & Joubert, E. (2003). Polyphenols from honeybush tea (*C. intermedia*). *Journal of Agricultural and Food Chemistry*, **53**, 3874-3879.

Kamara, B.I., Brand, D.J., Brandt, E.V. & Joubert, E. (2004). Phenolic metabolites from honeybush tea (*Cyclopia subternata*). *Journal of Agricultural and Food Chemistry*, **52**, 5391-5395.

Kawano, S., Watanabe, H. & Iwamoto, M. (1992). Determination of sugar content in intact peaches by near infrared spectroscopy with fibre optics in interactance mode. *Journal of the Japanese Society of Horticultural Science*, **61**, 445-451.

Karoui, R., Mouazen, A.M., Dufour, E., Pillonel, L., Schaller, E., Picque, D., Baerdemaeker, J. & Bosset, J.-O. (2006). A comparison and joint use of NIR and MIR spectroscopic methods for the determination of some parameters in European Emmental cheese. *European Food Research and Technology A*, **223**, (1), 44-49.

Laasonen, M., Harmia-Pulkkinen, T., Simard, C.L., Michiels, E., Rasanen, M. & Vuorela, H. (2002). Fast identification of *Echinacea purpurea* dried roots using near-infrared spectroscopy. *Analytical Chemistry*, **74**, 2493-2499.

Lima, F.S.G., Araújo, M.A.S. & Borges, L.E.P. (2004). Determination of lubricant base oil properties by near infrared spectroscopy using different sample and variable selection methods. *Journal of Near Infrared Spectroscopy*, **12**, 159-166.

Ludwig, B., Schmilewski, G. & Terhoeven-Urselmans, T. (2006). Use of near infrared spectroscopy to predict chemical parameters and phytotoxicity of peats and growing media. *Scientia Horticulturae*, **109**, (1), 86-91.

Manzocco, L., Anese, M. & Nicoli, M.C. (1998). Antioxidant properties of tea extracts as affected by processing. *Lebensmittel-Wissenschaft und-Technologie*, **31**, 694-698.

Manley, M., Joubert, E. & Botha, M. (2006). Quantification of the major phenolic compounds, soluble solid content and total antioxidant activity of green rooibos (*Aspalathus linearis*) by means of near infrared spectroscopy. *Journal of Near Infrared Spectroscopy*, **14**, 213-222.

Marnewick, J.L.M., Gelderblom, W.C.A. & Joubert, E. (2000). An investigation on the antimutagenic properties of South African herbal teas. *Mutation Research*, **471**, 157-166.

Marnewick, J., Joubert, E., Joseph, S., Swanevelder, S., Swart, P. & Gelderblom, W. (2005). Inhibition of tumour promotion in mouse skin by extracts of rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*), unique to South African herbal teas. *Cancer Letters*, **224**, 193-202.

Nicoli, M.C., Anese, M., Parpinel, T., Franceschi, S. & Lerici, C.R. (1997). Loss and/or formation of antioxidants during food processing and storage. *Cancer Letters*, **114**, 71-74.

Núñez-Sellés, A.J., Vélez Castro, H.T., Agüero-Agüero, J., González-González, J., Naddeo, F., De Simone, F. & Rastrelli, L. (2002). Isolation and quantitative analysis of phenolic antioxidants, free sugars, and polyols from mango (*Mangifera indica* L.) stem bark aqueous decoction used in Cuba as a nutritional supplement. *Journal of Agricultural and Food Chemistry*, **50**, 762-766.

Osborne, B.G. (1991). Measurement of the hardness of wheat endosperm by near infrared spectroscopy. *Postharvest News and Information*, **2**, 331-334.

Pettigrew, J. (1997). The beauty of tea. *Tea & Coffee Trade Journal*, **169**, 66-71.

Rannou, H. & Downey, G. (1997). Discrimination of raw pork, chicken and turkey meat by spectroscopy in the visible, near- and mid-infrared ranges. *Analytical Communications*, **34**, 401-404.

Re, R., Pellegrini, N., Proteggente, A., Yang, M. & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, **26**, 1231-1237.

Richards, E.S. (2002). The antioxidant and antimutagenic activities of *Cyclopia* species and activity-guided fractionation of *C. intermedia*. MSc in Food Science Thesis, Stellenbosch University, South Africa.

Rotelli, A.E., Guardie, T., Juárez, A.O., De La Rocha, N.E. & Pelzer, L.E. (2003). Comparative study of flavonoids in experimental models of inflammation. *Pharmacological Research*, **48**, 601-606.

Schulz, H., Pfeffer, S., Quilitzsch, R., Steuer, B. & Reif, K. (2002). Rapid and non-destructive determination of the echinacoside content in *Echinacea* root by ATR-IR and NIR spectroscopy. *Planta Medica*, **68**, 926-929.

Singleton, V.L. & Rossi, Jr., J.A. (1965). Colorimetry of total phenolics with phospho-molybdicphosphotungstic acid reagent. *American Journal of Enology and Viticulture,* **16**, 144-158.

Stark, E., Luchter, K. & Margoshes, M. (1986). Near-infrared analysis (NIR): a technology for quantitative and qualitative analysis. *Applied Spectroscopy Reviews*, **22**, 335-399.

Suematso, S., Hisanobu, Y., Saigo, H., Matsuda, R., & Komatsu, Y. (1995). A new extraction procedure for determination of caffeine and catechins in green tea. *Nippon Syokuhin Kagaku Kaishi*, **42**, 419-424 (as cited by Jung *et al*., 2006).

Tamil-Selvi, A., Joseph, G.S. & Jayaprakasha, G.K. (2003). Inhibition of growth and aflatoxin production in *Aspergillus flavus* by *Garcinia indica* extract and its antioxidant activity. *Food Microbiology*, **20**, 455-460.

Terblanche, S.E. (1982). *Report on Honeybush Tea*. University of Port Elizabeth, South Africa.

Van der Merwe, J.D. (2005). A comparative study on protection of *Cyclopia* spp. (honeybush), *Aspalathus linearis* (rooibos) and *Camellia sinensis* tea against aflatoxin B_1 induced mutagenesis in the *Salmonella* mutagenicity assay: Possible mechanisms involved. MSc in Food Science Thesis, Stellenbosch University, South Africa.

Van der Merwe, J.D., Joubert, E., Richards, E.S., Manley, M., Snijman, P.W., Marnewick, J.L. & Gelderblom, W.C.A. (2006). A comparative study on the antimutagenic properties of aqueous extracts of *Aspalathus linearis* (rooibos), different *Cyclopia* spp. (honeybush) and *Camellia sinensis* teas. *Mutation Research, Genetic Toxicology and Environmental Mutagenisis,* **611**, 42-53.

Wang, Z.Y., Cheng, S.J., Zhou, Z.C., Mohammad, A., Wasiuddin, A.K., Bickers, D.R. & Mukhatar, H. (1989). Antimutagenic activity of green polyphenols. *Mutation Research,* **223**, 273-285.

Wang, H., Provan, J.H. & Helliwell, K. (2000). Tea flavonoids: their functions, utilization and analysis. *Trends in Food Science and Technology*, **11**, 152-160.

Wang, L., Lee, F.C.S. & Wang, X. (2007). Near-infrared spectroscopy for classification of licorice (*Glycyrrhizia uralensis* Fisch) and prediction of the glycyrrhizic acid (GA) content. Lebensmittel-Wissenschaft und -Technologie, **40**, 83-88**.**

Woo, Y., Kim, H., Cho, J. & Chung, H. (1999). Discrimination of herbal medicines according to geographical origin with near infrared reflectance spectroscopy and pattern recognition techniques. *Journal of Pharmaceutical and Biomedical Analysis*, **21**, 407-413.

Woo, Y., Cho, C., Kim, H., Yang, J. & Seong, K. (2002). Classification of cultivation area of ginseng by near infrared spectroscopy and ICP-AES. *Microbiological Journal***, 73**, 299-306.

Zhang, M.H., Luypaert, J., Fernàndez Pierna, J.A., Xu, Q.S. & Massart, D.L. (2004). Determination of total antioxidant capacity in green tea by near-infrared spectroscopy and multivariate calibration. *Talanta,* **62,** 25-35.

Zhao, J., Chen, Q., Huang, X. & Fang, C.H. (2006). Qualitative identification of tea categories by near infrared spectroscopy and support vector machine. *Journal of the Pharmaceutical and Biomedical Analysis*, **41**, 1198-1204.

CHAPTER 2

LITERATURE REVIEW

CONTENTS

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LITERATURE REVIEW

1. INTRODUCION

The growing awareness of health issues among consumers has resulted in a thriving market for natural products with medicinal value. A large number of polyphenol-enriched extracts are being produced by the nutraceutical and cosmetic industries (Andersen *et al*., 2005; Wright, 2005). The consumption of tea as a health beverage, due to its reported benefits, has risen dramatically worldwide. The increasing interest in the health benefits of tea has additionally led to the inclusion of tea extracts in dietary supplements and functional foods (Higdon & Frei, 2003). Honeybush (*Cyclopia* spp.) is a South African herbal plant which is often used for the preparation of a tea and contains high levels of the phenolic compound, mangiferin. Mangiferin has been found to contribute to its antioxidant (Richards, 2002; Joubert *et al*., 2004) and antimutagenic (Richards, 2002; Van der Merwe, 2005) activites.

This literature review gives an overview of *Cyclopia* spp. including their polyphenolic composition and factors that will affect its retention, due to possible chemical changes, e.g. storage. Procedures used for preparation and processing of the plant material and extraction of the phenolic compounds are discussed. Quality control analysis techniques, i.e. near infrared (NIR) spectroscopy and UV/Vis colourimetric methods opposed to HPLC analysis are discussed. As the application of NIR spectroscopy for quality control purposes is still fairly new to the herbal industry in South Africa its principles will be discussed briefly.

2. NUTRACEUTICALS

2.1 Background

Safety, taste, value for money and convenience are some factors that are considered by consumers before purchasing a food product (Delgado-Vargas & Paredes-López, 2003). Consumers today, however, are looking for foods that additionally provide health benefits beyond their traditional nutritional value or for foods with physiologically beneficial components added. Functional foods differ from conventional foods in that they contribute to disease prevention and increased body functioning (Goldberg, 1994; Hasler, 1998; Delgado-Vargas & Paredes-López, 2003). According to The International Life Sciences Institute (ILSI), a food can be regarded as functional if it has been scientifically proven to either improve health or reduce the risk of sickness (Leskinen, 2002). Reducing the intake of fat, increasing fibre intake and lowering cholesterol are important for the public's

perception of health products (Sloan, 2000; Sloan, 2001). Currently it is thought that incorrect diet contributes to six out of ten leading causes of death and up to 70% of certain cancers may be attributed to diet. Markets for vitamin and herbal supplements are growing rapidly. Products containing vitamin E, omega-3 fatty acids, folic acid, calcium, ascorbic acid and vitamins B_6 and B_{12} are common commodities added in foods, as they are perceived as important components for health (Hollingsworth, 2000). Although nutraceuticals tend to be more costly, consumers are willing to pay the price as they are perceived to be healthy (Anon., 2006b). Some functional food components within the class flavonoids are listed in Table 1.

Table 1 Examples of functional food components within the class flavonoids (Anon., 2004)

Component	**Source**	**Potential benefit**
Anthocyanidins, i.e. cyanidin, malvidin	berries, cherries, red grapes	boosts cellular antioxidant effect; may contribute to maintenance of brain function
Flavanols, i.e. catechins, epicatechins, epigallocatechin, procyanidins	tea, cocoa, apples, grapes	may contribute to maintenance of heart health
Flavanones, i.e. hesperetin, naringen	citrus foods	neutralise free radicals, which may damage cells; boosts cellular antioxidant defences
Flavonols, i.e. quercetin, kaempferol, myricetin	apples, onion, tea, broccolli	neutralise free radicals which may damage cells; boosts cellular antioxidant defences
Proanthocyanidins	cranberries, cocoa, apples, strawberries, grapes, wine, peanuts, cinnamon	may contribute to maintenance of urinary tract health and heart health

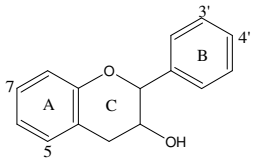
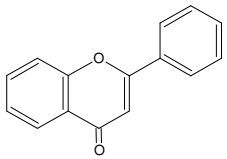
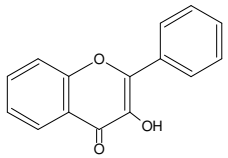
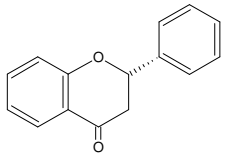
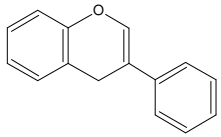
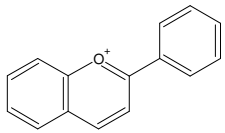
2.2 Phenolic compounds and its beneficial properties

Phenolic compounds are secondary plant phenolics distributed widely in leaves, seeds, bark and flowers of plants, offering protection against ultraviolet rays and pathogens (Harborne & Williams, 2000). These phenolics are also important constituents in the human diet as they are found in fruit, vegetables, seeds as well as teas and wine. Extensive research has therefore been undertaken to evaluate their role in human health (Siess *et al*., 2000). Phenolic secondary metabolites play an important role in the quality of plant-derived food, as they are relevant to the appearance, taste, and health promoting properties (Parr & Bolwell, 2000; Tomás-Barberán & Espín, 2001).

Flavonoids comprise the most common group of plant polyphenols and contribute significantly to the flavour and colour of fruit and vegetables (Tomás-Barberán & Espín, 2001) Table 2. The beneficial health effects of flavonoids are mainly attributed to their antioxidant and metal chelating abilities (Heim *et al*., 2002). Higher eukaryotic aerobic organisms cannot exist without oxygen, but oxygen is inherently dangerous to their existence and this is referred to as the paradox of life, or the 'Oxygen Paradox' (Davies, 1995). It appears that the common products of life in an aerobic environment (the superoxide anion radical, hydrogen peroxide and the extremely reactive hydroxyl radical) are responsible for oxygen toxicity. Intake of dietary antioxidants is essential due to the incomplete efficiency of endogenous defence systems and exposure to factors such as cigarette smoke, air pollution, UV radiation and high polyunsaturated fatty acid diet which increase oxidative stress. Dietary antioxidants can diminish the effects of oxidative damage during the human life span (Halliwell, 1994; Harborne & Williams, 2000). The potentially cancer-inducing oxidative damage may be prevented or limited by dietary antioxidants found in fruit and vegetables. Studies to date indicate that phytochemicals can have complementary and overlapping mechanisms of action, including scavenging of oxidative agents, stimulation of immune system and regulation of gene expression (Dasgupta & De, 2007). Other beneficial biological activities of flavonoids include anti-bacterial and anti-viral activity, anti-inflammatory, anti-angionic, analgesic, anti-allergic effects, hepato-protective, cytostatic, apoptotic, antimutagenic/anticancer, estrogenic and anti-estrogenic properties (Rice-Evans *et al*. 1996; Larson, 1998; Harborne & Williams, 2000; Heim *et al*., 2002; Dasgupta & De, 2007). Phenolic compounds could also play an important role in lowering low-density lipoprotein (LDL) peroxidation and thereby aid in the prevention of atherosclerosis (De Whalley *et al*., 1990; Harborne & Williams, 2000).

More than 5000 different flavonoids have been described so far with the six major subclasses of flavonoids including the flavones (e.g. apigenin, luteolin), flavonols (e.g. quercitin, myricetin);

Table 2 Basic structures of dietary flavonoids, substitution patterns and their dietary sources (Heim *et al*., 2002)

Class	General structure	*Flavonoid*	Substitution pattern	Dietary source
Flavanol		(+) catechin	5,7,3',4'-OH	tea (*Camellia sinensis*)
		(-) epicatechin	5,7,3',4'-OH	tea
		epigallocatechin gallate	5,7,3',4',5'-OH, 3-gallate	tea
Flavone		chrysin	5,7-OH	fruit skins
		apigenin	5,7,4'-OH	parsley, celery
		rutin	5,7,'3,4'-OH,3-rutinose	red wine, buckwheat, citrus
		luteolin	5,7,3',4'-OH	red pepper
		luteolin glucoside	5,4'-OH,4',7-glucose	
Flavonol		kaempferol	5,7,4'-OH	leek, broccoli, endives, grapefruit, black tea
		quercetin	5,7,3',4'-OH	onion, lettuce, broccoli, tomato, tea, red wine, berries, olive oil
		myricetin	5,7,3',4',5'-OH	cranberry grapes, red wine
		tamatixetin	5,7,3'-OH, 4'-OMe	
Flavanone (dihydroflavone)		naringin	5,4'-OH,7-rhamnoglucose	citrus, grapefruit
		naringenin	5,7,4'-OH	citrus fruits
		taxifolin	3,5,7,3',4'-OH	citrus fruits
		eriodictyol	5,7,3',4'-OH	lemons
		hesperidin	3,5,3'-OH,4'-OME,7-rutinose	oranges
Isoflavone		genistin	5,4'-OH, 7-glucose	soybean
		genistein	5,7,4'-OH	soybean
		daidzin	4'-OH,7-glucose	soybean
		daidzein	7,4'-OH	soybean
Anthocyanidin		apigenidin	5,7,4'-OH	coloured fruits
		cyanidin	3,5,7,4'-OH,3,5-OMe	cherry, raspberry, strawberry

flavanones (e.g. naringen, hesperidin); catechins or flavanols (e.g. epicatechin, gallocatechin); anthocyanidins (e.g. cyanidin, pelargonidin), and isoflavones (e.g. genistein, daizein). Most of the flavonoids present in plants are attached to sugars (glycosides) or they are found as aglycones (Ross & Kasum, 2002). The type and number of sugar residues along with chain branching result in a large number of individual glycosides (Rice-Evans *et al*. 1996; Wang & Helliwell, 2001).

Plant extracts are often used for wound-healing, anti-aging and disease treatments (Hsu, 2005). In a recent study, Marnewick *et al.* (2005) found that honeybush extracts inhibited tumour promotion in mouse skin. Tea catechins and polyphenols are effective scavengers of reactive oxygen species in vitro (Higdon & Frei, 2003). They may also function indirectly as antioxidants through their effects on transcriptase factors and enzyme activities. The increased public awareness of the positive health characteristics (anticarcinogenic, antimutagenic and cardioactive effects which is mostly associated due to their antioxidant properties) of tea, is generally attributed to the high flavonoid content of leaves and extracts (Khokhar & Magnusdottir, 2002).

Antioxidants are thought to decrease the possibility of these diseases by lowering the concentration of free radicals and in doing so reduce the chance that one of these radicals will cause an initiation event (Parr & Bolwell, 2000). For a polyphenol to be defined as an antioxidant two conditions need to be satisfied: 1) when present in low concentrations relative to the substrate to be oxidised it can delay, retard, or prevent the autoxidation or free radical-mediated oxidation and 2) the resulting radical formed after scavenging must be stable, i.e. through intramolecular hydrogen bonding on further oxidation (Halliwell, 1990; Shahidi *et al*., 1992).

Addition of antioxidants is a method of increasing the shelf life of food products, especially of fats, oil and food products containing fat (Krishnakumar & Gordon, 1996; Rice-Evans *et al*., 1996; Benavente-García *et al*., 1997; Percival, 1998; Spigno & De Faveri, 2007). Edible fats, oils and fat-containing products undergo oxidation, both during production and storage, causing a sequence of unfavourable changes (Gramza & Korczak, 2005). Rice-Evans *et al*. (1996) conclude that the specific mode of inhibition is not clear but they may act by: 1) chelating copper ions via ortho dihydroxy phenolic structure; 2) scavenging lipid alkoxyl and peroxyl radicals by acting as chain breaking antioxidants, as hydrogen donars; and 3) regenerating α-tocopherol through reduction of the α-tocopherol radical.

For increased shelf life, Krishnakumar & Gordon (1996) advised: 1) exclusion of oxygen by packing under vacuum; 2) exclusion of energy by storage at low temperature and/or the dark; and 3) addition of exogenous antioxidants. The use of synthetic antioxidants such as BHT and BHA is restricted in foods due to their toxicological effects on various species and suspected carcinogenic

potential (Krishnakumar & Gordon, 1996). The search for natural and safe antioxidants, especially of plant origin has greatly increased over the years and numerous examples are to be found in literature. Peschel *et al*. (2006) demonstrated the possibility to recover high amounts of phenolics with antioxidant properties from fruit and vegetable waste with application by both the food and cosmetic industries. However, more research needs to be undertaken into their safety, as polyphenols remain relatively unresearched (Melton, 2006).

2.3 Legislation

Introducing a new functional food brand is difficult in today's market. The new product not only has to comply with stringent laws but also enter a very competitive and increasingly growing market (Delgado-Vargas & Paredes-Lopez, 2003). In the USA the term 'functional food' was not recognised until 1998 (Hasler, 1998). Health claims, about the use of a dietary supplement or food to prevent, treat or cure a specific disease are illegal unless authorised by the Food and Drug Administration (Gise & Katz, 1997). They should also be based on sound and accurate scientific criteria, which should include rigorous studies of safety and efficacy (Hasler, 2002). The differences between health claims and structure-function claims should also allow the consumer to understand the differences in the scientific bases of these claims. The main scepticism regarding functional foods is in the various health claims and also the poor and inadequate control of their claimed properties (Arvanitoyannis & Van Houwelingen-Koukaliaroglou, 2005). Legislation is progressing at an extremely low pace at a time when 150 million Americans have reportly spent over $20.5 billion on functional, nutraceutical and dietary supplements (Burdock *et al*., 2006). An increasing number of dietary supplement manufacturers have increased their resources to obtain GRAS (Generally Recognised As Safe) status for their supplements. Currently only Japan, the United Kingdom and Scandinavian countries have made progress in this regard (Arvanitoyannis & Van Houwelingen-Koukaliaroglou, 2005).

The FDA regulates food for its intended use and the nature of claims made on their packaging. Five types of health related statements are allowed on food and dietary supplement labels (Anon., 2004). In South Africa, a framework exists for labelling of health claims, but they should be based on scientific evidence before allowed on food labels (Reid, 1997). Legislative authorities' main concern is that disease-related claims may mislead consumers (Hughson, 1995). Manufacturers will have difficulty gaining approval for health claims without substantive clinical proof of a nutrient's efficacy in a product or its efficacy to cure disease.

2.4 The future

Herbs and herbal extracts are important for future product development and those with the highest pharmaceutical potential are *Echinacea*, garlic and ginseng (Sloan, 1999; Sloan, 2000; Sloan, 2001; Mermelstein, 2002). In Japan where a long tradition of using food for health purposes exists (Woollen, 1990), foods are more targeted towards students, e.g. Coca Cola can be enriched with ginseng and vitamins, and sweets with catechins (usually found in green tea) are manufactured (Brower, 1998; Anon., 2000). In South Africa, lollipops (Fitchet, 2005) and tea centres with carob coating (Anon., 2006d) containing rooibos and honeybush extracts, have recently been introduced. Functional waters with components such as sage, camomile and peppermint are also becoming very popular (Anon., 2003). A variety of bars and beverages enhanced with botanicals could pose a risk to certain consumers due to the safety issues concerning herbs and complex herb-drug interaction e.g. the utilization of St John's wort for treatment of mild depression also inactivates several drugs (Hasler, 2002). Furthermore, numerous bioavailability and stability issues upon addition of nutrients to foods and beverages have become more prominent recently, e.g. the concern over what happens to potency of echinacea over time (Chaudhari, 1999).

Food substances can regulate the action of specific genes causing chronic diseases (Delgado-Vargas & Paredes-Lopez, 2003). The reduction in the amount of human gene products may prevent the onset of some chronic diseases. It is now clear that new food products have to be targeted towards the prevention/treatment of the most important diseases worldwide (Chaudhari, 1999; Hasler, 2002). However, it was found that South Africans believed strongly that certain food types are better and actually make you healthier (mean 85%), but far fewer believed (mean 64%), that food can influence the development of disease.

The application of biotechnology techniques for development of functional food plants with higher levels of bioactive components (phytochemicals) or increased availability of nutrients will greatly benefit most developing countries and improve health (Niba, 2003).

3. *CYCLOPIA* SPP. (honeybush)

3.1 Background

Honeybush (*Cyclopia* spp.), of which some 24 species have been identified, is an indigenous shrub from the Fabaceae family (Leguminosae) growing in the Cape fynbos biome in South Africa (Schutte, 1995). Traditionally it has been used for its medicinal properties (Watt & Breyer-Brandewijk, 1932). Nowadays a herbal infusion, referred to as honeybush tea, having a pleasant, mildly sweet taste and honey-like flavour is made from it (Du Toit *et al*., 1998). It is also becoming internationally known as a

healthier substitute for green tea (*Camellia sinensis*) due to its very low tannin (Terblanche, 1982) and negligible caffeine content (Greenish, 1881). It is therefore also suitable for children as well as sufferers of digestive and heart problems (Terblanche, 1982). "Heuningtee, Bergtee, Boertee, Bossietee or Bushtea" are some of the names that this tea is also referred to. During the flowering season, usually during May or September, the plants develop deep yellow flowers, which bear the characteristic honey-like aroma. Previously the plants were harvested during the flowering season, however, nowadays harvesting mainly takes place during summer and early autumn (E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, personal communication, 2005).

In the 1990's honeybush tea was consumed mostly locally, but research on the antioxidant, health properties and cultivation catalysed the growth of the industry (E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, personal communication, 2005). Sales figures have also shown a significant increase from 50 tons sold in 1999 to as much as 250 tons sold in 2006. Approximately 80-90% of honeybush tea produced is exported to Europe, the United Kingdom, the United States of America and Japan. The commercial supplies of honeybush are mostly obtained from *C. intermedia* ("bergtee"), *C. subternata* ("vleitee") and *C. genistoides* ("heuningtee") (E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, personal communication, 2005).

Today, a large majority of the honeybush plant material, especially *C. intermedia*, is still being collected from the wild. Cultivation was, however, inevitable with the rapid growth of the industry which forced the collectors to travel further into very poor accessible areas. The demand for the product to be more uniform also increased. Currently all *Cyclopia spp.* are deemed suitable for production of tea, although the quality of the tea has been found to vary. Some species are also only available in very small quantities in the wild. It is mainly the leaves and stems of the *Cyclopia spp.* (aerial parts) that are processed and used to manufacture the herbal infusion (Du Toit *et al.,* 1998).

3.2 Phenolic composition of honeybush

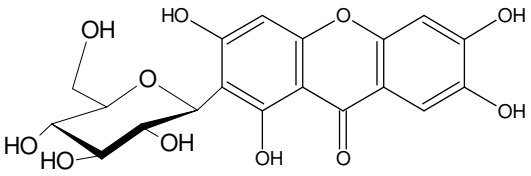
The phenolic composition among different *Cyclopia* spp. (De Nysschen *et. al*., 1996; Ferreira *et al*., 1998; Joubert *et al*., 2003; Kamara *et al*., 2003; Kamara *et al*., 2004), as well as types within the species (Joubert *et al.,* 2003*)* differs qualitatively and quantitatively. De Nysshen *et al*. (1996), screened methanol extracts from leaves of 22 unprocessed *Cyclopia* spp. and reported the major components, i.e. a xanthone C-glycoside, mangiferin, and the flavanone, hesperetin. However, quantitative analysis of four *Cyclopia* species showed hesperidin and not hesperetin is the major flavanone (Joubert *et al*., 2003).

From the first investigation on fermented *C. intermedia,* the flavanones naringenin, eriodictyol, hesperetin and hesperidin, the flavone luteolin, the isoflavones formononetin, afrormosin, calycosin, pseudobaptigen and fujikinetin, the coumestans medicagol, flemichapparin and sophoracoumestan B, the xanthones mangiferin and isomangiferin and the phenolic acid *p*-coumaric acid were all identified (Ferreira *et al*., 1998). Subsequent analysis of the methanol extract revealed the minor compounds: four flavanone glycosides, five flavonol glycosides, two isoflavones, i.e. wistin and a diglycosylated isoflavone, two flavones, tyrosol and a methoxy analogue, a diglycosylated phenyl ethanol and a diglycosylated benzaldehyde (Kamara *et al.* 2003).

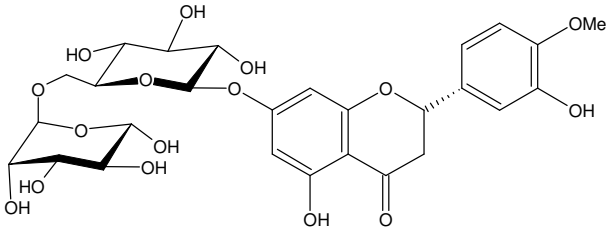
Unfermented (green) *C. subternata* has been found to possess a different phenolic profile in comparison to *C. intermedia*. Analysis of acetone and methanol extracts revealed mangiferin, hesperidin, luteolin and *p*-coumaric acid, which are also present in *C. intermedia.* Apart from these, the flavanones narirutin and eriocitrin, the flavones 5-deoxyluteolin and scolymoside, the isoflavone orobol, the flavonol C-6-glucosylkaempferol, a glycosylated flavan, and the flavanol epigallocatechin gallate were also present. The highly effective radical scavenger, epigallocatechin 3-O-gallate (Zhao *et al*., 1989) is associated with bio-antimutagenic activity (Kada *et al*., 1985). Other compounds isolated included 4-glucosyltyrosol and shikimic acid (Kamara *et al*. 2004).

The major phenolic compounds identified in unfermented *C. genistoides* were mangiferin (Figure 1a), isomangiferin and hesperidin (Figure 1b) (Joubert *et al*., 2003). By using DAD-HPLC and LC-MS the presence of narirutin and luteolin were demonstrated (Joubert *et al*., 2007).

The total polyphenol (TP) content of the hot water soluble solids of green *C. genistoides* are higher than that of *C. intermedia* (Du Toit & Joubert, 1998a; Joubert *et al*., 2007), but is lower than that of *C. subternata* (Joubert *et al*., 2007). It has also been proposed that growth and the leaf to stem ratio of the plant material used during production of honeybush tea influences the phenolic composition of the final product (Joubert *et al*., 2003).



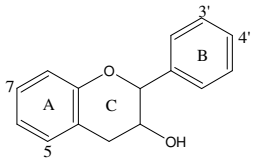
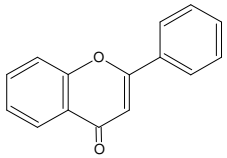
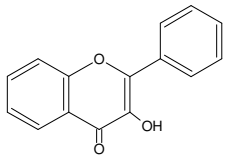
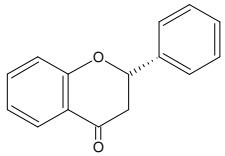
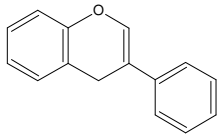
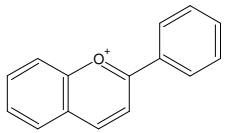
a)



b)

Figure 1 Molecular structures of a) mangiferin and b) hesperidin present in *Cyclopia* spp.

Table 2 Basic structures of dietary flavonoids, substitution patterns and their dietary sources (Heim *et al*., 2002)

Class	General structure	*Flavonoid*	Substitution pattern	Dietary source
Flavanol		(+) catechin	5,7,3',4'-OH	tea (*Camellia sinensis*)
		(-) epicatechin	5,7,3',4'-OH	tea
		epigallocatechin gallate	5,7,3',4',5'-OH, 3-gallate	tea
Flavone		chrysin	5,7-OH	fruit skins
		apigenin	5,7,4'-OH	parsley, celery
		rutin	5,7,'3,4'-OH,3-rutinose	red wine, buckwheat, citrus
		luteolin	5,7,3',4'-OH	red pepper
		luteolin glucoside	5,4'-OH,4',7-glucose	
Flavonol		kaempferol	5,7,4'-OH	leek, broccoli, endives, grapefruit, black tea
		quercetin	5,7,3',4'-OH	onion, lettuce, broccoli, tomato, tea, red wine, berries, olive oil
		myricetin	5,7,3',4',5'-OH	cranberry grapes, red wine
		tamatixetin	5,7,3'-OH, 4'-OMe	
Flavanone (dihydroflavone)		naringin	5,4'-OH,7-rhamnoglucose	citrus, grapefruit
		naringenin	5,7,4'-OH	citrus fruits
		taxifolin	3,5,7,3',4'-OH	citrus fruits
		eriodictyol	5,7,3',4'-OH	lemons
		hesperidin	3,5,3'-OH,4'-OME,7-rutinose	oranges
Isoflavone		genistin	5,4'-OH, 7-glucose	soybean
		genistein	5,7,4'-OH	soybean
		daidzin	4'-OH,7-glucose	soybean
		daidzein	7,4'-OH	soybean
Anthocyanidin		apigenidin	5,7,4'-OH	coloured fruits
		cyanidin	3,5,7,4'-OH,3,5-OMe	cherry, raspberry, strawberry

3.2.1 Mangiferin content

Unfermented *C. genistoides* contains the highest levels of the xanthones, mangiferin (3.61 g.100 g^{-1}) and isomangiferin (0.54 g.100 g^{-1}) Joubert *et al.*, 2003). The lowest levels of mangiferin (1.04 g.100 g^{-1}) were found in *C. sessiliflora*. Joubert *et al.*, (2003) also reported that mangiferin contents were found to differ among two types of *C. genistoides* with the Overberg type containing higher levels (6.37 g.100 g^{-1}) than the West Coast type (4.93 g.100 g^{-1}). Richards (2002) obtained a xanthone (mangiferin and isomangiferin) content of (1.69 g.100 g^{-1}) and Joubert *et al*. (2007) reported a mangiferin content of (0.44 g.100 g^{-1}) in leaf and stem samples.

3.2.2 Hesperidin content

Unfermented *C. intermedia* contain the highest levels of hesperidin, while its levels were similar in fermented *C. intermedia* and *C. subternata* (Joubert *et al*., 2007). Hesperidin contents were also found to differ between two types of *C. genistoides* with the Overberg type containing a hesperidin value of (2.23 g.100 g^{-1}) and that of the West Coast type being (5.21 g.100 $g^{-1)}$ (Joubert *et al*., 2003). Botha (2005) found low concentrations of hesperidin in water extracts from *C. genistiodes* due to its poor solubility, but it was found to be readily soluble in extraction with methanol. The lowest level of hesperidin (0.29 g.100 g^{-1}) was found in *C. sessiliflora*.

3.3 Potential of honeybush tea as a functional beverage

Honeybush tea has been associated with several positive beneficial health effects. It is mostly based on anecdotal evidence which suggests that honeybush tea is a diuretic, can increase appetite, prevent stomach ulcers through the stimulation of excess mucous production and stimulate milk production in lactating women (Watt & Breyer-Brandwijk, 1932). Several trace elements such as iron, potassium, calcium, sodium and magnesium occur. Futhermore, it has also been used to treat colic in babies, and as a cough syrup in cases of chronic tonsillitis and lung infection (Rood, 1994). Studies on its radical scavenging activity and antimutagenic activity could fulfil the requirements for honeybush to be regarded as a functional food or nutraceutical (Hubbe, 2000; Marnewick *et al*., 2000). It could also be considered as being superior to the traditional *Camellia sinensis* teas due to the low tannin content (Terblanche, 1982) and negligeble caffeine content (Greenish, 1881).

The xanthone, mangiferin, is a common constituent in traditional herbal medicine and has been found to exhibit antioxidant properties (Ferreira *et al*., 1998; Hubbe, 2000). According to Richards (2002) mangiferin has been noted to exhibit a moderate antioxidant effect. It is therefore extracted and sought after due to its strong antioxidant activities. Hesperidin falls under the flavonoid group of

flavanones, and although this compound has been found in smaller concentrations and is a poor antioxidant in vitro (Joubert *et al*., 2007), its anti-inflammatory properties make it a valuable addition in nutraceutical products (Guardia *et al*., 2001; Rotelli *et al*., 2003).

4. PREPARATION OF PLANT MATERIAL FOR HONEYBUSH MANUFACTURING

4.1 Processing

The manufacture of honeybush tea in South Africa is still based on traditional methods. Processing involves shredding, "fermentation" at elevated temperatures and sun or oven-drying (Du Toit & Joubert, 1999). The tea is cut to ensure disruption of the cell integrity and facilitates fermentation. Traditional heap fermentation, an uncontrolable process, has been replaced by high temperature oven fermentation E. Joubert, ARC, Infruitec-Nietvoorbij, Stellenbosch, South Africa, personal communication, 2005). Fermentation refers to the enzymatic and chemical changes including oxidation step used in processing for the development of the characteristic red-brown to dark brown colour and is essential for the development of the desired sensory properties e.g. the sweet flavour of the beverages (Du Toit & Joubert, 1998b). Unfermented tea refers to processed tea (shredded and dried), but more specifically where the processing did not involve chemical oxidation (fermentation) resulting in loss of phenolic compounds.

A reduction in TP content (Du Toit & Joubert, 1998a; Hubbe, 2000; Joubert *et al.,* 2007), mangiferin content and antioxidant capacity of honeybush extracts (Hubbe, 2000; Joubert *et al*., 2007) occurs with fermentation. This led to the development of green honeybush (De Beer & Joubert, 2002) as a high antioxidant product alternative to the herbal tea market or the agroprocessing industry (E. Joubert, ARC, Infruitec-Nietvoorbij, Stellenbosch, South Africa, personal communication, 2005).

The decreasing antiradical effect observed with fermentation is due to the oxidation of phenolic compounds during fermentation, resulting in a loss of hydroxyl groups, which is essential for effective radical scavenging (Hubbe, 2000). According to Hubbe (2000) aqueous extracts and crude phenolic fractions of *C. intermedia* were *ca*. 2.4 and 5.9 times more effective in donating H to the accepting free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical than their respective fermented equivalents. Antioxidants (AH) act as H-donors to free radicals and in doing so inhibit their activity (Kurechi & Kato, 1982). Crude fermented phenolic fractions of fermented *C. intermedia* with lowest total polyphenol contents were the least effective scavengers. Mangiferin and isomangiferin (Hubbe, 2000) have much higher antioxidant acitivity than hesperidin in the DPPH and superoxide radical scavenging assays, thus indicating it to be more susceptible to oxidation. There is also a possibility of polymerisation of the smaller monomeric polyphenols to form insoluble compounds, leading to

reduced activity of the fermented plant material (Du Toit *et al*., 1998a). Aqueous extracts of unfermented honeybush tea has also been observed to exhibit higher antimutagenicity (Marnewick *et al*., 2000; Van der Merwe *et al*., 2006).

4.2 Chemical changes during enzymatic browning

Appearance of foods, significantly influenced by colour, is one of the first attributes made by consumers in evaluating food quality (Marshall *et al.,* 2000). Food colour can be influenced by naturally occurring pigments such as chlorophylls, carotenoids and anthocyanins (Mackinney & Little, 1962; Counsell, 1981), or by enzymatic and nonenzymatic reactions (Sapers, 1993). The reactions responsible for causing browning discolourations in foods are more complex and cannot be classified as merely enzymatic/nonenzymatic reactions due to a number of secondary reactions that might occur (Sapers, 1993). This is also evident by the range of colours produced by such reactions.

The rearrangement of groups and polymerisation need to take place before colourless polyphenols can convert to brown compounds. Potatoes which have been peeled for e.g. may undergo red, brown, or even black discolourations which are the result of enzymatic browning reactions, but nonenzymatic "after-cooking darkening" which are induced by heating during steam or lye peeling, also may contribute to the discolouration of peeled surfaces. Enzymatic browning is catalysed by the enzyme polyphenol oxidase (PPO) (Martinez & Whitaker, 1995). It is present in most foods as a mixed function oxidase that participates only in the beginning phase of oxidation as it catalyses the change of monophenols to diphenols which are then in turn changed to highly reactive coloured o-chinones. These then react with other o-quinones, amino acids, reducing sugars, etc. to form polymers that precipitate, leading to dark, insoluble polymers called melanins. These form barriers on the surface and possess antimicrobial properties.

Polyphenols are substrates of PPO (Sapers, 1993; Robertson & Christensen, 1996) of which examples are given in Table 3. However, enzymatic browning (Figure 2) does not occur in intact plant cells because the phenolic compounds in cell vacuoles are separated from the polyphenol oxidase, which are present in the cytoplasm. It is only when the plant material tissues are disrupted that the compounds come into contact with the enzyme. The substrate specificity of polyphenol oxidase varies with the source of the enzyme. In general, phenolic compounds and polyphenol oxidase are directly responsible for enzymatic browning reactions during postharvest handling and processing. In addition to serving as substrates to polyphenol oxidase, they also serve as inhibitors of polyphenol oxidases.

Consequences of enzymatic browning can be both desirable and undesirable (Schwimmer, 1980; Martinez & Whitaker, 1995). In some instances, such as cocoa and tea, browning is important to the

flavour development, so the tissues are purposely damaged (Robertson & Christensen, 1996; Du Toit & Joubert, 1998b; Marshall *et al*., 2000). In cases where enzymatic oxidation is undesirable, inactivation of polyphenol oxidases has been brought about by heat treatments such as blanching. Brief exposure of freshly picked green tea leaves to steam for production of green tea results in the inactivation polyphenol oxidase, thereby preserving the antioxidant activities of the polyphenols (Wang & Helliwell, 2000; Hsu, 2005). Techniques attempting to remove one or more of these essential components (oxygen, enzyme, copper or substrate) from the reaction are undertaken (Martinez & Whitaker, 1995). Removal of oxygen can be brought about by vacuum treatment and chelating agents

Table 3 Phenolic substrates of polyphenol oxidases PPO in fruit and vegetables (adapted from Anon. (2004))

Source	**Phenolic substrates**
Apple	Catechol, chlorogenic acid (flesh), catechin (peel), *ρ*-coumaric acid, flavonol glycosides
Banana	3,4-dihydroxyphenylethylamine (Dopamine), leucodelphine, leucocyanidin
Cocoa	Catechins, leucoanthocyanidins, anthocyanins, complex tannins
Coffee beans	Chlorogenic acid, caffeic acid
Lettuce	Tyrosine, caffeic acid, chlorogenic acid derivatives
Mango	Dopamine-HCL, 4-methyl catechol, caffeic acid, catechol, catechin, chlorogenic acid, tyrosine, *ρ*-cresol
Tea	Flavonols, catechins, tannins, cinnamic acid derivatives

are effective in removing copper. Drying of rooibos tea under vacuum conditions to ensure low oxygen levels has been patented (E. Joubert, ARC, Infruitec-Nietvoorbij, Stellenbosch, South Africa, personal communication, 2005).

It has also been noted that many foods undergo browning due to enzymatic reactions that take place not only during processing, but also storage (Sapers, 1993; Marshall *et al.,* 2000). Such chemical changes that influence the quality of the final food product (good or bad) are therefore of great importance. Storage can therefore decrease the flavonoid content, but studies have also revealed increases in flavonoid content or the introduction of the occurrence of flavonoids with novel properties (Nicoli *et al.,* 1997; Manzocco *et al.,* 1998). Lachman et al. (2003) investigated the effect of storage on total polyphenol and main flavonoid antioxidant content in different types of onion (*Allium cepa* L.) varieties. Significant varietal differences were found of the polyphenolic compounds with the highest concentration in the red variety (108.300 $mg.kg^{-1}$ DM), and the lowest in white variety (26.445 $mg.kg^{-1}$ DM). The yellow variety contained 65.210 $mg.kg^{-1}$ DM. The total polyphenol content of red and yellow varieties increased during 36-weeks storage, especially during storage at 22ºC.

Post harvest investigations on a medicinal herbal plant called skullcap (*Scutellaria lateriflora*) growing in Australia was undertaken (Wills & Stuat, 2004). The major compound baicalin comprised 40-50% of the total flavonoids. The need for careful handling was evident due to brown discolouration of leaves caused by incorrect handling (Wills & Stuart, 2004). Storage of dried skullcap powder at 5ºC at ambient humidity resulted in a decrease in flavonoid content of 0.85 mg/day. This was attributed to an increase in moisture content that triggered an increased enzyme activity. In a study by Aquino-Bolaños & Mercado-Silva (2004), the activity of PPO on development of browning in cut jicama during storage was investigated. After a week of storage at 10 or 20ºC, the phenolic content increased from 0.37 to 1.04 $g.100\ g^{-1}$ while the lignin content in fresh tissue increased from 16.5 to 52.22 $g.100\ g^{-1}$. This could be seen as a response mechanism to injury. The PPO was greater in damaged external tissue than in the internal tissue; they were also influenced by temperature. Results indicated that browning of cut jicama at 20ºC was related to the process of lignfication in which the peroxidase enzyme plays an important role.

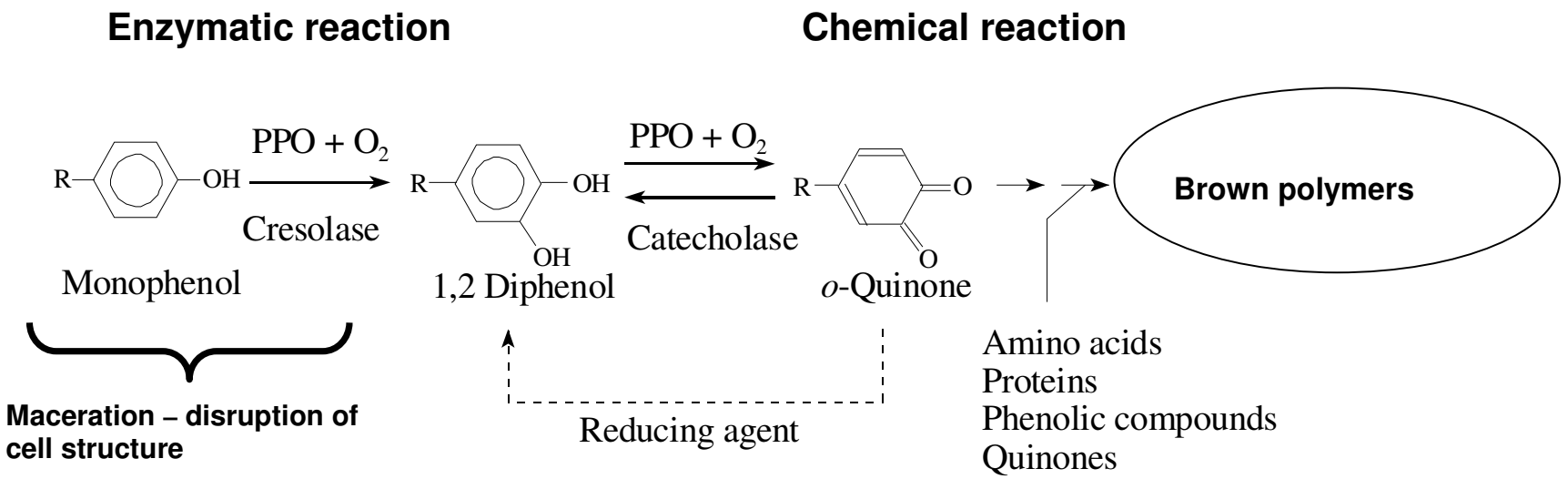


Figure 2 Enzymatic browning reaction (adapted from Sapers (1993)).

5. EXTRACTION PROCEDURES

Customer's awareness for "non-chemical" ingredients in health products provides the cosmetic and pharmaceutical industries with opportunities to commercialise compounds isolated from plant material and waste products. Limiting factors are the effectiveness of recovery and extraction, the marketability of resulting extracts and the practical suitability for the food, cosmetic or pharmaceutical products. Peschel *et al*. (2006) demonstrated the possibility to recover high amounts of phenolics with antioxidant properties from fruit and vegetable wastes for food and cosmetic applications. Antioxidants have also been recently extracted from wine making wastes (grape stalks and marc) by Spigo & De Faveri (2007). This also proved to be a good low-cost antioxidant source. Nwuha *et al*. (1999) recovered valuable bioactive components (green tea catechins) from industrial tea waste.

The decision as to the choice of extraction method is of great importance as it is essential to extract not only the desired chemical compounds from the plant material, but also to extract adequate quantities (Huie, 2002). The types of phenolic compounds and whether the objective is quantitative or qualitative recovery are further considerations that determine extraction procedures. The diversity and complexity of the chemical composition of plant material make it difficult to predict optimum extraction conditions. George *et al*. (2001) advises that the choice of solvent is also dependent on the end purpose of the extract. If for example, the purpose of extraction is to isolate chemical components (without any bioassay), toxicity of the solvent is not critical, as the extract can be made solvent-free before subsequent isolation procedures.

The solubility of phenolic compounds in the extraction solvent plays a key role in the quantities extracted. Hot water is frequently used in extraction systems (Bergeron *et al.,* 2005; Ivanova *et al*., 2005) and so too are organic solvents such as methanol, ethanol or acetonitrile, or mixtures of these solvents with water depending on the polarity of the compounds (Lin *et al*., 1996; Waterman & Mole, 1993; Ong, 2004; Bergeron *et al*., 2005; Gálvez *et al*., 2005; Lin & Giusti, 2005). It is the presence of the attached sugars which results in the phenolic compounds to be more water soluble and therefore combinations of the above solvents with water are more effective for glycosides (Escribano-Bailón & Santos-Buelga, 2003). Wang & Helliwell (2001) found 60% aqueous ethanol as the best solution for hydrolysing flavonoids in tea leaves. Flavonoids were most efficiently extracted from skullcap powder by using 40-60% aqueous ethanol with about 70% of the flavonoids passing into the solvent (Wills & Stuart, 2004).

Acetonitrile-water (1:1, v/v) has also been proposed to be an efficient extraction solvent to quantitatively extract catechins from green tea leaves without epimerisation of the major catechins (Suematso *et al.*, 1995). Small differences of the extraction efficiencies of (-)-epigallocatechin (ECG)

and (-)-epicatechin (EC) were observed with changes in tea concentration or using buffer solutions between pH 6.4 to 6.7 as extractant. The concentration of the tea had a greater impact though on extraction efficiency of gallate catechins compared to changes in pH.

Studies by Wang *et al*. (2000) and Fernandez *et al*. (2002) employing acetonitrile:water (1:1 and 60:40 respectively), showed acetonitrile as a good extraction solvent for tea biochemicals (catechins, gallic acid and xanthine alkoids). According to Perva-Uzunalié *et al*. (2006) extraction efficiency of catechins from green tea was vastly reduced (3%) when using pure acetone and acetronitrile. However, by using mixtures of different aqueous solvents (acetone, ethanol, methanol and acetonitrile), an extraction efficiency varying from 60% to almost 100% was obtained.

Xanthones have been successfully extracted from dry tree trunk and root bark of the shrub *Trema orientalis* with boiling ethanol-water (1:1) and ethyl acetate (Tchamo *et al*., 2001). Mangiferin has been found to be the only water-soluble xanthone (Harborne, 1998), but improved extraction efficiency is obtained using solvents of lower polarity (Markham, 1975). Methanol is used as extraction solvent for mangiferin quantification from various plant materials e.g. *Gnidia involucrata* (Ferrari *et al*., 2000) and Traditional Chinese medicines (*Cratoxylum* spp.*)* (Tang *et al*., 2004). Methanol has also been used in the initial extraction step of mangiferin from aerial parts of *Swertia puntata*, another traditional medicine (Menković *et al*., 2002). A patented product, Vimang® containing high levels of mangiferin is produced from mango stem bark by decoction (Núñez-Sellés *et al*., 2002). Kitanov & Nedialkov (1998) extracted both mangiferin and isomangiferin from *Hypericum* species using ethanol.

Citrus peels, with a rich source of natural flavonoids, including hesperidin, are also extracted due to their high polyphenol content (Li *et al*., 2006) using ethanol (food-grade solvent). The main parameters that affected the yield of phenolics were found to be the condition of the peels, temperature of the extraction, solvent concentration and also the species of the citrus. Methanol was previously used, but found to be a harsh and toxic organic solvent, whereas ethanol is more acceptable for use in the food industry. Several extraction conditions were investigated by Calabrò *et al*. (2004) to obtain the most efficient method to extract flavonoids from oranges including hesperidin. It was found that hesperidin was best extracted with ether and ethyl acetate at 40ºC for 20 min. Sonication was performed on powdered Daflon® tablets with methanol:DMSO (1:1) at 30ºC for 30 min and the results indicated a very efficient system for the complete extraction of hesperidin in these tablets (Kanaze *et al*., 2003). Hesperidin was also isolated in Buchu leaves and tablets following extraction with methanol and 10% dimethylsulfoxide added (El-Shafae, & El-Domiatry, 2001).

Other factors that may affect the quantity of the component to be extracted are the physical form of the plant material, the mass ratio (solvent-to-solid ratio), temperature of the solvent, contact time as it

affects diffusion of the soluble compounds from the plant matrix. It was observed that the particle size of ground tea leaves significantly influenced the yield of flavanols. Sharma *et al*. (2005a) found that it was due to the flatness of the particles that have higher open surface, which leads to more extraction of catechins. According to Eloff (1998), powdering of the plant material increased the surface area accessible to solvents and therefore resulting in better extraction. Dried material is preferred in cases where there is a delay between collection and processing. Fewer problems of dried material with large scale- extraction occur. Unlike the fresh material, in which problems arise in liquid-liquid extraction from the interfering water content (Eloff, 1998). Agitation (mass transfer) is also employed to increase the diffusion rate of polyphenols out of the leaf/plant material and into surrounding areas by decreasing concentration of the surrounding fluid (Yoshida *et al*., 1999). In a study done by Pinelo *et al*. (2005) on grape pomace, a linear relationship was found between the solvent-to-solid ratio and solid yields despite the solvent used. The enhancing effect of increasing the temperature on the solubility of the solute and diffusion coefficient was also demonstrated.

In order for accurate quantification of napthodianthrones and other constituents in St. John's wort, Williams *et al*. (2006) found that it depended on the selection of appropriate extraction conditions. The highest level of polar and nonpolar constituents was achieved through Soxhlet extraction over a period of 24 hours. Overall, longer extraction times and multiple solvent extracts resulted in more complete extractions. Soxhlet extraction is however unsuitable for thermo-labile compounds (George *et al*., 2001).

Table 4 is a summary of different extraction methods employed in obtaining phenolic components from various plants with emphasis on mangiferin and hesperidin. A summary of extraction conditions for with special reference to green honeybush is presented in Table 5.

Although solvent extraction is a traditional method used for extraction of natural plant products, it is labour-intensive, time-consuming and requires large volumes of solvents (Da Costa *et al*., 1999). Extraction with water as solvent takes longer and if not evaporated properly could result in microbial contamination (George *et al*., 2001). Reduced solvent methods include methods such as microwave-assisted extraction (MAE), solid-phase extraction (SPE) and solid-phase micro extraction (SPME) (Raynie, 2006). Alternatives to the conventional solid-liquid extraction to obtain optimum extraction of the active components or group of compounds also include pressurised fluid extraction (PFE) (also referred to as accelerated solvent extraction) and supercritical fluid extraction (SFE) (Da Costa *et al*., 1999; Huie, 2002; Escribano-Bailón & Santos-Buelga, 2003; Chen *et al*., 2004; Bergeron *et al*., 2005; Raynie, 2006). It was concluded that these methods remove compounds in similar or slightly higher yields from plant material and also in a much shorter period with smaller amounts of solvent (Da Costa

et al., 1999; Teixeira & Da Costa, 2005). Degradation as a result of lengthy exposure at elevated temperatures is avoided and extracts with fewer unwanted analytes are obtained by careful manipulation. These methods, however, did not give superior extraction of total flavonoids from *Scutellaria lateriflora* as compared to standard hot water or 70% ethanol extraction (Bergeron *et al*., 2005). In SFE the use of modifiers, such as ethanol or methanol can improve the solubility of the more polar compounds, but sometimes this is still inefficient. A novel packed-column extractor coupled with an absorption system was used to improve the quality of oleoresin oils (containing polyphenols) from Oolong and green teas. Carbon dioxide and ethanol were used in this extraction as co-solvent (Chang *et al*., 2001). This method is also referred to as supercritical carbon dioxide extraction ($SCCO_2$).

Extraction with microwave-assisted extraction (MAE) using tea leaves proved to be more efficient as higher percentages of polyphenols where extracted in a shorter time in comparison to other conventional methods (Pan, 2003). Although MAE shares several similarities to SFE and PLE, significant differences also exist. MAE may use either an open or closed vessel approach. It also allows the usage of acids and bases for making digestion possible. However, usage is directed more towards the food industry (Raynie, 2006). Another novel method for the extraction of xanthones and flavanones from root bark of the osange orange tree (*Maclura pomifera)* is matrix solid phase dispersion (MSPD), a patented process that permits simultaneous disruption and extraction of semi-solid and solid samples (Teixeira & Da Costa, 2005). This method proved to be a more efficient and rapid extraction method than solid-liquid extraction.

Table 4 Summary of methods employed for extraction of polyphenols from plant materials

Plant Material	Compound	Plant:solvent ratio	Extraction solvent	Extraction Method / Temperature	Extraction time	Reference
Camellia sinensis (green tea)	Catechins Tea alkaloids	0.2 g : 5 mL	Methanol	Shaking/vortex Centrifugation	30 min	Sharma *et al*., 2005a
Black tea	Theoflavins Thearubigins Theabrownins	0.2 g : 200 mL	Boiling water	Stirring on hot plate (90°C)	10 min	Yao, *et al*., 2006
Citrus Bergamia	Hesperidin Narirutin Eriocitrin	1 g : 50 ml	Ether/Hexane Ethyl/Methanol	Boiling	20 min	Calabrò *et al*., 2004
Hypericum species	Mangiferin Isomangiferin	1 g : 50 ml	Ethanol	N/A	2 hours	Kitanov & Nedialkov, 1998
Menthapiperita (Lamaiceae)	Flavonoids	3 g : 50 ml	Ethanol	Agitation	15 min	Yoshida *et al*., 1999
Orthosiphon stamineus	Flavonoids	10 g : 100 ml	Acetone Chloroform Methanol	Waterbath (40ºC)	2, 4, 8 hours	Akowuah *et al*., 2005
Camellia sinensis (green tea)*;* Black tea	Flavonols	1 g : 40 ml	Ethanol	Refluxing	2 hours	Wang & Helliwell, 2001
Citrus peels	Flavonoids	2 g : 16 ml	Ethanol	Centrifugation	6 hours	Li *et al*., 2006
Traditional Chinese medicines (*Cratoxylum* spp.*)*	Mangiferin	1 g : 50 ml	Methanol	Continuous Stirring	3x (30 min)	Tang *et al*., 2004
Gnidia involucrata	Mangiferin	250 g : 1.5 L	Methanol	N/A	N/A	Ferrari *et al*., 2000
Swertia punctata	Mangiferin	42 g : 150 ml	Methanol	(2 x 150 ml) at room temp.	24 hours	Menković *et al*., 2002

Table 5 Extraction conditions for mangiferin, xanthone and hesperidin content for green honeybush

Cyclopia species	Compound	Plant Material	Plant:solvent ratio	Extraction solvent	Extraction Method / Temperature	Extraction time	Reference
C. genistoides	Mangiferin Hesperidin	Leaves+stems	2 g:150 ml	Deioinised water	Magnetic/Stirring	2 min	Botha, 2005
	Mangiferin Hesperidin	Leaves+stems	5 g: 50 ml	Methanol	Waterbath (64°C)	30 min	Botha, 2005
C. subternata	Mangiferin Hesperidin	Leaves+stems	3 kg: 5 L	Acetone	N/A	24 hours	Brand, 2002
	Mangiferin Hesperidin	Leaves+stems	8 kg: 5 L	Methanol	N/A	24 hours	Brand, 2002
	Mangiferin	Leaves		Methanol	Room temp	4 hours	De Nysschen et al., 1996
		Leaves+stems	100 g: 1000 ml	Deioinised water	Steeping	5 min	Van der Merwe, 2005
	Xanthone(M+I)[a]	Leaves+stems	100 g: 1000 ml	Deioinised water	Steeping	5 min	Richards, 2002
	Hesperidin	Leaves + stems	100 g: 1000 ml	Deioinised water	Steeping	5 min	Richards, 2002
		Leaves + stems	100 g: 1000 ml	Deioinised water	Steeping	5 min	Van der Merwe, 2005

[a] Mangiferin and isomangiferin co-eluted

6. METHODS FOR QUALITY CONTROL AND QUANTIFICATION

Quality control in the food, nutraceutical and pharmaceutical industries can never be overemphasised. It is a very important aspect and careless mistakes cannot be afforded when the health of an individual is at stake. According to a World Health Organisation survey, 70-80% of the world's population relies on non-conventional medicine from herbal sources in their primary healthcare (Chan, 2003; Anon., 2006a). There has also been an increase in over-the-counter (OTC) health foods, nutraceuticals and medicinal products from plants. This is an indirect indication that the public is not satisfied with orthodox medical treatment. Such an increase in the popularity is also accompanied by concerns and fears over the professionalism of practitioners and the quality, efficacy and safety of the treatment methods and products from herbal and natural sources that are widely available on the market. There have been several news-catching episodes, which indicated adverse effects – some life threatening, as a result of taking herbal products. These products could be contaminated with excessive or banned pesticides, microbial contaminants, heavy metals, and chemical toxins or adulterated with orthodox drugs. The excessive use of banned pesticides and microbial contaminants could be related to the source of these herbal materials (Anon., 2006a). Chemical toxins may also come from unfavourable or wrong storage conditions or chemical treatment as a result of storage. However, some of these environmental related factors can be controlled by implementing standard operating procedures (SOP) leading to Good Agricultural Practice (GAP), Good Laboratory Practice (GLP), Good Supply Practice and Good Manufacturing Practice (GMP) for producing these medicinal plants from herbal or natural sources.

Wilkensen (1998) reported the following aspects that need to be taken into account when assessing the quality of herbal products for nutraceutical development: the macroscopic and microscopic properties; its botanical name and also the family; the geographical source; methods undertaken during drying and/or extraction (this includes solvents used); and consistent analytical data (e.g. thin layer chromatography (TLC) and high performance liquid chromatography (HPLC)). Standardisation and quality control of herbal products can further be implemented by using marker or pharmacologically active components (He, 2000; Liang *et al*., 2004; Ong, 2004). A chemical fingerprint used for the identity and quality of a given species was obtained for 10 phenolic compounds from ethanol extracts in peppermint *Mentha* x *piperita* (Areias *et al*., 2001) and flavonoids in *Ginko biloba* (Deng & Zito, 2003). Other examples of marker compounds are hyperoside in St. John's Wort (Rager *et al*., 2002), harpagoside in Devil's Claw (Guillerault *et al*., 1994), baicalein in *Scutellaria lateriflora* L. (Wills & Stuart, 2004), diosmin in buchu leaves (*Barosma betulina*) (El-Shafae, & El-Domiatry, 2001),

echinacosides in Echinacea (Perry *et al*., 2001) and squalene in mate tea leaves (*Ilex paraguariensis*) (Esmelindro *et al*., 2005).

It has also been postulated that phytochemicals (which include phenolic compounds, saponins, alkoloids and other classes of natural products occurring in plant extracts) in herbal plant material differ significantly not only among species but even the same plant extracts (He, 2000). Factors such as the harvesting season, geographical area, climate, drying process, age and storage conditions are all factors contributing to this variation and have made it a challenge for the food and phytopharmaceutical industries in the quality control of herbal material (He, 2000; Du Pont *et al*., 2000; Laasonen *et al*., 2002; Chan, 2003; Franke *et al*., 2004; Perva-Uzunalie *et al*., 2006; Yao *et al*., 2006). Howard *et al*., (2002) found the antioxidant activity and phenolic content of spinach to be influenced by genetics, biotic and abiotic stresses and seasonal growing conditions.

Due to the increased competitiveness in the food industry, authentication and adulteration of products are two other important aspects of quality control for manufacturers. Products must conform to the description provided by the producer/ processor and no adulterants are to be added (Downey, 1995; Downey, 1996a; Downey, 1998). The popularity and scarcity of certain medicinal herbs may encourage the sale of poor quality herbs. According to Chan (2003), substitution or adulteration with more toxic herbs may occur erroneously e.g. when the herb is incorrectly identified or deliberately adulterated when for economic reasons, a cheaper herb is supplied to replace a safer more expensive one. Dependence on chemical analysis for identification purposes leads to problems due to the levels of active (secondary metabolites) components that are affected by physiological conditions e.g. harvesting period, and the fact that closely related species may contain similar chemical components from which a definite botanical identification is not always possible.

Classical cytogenetic methods include chromosome counting and karyotyping may be useful to differentiate medicinal materials. Proteins have also been used as identification markers, but drawbacks include: varying protein patterns in different tissues, developmental stages and environment as a consequence of temporal and special gene expression and their degradation after long periods of storage. DNA markers are more reliable as they are less affected by age, physiological conditions as well as environmental factors. The DNA can be extracted from the leaves, stems or roots and a small amount is needed. The European Agency for the Evaluation of Medical Products states that the identification of herbal drugs is one of the first tests undertaken in ensuring quality, safety, and efficacy of herbal medicinal products. Testing should also be able to discriminate between related species and/or potential adulterants (Anon., 2001).

6.1 Quality evaluation of honeybush

Local extract manufacturers make use of TP content and TAA, as determined with the Folin-Ciocalteau (FC) and ABTS (Re *et al*., 1999) assays, respectively, to standardise their products (E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, personal communication, 2005). HPLC has also become a useful tool in quantifying compounds present in sample extracts. Currently, the standards and requirements for the export of honeybush tea (Anon., 2000) do not include any of the requirements for the evaluation of herbal products. Discussed in the previous section Table 6 gives a summary of analytical methods used in studies on honeybush and polpyphenol extracts to quantify phenolic compounds and determine the antioxidant activity of extracts.

6.2 High-performance liquid chromatography (HPLC)

Since as early as the 1970's, HPLC has been used for the determination of flavonoids and has become a popular method for separating, screening and quantitative analysis of plants, food products (Santos-Buelga *et. al*., 2003) and also herbal medicines (Liang *et. al* 2004; Wills & Stuart, 2004). High reproducibility and accuracy were obtained by performing HPLC on different tea samples (Sharma *et al*., 2005b). A simple gradient elution method efficiently separated various tea biochemicals (catechins, gallic acid and xanthine alkoids) using acetonitrile and 0.1% ortho-phosphoric acid in water was used. HPLC has also been used to quantify the xanthone mangiferin (De Nysschen *et al*., 1996; Joubert *et al*., 2003; Richards, 2002; Van der Merwe, 2005; Botha, 2005), isomangiferin (Joubert *et al*., 2003), hesperidin (Joubert *et al*., 2003; Richards, 2002; Van der Merwe, 2005), eriocitrin, hesperetin, narirutin and luteolin (Van der Merwe, 2005) in honeybush. Interestingly, separation systems for flavonoids in foods have been focused toward measurement of all (usually several subclasses) of prominent flavonoids (Merken & Beecher 2000).

Not only can HPLC be widely used in quantification of various flavonoids and other phenolics, it can also easily be adapted for identification of individual compounds and therefore has the advantage of generating a chemical fingerprint used for the identity and quality of the given species. Such a fingerprint was obtained for 10 phenolic compounds from ethanol extracts in peppermint *Mentha* x *piperita* (Areias *et al*., 2001). HPLC pattern of a standardised extract can also prevent the problem of adulteration due to misidentification of the herb in the field (Anon., 2006a). The Therapeutic Goods Adminstration (TGA) of Australia utilised the characteristic profiles for rapid indentification of HPLC in fingerprinting skullskap (*Scutellaria lateriflora* L.), a medicinal plant (Wills & Stuart, 2004). Flavonols have also been quantified in green and black tea leaves (Wang & Helliwell, 2001). A new faster method has made the simulataneous analysis of the eleven main flavonoid aglycones (flavanols,

flavonols, flavanones and flavones) in food samples under 15 min possible (Repollés *et al*., 2006). The disadvantages of this technique are that it is expensive due to the high cost of equipment and solvents and is also time consuming (Harborne, 1998).

6.3 Colorimetric methods

A variety of spectrophotometric and colorimetric methods exist which can be used for the quantification of various phenolic groups. For colorimetric determination the compounds are reacted with chemicals to form colour complexes that exhibit a selective light absorptive capacity, which are measured in the visible range with a spectrophotometer. This measurement will then determine the concentration of the compound responsible for the colour (Boltz & Mellon, 1966). Different compounds or groups of flavonoids will react differently with different chemicals. This is due to their molecular structure. The quantification of polyphenols can either be a class of compounds such as flavonoids, a subclass such as flavanones or individual compounds such as hesperidin (Harborne, 1998). Since the total polyphenol content and xanthone content are of interest for this research project, the principles behind the methodology used in their quantification will be discussed. Table 6 is a summary of conventional analytical methods used for determining the polyphenol composition of honeybush previously used.

6.3.1 Quantification of total polyphenols

The TP content of honeybush tea extracts, in view of a quality parameter, can routinely be determined using the Folin Ciocalteau colorimetric method (Joubert, 1988; Joubert, 1996; Du Toit & Joubert, 1998a; Joubert *et al*., 2004). The principle for the Folin Ciocalteau method is an electron transfer assay that gives reducing capacity (Prior *et al*., 2005). It is based on the reducing power of phenolic hydroxyl groups, which detect all the phenols with varying sensitivities (Singleton & Rossi, 1965). The intensity of the resulting blue complex can then be estimated with a spectrophotometer (λ_{max} at 725 nm). Several adaptations of the original method have been used, but the use of the original method is recommended (Prior *et al*., 2005). Compared to other methods, analyses using this method are simple, convenient and require available equipment commonly used. The phenols react to different degrees and expression of the results in terms of a standard, i.e. gallic acid is necessarily arbitrary. The reaction is also independent, quantitative, and predictable. The analyses of a mixture of phenols can therefore be recalculated in terms of any other standard (Singleton & Rossi, 1965). Interference can be caused by readily oxidized substances such as ascorbic acid, sulfite, sulfur dioxide and even high sugar content (Singleton *et al*., 1999). TP content determination has been applied to a wide variety of plant extracts

and wine (Zheng & Wang, 2001; Liu *et al*., 2002; Borbalán *et al*., 2003; Costin *et al*., 2003; Baydar *et al*., 2004; Ivanova *et al*., 2005).

Table 6 Analytical methods used for determining polyphenol composition of *Cyclopia* extracts

Parameter	*Method*	*References*
Total Polyphenol content	Folin-Ciocalteu assay colorimetric method[a]	Joubert, 1996; Du Toit & Joubert, 1998a,b; Du Toit & Joubert, 1999; Marnewick *et al.*, 2000; Richards, 2002; Marnewick *et al*., 2003; Joubert *et al*., 2006; Manley *et al*., 2006;
Xanthones (M+I)[b]	HPLC[c]	Richards, 2002
Mangiferin (M)	HPLC	De Nysschen *et al*., 1996; Joubert *et al*., 2003; Van der Merwe, 2005; Joubert *et al*., 2003
Isomangiferin (I)	HPLC	
Flavanones		
Hesperidin	HPLC	Joubert *et al*., 2003; Joubert *et al*., 2006
Narirutin	HPLC	Van der Merwe, 2005
Antioxidant activity	ABTS[d]	Richards, 2002; Schulz *et al*., 2003a; Joubert *et al*., 2006; Manley *et al*., 2006;

[a] Singleton & Rossi, 1965
[b] Mangiferin and isomangeferin co-eluted
[c] Reverse-phase-high performance liquid chromatography
[d] Radical cation 2,2'-azinobis(3-ethyl-benzothiazoline-6-sulphonate) decolourisation assay (Re *et al*., 1999)

6.3.2 Xanthone determination by aluminium chloride method

The determination of certain classes of phenolic compounds is made possible by using more specific colorimetric methods. The principle of the $AlCl_3$ colorimetric method is based on the fact that $AlCl_3$ forms acid stable yellow complexes with the C4 keto group and either the C3 or C5 hydroxyl group of flavones and flavonols (Jurd, 1962; Mabry *et al*., 1970; Chang *et al*., 2002). A possible complexation is depicted in Figure 3. Should the flavones and flavonols lack a free C5 or C3 hydroxyl group, no complexes will form after the addition of the $AlCl_3$ solution (Jurd, 1962). $AlCl_3$ can also form acid labile complexes with the orthodihydroxyl groups on the A or B ring of flavonoids (Chang *et al*.,

2002). Flavanones, having a C5 hydroxyl group, form complexes with $AlCl_3$, but they give insignificantly low absorbance at 415 nm (Chang *et al*., 2002).The molecular structure of mangiferin, particularly the keto group adjacent to a hydroxyl group, allows complexation with $AlCl_3$ and the spectra of xanthones have been observed to undergo characteristic bathochromic shifts with $AlCl_3$. These differ according to the number and position of the hydroxyl substituents (Harborne, 1998).

The complex formation results in significant bathochromic shifts of Bands I and II of flavonoids (Mabry *et al*., 1970). Detection of the flavonoids is in the ultra-violet region since phenols absorb in this region. Two absorption bands are characteristic of flavonoids. Band II, has a maximum in the 240-285 nm range and is believed to arise from the A-ring. Band I have a maximum in the 300-500 nm range, and is thought to arise from the B-ring.

Joubert *et al*. (2006) found a good correlation (r = 0.9) between the mangiferin content of methanol extracts from *C. genistoides,* determined by aluminium chloride complexation and HPLC analysis. Quantification of mangiferin was made possible due to the high quantity of this compound compared to the other phenolic compounds in *C. genistoides* (Joubert *et al*., 2003; Van der Merwe, 2005; Botha, 2005). However, a moderate correlation (r = 0.7) was obtained for comparison of the mangiferin content in water extracts analysed by HPLC and the aluminium chloride assay. This was attributed to the lower mangiferin content and greater interference by hesperidin in the water extract. Mangiferin has been noted to be slightly less soluble in methanol than in water, while hesperidin is readily soluble in methanol and not in water. The possible use of this method for determination of xanthones (mangiferin and isomangiferin) with minor interference of other phenolic compounds should be investigated for other *Cyclopia* species of commercial interest, since they contain substantially less xantones than *C. genistoides* (Joubert *et al*., 2007).

6.4 Colour as a quality parameter

The ethics, safety, sensory characteristics and acceptability of food are all affected by colour (Clydesdale, 1993). It is the most important attribute used to evaluate the quality of a product and its possible taste without touching. Judgments where safety of food is concerned based on colour is made prior to choosing/eating the food. Colour has been found to interfere with judgment of flavour intensity and identification and results show that it dramatically influences the pleasantness and acceptability of foods. A radical shift, for example in the colour of food, even though no change in flavour or texture, could make the food completely unacceptable to consumers (Daood, 1993). Colour, in a quantitive sense, has also been found to replace sugar and still maintains sweetness perception in flavoured foods (Clydesdale, 1993).

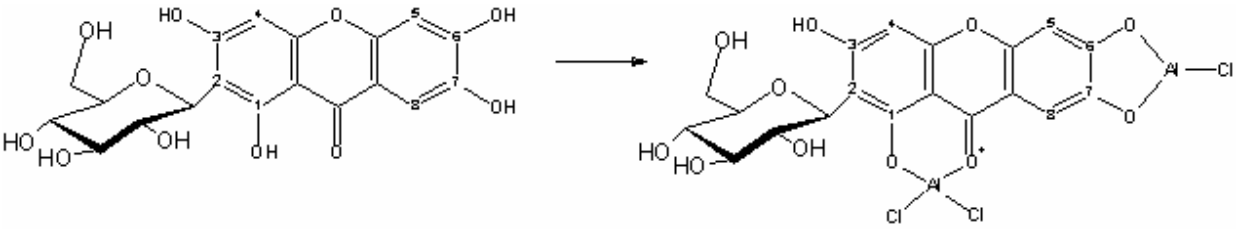


Figure 3 Possible complexation of mangiferin with $AlCl_3$.

Colour measurements are done using the "1976 CIE L*, a*, b* colour space" (official abbreviation – CIELAB). The three attributes of colour are saturation, lightness and hue. The parameter L* (refers to the lightness coordinate), a* (the red/green coordinate), with +a* indicating red, and -a* indicating green, b* (the yellow/blue coordinate), with +b* indicating yellow and -b* indicating blue (Clydesdale, 1978; Sharma & Goike, 2003.

The a* and b* measurements are used to calculate the hue angle (H), which is given by the equation $\tan^{-1}$ b*/a and the saturation index (chroma) given by square root of (a^{*2} + b^{*2}) (McLaren 1980). Both a* and b* measurements are taken into account during calculation of H, which accounts for the increased discriminating ability of the H value in comparison to individual objective colour measurements. Hue is the single colour function described by what most people of as colour, i.e. red, yellow, green or blue (Clydesdale, 1978) (Figure 4).

CIELAB colour measurements have been used to determine quality parameters ranging from providing efficient and reliable flower colours, which can be precisely, communicated (Gonnet, 1993) to the colour measurement of rooibos tea extracts, which differ in solid concentration and turbidity (Joubert, 1995). Commercially-produced tea samples bearing the highest colour rating had significantly higher *a values, thus indicating the importance of the red tint in rooibos tea extracts in quality evaluation.

According to Du Toit & Joubert (1998a), leaf colour of fermented *C. genistoides* and *C. intermedia* was significantly ($P<0.05$) influenced by the drying method used, but the difference in the b* values between the two drying conditions was insignificant ($P>0.05$). The extract colour was also not significantly ($P>0.05$) affected. Drying temperature was found not to have a significant ($P>0.05$) effect on the dry leaf colour of *C. genistoides* and *C. intermedia*, except on the a* values of *C. genistoides*

(dried at 60 and 70ºC). The loss of red colour was attributed to the formation of brown polymerisation products due to the high drying temperatures.

The effect of different pre-treatments (bruising, hot and cold water) on effect of fermentation of honeybush tea (*C. maculata*) was investigated by Du Toit & Joubert, 1998a). Colour measurements therefore have the potential to give us an indication as to the polyphenol content in the plant material based on visual appearace. However, up to now the effect of using pre-treatments on green honeybush has not been undertaken.

A naturally occurring colour pigment is chlorophyll. It is found in organisms capable of photosynthesis and is therefore a vital component of all green vegetables (Counsell, 1981). Naturally occurring chlorophylls are organic molecules, which contain a central atom of magnesium. There are two major forms of chlorophyll (types a and b), which differ in the position of the centrally located metal atom. This compound has been noted to undergo degradation during processing and storage (Figure 5). Chlorophyll degradation depends on temperature, pH, time, enzyme action, oxygen and light. According to Lajolo & Lanfer Marques (1982), for a_w >0.32, the most important mechanism of chlorophyll degradation is the transformation into pheophytin.

Furthermore, depending on the conditions of the dehydration process, dried products are found to vary in the content and composition of their chlorophylls. Under normal conditions of drying chlorophylls *a* and *b* make up the highest proportion of the pigments in dried products such as celery, spinach, green peas, etc. However, on the other hand, pheophytin and pheophorbides are predominant in vegetables which have been dried by severe heating or natural sun-drying, without a pre-drying blanching step (Daood, 1993). Canjura & Swartz (1991) found the highest conversion level of chlorophylls to chlorophyllides at 65ºC for 30 min in hot water. Prolonging the treatment, results in the increase in concentration of degradation compounds (pheophytins and pheophorbides) and a significant decrease of the concentration of chlorophylls and chlorophyllides.

Different methodologies have been used to preserve the green colour of fruits and vegetables. Techniques employed include use of salts, control of thermal treatment and changes in pH (Delgado-Vargas & Paredes-López, 2003). After crop harvesting, chlorophyll is degraded with the rate depending on the plant material and processing conditions. It can occur within a few hours or over several weeks (Ryan-Stoneham & Tong, 2000). In plant tissue, chlorophyll is released from the protein complex followed by phytyl elimination. Degradation proceeds by oxidation of the ring structure to chlorines and ultimately results in the formation of colourless end products.

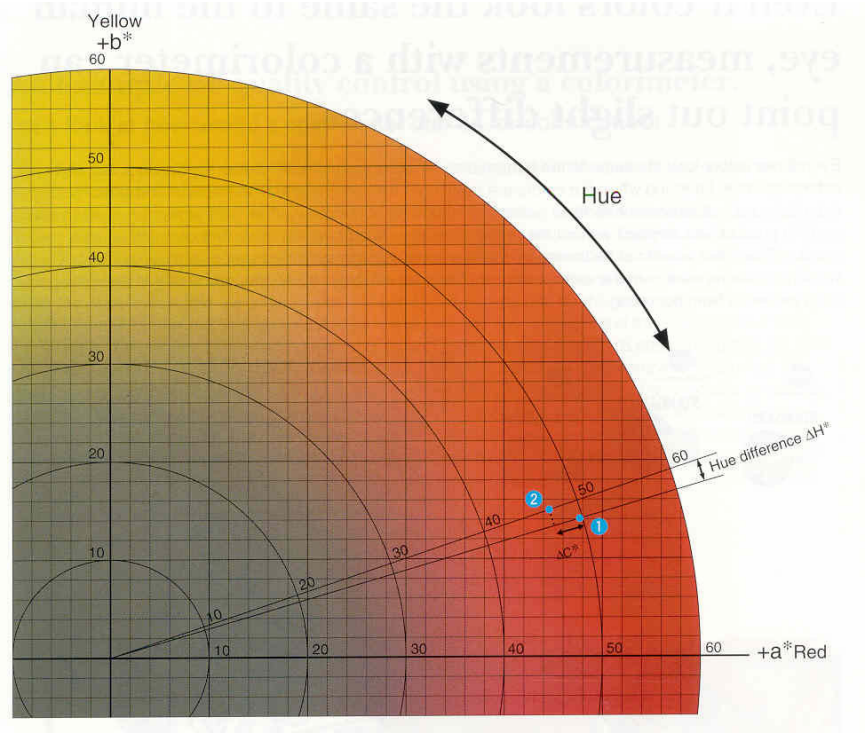


Figure 4 Colour tone (value and chroma) (Anon., 1998).

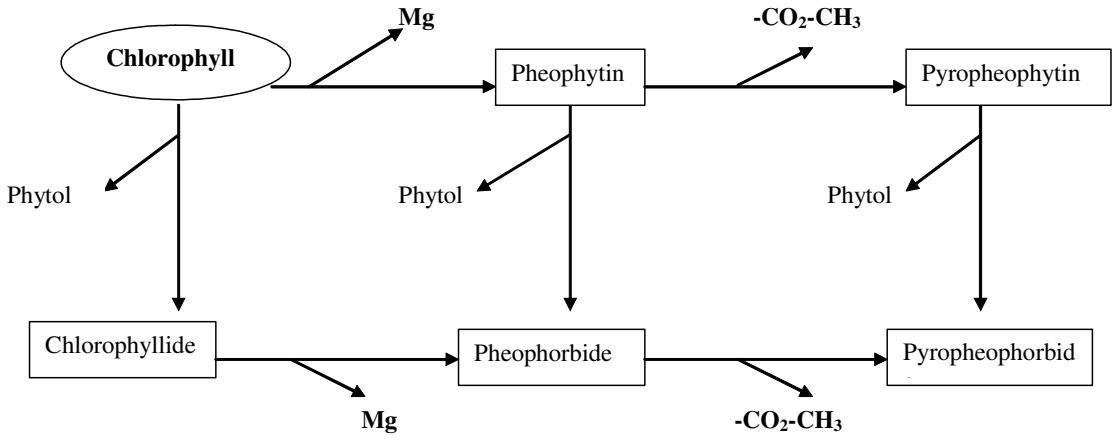


Figure 5 Chlorophyll degradation (adapted from Delgado-Vargas & Paredes-López (2003)).

The effect of colour as a quality parameter on green peppers was undertaken by Sigge *et al*. (1999). Colour measurements were used to evaluate the effect of drying conditions on dehydrated green peppers stored at different temperatures. Optimum retention of colour, using a*, C, and H as criteria, was found to remain constant at lower temperatures (55 and 60ºC) with increasing relative humidity and to decrease at higher temperatures (70 and 75ºC).

In a follow up study by Sigge *et al*. (2001) on dehydrated green peppers, storage temperatures significantly affected ($P<0.05$) the visual rankings given by a sensory panel. As the storage period progressed, the colour differences between samples stored at different temperatures became more evident and the panel could distinguish between them. Samples that had been stored at 0ºC retained their green colour better than those stored at 10 and 20ºC. Samples stored at low temperatures (0ºC) are therefore beneficial for the retention of the perceived green of colour of dried green peppers. Furthermore, the best correlation ($r = 0.74$) between visual colour evaluation and the objective colour measurements was obtained for a* (greenness) measurements. Samples stored at 0ºC and 10ºC resulted in less colour deterioration than those stored at 20ºC, indicated by the smaller increase in a* measurements for storage at 0ºC or 10ºC (-8.216) and 20^{o}C (-3.733) respectively. The results therefore corresponded to the observations made by visual ranking that storage at lower temperatures (0 and 10ºC) resulted in better retention of colour than storage at 20ºC. It was also showed that it is possible to ensure colour retention at low temperatures (e.g. 10ºC) without unnecessarily storing at 0ºC and incurring greater energy requirements. The H values decreased at all the conditions during storage, indicating that a shift had taken place from the yellowish-green region from the (L*a*b*) colour solid (Anon., 1998) and towards the yellow region.

Huag *et al*. (2005) investigated the effects of microwave and oven heating on the preservation quality of green tea. Tea processed by microwave heating had chlorophyll content and it was more stable than when oven heating was used. A strong possibility exists that this method could prevent the browning changes by reducing the decomposition of chlorophyll.

7. NEAR INFRARED (NIR) SPECTROSCOPY

7.1 Background

Near infrared (NIR) spectroscopy has become a favourable alternative quality control method in the food industry due to its valuable advantages over other analytical techniques. This method is fast, easy-to-use and non-destructive (Day & Fearn, 1982; Schulz, 2004). It is environmentally friendly as no harsh chemicals are required and it produces a complete spectrum of constituents in the sample. The analysis can be carried out on-line (Osborne *et al*., 1993a; Hoyer, 1997) as well as in-line (Kamatsu *et*

al., 1995; Singh Sahni *et al*., 2004). Most of the time little or no sample preparation or pre-treatment is needed (Day & Fearn, 1982; Osborne, 2000). An ingenious advantage of NIR spectroscopy is that it allows several constituents to be measured simultaneously (Osborne, 2000). It can also be conveniently used to analyse liquids, slurries and solids (Williams & Stevenson, 1990; Blanco & Villarroya, 2002).

Usually, flavonoids found in medicinal and spice plants are determined by HPLC, but this procedure is very time-consuming (Schulz, 1998). Often the chemical method requires a large sample size to perform the analysis and instruments such as HPLC and mass spectrometry are expensive and may not be available in some of the analytical laboratories. The NIR instrumentation itself is usually rugged and has reportedly been used inside and outside the laboratory (Stark *et al*., 1986). Portable as well as hand held instruments have also been developed (Blanco & Villarroya, 2002).

7.2 Calibration development

There is, however, a prerequisite for NIR analysis, i.e. the development of a separate reliable calibration model for each constituent to be measured (Williams, 2001). The aim of this is to develop a model that correlates the NIR spectra to the values obtained by the respective reference methods (Beebe & Kowalski, 1987).

7.2.1 Calibration data set

It is crucial that a sufficient number of samples are to be used for the calibration development. The samples should also include all possible variations likely to occur in future samples as failure to do so would lead to outliers (Næs & Isaksson, 1994). Possible sources of variation include growing location, chemical composition, season, temperature and possibly humidity of the work environment (Williams, 2001).

Sample preparation, if necessary, is important and should not alter the sample composition (Williams & Stevenson, 1990). It can account for up to 60-70% of the overall error of testing. This often involves grinding to reduce the particle size of the sample, the preparation of a uniform slurry or other forms of preparation which make direct testing or testing in a sample cell easier. Chemical composition is also important due to the interactions among the constituents during sample preparation and wavelength selection. Moisture content is an important compound in the food and agricultural industries, which may affect NIR calibration development (Williams, 2001). Strong absorbance by water in the 1450 and 1930 nm areas dominate the spectra of foods containing high moisture content. In such instances, were the NIR wavelength selections are affected by water absorbance bands, the moisture content must be included in the reference analyses (Williams & Stevenson, 1990). Should the

moisture content of the sample change during processing, it is important to take that into account to ensure that the right NIR prediction values are correlated to the correct reference method (Williams, 2001). Accuracy of analysis for both moisture and other compounds is therefore essential for effective calibration.

7.2.2 Reference data

Reference data during calibration is necessary because the NIR instrument reports the data in terms of the reference data. Reference methods are those that have been used prior to the development of the NIR spectroscopy technique. These methods include, e.g. the Kjeldahl test which measures the protein content, the Dumas (combustion analysis) procedure which determines the nitrogen present (Williams, 2001) and HPLC which has also been used to measure compounds such as mangiferin in honeybush tea (Kamara *et al*., 2004). Furthermore, these tests are also found to be more accurate. Not only is the accuracy and reproducibility of the reference methods important, it should also be attached to the correct NIR spectral data (Williams, 2001).

7.2.3 Chemometrics

Due to the large amounts of data that are obtained from NIR instruments, the spectral data also contains noise, uncertainties, variabilities, non-linearities and unrecognised features. Chemometrics are therefore required to extract as much relevant information as possible from the spectral data (Wold, 1995). Pretreatments of spectral data reduce noise and also aid in correcting baseline variations (Katsumoto *et al*., 2001; Delwiche & Reeves, 2004). There is also a need for reduction of variables to a few uncorrelated variables that contain only the relevant information (Blanco & Villarroya, 2002). Principal component analysis (PCA) is such a method and can thus be used as new variables, instead of the original data, in subsequent calculations (Cowe *et al*., 1985; Blanco & Villarroya, 2002).

A variety of multivariate analysis techniques exist to extract the analytical information from NIR spectra. These methods are used in the development of models to predict the properties of unknown samples. Qualitative (Downey, 1996a) and quantitative (Blanco & Villarroya, 2002) calibration models can be developed. Independent validation is then usually used to determine the ability of a calibration model to accurately predict the consitutent in question (Næs & Isaksson, 1994).

7.2.4 Statistical evaluation

Statistics performed to evaluate the accuracy of the calibration model include the standard error of prediction (SEP) (Osborne *et al*., 1993a) or the standard error of cross-validation (SECV); bias; coefficient of determination (R^2); and the ratio of standard error of performance to standard deviation (RPD), (Williams, 2001). The coefficient of correlation or (*r*) can be used to show how well the NIR predicted data correlates with the reference data over the range of composition. A high value (>0.95) indicates a good correlation, while a low value (<0.80) indicates that it is not possible to obtain consistent high accuracy by NIR spectroscopy analysis. The SEP should be as close as possible to the standard error of laboratory (SEL). This can be used to evaluate the accuracy of the calibration by indicating the variability in deviations of *x* (the reference data) from *y* (the NIR data). The bias then gives the average amount by which the results differ. The SEP and bias together indicate and evaluate the accuracy of the prediction model. According to Williams (2001), both the SEP and bias should be as low as possible with a slope close to 1.0. If cross-validation is done, the accuracy of the calibration can be indicated using the SECV. A value of 5.0 or more is recommended for the RPD value for use in NIR spectroscopy quality control, but for screening purposes a value of more than 3.1 would be sufficient.

7.3 Instrumentation

NIR spectroscopy equipment can include a variety of devices, depending on the characteristics of the sample to be analysed and the analytical conditions (Blanco & Villarroya, 2002). All of the instruments possess the same building blocks, i.e. a radiation source, wavelength selectors, sample presentation modes and a detector. Diffuse transmittance modes are used to analyse liquid samples and also to measure solid samples such as cheese, meat and whole grain (Penner, 1994). Diffuse reflectance mode measurements are used to analyse solid or granular samples. Diffuse reflectance and transflectance mode in combination can be used to measure turbid liquids. The transmittance and reflectance modes can also be combined to form an interactance mode that will illuminate and detect laterally separated points on the samples surface. This is advantageous for analysis of large samples such as a whole fruit (Osborne, 2000). It is therefore important to choose the correct instrument for a specific application.

7.4 Application of NIR spectroscopy analysis

A major advantage of NIR spectroscopy is that it can be applied in vast areas in the food industry. NIR spectroscopy has helped overcome difficulties in the design of novel food products by giving a better understanding of the microstructural quality of food emulsions (Haiduc *et al*., 2007). Analysis of the

material can start right at the beginning on the raw material which ensures that the final product will meet the given expectations (Williams & Stevenson, 1990). Product analysis during critical stages is also possible. It can therefore be used at any processing point during manufacture. Analysis of by-products to determination their value for future sales is also possible. A large number of samples can therefore be rapidly tested at time of arrival or delivery (Williams, 2001).

The use of NIR spectroscopy in the food industry was reported as early as 1938 on gelatine studies (Ellis & Bath, 1938). Although it was originally limited to grain analysis, today both qualitative and quantitative applications are widely used. Qualitative NIR spectroscopy analysis has made the confirmation of authenticity of commodities such as meat (Downey & Beauchêne, 1997) and fish (Downey, 1996b), tea (Budínová *et al*., 1998), coffee (Downey *et al*., 1994; Downey & Boussion, 1996; Downey *et al*., 1997), rice (Osborne *et al*., 1993b), oils (Sato, 1994) and wheat (Downey *et al*., 1986; Sorvaniemi *et al*., 1993; Downey, 1996a; Downey, 1998; Manley *et al*., 2002) possible. Quantitative analysis has also been employed for the authentication of food products, e.g. the adulteration of milk with non-milk fat (Sato, 1994) and milk fat with foreign fats (Sato *et al*., 1990). Camellia oil is also often the target for adulteration because it is an expensive product with high nutritional and medicinal value. Wang *et al*. (2006) discriminated between pure camellia oils and samples adulterated with soybean oils using NIR spectroscopy.

Increasingly diverse fields are using this analytical method not only for quality control, but also for process management purposes (Osborne *et al*., 1993a; Wetzel, 1998) as listed in Table 7.

NIR spectroscopy has become a valuable discrimination and classification tool especially in the tea, herbal product and medicinal industries (Table 8). Similary it has been applied extensive in quantitative analysis within these industries (Table 9).

Table 7 List of commodities to which NIR spectroscopy has been applied

Field	Reference
Biodiesel	Felizardo *et al*., 2007
Cereal and cereal products	Osborne 1991; Windham *et al*., 1993; Lee *et al*., 2005; Cho & Skidmore, 2006
Cheese	Karoui *et al*., 2006
Coffee	Pizarro *et al*., 2004
Cosmetics	Grunewald *et al*., 1998; Schulz & Albroscheit, 1998
Dairy industry	Rodriguez-Otero *et al*., 1997; Wüst & Rudzik, 2003; Blazquez *et al*., 2004
Diagnostic medicine	Hock *et al*., 1997; Schrader *et al*., 1999; Hirosawa *et al*., 2002; Suto *et al*., 2004
Distillation	Gomez-Cordoves & Bartolome, 1993
Essential oils	Schulz *et al*., 1998; Steuer *et al*., 2001; Schulz *et al*., 2003b
Feeds and forages	Murray & Hall, 1983; Volkers *et al*., 2003; Coûteaux *et al*., 2005
Fossil and other fuels	Lima *et al*., 2004
Fruit and vegetables	Kawano *et al*., 1992; Cai & Corke, 2001; Walsh *et al*., 2004; Xiabo *et al*., 2007
Growth media	Ludwig *et al*., 2006
Honey	García-Alvarez *et al*., 2000
Meat and fish	Wold & Isaksson, 1997; Pink *et al*., 1999; Cozzolino & Murray, 2004
Manure	Reeves, 2001
Oils	Hein *et al*., 1998; Houmøller *et al*., 2006
Pharmaceutical	Blanco *et al*., 1998; Otsuka, 2006
Soils	Demattê *et al*., 2004
Soybeans	Kovalenko *et al*., 2006
Spreads	Heussen *et al*., 2007
Tea	Grant *et al*., 1987; Hall *et al*., 1988
Textiles	Cleve *et al*., 2000
Tobacco and cigarette smoke	Hana *et al*., 1997; Parrish *et al*., 2001
Wine and grapes	Baumgarten, 1987; Manley *et al*., 2001; Chauchard *et al*., 2004; Cozzolino *et al*., 2004)

Table 8 Qualitative NIR spectroscopy applications for various teas and medicinal plants

Type of qualification	Description of application	References
Discrimination	unfermented and fermented rooibos	Schulz *et al*., 2003a
Discrimination	black teas with different qualities	Osborne & Fearn, 1988
Discrimination	herbal medicines according to geographical origin	Woo *et al*., 1999a
Discrimination	chinese herbal medicines	Chan *et al*., 2007
Identification	herbal products	Woo *et al*., 1999b; Laasonen, 2003; Schulz *et al*., 2002; Wang *et al*., 2007
Identification	green, black and Oolong tea	Chen *et al*., 2006b
Detection	adulteration of *Echinacea purpurea* with other species	Laasonen *et al*., 2002
Classification	green rooibos and green honeybush (*C. genistoides)*	Botha, 2005
Classification and identification	Plant species, geographical origin, cultivation area and processing methods	Ren & Chen, 1999; Laasonen, 2002; Fuzzati, 2004; Chan *et al*., 2007; Woo *et al*., 1999a; Woo *et al*. 2002; Schulz *et al*., 2003a

Table 9 Quantitative NIR spectroscopy applications for various teas and medicinal plants

Sample	Parameter	References
Green rooibos tea	aspalatin	Schulz *et al*., 2003a
	aspalatin, soluble solid content, total antioxidant activity	Manley *et al*., 2006
Green tea	total antioxidant capacity	Zhang *et al*., 2004
	caffeine content, epigallocatechin gallate, epicatechin, total antioxidant capacity	Luypaert *et al*., 2003
	total polyphenol content, epicatechin, caffeine content, epigallocatechin gallate, gallic acid, theobromine, nitrogen content	Ikegaya *et al*., 1985; Ikegaya *et al*., 1987; Schulz *et al*., 1999
	total polyphenol content, caffeine content	Chen *et al*., 2006a
	catechins	Zhao *et al*., 2006
Black tea	quality, theoflavin content, moisture	Hall *et al*., 1988
	caffeine content	Schmidt *et al*., 1998
	theoflavin and thearubigins content	Zhao *et al*., 2006
Green honeybush *(Cyclopia genistoides)*	mangiferin and hesperidin	Joubert *et al*., 2006
Licorice *(Glycyrrhizia uralensis)*	glycyrrhizic acid (GA)	Wang *et al*., 2007
Chinese herbal medicine	moisture content	Chan *et al*., 2007
Herb leaves	foliar nitrogen content	Cho & Skidmore, 2006
	chlorophyll content	
St. John's Wort extracts	hyperforin, I3,118-biapigenin	Rager *et al*., 2002
Plant material (grasses, shrubs)	quality, total extractable polyphenolics	Coûteaux *et al*., 2005
Red beet stems and leaves *(Amaranthus)*	betacyanins; pigment degradation, postharvest treatments, moisture content	Cai & Corke, 2001

8. CONCLUSION

The current awareness among consumers with regards to diet, health and delaying of ageing has boosted the functional and nutraceutical market.

Although lower quantities of mangiferin and hesperidin have been isolated from *C. subternata* in comparison to other *Cyclopia* spp. it is still a very good potential source of phenolic compounds and can be used in extracts by the nutraceutical and cosmetic industries. The importance of standardisation of marker or active compounds in nutraceutical products has been observed to be essential. Cheaper and more rapid methods are required for the routine screening for the quantification of phenolic compounds of not only in the plant material, but also in the final product.

Near infrared spectroscopy has therefore become a good candidate due to its fast analysis and low running cost. Its vast application for quantification of a range of phenolic compounds and antioxidant activity in herbal/medicinal plants indicates its ability to also predict mangiferin and hesperidin content in honeybush tea, as well as the total polyphenols, antioxidant activity and soluble solids. The effect of choosing a suitable extraction is therefore of great importance, but implementation of GMP practices should be adhered to at all times to ensure quality and safety of the final product.

UV/Vis colorimetric methods have the potential to be used as alternative cheaper methods to HPLC and NIR spectroscopy, as most laboratories possess spectrophotometers, but not all have the capital to purchase an NIR instrument. The acceptable levels of mangiferin in *C. subternata* make the use of aluminium chloride colorimetric method, to estimate the compound, possible. These methods are however not as accurate and might not be convenient for routine analysis.

9. REFERENCES

Akowuah, G.A, Ismail, Z., Norhayati, I. & Sadikun, A. (2005). The effects of different solvents of varying polarities on polyphenols of *Orthosiphon stamineus* and evaluaution of the free radical-scavenging activity. *Food Chemistry*, **93**, 311-317.

Andersen, M.L., Lauridsen, R.K. & Skibsted, L.H. (2005). Power of phenolic compounds. *Functional Foods and Nutraceuticals*, March 2005, 44-48.

Anonymous (1998). Precise Colour Communication. P. 11. Minolta Pamphlet PCC 807E-E1.

Anonymous (2000). What about functional foods? *South African Food Review*, September 2000, 39.

Anonymous (2001). CPMP/QWP/2820/00 (EMEA/CVMP/815/00). Note for guidance on specifications: Test procedures and acceptance criteria for herbal drugs, herbal drug preparations and herbal medicinal products.

Anonymous (2003). Beverage review of flavours and functionals. *South African Food Review*, March

2003, 19.

Anonymous (2004). International Food Information Council (IFIC) Foundation: Background of functional foods [http://ificinfo.health.org] 22 August 2007.

Anonymous (2006a). Dietary supplements – popularity, quality, safety, efficacy. [WWW document] URL: http://www.buffalo.edu/shlevy/dietsuppqualitysafetyefficacy.htm 19 July 2006.

Anonymous (2006b). Dit lyk gesond. *Finweek*, 25 Mei, 81-86.

Anonymous (2006c). Some projects in the rest of Africa. *Food & Beverage Reporter*, Jan/Feb 2006, 45.

Aquino-Bolaños, E.N. & Mercado-Silva. E. (2004) Effect of polyphenol oxidase and peroxidase activity, phenolics and lignin content on the browning of cut jicama. *Postharvest Biology and Technology,* **33,** 275-283.

Arvanitoyannis, I. S. & Van Houwelingen-Koukaliaroglou, M. (2005). Functional foods: A survey of health claims, pros and cons, and current legislation. *Critical Reviews in Food Science and Nutrition*, **45**, 385-404.

Areias, F.M., Valentão, P., Andrade, P.B., Ferreres, F. & Seabra, R.M. (2001). Phenolic fingerprint of pepperment leaves. *Food Chemistry,***73**, 307-311.

Atoui, A.K., Mansouri A., Boskou, G. & Kefalas, P. (2004). Tea and herbal infusions: Their antioxidant activity and phenolic profile. *Food Chemistry*, **89**, 27-36.

Baumgarten, G.F. (1987). The determination of alcohol in wines by means of near infrared technology. *South African Journal of Enology and Viticulture*, **8**, 75-77.

Baydar, N.G., Özkan, G. & Sagdiç, O. (2004). Total phenolic contents and antibacterial activities of grape (*Vitis vinifera* L.) extracts. *Food Control*, **15**, 335-339.

Beebe, K.R. & Kowalski, B.R. (1987). An introduction to multivariate calibration and analysis. *Analytical Chemistry*, **59**, 1007-1015.

Benavente-García, O., Castillo, J., Marin, F.R., Ortuño, A. & Del Río, J.A. (1997). Uses and properties of *Citrus* flavonoids. *Journal of Agricultural and Food Chemistry*, **45**, 4505-4515.

Bergeron, C., Gafner, S., Clausen, E. & Carrier, D.J. (2005). Comparison of the chemical composition of extracts from *Scutellaria lateriflora* using accelerated solvent extraction and supercritical fluid extraction versus standard hot water or 70% ethanol extraction. *Journal of Agricultural and Food Chemistry*, **53**, 3076-3080.

Blanco, M., Coello, J., Iturriaga, H, Maspoch, S. & De la Pezuela, C. (1998). Near infrared spectroscopy in the pharmaceutical industry. *Analyst*, **124**, 135-150.

Blanco, M. & Villarroya, I. (2002). NIR spectroscopy: A rapid-response analytical tool. *Trac-trends in Analytical Chemistry*, **21**, 240-250.

Blazques, C., Downey, G., O'Donnell, C., O'Callaghan, D. & Howard, V. (2004). Prediction of moisture, fat and inorganic salts in processed cheese by near infrared reflectance spectroscopy and multivariate data analysis. *Journal of Near Infrared Spectroscopy*, **12**, 149-157.

Boltz, D.F. & Mellon, M.G. (1966). Visible spectrometry. In: Standard methods of chemical analysis, Vol. 3, 6th ed. (edited by F.J. Welcher). Pp. 322. New York, USA: D. van Nostrand Company, Inc.

Borbalán, Á.M.A., Zorro, L., Guillén, D.A. & Barroso, C.G. (2003). Study of the polyphenol content of red and white varieties by liquid chromatography – mass spectrometry and its relationship to antioxidant power. *Journal of Chromatography A,* **1012**, 31-38.

Brand, D.L. (2002). Structure and synthesis of polyphenols from *Cyclopia subternata.* MSc Thesis, University of the Free State, South Africa.

Botha, M. (2005). Use of near infrared spectroscopy (NIRS) and spectrophometric methods in quality control green rooibos (*Aspalatus linearis*) and honeybush (*Cyclopia genistoides*). MSc in Food Science Thesis, Stellenbosch University, South Africa.

Brower, V. (1998). Nutraceuticals: poised for a healthy slice of the healthcare market? *Nature Biotechnology,* **16**, 728-731.

Budínová, G., Vlácil, D., Mestek, O. & Volka, K. (1998). Application of infrared spectroscopy to the assessment of authenticity of tea. *Talanta*, **47**, 255-260.

Burdock, G. A., Carabin, I.G. & Griffiths, J.C. (2006). The importance of GRAS to the functional food and nutraceutical industries. *Toxicology*, **221**, 17-27.

Cai, Y.Z. & Corke, H. (2001). Effect of postharvest treatments on *Amaranthus* betacyanin degradation evaluated by visible/near-infrared spectroscopy. *Journal of Food Science*, **66**, 1112-1118.

Calabrò, M.L., Galtieri, V., Cutroneo, P., Tommasini, S., Ficarra, P. & Ficarra, R. (2004). Study of the extraction procedure by experimental design and validation of a LC method for determination of flavonoids in Citrus bergamia juice. *Journal of Pharmaceutical and Biomedical Analysis*, **35**, 349--363.

Canjura, F.L. & Schwartz, S. J. (1991). Separation of chlorophyll compounds and their polar derivatives by high-performance liquid chomatography. *Journal of Agricultural and Food Chemistry,* **39**, 1102-1105.

Chan, K. (2003). Some aspects of toxic contaminants in herbal medicines. *Chemosphere*, **52**, 1361-1371.

Chan, C.-O., Chu, C.-C., Mok, D.K.-W. & Chau, F.-T. (2007). Analysis of berberine and total alkaloid content in *Cortex Phellodendri* by near infrared spectroscopy (NIRS) compared with high-performance liquid chromatography coupled with ultra-visible spectrometric detection. *Analytica Chimica Acta*, **592**, 121-131.

Chang, C.J., Chiu, K.L., Chen, Y.L. & Yang, P.W. (2001). Effect of ethanol content on carbon dioxide extraction of polyphenols from tea. *Journal of Food Composition and Analysis*, **14**, 75-82.

Chang, C.C., Yang, M.H., Wen, H.M. & Chern, J.C. (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *Journal of Food and Drug Analysis*, **10**, 178-182.

Chaudhari, R. (1999). Foods of the Future: The impact of functional foods in the cereal industry. *Cereal Foods World*, **44**, 94-95.

Chauchard, F., Cogdill, R., Roussel, S., Roger, J.M. & BellonMaurel, V. (2004). Application of LS-SVM to non-linear phenomena in NIR spectroscopy: Development of a robust and portable sensor for acidity prediction in grapes. *Chemometrics and Intelligent Laboratory Systems*, **71**, 141-150.

Chen, P.-Y., Tu, Y.-X., Wu, C.-T., Jong, T-T. & Chang, C.-M. (2004). Continuous hot pressurised solvent extraction of 1,1-diphenyl-2-picrylhydrazyl free radical scavenging compounds from Taiwan yams (*Dioscorea alata*). *Journal of Agricultural and Food Chemistry*, **52**, 1945-1949.

Chen, Q., Zhao, J., Huang, X., Zhang, H. & Liu, M. (2006a). Simultaneous determination of total polyphenols and caffeine contents of green tea by near-infrared reflectance spectroscopy. *Microchemical Journal*, **83**, 42-47.

Chen, Q., Zhao, J., Huang, X., Zhang, H. & Wang, X. (2006b). Feasibility study on qualitative and quantitative analysis in tea by near-infrared reflectance spectroscopy with multivariate calibration, *Analytica Chemica Acta*, **572**, 77-84.

Cho, M.A. & Skidmore, A.K. (2006). A new technique for extracting the red edge position from hyperspectral data: The linear extrapolation method. *Remote Sensing of Environment*, **101**, 181-193.

Cleve, E., Bach, E. & Schollmeyer, E. (2000). Using chemometric methods and NIR spectrophotometry in the textile industry. *Analytica Chimica Acta*, **420**, 163-167.

Clydesdale, F.M (1978). Colorimetry-methodology and explanations. *Critical reviews in food science and nutrition*, **10**, 243-301.

Clydesdale, F.M (1993). Colour as a factor in food science. *Critical reviews in food science and* nutrition, **33**, 83-101.

Costin, J.W., Barnett, N.W., Lewis, S.W. & McGillivery, D.J. (2003). Monitoring the total phenolic/antioxidant levels in wine using flow injection analysis with acidic potassium

permanganate chemiluminescence detection. *Analytica Chimica Acta*, **499**, 47-56.

Counsell, J.N. (1981). Some other natural colours and their applications. In: *Natural colours for food and other uses* (edited by Counsell, J.N.) Pp. 123-129. London: Applied Science Publishers Ltd.

Coûteaux, M.-M., Sarmiento, L., Hervé, D. & Acevedo, D. (2005). Determination of water-soluble and total extractable polyphenolics in biomass, necromass and decomposing plant material using near-infrared reflectance spectroscopy (NIRS). *Soil Biology and Biochemistry*, **37**, 795-799.

Cowe, I.A., McNicol, J.W. & Cuthbertson, D.C. (1985). Principal component analysis: A chemometric approach to the analysis of near infrared spectra. *Analytical Proceedings*, **27**, 61-63.

Cozzolino, D., Kwiatkowski, M.J., Parker, M., Cynkar, W.U., Dambergs, R.G., Gishen, M. & Herderich, M.J. (2004). Prediction of phenolic compounds in red wine fermentations by visible and near infrared spectroscopy. *Analytica Chimica Acta*, **513**, 73-80.

Cozzolino, D. & Murray, I. (2004). Identification of animal meat muscles by visible and near infrared reflectance spectroscopy. *Lebensmittel-Wissenschaft und Technologie*, **37**, 447-452.

Da Costa, C.T., Margolis, S.A., Benner, B.A. & Horton, D. (1999). Comparison of methods forextaction of flavanones and xanthones from the root bark of the osage orange tree using of flavanones and xanthones from the root bark of the osage orange tree using liquid chromatography. *Journal of Chromatography A*, **831**, 167-178.

Daood, H.G. (1993). Chlorophyll. In: *Encyclopedia of Food Science*, *Food Technology and Nutrition*, (edited by Macrae, R. Robinson, R.K., Stadler, M.J.). Pp 904-911. Academic Press, London.

Day, M.S. & Fearn, F.R.B. (1982). Near infra-red reflectance as an analytical technique, Part 1. History and development. *Laboratory Practice*, **31**, 328-330.

Dasgupta, N. & De, B. (2007). Antioxidant activity of some leafy vegetables of India: a comparative study. *Food Chemistry*, **101**, 471-474.

Davies, K.J.A. (1995). Oxidative stress: the paradox of life. In: *Free Radicals and Oxidative Stress*: *Environmental, Drugs and Food Additives* (edited by C. Rice-Evans, B. Halliwell, & G.G. Lunt.). P.1. London: Portland Press.

De Beer, S.W. & Joubert, E. (2002). Preparation of tea-like beverages. South African Patent 2002/2808.

Delgado-Vargas, F. & Paredes-López, O. (2003). *Natural Colorants for Food and Nutraceutical uses*. Pp. 221-224, 291- 298. Boca Raton: CRC Press.

Delwiche, S.R. & Reeves, J.B. (2004). The effect of spectral pretreatments on the partial least squares modelling of agricultural products. *Journal of Near Infrared Spectroscopy,* **12**, 177-182.

Demattê, J.A.M., Campos, R.C., Alves, M.C., Fiorio, P.R. & Nanni, M.R. (2004). Visible NIR

reflectance: A new approach on soil evaluation. *Geodema*, **121**, 95-112.

Deng, F. & Zito, S.W. (2003). Development and validation of a gas chromatographic-mass spectrometric method for simultaneous identification and quantification of marker compounds including bilobalide, ginkgolides and flavonoids in *Ginkgo biloba* L. extract and pharmaceutical preparations. *Journal of Chromatography A,* **986**, 121-127.

De Nysschen, A.M., Van Wyk, B-E., Van Heerden, F.R. & Schutte, A.L. (1996). The major phenolic compounds in the leaves of *Cyclopia* species (honeybush tea). *Biochemical Systematics and Ecology*, **24**, 243-246.

De Whalley, C.V., Rankin, S.M, Houldt, J.S.R., Jessup, W. & Leake, D.S. (1990). Flavonoids inhibit the oxidative modification of low density lipoproteins by macrophages. *Biochemical Pharmacology*, **39**, 1743-1750.

Downey, G. (1995). Food quality and authenticity measurement. *Farm and Food*, July/September 1995, 21-24.

Downey, G. (1996a). Authentication of food and food ingredients by near infrared spectroscopy. *Journal of Near Infrared Spectroscopy*, **4**, 47-61.

Downey, G. (1996b). Non-invasive and non-destructive percutaneous analysis of farmed salmon flesh by near infra-red spectroscopy. *Food Chemistry*, **55**, 305-311.

Downey, G. (1998). Direct Measurement of food quality and authenticity. *Analysis and Control*, **1**, 27--30.

Downey, G. & Beauchêne, D. (1997). Authentication of fresh vs. frozen-then-thawed beef by near infrared reflectance spectroscopy of dried drip juice. *Lebensmittel-Wissenschaft und*
- Technologie, **30**, 721-726.

Downey, G. & Boussion, J. (1996). Authentication of coffee bean variety by near infrared reflectance spectroscopy of dried extract. *Journal of the Science of Food and Agriculture,* **71**, 41-49.

Downey, G., Boussion, J. & Beauchêne, D. (1994). Authentication of whole and ground coffee beans by near infrared reflectance spectroscopy. *Journal of Near Infrared Spectroscopy*, **2**, 8592.

Downey, G., Briandet, R. Wilson, R.H. & Kemsley, E.K. (1997). Near- and mid-infrared spectroscopies in food and authentication: Coffee varietal identification. *Journal of Agricultural and Food Chemistry*, **45**, 4357-4361.

Downey, G., Byrne, S. & Dwyer, E. (1986). Wheat trading in the Republic of Ireland: The utility of a hardness index derived by near infrared reflectance spectroscopy. *Journal of the Science of Food and Agriculture*, **37**, 762-766.

Du Pont, M.S., Mondin, Z., Williamsen, G. & Price, K.R. (2000). Effect of variety, processing and

storage on the flavonoid content and composition of lettuce and endive. *Journal of Agricultural and Food Chemistry*, **48**, 3957-3964.

Du Toit, J. & Joubert, E. (1998a). Effect of drying conditions on the quality of honeybush tea (*Cyclopia*). *Journal of Food Processing Preservation,* **22**, 493-507.

Du Toit, J. & Joubert, E. (1998b). The effect of pretreatment on the fermentation of honeybush tea (*Cyclopia maculata*). *Journal of the Science of Food and Agriculture*, **76**, 537-545.

Du Toit, J. & Joubert, E. (1999). Optimization of the fermentation parameters of honey bush tea (*Cyclopia*). *Journal of Food Quality*, **22**, 241-256.

Du Toit, J., Joubert, E. & Britz, T.J. (1998). Honeybush tea- A rediscovered indigenous South African herbal tea. *Journal of Sustainable Agriculture*, **12**, (2/3).

Ellis, J.W. & Bath, J. (1938). Alterations in the infrared absorption spectrum of water in gelatin. *Journal of Chemical Physics*, **6**, 723-729.

El-Shafae, A.M. & El-Domiatry, M.M. (2001). Improved LC methods for the determination of diosmin and/or hesperidin in plant extracts and pharmaceutical formulations, *Journal of Pharmaceutical and Biomedical Analysis*, **26**, 539-545.

Eloff, J.N. (1998). On expressing the antibacterial activity of plant extracts – a small first step in applying scientific knowledge to rural primary health care. *South African Journal of Science*, **96**, 116-118.

Escribano-Bailón, M.T. & Santos-Buela, C. (2003). Polyphenol extraction from foods. In: *Methodsin Polyphenol Analysis* (edited by C. Santos-Buela & G. Williamson). Pp. 1-16. Gateshead, UK: Anthenaeum Press Ltd.

Esmelindro, M.C., Toniazzo, G., Lopes, D., Oliveira, D. & Dariva, C. (2005). Effects of processing conditions on the chemical distribution of mate tea leaves extracts obtained from CO_2 extraction at high pressures. *Journal of Food Engineering*, **70**, 588-592.

Felizardo, P., Baptista, P., Menezes, J.C. & Correira, M.J.N. (2007). Multivariate near infrared spectroscopy models for predicting the methanol and water content in biodiesel. *Analytica Chimica Acta*, **595**, 107-113.

Fernandez, P.L., Fernando, P., Martin, J., Gonzalez, A.G., (2002). Study of catechin and xanthine tea profiles as geographical tracers. *Journal of Agricultural and Food Chemistry*, **50**, 1833- 1839.

Ferrari, J., Terreaux, C., Sahpaz, S., Msonthi, J.D., Wolfender, J-L. & Hostettmann, K. (2000). Benzophenone glycosides from *Gnidia involucrate*. *Phytochemistry*, **54**, 883-889.

Ferreira, D., Kamara, B.I., Brandt, E.V. & Joubert, E. (1998). Phenolic compounds from *Cyclopia intermedia* (Honeybush tea). *Journal of Agricultural and Food Chemistry*, **46**, 3406-3410.

Fitchet, T. (2005). Functional Foods: suckers for health. *South African Food Review*, August 2005, 18-20.

Franke, A.A., Custer, L. J., Arakaki, C. & Murphy, S.P (2004). Vitamin C and flavanoid levels of fruits and vegetables consumed in Hawaii. *Journal of Food Composition and Analysis*, **17**, 1-35.

Fuzzati, N. (2004). Analysis methods of ginsenosides. *Journal of Chromatography B,* **812**, 119-133.

Gálvez, M., MartínCordero, C., Houghton, P.J. & Ayuso, M.J. (2005). Antioxidant activity of methanol extracts obtained from Plantago species. *Journal of Agricultural and Food Chemistry*, **53**, 1927-1933.

García-Alvarez, M., Huidobro, J.F., Hermida, M. & Rodríguez-Otero, J.L. (2000). Major components of honey analysis by near-infrared transflectance spectroscopy. *Journal of Agricultural and Food Chemistry*, **48**, 5154-5158.

George, J., Laing, M.D. & Drewes, S.E. (2001). Phytochemical research in South Africa. *South African Journal of Science*, **97**, 93-105.

Gise, J. & Katz. F. (1997). Ethical marketing of functional foods. *Food Technology*, **51**, 58-61.

Goldberg, I. (1994). *Functional foods, Designer foods, Pharmafoods, Nutraceuticals*, Pp. 3-7. New York: Chapman & Hall.

GómezCordovés, C. & Bartolome, B. (1993). Application of princical component analysis to simple determinations of brandies as a means of verifying quality. *Zeitschrift für Lebensmittel Untersuchung und Forschung*, **197**, 260-263.

Gonnet, J.-F. (1993). CIELab measurement, a precise communication in flower colour: an example with carnation (*Dianthus caryophyllus*) cultivars. *Journal of Horticultural Science*, **68**, 499-510.

Gramza, A. & Korczak, J. (2005). Tea constituents (*Camellis sinensis* L.) as antioxidant in lipid systems. *Trends in Food Science and Technology*, **16**, 315-358.

Grant, A., Franklin, J.G. & Davies, A.M.C. (1987). Near infra-red analysis: The use of multivariate statistics for the investigation of variables in sample preparation and presentation of tea leaf. *Journal of the Science of Food and Agriculture*, **42**, 129-139.

Greenish, H.G. (1881). Cape tea. *The Pharmaceutical Journal and Transactions*, **3rd series** (550), 549-551.

Griffiths, P.R. (1987). Laboratory Methods in Vibrational Spectroscopy (Edited by J.H. van der Maas & R.G.J. Miller), 3rd ed. p. 121. Chichester, UK: John Wiley & Sons (As cited by Katsumoto *et al*., 2001).

Griffiths, P.R., Pierce, J.A. & Hongjin, G. (1987). Computer-enhanced Analytical Spectroscopy (Edited by H.L.C. Meuzelaar & T.L. Isenhour), 3rd ed. p. 29. New York, USA: Plenum Press (As

cited by Katsumoto *et al*., 2001).

Grunewald, H., Kurowski, C., Timm, D., Grummisch, U. & Meyhack, U. (1998). Rapid nondestructive raw material identification in the cosmetic industry with near infrared spectroscopy. *Journal of Near Infrared Spectroscopy*, **6**, 215-222.

Guardia, T., Rotelli, A.E., Juarez, A.O., Pelzer, L.E. (2001). Anti-inflammatory properties of plant flavonoids. Effects of rutin, quercetin and hesperidin on adjuvant arthritis in rats. *Farmaco*, **56**, 683-687.

Guillerault, L., Ollivier, E., Elias, R. & Balansard, G. (1994). Determination of harpagide, 8-paracoumaroyl harpagide and harpagoside by high-performance liquid chromatography in *Harpagophytum procumbens* drugs and in a commercial extract. *Journal of Liquid Chromatography*, **17**, 2951-2960.

Haiduc, A.M., van Duynhoven, J.P.M, Heussen, P., Reska, A.A. & Reiffers-Magnani, C. (2007). Multivariate modelling of the microstructural quality of food emulsions based on NMR. *Food Research International*, **40**, (4), 425-434.

Hall, M.N., Robertson, A. & Scotter, C.N.G. (1988). Near-infrared reflectance prediction of quality, theoflavin content and moister content in black tea. *Food Chemistry*, **27**, 61-65.

Halliwell, B. (1990). How to characterise a biological antioxidant. *Free Radical Research Communication*, **9**, 1-32.

Halliwell, B. (1994). Free radicals, antioxidants and human disease: curiosity, cause, or consequence. *The Lancet*, **344**, 721-724.

Hana, M., McClure, W.F., Whitaker, T.B., White, M.W. & Bahler, D.R. (1997). Applying artificial neural networks: Part II. Using near infrared data to classify tobacco types and identify native grown tobacco. *Journal of Near Infrared Spectroscopy*, **5**, 19-25.

Harborne, J.B. (1998). *Phytochemical methods. A Guide to Modern Techniques of Plant Analysis*. 3rd ed. Pp. 1-39, 40-106. London, UK: Chapman & Hall.

Harborne, J.B. & Williams, C.A. (2000). Advances in flavonoid research since 1992. *Phytochemistry*, **55**, 481-504, 619-644.

Hasler, C.M. (1998). Functional foods: Their role in disease prevention and health promotion. *Food Technology*, **52**, 63-70.

Hasler, C.M. (2002). Functional foods: Benefits, concerns, and challenges – a position paper from the American Counsil on Science and Health. *The Journal of Nutrition*, **132**, 3772-3781.

He, X.G. (2000). On-line identification of phytochemical constituents in botanical extracts by combined high-performance liquid chromatographic-diode array detection-mass spectrometric

techniques. *Journal of Chromotography A*, **880**, 203-232.

Heim, K.E., Tagliaferro, A.R. & Bobilya, D.J. (2002). Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *The Journal of Nutritional Biochemistry*, **13**, 572-584.

Hein, M., Henning, H. & Isengard, H.-D. (1998). Determination of total polar parts with new methods for the methods for the quality survey of frying fats and oils. *Talanta*, **47**, 447-454.

Heussen, P.C.M., Janssen, H.-J., Samwel, I.B.M. & Duynhoven, J.P.M. (2007). The use of multivariate modelling of near infra-red spectra to predict the butter fat content of spreads. *Analytica Chimica Acta*, **595**, 176-181.

Higdon, J.V. & Frei, B. (2003). Tea catechins and polphenols: Health effects, metabolism, and antioxidant functions. *Critical reviews in Food Science and Nutrition*, **43**, 89-55.

Hirosawa, N., Sakamoto, Y., Katayama, H. & Tonooka, S. (2002). In vivo investigation in rat mammary gland tumors by near-infrared spectroscopy. *Analytical Biochemistry*, **305**, 156-165.

Hock, C., Villringer, K., MüllerSpahn, F., Wenzel, R., Heekeren, H., SchuhHofer, S., Hofmann, M., Minoshima, S., Schwaiger, M., Dirnagl, U. & Villringer, A. (1997). Decrease in parietal cerebral hemoglobin oxygenation during performance of a verbal fluency task in patients with Alzheimer's disease monitored by means of near infrared spectroscopy (NIRS) correlation with simultaneous rCPFPET measurements. *Brain Research*, **755**, 293-303.

Hollingsworth, P. (2000). Marketing trends fueling healthful foods success. *Food Technology,* **54**, 54-59.

Houmøller, L.P., Kristensen, D. & Rosager, H. (2006). Determination of SFC, FFA, and equivalent reaction time for enzymatically interestified oils using NIRS. *Talanta*, **71**, 868-873.

Howard, L.R. Pandjaitan, N. Morelock, T. & Gil, M.I. (2002). Antioxidant capacity and phenolic content of spinach as affected by genetics and growing season. *Journal of the Agricutural and Food Chemistry*, **50**, 5891-5896.

Hoyer, H. (1997). NIR on-line analysis in the food industry. *Process Control and Quality*, **9**, 143-152.

Hsu, S. (2005). Green tea and the skin. *Journal of the American Academy of Dermatology*, **52**, 1049-1059.

Huang, Y., Sheng, J., Yang, F. & Hu, Q.H. (2005). Effect of enzyme inactivation by microwave and oven heating on the preservation quality of green tea. *Journal of Food Engineering*, **in press**.

Hubbe, M.E. (2000). Evaluation of antioxidant and free radical scavenging activities of honeybush tea (*Cyclopia)*. M.Sc. in Food Science Thesis, University of Stellenbosch, South Africa

Hughson, L. (1995). Nutraceuticals: foods with a future. *South African Food Review*, April/May 1995, 21-22.

Huie, C.W. (2002). A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants. *Analytical and Bioanalytical Chemistry*, **37**, 23-30.

Ikegaya, K., Hino, A., Uozumi, J., Takayanagi, H., Anan, K. & Iwamato, M. (1985). Near-infrared spectrophotometric analysis of total nitrogen content in green tea. *Nippon Shokuhin Kogyo Gakkaaishi*, **32**, 553-559 (As cited by Schulz, 2004).

Ikegaya, K., Iwamoto, M., Uozumi, J. & Nishinari, K. (1987). Near infrared spectra of caffeine and its related compounds and their application to determination of caffeine content in green tea. *Nippon Shokuhin Kogyo Gakkaishi*, **34**, 254-258 (As cited by Schulz, 2004).

Ivanova, D., Gerova, D., Chervenkov, T. & Yankova, T. (2005). Polyphenols and antioxidant capacity of Bulgarian medicinal plants. *Journal of Ethnopharmacology,* **96**, 145-150.

Iwamoto, M., Kawano, S. & Ozaki, Y. (1995). An overview of research and development of near infrared spectroscopy in Japan, *Journal of Near Infrared Spectroscopy*, **3**, 179-189.

Joubert, 1988. Effect of batch extraction conditions on yield of soluble solids from rooibos tea. *International Journal of Food Science and Technology*, **23**, 43-47.

Joubert, E. (1995). Tristimulus colour measurement of rooibos tea extracts as an objective colour quality parameter. *International Journal of Food Science and Technology*, **30**, 783-792.

Joubert, 1996. HPLC quantification of the dihydrochalcones, aspalathin and nothofagin in rooibos tea (*Aspalathus linearis*) as affected by processing. *Food Chemistry*, **55**, 403-411.

Joubert, E., Manley, M. & Botha, M. (2006). Use of NIRS for the quantification of mangiferin and hesperidin contents from dried green honeybush (*Cyclopia genistoides*) plant material. *Journal of Agricultural and Food Chemistry*, **54**, 5279-5283.

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

Joubert, E., Richards, E.S., Van der Merwe, J.D., De Beer, D., Manley, M. & Gelderblom, W.C.A. (2007). Effect of species variation and processing on phenolic composition and in vitro antioxidant activity of aqueous extracts of *Cyclopia* spp. (honeybush tea. *Journal of Agricultural and Food Chemistry* (in press).

Joubert, E., Winterton, P., Britz, T.J. & Ferreira, D. (2004). Superoxide anion and a,a-diphenol-b-picrylhydrazyl radical scavenging capacity of rooibos (*Aspalathus linearis*) aqueous extracts, crude phenolic fractions, tannin and flavonoids. *Food Research International*, **37**, 133-138.

Jurd, L. (1962). Spectral properties of flavonoid compounds. In: *The Chemistry of Flavonoid Compounds* (edited by T.A. Geissman). Pp. 107-155. Oxford, UK: Pergamon Press.

Kada, T., Kaneko, K. Matsuzaki, S., Matsuzaki T. & Hara, Y. (1985). Detection and chemical identification of natural bio-antimutagens. A case of the green tea factor. *Mutation Research*, **150**, 127-132.

Karoui, R., Mouazen, A.M., Dufour, E., Pillonel, L., Schaller, E., Picque, D., Baerdemaeker, J. & Bosset, J.-O. (2006). A comparison and joint use of NIR and MIR spectroscopic methods for the determination of some parameters in European Emmental cheese. *European Food Research and Technology A*, **223**, 44-49.

Kamara, B.I., Brand, D.J., Brandt, E.V. & Joubert, E. (2004). Phenolic metabolites from honeybush tea (*Cyclopia subternata*). *Journal of Agricultural and Food Chemistry*, **52**, 5391-5395.

Kamara, B.I., Brandt, E.V., Ferreira, D. & Joubert, E. (2003). Polyphenols from honeybush tea (*Cyclopia intermedia*). *Journal of Agricultural and Food Chemistry*, **53**, 3874-3879.

Kanaze, F.I., Gabrieli, C., Kokkalou, E., Georgarakis, M. & Niopas, I. (2003). Simultaneous reverse-phase high-performance liquid chromatography method for the determination of diosmin, hesperidin and naringin in different citrus fruit juices and pharmaceutical formulations. *Journal of Pharmaceutical and Biomedical Analysis*, **33**, 243-249.

Katsumoto, Y., Jiang, J-H., Berry, R.J. & Ozaki, Y. (2001). Modern pretreatment methods in NIR spectroscopy. *Near Infrared Analysis*, **2**, 29-36.

Kawano, S., Watanabe, H. & Iwamto, M. (1992). Determination of sugar content in intact peaches by near infrared spectroscopy with fibre optics in interactance mode. *Journal of the Japanese Society of Horticultural Science*, **61**, 445-451.

Khokhar, S. & Magnusdottir, S.G.M. (2002). Total phenol, catechin, and caffeine contents of teas commonly consumed in the United Kingdom. *Journal of Agricultural and Food Chemistry*, **50**, 565-570.

Kitanov, G.M. & Nedialkov, P.T. (1998). Mangiferin and isomangiferin in some *Hypericum* species, *Biochemical Systematics and Ecology*, **26**, September 1998, 647-653.

Kovalenko, I.V., Rippke, G.R. & Hurburgh, C.R. (2006). Measurement of soybean fatty acids by near-infrared spectroscopy: linear and nonlinear calibration. *Journal of the American Oil Chemist's Society*, **83**, 421-427.

Krishnakumar, V. & Gordan, I. (1996). Antioxidants – trends and developments. *International Food Ingredients*, **12**, 41-44.

Kurechi, T. & Kato, T. (1982). Studies on the antioxidants. XVI. Synergistic reaction between butylated hydroxyanisole and butylated hydroxytoluene in hydrogen donation to 2,2-diphenyl-1-picrylhydrazyl. *Chemical and Pharmaceutical Bulletin*, **30**, 2964-2970.

Laasonen, M. (2003). Near infrared spectroscopy, a quality control tool for the different steps in the manufacture of herbal medicinal products. MSc. Thesis, University of Helsinki, Finland.

Laasonen, M., Harmia-Pulkkinen, T., Simard, C.L. Michiels, E., Rasanen, M. & Vuorela, H. (2002). Fast identification of *Echinacea purpurea* dried roots using near-infrared spectroscopy. *Analytical Chemistry*, **74**, 2493-2499.

Lachman, J., Proněk, D., Hejtmánkova, A., Dudjak, J., Pivec, V., & Faitová, K. (2003). Total polyphenol and main flavonoid antioxidants in different onion (*Allium cepa* L.) varieties. *Horticultural Science,* **30**, 142-147.

Lajolo, F.M. & Lanfer Marques, U.M. (1982). Cholrophyll degradation in a spinach system at low and intermediate water activities. *Journal of Food Science*, **47**, 1995-1998.

Larson, R.A., (1998). The antioxidants of higher plants. *Phytochemistry,* **27**, 969-978.

Lee, K.-M., Herrman, T.J., Lingenfelser, J. & Jackson, D.S. (2005). Classification and prediction of maize hardness-associated properties using multivariate statistical analyses. *Journal of Cereal Science*, **41**, 85-93.

Leskinen, K. (2002). Functional Food Challenge: Positioning and communicating innovative food brands in Finland. INNOMARKET, **13.** [WWW document] URL: http://www.tukkk.fin/innomarket/reports. 12 July 2006.

Li, B.B., Smith, B. & Hossain, M.Md. (2006). Extraction of phenolics from citrus peels. *Separation and Purification Technology*, **48**, 182-188.

Liang, Y.Z., Xie, P. & Chan, K. (2004). Quality control of herbal medicines. *Journal of Chromatography B*, **812**, 53-70.

Lima, F.S.G., Araújo, M.A.S. & Borges, L.E.P. (2004). Determination of lubricant base oil properties by near infrared spectroscopy using different sample and variable selection methods. *Journal of Near Infrared Spectroscopy*, **12**, 159-166.

Lin, F. & Giusti, M.M. (2005). Effects of solvent polarity and acidity on the extraction efficiency of isoflavones from soybeans *(Glycine max). Journal of Agricultural and Food Chemistry*, **53**, 3795-3800.

Lin, S.-J., Tseng, H.-H., Wen, K.-C. & Suen, T.-T. (1996). Determination of gentiopicroside, mangiferin, palmatine, bererine, baicalin, wogonin and glycyrrhizin in the traditional Chinese medical preparation Sann-Joong-Kuey-Jian-Tang by high-performance liquid chromatography. *Journal of Chromatography* A., **730**, 17-23.

Liu, M., Li, X.Q., Weber, C., Lee, C.Y., Brown, J. & Liu, R.H. (2002). Antioxidant and antiproliferative activities of raspberries. *Journal of Agricultural and Food Chemistry,* **50**, 2926-

2930.

Ludwig, B., Schmilewski, G. & Terhoeven-Urselmans, T. (2006). Use of near infrared spectroscopy to predict chemical parameters and phytotoxicity of peats and growing media. *Scientia Horticulturae*, **109**, 86-91.

Luypaert, J., Zhang, M.H. & Massart, D.L. (2003). Feasibility study for the use of near infrared spectroscopy in the qualitative and quantitative analysis of green tea, *Camellia sinensis* (L.). *Analytica Chimica Acta*, **478**, 303-312.

Mackinney, G. & Little, A.C. (1962). The natural coloring matters. In: *Colour of Foods*. Pp. 212-216. Westport, Connecticut: The AVI Publishing Company, Inc.

Manley, M., Joubert, E. & Botha, M. (2006). Quantification of the major phenolic compounds, soluble solid content and total antioxidant activity of green rooibos (*Aspalathus linearis*) by means of near infrared spectroscopy. *Journal of Near Infrared Spectroscopy*, **14**, 213-222.

Manley, M., Van Zyl, L. & Osborne, B.G. (2002). Using Fourier transform near infrared spectroscopy in determining kernel hardness, protein and moisture content of whole wheat flour. *Journal of Near Infrared Spectroscopy*, **10**, 71-76.

Manley, M., Van Zyl, L. & Wolf, E.E.H. (2001). The evaluation of the applicability of Fourier transform near-infrared (FT-NIR) spectroscopy in the measurement on analytical parameters in must and wine. *South African Journal of Enology and Viticulture*, **22**, 93-100.

Manzocco, L., Anese, M. & Nicoli, M.C. (1998). Antioxidant properties of tea extracts as affected by processing. *Lebensmittel-Wissenschaft und-Technologie*, **31**, 694-698.

Mabry, T.J., Markham, K.R. & Thomas, M.B. (1970). *The systematic Identification of Flavonoids*. Pp. 41-61. New York, USA: Springer-Verlag.

Markham, K.R. (1975). Isolation of techniques for flavonoids. In: *The Flavonoids* (edited by J.B. Harborne, T.J. Mabry & H. Mabry). Pp. 144. London, UK: Chapman and Hall.

Marnewick, J.L., Gelderbloem, W.C.A. & Joubert, E. (2000). An investigation on the antimutagenic properties of South African herbal teas. *Mutation Research*, **471**, 157-166.

Marnewick, J.L., Joubert, E., Swart, P., Van der Westuizen, F. & Gelderblom, W. (2005).

Marnewick, J., Joubert, E., Joseph, S., Swanevelder, S., Swart, P. & Gelderblom, W.C.A. (2003). Modulation of hepatic drug metabolizing enzymes and oxidative status by rooibos (*Aspalathus lienaris*) and honeybush (*Cyclopia intermedia*), green and black (*Camellia sinensis*) tea in rats. *Journal of Agricultural and Food Chemistry*, **51**, 8113-8119.

Marshall, M.R., Jeongmok, K. & Cheng, I.W. (2000). Enzymatic browning in fruits, vegetables and seafoods. Food Agricultural organization. [WWW document] URL: http://www.FAO.html. 18 July

2006.

Martinez, V.M. & Whitaker, J.R. (1995). The biochemistry and control of enzymatic browning. *Trends in Food Science and Technology*, **6**, 195-200.

McLaren, K. (1980). Food colorimetry. In: *Developments in Food Colors*, **1**, (edited by J.C. Walford). Pp. 27-45. Applied Science Publishers, London.

Melton, L. (2006). The antioxidant myth. *Newscientist*, **191** (2563), 40-43.

Menković, N., Ŝavikin-Fodulović, K. Bulatović, V., Aljačić, I., Juranić, N., Macura., S. Vajs, V. & Milosavljević, S. (2002). Xanthones from Swertia punctata. *Phytochemistry*, **61**, 415-420.

Merken, M.H. & Beecher, G.R. (2000). Measurement of food flavonoids by high-performance liquid chromatography: A Review. *Journal of Agricultural and Food Chemistry*, **48**, 577-599.

Mermelstein, N.H. (2002). A new look into the future of food science and technology. *Food Technology*, **56**, 46-53.

Murray, I. & Hall, P.A. (1983). Animal feed evaluation by use of near infrared reflectance (NIR) spectrocomputer. *Analytical Proceedings*, **20**, 75-78.

Næs, T. & Isaksson, T. (1994). Selection of samples for calibration. *NIR news*, **5**, 16.

Niba, L.L. (2003). The relevance of biotechnology in the improvement of functional foods for improved nutrition and health quality in developing countries. *African Journal of Biotechnology*, **2,** 631-635.

Nicoli, M.C., Anese, M., Parpinel, T., Franceschi, S. & Lerici, C.R. (1997). Loss and/or formation of antioxidants during food processing and storage. *Cancer Letters*, **114**, 71-74.

Núñez-Sellés, A.J., Vélez Castro, H.T., Agüero-Agüero, J., González-González, J., Naddeo, F., De Simone, F. & Rastrelli, L. (2002). Isolation and quantitative analysis of phenolic antioxidants, free sugars, and polyols from mango (*Mangifera indica* L.) stem bark aqueous decoction used in Cuba as a nutritional supplement. *Journal of Agricultural and Food Chemistry*, **50**, 762-766.

Nwuha, V., Nakajima, M., Tong, J. & Ichikawa, S. (1999). Solubility study of green tea extracts in pure solvents and edible oils. *Journal of Food Engineering*, **40**, 161-165.

Ong, E.S. (2004). Extraction methods and chemical standardisation of botanicals and herbal preparations. *Journal of Chromatography B*, **812**, 23-33.

Osborne, B.G. (1991). Measurement of the hardness of wheat endosperm by near infrared spectroscopy. *Postharvest News and Information*, **2**, 331-334.

Osborne, B.G. (2000). Near-infrared spectroscopy in food analysis. In: *Encyclopedia of analytical Chemistry* (edited by R.A. Meyers). Pp. 1-13. UK: John Wiley & Sons Ltd.

Osborne, B.G. & Fearn, T. (1988). Discriminant analysis of black tea by near infrared reflectance

spectroscopy. *Food Chemistry*, **29**, 233-334.

Osborne, B.G., Fearn, T. & Hindle, P.H. (1993a). *Practical NIR Spectroscopy with Practical Applications in Food and Beverage Analysis*, 2nd ed. Pp. 1-220. Harlow, UK: Longman Scientific and Technical.

Osborne, B.G., Mertens, B., Thompson, M. & Fearn, T. (1993b). The authentication of Basmati rice using near infrared spectroscopy. *Journal of Near Infrared Spectroscopy*, **1**, 77-83.

Otsuka, M. (2006). Chemoinformetrical evaluation of granule and tablet properties of pharmaceutical preparations by near-infrared spectroscopy. *Chemometrics and Intelligent Laboratory Systems*, **82**, 109-114.

Pan, X. Niu, G. & Liu, H. (2003). Microwave-assisted extraction of tea polyphenols and tea caffeine from green tea leaves, *Chemical Engineering and Processing*, **42**, 129-133.

Parr, A.J., & Bolwell, G.P. (2000). Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. *Journal of the Science of Food and Agriculture*, **80**, 985-1012.

Parrish, M.E., Lyons-Hart, J.L., & Shafer, K.H. (2001). Puff-by-puff and intrapuff analysis of cigarette smoke using infrared spectroscopy. *Vibrational Spectroscopy*, **27**, 29-42.

Penner, M.H. (1994). Basic principles of spectroscopy. In: Introduction to the Chemical Analysis of Foods (edited by S.S. Nielsen). Pp. 317-324. Boston, MA: Jones and Barlett Publishers.

Percival, M. (1998). Antioxidants. *Clinical Nutrition Insight*, **31**, 1-4 (as cited by Atoui *et al*., 2004).

Perry, N.B., Burgess, E.J. & Glennie, V.I. (2001). Echinacea standardization: Analytical methods for phenolic compounds and typical levels in medicinal species. *Journal of Agricultural and Food Chemistry*, **49**, 1702-1706.

Perva-Uzunalié, A., Škerget, M., Kinez, Ž. & Weinreich, B. (2006). Extraction of active ingredients from green tea (*Camellia sinensis*): Extraction efficiency of major catechins and caffeine. *Journal of Food Chemistry*, **96**, 597-605.

Peschel, W., Sánchez-Rabaneda, F. Diekmann, W., Plescher, A., Gartzía, I., Jiménez, D., Lamuela-Raventós, R., Buxaderas, S. & Codina, C. (2006). An industrial approach in the search of natural antioxidants from vegetable and fruit wastes. *Food Chemistry*, **97**, 137-150.

Pinelo, M., Rubilar, M., Jerez, M., Sineiro, J. & Núñez, M.J. (2005). Effect of solvent, temperature, and solvent-to-solvent ratio on the total phenolic content and antiradical activity of extracts from different compounds of grape pomace. *Journal of Agricultural and Food Chemistry*, **53**, 2111-2117.

Pink, J., Naczk, M. & Pink, D. (1999). Evaluation of the quality of frozen minced red hake: use of Fourier transform near-infrared spectroscopy. *Journal of Agricultural and Food Chemistry*, **47**,

4280-4284.

Pizarro, C., Esteban-Diez, I., Nistal, A.-J. & Conzález-Sáiz, J.-M. (2004). Influence of data preprocessing on the quantitative determination of the ash content and lipids in roasted coffee by near infrared spectroscopy. *Analytica Chimica Acta*, **509**, 217-227.

Prior, R.L., Wu, X. & Schaich, K. (2005). Standardization methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry*, **53**, 4290-4302.

Rager, I., Roos, G., Schmidt, P.C. & Kovar, K-A. (2002). Rapid quantification of constituents in St. John's wort extracts by NIR spectroscopy. *Journal of Pharmaceutical and Biomedical Analysis*, **28**, 439-446.

Raynie, D.E. (2006). Modern extraction techniques. *Analytical Chemistry*, **78**, 3997-4004.

Re, R., Pellegrini, N., Proteggente, A., Yang, M. & RiceEvans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology & Medicine*, **26**, 1231-1237.

Reeves, J.B. (2001). Near infrared diffuse reflectance spectroscopy for the analysis of poultry manures. *Journal of Agricultural and Food Chemistry*, **49**, 2193-2197.

Reid, R. (1997). Wanted: Ethics, co-operation and caution. *South African Food Review*, **24**, 7.

Ren, G. & Chen, F. (1999). Simultaneous quantification of ginsenosides in American ginseng (*Panax quiquefolium*) root powder by visible/near-infrared reflectance spectroscopy. *Journal of Agricultural and Food Chemistry*, **47**, 2771-2775.

Repollés, C. Herrero-Martínez, J.M. & Ràfols, C. (2006). Analysis of prominent flavonoid aglycones by high-performance liquid chromatography using a monolytic type column. *Journal of Chromatography* A, **1131**, 51-57.

Rice-Evans, C.A., Miller, N.J. & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical and Medicine*, **20**, 933-956.

Richards, E.S. (2002). The antioxidant and antimutagenic activities of *Cyclopia* species and activity-guided fractionation of *C. intermedia*. MSc in Food Science Thesis, Stellenbosch University, South Africa.

Robertson, C. & Christensen, G. (1996). Enzymatic browning inhibitors: Factors which interfere or prevent polyphenoloxidase from oxidizing phenolic compounds in tissues and creating brownish polymers. Food Resource. [WWW document]. http://food.oregonstate.edu/flavor/phenol.html. 23 August 2006.

Rodriguez-Otero, J.L., Hermida, M. & Centeno, J. (1997). Analysis of dairy products by near-infrared

spectroscopy: a review, *Journal of Agricultural and Food Chemistry*, **45**, 2815-2819.

Rood, B. (1994). *Uit die veldapteek*. P 51. Cape Town: Tafelberg-Uitgawers Beperk.

Ross, J.A. & Kasum C.M. (2002) Dietary flavonoids: Bioavailability, metabolic effects and safety. *Annual review of Nutrition,* **22**, 19-34.

Rotelli, A.E., Guardia, T., Juárez, A.O., De la Rocha, N.E. & Pelzer, L.E. (2003). Comparative study of flavonoids in experimental models of inflammation. *Pharmacological Research*, **48**, 601-606.

Ryan-Stoneham, T. & Tong, C.H. (2000). Degradation kinetics of chlorophyll in peas as a function of pH. *Journal of Food Science,* **65**, 1296-1301.

Santos-Buelga, C., García-Viguera, C. & Tomás-Barberán, F.A. (2003). Online identification of flavonoids by HPLC coupled to diode array detection. In: Methods in Polyphenol Analysis (edited by C. Santos-Buelga & G. Williamson). Pp. 92-127. Gateshead, UK: Athenaeum Press Ltd.

Sapers, G.M. (1993). Browning of foods: control by sulphites, antioxidants and other means. *Food Technology,* **47**, 75-84.

Sato, T. (1994). Application of principal component analysis on near-infrared spectroscopic data of vegetable oils for their classification. *Journal of the American Oil Chemists' Society*, **71**, 293298.

Sato, T., Kawano, S. & Iwamoto, M. (1990). Detection of foreign fat adulteration of milk fat by near infrared spectroscopic method. *Journal of Dairy Science*, **73**, 3408-3413.

Schrader, B., Dippel, B., Erb, I., Keller, S., Löchte, T., Schulz, H., Tatsch, E. & Wessel, S. (1999). NIR Raman spectroscopy in medicine and biology: results and aspects. *Journal of Molecular Structure*, **480-481**, 21-32.

Schmidt, A., Davies, B.P. & Schulzki, G. (1998). Near-IR reflectance spectroscopy (NIRS) for the determination of low caffeine content in black tea. Bruker Report, **146**, 10-13 (As cited by Schulz, 2004).

Schulz, H. (2004). Analysis of coffee, tea, cocoa, tobacco, spices, medicinal and aromatic plants, and related products. In: Near-infrared Spectroscopy in Agriculture (edited by C. Roberts, J. Workman & J. Reeves). Pp. 345-376. Madison, USA: American Society of Agronomy, Crop Science of America and Soil Science of America.

Schulz, H. & Albroscheit, G. (1998). High-performance liquid chromatographic characterization of some medicinal plant extracts used in cosmetic formulas. *Journal of Chromatography A*, **442**, 353-361.

Schulz, H., Drews, H.H., Quilitzsch, R. & Krüger, H. (1998). Application of near infrared spectroscopy for the quantification of quality parameters in selected vegetable and essential oil plants. *Journal of Near Infrared Spectroscopy*, **6**, 125-130.

Schulz, H., Engelhardt, U.E. Wegent, A., Drews, A. H-H. & Lapczynski, S. (1999). Application near-infrared reflectance spectroscopy to the simultaneous prediction of alkaloids and phenolic substances in green tea leaves. *Journal of Agricultural and Food Chemistry*, **47**, 5064-5067.

Schulz, H., Pfeffer, S., Quilitzsch, R., Steuer, B. & Reif, K. (2002). Rapid and nondestructive determination of the echinacoside content in *Echinacea* root by ATR-IR and NIR spectroscopy. *Planta Medica*, **68**, 926-929.

Schulz, H., Joubert, E. & Schütze, W. (2003a). Quantification of quality parameters for reliable evaluation of green rooibos (*Aspalathus linearis). European Food Research and Technology*, **216**, 539-543.

Schulz, H., Quilitzsch, R. & Krüger, H. (2003b). Rapid evaluation and quantitative analysis of thyme, origano and chamomile essential oils by ATRIR and NIR spectroscopy. *Journal of Molecular Structure*, **661-662**, 299-306.

Schutte, A.L. (1995). A taxonomic study of the tribes *Podalyrieae* and *Liparieaea (Fabaceae)*. PhD Dissertation, Rand Afrikaanse University, Johannesburg, South Africa.

Schwimmer, S. (1980). Influence of water activity on enzyme reactivity and stability. *Food Technology*, **34**, 64-74, 82-83.

Seiss, M-H., Le Bon, A-M., Canivenc-Lavier, M-C. & Suschetet, M. (2000). Mechanisms involved in the chemoprevention of flavonoids. *Biofactors*, **12**, 193-199.

Shahidi, F. Wanasundra, P.K.J. Phenolic antioxidants (1992). *Critical Reviews of Food Science and Nutition*, **32**, 67-103.

Sharma, A. & Goike, T. (2003). CIELAB: Measuring colour on many different media. *American Printer.* [WWW document] http://Americanprinter.com.

Sharma, V., Gulati, A., Ravindranath, S.D. (2005a). Extractability of tea catechins as a function of manufacture procedure and temperature of infusion. *Food Chemistry*, **93**, 141-148.

Sharma, V., Gulati, A., Ravindranath, S.D., & Kumar, V. (2005b). A simple and convenient method for analysis of tea biochemicals by reverse phase HPLC. *Journal of Food Composition and Analysis*, **18**, 583-594.

Sigge, G.O., Hansmann, C.F. & Joubert, E. (1999). Dehydration conditions of green bell peppers (*Capsicum annum* L.). Quality criteria. *Journal of Food Quality,* **22**, 439-452.

Sigge, G.O., Hansmann, C.F. & Joubert, E. (2001). Effect of storage conditions, packaging material and metabisulphite treatment on the colour of dehydrated green bell peppers (*Capsicum Annum* L.). *Journal of Food Quality,* **24,** 205-218.

Singh Sahni, N., Isaksson, T. & Næs, T. (2004). In-line near infrared spectroscopy for use in products

and process monitoring in the food industry. *Journal of Near Infrared Spectroscopy,* **12**, 77-83.

Singleton, V.L., Orthofer, R. & Lamuela-Raventós, R.M. (1999). Analysis of total phenolics and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. In: *Methods in Enzymology*, Vol 299 (edited by L. Parker). Pp. 152-178. USA: Academic Press.

Singleton, V.L. & Rossi, Jr., J.A. (1965). Colorimetry of total phenolics with phosphomolybdicphosphotungstic acid reagent. *American Journal of Enology and Viticulture*, **16**, 144-158.

Sloan. A.E. (1999). Top ten trends to watch and work on for the millennium. *Food Technology,* **53,** 40-60**.**

Sloan. A.E. (2000). The top ten functional food trends. *Food Technology,* **54**, 33-62.

Sloan. A.E. (2001). Top ten trends to watch and work on. 3rd biannual report. *Food Technology*, **55**, 38-56.

Sorvaniemi, J., Kinnunen, A., Tsados, A. & Mälkki, Y. (1993). Using partial least squares regression and multiplicative scatter correction for FT-NIR data evaluation of wheat flours. *Lebensmittel-Wissenschaft und -Technologie*, **26**, 251-258.

Spigno, G. & DeFaveri, D.M. (2007). Antioxidants from grape stalks and marc: Influence of extraction procedure on yield, purity and antioxidant power of the extracts. *Journal of Food Engineering*, **78**, 793-801.

Stark, E., Luchter, K. & Margoshes, M. (1986). Near-infrared analysis (NIRA): A technology for quantitative and qualitative analysis. *Applied Spectroscopy Reviews*, **22**, 335-399.

Steuer, B., Schulz, H, & Läger, E. (2001). Classification and analysis of citrus oils by NIR spectroscopy. *Food Chemistry*, **72**, 113-117.

Suematso, S., Hisanobu, Y., Saigo, H., Matsuda, R. & Komatsu. Y. (1995). A new extraction procedure for determination of caffeine and cathechins in green tea. *Nippon Syokuhin Kagaku Kaishi*, **42**, 419-424 (As cited by Yoshida *et al*., 1999).

Suto, T., Fukuda, M., Ito, M., Uehara, T. & Mikuni, M. (2004). Multichannel near infrared spectroscopy in depression and schizophrenia: Cognitive brain activation study. *Biological Psychiatry*, **55**, 501-511.

Tang, S.Y., Whiteman, M., Peng, Z.F., Jenner, A., Yong, E.L. & Halliwell, B. (2004). Characterization of antioxidant and antiglycation properties and isolation of active ingredients from traditional Chinese medicines. *Free Radical Biology & Medicine*, **36**, 1575-1587.

Tchamo, D.N., Cartier, G., Dijoux-Franca, M.-G., Tsamo, E. & Mariotte, A.-M. (2001). Xanthones and other constituents of *Trema orientalis. Pharmaceutical Biology*, **39**, 202-205.

Terblanche, S.E. (1982). Report on honeybush tea. University of Port Elizabeth, South Africa.

Teixeira, D.M. & Da Costa, C.T. (2005). Novel methods to extract flavanones and xanthones from the root bark of *Maclura pomifera. Journal of Chromatography A*, **1062**, 175-181.

Tomás-Barberán, F.A. & Espín, J.C. (2001). Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. *Journal of the Science of Food and Agriculture*, **81**, 853-876.

Van der Merwe, J.D. (2005). A comparative study on the protection of *Cyclopia spp*. (honeybush), *Aspalathus linearis* (rooibos) and *Camellia sinensis* tea against aflatoxin B1 induced mutagenesis in the Salmonella mutagenicity assay: Possible mechanisms involved. MSc in Food Science Thesis, Stellenbosch University, South Africa.

Van der Merwe, J. D., Joubert, E., Richards, E. S., Manley, M., Snijman, P. W., Marnewick, J. L., Gelderblom, W. C. A. (2006). A comparative study on the antimutagenic properties of aqueous extracts of *Aspalathus linearis* (rooibos), different *Cyclopia* spp. (honeybush) and *Camellia sinensis* teas. *Mutation Research*, **611**, 42-53.

Volkers, K.C., Wachendorf, M., Loges, R., Jovanovic, N.J. & Taube, F. (2003). Prediction of the quality of forage maize by near-infrared reflectance spectroscopy. *Animal Feed Science and Technology*, **109**, 183-194.

Walsh, K.B., Golic, M. & Greensill, C.V. (2004). Sorting of fruit using near infrared spectroscopy: application to a range of fruit and vegetables for soluble solids and dry matter content. *Journal of Near Infrared Spectroscopy*, **12**, 141-148.

Wang, H., & Helliwel, K. (2000). Epimerisation of catechins in green tea infusions. *Food Chemistry*, **70**, 337-344.

Wang, H. & Helliwel, K. (2001). Determination of flavonols in green and black tea infusions by high-performance liquid chromatography. *Food Research International*, **34**, 223-227.

Wang, H.F., Helliwel, K., & You, X. (2000). Isocratic elution system for the determination of catechins, caffeine and gallic acid in green tea using HPLC. *Food Chemistry,* **68**, 115-121.

Wang, L., Lee, F.S.C. & Wang, X. (2007). Near-infrared spectroscopy for classification of licorice (*Glycyrrhizia uralensis* Fisch) and prediction of the glycyrrhizic acid (GA) content. *Lebensmittel-Wissenschaft und -Technologie*, **40**, 83-88.

Wang, L., Lee, F.S.C. & Wang, X. & He, Y. (2006). Feasibility study of quantifying and discriminating soybean oil adulturation in camellia oils by attenuated total reflectance MIR and fiber optic diffuse reflectance NIR. *Food Chemistry*, **95**, 529-536.

Waterman P. & Mole, S. (1993). Extraction and chemical quantification. In: *Analysis of Phenolic Plant Metabolites*. Pp. 66-74. Blackwell Scientific Publications.

Watt, J.M. & Breyer-Brandwijk, M.G. (1932). *The Medicinal and Poisonous Plants of Southern Africa.* Livingstone, Edinburgh, Scotland. Pp. 70.

Wetzel, D.L.B. (1998). Analytical near infrared spectroscopy. In: *Instrumental Methods in Food and Beverage Analysis* (Edited by D.L.B. Wetzel & G. Charalambous). Pp. 141-194. Amsterdam: Elsevier Science B.V.

Wilkensen, J.A. (1998). The potential of herbal products for nutraceutical and pharmaceutical development. Presented at the International Business Communications (IBC), Fifth Annual Conference, "Functional Foods 1998", Copthorne Tara Hotel, London, 7-8 September 1998.

Williams, P.C. (2001). Implementation of near-infrared technology. In: *Near-infrared Technology in the Agricultural and Food Industries*, 2nd ed. (edited by P. Williams & K. Norris). Pp. 145-169. St Paul, USA: American Association of Cereal Chemists.

Williams, P.C. & Stevenson, S.G. (1990). Near-infrared reflectance analysis: food industry applications. *Trends in Food Science & Technology*, **1**, 44-48.

Williams, F.B., Sander, L.C., Wise, S.A. & Girard, J. (2006). Development of evaluation of methods for determination of naphthodianthrones and flavonoids in St. John's wort. *Journal of Chromatography A*, **1115**, 93-102.

Wills, R.B.H. & Stuat, D.L. (2004). Generation of High Quality Australian Skullcap Products. In: A report for the Rural Industries Research and Development Corporation. RIRDC Publication no 04/020.

Windham, W.R., Gaines, C.S. & Leffler, R.G. (1993). Effect of wheat moisture content on hardness scores determined by near-infrared reflectance and on hardness score standardization. *Cereal Chemistry*, **70**, 662-666.

Wold, S. (1995). Chemometrics: what do we mean with it, and what do we want from it? *Chemometrics and Intelligent Systems*, **30**, 109-115.

Wold, J.P. & Isaksson, T. (1997). Nondestructive determination of fat and moisture in whole Atlantic salmon by near infrared diffuse reflectance spectroscopy. *Journal of Food Science*, **62**, 734-736.

Woo, Y., Cho, C., Kim, H., Yang, J. & Seong, K. (2002). Classification of cultivation area of ginseng by near infrared spectroscopy and ICP-AES. *Microchemical Journal*, **73**(3), 299-306.

Woo, Y., Kim, H., Cho, J. & Chung, H. (1999a). Discrimination of herbal medicines according to geographical origin with near reflectance spectroscopy and pattern recognition techniques. *Journal of Pharmaceutical and Biomedical Analysis,* **21**, 407-413.

Woo, Y., Kim, H., Cho, J. & Cho, J. (1999b). Identification of herbal medicines using pattern recognition techniques with near-infrared reflectance spectra. *Microchemical Journal*, **63**, 61-70.

Woollen, A. (1990). Functional foods – a new market? *South African Food Review*, August/September 1990, 63-64.

Wright, R. (2005). Antioxidant avalanche. *Nutraceuticals World*, March 2005, 46, 48, 50, 52, 54, 56-57.

Wüst, E. & Rudzik, L. (2003). The use of infrared spectroscopy in the dairy industry. *Journal of Molecular Structure*, **661-662**, 291-298.

Xiabo, Z., Jiewen, Z., Xingyi, H. & Yanxiao, L. (2007). Use of FT-NIR spectrometry in non-invasive measurement of soluble solid content (SSC) of 'Fuji' apple based on different PLS models. *Chemometrics and Intelligent Laboratory Systems*, **87**, 45-51.

Yao, L.H., Jiang, Y.M., Caffin, N. D. (2006). Phenolic compounds in tea from Australian supermarkets. *Food Chemistry,* **96**, 614-620.

Yoshida, Y., Masaaki, K., & Goto, T. (1999). Efficiency of the extraction of catechins from green tea. *Food Chemistry, **67***, 429-433.

Zhang, M.H., Luypaert, J., Fernàndez Pierna, J.A., Xu, Q.S. & Massart, D.L. (2004). Determination of total antioxidant capacity in green tea by near-infrared spectroscopy and multivariate calibration. *Talanta,* **62,** 25-35.

Zhao, J., Chen, Q., Huang, X. & Fang, C.H. (2006). Qualitative identification of tea categories by near infrared spectroscopy and support vector machine. *Journal of the Pharmaceutical and Biomedical Analysis*, **41**, 1198-1204.

Zhao, B., Li, X. He, R., Cheng, S. & Wenjuan, X. (1989). Scavenging effect of extracts of green tea and natural antioxidants on active oxygen radicals. *Cell Biophysics*, **14**, 75-185.

Zheng, W. & Wang, S.Y. (2001). Antioxidant activity and phenolic compounds in selected herbs. *Journal of Agricultural and Food Chemistry*, **49**, 5165-5170.

CHAPTER 3

EVALUATION OF EXTRACTION PROCEDURES FOR MANGIFERIN AND HESPERIDIN AND THE APPLICATION OF NEAR INFRARED (NIR) SPECTROSCOPY FOR THE QUANTIFICATION OF MANGIFERIN AND XANTHONE CONTENTS IN GREEN HONEYBUSH (*CYCLOPIA SUBTERNATA*) PLANT MATERIAL

Abstract

Extraction efficiency of soluble solids (SS), total polyphenols (TP) and xanthones ($AlCl_3$ assay) from dried, green *Cyclopia subternata*, as affected by mass-solvent ratio, extraction time and solvents, was investigated. In addition the effect of solvent composition on extraction of mangiferin and hesperidin was determined. Extraction of 5 g plant material as opposed to 0.5 and 1 g resulted in lower recoveries of SS, TP and xanthones ($P<0.05$). Extraction of SS and TP increased during the initial 20 min of contact time, where after it remained constant ($P>0.05$). Water, 33% acetonitrile, ethanol (50, 80 and 100%), methanol (50 and 100%) and 70% acetone were investigated as extraction solvents. Extraction for 30 min with 33% acetonitrile on a steam bath or 50% ethanol at 64°C proved to be the most effective for extraction of SS, TP and xanthones, while 33% acetonitrile was most effective in extracting hesperidin from *C. subternata*. Both solvents were less effective than 70% acetone in extracting mangiferin. The total antioxidant activity (TAA) of *C. subternata*, as determined for water extracts, correlated poorly ($r = 0.54$) with the mangiferin content determined by HPLC. A moderate correlation ($r = 0.85$) was, however, obtained for TAA and TP content.

The potential of using the aluminium chloride ($AlCl_3$) colorimetric method to determine the mangiferin content in green *C. subternata* was also undertaken. A moderate correlation ($r = 0.87$) was found for the xanthone content of the plant material determined using the $AlCl_3$ colorimetric method and mangiferin content quantified by HPLC ($y = 1.2x + 0.54$) when extracted with hot water. When using 33% acetonitrile a weaker correlation ($r = 0.74$; $y = 1.3x + 0.87$) was found between the xanthone and mangiferin content. The xanthone content (determined by $AlCl_3$) of the plant material as extracted by the two solvents, correlated well ($r = 0.91$). Good correlations were also obtained, when comparing extractions with water and 33% acetonitrile, for determination of the SS (0.94) and mangiferin contents ($r = 0.97$) of the plant material.

Near infrared (NIR) spectroscopy was applied to develop calibration models to predict mangiferin and xanthone contents of dried, green *C. subternata* plant material. Samples included stems, leaves or stems+leaves to extend the variation range of the samples. The NIR spectroscopy calibration models

developed for mangiferin and (SEP = 0.21 g.100 g^{-1}; r = 0.82) and xanthone (SEP = 0.27 g.100 g^{-1}; r = 0.81) are suitable for screening purposes.

Introduction

The antioxidant properties of foods containing polyphenols have resulted in an increasing interest in the development of polyphenol-rich extracts for use in the nutraceutical, pharmaceutical and cosmetic industries (Andersen *et al*., 2005; Wright, 2005). An increasing body of scientific evidence supports the role of phytopharmaceuticals in prevention and treatment of diseases linked to oxidative stress (Sloan, 2002). Phenolic compounds play an important role in regulating elevated levels of oxidative stress associated with diseases such as cancer, arteriosclerosis, coronary heart disease and rheumatoid arthritis (Davies *et al*., 1995; Moure *et al*., 2001; Melton, 2006).

Honeybush (*Cyclopia* spp.) has been identified as a source of polyphenols and is being used for manufacturing of commercial extracts. Antimutagenic (Marnewick *et al*., 2000; Richards, 2002) and antioxidant activity (Hubbe, 2000; Richards, 2002) have been demonstrated in support of its potential health-promoting properties.

Mangiferin and hesperidin are the major phenolic compounds in the honeybush plant material. The highest levels of mangiferin is present in green *Cyclopia genistoides* (4.93 to 6.37 g.100 g^{-1}), while it also contain substantial quantities of hesperidin (2.23 to 5.21 g.100 g^{-1} (Joubert *et al*., 2003), making it the sought-after *Cyclopia* spp. for manufacture of a polyphenol-enriched extract. On the other hand, green *C. subternata* extracts, although containing lower quantities, i.e. *ca.* 1.19 g.100 g^{-1} mangiferin and 0.42 g.100 g^{-1} hesperidin (Joubert *et al*., 2007a), is used as herbal tea. As pharmacologically active compounds (Simova *et al*., 1986; Banjeri, *et al*., 1994; Peres *et al*., 2000; Guardia *et al*., 2001) it is important that these are included in future quality evaluation procedures of *C. subternata*, irrespective of it being used as a herbal tea or for extract manufacture.

Minimum levels of compounds of interest need to be standardised to ensure consistency and efficacy of herbal extracts (He, 2000; Huie, 2002). The quality evaluation of herbal medicines relies on the content of marker or pharmacologically active compounds (Liang *et al*., 2004), e.g. baicalein in *Scutellaria lateriflora* L. (Wills & Stuart, 2004), hyperoside in St. John's Wort (Rager *et al*., 2002), harpagoside in devil's claw (*Harpagophytum procumbens*) (Guillerault *et al*., 1994), diosmin in buchu leaves (*Barosma betulina*) (El-Shafae & El-Domiatry, 2001) and squalene in mate tea leaves (*Ilex paraguariensis*) (Esmelindro *et al*. 2005). To date, there are no quality standards relating to marker compounds or bioactive compounds for honeybush (Anon., 2000; E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, personal communication, 2005).

Although HPLC was found to be an accurate method for the quantification of individual compounds, local processers and extract manufacturers do not make use of it, mainly due to the cost involved. It is also time-consuming, labour intensive and requires large quantities of reagents (Huie, 2002; Laasonen, 2003). Mangiferin and hesperidin are present in varying concentrations in the plant material and if they are of interest, screening of the plant material to ensure that it contains minimum levels, would be required. It is therefore critical to employ rapid analytical methods for quality control of the raw material, as well as the final product.

Near infrared (NIR) spectroscopy is a rapid, easy to use, non-destructive technique (Day & Fearn, 1982; Schulz, 2004). It also has low running costs due to minimum sample preparation and as it does not require harsh chemicals. Near infrared spectroscopy has been successfully applied to quantify chemical compounds in herbal and medicinal plant species, e.g. echinacosides in *Echinacea* roots (Schulz *et al*., 2002) and harpagoside in devil's claw root (Joubert *et al*., 2005). Near infrared spectroscopy has also been used in the quantification of mangiferin and hesperidin contents of green honeybush (*C. genistiodes)* (Joubert *et al*., 2006), as well as the soluble solid content, total polyphenol content and antioxidant activity of green rooibos (*Aspalatus linearis*), (Manley *et al*., 2006). Presently, no calibration models exist for the prediction of mangiferin and hesperidin contents in dried, green *C. subternata*. The focus of this study will be on mangiferin.

Extract manufacturers might, however, find the high initial capital cost of purchasing an NIR instrument excessive in spite of the low running cost. Spectrophotometers (UV/Vis), on the other hand, are usually available in most quality control laboratories, and spectrophotometric assays could potentially be an alternative method of analysis to HPLC or NIR spectroscopy.

Colorimetric methods have also been used to determine specific classes of phenolic compounds. The aluminium chloride ($AlCl_3$) method is relatively specific for flavones and flavonols (Jurd, 1962; Mabry *et al*., 1970; Chang *et al*., 2002). The aluminium chloride forms a complex with the C-4 keto group, the C-3 or C-5 hydroxyl group, and also the orthodihydroxyl groups on the A- or B-ring of flavonoids (Chang *et al*., 2002). Joubert *et al*. (2007b) showed that mangiferin, with a conjugated carboxyl chromophore and C-1 hydroxyl group, as well as the C-6 and C-7 hydroxyl groups, complexed $AlCl_3$ and that the $AlCl_3$ assay could be used to estimate the mangiferin content of *C. genistoides.* A correlation of 0.91 between the mangiferin content determined by $AlCl_3$ method and mangiferin content quantified by HPLC was obtained for methanol extracts. Another xanthone present in *C. subternata* plant material, but in very low quantities, i.e. isomangiferin (Joubert *et al*., 2007b) could also form complexes with $AlCl_3$. Reaction with $AlCl_3$ will therefore estimate a xanthone content and not only mangiferin. Hesperidin lacks the orthodihydroxyl groups on the A- or B-ring and would

therefore have very low reactivity towards $AlCl_3$ (Mabry *et al*., 1970; Chang *et al*., 2002). Minor interferences of other phenolic components, such as flavones and flavonols in extracts of *C. subternata* could, however, pose a challenge. With the much lower mangiferin content than *C. genistoides* and possible interference by other phenolic compounds, the question therefore arises whether the $AlCl_3$ assay could be used to screen the mangiferin (or xanthone) content of *C. subternata*.

With the low mangiferin content of *C. subternata*, it is essential that extraction conditions be optimised for mangiferin, and especially so, when the results obtained by such a method is used as the reference data for developing a quantitative calibration model for NIR spectroscopy. Joubert (1988; 1990) found that by increasing the temperature of the water, the contact time and the solvent-to-solid ratio from 5:1 to 10:1, resulted in a higher total polyphenol content extracted from fermented rooibos tea. Extraction conditions such as extraction time, mass-solvent ratio and solvent composition have, however, not as yet been optimised for *C. subternata*. According to Waterman & Mole (1993) phenolics tend to leach out of a sample faster if they are very soluble in the extraction solvent. Extraction processes have generally been found to take longer from dry compared to fresh material because the components need to be rewetted and essentially get 'unstuck' before entry into the extract. It has been recommended for solvent selection that a mixture of water and either methanol, ethanol or acetone be used.

Peschel *et al*. (2006) identified other limiting factors, i.e. effective recovery of sought-after compounds, the marketability of resulting extracts, and the practical suitability for food, cosmetic and pharmaceutical products.

Quality parameters that are used by the local extract manufacturers, in the absence of mangiferin quantification, is the total polyphenol (TP) content of extracts as determined by the Folin-Ciocalteau assay (Singleton & Rossi, 1965) and the total antioxidant activity (TAA) as determined with the ABTS radical cation method of Re *et al*. (1999) (E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, personal communication, 2005). Both methods can be considered antioxidant capacity methods (Huang *et al*., 2005; Prior *et al*., 2005), their correlation would depend on the extract phenolic composition (Heinonen *et al*., 1998; Luximon-Ramma *et al*. (2002). The poor correlation of water extracts of *C. genistoides* ($r = 0.27$) was attributed to the difference in relative reactivity of mangiferin and hesperidin in the respective assays (Joubert *et al*., 2007b). As *C. subternata* has been found to contain lower quantities of the major polyphenols in honeybush, these aspects might need to be reassessed.

The objectives of this study were to determine:

- optimum extraction conditions for mangiferin from dried, green *C. subternata*;

- the potential of the $AlCl_3$ colorimetric method to determine the xanthone (mangiferin and isomangiferin) content of green *C. subternata*;
- the relationship between the mangiferin content and the TAA of green *C. subternata*;
- the relationship between the TAA and TP content of green *C. subternata*; and
- the potential of NIR spectroscopy to quantify mangiferin and xanthone contents of green *C. subternata*.

Materials and methods

Chemicals

Solvents for sample preparation were of analytical grade (Analar) and solvents used for chromatographic separations were of HPLC grade. Mangiferin, hesperidin (97%), dimethyl sulfoxide (DMSO) (99.5%), potassium persulphate, gallic acid, acetonitrile R Chromasolv® (Riedel-de Haën) and glacial acetic acid (99.8%) (Riedel-de Haën) were purchased from Sigma-Aldrich (Cape Town, South Africa). Folin Ciocalteau's phenol reagent, methanol and sodium carbonate were purchased from Merck Chemicals (Pty) Ltd (Cape Town, South Africa). Ethanol (99%) and acetone were purchased from Illovo (Cape Town, South Africa) and Saarchem (Johannesburg, South Africa), respectively. A Modulab Water Purification System (Separations, Cape Town, South Africa) was used to prepare deionised water. For the purpose of HPLC analysis, the deionised water was further purified by means of a Milli-Q 185 Académic Plus water purification system (Microsep (Pty) Ltd, Bellville, South Africa).

Plant material

For investigation of extraction conditions *C. subternata* plant material, comprising shoots (leaves and stems) was harvested on Helderfontein (Stellenbosch, South Africa), an experimental farm of ARC Infruitec-Nietvoorbij, during February 2003. The fresh plant material was dried intact at 40ºC in a temperature-controlled drying tunnel to *ca.* 8-10% moisture content and ground with a Retsch mill (1 mm sieve). For the comparison of water and 33% acetonitrile extracts a large selection of plant material samples (n = 197) was made up from plant material harvested during 2004 and 2005 at Kanetberg (Barrydale district, South Africa) and samples obtained from the collection of ARC Infruitec-Nietvoorbij . The fresh plant material was prepared as before. The plant material harvested during 2004 and 2005 was obtained from an experimental trial, investigating different harvesting practices. Samples consisting of either leaves or stems were also prepared by separating the leaves from the stems after

drying and grounding separately. These samples were included to extend the range of mangiferin and hesperidin contents in the sample set.

Effect of mass-solvent ratio on extraction efficiency

Approximately 0.5, 1.5, 5 and 10 g of dried, ground plant material were weighed off in triplicate in 100 ml volumetric flasks to which 50 ml of boiling deionised water was added and the flasks placed on a steam bath for 30 min. After cooling to room temperature, the volumetric flasks were filled to volume. The extracts were subsequently filtered through Whatman No. 4 filter paper (Whatman International Ltd, Maidstone, England) and the respective filtrates were analysed for soluble solids content. Aliquots of each extract were then frozen at -18°C until analysed (TP content; $AlCl_3$).

Effect of solvent extraction time on extraction efficiency

Dried, ground plant material (*ca.* 5 g) was weighed off in triplicate in 100 mL volumetric flasks. Extractions were performed with (1) 50 mL boiling deionised water, (2) 15 mL HPLC grade acetonitrile + 30 mL boiling deionised water (33% acetonitrile), and (3) 50 mL 80% ethanol for 10, 20, 30, 40 and 60 min in separate volumetric flasks, respectively. The flasks containing water and 33% acetonitrile were placed on a steam bath and the flasks containing 80% ethanol were placed in a water bath (*ca.* 64ºC). After cooling to room temperature, the volumetric flasks were filled to volume with deionised water for the water and 33% acetonitrile extracts. For extraction with 80% ethanol, it was filled to volume with 80% ethanol. The extracts were then filtered through a Whatman No. 4 filter paper. Aliquots of each extract were kept at -18ºC until analysed (TP content). HPLC analyses were performed immediately after the extraction procedures were completed. The experiment was repeated on three consecutive days. Table 1 summarises the experimental procedures.

Effect of type of solvent on extraction efficiency

Dried, ground plant material (*ca.* 5) g was weighed off and extractions were performed for 30 min irrespective of the solvent. Extraction with water was carried out on a steam bath, while extraction with 33% acetonitrile (acetonitrile/water (33:67), v/v) was performed on both a steam and water bath (*ca.* 64°C). The water bath (*ca.* 64°C) was used for the other solvents, i.e. 50, 80 and 100% ethanol; 50 and 100% methanol; and 70% acetone. After cooling to room temperature water or the relevant concentration of each organic solvent was used to fill the respective volumetric flasks (100 mL) to volume. The experiment, comprising nine solvents, was repeated on three separate days. Aliquots of

each extract were stored at -18°C until analysed (TP content, $AlCl_3$). Table 2 summarises the different solvents and methods used during the execution of the experiment.

Correlation of water and 33% acetonitrile extracts

For the purpose of evaluating the correlation between water and 33% acetonitrile extracts in terms of TP content, TAA, $AlCl_3$ assay values, and mangiferin and hesperidin content by HPLC *ca.* 5 g dried, ground plant material of *C. subternata* samples (n = 60 for water extracts; n = 197 for acetonitrile extracts) was extracted. Boiling deionised water (50 mL) was added to the plant material and steeping for 30 min on a steam bath in a 100 mL volumetric flask. The extract was cooled to room temperature, filled to volume and filtered through a Whatman No. 4 filter paper. For the preparation of 33% acetonitrile extracts, the same procedure described earlier (plant material extracted with 15 mL acetonitrile to which 30 mL boiling deionised water was added and steeped for 30 min on a steam bath in a 100 mL volumetric flask) was followed. Aliquots of each extract were stored at -18ºC until analysed (TP content, TAA, $AlCl_3$, HPLC).

Table 1 Summary of the different solvents used for the experimental procedure to evaluate the effect of solvent x extraction time on extraction efficiency

Solvent	**Volume organic solvent added (mL)**	**Volume of boiled water added (mL)**	**Solvent used to fill to volume (100 mL)**	**Heating method**
Water		50	water	steam bath
Acetonitrile (33%)	15	30	water	steam bath
Ethanol (80%)	50		80% ethanol	water bath (*ca.* 64°C)

Table 2 Summary of the different solvents and methods employed for the experimental procedure to evaluate the effect of using different solvents on extraction efficiency.

Solvent	Volume of solvent added (mL)	Volume of boiling water added (mL)	Solvent used to fill to volume (100 mL)	Heating method
Water		50	water	steam bath
Acetonitrile (33%)	15	30	water	steam and water bath
Ethanol (50, 80, 100%)	50	_	ethanol	water bath
Methanol (50, 100%)	50	_	methanol	water bath
Acetone (70%)	50	_	acetone	water bath

Determination of soluble solids content of C. subternata extracts

The soluble solids (SS) content of the water, 33% acetonitrile and other solvent extracts was determined gravimetrically (in duplicate) by evaporating the extract (15 mL) in nickel moisture dishes on a steam bath until dry. The samples were subsequently dried at 100°C for 60 min and the results were expressed as mg.100 mL^{-1} extract. Volumes of extract used for determination of soluble solids content for the effect of mass-solvent ratio on extraction efficiency experiment were 20 mL for 0.5 g.100 mL water extracts, 15 mL for 1 g.100 mL water extracts and 10 mL for 5 g.100 mL water extracts.

Moisture content determination

The moisture content of the dried, green plant material was determined gravimetrically (in duplicate) by weighing off *ca.* 2-3 g of sample in nickel moisture dishes. The samples were then subsequently dried at 70°C for 16 hours in a vacuum oven.

Quantification of mangiferin and hesperidin by HPLC analysis

All extracts, except the water extract, was filtered "as-is" through a 0.45 µm Millpore Milex-HV hydrophilic PVDF syringe filter (25 mm diameter) (Microsep (Pty) Ltd). A 300 µl aliquot of DMSO was added to 3 mL water extract before filtration to enhance solubility of hesperidin. Quantification of mangiferin and hesperidin in the extracts (10 µl) were performed in duplicate by reversed-phase HPLC,

using a LaChrom (Merck/Hitachi) HPLC system (Merck, Cape Town, South Africa) which consisted of: an L7000 interface, L7400 UV detector, L7100 pump and a L7200 autosampler. Separation was performed on a Luna Phenyl Hexyl column with a pore size of 3 µm using the solvent gradient method (Program I) of Joubert *et al*. (2003). The solvents used for separation were 2% acetic acid and acetonitrile at a flow rate of 1 $mL.min^{-1}$. A Phenomenex Degasser Model DG-4400 from (Separations, Cape Town, South Africa) was used to degas the solvents in-line. Measurements were done at 320 nm (for mangiferin and isomangiferin) and 288 nm (for hesperidin), respectively. A standard dilution series of mangiferin (concentration range 0.15-10.9 µg injected; $R^2 = 1$) and hesperidin (concentration range 0.1-6 µg injected; $R^2 = 1$) were prepared and analysed weekly. Isomangiferin was quantified in terms of mangiferin. Integration of the peak area was done with the LaChrom Multisystem Software D700.

Determination of total polyphenol content of C. subternata

The TP content of *C. subternata* water and solvent extracts was determined in triplicate using a 20:1 reduced version (with a final volume of 5 mL) of the Folin Ciocalteau method of Singleton & Rossi (1965). Gallic acid was used for the preparation of the calibration curve (concentration range 10-100 $\mu g.mL^{-1}$; $R^2 = 1$) and the total polyphenol content of the samples used was expressed as mg gallic acid equivalents (GAE) 100 mL^{-1} extract. Extracts were diluted to ensure the absorbance falls within the range of the calibration curve. Measurements were performed in 1 cm path length disposable cuvettes (Plastibrand PS-cuvette, Sigma-Aldrich, Cape Town, South Africa) at 765 nm using a GBC UV/Vis 911A spectrophotometer (Wirsam, Cape Town, South Africa).

Aluminum chloride colorimetric measurement of xanthone (mangiferin and isomangiferin) content

The disposable glass tubes, including the plastic caps, were pretreated with 5% EDTA solution overnight (Merck Chemicals (Pty) Ltd, Cape Town, South Africa) wherafter it was thoroughly rinsed with deionised water and dried before use. Mangiferin (*ca*. 20 mg dissolved in 10 mL DMSO) was diluted with 80% ethanol to prepare a standard series (concentration range 20.14-200.70 $ug.mL^{-1}$; $R^2 = 1$) for the preparation of a calibration curve. The standard solutions and samples (0.5 mL) were mixed with 1.5 mL ethanol, 0.1 mL 10% $AlCl_3$ (m/v) and 3 mL 0.03 M potassium acetate. The standard and sample blanks were prepared identically, but the $AlCl_3$ solution was substituted with water. The reaction mixture was vortexed followed by incubation at 30ºC in a temperature controlled laboratory oven for an hour. The absorbance was measured in disposable plastic cuvettes (1 cm path length; Plastibrand PS-cuvette, Sigma-Aldrich, Cape Town, South Africa) with a GBC UV/Vis 911A spectrophotometer (Wirsam, Cape Town, South Africa) at 410 nm. The extracts were diluted before

being added to the reaction mixture to fit the calibration curve (0.2-0.8). The mangiferin content of the extracts was expressed as mg.100 mL^{-1} extract.

Total antioxidant activity of water extracts

The total antioxidant activity (TAA) of *C. subternata* water extracts was determined using the ABTS decolourisation assay of Re *et al*. (1999). The ABTS radical cation ($ABTS^{\cdot+}$) was created by reacting 7 mmol ABTS aqueous solution with 2.45 mmol potassium persulfate and left overnight in the dark, followed by dilution with ethanol to obtain an absorbance of 0.70 (± 0.02) at 734 nm. The extracts were diluted with deionised water to give a 40-60% inhibition of the $ABTS^{\cdot+}$ solution after 4 min at 37°C. The TAA of the samples was calculated from a calibration curve prepared with Trolox (concentration range 0.003-0.02 µmol.mL^{-1}; R^2 = 1). Measurements were taken in 1 cm path length disposable cuvettes (Plastibrand PS-cuvette, Sigma-Aldrich, Cape Town, South Africa), using a GBC UV/Vis 911A spectrophotometer (Wirsam, Cape Town, South Africa).

Near infrared spectroscopy measurements

Dried, green *C. subternata* plant material (n =197)

Near infrared spectroscopy measurements were performed using a Büchi NIRLab N-200 Fourier transform near infrared (FT-NIR) spectrophotometer (Büchi Labortechnik, AG, Flawil, Switzerland) with NIRLabWare (version 3.0) measurement software (Büchi Labortechnik, AG, Flawil, Switzerland). Near infrared spectroscopy measurements were performed in diffuse reflectance mode. The dried, ground plant material was presented to the instrument in rotating glass Petri dishes and NIR spectra was collected from 1000-2500 nm (10 000-4000 cm^{-1}) at a resolution of 8 cm^{-1} resulting in 1557 data points as a data point is collected every 3.86 cm^{-1}.

Near infrared spectroscopy calibration development

The Unscrambler® (v9.2) software (Camo Process AS, Oslo, Norway) was used for Partial Least Square (PLS) regression model development on spectral data pretreated with multiplicative scatter correction and Savitsky-Golay first derivative (22 point segment). The calibration models were validated by means of independent validation. The standard error of prediction (SEP), the coefficient of determination (R^2) and the RPD (Williams, 2001), which is the ratio of SEP to the standard deviation (SD) of the validation set (used to indicate the efficiency of a calibration), were used to evaluate the accuracy of the calibration models. The aim of model development is to obtain a calibration model

with a low SEP, a high R^2 (above 0.83) and a RPD value higher than 5. The value of SEP should also be as close as possible to the standard error of laboratory (SEL).

Statistical analysis

One-way analysis of variance (ANOVA) was performed with SAS version 8.2. The student's t-LSD (Least Significant Differences) was calculated at a 5% level ($P<0.05$) to compare treatment means. Correlations were undertaken with the parametric Pearson r value and also the nonparametric Spearman correlation. Graphs were compiled using Statistica version 7.1 (StatSoft, Inc., Tulsa, OK, USA).

Results

Effect of mass-solvent ratio on extraction efficiency

From Figure 1a it is evident that there was no significant difference ($P>0.05$) between 0.5 and 1 g, and between 5 and 10 g plant material in their water SS content. By using 0.5 and 1 g plant material, significantly better extraction ($P<0.05$) of SS was obtained. Significantly less ($P<0.05$) TP was extracted when the mass-solvent ratio was increased to 5 g plant material (Figure 1b). Recovery of xanthones from 0.5 and 1 g plant material was not significantly different ($P>0.05$), but higher mass-solvent ratios resulted in significantly and progressively lower recoveries (Figure 1c). Slightly different trends were obtained for the TP content and xanthone content of the SS. The TP content (Figure 2a) and xanthone (Figure 2b) content of the SS, extracted from 0.5 and 1 g plant material, as well as the TP content of the SS from 5 and 10 g plant material, were not significantly different ($P>0.05$). The xanthone content, however, was again significantly lower ($P<0.05$) when using 10 g plant material compared to 5 g for extraction.

Effect of solvent extraction time on extraction efficiency

No significant difference ($P>0.05$) was observed between the SS contents of the plant material obtained with water and 33% acetonitrile extractions on a steam bath, but both gave significantly higher SS contents ($P<0.05$) than 80% ethanol extraction (Figure 3a). However, the TP content of the plant material, obtained with the three respective solvents, differed significant (Figure 3b), with 33% acetonitrile and 80% ethanol extraction, respectively recovering the most and least TP from the plant material. Increasing extraction times did not increased the SS contents (Figure 4a). The TP content showed a different trend, with a substantial increase in extraction of TP by al three solvents after 20

min, compared with 10 min extraction (Figure 4b). No significant differences in extraction times of 20 min and longer were observed.

Effect of type of solvent on extraction efficiency

Extraction solvent significantly affected the soluble solids recovered from the plant material (Figure 5a). The most effective solvent was 33% acetonitrile, irrespective of whether extraction was carried out on a steam bath, or at a lower temperature in a water bath. The latter did not give significantly better extraction of SS than 50% ethanol. Water (steam bath) alone was significantly less effective than 33% acetonitrile, 50% methanol and 50% ethanol, while as effective as 70% acetone, but more effective than 100% ethanol, 100% methanol and 80% ethanol. Overall 100% ethanol had the lowest SS recovery from the plant material.

Extraction with 33% acetonitrile (steam bath and water bath) and 50% ethanol gave the same extraction of TP (Figure 5b). For the other solvent the same trend as for the SS was obtained, except that water (steam bath) extracted significantly less ($P<0.05$) TP than 70% acetone from the plant material. The highest recovery of xanthones from the plant material, however, was achieved with 70% acetone and 50% ethanol (Figure 5c). Acetonitrile (33%) (both water bath and steam bath) was as effective as 50% methanol, 100% methanol, 80% ethanol. The least efficient extraction was observed with 100% ethanol.

Considering the solvent efficacy based on the composition of the soluble solids, no significant difference ($P>0.05$) for TP content was found between the 50% ethanol, 50% methanol, 33% acetonitrile (water and steam bath) and 70% acetone extractions (Figure 6a). The lowest total polyphenol content was again obtained by extraction with 100% ethanol. In contrast, extraction with 100% ethanol resulted in SS with the highest xanthone content with extraction with water (steam bath) and 33% acetonitrile (steam bath) being the least efficient (Figure 6b).

Extraction with 70% acetone gave the highest amount of mangiferin based on both the plant material (Figure 7a) and soluble solids basis (Figure 7b). Acetonitrile (33%) (steam bath and water bath), 50% methanol and 50% ethanol were equally effective in extracting mangiferin. Once again 100% ethanol was the least effective extraction solvent (Figure 7a). However, no significant difference was obtained in mangiferin content of the soluble solids when 70% acetone, 80% ethanol, 100% ethanol and 100% methanol were used (Figure 7b). In this case water (steam bath) overall gave soluble solids with the lowest mangiferin content.

Ethanol (100%) was found to significantly extract the highest quantity of hesperidin from the plant material ($P<0.05$) (Figure 8a), while extraction with acetonitrile (33%) (steam bath) yielded the soluble

solids with the highest hesperidin content (P<0.05) (Figure 8b). In both cases extraction with water (steam bath) were the least efficient.

A general observation is that extracts, obtained with 80% and 100% methanol and 100% ethanol, were greener in colour than the other extracts.

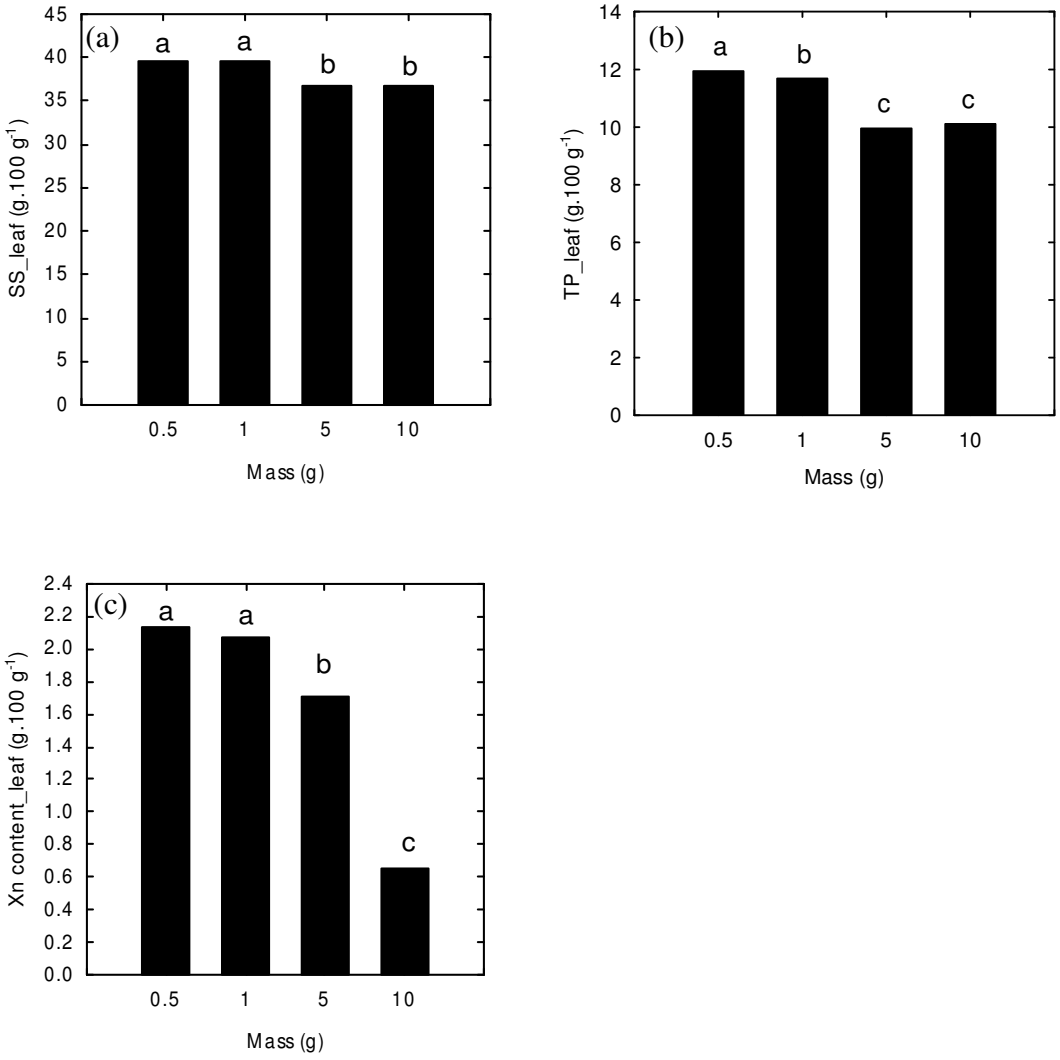


Figure 1 Effect of plant material mass on (a) soluble solids (SS) content, (b) total polyphenol (TP) content (Folin-Ciocalteau assay) and (c) xanthone (Xn) content ($AlCl_3$ method of dried green *C. subternata* plant material (expressed as g.100 g^{-1}). Water (50 ml) was used as extraction solvent.

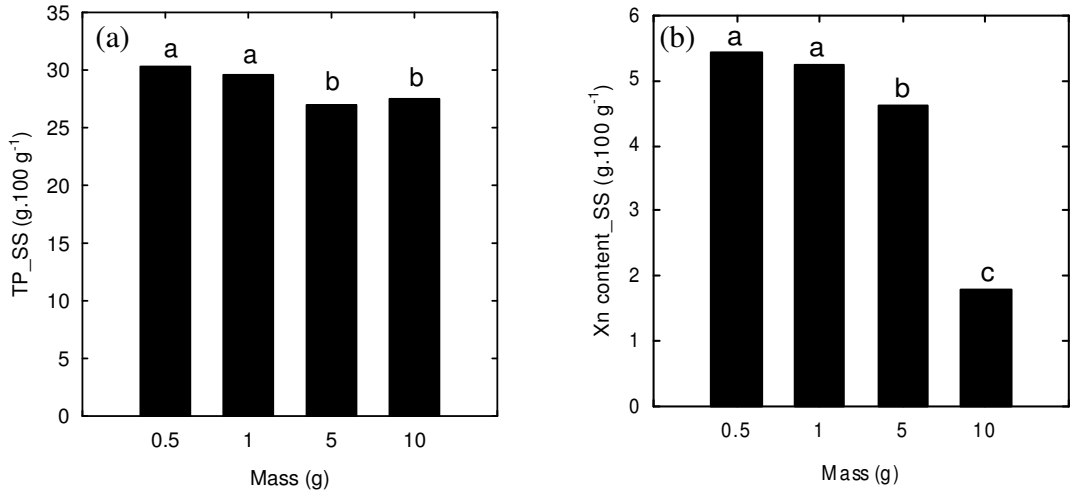
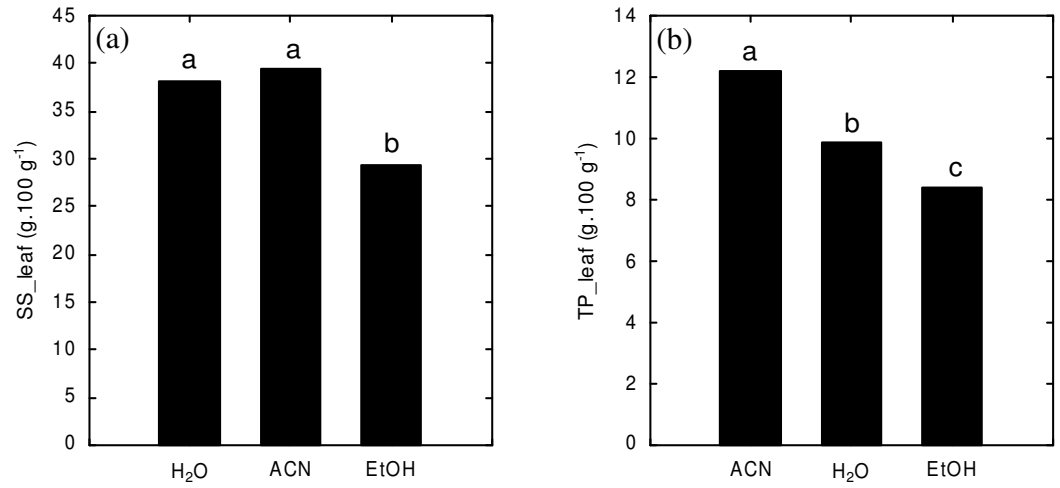


Figure 2 Effect of plant material mass on (a) total polyphenol (TP) content (Folin-Ciocalteau assay), and (b) xanthone content ($AlCl_3$ method) of the soluble solids (expressed as g.100 g^{-1}) of green *C. subternata,* extracted with 50 ml water.



(a) (b)

Figure 3 Effect of solvent on extraction of (a) soluble solids (SS) content and (b) total polyphenol (TP) content (Folin-Ciocalteau assay) of the plant material (expressed as g.100 g^{-1}). Solvents used were water (H_2O), 33% acetonitrile (ACN) and 80% ethanol (EtOH). ACN and H_2O extractions were performed on a steam bath and EtOH extraction in a water bath at 64°C.

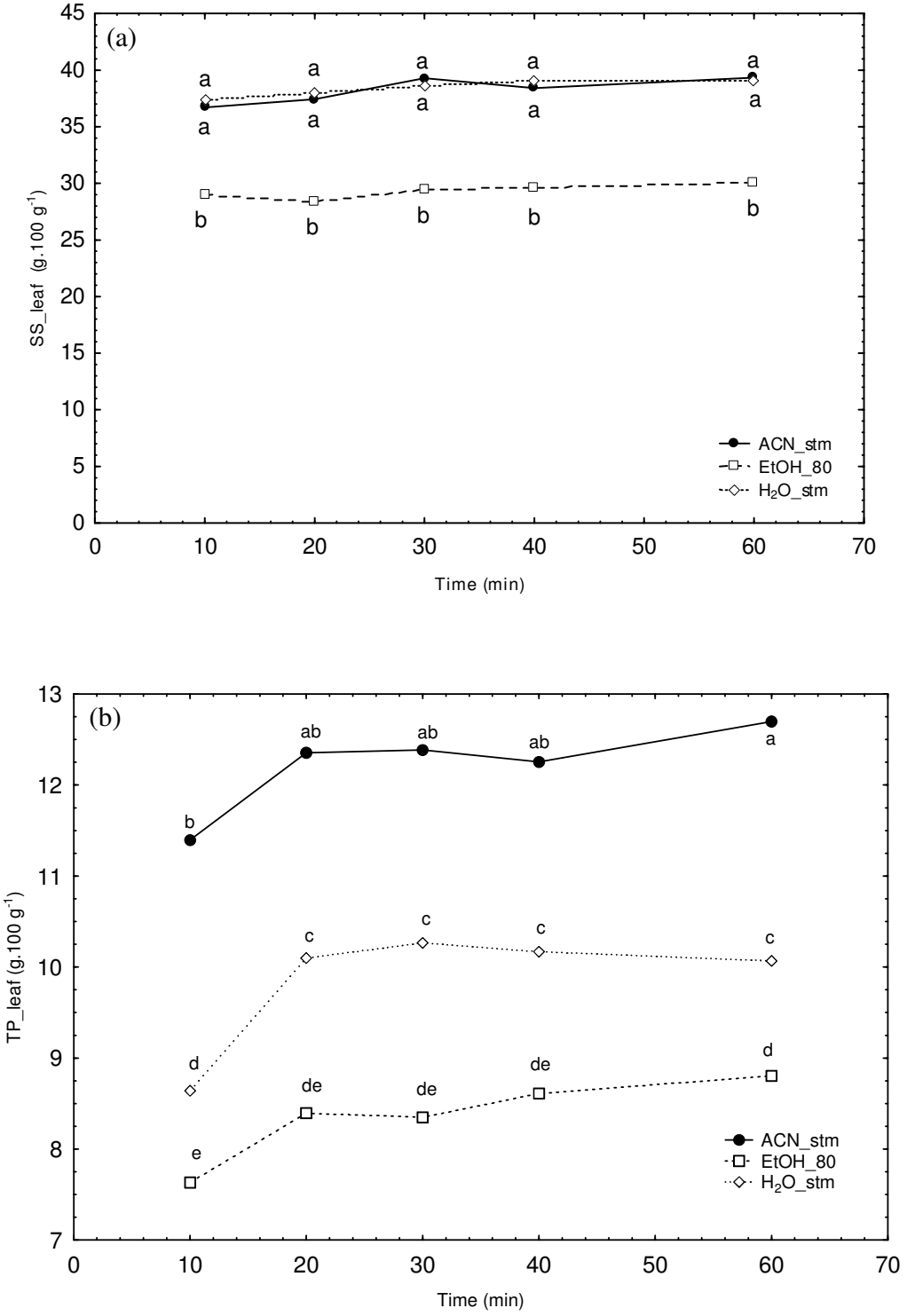
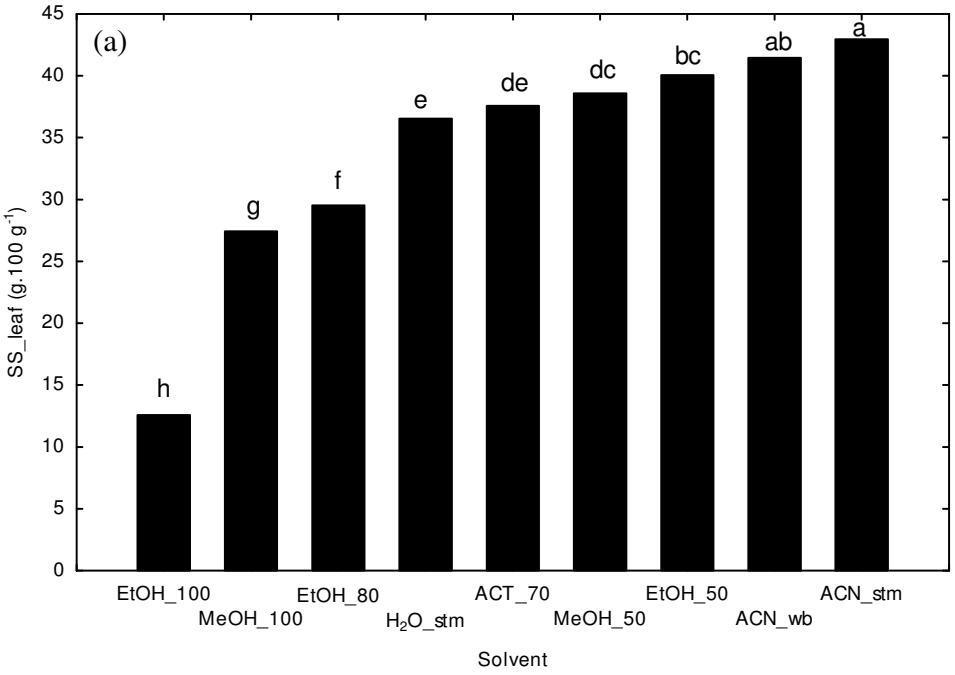


Figure 4 Effect of solvent and extraction time on the (a) soluble solid content (SS) and (b) total polyphenol (TP) content (Folin-Ciocalteau assay) of the plant material (expressed as g.100 g^{-1}). Solvents used were water (H_2O), 33% acetonitrile (ACN), and 80% ethanol (EtOH). ACN and H_2O extractions were performed on a steam bath and EtOH extraction in a water bath at 64°C.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40
35
30
25
20
15
10
5
0
h
g
f
e
de
dc
bc
ab
a
EtOH_100
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EtOH_80
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MeOH_50
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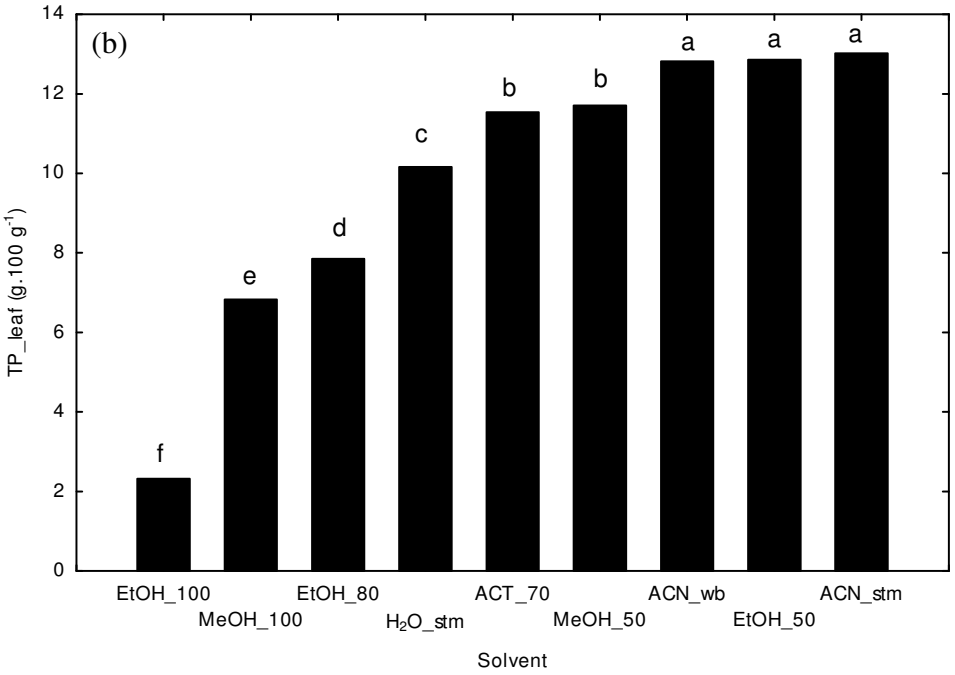
ACN_wb
ACN_stm
Solvent


(b)
TP_leaf (g.100 g-1)
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12
10
8
6
4
2
0
f
e
d
c
b
b
a
a
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Solvent

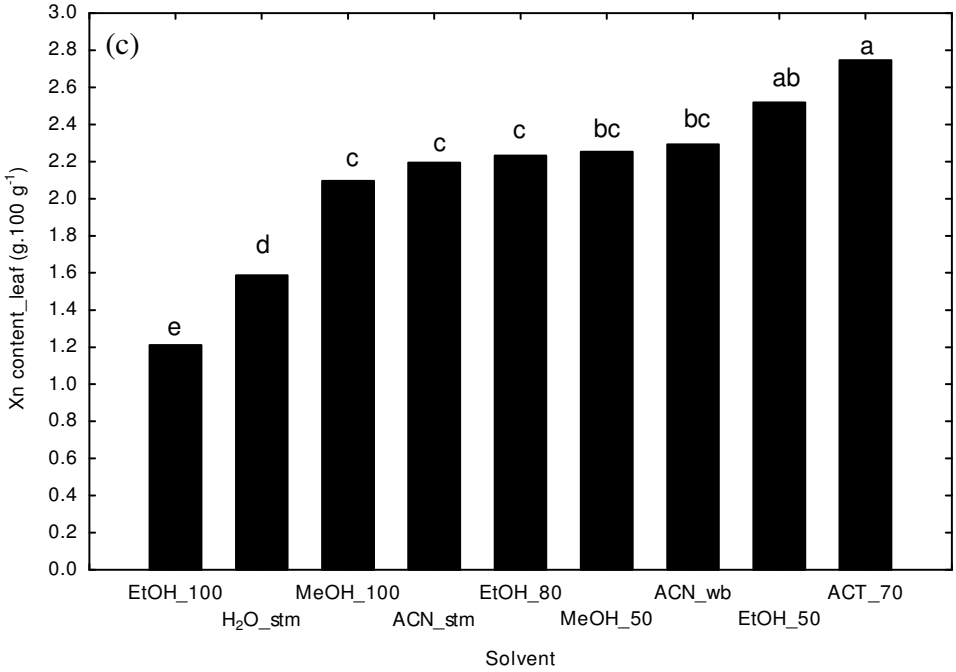


Figure 5 Solvent extraction efficiency in terms of (a) soluble solid content (SS), (b) total polyphenol (TP) content (Folin-Ciocalteau assay) and (c) xanthone (Xn) content ($AlCl_3$ method) of the plant material (expressed as g.100 g^{-1}). Solvents used were water (H_2O), 33% acetonitrile (ACN), 50% ethanol (EtOH_50), 80% ethanol (EtOH_80), 100% (EtOH_100), 50% methanol (MeOH_50), 100% methanol (MeOH_100) and 70% acetone (ACT_70). Extraction with H_2O: water, stm = steambath; wb = water bath. All other extractions were performed on a water bath at 64°C.

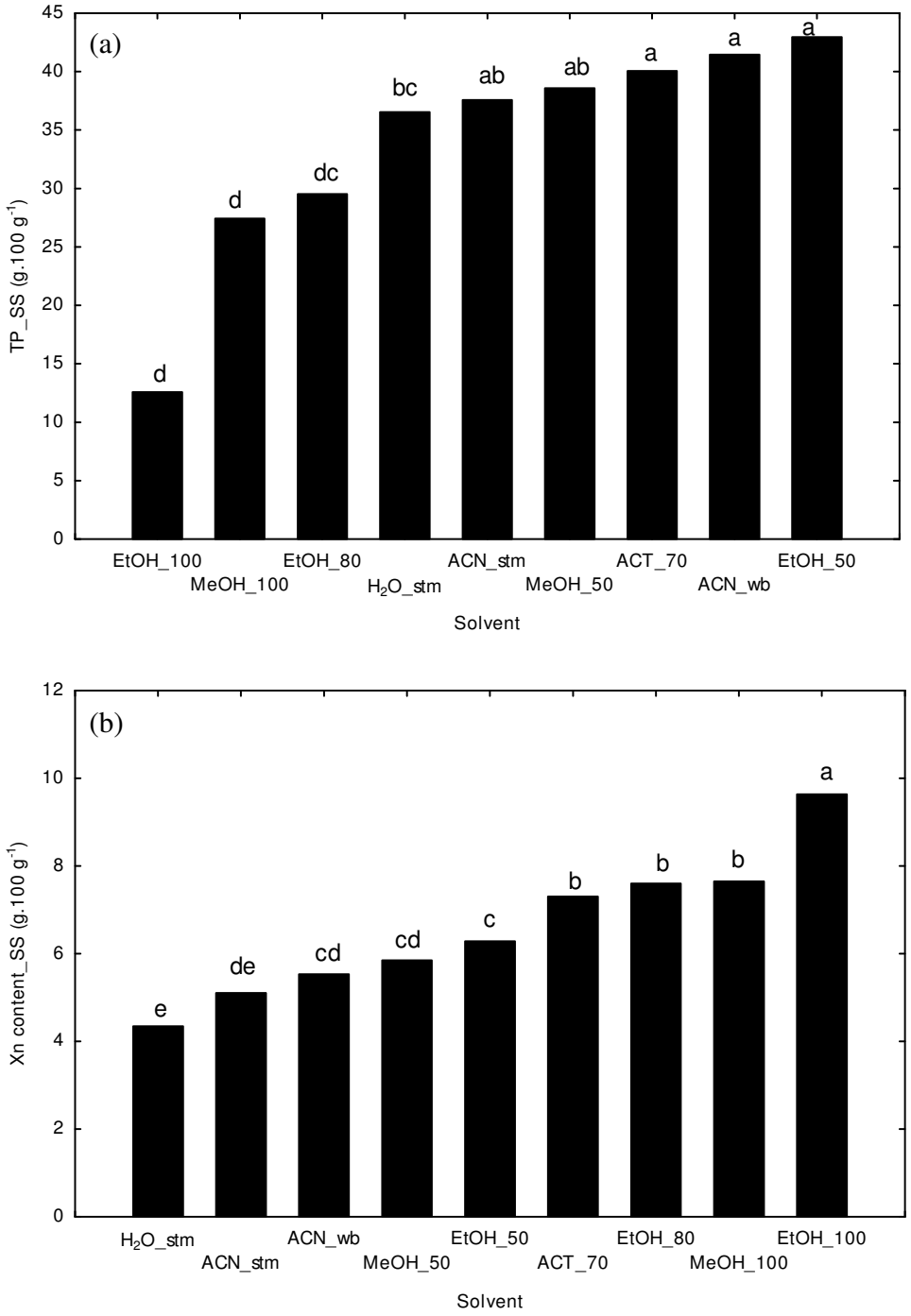


Figure 6 Effect of solvent on the (a) total polyphenol content (TP) Folin-Ciocalteau assay and (b) xanthone content (Xn) ($ALCl_3$ method) of the soluble solids (expressed as g.100 g^{-1}). Solvents used were water (H_2O), 33% acetonitrile (ACN), 50% ethanol (EtOH_50), 80% ethanol (EtOH_80), 100% (EtOH_100), 50% methanol (MeOH_50), 100% methanol (MeOH_100) and 70% acetone (ACT_70). Extraction with H_2O: water, stm = steambath; wb = water bath. All other extractions were performed on a water bath at 64°C.

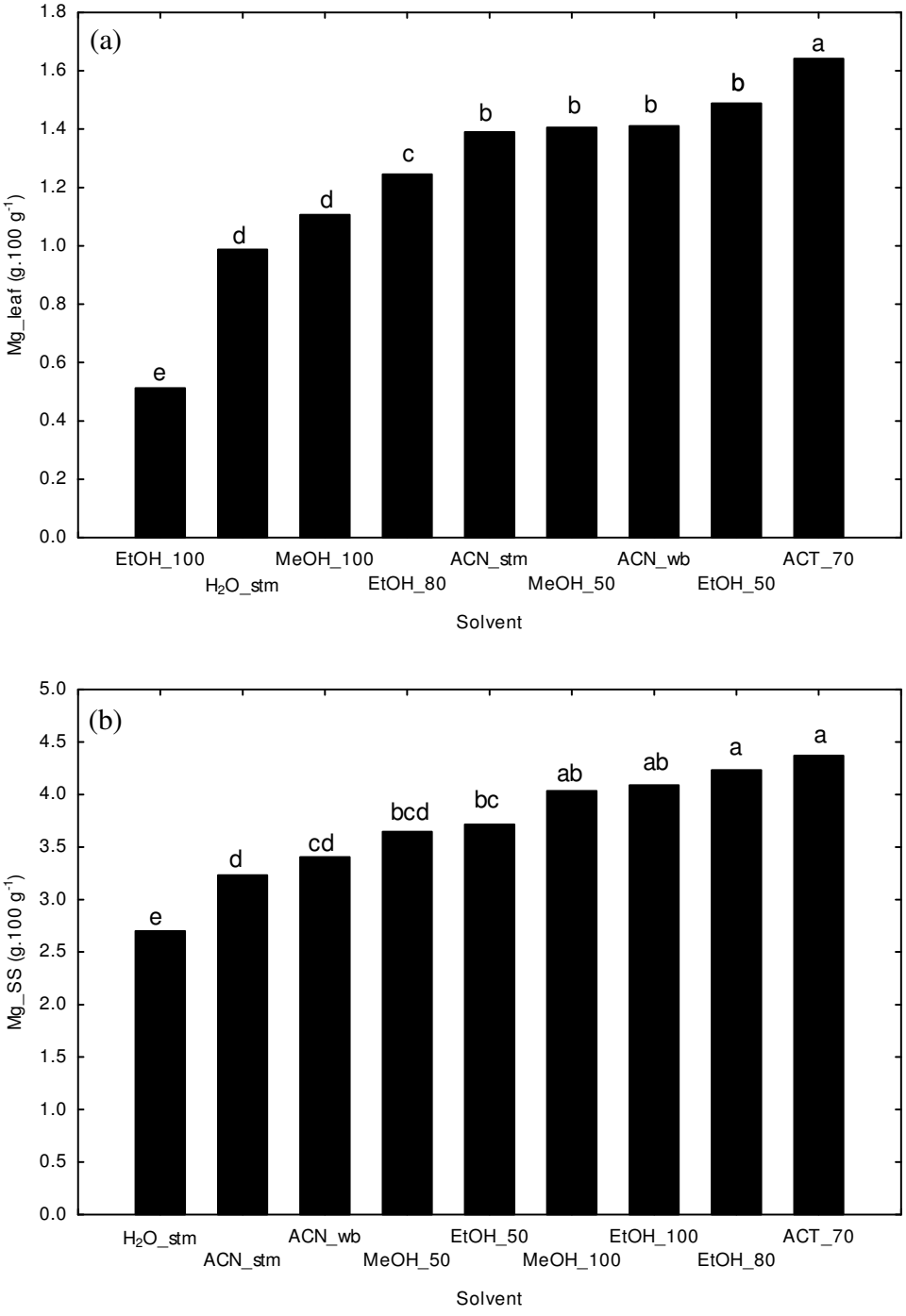
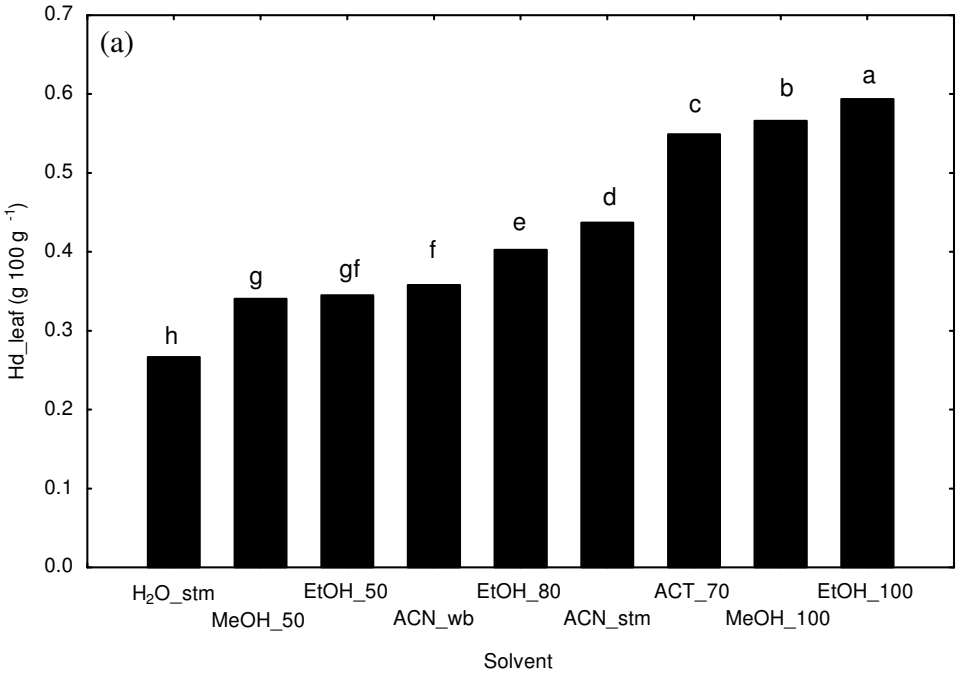


Figure 7 Solvent extraction efficiency in terms of mangiferin (Mg) content of (a) the plant material and (b) the soluble solids (SS) (expressed as g.100 g^{-1}). Solvents used were water (H_2O), 33% acetonitrile (ACN), 50% ethanol (EtOH_50), 80% ethanol (EtOH_80), 100% (EtOH_100), 50% methanol (MeOH_50), 100% methanol (MeOH_100) and 70% acetone (ACT_70). Extraction with H_2O: water, stm = steambath; wb = water bath. All other extractions were performed on a water bath at 64°C.



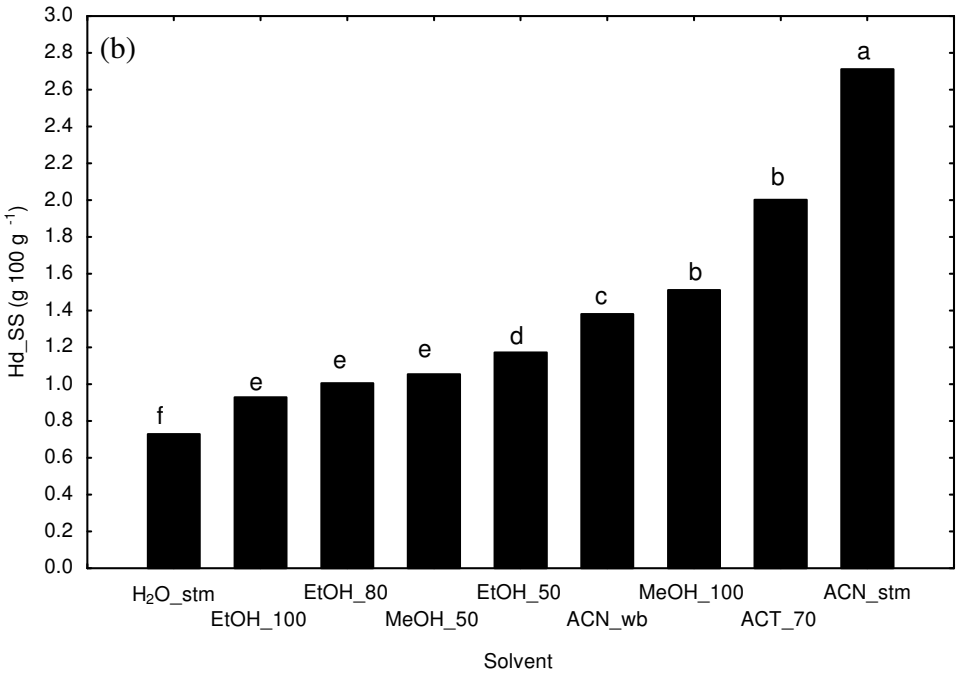


Figure 8 Effect of solvent extraction on hesperidin (Hd) content of (a) the plant material and (b) soluble solids (SS) (expressed as g. 100 g^{-1}). Solvents used were water (H_2O), 33% acetonitrile (ACN), 50% ethanol (EtOH_50), 80% ethanol (EtOH_80), 100% (EtOH_100), 50% methanol (MeOH_50), 100% methanol (MeOH_100) and 70% acetone (ACT_70). Extraction with H_2O: water, stm = steambath; wb = water bath. All other extractions were performed on a water bath at 64°C.

Correlation between water and 33% acetonitrile extracts (n = 60) based on mangiferin, xanthone, hesperidin, TP and SS contents in green C. subternata

A good correlation was observed between the HPLC mangiferin content (r = 0.97) of the 33% acetonitrile and water extracts (Figure 9a) as well as for the HPLC xanthone content (r = 0.91) determined for the two respective extracts (Figure 9b). A poor correlation (r = 0.59) was observed between of the water and 33% acetonitrile extracts for the TP content of the plant material (Figure 9c) and a good correlation (r = 0.94) between the SS content of the water and 33% acetonitrile extracts (Figure 9d).

Correlation between xanthone determined with $AlCl_3$ colorimetric method and mangiferin as well as xanthone contents quantified with HPLC of green C. subternata

A moderate correlation was obtained (r = 0.87) between the xanthone (mangiferin + isomangiferin) content determined using the $AlCl_3$ colorimetric method and mangiferin quantified by HPLC of green *C. subternata* when extracted with water (Figure 10a). When extracted with acetonitrile a correlation of 0.74 was obtained (Figure 10b). Similar correlations was also obtained (r = 0.89) between the xanthone content ($AlCl_3$) and xanthone quantified by HPLC of green *C. subternata* when extracted with water (Figure 11a) and acetonitrile (r = 0.75) (Figure 11b), respectively.

Correlation between TAA and TP, mangiferin and mangiferin+isomangiferin+hesperidin contents of green C. subternata

Using water as extraction solvent, a moderate correlation (r = 0.85) was found between the TAA of the green *C. subternata* and their TP content (Figure 12a). Poor correlations were obtained between TAA and the mangiferin content (HPLC) (r = 0.53) (Figure 12b) and the combined mangiferin+isomangiferin+hesperidin (r = 0.59) contents (HPLC) when extracted with water content (Figure 12c).

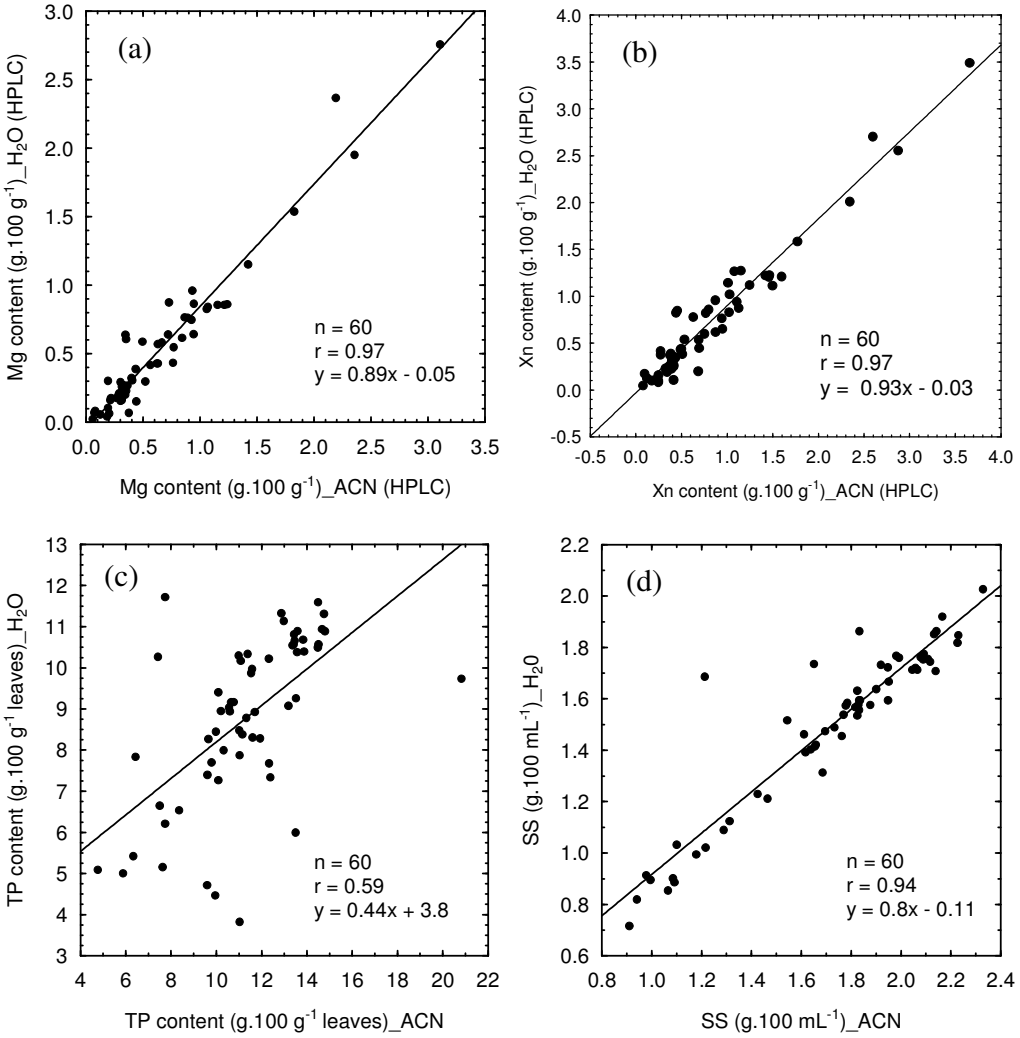


Figure 9 Correlation of (a) mangiferin (Mg) content quantified by HPLC, (b) xanthone (Xn) (mangiferin + isomangiferin) content quantified by HPLC, (c) total polyphenol (TP) content (Folin-Ciocalteau assay) and (d) soluble solids (SS) content of green *C. subternata* as determined using water (H_2O) and 33% acetonitrile (ACN) for extraction. Results were expressed as g.100 g^{-1} of the plant material, except for soluble solids (g.100 mL^{-1}).

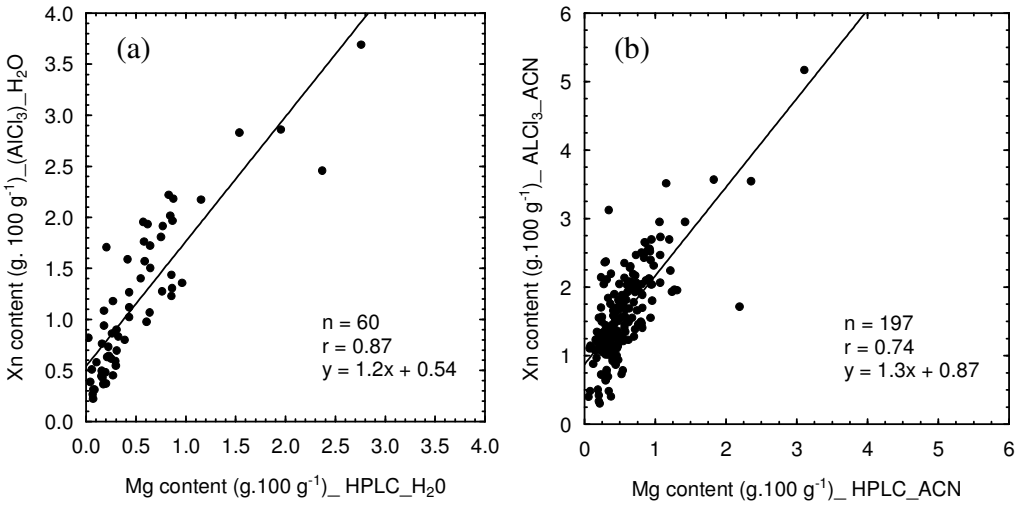


Figure 10 Correlation between xanthone (Xn) (mangiferin+isomangiferin) content determined with $AlCl_3$ method with mangiferin (Mg) content quantified by HPLC of green *C. subternata* as determined using (a) water (H_2O) and (b) 33% acetonitrile (ACN) for extraction. Results were expressed as g.100 g^{-1} of the plant material.

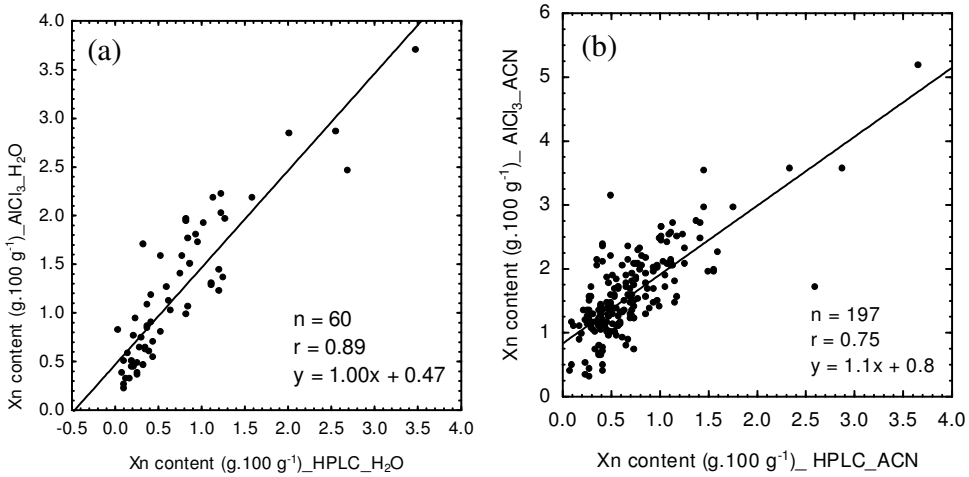


Figure 11 Correlation of xanthone (Xn) (mangiferin+isomangiferin) content determined with $AlCl_3$ method with xanthone (mangiferin+isomangiferin) content quantified by HPLC of green *C. subternata* as determined using (a) water (H_2O) and (b) 33% acetonitrile (ACN) for extraction. Results were expressed as g.100 g^{-1} of the plant material.

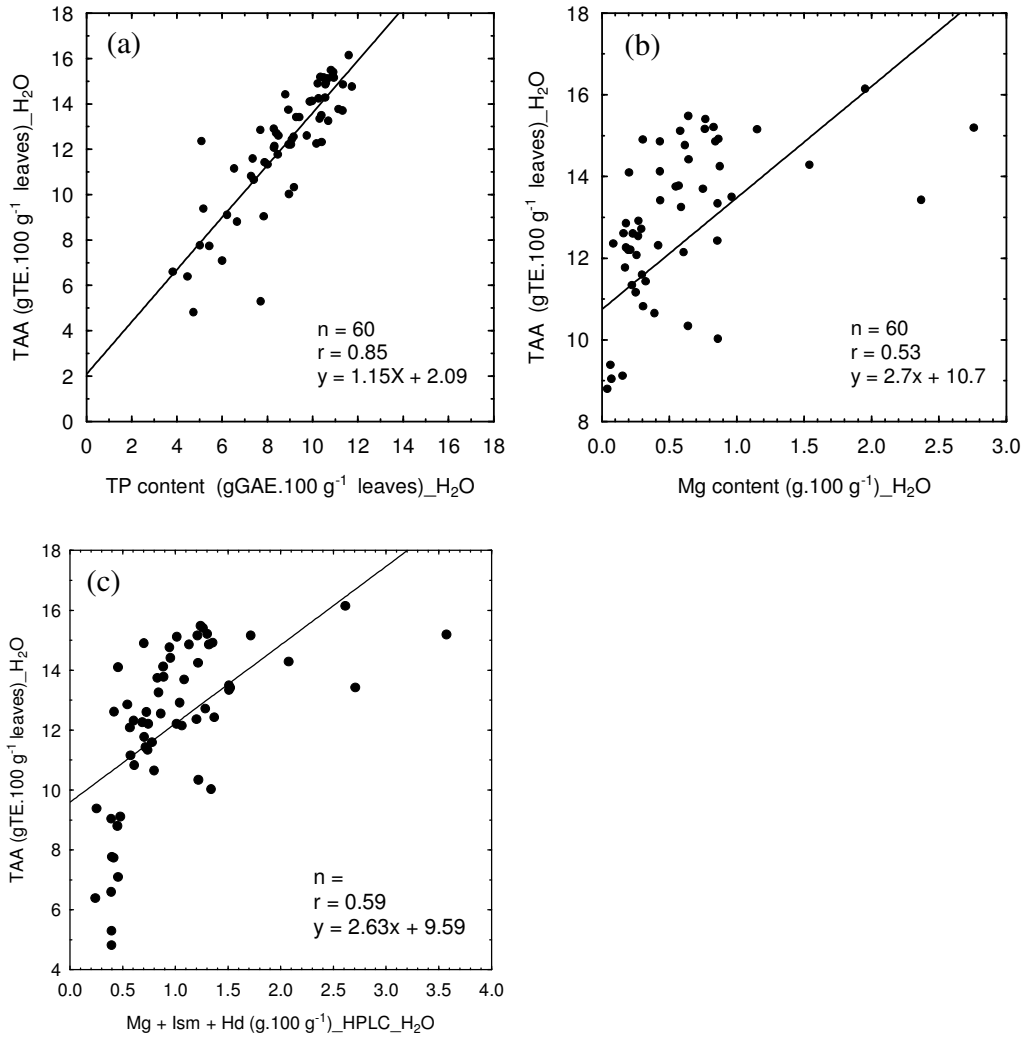


Figure 12 Correlation between total antioxidant activity (TAA) (ABTS assay) and (a) total polyphenol content (TP) (Folin-Ciocalteau assay), (b) mangiferin (Mg) content quantified by HPLC and (c) mangiferin (Mg)+ isomangiferin (Ism)+ hesperidin (Hd) content quantified by HPLC. Results were expressed as g.100 g^{-1} of the plant material and extractions were performed with water (H_2O).

NIR spectroscopy calibration models for mangiferin and xanthone contents of dried, green *C. subternata* plant material

A summary of the reference data for mangiferin and hesperidin contents of the dried, green honeybush plant material in *C. genistoides* and the mangiferin and xanthone content of *C. subternata* is given in Table 3. The distribution of mangiferin and xanthone contents is depicted in Figure 13. A positive skew distribution was noticed for both the histograms due to the longer tails. In both cases, a longer tail in the positive direction was obtained and thus indicated that the distribution of the samples was mostly with lower mangiferin content. If, however, a Guassian distribution had been obtained, more accurate predictions near the mean and less accurate predictions for samples near the extremes of the ranges would be possible (Williams, 2001). Typical raw (no pre-treatment) NIR spectra of dried, ground, green honeybush *C. subternata* plant material are shown in Figure 14.

The validation results of the mangiferin calibration model (SEP = 0.21 g.100 g^{-1}; r = 0.82; RPD = 1.62) indicated that it can be used for screening and approximate predictions (Table 4, Figure 15a), (Williams, 2001). The xanthone calibration model (SEP = 0.27 g.100 g^{-1}; r = 0.81; RPD = 1.52) also gave similar results (Figure 15b). These values compared relatively well with their SEL values obtained (0.03 g. 100 g^{-1} for mangiferin) and (0.07 g.100 g^{-1}) for xanthones (Table 3). A small bias (0.007%) and correlation coefficient (r) of 0.82 was observed for mangiferin. The respective values for xanthones were 0.001% and 0.81 (Table 3).

Table 3 Reference data of dried, green honeybush plant material for mangiferin and hesperidin contents of dried, green *C. genistoides* and mangiferin and xanthone contents of dried *C. subternata*

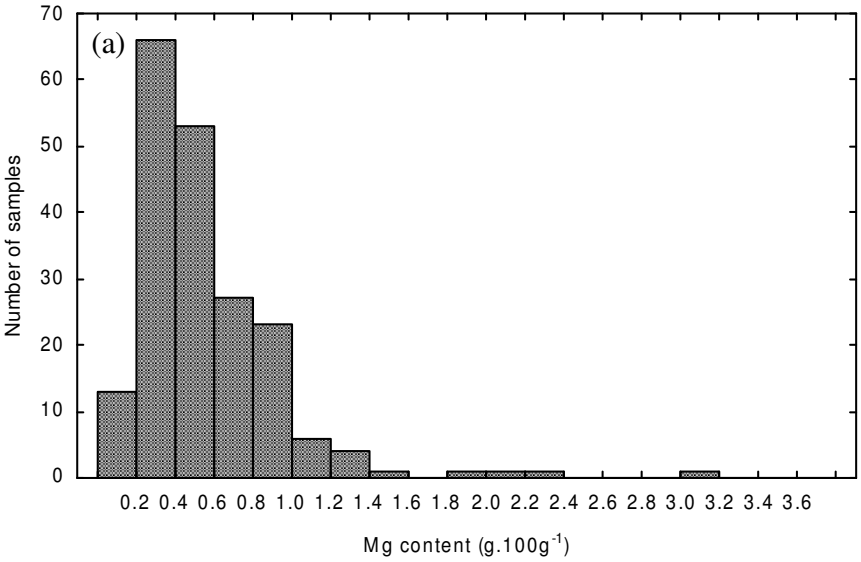
	***C. genistoides*[a]**		***C. subternata***	
	Mangiferin	**Hesperidin**	**Mangiferin**	**Xanthone**
n	240	240	197	197
Calibration set				
Range (g.100 g^{-1})	0.70-7.21	0.60-4.80	0.06-3.11	0.08-3.66
Mean (g.100 g^{-1})	3.64	2.04	0.57	0.73
SD[b]			0.40	0.49
n	160	160	132	132
Validation set				
Range (g.100 g^{-1})	1.17-7.18	0.70-3.81	0.07-2.19	0.11-2.60
Mean (g.100 g^{-1})	3.68	1.98	0.51	0.65
n	80	80	65	65
SD[c]	0.90	0.72	0.34	0.41
SEL[d]	0.08	0.03	0.03	0.07

[a]Joubert *et al*. (2006)
[b]Standard deviation of reference data of validation set
[c]Standard deviation of reference data of validation set
[b]Standard error of laboratory of reference data of complete data set

Table 4 NIR spectroscopy validation results for the prediction of mangiferin and hesperidin contents of dried, green *C. genistoides* and mangiferin and xanthone contents of dried, green *C. subternata*

	***C. genistoides*[a]**		***C. subternata***	
	Mangiferin	**Hesperidin**	**Mangiferin**	**Xanthone**
SEP[b] (g.100 g^{-1})	0.46	0.38	0.21	0.27
R^2	0.74	0.72	0.67	0.66
Bias	-0.04	0.02	0.007	0.001
PLS factors[c]	4	6	8	8
RPD[d]	1.96	1.90	1.62	1.52

[a]Joubert *et al*. (2006)
[b]Standard error of prediction
[c]Number of PLS factors
[b]Ratio of stardard error of prediction to standard deviation of validation set



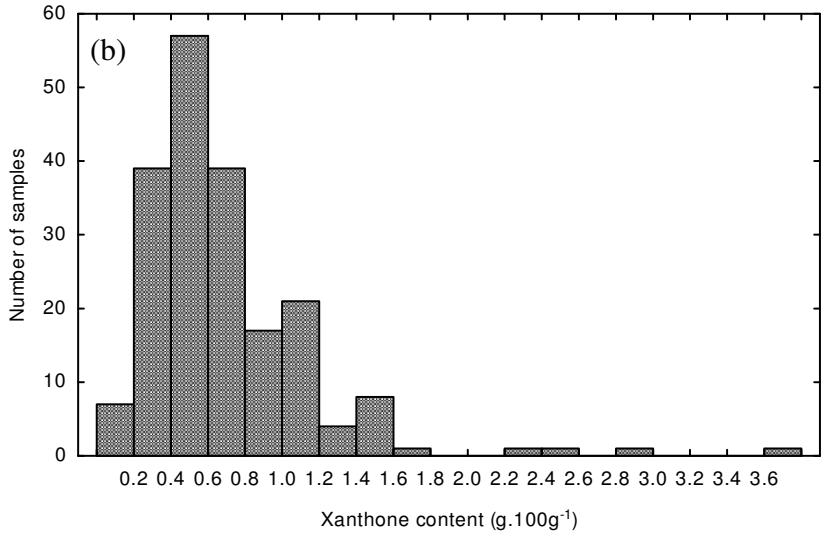


Figure 13 Histogram of the distribution of a) mangiferin and b) xanthone contents of the ground, dried, green *C. subternata* plant material samples (calibration and validation sets combined).

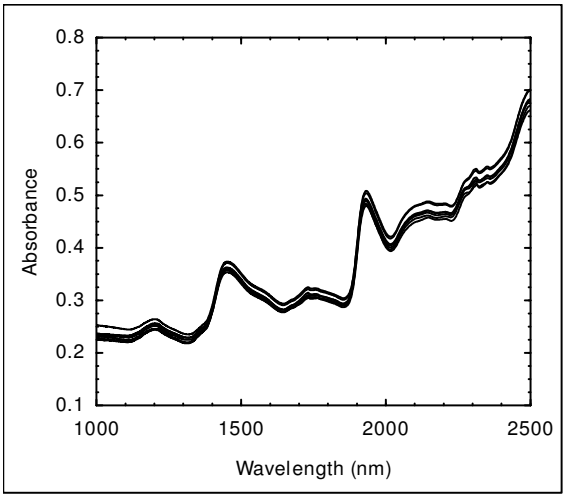


Figure 14 Typical NIR spectra of ground, dried, green *C. subternata* plant material.

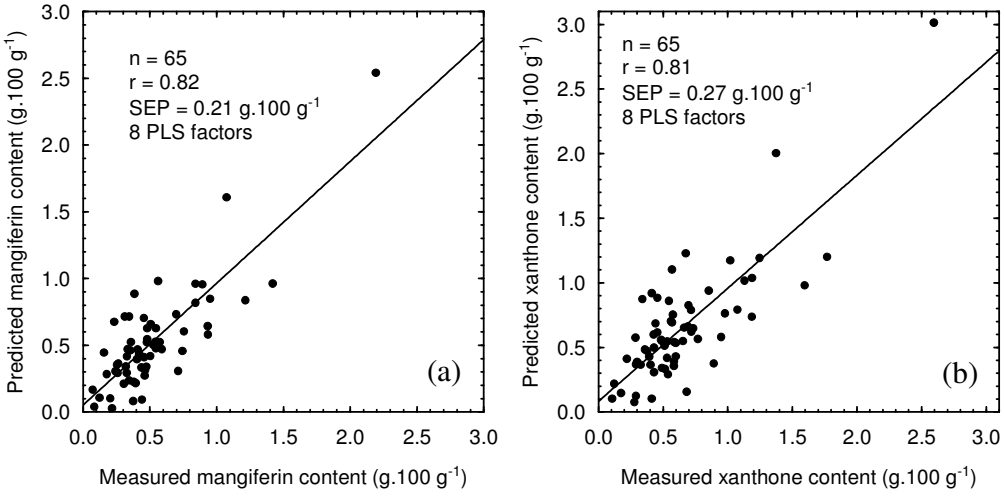


Figure 15 Validation plots of (a) the predicted mangiferin content versus the measured (HPLC) mangiferin content and (b) the predicted xanthone content versus the measured (HPLC) xanthone content for the calibration models for green *C. subternata* plant material.

Discussion

Cyclopia subternata, one of the cultivated *Cyclopia* spp., and thus of commercial importance, is used mainly as a herbal tea. Presently, no quality control standards based on composition exist for *Cyclopia* spp., nor do rapid and/or simple screening methods for *C. subternata* exist should industry wishes to implement such standards. The applicability of the $AlCl_3$ colorimetric assay for determination of the xanthone content (Joubert *et al*., 2007b) and the use of NIR spectroscopy for estimating the mangiferin content (Joubert *et al*., 2006) of *C. genistoides* have been demonstrated. Since *C. subternata* aqueous extracts contain up to eight times less mangiferin than *C. genistoides* (Joubert *et al*., 2007a) and as other compositional differences affect the applicability of assays, application of both the $AlCl_3$ colorimetric assay and NIR spectroscopy needed to be tested and confirmed for *C. subternata.* The low mangiferin content of *C. subternata* also required optimisation of extraction procedures for accurate quantification. The extraction method used, should, however, be easy to apply by industry, i.e. without requiring expensive extraction equipment.

In this study it was shown that extraction of 0.5 and 1 g plant material with hot water not only gave better recovery of SS, TP and xanthones, but that it also affected the TP and xanthone contents of the extracted SS (Figures 1 & 2). A higher mass to volume ratio would result in a faster decline in the concentration gradient between the solid and solvent, thereby slowing mass transfer and thus the rate of extraction (Schwartzberg *et al*., 1982). Since ground (1 mm sieve) plant material was extracted, the diffusion distance was small and not expected to be rate limiting. As none of the extracts were stirred during extraction, movement of the soluble matter from the surface of the plant material particles was thus through natural convection.

The extracts prepared from 0.5 or 1 g plant material gave very small HPLC peak areas for mangiferin and especially isomangiferin. Even if the extracts were injected undiluted, it still hindered accurate quantification. All subsequent extractions were therefore carried out with 5 g plant material to obtain larger peak areas. Improvement of extraction efficiency when using 5 g plant material, required further improvement of extraction procedure. By decreasing solvent polarity, i.e. using 33% acetonitrile, extraction of soluble solids (Figure 3a) did not improve, but better extraction of TP than with water was obtained (Figure 3b).

Considering extraction time, for maximum recovery of total polyphenols, extraction should take place for at least 20 min (Figure 5). The amount of SS recovered remained stable for the extraction periods from 20 to 60 min. For the SS recovery from the plant material to remain stable, while the amount of TP extracted increased at the same time, some unknown soluble matter must have been rapidly solubilised and extracted when the solvent added. This must have been followed by a decline

during the first 20 min of extraction. Li *et al*. (2006) found that at high extraction temperatures pectic polysaccharides are extracted from cell walls which weakened it. The solvent containing water possibly got in contact with the phenolic materials easier and improved the recovery. Wetting of the plant material for solubilisation of solids, followed by swelling and the ”opening up” of the plant matrix that would allow easier movement of soluble solids from the matrix would explain the substantial increase in TP extraction between 10 and 20 min extractions, irrespective of whether water, 33% acetonitrile or 80% ethanol were used. Although recoveries did not improve with longer extraction periods, 30 min was chosen as a safe margin for subsequent extractions.

Standardising on 5 g plant material and 30 min extraction time, extraction efficiency of other solvents was investigated. Water, 33% acetonitrile and 80% ethanol were also included for direct comparison. Overall 100% ethanol gave the poorest extraction of SS, TP, xanthones ($AlCl_3$) and mangiferin from green *C. subternata*. However, it extracted SS with the highest hesperidin and xanthone ($AlCl_3$) contents, as well as the highest mangiferin content. In the latters case 100% methanol, 80% ethanol and 70% acetone were equally effective ($P>0.05$). These results, together with SS containing the lowest TP content, indicated that it selectively extracted polyphenols. The polymeric polyphenols and other polymers would require an extraction solvent with high water content.

Botha (2005) found mangiferin to be slightly less soluble in methanol than in water, while the hesperidin more readily soluble in methanol than in water. Results from the present study showed 100% methanol to be as effective as water in extracting mangiferin ($P>0.05$) and 100% methanol significantly more effective than water in extracting hesperidin. Li *et al*. (2006) found that the presence of water in 72% ethanol aided extraction of polyphenols.

When investigating solvent efficacy, 33% acetonitrile was more effective than 80% ethanol in extracting SS and TP from *C. subternata*, but contrary to the results obtained from experiment evaluating extraction time, it also gave higher recovery of SS than water. Best extraction of xanthones and mangiferin from the plant material was obtained with 70% acetone. Since acetone leads to poor HPLC peak shape, it would require additional preparation steps before analysis of the extract, i.e. evaporating and redissolving of the residue in another solvent. For that reason 33% acetonitrile would be the preferred solvent.

When choosing a solvent for production of extracts, other considerations than those considered when optimising a procedure for an analytical method, are important. Industry would also require high soluble solids yield and/or selectivity for certain compounds depending on the final application of the extract. Total polyphenol and antioxidant activity are presently the only quality criteria. Ethanol (50%)

would be a better solvent than hot water, currently used by industry, to ensure good recovery of TP, xanthones ($AlCl_3$) and mangiferin from green *C. subternata*.

The extraction solvents should be used according to good manufacturing practices (GMP) and the resultant residues or derivatives which could subsequently be present in "technically unavoidable" quantities should present no danger to human health (Anon., 1991). The above-mentioned rule applies to extraction with, i.e. ethanol and acetone. For extraction with other solvents, conditions of use have been specified for maximum residue limits in extracted foodstuffs of food ingredients. The United States Food and Drug Administration also has a "Guidance for Industry" list for the appropriate use of residual solvents (Anon., 2006). The solvents are divided into three classes: class1 are solvents which are known to cause unacceptable toxicities and should be avoided; class 2 are solvents associated with less severe toxicity; and solvents of class 3, which are the least toxic solvents, should be used where practical. Acetonitrile, falls under class 2, with a permitted daily exposure (PDE) of 0.1 $mg.day^{-1}$ (corresponding to 10 ppm). Class 3 solvents, i.e. ethanol, have low toxic potential to humans and a PDE of 50 mg or more per day (corresponding 5000 $mg.kg^{-1}$). There are, however, no long-term toxicity or carcinogenicity studies for many of the solvents in class 3.

In spite of 33% acetonitrile resulting in a significantly more efficient extraction compared to water a good correlation was still obtained between the mangiferin, xanthone and SS contents extracted with acetonitrile and water, respectively.

The moderate correlation observed between TAA and TP content of water extracts was in agreement with results obtained with other plant materials (Cai, *et al*., 2004; Gorinstein *et al*., 2004; Soong & Barlow, 2004; Djeridane *et al*., 2005; Joubert *et al*., 2007). In contrast Dasgupta & De (2007) observed no correlation between antioxidant activity and the total polyphenol content of leafy vegetables. Richards (2002) found a good correlation between the TAA and the TP content ($r = 0.98$) of *Cyclopia* spp. determined in water extracts.

The water extracts resulted in good correlation between the mangiferin (Figure 10a) and xanthone contents (Figure 11a) quantified by HPLC and the xanthone content determined using $AlCl_3$ colorimetric method. The corresponding correlations observed for the acetonitrile extracts where slightly worse (Figures Figures 10b & 11b). Mangiferin values (0.223 to 3.69 $g.100\ g^{-1}$) estimated by $AlCl_3$ were higher than those quantified by HPLC (0.023 to 2.7 $g.100\ g^{-1}$). The overestimation could also be due to compounds such as hesperidin (0.001 to 1.03 $g.100\ g^{-1}$) for water extracts and (0.05 to 0.69 $g.100\ g^{-1}$) for acetonitrile-water extracts. It has a low reactivity towards $AlCl_3$, but it might still make a small contribution when present in higher quantities in extracts (Joubert *et al*., 2007b). Another contribution towards the xanthone content could have been due to isomangiferin, another xanthone also

present in green *C. subternata* (Brandt, 2004). Isomangiferin was quantified in this study and was found to be present in quantities of 0.02 to 0.74 g.100 g^{-1} for water extracts and 0.01 to 0.06 g.100 g^{-1} for 33% acetonitrile extracts. Eriodyctiol and luteolin, which possess a C-4 keto and a C-5 hydroxyl group or C-6 and C-7 hydroxyl groups, give strong absorbsances (Chang *et al*., 2002) and although present in very low quantities (Van der Merwe, 2005) could make a small contribution.

The correlation between TAA and mangiferin of the *C. subternata* water extracts was lower (r = 0.53) than that observed (r = 0.75) by Joubert *et al*. (2007b) for *C. genistoides*. It should be taken into consideration that the mangiferin content in *C. subternata* is lower than that of other *Cyclopia* spp. with a contribution of *ca*. 12% (Richards, 2002) to the ABTS radical scavenging activity. The lower mangiferin content and lower TAA activity of the water extracts could therefore have contributed to the lower correlation.

The addition of isomangiferin and hesperidin to the mangiferin content did not improve the correlation (r =0.59). This could be attributed to the fact that mangiferin displays a moderate activity in the ABTS assay (Richards, 2002) and is highly reactive in comparison to hesperidin which contributed only *ca.* 0.3% of the ABTS radical scavenging ability.

The calibration model for mangiferin content was weaker in comparison to that obtained by Joubert *et al*. (2006). The latter study used a slightly larger sample set (n = 240) compared to that in this study (n = 197). Although smaller SEP values were obtained in comparison to Joubert *et al*. (2006) the larger RPD values obtained in the latter study suggested more robust calibration models. The smaller range in mangiferin content (0.06-3.11 g.100 g^{-1}) of the *C. subernata* sample set compared to that (0.70-7.21 g.100 g^{-1}) of the *C. genistoides* sample set (Joubert *et al*., 2006) resulted in a less robust calibration and subsequently the prediction ability of the model was impeded. The similarity in results obtained for the prediction of mangiferin and xanthone content might be due to the solubility of these compounds. Both compounds are xanthones and react similarly in solvents. However, isomangiferin is less abundant than mangiferin and could have slightly affected the accuracy of the calibration.

Conclusions

Extractions prepared on 5 g plant material for 30 min was found to be the most appropriate extraction procedure to be used to accurately quantify the mangiferin and xanthone contents. Best extraction of xanthones and mangiferin from the plant material was obtained with 70% acetone, but 33% acetonitrile was found to be the preferred solvent for accurate HPLC analysis. Extraction procedures and solvents to be used for industrial production of extracts would, however, require different procedures that would result in high soluble solids yield and/or selectivity for certain compounds depending on the final

application of the extracts. Better correlations was observed between the mangiferin and xanthone contents quantified by HPLC and $AlCl_3$ colorimetric method, respectively, as determined in water extracts than in acetonitrile extracts. The correlation between TAA and mangiferin of the *C. subternata* water extracts was lower than that observed for *C. genistoides* due to its lower mangiferin content and its lower contribution to the ABTS radical scavenging activity.

NIR spectroscopy calibration models can only be used for screening purposes. Although the isomangiferin content is present in much smaller quantities than mangiferin, both contributed towards the xanthone content and accuracy of the calibration models. Improvement of the calibration model could be achieved by adding more samples with higher mangiferin content to give a more even distribution of the mangiferin and xanthone contents.

References

Andersen, M.L., Lauridsen, R.K. & Skibsted, L.H. (2005). Power of phenolic compounds. *Functional Foods and Nutraceuticals*, **March 2005**, 44-48.

Anonymous (2000). Agricultural Product Standards Act. Act no. 119 of 1990, Regulation no. 1177 of 24 November 2000.

Anonymous (1991). European Communities (Extraction solvents in Foodstuffs) Regulations. S.I. No. 334/1991: [URL document]. http://irishstatutebook.ie.ZZSI334Y1991.html

Anonymous (2006). Unites States Food and Drug Administration. Guidance for Industry. Q3C Impurities: residual solvents. [URL document]. http://fda.gov/cder/guidance/index.htm

Banjeri, A., Deshpande, A.D., Prabhu, B.R. & Pradhan, P. (1994). Tomentonone, a new xanthonoid from the stem bark of *Calophyllum Tomentosum*. *Journal of Natural Products*, **57**, 396-399.

Botha, M. (2005). Use of near infrared spectroscopy (NIRS) and spectrophotometric methods in quality control of green rooibos (*Aspalatus linearis*) and honeybush (*Cyclopia genistoides*). MSc in Food Science Thesis, Stellenbosch University, South Africa.

Brandt, E.V. (2004). Identification of naturally occurring phenolic substances in the indigenous honeybush tea plant. Project 282006 Progress Report. ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa.

Cai, Y., Lou, Q., Sun, M. & Corke, H. (2004). Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sciences*, **74**, 2157-2184.

Chang, C.C. Yang, M.H., Wen, H.M. & Chern, J.C. (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *Journal of Food and Drug Analysis*, **10**, 178-182.

Dasgupta, N. & De, B. (2007). Antioxidant activity of some leafy vegetables of India: a comparative study. *Food Chemistry*, **101**, 471-474.

Davies, K.J.A. (1995). Oxidative stress: the paradox of aerobic life. In: *Free Radicals and Oxidative Stress: Environmental, Drugs and Food Additives* (edited by C. Rice-Evans, B. Halliwell, & G.G. Hunt). p.1. London: Portland Press.

Day, M.S. & Fearn, F.R.B. (1982). Near infra-red reflectance as an analytical technique, Part 1. History and development. *Laboratory Practice*, **31**, 328-330.

Djeridane, A. Yousfi, M., Nadjimi, B. Boutassouna, D. & Stocker, P. & Vidal, N. (2005). Antioxidant activity of some Algerian medicinal plants extracts containing phenolic compounds. *Journal of Food Chemistry*, **97**, 654-660.

El-Shafae, A.M. & El-Domiatry, M.M. (2001). Improved LC methods for the determination of diosmin and/or hesperidin in plant extracts and pharmaceutical formulations. *Journal of Pharmaceutical and Biomedical Analysis,* **26**, 539-545.

Esmelindro, M.C., Toniazzo, G., Lopes, D., Oliveira, D. & Dariva, C. (2005). Effects of processing conditions on the chemical distribution of mate tea leaves extracts obtained from CO_2 extraction at high pressures. *Journal of Food Engineering*, **70**, 588-592.

Gorinstein, S., Cvikrová, Machackova, I., Haruenkit, R., Park, Y.-S., Jung, S.-T., Yamamoto, K., Ayala, A.L.M, Katrich, E. & Trakhtenberg, S. (2004). Characterisation of antioxidant compounds in Jaffa sweeties and white grapefruits. *Food Chemistry*, **84**, 503-510.

Guardia, T., Rotelli, A.E., Juarez, A.O., Pelzer, L.E. (2001). Anti-inflammatory properties of plant flavonoids. Effects of rutin, quercetin and hesperidin on adjuvant arthritis in rats. *Farmaco*, **56**, 683-687.

Guillerault, L., Ollivier, E., Elias, R. & Balansard, G. (1994). Determination of harpagoside, 8-paracoumaroyl harpagide and harpagoside by high performance liquid chromatography in *Harpaophytum procumbens* drugs and in a commercial extract. *Journal of Liquid Chromatography*, **17**, 2951-2960.

He, X.G. (2000). On-line identification of phytochemical constituents in botanical extracts by combined techniques. *Journal of Chromatography A*, **880**, 203-232.

Heinonen, I.M., Lehtonen, P.J. & Hopia, A.I. (1998). Antioxidant activity of berry and fruit wines and liquors. *Journal of Agricultural and Food Chemistry*, **46**, 25-31.

Huang, D., Ou, B. & Prior, R.L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry*, **53**, 1841-1856.

Hubbe, M.E. (2000). Evaluation of antioxidant and free radical scavenging activities of honeybush tea (*Cyclopia)*. MSc in Food Science Thesis, Stellenbosch University, South Africa.

Huie, C.W. (2002). A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants. *Analytical and Bioanalytical Chemistry*, **373**, 23-30.

Joubert, E. (1988). Effect of batch extraction conditions on yield of soluble solids from rooibos tea. *International Journal of Food Science and Technology*, **23**, 43-47.

Joubert, E. (1990). Effect of batch extraction conditions on extraction of polyphenols from rooibos tea (*Aspalathus linearis*). *International Journal of Food Science and Technology*, **25**, 339-343.

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

Joubert, E., Manley, M., Gray, B.R. & Schulz, H. (2005). Rapid measurement and evaluation of the effect of drying conditions on harpagoside content in *Harpagophytum procumbens* (Devil's Claw) root. *Journal of Agricultural and Food Chemistry*, **53**, 3493-3502.

Joubert, E., Manley, M. & Botha, M. (2006). Use of NIRS for the quantification of mangiferin and hesperidin contents from dried green honeybush (*Cyclopia genistoides*) plant material. *Journal of Agricultural and Food Chemistry*, **54**, 5279-5283.

Joubert, E., Richards, E.S., Van der Merwe, J.D., De Beer, D., Manley, M. & Gelderblom, W.C.A. (2007a). Effect of species variation and processing on phenolic composition and in vitro antioxidant activity of aqueous extracts of *Cyclopia* spp. (honeybush tea). *Journal of Agricultural and Food Chemistry (*in print*)*

Joubert, E., Manley, M. & Botha, M. (2007b). Evaluation of spectrophotometric methods for screening of green rooibos (*Aspalathus linearis*) and green honeybush (*Cyclopia genistoides*) extracts for high levels of bio-active compounds. *Phytochemical Analysis,* in print.

Jurd, L. (1962). Spectral properties of flavonoid compounds. In: *The Chemistry of Flavonoid Compounds* (edited by T.A. Geissman). Pp 107-155. Oxford, UK: Pergamon Press.

Laasonen, M. (2003). Near infrared spectroscopy, a quality control tool for the different steps in the manufacture of herbal medicinal products. Msc Thesis, University of Helsinki, Finland.

Liang, Y.Z., Xie, P. & Chan, K. (2004). Quality control of herbal medicines. *Journal of Chromatography B,* **812**, 53-70.

Li, B.B., Smith, B. & Hossain, M.Md. (2006). Extraction of phenolics from citrus peels. *Separation and Purification Technology*, **48**, 182-188.

Luximon-Ramma, A., Bahorun, T., Soobrattee, M.A. & Aruoma, O.I. (2002). Antioxidant activities of phenolic, proanthocyanidin, and flavonoid components in extracts of *Cassia fistula*. *Journal of Agricultural and Food Chemistry*, **50**, 5042-5047.

Mabry, T.J., Markham, K.R. & Thomas, M.B. (1970). The systematic identification of flavonoids. Pp. 41-61. New York, USA: Springer-Verlag.

Manley, M., Joubert, E. & Botha, M. (2006). Quantification of the major phenolic compounds, soluble solid content and total antioxidant activity of green rooibos (*Aspalathus linearis*) by means of near infrared spectroscopy. *Journal of Near Infrared Spectroscopy*, **14**, 213-222.

Marnewick, J.L., Gelderblom, W.C.A. & Joubert, E. (2000). An investigation on the antimutagenic properties of South African herbal teas. *Mutation Research*, **471**, 157-166.

Melton, L. (2006). The antioxidant myth. *New Scientist*, **8**, 40-43.

Moure, A., Cruz, J.M. Franco, D. Dominguez, J.M., Sineiro, J., Dominguez, H., Núñez, M.J. & Parajo, J.C. (2001). Natural antioxidants from residual sources. *Food Chemistry*, **72**, 145-171.

Peres, V., Nagem, T.J. & De Oliveira, F.F. (2000). Tetraoxygenated naturally occurring xanthones. *Phytochemistry*, **55**, 683-710.

Peschel, W., Sánchez-Rabaneda, F. Diekmann, W., Plescher, A., Gartzía, I., Jiménez, D., Lamuela-Raventós, R., Buxaderas, S. & Codina, C. (2006). An industrial approach in the search of natural antioxidants from vegetable and fruit wastes. *Food Chemistry*, **97**, 137-150.

Prior, R.L., Wu, X. & Schaich, K. (2005). Standardization methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry*, **53**, 4290-4302.

Rager, I., Roos, G., Schmidt, P.C. & Kovar, K.-A. (2002). Rapid quantification of constituents in St. John's wort extracts by NIR spectroscopy. *Journal of Pharmaceutical and Biomedical Analysis*, **28**, 439-446.

Re, R., Pellegrini, N., Proteggente, A., Yang, M. & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, **26**, 1231-1237.

Richards, E.S. (2002). The antioxidant and antimutagenic activities of *Cyclopia* species and activity-guided fractionation of *C. intermedia*. MSc in Food Science Thesis, Stellenbosch University, South Africa.

Schulz, H. (2004). Analysis of coffee, tea, cocoa, tobacco, spices, medicinal and aromatic plants, and related products. In: Near-infrared Spectroscopy in Agriculture (edited by C. Roberts, J. Workman & J. Reeves). Pp. 345-376. Madison, USA: American Society of Agronomy, Crop Science of

America and Soil Science of America.

Schulz, H., Pfeffer, S., Quilitzsch, R., Steuer, B. & Reif, K. (2002). Rapid and nondestructive determination of the echinacoside content in *Echinacea* root by ATR-IR and NIR spectroscopy. *Planta Medica*, **68**, 926-929.

Schwartzberg, H.G. & Chao, R.Y. (1982). Solute diffusivities in leaching processes. *Food Technology*, **36** (2), 73-86.

Simova, M., Tomov, E., Pangarova, T. & Pavlova, N. (1986). Determination of phenolic compounds in *Colladonia triqeutra* L. *Journal of chromatography*, **351**, 379-382.

Singleton, V.L. & Rossi, Jr., J.A. (1965). Colorimetry of total phenolics with phosphomolybdicphosphotungstic acid reagent. *American Journal of Enology and Viticulture*, **16**, 144-158.

Sloan, A.E. (2002). The top 10 functional food trends: the next generation. *Food Technology*, **56**, 32-56.

Soong, Y.-Y. & Barlow, P.J. (2004). Antioxidant activity and phenolic content of selected fruit seeds. *Food Chemistry*, **88**, 411-417.

Van der Merwe, J.D. (2005). A comparative study on the protection of *Cyclopia spp*. (honeybush), *Aspalathus linearis* (rooibos) and *Camellia sinensis* tea against aflatoxin B1 induced mutagenesis in the Salmonella mutagenicity assay: Possible mechanisms involved. MSc in Food Science Thesis, Stellenbosch University, South Africa.

Waterman P. & Mole, S. (1993). Extraction and chemical quantification. In: *Analysis of Phenolic Plant Metabolites*. Pp. 66-74. Oxford, UK: Blackwell Scientific Publications.

Williams, P.C. (2001). Implementation of near-infrared technology. In: *Near-infrared technology in the Agricultural and Food Industries*, 2nd ed. (edited by P. Williams & K. Norris). Pp. 145-169. St Paul, USA: American Association of Cereal Chemists.

Wills, R.B.H. & Stuart, D.L. (2004). Generation of High Quality Australian Skullcap Products. In: A report for the Rural Industries Research and Development Corporation. RIRDC Publication no 04/020.

Wright, R. (2005). Antioxidant avalanche. *Nutraceutical World*, **March 2005**, 46, 48, 50, 52, 54, 56-57.

CHAPTER 4

EFFECT OF PRE-DRYING TREATMENTS AND STORAGE ON THE COLOUR AS WELL AS MANGIFERIN AND HESPERIDIN CONTENTS OF GREEN HONEYBUSH (*CYCLOPIA SUBTERNATA*)

Abstract

The effect of various pre-drying treatments and storage temperatures on the colour, soluble solids (SS), total polyphenol (TP), mangiferin and hesperidin contents of green *Cyclopia subternata* was investigated. The treatments comprised drying of intact leaves (control), maceration + drying (T2), steaming + maceration + drying (T3), and maceration + steaming + drying (T4). All treatments significantly ($P>0.05$) affected the colour parameters (L*, a*, b*, chroma and hue) compared to the control. T3 retained the green colour of the leaves the best ($P<0.05$) compared to T4 and T2. The latter treatment was also found to significantly ($P<0.05$) reduce the SS and TP contents of the leaves. The mangiferin and hesperidin contents were, however, not affected ($P>0.05$). Delaying the steam treatment after maceration for 20 min aggravated the detrimental colour changes ($P<0.05$), but it had no effect on the SS, TP, mangiferin and hesperidin contents. Steaming before maceration as opposed to maceration without steaming was found to decrease the rate of colour change over 60 min after treatment ($P<0.05$). Storage of control and T3 samples for 6 months at 0°C and 30°C did not significantly affect the SS, mangiferin or hesperidin contents of the leaves ($P>0.05$). Storage at 30°C, irrespective of pre-treatment, resulted in detrimental colour changes.

Introduction

Traditional processing of honeybush involves "high temperature fermentation", i.e. chemical oxidation necessary for browning and formation of the sweet flavour of honeybush tea (Du Toit & Joubert, 1999). This process is accompanied by reduction of water soluble solids (SS), total polyphenol (TP) (Du Toit & Joubert, 1999; Joubert *et al*., 2007), mangiferin and hesperidin contents of honeybush (Joubert *et al*., 2007). With the growing interest of consumers in health-promoting foods and beverages, manufacture of green honeybush tea for the herbal tea market became a reality. Green honeybush is also preferred for preparation of extracts for the food, nutraceutical and cosmetic industries, since it has greater antioxidant (Hubbe, 2000; Joubert *et al*., 2007), antimutagenic (Van der Merwe *et al*., 2006) and phytoestrogenic properties (Verhoog *et al*., 2007).

Retention of the green colour of honeybush is important, because it is an indication that minimum compositional changes take place during its manufacture, i.e. shredding and drying. Steaming, followed

by vacuum drying, was one of the processes proposed to prevent oxidative changes and thus loss of bioactive compounds (De Beer & Joubert, 2002). Due to the cost of vacuum drying, the plant material is in most cases only shredded and dried without any attempt to prevent degradation reactions that could take place. However, one processor does employ steaming directly after shredding (E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, personal communication, 2005).

Discolouration of the leaves is initiated when the tea shoots are shredded into small pieces of less than 4 mm. The cut surfaces are exposed to oxygen and disruption of the organised structures within a cell that separates enzymes and substrates, occur. Phenolic oxidation (Martinez & Whitaker, 1995) and enzymatic chlorophyll degradation, leading to pheophorbide (olive brown) formation (Ihl *et al*., 1998), could be expected to occur. Shredded leaves of *C. subternata* rapidly change to a greenish-brown colour. However, it is not known what compositional changes and to what extent they take place in the interim period between shredding and drying. Incorrect storage conditions could also be detrimental for colour retention. Factors such as storage temperature (Sigge *et al*., 2001; Pandrangi & LaBorde, 2004), water activity of the dried plant material (Lajollo *et al*., 1971) and light (Mangos & Berger, 1997) plays a role in retention of green colour. To date, no long-term storage experiments to determine the stability of honeybush have been carried out.

The objectives of this study were to investigate the effect of different pre-drying treatments and storage temperatures on SS, TP, mangiferin and hesperidin contents, as well as the effect of the pretreatments on colour retention of dried, green *C. subternata*.

Materials and methods

Chemicals

Chemicals used were as described in Chapter 3 (p. 81).

Plant material

Cyclopia subternata shoots were harvested on three occasions, i.e. July 2005, February 2006 and September 2006 from a commercial plantation established in 2001 at Kanetberg in the Barrydale district. Upon arrival at ARC Infruitec-Nietvoorbij, Stellenbosch, the plant material was immediately placed in cold storage (<10°C) until used. The period of cold storage depended on the duration of each experiment. Plant material was also harvested in July 2005 at Helderfontein, the experimental farm of the ARC Infruitec-Nietvoorbij in Stellenbosch.

Effect of pre-drying treatments

Leaves from approximately 1.8 kg of shoots were manually stripped from the stems and mixed thoroughly to obtain a homogenous sample. This sample was subsequently divided into 4 sub-samples of *ca*. 200 g each and each sub-sample was used for a specific treatment. The treatments comprised (a) drying of the intact leaves, which were spread open on 30 mesh stainless steel mesh trays (370 x 310 mm), at 40ºC and at 30% relative humidity (RH) in a purpose built dehydrator (Sigge *et al*., 1999) for 4 h to a moisture content of *ca*. 10% (T1, which served as the control); (b) maceration in a Waring blender for 20 s (a handful of the leaves at a time), followed by drying at 40°C/30% RH (T2); (c) steaming of the leaves for 60 s at >90ºC, followed by maceration and drying (T3); and (d) maceration, followed by steaming and drying (T4) (Table 1). During steaming the leaves were spread open in a thin layer on the stainless steel mesh trays and placed in a cabinet-type steam blancher. The trays were placed in the preheated dehydrator as soon as possible after steaming.

The experiment was repeated seven times; 4 batches of plant material from Kanetberg (harvested Jul 2005) and 3 batches of plant material from Helderfontein. Colour measurements were done on T2, T3 and T4 samples after completion of treatment (M0). Samples from T1 were not subjected to colour measurement at this stage as its physical form (intact leaves) was different to the others. The samples were subsequently stored for a month in plastic air tight containers at room temperature (25ºC) in the dark, before colour measurements were repeated on T2, T3 and T4 samples (M1). The samples, including those of T1, were then milled with a Retsch mill (1mm sieve) to a fine powder. Colour measurements were taken again, including the samples from T1, where after the samples were stored in plastic containers for another month at 0°C before analysis (SS, TP and HPLC analysis).

Table 1 Summary of treatments and the order in which the processing steps were performed

Treatment	**Processing steps**[a]			
	Macerated	**Dried intact**	**Steamed**	**Dried**
T1[b]		1		
T2[b]	1			2
T3[b]	2		1	3
T4[b]	1		2	3

[a]1, 2, 3: Order of processing steps performed
[b]T1: Intact leaves dried at 40ºC/30% RH (control)
[b]T2: Leaves macerated and dried
[b]T3: Leaves steamed for 60 s at >90ºC, macerated and dried
[b]T4: Leaves macerated, steamed without delay and dried

Effect of delayed steaming between maceration and drying

The plant material was prepared for maceration as described earlier. Approximately 200 g of leaves was macerated for 20 s with a Waring blender (a handful of leaves was macerated at a time), immediately divided into two sub-samples and respectively treated as follows: (a) steaming without delay for 60 s at >90ºC, followed by drying at 40°C/30% RH (T4/0); and (b) delayed steaming, i.e. the macerated leaves were allowed to stand for 20 min before it was steamed and dried (T4/20).

The experiment was repeated 5 times on each of two consecutive days, giving 10 replicates in total. Colour measurements of the dried, macerated leaves were taken at completion of the experiment. Samples were stored for one month and then milled (Retsch mill, 1 mm sieve) for re-evaluation of colour and compositional analysis.

Effect of steaming on colour change of macerated fresh leaves over time

The plant material was prepared for maceration as described earlier. No steaming and drying took place after maceration (T2/M). Colour measurements of the moist macerated leaves commenced without delay over a period of 60 min. Measurements were taken every 2 min for the first 30 min, where after measurements were taken every 15 min. For the second treatment (T3/M), *ca.* 200 g leaves were first steamed for 60 s at >90º C before maceration. Colour measurements were carried out as for T2/W. The experiment was repeated in triplicate on two consecutive days, giving 6 replicates in total.

Effect of storage temperature

The leaves, stripped of *ca*. 1.8 kg shoots and mixed thoroughly, were divided into two sub-samples. The leaves of the first sub-sample were dried intact at 40˚C/30% RH (T1/S). The intact leaves of the second sub-sample were steamed for 60 s at >90˚C. The leaves were macerated, a handful at a time, with a Waring blender for *ca*. 20 s, before drying at 40˚C/30% RH (T3/S). The experiment was repeated 3 times. Samples were milled into a fine powder (Retsch mill, 1 mm sieve) and glass jars were filled with the ground plant material of both treatments (T1/S and T3/S).

Samples of each treatment were stored for a period of 6 months at 0˚C and at 30˚C in the dark in temperature controlled rooms of which colour measurements were performed when storage commenced (0 months) and after 1, 2, 3 and 6 months, respectively. Jars for each time point x storage temperature x treatment x replicate were prepared. The colour of the control sample was measured when storage commenced. After colour measurement the samples were stored in a freezer room at -20°C until HPLC analysis. Colour measurements were subsequently performed following 1, 2, 3 and 6

months of storage, where after they were stored at -20°C until completion of the storage experiment and HPLC analysis of all samples could be carried out.

Preparation of acetonitrile-water extracts

Acetonitrile-water extracts were prepared from milled green honeybush (*C. subternata*) using the procedure (*ca*. 5 g plant material extracted with 33% acetonitrile-water on a steam bath for 30 minutes) previously described in Chapter 3 (p. 82).

Soluble solids (SS) content of C. subternata extracts

The SS content of the acetonitrile-water extracts were determined gravimetrically as described in Chapter 3 (p. 84). Fifteen ml of each extract was evaporated in duplicate. Results were expressed on a dry basis.

Quantification of mangiferin and hesperidin by HPLC analysis

Mangiferin and hesperidin contents of the *C. subternata* ectracts were determined by HPLC according to the procedure as described in Chapter 3 (p. 84). Results were expressed on a dry basis.

Total polyphenol (TP) content of C. subternata acetonitrile-water extracts

The TP content of *C. subternata* acetonitrile-water extracts was determined as described in Chapter 3 (p. 85). Results were expressed on a dry basis.

Moisture content

The same procedure was followed as described in Chapter 3 (p. 84).

Colour measurement of honeybush leaves

CIELAB colour parameters, L*, a* and b*, were measured with a Colorgard 2000/05 (BYK-Gardner, Geretsried, Germany) in reflectance mode. Calibration was done with a black zero reference standard (Gardner No. 05-1528) and a white tile (L* = 94.53; a* = -0.99; b* = 0.92) supplied by Gardner. Calibration was done with a quartz plate in place to compensate for the quartz sample cup. The CIE L*a*b* scale was used with the CIE 1931 standard colorimetric observer under illuminant C (geometry is 45° illumination and 0° viewing). Chroma and hue angle (°) were calculated as follows (Hunt, 1977):

$$\text{Chroma} = \left[(a^*)^2 + (b^*)^2\right]^{1/2} \quad (1)$$

$$\text{Hue angle} = \tan^{-1}(b^*/a^*) \quad (2)$$

Negative values of the hue angle were converted to positive values by adding 180° so that it could fall in the 90° to 180° quadrant (+b* = yellow; -a* = green). In the text it will be referred to as hue. The quartz sample cup was filled ¾, a black cover was placed over the sample cup to exclude any external light and measurements were done from the bottom by illuminating a 10 mm diameter area. The colour of milled samples was measured once, while the colour of the macerated (not milled) samples was measured five times, taking into account the small area of illumination for colour measurement. Before each consecutive measurement, the macerated dried leaves were poured out, mixed and the sample cup refilled to ensure that good average colour values for a pretreatment could be obtained.

Colour measurements of the moist leaves of pretreatments T2/M and T3/M done over 60 min, required the sample cup to be removed from the sensor between measurements to prevent gradual heating of the sample and acceleration of degradation reactions. To ensure that the same sample area was illuminated again, the sample cup was positioned with the mark on the cup aligned with the one on the sensor. All measurements of a pre-treatment x replicate were completed before preparation and colour measurement of the next sample commenced.

Statistical analysis

Analysis of variance was used to determine whether the difference between treatment means was significant. In cases where significant ($P<0.05$) differences were found, LSD of the Students t-test ($P=0.05$) was calculated to compare treatment means. Data were tested for normality using Shapiro-Wilk procedure. Colour and compositional data for the storage experiment were fitted to the models, $y = a + bLn(x + 1)$ and $y = a + bx$, respectively to calculate the rate of change during storage. The SAS software package version 9.1 (SAS Institute Inc., Cary, NC) was used for all statistical analyses and curve fitting.

Results

Effect of pre-drying treatments

Treatment was found to have a significant effect ($P<0.05$) on a* (+a* indicating redness; -a* indicating greenness) (Figure 1). The visual inspection indicated T2 to be slightly brown-green and T3 to retain the green colour the best. A positive a* was obtained for T2, while T3 was found to possess the most

negative a* value. Significant differences (P<0.05) were also found for b* and chroma values between T2 and T3, and T2 and T4. T3 and T4 did not differ significantly (P>0.05). Hue180 was also significantly affected by treatment with the lowest hue value for T2, and the highest for T3.

Figure 2 depicts the colour data for the samples of T2, T3 and T4 before (M0) and after 1 month storage (M1) at room temperature in the dark. Only b* and chroma showed significant differences (P<0.05) as a result of the one-month-storage period (Table 2).

Milling of the samples changed all colour data parameters significantly (P<0.05). The data averaged over treatment is given in Table 3. Milling not only increased the absolute a* values for T2, T3 and T4, but +a* (positive) for T2 and T4 changed to -a* (negative) (Figure 3). For all other colour parameters milling increased the values obtained for the individual treatments (data not shown). Even after averaging the data of the samples obtained before and after milling T3 had the most negative a* and the highest hue of T2, T3 and T4 (Table 4). Figure 4 depicts the colour data of T1, T2, T3 and T4 for the milled dried leaves. Significantly higher values for L*, b* and chroma (P<0.05) were obtained for T1. In the case of a* and hue T1 was only significantly different from T2.

Significantly lower SS and TP contents of the leaves were obtained for T2 (P<0.05) (Table 5). However no significant differences (P>0.05) were noted for the mangiferin and hesperidin contents of the leaves and SS, as well as the TP content of the SS.

Table 2 Effect of treatment on the CIELAB colour values of dried *C. subternata* leaves before (M0) and after 1 month storage (M1) at room temperature (results are the averages for T2, T3 and T4)

Month	**Colour Parameter**[a]				
	L*	**a***	**b***	**Chroma**	**Hue180**
M0[b]	33.2a	-0.5 a	16.5 b	16.6b	89.9 a
M1[c]	33.3 a	-0.02 a	17.7 a	17.8a	91.4 a

[a]Means in a column with the same letter are not significantly different (P>0.05) (colour measurements done on macerated, but not yet milled leaves)
[b]Leaves after treatment and no storage
[c]Leaves after treament and 1 month storage at room temperature

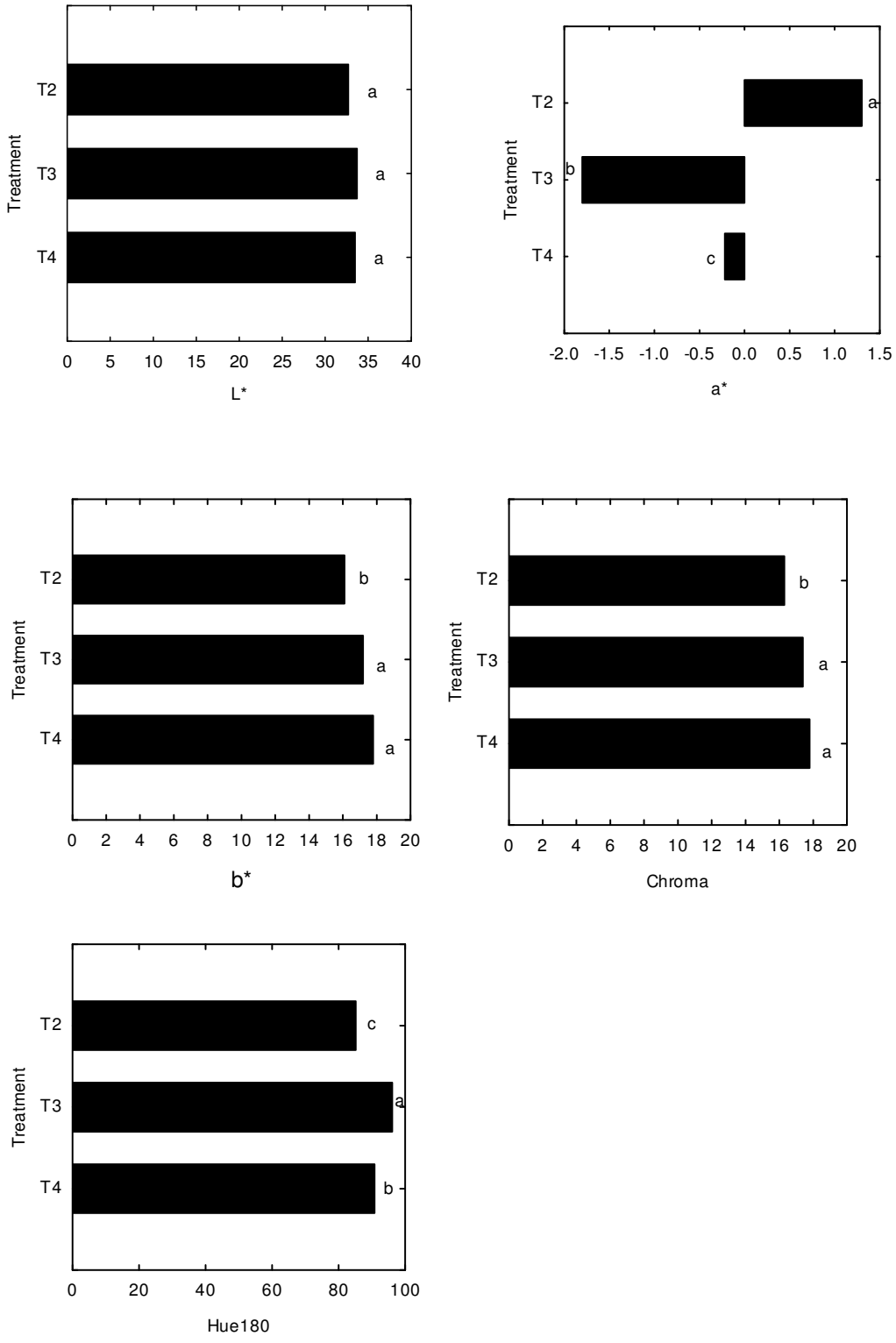


Figure 1 Effect of treatment on CIELAB colour values. Details of treatments are given in Table 1. Leaves were only macerated and not yet milled.

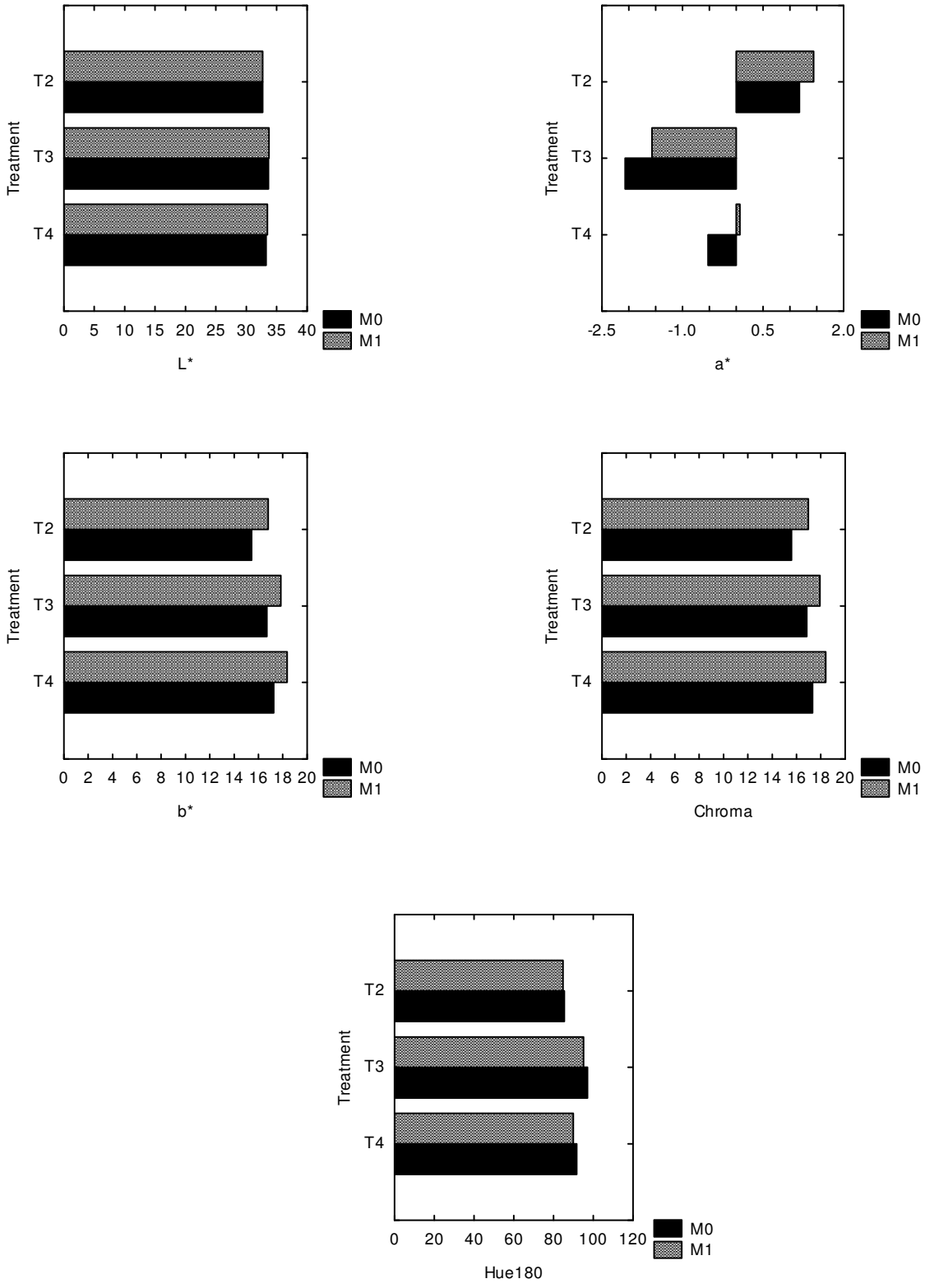


Figure 2 CIELAB colour values for treatments T2, T3 and T4 before (M0) and after 1 month (M1) storage at room temperature. Details of treatments are given in Table 1. Leaves were only macerated and not yet milled.

Table 3 Effect of treatment on CIELAB colour values of dried *C. subternata* after one month storage at room temperature (results are the averages for T2, T3 and T4)

Treatment	Colour Parameter[a]				
	L*	**a***	**b***	**Chroma**	**Hue180**
Macerated[b]	33.3 b[b]	-0.01 a	17.7 b	17.8 b	89.9 b
Milled[c]	49.2 a[a]	-4.4 b	27.7 a	28.1 a	98.8 a

[a]Means in a column with the same letter are not significantly different (P>0.05)
[b]Leaves were macerated, but not milled
[c]Leaves were macerated and milled

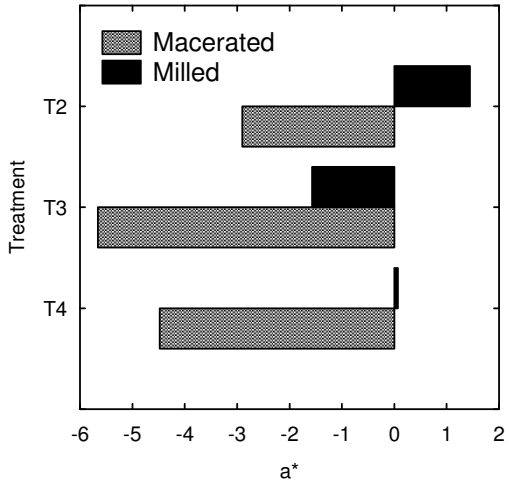


Figure 3 CIELAB colour value (a*) for macerated, but not yet milled, as well as for milled leaves of treatments T2, T3 and T4 after storage at room temperature for one month. Details of treatments are given in Table 1.

Table 4 Effect of treatment on the CIELAB colour values of dried *C. subternata* after one month storage at room temperature (results are the averages of data before and after milling)

Treatment	Colour Parameter[a]				
	L*	**a***	**b***	**Chroma**	**Hue180**
T2[b]	40.3 b	-0.8 a	22.1 b	22.3 b	90.4 c
T3[c]	42.1 a	-3.6 b	22.9 ab	23.2 ab	98.3 a
T4[d]	41.4 a b	-2.2 c	23.0 a	23.3 a	94.6 b

[a]Means in a column with the same letter are not significantly different (P>0.05)
[b]Leaves were macerated and dried at 40°C/30% RH
[c]Leaves were steamed, macerated and dried at 40ºC
[d]Leaves were macerated, steamed and dried at 40ºC

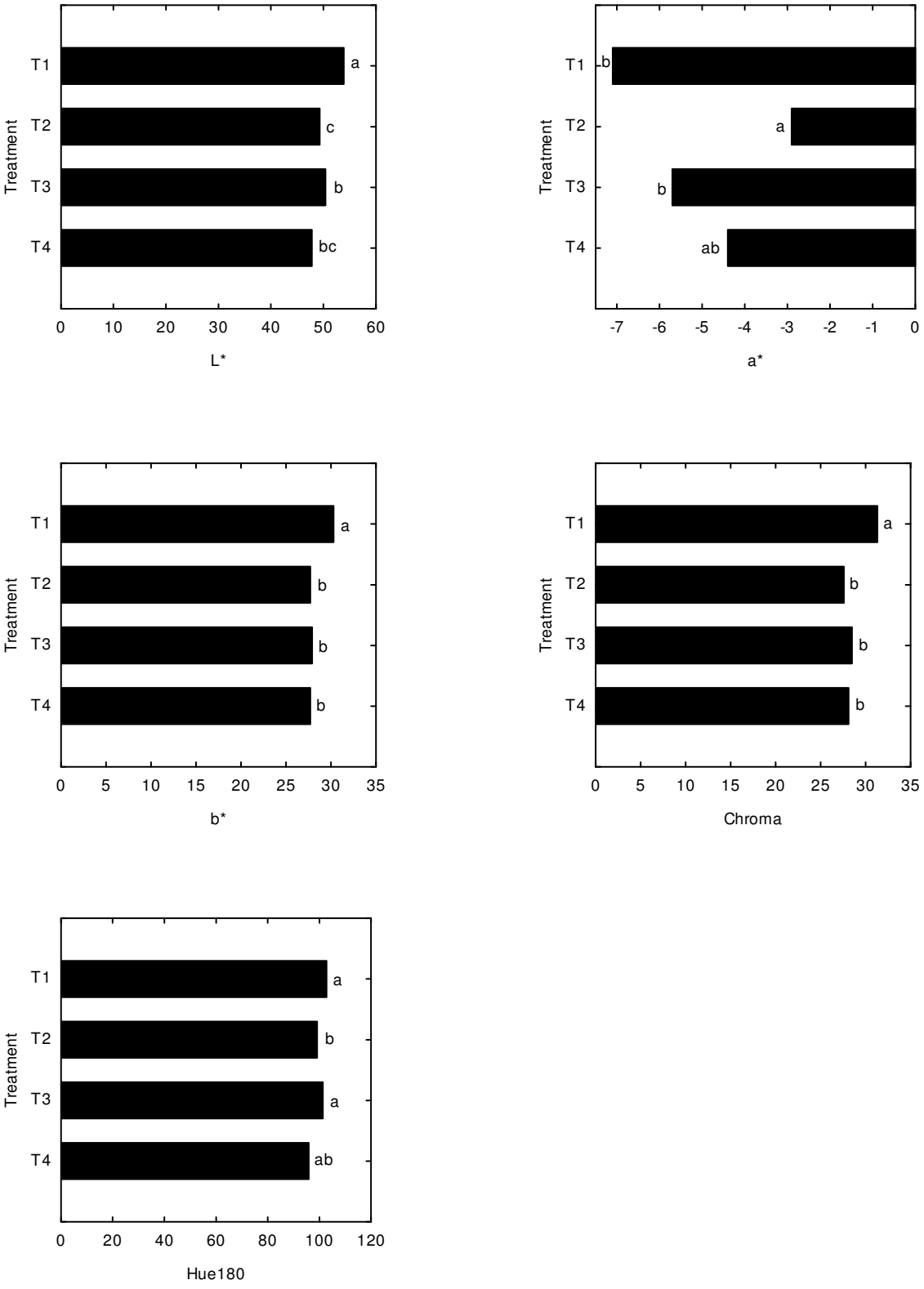


Figure 4 CIELAB colour values of different treatments. Colour measurements were performed after storage for 1 month at room temperature on milled leaves. Details of treatments are given in Table 1.

Table 5 Effect of treatment on the compositional data of *C. subternata*

Parameter	Treatment[a]			
	T1	**T2**	**T3**	**T4**
SS[b]_Leaf	42.9 a	40.1 b	42.1 a	41.8 a
TP[c]_SS	29.7 a	29.1 a	29.7 a	29.3 a
TP_Leaf	12.7 a	11.7 b	12.5 a	12.2 a
Mg[d]_Leaf	0.9 a	0.7 a	0.8 a	0.8 a
Hd[e]_Leaf	0.1 a	0.1 a	0.1 a	0.1 a
Mg_ SS	2.2 a	1.9 a	2.0 a	2.0 a
Hd_SS	0.3 a	0.3 a	0.2 a	0.2 a

[a]Means in a row with the same letter are not significantly different (P>0.05)
[b]Soluble solids content (g.100 g^{-1})
[c]Total polyphenol content (g.100 g^{-1})
[d]Mangiferin content (g.100 g^{-1})
[e]Hesperidin content (g.100 g^{-1})

Effect of delayed steaming between maceration and drying

All colour parameters differed significantly (P<0.05) between the two treatments. The 20 min delay before steaming of the macerated leaves commenced (T4/20) resulted in lower L*, b*, chroma and hue values, and the a* value was less negative compared to that of the leaves that were steam without delay (T4/0) (Table 6). Treatment, however, did not affect compositional data (Table 7). Milling had a significant effect on all colour parameters. The values, averaged over T4/0 and T4/20, are given in Table 8.

Effect of steaming on colour change of macerated fresh leaves over time

For this experiment the macerated leaves were not dried after treatment to allow the colour changes of the leaves while moist to be followed. Rapid colour changes were observed for all CIELAB parameters of the leaves which had been macerated but not steamed (T2/M) (Figure 5). L*, b* and chroma of the leaves steamed before maceration (T3/M) remained relatively stable over the first 60 min after treatment, but changes in a* and hue were observed for T3/M (Figure 5). Steaming lowered L* initially, after which it remained stable. The T2/M leaves had initially a higher L*, but within *ca.* 10 min, it decreased to less than that of the T3/M sample. Comparing the colour data of the leaves at 0 and 60 min (Table 9) showed that L*, b*, chroma and hue of T2/M were significantly (P<0.05) lower after 60 min. The a* value changed from negative (-6.43) to positive (1.66) (P<0.05). Only the a* and hue values of the T3/M leaves changed significantly (P<0.05) during the 60 min.

Table 6 The effect of delayed steaming on CIELAB colour values of *C. subternata* leaves

Treatment	Colour Parameter[a]				
	L*	**a***	**b***	**Chroma**	**Hue180**
T4/0[b]	37.7 a	-2.9 b	23.2 a	23.4 a	96.3 a
T4/20[c]	35.6 b	-0.9 a	21.5 b	21.6 b	90.9 b

[a]Means in a column with the same letter are not significantly different (P>0.05)
[b]Leaves were macerated, steamed for 60 s at >90ºC without delay and dried at 40°C/30% RH
[c]Leaves were macerated, left for 20 min, steamed and dried

Table 7 Effect of delayed steaming on compositional data of *C. subternata* leaves

Parameter[a]	Treatment	
	T1[b]	**T2[c]**
SS[d]	42.763 a	41.052 a
TP[e]	10.767 a	10.259 a
Mg[f]	0.902 a	0.805 a
Hd[g]	0.049 a	0.041 a

[a]Means within a row with the same letter are not significantly different (P>0.05)
[b]Leaves macerated, steamed for 60 s at >90ºC without delay and dried at 40°C/30% RH
[c]Leaves macerated, left standing for 20 min, steamed and dried
[d]Soluble solids content (g.100 g^{-1})
[e]Total polyphenol content (g.100 g^{-1})
[f]Mangiferin content (g.100 g^{-1})
[g]Hesperidin content (g.100 g^{-1})

Table 8 Effect of milling on CIELAB colour values of *C.subternata* leaves subjected to immediate (T4/0) and delayed steaming (T4/20), following 1 month storage at room temperature (results are the averages for T2, T3 and T4)

	Colour Parameter[a]				
	L	**A***	**b***	**Chroma**	**Hue180**
Not Milled[b]	29.3 b	0.3 a	16.5 b	16.5 b	88.7 b
Milled[c]	43.9 a	-4.3 b	28.2 a	28.5 a	98.5 a

[a]Means in a column with the same letter are not significantly different (P>0.05)
[b]Leaves were only macerated and not milled
[c]Leaves were milled

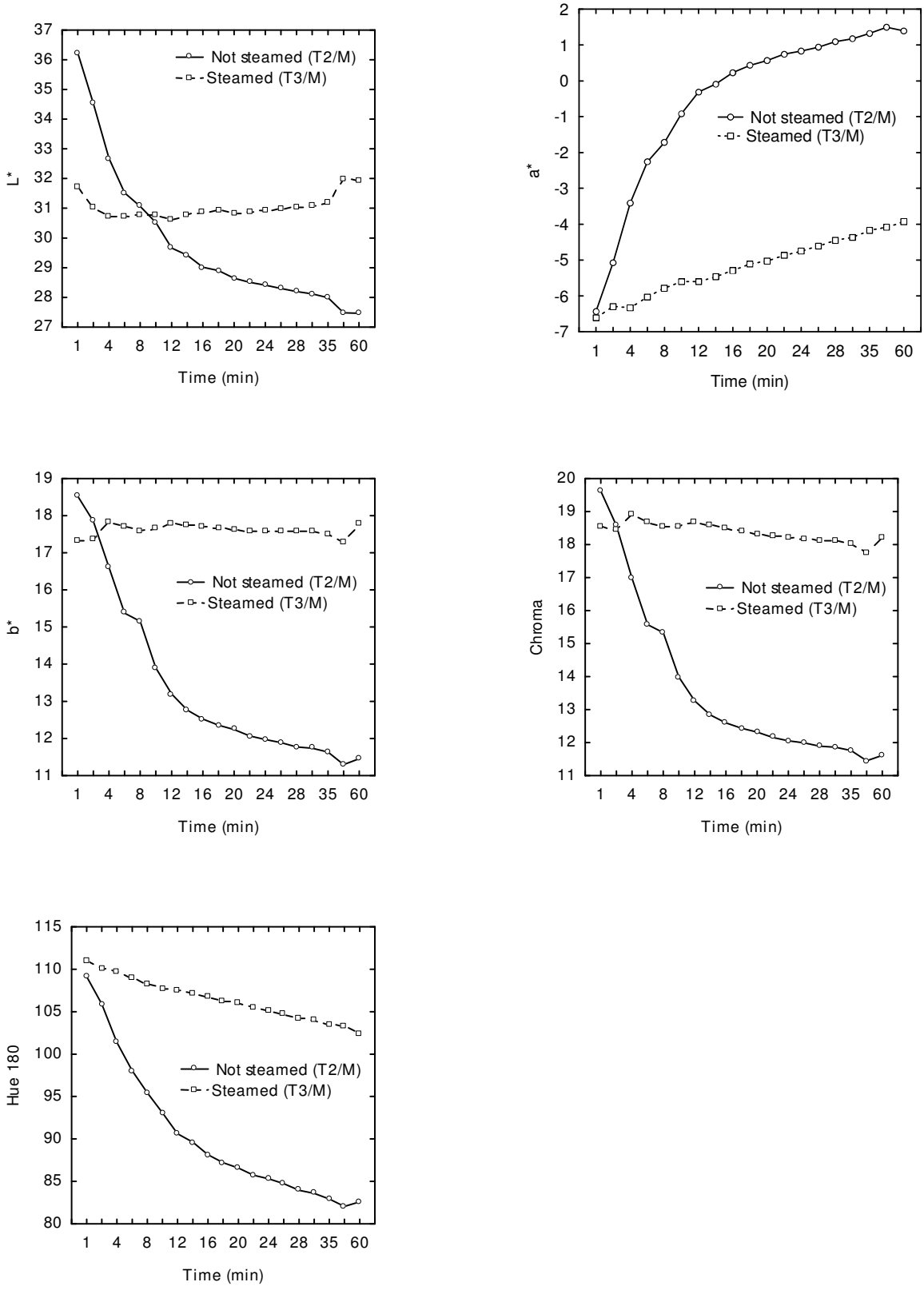


Figure 5 The effect of steaming (T2/M - no steaming after maceration; T3/M - steaming for 60 s at >90°C before maceration) on colour changes of *C. subternata* leaves. Colour measurements were done on the moist leaves.

Table 9 The effect of steaming on colour changes of *C. subternata* leaves

Treatment	Time (min)	Colour Parameters [a]				
		L*	**a***	**b***	**Chroma**	**Hue180**
T2/M: Not steamed[b]	**0**	36.24 a	-6.43 c	18.53 a	19.62 a	109.113 a
	60	27.29 c	1.66 a	11.46 b	11.68 b	81.21 c
T3: Steamed[c]	**0**	31.74 b	-6.61 c	17.32 a	18.56 a	111.01 a
	60	31.76 b	-3.62 b	17.89 a	18.26 a	101.35 b

[a]Values in a column with the same letter are not significantly different (P>0.05)
[b]T2/M: No steam treatment after maceration
[c]T3/M: Steam treatment for 60 s at >90°C before maceration

Effect of storage temperature

For this experiment, samples of the best treatment in terms of colour retention, i.e. T3 was subjected to storage for 6 months. The control treatment (T1/S) was included as reference. The moisture content of the samples was 5.59±0.87% (mean±SD) before storage and 5.42±0.49% after storage. Treatment had a significant effect on all the colour parameters, but not on the compositional data (Table 10). The changes in colour parameters (Figure 6) and compositional data (Figure 7) for the treatments as affected by storage time and temperature showed that storage at 30°C, irrespective of treatment resulted in changes in the colour parameters. Hue and a* of T3/S showed an initial change (after 1 month) where after the values remained relatively stable. The TP content of both T1/S and T3/S at both storage temperature increased over time. The rate of change was evaluated and the data summarised in Table 11. The rate of change of none of the colour parameters, irrespective of treatment, was significant (P>0.05) with storage at 0°C. Storage at 30°C did not result in a significant (P>0.05) rate of change for b* and hue values of T1/S and T3/S, respectively. The rate of change in the acetonitrile-water soluble TP content of the leaves was found to be significant (P<0.05) for both treatments and storage temperatures. However, when averaged over time and treatment, no significant effect (P>0.05) was found for temperature (Table 12).

Table 10 Effect of treatment (T1/S and T3/S) on CIELAB colour parameters and compositional data of *C. subternata* leaves after 6 months storage

Parameter[a]	**Treatment**	
	T1/S[b]	**T3/S**[c]
L*	52.36a	47.36b
a*	-10.18b	-7.04a
b*	32.95a	30.38b
Chroma	34.53a	31.33b
Hue180	107.08a	102.83b
SS[d]	49.39a	47.36a
TP[e]	13.36a	12.98a
Mangiferin[f]	1.77a	1.72a
Hesperidin[g]	0.04 a	0.03 a

[a]Means in a row with the same letter are not significantly different (P>0.05)
[b]T1: Dried intact
[c]T3: Steamed for 60 s at >90^{o}C, macerated and dried at 40°C/30% RH
[d]Soluble solids content (g.100 g^{-1})
[e]Total polyphenol content (g.100 g^{-1})
[f]Mangiferin content (g.100 g^{-1})
[g]Hesperidin content (g.100 g^{-1})

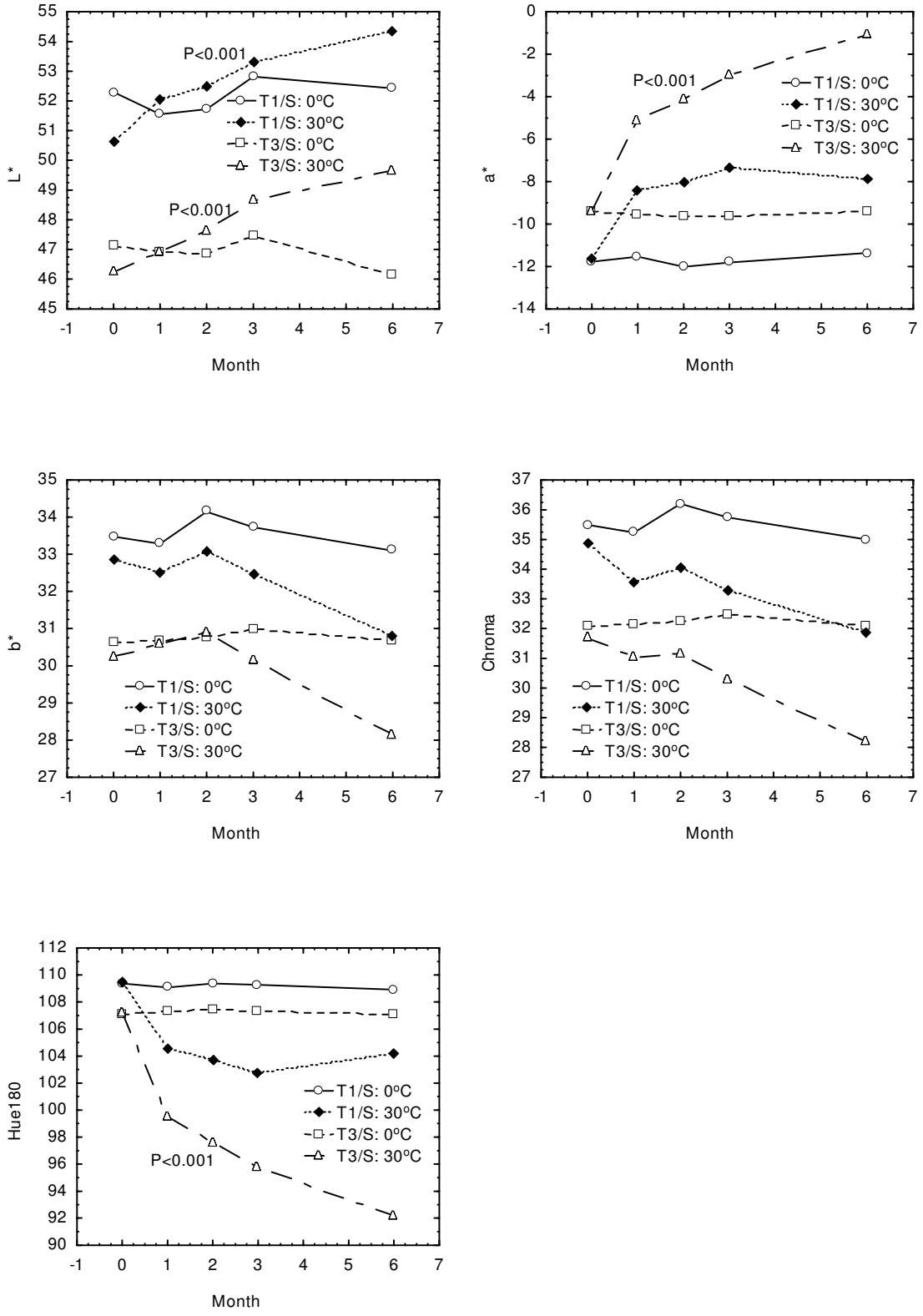


Figure 6 Effect of storage on CIELAB colour values of *C. subternata* milled leaves during 6 months storage at 0ºC and 30ºC. (T1/S: dried intact, T3/S: steamed, macerated and dried).

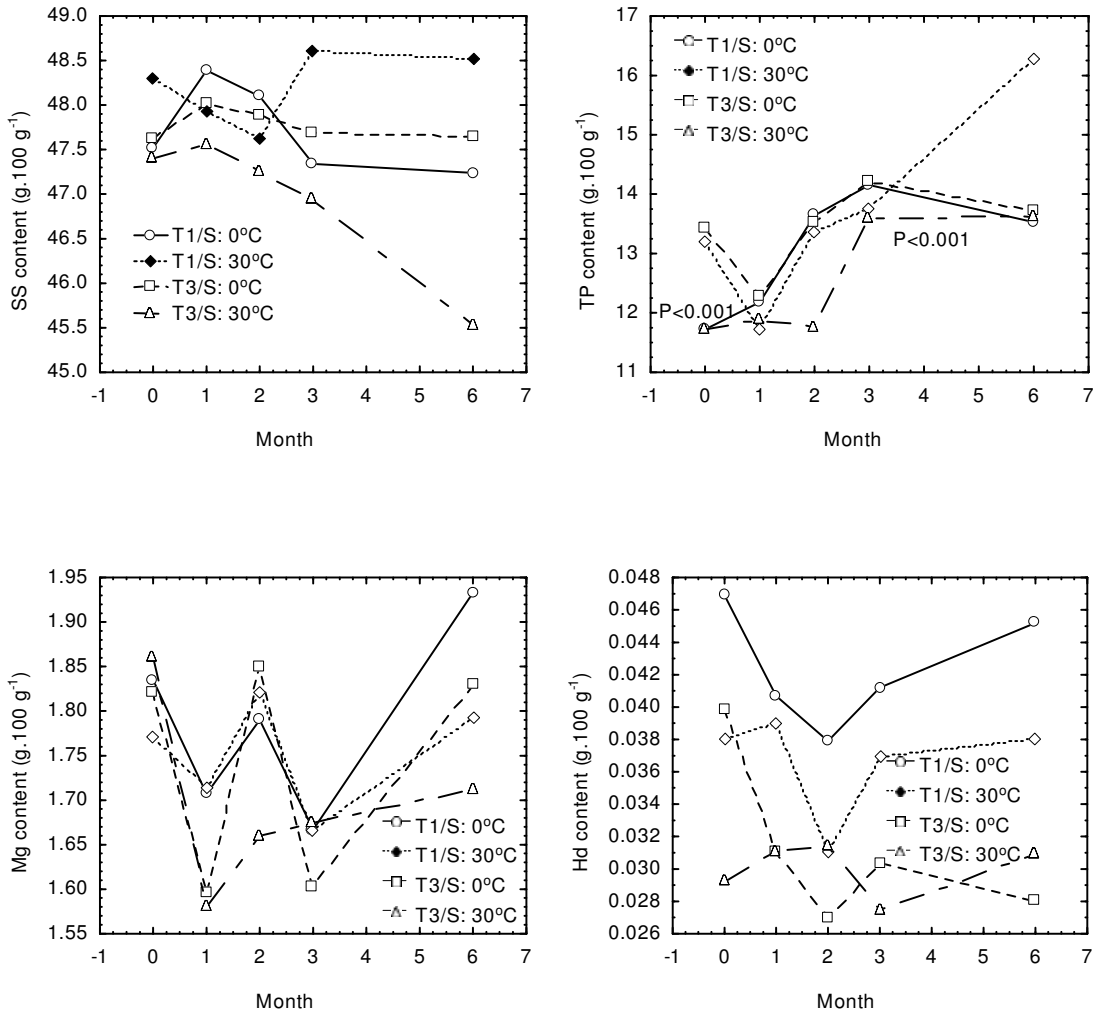


Figure 7 Effect of treatment on compositional data of *C. subternata* milled leaves during 6 months storage at 0ºC and 30ºC. (T1/S: dried intact, T3/S: steamed, macerated and dried).

Table 11 Effect of treatment and storage temperature on the rate of change in CIELAB colour values and compositional data for *C. subternata* leaves

Parameter	Treatment[a]	Storage temperature	Rate of change	Significance
L*[b]	T1/S	0°C	0.238	0.310
		30°C	1.888	<0.001
	T3/S	0°C	-0.328	0.194
		30°C	1.828	<0.001
a*[b]	T1/S	0°C	0.128	0.679
		30°C	1.963	0.004
	T3/S	0°C	-0.015	0.937
		30°C	4.158	<0.001
b*[b]	T1/S	0°C	-0.066	0.852
		30°C	-0.891	0.024
	T3/S	0°C	0.068	0.795
		30°C	-0.951	0.055
Chroma[b]	T1/S	0°C	-0.103	0.812
		30°C	-1.396	0.002
	T3/S	0°C	0.068	0.824
		30°C	-1.651	0.002
Hue180[b]	T1/S	0°C	-0.162	0.588
		30°C	-2.819	0.008
	T3/S	0°C	-0.006	0.978
		30°C	-7.531	<0.001
SS[d]	T1/S	0°C	-0.121	0.097
		30°C	0.490	0.487
	T3/S	0°C	-0.025	0.560
		30°C	-0.338	0.0098
TP[e]	T1/S	0°C	0.309	<0.001
		30°C	0.635	0.019
	T3/S	0°C	0.147	0.016
		30°C	0.361	<0.001
Mangiferin[f]	T1/S	0°C	0.002	0.382
		30°C	0.000347	0.868
	T3/S	0°C	0.000965	0.667
		30°C	-0.000837	0.596
Hesperidin[g]	T1/S	0°C	0.000012	0.938
		30°C	-0.00000144	0.992
	T3/S	0°C	-0.0001446	0.055
		30°C	0.00000894	0.884

[a]T1/S: dried intact; T3/S: steamed for 60 s at >90°C, macerated and dried at 40°C/30% RH
[b]Colour values calculated according to y= a + b Ln (x+1).
[c]Compositional values calculated according to y= a + b x.
[d]Soluble solids content (g.100 g^{-1})
[e]Total polyphenol content (g.100 g^{-1})
[f]Mangiferin content (g.100 g^{-1})
[g]Hesperidin content (g.100 g^{-1})

Table 12 Effect of storage temperature on compositional data of *C. subternata* leaves stored for 6 months (results were averages over storage time and treatment)

	Temperature (0°C)	Temperature (30°C)
SS[ab]	47.75 a	49.01 a
TP[c]	13.24 a	13.09 a
Mg[d]	1.77 a	1.72 a
Hd[e]	0.037 a	0.033 a

[a]Means in a row with the same letter are not significantly different (P > 0.05)
[b]Soluble solids content (g.100 g^{-1})
[c]Total polyphenol content (g.100 g^{-1})
[d]Mangiferin content (g.100 g^{-1})
[e]Hesperidin content (g.100 g^{-1})

Discussion

Loss of colour of food is perceived as a loss of quality by consumers. Undesirable chemical changes are in many instances associated with colour changes. Processing of traditional honeybush with a sought-after dark brown colour is accompanied, not only by changes to its sensory properties, but also a decrease in water extractable SS, TP, mangiferin and hesperidin contents, as well as antioxidant (Joubert *et al*., 2007), antimutagenic (Van der Merwe *et al*., 2006) and phytoestrogen (Verhoog *et al*., 2007) activities. This is brought about as a result of the severe heat treatment (high temperature-long time) employed in the "fermentation" process. In the case of the manufacture of green honeybush, retention of the green colour is considered essential for quality. It could also be an indication that detrimental chemical changes, especially degradation of bioactives, were limited during manufacture.

Since the shoots are shredded into small pieces to prepare particle sizes suitable for the tea retail market, extensive cell damage and exposure to oxygen occur. This leads to rapid discolouration and loss of green colour which is believed to be a result of polyphenol oxidation and/or chlorophyll degradation. Blanching are used to preserve the green colour of vegetables (Ihl *et al*., 1998; Viňa *et al*., 2007) and tea (Gulati *et al*., 2003; Huang *et al*., 2005; Sharma *et al*., 2005) by inactivating enzymes such as chlorophyllase (Schwartz & Von Elbe 1983) and polyphenol oxidase (PPO) (Gulati *et al*., 2003). Of the different blanching techniques such as heating by steam, boiling water, microwave (Ihl *et al*., 1998), oven (Huang *et al*., 2005; Sharma *et al*., 2005) or high intensity pulsed electric field (PEF) (Yin *et al*., 2007) that can be employed, steaming is routinely applied. The use of boiling water is not practical due to leaching of water SS, oven heating is slow and microwave and PEF heating would

require specialised equipment. Heating with steam was chosen as the herbal tea industry in South Africa is familiar with its use, although employed as a pasteurisation technique. Drying at 40°C was selected since higher drying temperatures would increase chlorophyll degradation (Maharaj & Sankat, 1996; Sigge *et al.,* 1999). The drying time of 4 h, used in the present study, was shown by Du Toit & Joubert (1998) to be adequate to reduce the moisture content of *Cyclopia* to at least 10%.

The conditions prevalent during the treatment of honeybush could support both chlorophyll degradation to pheophorbide via pheophytin or via chlorophyllide. The heat of the steaming process could lead to substitution of magnesium in the porphyrin ring with two hydrogen atoms and the formation of pheophytin when the pigments are exposed to acids as a result of cell disruption (Heaton *et al*., 1996). This conversion to pheophytin is accompanied by an undesirable colour change in leafy vegetables from bright green to olive brown (Schwartz & Lorenzo, 1990). Enzymatic activity initiated by maceration would cleave the phytol chain leading to chlorophyllides and eventually pheophorbides (Heaton *et al*., 1996). Degradation of chlorophyll a and b both follow first-order degradation kinetics (Weemaes *et al*., 1999).

In this study only leaves and some very fine green stems attached to the leaves were used for experimentation; solely because of practical reasons. In the first instance, the small sample sizes required that a Waring blender be used for maceration, instead of the mechanised fodder cutter normally employed. Secondly, some of the thick stems of old shoots are brown, which, if included would have "masked" colour changes observed. Under normal manufacturing conditions these thick stems are removed from the dried tea by sieving.

Intact leaves (T1), dried under the same conditions as treatments T2, T3 and T4, were used as control. Since the cellular structures were not damaged, enzyme activity was prevented and the best leaf colour could be obtained, without resorting to freeze-drying. It is also important to note that, although the intact leaves were included as control, their colour data could not be compared with the macerated leaves obtained with T2, T3 and T4, due to differences in physical size of the leaves. Physical size greatly affects colour measurement, especially L* due to increased reflectance (MacDougall, 2002). Milling can overcome this problem, but it leads to loss of information of surface colour. For this reason the colour was determined both before (without control) and after milling (control included).

Steaming of intact leaves before maceration (T3) proved to retain the green colour the best, based on the most negative a* and the highest hue values, as well as visual observation. The hue angle was higher than 90°, falling in the green/yellow quadrant of the colour solid (Clydesdale, 1978). T3 being the least detrimental to the green colour of the leaves was to be expected, since enzymes were

inactivated before the leaves were macerated. The extent of inactivation of PPO and chlorophyllase would depend on the plant material. Treating sweet potato PPO at 90°C/1 min completely inactivated the enzyme (Lourenco *et al*., 1992), while PPO in *Camellia sinensis* leaves required 4 min of steam treatment (Gulati *et al*. 2003). Chlorophyllase of strawberry has considerable resistance to heat treatment (Martinez *et al*., 1995), whereas steaming of Tencha leaves for 18 s reduced its activity to about 1/18 of that in fresh leaves (Katsunori *et al*., 2001).

Treatment T4 was more detrimental to retention of the green colour than T3 as a result of maceration before steaming. This was visually observed and confirmed by the substantially smaller -a* value and lower hue value. The latter fell just in the green/yellow quadrant of the colour solid. The b* and chroma values of T3 and T4 were, however, not significantly different. This is a further indication that T4, with further optimisation, could be a viable treatment option for industry. T3 would not be feasible, as it would require steaming of whole shoots.

Maceration of the leaves, followed by drying (T2), was the least effective treatment as indicated by the positive a* (no visual green) and lowest hue (<90°, falling in the red/yellow quadrant of the colour solid). Maceration would initiate enzymatic degradation reactions, which would then be accelerated by the mild heat of the drying process. PPO can be active over a wide temperature range, i.e 20 to 60°C (Schomberg *et al*., 1994). The optimum temperature of strawberry chlorophyllase has been found to be 40°C (Martinez *et al*., 1995). Evaporation during drying would keep the macerated leaves at a lower temperature than that of the circulating air, until the dry state was reached. Enzyme activity would thus be possible, if the leaves did not receive an adequate steam treatment, especially if the water activity (a_w) is high enough. The a_w of the leaves would decrease during drying, also decreasing enzyme activity. LaJollo *et al*. (1971) showed that chlorophyll was mostly converted to pheophytin at 37°C and an $a_w > 0.32$. At an $a_w < 0.32$ the rate of pheophytin formation was low and other products formed.

A delay in steaming after maceration was detrimental to colour retention as indicated by the colour parameters, especially a* and hue. By also following the rate of colour change after maceration (T2/M) over 60 min, it was shown that a* changed from negative to positive after approximately 14 min. This would mean that steaming should be carried out without delay after shredding of the shoots.

For the storage experiment T3 was used to ensure that the starting material was adequately green so that any change in a* and hue would mostly occur whilst still green. T1 was included as control. Treatment again affected the colour parameters, consistent with the results of the first experiment, comparing the efficacy of treatments. Notable was the substantial difference in the rate of change of a* and hue between T1 and T3 at 30°C, with T3 samples having a substantially higher rate (change in a* and hue 2.1 and 2.7 faster). At 0°C the rate of change of these colour parameters was not significant for

both treatments. This difference in rate of change between T1 and T3 would suggest that treatment also introduced qualitative changes in chlorophyll composition. Chlorophyllide, also a green pigment, but a degradation product of chlorophyll, degrades faster than chlorophyll (Canjura *et al*., 1991). Using spinach, they demonstrated that chlorophyllide degraded two times faster than chlorophyll a, and chlorophyll b degraded four times faster than chlorophyll b at high temperatures. It is thus possible that higher levels of chlorophyllide formed when the samples were steamed. Their more rapid degradation would explain the difference between T1 and T3.

The effect of milling on a* and hue of the samples is of practical interest, as milling would give an indication of the internal state of the dry leaves. The a* either changed from positive to negative or was more negative after milling. Hue also changed to a value indicating "greenness". The higher b* value of milled samples indicated more yellowness. The change in a* and b* with milling was also reported by Berset & Caniaux (1983) for dried parsley. Large-scale milling would only be feasible if the plant material is intended for extract preparation.

Treatment had no effect on the SS, TP, mangiferin and hesperidin contents, indicating high stability for oxidation by PPO, or low enzyme activity. Except for TP becoming more soluble upon storage, storage also had no effect on these parameters.

Conclusions

By steaming green *C. subternata* directly after maceration, its colour retention can be improved. Good stability was shown for mangiferin and hesperidin during manufacture and storage of *C. subternata.*

References

Berset, C. & Caiaux, P. (1983). Relationship between colour evaluation and chlorophyllian pigment content in dried parsley leaves. *Journal of Food Science*, **48**, 1854-1857 & 1877.

De Beer, S.W. & Joubert, E. (2002). Preparation of tea-like beverages. South African Patent 2002/2808.

Canjura, F.L., Schwartz, S.J. & Nunes, R.V. (1991). Degradation kinetics of chlorophylls and chlorophyllides. *Journal of Food Science*, **56**, 1639-1643.

Clydesdale, F.M (1978). Colorimetry - methodology and explanations. *Critical Reviews in Food Science and* N*utrition*, **10**, 243-301.

Du Toit, J. & Joubert, E. (1998). Effect of drying conditions on the quality of honeybush tea (*Cyclopia*). *Journal of Food Processing Preservation,* **22**, 493-507.

Du Toit, J. & Joubert, E. (1999). Optimization of the fermentation parameters of honeybush tea

(*Cyclopia*). *Journal of Food Quality*, **22**, 241-256.

Gulati, A., Rawat, R., Singh, B. & Ravindranath, S.D. (2003). Application of microwave energy in the manufacture of enhanced-quality green tea. *Journal of Agricultural and Food Chemistry*, **51**, 4764-4768.

Heaton, J.W., Yada, R.Y. & Marangoni, A.G. (1996). Discolouration of coleslaw is caused by chlorophyll degradation. *Journal of Agricultural and Food Chemistry*, **44**, 395-398.

Huang, Y., Sheng, J., Yang, F. & Hu, Q. (2007). Effect of enzyme inactivation by microwave and oven heating on preservation quality of green tea. *Journal of Food Engineering*, **78**, 687-692.

Hubbe, M.E. (2000). Evaluation of antioxidant and free radical scavenging activities of honeybush tea (*Cyclopia)*. M.Sc in Food Science Thesis, University of Stellenbosch, South Africa

Hunt, R.W.G. (1977). The specification of colour appearance. Concepts and terms. *COLOR Research and Application*, **2**, 55-68.

Ihl, M., Monslaves, M., Bifani, V. (1998). Chlorophyllase inactivation as a measure of blanching efficacy and colour retention of artichokes (Cynara scolymus L.). *Lebensmitteln–Wissenschaft und –Technologie*, **31**, 50-56.

Joubert, E., Richards, E.S., Van der Merwe, J.D., De Beer, D., Manley, M. & Gelderblom, W.C.A. (2007). Effect of species variation and processing on phenolic composition and in vitro antioxidant activity of aqueous extracts of *Cyclopia* spp. (honeybush tea). *Journal of Agricultural and Food Chemistry (*in print).

Katsunori K., Takafumi, H., Masaki, T., Tomomi, U. (2001). Changes in chlorophyll pigments and chlorophyllase activity during manufacture of Tencha. *Journal of the Japanese Society for Food Science and Technology*, **48**, 744-750 (J-East Abstract).

Lajollo, F.M., Tannenbaum, S.R. & Labuza, T.P. (1971). Reaction at limited water concentration-chlorophyll degradation. *Journal of Food Science*, **36**, 850-853.

Lourenco, E.J., Neves, V.A. & Da Silva, M.A. (1992). Polyphenol oxidase from sweet potato: purification and properties. *Journal of Agricultural and Food Chemistry*, **40**, 2369-2373.

MacDougall, D.B. (2002). *Colour in Food: Improving Quality*. Baco raton, FL, USA: CRC Press, p. 98.

Maharaj, V. & Sankat, C.K. (1996). Quality changes in dehydrated dasheen leaves; effect of pre-treatments and drying conditions. *Food Research International*, **29**, 563-568.

Mangos, T.J. & Berger, R.G. (1997). Determination of major chlorophyll degradation products. *Zeitschrift für Lebensmittel-Untersuchung und –Forschung A*, **204**. 345-350.

Martinez, G.A., Civello, P.M., Chaves, A.R., Anon, M.C. (1995). Partial characterization of chlorophyllase from strawberry fruit (*Frangaria ananassa*, Duch.). *Journal of Food Biochemistry*, **18**, 213-226 (IBIDS Abstract).

Martinez, V.M. & Whitaker, J.R. (1995). The biochemistry and control of enzymatic browning. *Trends in Food Science and Technology*, **6**, 195-200.

Pandrangi, S. & LaBorde, L.F. (2004). Retention of folate, carotenoids, and other quality characteristics in commercially packaged fresh spinach. *Journal of Food Science*, **69**, 702-707.

Schomburg, D., Salzmann, M. & Stephan, D. (1994). Class 1.13-10.97: Oxidoreductases. Enzyme handbook Volume 8. Berlin, Germany: Springer-Verlag.

Schwartz, S.J. & Lorenzo, T.V. (1990). Chlorophylls in foods. *Critical Reviews in Food Science and Nutrition*, **29**, 1-17.

Schwartz, S.J. & Von Elbe, J.H. (1983). Kinetics of chlorophyll degradation to pyropheophytin in vegetables. *Journal of Food Science*, **48**, 1303-1306.

Sharma, V., Gulati, A., Ravindranath, S.D. (2005). Extractability of tea catechins as a function of manufacture procedure and temperature of infusion. *Food Chemistry*, **93**, 141-148.

Sigge, G.O., Hansmann, C.F. & Joubert, E. (1999). Optimising the dehydration conditions of green bell peppers (*Capsicum annum* L.): quality criteria. *Journal of Food Quality,* **22**, 439-452.

Sigge, G.O., Hansmann, C.F. & Joubert, E. (2001). Effect of storage conditions, packaging material and metabisulphite treatment on the colour of dehydrated green bell peppers (*Capsicum Annum* L.). *Journal of Food Quality,* **24,** 205-218.

Van der Merwe, J.D., Joubert, E., Richards, E.S., Manley, M., Snijman, P.W., Marnewick, J.L. & Gelderblom, W.C.A. (2006). A comparative study on the antimutagenic properties of aqueous extracts of *Aspalathus linearis* (rooibos), different *Cyclopia* spp. (honeybush) and *Camellia sinensis* teas. *Mutation Research*, **611**, 42-53.

Verhoog, N.J.D., Joubert, E. & Louw, A. (2007). Screening of four *Cyclopia* (honeybush) species for putative phyto-oestrogenic activity by oestrogen receptor bindings assays. *South African Journal of Science*, **103**, 13-21.

Viňa, S.Z., Oliviera, D.F., Marani, C.M., Ferreyra, R.M., Mugridge, A., Chaves, A.R. & Mascheroni, R.M. (2007). Quality of Brussels sprouts (*Brassica oleracea* L. *gemmifera* DC) as affected by blanching method. *Journal of Food Engineering*, **80**, 218-225.

Weemaes, C.A., Ooms, V., Van Loey, A.M., Hendrickx. M.E. (1999). Kinetics of chlorophyll degradation and colour loss in heated broccoli juice. *Journal of Agricultural and Food Chemistry*, **47**, 2404-2409.

Yin, Y., Han, Y., Liu, J. (2007). A novel protecting method for visual green colour in spinach puree treated by high intensity pulsed electric fields. *Journal of Food Engineering*, **79**, 1256-1260.

CHAPTER 5
GENERAL DISCUSSION AND CONCLUSION

Honeybush (*Cyclopia* spp.) is a herbal plant containing the valuable xanthone, mangiferin and the flavonoid hesperidin. Mangiferin is one of the major contributors to the total antioxidant activity of extracts and plant material should contain the highest amounts of these compounds possible for the development of polyphenol-enriched extracts for use by the food, cosmetic and nutraceutical industries. Both or one of the compounds, preferably mangiferin, can also be used as a quality standard for green honeybush. Minimum levels would be required in such a case. Other potential quality standards could be total polyphenol (TP) and soluble solids (SS) content and total antioxidant activity (TAA). Screening of green honeybush tea would be advisable to ensure that the tea meets the minimum standards. In a previous study by Botha (2005) it was demonstrated that NIR spectroscopy could be used for rapid screening of the mangiferin and hesperidin contents in reflectance mode, but that transmittance NIR spectroscopy calibration models developed for TP content, SS content and TAA of water extracts did not give acceptable results. Quantification of the mangiferin content of methanol extracts of *C. genistoides* with the aluminium chloride colorimetric method correlated well with values obtained with HPLC.

Cyclopia subternata water extracts contain low quantities of mangiferin and hesperidin, compared to that of *C. genistoides*, yet are of comparable TAA (Joubert *et al*., 2007), suggesting also the presence of other phenolic compounds with potent TAA. These quantitative and qualitative differences between the two *Cyclopia* species indicated that methods suitable for *C. genistoides* would not necessary be suitable for *C. subternata,* which prompted the present study.

For more accurate quantification of mangiferin, present in lower quantities in *C. subternata*, the reference HPLC method as used by Botha (2005) was improved by changing from a C12 to a phenyl-hexyl phase. This column has been found to possess unique selectivity for aromatic compounds such as mangiferin and hesperidin and thereby resulting in sharper peaks for better quantificantion. The absorbance measurement for the determination of mangiferin was also changed from 288 nm to 320 nm to improve sensitivity. The extraction procedure itself was optimised to extract the maximum amount of both mangiferin and hesperidin. Parameters that were investigated were the effect of extraction solvent, extraction time and also the mass to solvent ratio during extraction.

Several of the solvents tested were equally effective in extracting mangiferin and hesperidin. Water, 100% ethanol and 100% methanol gave the least effective extraction, while 70% acetone and 33% acetonitrile-water (steam bath) were most effective for extraction of mangiferin and hesperidin,

repectively. However, HPLC analysis of 70% acetone was not effective as the acetone content was found to produce a poor peak shape. Acetonitrile-water, being second most effective in extraction of mangiferin and giving only slightly lower values than 70% acetonitrile, was thus chosen as the most effective solvent for the extraction of both mangiferin and hesperidin.

An extraction time of 30 min was chosen, based on the results obtained for TP content. Future studies should investigate the stability of mangiferin and hesperidin in a typical extract under similar heating conditions to that used for extraction. Standarisation of the use of 5 g plant material for extraction, although giving slightly less effective extraction, was governed by the need to obtain extracts containing reasonable quantities of mangiferin and hesperidin for HPLC analysis.

Honeybush is mainly consumed in the form of an infusion (cup of tea) and a water extract is produced by a local extract manufacturer for the food industry. TP content and TAA are used to standardise their product. It was thus of importance that the correlation of TAA with TP content be investigated. A moderate correlation (r = 0.85) was obtained for TAA and TP content. However, TAA and mangiferin content correlated poorly (r = 0.54). This indicates that plant material having a high mangiferin content would not necessarily result in a water extract with a high TAA. By including isomangiferin, the correlation improved slightly.

The complexation of $AlCl_3$ as a rapid quantification method for mangiferin was also investigated as the method showed promise when applied to green *C. genistoides* extracts (Botha, 2005). The aluminium ion can complex with the mangiferin at two sites. Hesperidin, on the other hand, has only one site. Botha (2005) showed that the addition of $AlCl_3$ to a mangiferin solution, resulted in hyperchromic and bathochromic shifts in its absorbance spectrum. Hesperidin, however, showed a very small shift in the absorbance spectrum. The interference of hesperidin on the mangiferin was therefore small. A moderate correlation (r = 0.87) was found for xanthone content of the plant material using the $AlCl_3$ colorimetric method and mangiferin content quantified by HPLC (y = 1.2x + 0.54) when the plant material was extracted with water. Using 33% acetonitrile-water, a weaker correlation (r = 0.74; y = 1.3x + 0.87) was found. Correlation of the xanthone content (determined by $AlCl_3$) of the two solvents, resulted in a good correlation (r = 0.91). Mangiferin values (0.023 to 3.69 g.100 g^{-1}) estimated by $AlCl_3$ were higher than those quantified by HPLC (0.023 to 2.7 g.100 g^{-1}). This over estimation could be due to the presence of hesperidin (although with a low reactivity towards $AlCl_3$) and also isomangiferin.

For the development of NIR spectroscopy calibration models a large sample set was used (n=197) to include samples from different cultivation areas, plants of different ages, plants harvested during different seasons and also samples consisting of different leaf:stem ratios. A positively skewed

distribution for the mangiferin content was obtained. This indicated that the majority of samples used had low mangiferin contents. The results obtained indicated that the NIR spectroscopy calibration models would be effective for screening purposes. Future studies should include more plant material samples with higher mangiferin content. This could most likely improve not only the robustness of the model, but quantification accuracy.

Another aspect of the present study was the investigation of the effect of different pre-drying treatments and storage temperatures on the SS, TP, mangiferin and hesperidin contents and colour retention of green *C. subternata*. With low levels of mangiferin and hesperidin present in *C. subternata*, their retention during processing is of importance. Since retention of the green colour is considered indicative of little or no detrimental compositional changes, especially of phenolic oxidative reactions, objective colour measurements were carried out. The present study, however, showed that although indicators of greenness, i.e. -a* and hue were affected by the pre-drying treatments and storage at 30°C for 6 months, no detrimental changes occurred in the SS, TP, mangiferin and hesperidin contents of the plant material. Storage at 0°C was also effective to prevent changes in the colour parameters. Use of this low temperature may, however, not be practical in industry, but could be useful for future studies. During this study, honeybush samples were stored under dark conditions and future studies could be undertaken to investigate the effect of storage under light conditions.

Interesting results were obtained for colour parameters for the respective treatments. Drying the intact leaves (with no treatment), followed by steaming of the plant material for 60 s at > 90ºC before maceration, was found to be the most effective pre-drying treatments for colour retention. Both would not be practical in industry, but good results could be obtained with steaming directly after maceration, confirming the suitability of the procedure currently used by one of the processors.

Results from the storage experiment at 30°C indicated that steaming, followed by maceration and drying, resulted in a faster rate of change of a* and hue than when intact leaves were dried. It is suggested that the difference in the rate of change between the treatments could be due to faster breakdown of chlorophyllides, formed during steaming and maceration. A future study should investigate this hypothesis. This would also require quantification of chlorophyll and its degradation products.

It is not known to what extent drying conditions can have an effect on the retention and extractability of *C. subternata* polyphenols. For this study, drying was standardised at 40°C and 30% relative humidity (RH), but different drying treatments still need to be investigated. Future work could potentially shed light on this topic. Other methods such as sonication and accelerated solvent extraction

could also be investigated as possible means to improve extraction of polyphenols from green *C. subternata.*

Application of NIR spectroscopy on dried extract, in addition to the plant material, could also be a possibility, and this should be investigated as it would give the quality control analyst a means of rapidly evaluating both the raw material and final product. Future studies could therefore focus on the use of NIR spectroscopy at different stages of processing of the plant material.

References

Botha, M. (2005). Use of near infrared spectroscopy (NIRS) and spectrophotometric methods in quality control of green rooibos (*Aspalatus linearis*) and honeybush (*Cyclopia genistoides*). MSc in Food Science Thesis, Stellenbosch University, South Africa.

Joubert, E., Richards, E.S., Van der Merwe, J.D., De Beer, D., Manley, M. & Gelderblom, W.C.A. (2008). Effect of species variation and processing on phenolic composition and in vitro antioxidant activity of aqueous extracts of *Cyclopia* spp. (honeybush tea). *Journal of Agricultural and Food Chemistry,* **56**, 954-963.