THE STABILITY OF ASPALATHIN, ISO-ORIENTIN AND ORIENTIN IN ROOIBOS ICED TEA

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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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December 2008

DELARATION

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ABSTRACT

The change in aspalathin, iso-orientin, orientin and total polyphenol (TP) content of a commercially produced fermented rooibos (FR) extract was monitored throughout production. Particular attention was paid to the effect of spray-drying on FR and unfermented rooibos (UR) extracts. The quality of commercial, South African rooibos iced teas made with FR extract was also investigated with respect to the aforementioned parameters. Subsequently, the effect of heating and storage on the phenolic composition and colour of experimental iced teas containing respectively FR, UR and nano emulsified unfermented rooibos (NEUR) extracts was investigated. The combined effect of pH (pH 3-7) and storage (5, 30 and 40ºC), as well as high (660 mg/L, 0-7 days at 30ºC; UR only) and low (0.5 mg/L) concentrations of H_2O_2, was determined on reconstituted FR, UR and NEUR extracts. Finally, eight rooibos iced teas (four variants; unflavoured and lemon-flavoured) were analysed for plant-like, hay-like, rooibos and lemon flavour, as well as astringency, using descriptive sensory analysis. The degree of consumer preference of the flavoured variants was determined using the nine point hedonic scale. In all cases, changes in individual flavonoid content were quantified using HPLC. The TP content of the iced teas and commercial extracts was determined using the Folin-Ciocalteu assay. Browning of the iced teas and reconstituted extracts was monitored spectrophotometrically (420 nm).

Aspalathin, iso-orientin and orientin were found to be present after all stages of the FR extract production process. Spray-drying, specifically, also did not reduce the content of these flavonoids, or the TP content, in FR and UR extracts. Despite the relatively good retention during the heating and storage of experimental rooibos iced teas, these flavonoids were either absent or present at extremely low levels in commercial iced teas. The latter suggested that either extremely low quantities of extract, no extract at all or extracts of poor quality, were used for the production of the analysed iced teas. Increased degradation was generally observed for sterilisation treatments compared to pasteurisation whilst losses during storage increased with time. The presence of citric acid, due to its pH-lowering effect, and ascorbic acid, due to its antioxidant activity, was integral to the retention of aspalathin, iso-orientin and orientin during heating, but less so during storage. The UR iced teas generally performed better than their FR counterparts, however, NEUR iced teas exhibited the greatest retention of the aforementioned flavonoids. Heating and storage resulted in browning of most iced teas, whilst the TP content increased slightly or remained unchanged.

Phenolic retention in FR and UR extracts decreased with increasing pH and temperature, with concomitant browning. However, between pH 5 and 7, the stability of aspalathin was superior in the NEUR extract formulation. The latter also greatly resisted absorbance changes at pH 3 and 4, despite a loss of aspalathin.

The phenolic content of UR extract was immediately reduced by high a concentration of H_2O_2, however, no significant ($P \geq 0.05$) changes in absorbance were detected, suggesting the formation of intermediate, colourless oxidation products. Formulations containing ascorbic acid experienced the greatest reductions. This was attributed to the iron reducing ability of this compound, as reduced iron accelerates the

rate of the Fenton reaction. At low levels of H_2O_2, only the FR extract exhibited a loss of phenolic compounds. The level of iron in this extract was the highest.

Despite having the greatest aspalathin and total flavonoid content, lemon flavoured unfermented rooibos iced tea (UF/LEMON) was disliked by consumers. Preference was directed away from the plant-like characteristic of this tea and towards rooibos flavour, characteristic of fermented rooibos iced tea. Iced tea comprising both FR and NEUR extract produced a product that 77% of consumers rated positively. Its slight hay-like flavour did not significantly ($P \geq 0.05$) reduce the liking of this product compared to fermented rooibos iced tea.

UITTREKSEL

Die verandering in die aspalatien-, iso-orientin-, orientin- en totale polifenoliese (TP)-inhoud van ’n kommersiële gefermenteerde rooibos (FR)-ekstrak is deur die loop van die produksieproses gemonitor. Die effek van sproeidroging op FR- en ongefermenteerde rooibos (UR)-ekstrakte het veral aandag geniet. Die kwaliteit van kommersiële, Suid-Afrikaanse rooibos ystee, wat FR-ekstrak bevat, is ook ondersoek met betrekking tot die voorafgenoemde parameters. Daaropvolgend is die effek van verhitting en opberging op die fenoliese samestelling en kleur van eksperimentele ystees wat onderskeidelik FR, UR en nano ge-emulsifiseerde ongefermenteerde rooibos (NEUR)-ekstrak bevat het, ondersoek. Die gesamentlike effek van pH (pH 3-7) en opberging (5, 30 en 40ºC), asook die effek van hoë (660 mg/L, 0-7 dae by 30ºC; slegs UR) en lae (0.5 mg/L) konsentrasies H_2O_2, op FR-, UR- en NEUR-ekstrak oplossings is ondersoek. Laastens is agt rooibos ystees (vier variasies; ongegeur and suurlemoengeur) geanaliseer vir plantagtige, hooiagtige, rooibos- en suurlemoengeur, sowel as frankheid, met behulp van beskrywende sensoriese analiese. Verbruikervoorkeur van die gegeurde variante is met behulp van die nege-punt hedoniese skaal bepaal. In alle gevalle is veranderings in die individuele flavonoïedinhoud deur middel van HPLC-analiese gekwantifiseer. Die TP-inhoud van die ystees en kommersiële ekstrakte is met die Folin-Ciocalteu toets bepaal. Verbruining van die ystees en ekstrakoplossings is spektrofotometries gemonitor (420 nm).

Aspalatien, iso-orientin en orientin het voorgekom na alle stappe van die FR-ekstrak produksieproses. Sproeidroging, spesifiek, het geen negatiewe uitwerking op die inhoud van hierdie flavonoïede of die TP-inhoud van die FR- en UR-ekstrakte gehad nie. Ten spyte van relatiewe goeie behoud tydens verhitting en opberging van eksperimentele rooibos ystees, is bevind dat hierdie flavonoïede afwesig of teenwoordig is in baie klein hoeveelhede in kommersiële rooibos ystees. Hierdie bevindings dui daarop dat baie klein hoeveelhede rooibos ekstrak, ekstrak van swak kwaliteit of geen ekstrak vir die vervaardiging van die geanaliseerde ystees gebruik is nie. In die algemeen is verhoogde degradasie opgemerk vir sterilisasie teenoor pasteurisasie, terwyl verliese tydens opberging oor tyd toegeneem het. Die teenwoordigheid van sitroensuur, as gevolg van sy pH-verlagende effek, en askorbiensuur, as gevolg van sy antioksidant-aktiwiteit, was van kernbelang vir die behoud van aspalatien, iso-orientin en orientin tydens verhitting, maar minder belangrik tydens opberging. Die UR-ystees het in die algemeen beter gevaar as hul ooreenstemmende FR ystees, hoewel behoud van die voorafgenoemde flavonoïede beste was in NEUR-ystees. Verhitting en opberging het bruinwording veroorsaak in die meeste ystees, terwyl die TP-inhoud effens toegeneem het, of onverandered gebly het.

Die behoud van fenoliese verbindings in FR- en UR-ekstrakte het afgeneem met verhoogde pH en temperatuur, gepaardgaande met bruinwording. Die stabiliteit van aspalatien was by verre die beste in NEUR-ekstrak formulasies tussen pH 5 en 7. Ten spyte van verliese van aspalatien, het laasgenoemde ekstrak min verandering in absorbansie by pH 3 en 4 getoon.

Byvoeging van ’n hoë konsentrasie H_2O_2 het ’n onmiddelike verlaging in die fenoliese inhoud van die UR-ekstrak tot gevolg gehad, hoewel geen betekenisvolle ($P \geq 0.05$) veranderinge in absorbansie opgemerk is nie, wat op die vorming van kleurlose, oksidasie tussenprodukte gedui het. Die grootste verliese

is opgemerk in formulasies wat askorbiensuur bevat het. Dis aan die yster reduserende rol van hierdie verbinding toegeskryf, aangesien gereduseerde yster die tempo van die Fenton reaksie verhoog. Die byvoeging van H_2O_2 in klein hoeveelhede het slegs verliese van fenoliese verbindings in die FR-ekstrak veroorsaak, wat die hoogste ysterinhoud gehad het.

Ongeag die hoë aspalatien- en totale flavonoïed-inhoud van suurlemoen gegeurde ongefermenteerde rooibos ystee (UF/LEMON), het die verbruikers nie daarvan gehou nie. Voorkeur was in die teenoorgestelde rigting van die plantagtige geur van hierdie ystee en was in die rigting van rooibosgeur, ’n eienskap van gefermenteerde rooibos ystee. Ystee wat beide NEUR- en FR-ekstrak bevat het, het ’n produk gelewer wat 77% van die verbruikers as positief ervaar het. Die effense hooiagtige geur het nie die voorkeur van hierdie ystee in vergelyking met gefermenteerde rooibos ystee beduidend ($P \geq 0.05$) verlaag nie.

I would like to dedicate this thesis to my parents, Gerhard, Carol and all the Viljoens for supporting me every step of the way!

ACKNOWLEDGEMENTS

My sincere thanks to the following for their invaluable contributions:

Prof Lizette Joubert of the Division Post-Harvest and Wine Technology, ARC Infruitec-Nietvoorbij, as study leader, for her guidance, support and assistance in the completion of this study as well as her aid in the preparation of this thesis. It was a pleasure to work with someone with such a passion for research!

Dr Dalene de Beer of the Division Post-Harvest and Wine Technology, ARC Infruitec-Nietvoorbij, for her HPLC expertise, assistance in the laboratory and encouragement throughout the study. Furthermore, I would like to thank her for her aid in the preparation of this thesis. It was a pleasure sharing in her knowledge!

Dr Marena Manley of the Department of Food Science, Stellenbosch University, for her willing assistance as co-study leader and especially in the preparation of this thesis.

Miss Nina Muller of the Department of Food Science, Stellenbosch University, for advice with sensory analysis and encouragement throughout the study.

For assistance in the laboratory, Christie Malherbe and George Dico.

Dr Chris Hansmann of the Division Post-Harvest and Wine Technology, ARC Infruitec-Nietvoorbij, for help with initial pasteurisation experiments and encouragement throughout the study.

Prof Trevor Britz of the Department of Food Science, Stellenbosch University, for recommendations and use of a laboratory-scale pressure cooker as well as a bench-top autoclave.

For the statistical analysis of results obtained, the author would like to thank Marde Booyse and Frikkie Calitz of the Division Agrimetry, ARC Infruitec-Nietvoorbij.

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

The author would like to thank the Raps Foundation, Freising-Weihenstephan, Germany, and THRIP (Project TP2007072000012) for research funding.

I would like to express my gratitude to the Stellenbosch University, Pick ‘n Pay, Ernst and Ethel Eriksen Trust and SAAFoST for bursaries.

Gawie Pistorius of Parmalat for support with respect to the formulation of rooibos iced tea and supply of UHT fruit juice.

Dr Bernd Weinreich, R & D Manager at Raps Foundation, Freising-Weihenstephan, Germany, for technical advice and supply of rooibos extracts.

Riaan van Breda and Andre Van Zyl of Afriplex for supply of rooibos extracts and concentrates.

Mane flavours (Mark Mulholland and Ronel Haller) and Inessence (Rosie Maguire) for supplying flavours for the rooibos iced teas.

For assistance during the sensory analysis of the rooibos iced tea: Nicola Manning, Petrus van Eeden, Bonnie van Wyk, John Achilles and the final year Food Science students of 2008.

The Department of Animal Science, Stellenbosch University, for the use of their freeze drier and Resia Swart for technical support.

Employees at the Division Post-Harvest and Wine Technology, ARC Infruitec-Nietvoorbij as well as staff and post-graduate students at the Department of Food Science, Stellenbosch University, for support.

Gerhard, my family and the Viljoens for their love, encouragement, interest and support.

Above all, I thank God, through whom all things are possible.

ABBREVIATIONS

ABTS	2,2’-Azinobis(3-ethylenebenzothiazoline-6-sulphonic acid
ACC	Antioxidant activity
ADA	American Dietetics Association
AE	Antiradical efficiency
ANOVA	Analysis of variance
ARC	Agricultural Research Council
B	Base (rooibos extract in deionised water)
BA	Base + ascorbic acid
BC	Base + citric acid
BCA	Base + citric acid + ascorbic acid
BDE	Bond dissociation energy
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytirisole
CD	Conjugated diene
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DSHEA	Dietary Supplement and Health Education Act
EC_{50}	Effective concentration
EDTA	Ethylene diamine tetraacetic acid
EGC	(-)-Epigallocatechin
EGCG	(-)-Epigallocatechin gallate
EU	European Union
F	Fermented rooibos iced tea
FDA	Food and Drug Administration
F/LEMON	Lemon flavoured fermented rooibos iced tea
FOSHU	Foods for Specified Health Use
FR	Fermented rooibos
FR/UR	Fermented rooibos/unfermented rooibos
FR/NEUR	Fermented rooibos/nano emulsified unfermented rooibos
FRAP	Ferric ion reducing antioxidant parameter
GAE	Gallic acid equivalents
GI	Glycaemic index
HAT	Hydrogen atom transfer
HPLC	High pressure liquid chromatography
HPLC-DAD	High pressure liquid chromatography-diode array detection
HTS	High temperature sterilisation

IC_{50}	Inhibitory concentration
ICPS	Inductive coupled plasma spectrophotometer
i.d.	Inner diameter
IP	Ionisation potential
MRC	Medical Research Council
N	Nano emulsified unfermented rooibos iced tea
NE	Nano emulsified unfermented rooibos extract in deionised water
NEUR	Nano emulsified unfermented rooibos
NF	Nano emulsified unfermented rooibos iced tea/fermented rooibos iced tea
NF/LEMON	Lemon flavoured nano emulsified unfermented rooibos iced tea/fermented rooibos iced tea
N/LEMON	Lemon flavoured nano emulsified unfermented rooibos iced tea
NTS	Normal temperature sterilisation
NEC	Nano emulsified unfermented rooibos extract in deionised water + citric acid
o.d.	Outer diameter
ORAC	Oxygen radical absorbance assay
PTFE	Poly(tetrafluoroethene)
PV	Peroxide value
QUID	Quantitative ingredient declaration
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Revolutions per minute
SD	Standard deviation
SET	Single electron transfer
TAC	Total antioxidant capacity
TBARS	Thiobarbituric acid reactive substances
TBHQ	Tertiary butyl hydroquinone
TEAC	Trolox equivalent antioxidant capacity
TOSC	Total oxidant scavenging capacity
TP	Total polyphenol
TPTZ	Tripyridyl-*s*-triazine
TRAP	Total radical-trapping antioxidant parameter
UF	Unfermented rooibos iced tea
UF/LEMON	Lemon flavoured unfermented rooibos iced tea
UHT	Ultra high temperature
UR	Unfermented rooibos
US	United States
UV	Ultraviolet

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The language and style of this thesis is in accordance with the requirements of the *International Journal of Food Science and Technology*. This dissertation represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has therefore been unavoidable. Figures were placed at the end of each chapter to minimise interruptions to the text, especially for the research chapters, which contained numerous figures.

CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

Much attention has been given to the characterisation of natural antioxidant compounds such as flavonoids (Bors *et al*., 1990; Das & Pereira, 1990; Hanasaki *et al*., 1994), carotenoids (Gey *et al*., 1993; Pellegrini *et al*., 1999) and tocopherols (Raskin *et al*., 2002) over the past decade. This is due to their likely role in reducing the risk of developing life-threatening disorders such as cancer (Klaunig & Kamendulis, 2004), cardiovascular heart disease (Frei, 1995; Virgili *et al*., 2001), Alzheimer's disease (Valko *et al*., 2007) and Parkinson's disease (Guo *et al*., 2007). This potential beneficial role follows reports linking the consumption of a diet containing plenty of antioxidant-rich fruits and vegetables (Hertog *et al*., 1992; Hertog *et al*., 1993) with reduced disease incidence (Hertog *et al*., 1995).

The abovementioned research has prompted consumers to take control of their diet (Bech-Larsen & Scholderer, 2007), leading to an increased demand for natural, healthy, antioxidant-containing food products, i.e. food products with benefits beyond basic nutrition. In 2003, estimated sales of functional foods were US$11.7 billion and US$10.5 billion in Japan and the United States alone (Bech-Larsen & Scholderer, 2007). The top European markets were the United Kingdom (US$2.6 billion), Germany (US$2.4 billion), France (US$1.4 billion) and Italy (US$1.2 billion). According to a global survey by ACNielsen (2005), European consumers are less inclined to believe that functional foods have any health benefits. In contrast, respondents in South Africa, Brazil, Chile and Mexico are most convinced of the value of foods promoting health benefits. In fact, South African consumers proved to be the most regular buyers of products in six of the ten functional food categories surveyed (cholesterol reducing oils and margarines, yoghurts and *Acidophilus* cultures/probiotics, bread with added supplements/vitamins, whole grain/high fibre products, cereal with added folate and fruit juices with added supplements/vitamins). The remaining four categories were fermented drinks and 'good' bacteria, soy milk, milk with added supplements/vitamins and iodine enhanced cooking salt.

In 2006, the global functional food sector reportedly grew by 7.4%, i.e. 5% more than the rest of the food industry (Starling, 2007). Due to a connection with the health promoting properties of brewed tea, the consumption of ready-to-drink tea beverages has also increased significantly (Anon., 2006). According to this source, iced teas have been particularly popular in South Africa, with 30% of all new product launches in the tea and coffee sector arising from comparable (ready-to-drink) products.

Rooibos is a caffeine-free (Blommaert & Steenkamp, 1978), low tannin alternative to black and green tea. It is brewed from the leaves and stems of the endemic South African shrub, *Aspalathus linearis*, and has an agreeable, sweet, honey-like aroma in its fermented form (Joubert & Ferreira, 1996). Extracts of traditional ("fermented" or oxidised) rooibos are popular as ingredients in the food and beverage sector, particularly for use in products such as ready-to-drink iced tea and confectionary (Joubert & Schulz, 2006). Apart from its natural appeal, rooibos is the only source of aspalathin (Koeppen & Roux, 1966), a β-dihydrochalcone with significant

antioxidant (Von Gadow *et al*., 1997a; Joubert *et al*., 2004; Joubert *et al*., 2005) and antimutagenic activity (Snijman *et al*., 2007). Its uniqueness adds to the novelty of this herbal tea.

In order to develop the characteristic red-brown colour and pleasant, honey-like aroma of traditional rooibos, the plant material is fermented (oxidised) (Joubert & De Villiers, 1997). However, fermentation reduces the antioxidant activity (Von Gadow *et al*., 1997b) and most notably, the aspalathin content of rooibos (Joubert, 1996). Oxidative products of aspalathin are thought to include the flavones iso-orientin and orientin, although these compounds are also naturally present in the plant material (Koeppen & Roux, 1966). The latter two compounds are less effective than aspalathin in scavenging the synthetic radical, $DPPH^{\bullet}$, and superoxide anion radical, a physiologically relevant radical (Joubert *et al*., 2004). Aspalathin-enriched extracts are made from unfermented (green) rooibos plant material and find application in the cosmetic as well as nutraceutical industry (Tiedtke & Marks, 2002; Otto *et al*., 2003). Despite its superior antioxidant content, food and beverage products containing unfermented rooibos extract (such as rooibos iced tea) are not currently available on the South African market.

Despite the positive qualities of natural compounds such as flavonoids, their application within functional foods is not without challenges (Bagchi, 2006). Due to factors such as inherent genetic variation (Van Heerden *et al*., 2003) and differences in climate (Areias *et al*., 2000), the composition and active ingredient content of plants may vary considerably. Beyond this, the flavonoid profile of products such as fruits and vegetables depends upon their processing history (Ewald *et al*., 1999; Nicoli *et al*., 1999). Heat, high pH and prolonged storage are but a few of the factors known to have a detrimental effect on the flavonoid content of food. Processed products are regularly of inferior flavonoid quality compared to their unprocessed counterparts (Ewald *et al*., 1999; Lee & Howard, 1999).

One of the dilemmas surrounding the production of ethically sound and safe functional foods is the stability of the functional ingredients in the foods during and after processing. To date, no information has been found with respect to the fate of the flavonoids/antioxidants in rooibos beverages after heat processing, such as pasteurisation, storage or changes in pH. Despite the possible negative effects of processing, pasteurisation (Blackburn, 2000) and pH adjustment (Zhu *et al*., 1997; Lambert & Stratford, 1999; Battey & Schaffner, 2001; Battey *et al*., 2002) are essential for the microbiological stability of products such as iced tea. Aseptically packaged beverages may be further exposed to changes in phenolic/antioxidant composition if they come into contact with hydrogen peroxide, a reactive oxygen species (ROS), employed as a package sterilising agent (Stefanovic & Dickerson, 1986). Hydroxyl radicals, generated via the reaction of hydrogen peroxide with iron, have been noted to have a destructive effect on flavonoids (Namiki, 1990; Morris *et al*., 1995). Reaction of green tea catechins and hydroxyl radicals has been reported to deliver numerous oxidation products (Valcic *et al*., 2000). From this, it may thus be concluded that the reaction of rooibos flavonoids (in an iced tea) with hydrogen peroxide (hydroxyl radicals) may lead to their oxidation. The pH of the beverage (faster at acidic pH values, compared to neutral pH values, in the presence of excess hydrogen peroxide) (Jeong & Yoon, 2005), as

well as the inclusion of reducing agents, such as ascorbic acid, is known to increase the rate of hydroxyl radical generation via the Fenton reaction (Aruoma, 1994) and thus causes enhanced flavonoid destruction.

The use of new technologies such as micro and nanoencapsulation are on the increase. These provide not only a means by which the antioxidant activity of food/beverages may be preserved (Gouin, 2004), but may offer added benefits such as improved bioavailability (Back *et al*., 2006). In most encapsulated products, carriers also act as barriers that stabilise the ingredients during processing, making them easier to handle or improving their functionality (Morris, 2006). With the aid of nanotechnology, the aforementioned deleterious effects of processing may be minimised or completely removed (Anon., 2007). This could be of particular interest/use in the development of functional food and beverage products as it may provide a means of improving and ensuring the stability of functional ingredients, such as tea extracts and flavonoids, during and after processing.

Based on the above, it is possible that rooibos flavonoids such as aspalathin will undergo marked changes during processes other than fermentation. No data are, however, currently available with respect to this issue. This study aims to answer questions surrounding the processing stability of selected rooibos flavonoids to provide the industry with much-needed information/guidelines which may assist in the production of high-quality (in terms of phenolic content) beverage products. Iced tea was chosen as the vehicle for study as it is an example of a processed rooibos product on the South African market. Furthermore, certainty is required with respect to the assumed flavonoid content and health value of this beverage, as this is the current driver of its popularity. The aim of this study was thus to elucidate the effect of thermal processing (pasteurisation, normal temperature sterilisation and high temperature sterilisation), pH, ascorbic acid addition and storage on the stability of rooibos iced tea as assessed in terms of phenolic composition (HPLC), colour (absorbance at 420 nm) and antioxidant activity (Folin-Ciocalteu assay). In addition, the effect of hydrogen peroxide on the aforementioned parameters was also investigated. The matrices comprised fermented and unfermented rooibos extract powders, as well as a nano emulsified unfermented rooibos extract. Finally, descriptive and consumer sensory analysis were performed on the iced teas in order to characterise the sensorial properties of these beverages as well as test the liking of unfermented rooibos iced tea compared to commercially available fermented rooibos iced tea.

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CHAPTER 2

LITERATURE REVIEW

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LITERATURE REVIEW

1. BACKGROUND

There is a considerable amount of epidemiological evidence suggesting that regular consumption of polyphenol-containing fruits and vegetables (five servings a day) decreases the risk of developing circulatory diseases and cancers (Hertog *et al*., 1992; Hertog *et al*., 1993; Whitehead *et al*., 1995). It is not known exactly which compounds in plants and plant-based foods are responsible for this protective effect (Bors *et al*., 1990; Weisburger, 1999), although phytochemicals including folate, selenium, carotenoids, vitamins (vitamin C and E), flavonoids, phytoestrogens and isothiocyanates are all thought to play a role (Duthie *et al*., 2003; Serafini, 2006).

The role of polyphenols in health was initially highlighted by the finding that the French population have a remarkably low incidence of coronary heart disease despite the consumption of a high fat diet. This phenomenon was dubbed the French Paradox (Renaud & De Lorgeril, 1992) and was attributed to the regular consumption of red wine, containing high levels of phenolic compounds with antioxidant activity. Amongst others, findings such as these have lead to an increased demand for flavonoid-containing products (Arvanitoyannis & Van Houwelingen-Koukaliaroglou, 2005). As a result of this demand, new fruit cultivars are constantly being bred to achieve higher flavonoid levels (Capocasa *et al*., 2008; Jacob *et al*., 2008) and beverages such as iced tea are increasingly supplemented with flavonoid extracts such as Teavigo®.

Little doubt remains with respect to the presence of antioxidant compounds in foods such as fruit and vegetables. Questions do, however, surround the quantity and quality of the antioxidants in food, as well as their bioavailability (Katan & De Roos, 2004). Factors that affect e.g. the flavonoid content of food include environmental factors (Areias *et al*., 2000; Høgedal & Mølgaard, 2000; Celiktas *et al*., 2007), as well as procedures such as thermal processing (Torregrosa *et al*., 2006) and storage (Manzocco *et al*., 1998a).

This review gives an overview on functional foods, antioxidants, methods widely used for determining total antioxidant capacity and the changes in phenolic composition and antioxidant activity of plants and plant extracts as a result of heat, other forms of processing and storage. This forms the background for a discussion of rooibos as a source of flavonoid antioxidants.

2. FUNCTIONAL FOODS

2.1 Definition and use

“Let food be thy medicine and medicine thy food” (Hippocrates, 400 B.C.).

There are a variety of terms that have been used to describe foods that can be used for health improvement and disease prevention (Hasler, 1996). The most prominent are “designer foods”, “nutraceuticals” and

"functional foods". In 1989, the term "designer foods" was used to describe "foods that naturally contain or are enriched with non-nutritive, biologically active chemical components of plants (e.g. phytochemicals) that are effective in reducing cancer risk". The term "nutraceuticals", however, refers to: "a food or parts of food that provide medical or health benefits, including the prevention and/or treatment of disease" (DeFelice, 1995). Lastly, functional foods have been described as those foods that "encompass potentially healthful products, including any modified food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains". A more accurate definition of functional foods is perhaps that of the American Dietetics Association (ADA). The latter organisation defines functional foods as "foods that have a potentially beneficial effect on health when consumed as part of a varied diet, on a regular basis, at effective levels" (Hasler *et al*., 2004). These foods include whole foods as well as fortified and enriched foods. In South Africa, fortification refers to the addtion of one or more specific micronutrients to a foodstuff with the purpose of preventing/correcting a demonstrated deficiency in the general population (Anon., 2007a). Enrichment is the addition of nutrients to food (whether or not it is normally contained in the food) for the purpose of adding nutritional value to the food (Anon., 2007a).

The increasing demand for functional foods can mostly be ascribed to mounting scientific evidence connecting the consumption of a healthy diet with reduced disease incidence, the increasing cost of healthcare and changing food regulations (Arvanitoyannis & Van Houwelingen-Koukaliaroglou, 2005). The concept of functional foods is, however, far from being universal, since the definition varies between countries and even between companies.

Functional foods may be differentiated from traditional foods based on their specified physiological advantages above the latter (Arvanitoyannis & Van Houwelingen-Koukaliaroglou, 2005). The key to the functionality of functional foods is their bioactive ingredients or health-enhancing dietary components. Functional foods are most commonly classified according to their bioactive ingredient composition: amino acids, fibre, herbs, minerals, omega-3 fatty acids, peptide-proteins, probiotics, phytochemicals and vitamins. Prebiotics and probiotics are probably the most well known of the functional foods, their claim to fame being the maintenance of "good bacteria" or "intestinal flora" and the promotion of colon health.

The development of foods and food components that provide benefits beyond their traditional nutritional value has created tremendous academic, commercial, regulatory and public interest (Frankel & German, 2006; Gulati & Ottaway, 2006). These products (nutraceuticals, functional and fortified foods) cover the areas of health promotion, optimal nutrition, enhanced performance (physical and mental) and disease reduction. Functional and fortified foods are those with a similar appearance to their traditional counterparts, whilst nutraceuticals may be used in specific doses (tablets, capsules or liquids commonly known as food/dietary supplements).

The unique features of functional foods are that they (Gulati & Ottaway, 2006):

(i) Are conventional or everyday foods.

(ii) Are consumed as part of the normal diet.

(iii) Are composed of naturally occurring components, sometimes in unnatural proportions.

(iv) Have a positive effect on the "target function" beyond nutritive value (basic nutrition).

(v) May enhance well-being and health and/or reduce the risk of disease or provide health benefits so as to improve the quality of life including physical, psychological and behavioural performances.

(vi) Have authorised and scientifically substantiated health claims.

The concept "target function" refers to genomic, biochemical, physiological, psychological or behavioural functions that are relevant to the maintenance of a state of well-being and health or to the reduction of the risk of disease (Cansev *et al*., 2008). Modulation of these functions should be quantitatively/objectively evaluated by measuring:

(i) Biological markers (for example metabolites, proteins, hormones and enzymes).

(ii) Physiological parameters (for example blood pressure, heart rate, gastrointestinal transit time and blood glucose level).

(iii) Changes in physical and intellectual performance (for example, omega-3 fatty acids are known to enhance brain function) (Cansev *et al*., 2008).

After 1994, development of the nutraceutical concept was largely driven by the United States (US) (Gulati & Ottaway, 2006). Introduction of the Dietary Supplement and Health Education Act (DSHEA) can be seen as the greatest single factor promoting the development of the nutraceutical industry as it offered considerable flexibility to the border between food and medicine. This was not the case in other countries/areas of the world. According to DSHEA, a dietary supplement may contain "a herb or other botanical" or "a concentrate, metabolite, constituent, extract or combination of any ingredient from the other categories". Under this Act, many botanicals and other substances have been sold as dietary supplements in the US, including some that are regulated in European Union (EU) countries.

2.2 Interest in antioxidants as functional ingredients

The increased life expectancy of many people today, has led to a dramatic increase in the incidence of diseases that generally occur later in life (Duthie *et al*., 2003). With respect to diseases such as cancer, the discovery of radical-induced damage in affected tissues has prompted scientists to investigate the role of free radicals in the progression of such diseases (Higdon & Frei, 2003). As a result, particular attention is being paid to nutrients or compounds capable of reducing or eliminating free radicals from food as well as from the human body, and therefore the interest in antioxidants.

As a result of its antioxidant and antimutagenic activity, tea (from *Camellia sinensis*) is a good example of a functional beverage that has attracted a significant amount of attention due to its connection with health-promoting properties (Tijburg *et al*., 1997). Tea is usually consumed after brewing tea leaves or tea bags in boiling water. It is also consumed in a ready-to-drink format, namely iced tea. Extracts of tea may be prepared in a variety of physical forms to meet the food industry's application requirements, such as concentrated infusions, dry powders or pure catechins (Wang *et al*., 2000).

Due to the inherent antioxidant properties of tea extracts, the latter can be used to market and improve the appeal of a great variety of products (Wang *et al*., 2000). Cereals, baked goods, ready-to-drink beverages and health foods are but a few examples of areas where green tea extracts may find application in

the food industry. Tea extracts may even be used to replace artificial antioxidants in products such as meat (Bañón *et al*., 2007). In foods such as ice-cream (Jiang *et al*., 1995), noodles and salad dressings (Yang *et al*., 1995), tea extracts add "health" appeal. Green tea extracts are also used to improve the shelf-life and flavour characteristics of Moon cakes, a traditional delicatessen enjoyed during the Chinese Middle Autumn Festival (Wang *et al*., 2000).

The manufacturers of toothpastes, mouthwashes, breath fresheners and chewing gum have also begun to incorporate tea extracts in their formulations (Yoshinori *et al*., 1987; Miki *et al*., 1991; Yasuda, 1992). The claims "slows tooth decay", "anti-caries" and "freshens breath" arise from the antibacterial (Sakanaka, 1991) and deodorising effect of tea catechins (Yasuda, 1992). Tea extracts are also being increasingly used in skin and hair-care products such as shampoos, moisturising creams, perfumes and sunscreens (Alexis *et al*., 1999). The tea extracts presumably have a soothing effect on the skin and protect it from the action of free radicals. Although such applications may seem novel, the Chinese have been using green tea extract in products such as toothpaste and shampoo for more than ten years (Wang *et al*., 2000). Future innovations may include green tea enriched fruit juices and beverages designed to "help smokers to stop smoking" (Wang *et al*., 2000).

2.3 Promises and problems

The greatest claimed problem with functional foods is the term itself, as it is considered misleading (Arvanitoyannis & Van Houwelingen-Koukaliaroglou, 2005). It is argued that all foods, whether they contain biologically active ingredients or not, are functional at some level – even if the food merely contributes kilojoules to the diet.

Although functional foods claim to contain specific functional ingredients, the levels of these ingredients are not regulated (Katan & De Roos, 2004). The actual level of a functional ingredient in a particular food will depend on the quantity and quality of the ingredient added to the product, as well as its stability during processing and storage. It is well documented that many food-borne antioxidants can be lost as a result of processes such as pasteurisation, sterilisation and dehydration (Manzocco *et al*., 1998a; Ewald *et al*., 1999). In addition, interactions with other components within the food matrix, such as protein, may render the antioxidants useless (Arts *et al*., 2001).

"The right dose differentiates a poison from a remedy" (Paracelsus)

Due to their "natural" or "health promoting" image, many consumers believe that functional food products may be consumed without giving any thought to dosage (Bagchi, 2006). This assumption neglects the fact that approximately one third of the world's pharmaceutical drugs were originally isolated from plant material (Bent & Ko, 2004). Eisenberg *et al*. (1998) found that the percentage of adults using herbal medicines in the United States (US) has increased from 3% in 1990, to over 12% in 1997. This self-medication trend is understandable, as the cost of healthcare is said to be "skyrocketing" (Hasler, 1996). Care should, however, be taken in the use of herbal medicines, especially in the light of recently discovered

herb-drug interactions, which can alter the efficacy of prescribed medicines (Zhou *et al*., 2007). Unfortunately, the fact that functional foods exist at the interface between foods and drugs (Hasler, 1996) has meant that, until recently, no provision has been made for them in the US food regulations (similar to those in South Africa). Passing of the DSHEA in 1994 saw a boom in the functional food industry as it exempted dietary supplements from the stringent regulations imposed upon drugs and most food additives (Hasler, 1996; Thomson *et al.*, 1999).

During the 1980's, Japan was the first country to introduce the term "functional foods" (Ohama *et al*., 2006). These foods are identified by the phrase Foods for Specified Health Use (FOSHU). To date, Japan is the only country that regulates the introduction of functional foods into the market. The latter also summarises the greatest problem with this food category, namely a lack of legislation governing the levels and efficacy of functional ingredients (Arvanitoyannis & Van Houwelingen-Koukaliaroglou, 2005). It is predicted that, unless such laws are implemented, dubious products will cause consumers to lose their faith in the product category as a whole and functional foods will merely be remembered as a fad of the 21st century.

Finally, the use of new technologies for the production of "improved" functional foods and ingredients necessitates re-evaluation of their safety (Weiss *et al*., 2006). Nanotechnology is a new technique/expertise that may be used to alter the functionality of food ingredients. With the help of this technology, the absorption (Back *et al*., 2006) and bioavailability of known compounds may be altered (Weiss *et al*., 2006), making current knowledge with respect to toxicity, obsolete. According to the Food and Drug Administration (FDA), foods that include ingredients that were produced with the help of nanotechnology are subject to the same standard safety tests as are currently standard for existing food products.

2.4 Pro-oxidant activity

Despite the fact that flavonoids are primarily researched for their positive effect on human health, the extensive usage of concentrated nutraceutical products has been associated with pro-oxidant activity and oxidative damage in the human body (Yoshino *et al*., 1999; Heim *et al*., 2002).

Pro-oxidant activity is, however, also important for normal cell functioning (Salganik, 2001). Reactive oxygen species (ROS), for example, play an integral role in the apoptosis of cancerous cells. Removal of pro-oxidants from individuals with cancer may thus not be desirable as the use of excessive antioxidants can inhibit production of $O_2^{\cdot -}$ and other ROS. The effect of flavonoids in the diet is thus likely dependent upon the health status of the individual (Salganik, 2001).

The availibility of iron in the human body will also play a determining role in pro-oxidant activity, due to its involvement in the Fenton reaction (a process that results in the formation of hydroxyl radicals) (Salganik, 2001). The availibility of iron in the human body is, however, dependent upon a number of factors such as disease status (Gutteridge & Halliwell, 1994) and life stage (Dai *et al*., 2008).

In the human body, iron is stored and transported in the proteins ferritin and transferrin, respectively (Dai *et al*., 2008). Saturation of these proteins with iron, due to diseases such as idiopathic

haemochromatosis (an inherited disease resulting in iron overload), results in greater plasma levels of "free" iron (Gutteridge & Halliwell, 1994). Free iron is a dangerous catalyst of oxidative reactions, whilst iron bound to ferritin is not available to catalyse free radical reactions (Gutteridge & Halliwell, 1994). Compounds such as ascorbic acid also affect an individual's iron status: iron absorption is improved in the presence of ascorbic acid (Gutteridge & Halliwell, 1994). Furthermore, the latter also plays a direct role in pro-oxidant activity by increasing the rate of hydroxyl radical formation via the Fenton reaction (Ratty & Das, 1988).

As is known for antioxidant activity, the number of OH groups on a flavonoid is thought to be directly proportional to its pro-oxidant activity (Cao *et al*. 1997). This is especially true when the OH groups occur predominantly on the B-ring (Hanasaki *et al*., 1994). Structural features such as an unsaturated 2,3-bond and a 4-keto group, which are important for antioxidant activity, also contribute to pro-oxidant activity under specific conditions (Cao *et al*., 1997).

Although the dangers of flavonoid-related pro-oxidant activity indirectly suggest abstinence from flavonoid supplements, evidence for the positive health effects of a diet rich in flavonoids remains. Further information with respect to the absorption and metabolism of these compounds is required, as current understanding of these processes in the human body is limited to a small number of flavonoids, and even here, knowledge is limited (Hollman & Katan, 1998). In all aspects of life, the human body strives for homeostasis (Tiwari, 2001), so perhaps it would be best to approach the intake of dietary constituents with an equal sense of balance.

2.5 New methods for the delivery of functional food ingredients

2.5.1 Microencapsulation

Encapsulation is a technique that can be used to protect functional ingredients from oxidation, evaporation or other compound-altering reactions in food (Karathanos *et al*., 2007). This technology has seen extensive application in the cosmetic and pharmaceutical industry but, until recently, has not been broadly applied in the food industry. Applications have mainly been limited to the flavour sector where encapsulation has facilitated the production of controlled flavour-release products and easily-manageable dry powders. Improvements gained through encapsulation include easier handling and processing, improved or altered solubility, increased shelf-life (reduced oxidation), masking of odour/taste/colour, controlled release of active ingredients, reduced volatility (as with flavours) and controlled initiation of reactions (Karathanos *et al*., 2007).

Microencapsulation may be defined as "the packing or coating of liquids, solids or gases with a thin protective layer or wall material, which inhibits volatilisation and protects against chemical deterioration" (McNamee *et al*., 1998). This process does not only improve the effectivity of food ingredients, but allows for their application in foods where it was previously not possible (Gouin, 2004). The most common technologies used to produce encapsulated food ingredients are spray-drying and extrusion.

Until recently, cyclodextrins have mostly seen application in the pharmaceutical industry where they are used as drug-complexing agents (Calabrò *et al*., 2005). Cyclodextrins increase the solubility and

bioavailability of therapeutic agents. Depending on the geometry of the guest molecule, either the entire molecule or a part thereof may be included in the cavity. Whilst inside this cavity, the guest molecule is protected from heat or light-induced changes (Granero *et al*., 2002; Karathanos *et al*., 2007). Apart from having a protective role, cyclodextrins also promote the growth and excretion of Bifidobacteria in the colon (Szejtli, 1998). The use of cyclodextrins in the food industry may thus be beneficial as Bifidobacteria are natural inhabitants of the human intestine and are associated with the maintenance of good colon health (Salminen & Isolauri, 2006).

2.5.2 Nanotechnology

Nanoencapsulation may be seen as an improvement on microencapsulation. Nanoencapsulation is the encapsulation of compounds, extracts or other materials within nano-sized micelles (Weiss *et al*., 2006). According to the National Nanotechnology Initiative, "Nanotechnology is the understanding and control of matter at dimensions of 1-100 nm". Nanoscale technology enables the design of solutions capable of controlled delivery as well as the application of sensitive ingredients in areas where they could not previously be used (Forssell *et al*., 2006). Food ingredients such as nanoparticulate lycopene and carotenoids are already commercially available, the bioavailability of which is typically higher than that of their traditional counterparts (Weiss *et al*., 2006). In a study performed by Back *et al*. (2005), a water-soluble, nanoencapsulated form of vitamin E was shown to improve the vitamin E status of cystic fibrosis patients. Conventional supplements cannot be used to improve the vitamin E status of such people as fat digestion and absorption are chronically impaired.

Nanotechnology may also be used to create solubilisates (emulsion-like solution in which the micelles are less than 30 nm in size and are completely water soluble) of functional food ingredients (Anon., 2007b). The advantage of such a solubilisate, compared to "bare" ingredients, includes high dermal penetration and intestinal absorption of the micellated active ingredients (Anon., 2007b). Due to higher absorption and bioavailability/penetration of active substances, the concentrations of the substances in the solubilisates can be reduced. In comparison to emulsions or liposomes, the solubilisate micelles are thermally, mechanically and pH-stable, as well as having lower susceptibility to microbiological damage (Anon., 2007b). The micelles are completely and irreversibly water soluble and completely transparent in aqueous solution. No special product/matrix design is required as the solubilisate may be incorporated directly into the final product (Back *et al*., 2005).

2.6 Legislation pertaining to food and functional food products/ingredients in South Africa

The "reduction of disease risk claims" which may be made on the labels of food/functional food products are subject to strict control in South Africa. The 19 foods and food constituents that have been identified in the draft regulation of the Foodstuffs, Cosmetics and Disinfectants Act (Act 54 of 1972, Regulation no. 642 of 20 July 2007) for which reduction of disease risk claims may be made are listed in Section 62 (Anon., 2007a). The latter claims are, however, subjected to the foodstuff meeting the requirements set out in Section 62, paragraph (b) to (g) and Table 2. For example, the exact claim "diets low in sodium may reduce

the risk of high blood pressure, a disease associated with many risk factors, in some individuals" may be made on the packaging of a foodstuff having a low sodium content. According to Section 57 of the Act, a low sodium content implies that the product contains 120 mg (or less) sodium/100g.

The release of the draft regulations of the Act has seen the addition of numerous food compounds/constituents to the list of items for which "reduction of disease risk claims" may be made. Claims pertaining to psyllium fibre, plant sterols and stanol esters, walnuts and omega-3 fatty acids are some of the additions to the Act. The Act does not, however, recognise the possible health promoting properties of any form/type of tea, but special mention has been made of iced tea.

2.6.1 Iced tea

"New products and uses are emerging and tea is consumed in different manners. Iced tea is now a convenient alternative to soft drinks and is found in grocery stores and fast food facilities" (Dufresne & Farnworth, 2001).

In Annexure 6 of the newly released draft regulations of the Foodstuffs, Cosmetics and Disinfectants Act (Act 54 of 1972, Regulation no. 642 of 20 July 2007), iced tea is considered a "foodstuff not considered essential for a healthy diet and for which no nutrient content, GI (glycaemic index), certain comparative, health, slimming or any other claim with a health or nutritional message will be permitted" (Anon., 2007a).

Iced tea has been included in Annexure 6 of the new draft regulation for three major reasons (Van der Riet, Y., senior medical natural scientist, South African Department of Health, personal communication, 2007):

(i) Many iced teas have a high sugar content.

(ii) Many iced teas contain non-nutritive sweeteners, the safety of which are still under investigation (aspartame was specifically mentioned).

(iii) Many of the iced teas on the market do not contain sufficient proportions of the beneficial ingredient(s). It is debateable whether all so-called iced teas of the South African market contain tea extract.

Products listed in Annexure 6 of the Act may not be advertised to children in any way (Section 52(2) and may not make any health claims (Section 59) (Anon., 2007a). Such products may, however, make a quantitative ingredient declaration (QUID, Section 28). In such a declaration, the percentage of the emphasised ingredient may be stated on the label: for example, total polyphenol (TP) content. This allows the consumer to discriminate between products on this basis, should he/she decide to do so.

Several iced teas containing fermented rooibos and green tea extracts are on the market in South Africa. The past few years have seen an escalation in the popularity of iced teas, judging from the increase in the branded products available to the consumer (Anon., 2006). Although not allowed to make any health claims, these products are associated with health benefits, due to the well-researched link between tea, antioxidant activity and health (Anon., 2006).

2.7 Future challenges

According to Gulati & Ottaway (2006), the future challenges of the nutraceutical market relate to quality, efficacy and safety. There are still many unknowns: the identification of the specific active components; the effect of absorption and metabolism as well as the effect of processing on the activity of the functional ingredients are but a few of the constraining factors. Botanical materials (fragmented parts of plants, algae, fungi, lichens or preparations of all the aforementioned) represent a large segment of the nutraceutical market. This fact presents a problem as botanicals are complex in nature and vary inherently in composition. In future, standardisation of functional ingredients may be essential in order to produce functional foods of consistent quality (Gulati & Ottaway, 2006).

3. FREE RADICALS AND ANTIOXIDANTS

3.1 Free radicals - general

According to Halliwell *et al*. (1995a) a free radical is "any species capable of independent existence that contains one or more unpaired electrons, an unpaired electron being one that is alone in an atomic or molecular orbital". Radicals, including ROS and reactive nitrogen species (RNS), are continuously produced by the body as a result of energy producing reactions (Serafini, 2006). Radicals may also be formed as a result of oxidative bursts by phagocytes or enzyme systems. Cigarette smoke, pollution and physical exercise constitute exogenous sources. The presence of radicals is essential for proper cell functioning. An excess of these molecules, however, lead to oxidative stress. Oxidative stress is a shift in the redox balance of a cell towards an oxidative state. Biological molecules, such as lipids, proteins, carbohydrates and deoxyribonucleic acid (DNA), may be damaged during oxidative stress, in which case, altered cell function results and cell death becomes unavoidable (Halliwell *et al*., 1995a; Halliwell *et al*., 1995b). Oxidative stress is associated with a variety of bodily disorders, including cancer and coronary heart disease (Frei, 1995; Halliwell *et al*., 1995a; Halliwell *et al*., 1995b).

Antioxidants may counteract the effects of ROS and RNS, either by preventing their generation or by reducing their numbers (Serafini, 2006). Examples of the above include metal chelation (no redox reaction) and enzyme catalysed oxidant removal, respectively. When antioxidants remove ROS or RNS via reduction, a redox reaction takes place (Serafini, 2006). During this reaction, the oxidant (ROS or RNS) reacts with the antioxidant instead of the substrate, protecting the latter. Non-enzymatic antioxidants may also be considered reductants (Serafini, 2006). Reductants take part in redox reactions where they are preferentially oxidised to the substrate.

3.2 Radical formation and the Fenton reaction

In 1934, Haber and Weiss proposed that $^{\bullet}OH$ could be formed from $O_2^{-\bullet}$ and H_2O_2 according to the reaction:

$$O_2^{-\bullet} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + O_2^{\bullet} \qquad [1]$$

The rate constant for this reaction is, however, low (Halliwell & Gutteridge, 1982). A modified version of the Haber-Weiss reaction, the Fenton reaction, makes use of iron to increase the rate of the reaction (Namiki, 1990; Morris *et al*., 1995).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + {}^{-}OH \quad [2]$$

From the above, it is evident that hydroxyl radical formation is largely dependent upon the availability of iron. Beverages such as tea and rooibos naturally contain both iron (Reyneke *et al*., 1949; Gallaher *et al*., 2006) and flavonoids (Blommaert & Steenkamp, 1978; Tijburg *et al*., 1997; Wiseman *et al*., 1997). During aseptic packaging, hydrogen peroxide is used to sterilise the surfaces of the beverage's packaging material (Stefanovic & Dickerson, 1986) in order to ensure product shelf-stability. Although the majority of the peroxide is removed from the packaging material prior to aseptic filling, a small residue may remain. A FDA regulation currently limits residual H_2O_2 to 0.5 mg/L in finished food products (Anon., 2000). Hydroxyl radical formation via Fenton-type reactions is thus theoretically possible in aseptically packaged flavonoid-containing beverages such as iced tea. The study by Zhu *et al*. (2000) supports this idea, as the addition of hydrogen peroxide to tea (*Camellia sinensis*) resulted in flavonoid losses.

3.2.1 The pH requirement of the Fenton reaction

The rate of the Fenton reaction is pH dependent (Hsieh & Hsieh, 1997) and the pH requirement of the reaction is dependent upon the concentration of hydrogen peroxide present (Jeong & Yoon, 2005). In the absence of externally supplied H_2O_2, the rate of the Fenton reaction is enhanced with increasing pH. Small amounts (< 0.1 mM) of hydrogen peroxide may be formed in bottled tea and canned coffee immediately after opening (Aoshima & Ayabe, 2007). This value varies between 0.15 and 0.6 mM, depending on the beverage (tea or coffee), upon exposure to air. Small amounts of hydrogen peroxide have also been detected in herbal teas after preparation with hot water (Aoshima *et al*., 2007). Storage at 25°C led to further production of hydrogen peroxide with greater amounts forming in teas incubated in a phosphate buffer (pH 7.4), a pH equivalent to that in the human intestines. Addition of thorn apple and hibiscus herbal tea to catechin enriched green tea decreased the production of hydrogen peroxide (Aoshima *et al*., 2007). This was ascribed to the pH-lowering effect of the fruit teas as the addition of citric, malic, succinic and fumaric acid also reduced H_2O_2 production (Aoshima & Ayabe, 2007).

In the presence of excess H_2O_2, however, the rate of the Fenton reaction is reduced with increasing pH (Jeong & Yoon, 2005). A low pH environment is required for the progression of the Fenton reaction, in the presence of H_2O_2, in order to prevent $Fe(OH)_3$ precipitation (Li *et al*., 2007). Citric acid is commonly used as an ingredient to lower the pH of beverages such as iced tea (Zhu *et al*., 1997; Lambert & Stratford, 1999; Battey & Schaffner, 2001; Battey *et al*., 2002). Despite the metal chelating ability of citric acid (Francis *et al*., 1992; Dodge & Francis, 2002), the Fenton reaction has been reported to occur in mixtures containing both hydrogen peroxide and citric acid, at pH values ranging from 3-8 (Zepp, 1992).

3.2.2 Flavonoids and the Fenton reaction

According to Aruoma (1994), the rate of the Fenton reaction can also be greatly accelerated by reducing agents such as ascorbic acid. This is due to the reduction of Fe^{3+} to Fe^{2+} by ascorbic acid. Ascorbic acid is, however, not the only compound capable of increasing the rate of the Fenton reaction in this manner. Laughton *et al*. (1989) and Puppo (1992) have shown that flavonoids accelerate hydroxyl radical generation in Fenton-type reactions, by reducing Fe^{3+} to Fe^{2+}. At low concentrations, flavonoids may act as pro-oxidants, whilst at high concentration they scavenge H_2O_2, thus acting as antioxidants (Joubert *et al*., 2005). The pro-oxidant activity of these compounds at a low concentration may be attributed to Fe^{3+} reduction (Aruoma *et al*., 1993), whilst H_2O_2 scavenging (and thus reduced production of $^{\bullet}OH$) is responsible for antioxidant activity.

Apart from the work by Zhu *et al.* (2000), very little information is available on the oxidation products resulting from the reaction between flavonoids and H_2O_2. Reaction of (−)-epigallocatechin gallate (ECGC) and (−)-epigallocatechin (EGC) with hydrogen peroxide led to oxidation and decarboxylation of the A-ring on the catechin molecule (Zhu *et al*., 2000) (Fig. 2.1). This type of oxidation product differs from that resulting from reaction with peroxyl radicals, where the B-ring was the site of antioxidant reactions (Valcic *et al*., 2000; Sang *et al*., 2003). Krishnamachari *et al*. (2000) showed that peroxy radical oxidation of quercetin led to the formation of a heterodimer. The presence of a free 3-OH and a B-ring 3′,4′-OH unit appears essential for dimer formation. Under different chemical oxidative conditions (alkaline ferricyanide), Gülsen *et al*. (2007) confirmed the formation of the same quercetin heterodimer. Different oxidation products may thus result from the use of different oxidants and the location of the main antioxidant site on flavonoids depends on the oxidant used.

3.3 Antioxidants - general

Due to its biological nature, food is inevitably prone to deterioration (Namiki, 1990). Biological changes brought about by microbes and chemical reactions trigger various forms of deterioration in food during production, processing, distribution and storage. Chemical changes are represented mostly by the enzymatic and non-enzymatic oxidation of lipids and phenolic substances. The latter results in undesirable changes in the flavour, nutritional value, appearance, physical character, colour and toxicity of food (Namiki, 1990).

Oxidation is a chemical reaction characterised by the loss of one or more electrons from an atom or molecule. It is associated with a reduction reaction during which another molecule gains the released electrons. During oxidation-reduction (redox) reactions the number of electrons lost by the reductant (electron donor) is equal to the number of electrons gained by the oxidant (electron acceptor) (Prior & Cao, 1999).

The airtight packaging or deoxygenation of food plays an important role in the extension of the shelf-life of many food products (Namiki, 1990; Shahidi & Wanasundara, 1992). The role of antioxidants in food (as additives or constituents) can, however, not be omitted. According to Halliwell *et al*. (1995b), an antioxidant is “any substance that, when present at low concentrations compared to that of an oxidisable substrate, significantly delays or prevents oxidation of that substrate”. Antioxidant reactions are thus

chemical reactions that involve the transfer of electrons from an antioxidant to the molecule being protected (which is reduced) (Prior & Cao, 1999). During this reaction, the antioxidant is oxidised. The oxidised form of the antioxidant may then become a pro-oxidant. Ascorbic acid has the ability to be both an antioxidant and pro-oxidant, easily donating electrons to another molecule (ascorbic acid becomes dehydroascorbic acid), after which it again needs to be reduced.

3.4 Classification of antioxidants

Antioxidants generally exhibit an effect on oxidation in two ways, namely by scavenging free radicals or by inhibiting/retarding their formation (Shahidi & Wanasandara, 1992; Gordon, 2001). Primary antioxidants (chain-breaking antioxidants) react directly with lipid radicals, converting them into more stable products. The antioxidant radicals are stabilised by kinetic or resonance factors, making them less reactive and thus less capable of participating further in radical propagation. Primary antioxidants are utilised in the period before oxidation occurs (induction period) and result in a reduced reaction rate during this time (Gordon, 2001). The duration of the induction period is directly proportional to the antioxidant concentration, until a certain maximum level. At this point, additional protection is not observed, or it is reduced. The effectivity of antioxidants added to substrates containing large amounts of radicals is also very reduced or negligible.

Secondary antioxidants may reduce the rate of radical chain initiation by retarding radical formation. This may occur via scavenging oxygen, chelating metal ions, absorbing ultraviolet (UV) radiation, deactivating singlet oxygen or converting hydroperoxides to non-radical species (Gordon, 2001).

3.5 Factors influencing the stability and activity of antioxidant compounds

3.5.1 Physical factors

Physical factors such as heating, irradiation, a high oxygen pressure or a large surface area in contact with oxygen are all factors which may accelerate the initiation of the oxidation process and the speed of radical propagation (Yanishlieva-Maslarova, 2001). The presence of radicals results in a faster depletion of added antioxidants. For this reason, food products are usually stored in light or air-impermeable packaging (vacuum packed) or kept at a low temperature. Oxidation may also alter the colour of a food or beverage product. For example, oxidation during maturation is known to result in a change in the colour of wine from red-blue to reddish-brown (Atanasova *et al.*, 2002).

3.5.2 Structure

Flavonoids can be divided into flavanols, flavonols, flavones, flavanones, anthocyanins, chalcones and dihydrochalcones. They are compounds that have a C_6-C_3-C_6 configuration (Machieux *et al.*, 1990). They have two aromatic rings joined by an aliphatic three-carbon chain and have OH, CH_3 or glycosyl groups attached at various points on the skeleton.

The most important factor influencing the antioxidant activity of a compound is the ability to form a stable free radical. Based on this feature, phenolic compounds are generally highly reactive antioxidants as they possess an aromatic ring system, capable of stabilising free radicals (Rice-Evans *et al.*, 1996). The

phenolic radicals are generally not very reactive, therefore preventing propagation of the free radical chain reaction.

There are varying opinions with respect to the identity of the structural groups that are responsible for the antioxidant activity of flavonoids (general structure in Figure 2.2). The antioxidant activity of flavonoids is generally related to the position and number of OH groups present (Rice-Evans *et al*., 1996). Three, distinct structural features contribute to the majority of the radical scavenging ability of flavonoids:

(i) The 3′,4′-OH group of the B-ring, participating in electron delocalisation.

(ii) The 2,3-double bond in the C-ring, in combination with a 4-keto group, is responsible for electron delocalisation on the B-ring. The antioxidant activity of flavonoids is thus directly related to structure, since the flavonoid radical formed (after interaction of a flavonoid and a free radical) is stabilised by the resonance effect of the aromatic nucleus (Fig. 2.3).

(iii) The 5-OH (A-ring) and 3-OH groups (C-ring), together with a 4-keto group (C-ring), maximises scavenging potential.

Based on the structural features mentioned above, quercetin is one of the most effective flavonoid antioxidants as it possesses a 3′,4′-OH group, a 2,3-double bond and a metal chelating area (5-OH and 3-OH groups on the A-ring and C-rings, respectively, in combination with the 4-keto group) (Rice-Evans & Miller, 1998). These groups are highlighted on the structures of quercetin and catechin in Figure 2.4.

Another structural feature capable of enhancing antioxidant activity, is the presence of an additional OH group at the 5′ position of a flavonoid. The presence of a single OH group in the B-ring, however, is not very effective. Kaempferol, with a single B-ring OH group, has only 27% of the antioxidant capacity of quercetin (two OH groups on the B-ring). The occurrence of two B-ring OH groups in the *meta*-position, with respect to each other, also does not contribute to enhanced antioxidant activity. The effect of this structural feature on the antioxidant activity of various flavonoid compounds is reflected by their Trolox equivalent antioxidant capacity (TEAC) values (Table 2.1). Morin, having B-ring OH groups in the *meta*-position has a TEAC value of 2.6 mM whereas quercetin, with an 3′,4′-OH arrangement, has a value of 4.7 mM (Table 2.1). The presence of a third OH group on the B-ring does not, however, increase antioxidant activity. The antioxidant activity of myricetin, with three OH groups on the B-ring, is lower than that of quercetin with only two OH groups (Table 2.1). Compared to kaempferol with only one OH group the TEAC value of myricetin (three OH groups) is higher. The TEAC values of myricetin and kaempferol are 3.1 mM and 1.3 mM, respectively (Table 2.1). This order, quercetin>myricetin>kaempferol, does not necessarily remain when other test systems are used. Husain *et al*. (1987) found that hydroxyl radical scavenging efficiency decreased in the order myricetin>quercetin>kaempferol, whereas Özyürek *et al*. (2008) reported that hydroxyl radical scavenging efficiency decreased in same order as that reported for the TEAC assay.

The presence of an OH group at C_3 (C-ring) causes the antioxidant activity of flavonols to be enhanced, compared to the corresponding flavones (4.7 mM for quercetin compared to 2.1 mM for luteolin) (Table 2.1). Saturation of the 2,3-double bond also decreases antioxidant activity. The effect of this structural change is seen when comparing the antioxidant activity of quercetin (flavonol, unsaturated double

bond) to that of the flavanone, taxifolin (Table 2.1). Glycosylation of flavonoids reduces their antioxidant activity compared to corresponding aglycones. For example, glycosylation of luteolin in the 4′-position (B-ring) results in a decrease in antioxidant activity from 2.1 mM to 1.7 mM. Similarly, the positioning of a glucose residue on the 3-position of the C-ring causes rutin to have a lower antioxidant capacity than quercetin (respectively, 2.4 mM and 4.7 mM).

The structure of dihydrochalcones differs from that of other flavonoids in that the C_3 structure of the C_6-C_3-C_6 configuration is not cyclisised (Rezk *et al*., 2002). In this case, keto-enol tautomeric transformation between the carbonyl group and the α-methylene may be involved in antioxidant activity (Rezk *et al*., 2002). The 2′-OH group on the A-ring of a dihydrochalcone (represented by phloretin, Fig. 2.5a) is essential for its antioxidant activity (Nakamura *et al*., 2003). After acting as a radical scavenger, an active dihydrochalcone may form a phenoxy radical, followed by loss of its symmetrical structure (Mathiesen *et al*., 1997). The dihydrochalcone may then change into a coplanar conformation due to the formation of an intramolecular hydrogen bond between the remaining OH group (B-ring) and the carbonyl group. Rezk *et al*. (2002) speculated that stabilisation of the radical formed, after hydrogen abstraction, may take part in the antioxidant activity of phloretin. The antioxidant activity of dihydrochalcones results from the 2,4,6-hydroxyacetophenone moiety (Fig. 2.5b), which is unique to dihydrochalcones.

3.5.3 Metal ions

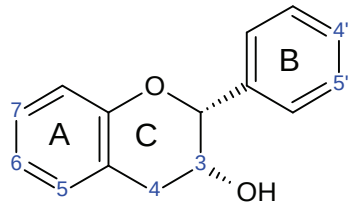
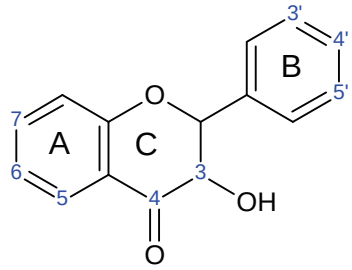
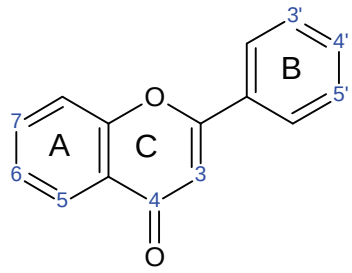
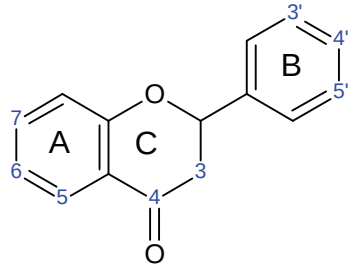
Many compounds, including antioxidants (e.g. flavonoids), possess the ability to complex metal ions (Hall, 2001). This process is known as chelation. In order to act as metal chelators, flavonoids must have a 3′,4′-OH configuration (B-ring), a C_4 keto and 3-OH arrangement in the C-ring, or a C_4 keto and 5-OH arrangement on the C- and A-rings, respectively. The metal binding sites on the general structure of flavonoids is illustrated in Figure 2.6.

It is assumed that the iron chelating ability of flavonoids plays an important role in their role as antioxidants via “site-specific scavenging” (Van Acker *et al*., 1998). Site specific scavenging refers to radical scavenging at the site of the antioxidant. Van Acker *et al*. (1996) suggest that chelation is a minor factor affecting the activity of powerful antioxidants, but that it makes a significant difference to the activity of less powerful flavonoids.

Despite the fact that metal ions are usually viewed in a negative light with respect to radical formation, Moridani *et al*. (2003) demonstrated that 2:1 flavonoid-metal complexes of Cu^{2+}, Fe^{2+} and Fe^{3+} were more effective superoxide radical scavengers than the flavonoids alone. The metal in the metal-flavonoid complex is responsible for the enhanced antioxidant activity as it effectively scavenges superoxide and dismutates it according to the reaction (Kostyuk *et al*., 2004):

$$Me^{(n+1)+} + O_2^{\bullet -} \rightarrow Me^{n+} + O_2 \qquad [3]$$

Table 2.1 Trolox equivalent antioxidant capacity (TEAC) and dietary sources of common flavonoids (Rice-Evans *et al*., 1996)

Family	Compound	Free OH substituents	Glycosylated position	Dietary sources	TEAC (mM)
Flavanol					
	(-)-Epicatechin gallate	5,7,3′,4′,3″,4″, 5”		Tea[a]	4.9 ± 0.02
	(-)-Epigallo-catechin gallate	5,7,3′,4′,5′,3″, 4″,5″		Tea	4.8 ±0.06
	(-)-Epigallo-catechin	5,7,3′,4′,5′		Tea	3.8 ±0.06
	(-)-Epicatechin	3,5,7,3′,4′		Tea	2.5 ± 0.02
	(+)-Catechin	3,5,7,3′,4′		Tea	2.4 ± 0.05
Flavonol					
	Quercetin	5,7,3′,4′		Onion, broccoli, red wine, tea	4.7 ± 0.1
	Myricetin	5,7,3′,4′,5′		Red wine, cranberry grapes,	3.1 ± 0.3
	Morin	5,7,3′,5′			2.6 ± 0.02
	Rutin	5,7,3′,4′	3-rut	Red wine, citrus, tomato skin	2.4 ± 0.06
	Kaempferol	5,7,4′		Broccoli, leek, black tea	1.3 ± 0.08
Flavone					
	Luteolin	5,7,3′,4′		Red pepper	2.1 ± 0.05
	Luteolin-4’-glucoside	5,7,3′	4′-glc		1.7 ± 0.09
	Apigenin	5,7,4		Parsley, celery	1.5 ± 0.08
	Chrysin	5,7		Fruit skins	1.4 ± 0.07
	Luteolin-3’,7-diglucoside	5,4′	3′,7-diglc		0.8 ± 0.04
Flavanone					
	Taxifolin	5,7,3′,4′		Citrus	1.9 ± 0.03
	Naringenin	5,7,4′		Citrus	1.5 ± 0.05
	Hesperetin	5,7,3′	4′-OMe		1.3 ± 0.08
	Hesperidin	5,3′	4′-OMe 7-rut	Oranges	1.1 ± 0.04

[a]*Camellia sinensis*

Figure 2.7 is a schematic representation of the proposed mechanism of superoxide radical scavenging by flavonoid-iron complexes.

The structures of fisetin (lacking an OH group at C_5 of the A-ring) and kaempferol (which lacks a 3′-OH group in the B-ring) are similar to that of quercetin. Quercetin and fisetin are capable of forming metal complexes. Despite lacking a 3′-OH group on the B-ring, kaempferol is also still capable of chelating metal ions. This suggests that, in the case of kaempferol, chelation takes place via the C-ring, whereas quercetin and fisetin may complex metal ions via the C- and B-ring.

3.5.4 pH of the solution

Lemańska *et al*. (2001) documented that the pH-dependent radical scavenging behaviour of hydroxyflavones is related to OH moiety deprotonation (Fig. 2.8). The radical scavenging capacity (measured using TEAC assay) of specific hydroxyflavones was found to increase upon deprotonation. This increase was attributed to increased ease of electron donation (oxidation). The factor which reflects the ease of electron donation, namely the ionisation potential, is greatly influenced by deprotonation.

Since deprotonated flavonoids are more easily oxidised than their protonated counterparts (Lemańska *et al*., 2001), pH plays an important role in flavonoid preservation. Guyot *et al*. (2007) found that the heat-induced degradation (above 80°C) of an oxidation product of phloridzin (a dihydrochalcone-*O*-glucoside found in apple) was more pronounced at higher pH values. In the same study, it was also noted that the colour of this phloridzin derivative (Fig. 2.9a) changed according to the pH of the medium. The compound had a bright yellow colour at pH 3, but became orange at higher pH values (pH 5). The intermediate (Fig. 2.9b), which formed prior to the phloridzin derivative (Fig. 2.9a), exhibited 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity comparable to that of ascorbic acid, Trolox and (-)-epicatechin. The difference in the structure between these two compounds was implicated: the derivative has only one free phenolic group whereas the intermediate has four phenolic groups, two of which are in the *ortho*-position.

3.5.5 Synergism

Synergism refers to a condition where the collective effect of two antioxidants in the same mix is more than the sum of their effects when tested separately (Cirico & Omaye, 2006.). Synergism is usually maximal when chain-breaking antioxidants are combined with “preventative inhibitors” such as metal chelators, hydroperoxide decomposers and singlet oxygen quenchers. Citric acid owes its synergistic effect to metal chelation, whereas ascorbic acid has a synergistic action with α-tocopherol. The latter is due to the fact that ascorbic acid is able to restore the antioxidant properties of α-tocopherol by reducing its oxidised form (Yanishlieva-Maslarova, 2001).

4. CHANGES IN PHENOLIC COMPOSITION AND FUNCTIONALITY DURING FOOD PROCESSING AND STORAGE

4.1 General

Thermal processing is one of the technologies used to preserve foods and make it available to the consumer throughout the year. In addition to the eradication of microorganisms, varying percentages of desirable characteristics such as colour, flavour (Torregrosa *et al*., 2006), antioxidants (Manzocco *et al*., 1998a; Sikora *et al*., 2008), nutrients (Ewald *et al*., 1999; Shi & LeMaguer, 2000; Shi *et al*., 2008) and texture may be destroyed during such treatments. Vitamin C is thermolabile and is thus often employed as an indicator of the extent of loss of other nutrients, pigments and aromatic substances in fruits and vegetables (Klimczak *et al*., 2007). Apart from processing, the vitamin C content of food also decreases during storage, depending upon factors such as storage temperature, oxygen concentration and light (Klimczak *et al*., 2007).

Despite the fact that processing may have an important effect on antioxidant activity, very little research has been conducted with respect to this topic. Food contains a variety of antioxidants derived from the raw materials themselves or added to the food product during processing. Although endogenous antioxidants have the benefit of being classified as "natural", they do not possess the same resistance to oxygen as synthetic antioxidants (Pokorný & Schmidt, 2001). Factors which further reduce oxygen resistance include heat, light and drying. Food processing techniques that may affect antioxidant activity include pasteurisation, blanching, sterilisation, boiling, evaporation, extrusion cooking, baking, frying, drying, smoking and fermentation (Pokorný & Schmidt, 2001).

Processing and storage do not, however, always result in foods with a reduced antioxidant capacity compared to their unprocessed counterparts. Some researchers have shown that processing may result in the formation of new antioxidant compounds with novel antioxidant properties that may maintain or enhance the antioxidant properties of the specific food (Nicoli *et al*., 1997a; Nicoli *et al*., 1997b). Changes taking place in the polyphenol composition of wine, during aging, has been greatly studied. Of interest are studies that showed that extended air exposure resulted in a gradual increase in the chain breaking antioxidant activity of wine (Manzocco *et al*., 1998b). Oxidative changes in phenolic composition therefore do not necessarily result in a decrease in *in vitro* antioxidant activity.

Very little information is available on the quantitative and qualitative changes in the phenolic composition of plant extracts after processing. Losses reported for vegetables are mostly ill-defined. Often, no distinction is made between actual degradation and losses as a result of leaching, e.g. processing-attributed losses of phenolic compounds in broccoli florets (Price *et al*., 1998a), asparagus (Makris & Rossiter, 2001), Swiss chard (Gil *et al*., 1998), green beans (Price *et al*., 1998b) and onions (Crozier *et al*., 1997; Price *et al*., 1997; Hirota *et al*., 1998; Ewald *et al*., 1999; Makris & Rossiter, 2001; Lombard *et al*., 2005) can be predominantly attributed to leaching.

The next section will deal with processing-induced changes in specific flavonoid classes. Anthocyanins will be excluded as they are mostly relevant in wine production.

4.2 Changes in content of specific flavonoid classes

4.2.1 Dihydrochalcones

Phloridzin (Fig. 2.10a) and phloretin (Fig. 2.10b) are dihydrochalcones specifically found in apples (Schieber *et al*., 2001; Oszmiański *et al*., 2008; Rupasinghe *et al*., 2008). Phloridzin, for example, comprises *ca*. 0.007-0.085 mg/g apple pomace whereas the TP content may vary between 4.22 and 8.67 mg/g, depending on the cultivar (Ćetković *et al*., 2008). A number of studies have been performed on the stability of these compounds during processing. For example, the recovery of phloridzin from baked muffins containing apple skin powder has been shown to be rather low, namely 44% (Rupasinghe *et al*., 2008), whilst microwave heating of Shampion type apples has been shown to be beneficial with respect to the preservation of phenolic compounds (Oszmiański *et al*., 2008). Due to the inactivation of polyphenol oxidase, phenolic oxidation was minimised, resulting in heated samples having a significantly increased phloretin-2′-glucoside content compared to unheated controls. Oszmiański *et al*. (2008) also demonstrated that the retention of phloretin-2′-glucoside in apple purée treated with vitamin C, prior to storage at 30°C, was greater than that in the control.

Investigating the stability of the sweetener neohesperidin dihydrochalcone (Fig. 2.10c) in blackcurrent jams, Tomás-Barberán *et al*. (1995) found no significant degradation under the temperature conditions prevailing during the manufacturing process (106ºC at 65º Brix) or after 18 months of storage at room temperature. In a carbonated lemonade beverage (pH 3.3), no change in the levels of this sweetener was found after one year of storage at room temperature (light and dark storage) or after three months of storage at 40°C (Montijano *et al*., 1997).

An investigation into the degradation of neohesperidin dihydrochalcone at temperatures of 30-60ºC and pH 1-7 indicated that maximal stability occurred in the region of pH 3 - 5 (Canales *et al*., 1993). In a similar study, the stability of an aqueous solution containing neohesperidin dihydrochalcone, heated at 50, 70 and 90ºC, was found to be maximal at pH 4.5 (Coiffard *et al*., 1998). The area of pH stability of neohesperidin dihydrochalcone thus appears to decrease with increased heating temperature.

4.2.2 Flavonols

The quercetin content of yellow banana peppers has been shown to decrease by as much as 45% after pasteurisation (internal temperature of 74ºC for 10 min) (Lee & Howard, 1999), whilst jam manufacture typically results in flavonol losses of around 20% (Häkkinnen *et al*., 2000; Zafrilla *et al*., 2001). Both red raspberry and strawberry flavonols have been shown to decrease by 15-20% (cooked for 30 min, temperature not specified) during processing. Quercetin and kaempferol were investigated, and in both studies, kaempferol exhibited greater heat sensitivity (20% loss compared to 6% loss of quercetin 3-glucoside) (Zafrilla *et al*., 2001). Furthermore, over the six month storage period which followed jam manufacture, loss of quercetin amounted to 40% and that of kaempferol to 50% (Zafrilla *et al*., 2001).

4.2.3 Flavanols

Several studies have investigated the stability of catechins, and more specifically green tea catechins. Catechins have been shown to undergo epimerisation (conversion to the corresponding isomers) during the

production of tea or tea beverages (Kiatgrajai *et al*., 1982; Komatsu *et al*., 1993). When placed in a hot aqueous solution, the epimerisation of tea catechins generally occurs at the C-2 position (Kiatgrajai, 1982). According to Wang & Helliwell (2000), epimerisation takes place more easily in tap water than purified water. The difference in pH and mineral ion complexity of these two types of water has been implicated. Green tea catechins may also undergo epimerisation during autoclaving (121ºC for 20 min). Epimerisation of epigallocatechin gallate to gallocatechin gallate has been implicated as the reason for the high levels of the latter in certain ready-to-drink teas that undergo thermal processing (Chen *et al*., 2001). When heated for longer periods (98ºC for 7 h), degradation, but not epimerisation of green tea catechins are observed. No mention was, however, made of any degradation products.

Wang *et al*. (2006) observed less severe epimerisation of tea catechins when a microwave reactor (100-165ºC for up to 120 min), instead of an autoclave, was used to replicate the UHT process. The reduced level of epimerisation was ascribed to the shorter processing time in the microwave reactor (shorter preheating and cooling times).

The stability of green tea catechins is pH dependent (Zhu *et al*., 1997). Catechins have been found to be extremely unstable in alkaline solutions (pH > 8), and degrade within minutes. The stability of the catechins, however, was found to increase with decreasing pH from pH 8 to pH 4. In a similar study, Su *et al*. (2003) found that increased pH and temperature enhanced the degradation of both green tea catechins and theaflavins.

Chen *et al*. (1998) investigated the role of acid type on catechin stability. Apart from being highly stable in an acidic environment, ascorbic acid was found to increase the stability of catechin mixtures incubated at 37°C. In contrast, the use of citric acid had no additional beneficial effect on the stability of the catechins.

4.2.4 Flavones

The levels of the flavones apigenin and luteolin in fresh celery increased between two and six hours after cutting (Viña & Chaves, 2007). The TP content of the celery was found to remain constant when stored at 0°C, increasing within two hours for samples stored at 10 and 20°C.

The luteolin content of pasteurised (internal temperature of 74ºC for 10 min) yellow banana peppers decreased by 45% due to processing (Lee & Howard, 1999), whilst the apigenin content of sweet potato leaves decreased by 49, 55 and 67% after 30, 60 and 120 s blanching (Chu *et al*., 2000).

4.3 Changes in antioxidant activity

Both increases and decreases in antioxidant activity have been reported as a result of processing and storage. In plants, many antioxidant compounds are present in a covalently bound form with insoluble polymers (Peleg *et al*., 1991). Since heat treatment may disrupt plant cell walls and liberate antioxidant compounds from inaccessible parts of the plant material (and insoluble polymers), the latter may be responsible for observed increases in antioxidant activity after processing. The formation of novel compounds, having greater antioxidant activity than their precursor compounds, may also play a role in such observations (Nicoli

et al 1997a). Processing and storage-related decreases in antioxidant activity, on the other hand, may result from leaching (Sikora *et al*., 2008) or destruction/reduction in the number of antioxidant compounds in the food matrix (Chen *et al*., 2001; Klimczak *et al*., 2007).

As previously mentioned, in many cases, losses of antioxidant compounds may be ascribed to non-destructive processes such as leaching, and not true flavonoid degradation. Some studies investigating increases and decreases in the antioxidant activity of foods and beverages, as a result of processing and storage, are discussed below.

The antioxidant activity [measured using the DPPH and 2,2'-azinobis(3-ethylenebenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging assays] of Shiitake mushrooms has been found to increase upon autoclave heating (Choi *et al*., 2006). This was attributed to disruption of the plant cell walls (by the heat treatment) and the more effective extraction of bound polyphenolic and flavonoid compounds from the heated plant material, compared to its raw counterpart. The concentration of free flavonoids was found to increase after heating, and more severe heating (121ºC for 30 min) produced greater increases in antioxidant activity (compared to heating at 100ºC for 15 min). Miglio *et al*. (2008) reported similar findings for selected vegetables cooked according to three methods: boiling; steaming and frying. An increase in the TEAC, ferric ion reducing antioxidant parameter (FRAP) and total radical-trapping antioxidant parameter (TRAP) values of carrots, courgettes and broccoli was observed for all three cooking methods. The increases were attributed to tissue softening and increased extractability of the antioxidant compounds.

Takeoka *et al*. (2001) reported that the processing of tomatoes resulted in tomato paste samples exhibiting greater antioxidant activity than fresh tomatoes. Dewanto *et al*. (2002a) also found that processing of tomatoes significantly increased the total antioxidant activity of the fruit, whilst the TP content was typically unaffected. In both cases, the increased antioxidant activity was attributed to the increased amount of lycopene, and other bound phytochemicals, that could be extracted from the tissue after thermal processing. Disruption of the tissue allowed for the release of phytochemicals from the matrix, making them more accessible for extraction compared to the phytochemicals from raw tomatoes (tissues intact). Furthermore, with respect to the polyphenol content, inactivation of oxidative and hydrolytic enzymes by the heat treatment could have prevented loss of phenolic compounds.

Compared to controls, Jeong *et al*. (2004) reported that heating of citrus peel at 150ºC (60 min) increased the TP content, radical scavenging activity and reducing power. This observation was attributed to destruction of the matrix and the liberation of phenolic compounds. Furthermore, inhibition of enzymatic oxidation was offered as another explanation for the increased antioxidant activity of the orange peel after heat treatment. Similarly, thermal processing of sweetcorn (115ºC for 25 min) increased its total antioxidant activity and TP content by 44% and 54%, respectively (Dewanto *et al*., 2002b). This was attributed to greater liberation of phenolic compounds in the processed sweetcorn, compared to the fresh product.

Storage of orange juice at 18, 28 and 38ºC for 2, 4 and 6 months resulted in a decrease in TP content, vitamin C content and antioxidant capacity (Klimczak *et al*., 2007). Similarly, modified atmosphere storage of fresh-cut spinach resulted in a decrease in the vitamin C content (Gil *et al*., 1999). No change in the

polyphenol content of the spinach was observed, indicating that the observed decrease in the antioxidant activity of the vegetable was most likely due to vitamin C loss.

5. IN VITRO TOTAL ANTIOXIDANT ACTIVITY METHODS

5.1 General

In vitro antioxidant tests are used as a means to assess the basic antioxidant properties of various compounds (Halliwell *et al*., 1995b). Such tests do not predict exactly how effective an antioxidant will be *in vivo*, as several other factors such as bioavailability and metabolism play a role. However, *in vitro* antioxidant capacity serves as an indication of *in vivo* antioxidant capacity; if a compound is poorly effective *in vitro*, it is unlikely to perform any better *in vivo*. *In vitro* antioxidant tests are simpler than *in vivo* tests and allow scientists to screen compounds for likely antioxidant activity before proceeding to more expensive and complex *in vivo* tests. Furthermore, *in vitro* antioxidant tests also allow scientists to evaluate the possible pro-oxidant ability of compounds on various molecular targets.

This section will only deal with *in vitro* antioxidant activity, and specifically with methods that have found wide-spread application as rapid screening methods to assess the potential of plant extracts as antioxidants, as well as evaluate the effect of processing and storage on antioxidant activity. The methods selected for discussion were chosen since they allow for the rapid assessment of large numbers of samples. A wide range of methods are currently used to assess the antioxidant potential of various food compounds and substances. Each of these methods has its advantages and limitations. Some of the commonly used *in vitro* antioxidant capacity assays are listed in Table 2.2.

5.2 Determination of antioxidant activity: method selection

As reviewed by Prior *et al*. (2005), a standardised method for the determination of antioxidant activity should meet the following criteria:

(i) Measures chemical reactions occurring in potential applications.

(ii) Simple to perform.

(iii) Uses a method with a defined endpoint and chemical mechanism.

(iv) Uses instrumentation that is readily available.

(v) Good reproducibility within and between days.

(vi) Adaptable for analysis of antioxidants of hydrophilic and hydrophobic nature as well as the use of different radical sources.

(vii) High through-put for routine analysis of quality control samples.

Performance characteristics of importance include the method's analytical range, recovery, repeatability, reproducibility and the recognition of interfering substances.

Table 2.2 *In vitro* antioxidant capacity assays (Huang *et al*., 2005)

Basis of assay	**Assay**
Hydrogen atom transfer reactions	
$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet}$	ORAC (oxygen radical absorbance assay)
$ROO^{\bullet} + LH \rightarrow ROOH + L^{\bullet}$	TRAP (total radical trapping antioxidant parameter)
	Crocin bleaching assay
	Inhibition of linoleic acid oxidation
	Inhibition of LDL oxidation
Electron transfer reaction	
$M(n) + E \text{ (from AH)} \rightarrow AH^{\bullet+} + M(n-1)$	TEAC (Trolox equivalent antioxidant capacity)
	FRAP (ferric ion reducing antioxidant parameter)
	DPPH (diphenyl-1-picrylhydrazyl)
	Copper (II) reduction capacity
	Total phenols assay using Folin-Ciocalteu reagent
Other	
	TOSC (total oxidant scavenging capacity)
	Inhibition of Briggs-Rauscher oscillation reaction

5.3 Differentiation between hydrogen atom transfer (HAT) and single electron transfer (SET) mechanisms

Antioxidants can deactivate radicals via two mechanisms, namely hydrogen atom transfer (HAT) and single electron transfer (SET) (Prior *et al*., 2005). Although both mechanisms deliver the same results, the kinetics and potential side-reactions for each mechanism differ. Bond dissociation energy (BDE) and ionisation potential (IP) are two major factors that determine the mechanism and efficiency of antioxidants.

Hydrogen atom transfer-based methods measure the ability of an antioxidant to quench free radicals by hydrogen atom donation (Prior *et al*., 2005):

$$X^{\bullet} + AH \rightarrow XH + A^{\bullet} \qquad \text{(AH = any H-donor)} \qquad [4]$$

The relative reactivity of a potential antioxidant in HAT methods depends on the BDE of the H-donating group of the potential antioxidant, dominating for compounds with a ΔBDE of ~ −10 kcal/mol and ΔIP < −36 kcal/mol (Prior *et al*., 2005). Hydrogen atom transfer-based reactions are solvent and pH dependent. They are usually rapid and the presence of reducing agents (including metal ions), which can result in mistakenly high reactivity.

Single electron transfer-based methods detect the ability of a potential antioxidant to transfer one electron to any compound. Metals, carbonyls and radicals may all be reduced (Prior *et al*., 2005):

$$X^{\bullet} + AH \rightarrow X^{-} + AH^{\bullet +} \qquad [5]$$

$$AH^{\bullet} \overset{H_2O}{\leftrightarrow} A^{\bullet} + H_3O^{+} \qquad [6]$$

$$X^{-} + H_3O^{+} \rightarrow XH + H_2O \qquad [7]$$

$$M(III) + AH \rightarrow XH^{+} + M(II) \qquad [8]$$

Single electron transfer and HAT mechanisms usually occur together in samples. The balance between the two is determined by antioxidant structure and pH. The relative reactivity of an antioxidant in SET methods is predominantly based upon the deprotonation (Lemanska *et al*., 2001) and IP (Wright *et al*., 2001) of the reactive functional group. Single electron transfer-based reactions are thus also pH dependent. It is generally true that IP values decrease with increasing pH, reflecting increased electron donating capacity with deprotonation. Compounds with an IP > −45 kcal/mol generally have a SET antioxidant mechanism.

Single electron transfer-based reactions are usually slow and require sufficient reaction time in order to achieve completion. Antioxidant capacity calculations are thus based on the percentage decrease in the product, rather than its kinetics. Secondary reactions become a significant source of interference in assays where $AH^{\bullet +}$ has a sufficient lifetime, and toxicity or mutagenicity may even result *in vivo* (Sartor *et al*.,

1999). Contaminants such as metal ions may interfere with SET methods and result in poor reproducibility or high variability in results (Prior *et al*., 2005).

As reviewed by Huang *et al*. (2005), assays based on electron transfer involve two components in the reaction mixture, antioxidants and oxidants (probe). The probe is an oxidant that abstracts an electron from the antioxidant, resulting in the probe changing colour. The extent of the colour change is proportional to the antioxidant concentration. The end point is achieved when the colour ceases to change. The change in absorbance (ΔA) of the sample is plotted against its concentration, resulting in a linear curve. The reducing capacity of the antioxidant, expressed as Trolox equivalents (TE) or gallic acid equivalents (GAE), is indicated by the slope of the curve. In order to make a correlation between the assay and the antioxidant capacity of the sample, its reducing capacity and antioxidant capacity are considered equivalent.

There are many different views on the exact identity (SET or HAT) of the various antioxidant assays. According to Huang *et al*. (2005), the TP, FRAP, DPPH and TEAC assays all make use of a SET mechanism. (Prior was one of the co-authors of this paper). However, in a follow up paper by Prior *et al*. (2005), only the FRAP assay is described as a true example of a SET reaction assay. The remaining assays were said to make use of both HAT and SET mechanisms.

5.4 Quantification of total antioxidant activity

It is important, especially if large numbers of samples are being analysed, that the methods used allow for high through-put. As such, there has been a move towards the adaption of TP (Zhang *et al*., 2006), DPPH (Fukumoto & Mazza, 2000), FRAP (Moyer *et al*., 2002) and ABTS (Oki *et al*., 2006) assays to microplate scale. Numerous other assays have also been adapted (Lussignoli *et al*., 1999; Huang *et al*., 2002). The smaller scale of such methods saves time, significantly reduces the amount of sample and chemical reagents required for the analysis and allows for the handling of a large number of samples in a shorter space of time (Zhang *et al*., 2006). Furthermore, smaller quantities of chemical waste are generated and the results are generally also more repeatable. The methods discussed below meet the criteria generally required to be used in quality control work and research, as they allow for rapid analysis of samples and a high sample throughput.

5.4.1 The Folin-Ciocalteu (total polyphenol) assay

The majority of the oxygen uptake capacity of plant-derived products such as tea and wine results from their polyphenol content (Singleton *et al*., 1999). Apart from compounds such as β-carotene, the antioxidants in food are generally polyphenols. Since polyphenols act as antioxidants, it is desirable to measure their quantities in plant-derived products. The most reliable method for the determination of the TP content of a sample makes use of an oxidation reaction (Marshall, 1992). Folin-Ciocalteu reagent, containing phosphomolybdate and sodium tungstate, is required for this assay. These two compounds are combined and boiled in the presence of acid, after which the addition of lithium sulphate delivers the yellow Folin-Ciocalteu reagent (Marshall, 1992).

The exact chemical nature of the reagent is not known, but it is thought to contain heteropoly-phosphotungstates-molybdates (Singleton *et al*., 1999). Electron reduction leads to the formation of blue complexes $(PMoW_{11}O_{40})^{4-}$. It is believed that the molybdenum is the more easily reducible part of the complex and that electron transfer occurs between the Mo(VI) and the reductants:

$$Mo(VI) + e^- \rightarrow Mo(V) \quad [9]$$

Phenolic compounds only react with the Folin-Ciocalteu reagent under alkaline conditions and the reaction mixture must thus be adjusted to a pH of *ca*. 10 using sodium carbonate (Singleton *et al*., 1999). Under these conditions, the dissociation of a phenolic proton leads to the formation of a phenolate anion which reduces the Folin-Ciocalteu reagent. The blue compounds formed during the reaction are independent of the structure of the phenolic compounds that react with the reagent. The possibility of coordination complexes forming between the phenolic compounds and metal ions is thus eliminated. The assay is relatively convenient, simple, and makes use of readily available laboratory apparatus. A large amount of comparable data has been produced with the help of this assay (Huang *et al*., 2005).

The intensity of the relatively stable blue complex that forms upon reaction of the oxidisable substances in the sample and the Folin-Ciocalteu reagent is measured at 765 nm (Singleton *et al*., 1999). The more intense the colour, the greater the polyphenol content of the sample. The results are expressed in gallic acid equivalents (GAE). Gallic acid is generally used as a standard as it is inexpensive, soluble in water and stable in the dry form (Singleton *et al*., 1999). Other standards have also been used, for example (+)-catechin (Vinson *et al*., 2001) and chlorogenic acid (Chun & Kim, 2004). [The latter are commonly occurring phenolics in fruits (generally) and chlorogenic acid-rich plums, respectively]. The phenolic content of a sample is determined from a standard curve, plotted from the absorbance and concentration of a standard gallic acid dilution series.

Due to the fact that the assay is independent, quantitative and predictable, the analysis of phenolic samples can be recalculated in terms of other standards (Singleton *et al*., 1999). The Folin-Ciocalteu assay is not only a measure of the phenolic compounds in a sample, but rather all compounds that are oxidisable under the reaction conditions. Phenolic amino acids, for example, may also react with the reagent during the assay. Provided due attention is given to possible sources of interference, informative results will be obtained (Singleton *et al*., 1999).

The TP assay should be considered supplementary to an analysis such as high pressure liquid chromatography (HPLC), which is highly specific and can separate samples into individual compounds. By combining the results of two such assays, it is possible to determine the contribution of a single component to the TP content of the reaction mixture (Singleton *et al*., 1999).

According to Prior *et al*. (2005), the main advantage and disadvantage of the TP assay are as follows:

(i) The method is simple and is useful for characterising and standardising botanical samples.

(ii) A number of substances may interfere with the assay, for example sugars, aromatic amines, sulphur dioxide, ascorbic acid, enediols and reductones, organic acids and Fe^{2+}.

5.4.2 Ferric ion reducing antioxidant parameter (FRAP) assay

The FRAP assay is a simple test that can be used to determine the "antioxidant power" of a solution (Benzie & Strain, 1996; Benzie & Strain, 1999; Pulido *et al.*, 2000). The reaction detects compounds with redox potentials of < 0.7 V (the redox potential of Fe^{3+} 2,4,6-tripyridyl-*s*-triazine (TPTZ)). At a low pH, the ferric ions (Fe^{3+}) in the reaction mixture are reduced to ferrous ions (Fe^{2+}), with the formation of a coloured, ferrous-tripyridyltriazine ($Fe^{2+}(TPTZ)_2$) complex (Benzie & Strain, 1996; Benzie & Strain, 1999). The oxidant does not, however, only comprise $Fe^{3+}(TPTZ)_2$. The presence of other Fe^{3+} ions in the oxidant is a potential problem in this assay. The presence of metal chelators in the food/extract to be sampled can also result in complex formation (Fe^{3+} and chelator) and misleading results (Benzie & Strain, 1996).

In order to obtain FRAP values, the change in the absorbance ($\Delta A = A_{4\text{min}} - A_{0\text{min}}$) of the sample at 593 nm is compared to that of a solution with a known ferrous ion (Fe^{2+}) concentration (Benzie & Strain, 1996; Benzie & Strain, 1999), i.e. one FRAP unit is defined as the reduction potential of 1 mol of Fe^{3+} to Fe^{2+}. Other standards such as Trolox have also been used (Capocasa *et al.*, 2008). The FRAP assay does not detect compounds that act by hydrogen transfer, and is thus not effective for measuring the antioxidant power of thiol or protein-rich foods (Prior *et al.*, 2005). The latter may result in a serious underestimation of the total antioxidant power of such foods. On the other hand, this may be advantageous when changes in antioxidants other than proteins are being investigated, since interference as a result of the latter will be low (Benzie & Strain, 1996).

The FRAP assay is an inexpensive, reproducible and a fast means of determining the reducing potential of samples (Prior *et al.*, 2005). The FRAP assay is non-specific and any redox half reaction with a less positive redox potential than the Fe^{3+}/Fe^{2+} reaction, can drive colour change. Test conditions favour complex formation. If any antioxidants are present, colour formation indicates the reducing ability of the sample. The test can be successfully used for antioxidant mixtures (typical of tea) as there is no apparent antioxidant interaction (Prior *et al.*, 2005).

It is argued that the ability to reduce iron has little relationship to the bodily radical quenching process mediated by most antioxidants (Prior *et al.*, 2005). The oxidation or reduction of radicals to ions does, however, arrest radical chain reactions. Since the FRAP assay is based on electron transfer alone, when used in combination with other assays, it can be used to distinguish the dominant mechanisms according to which antioxidants work.

The disadvantages and advantages of the FRAP assay may be summarised as follows (Prior *et al.*, 2005):

(i) The FRAP assay assumes that the reducing reactions are complete within 4-6 min, which is not always correct. The results of the FRAP assay vary considerably depending on the duration of analysis.

(ii) The reducing capacity of thiol antioxidants, such as glutathione, cannot be measured.

(iii) The FRAP assay only measures reducing capacity, which is not relevant to antioxidant activity physiologically.

(iv) The FRAP assay is simple, fast, inexpensive and does not require specialised equipment.

(v) The assay may be performed using automated, semi-automated and manual methods.

5.4.3 The 2,2'-azinobis(3-ethylenebenzothiazoline-6-sulphonic acid) (ABTS) assay

This assay was first described by Miller *et al*. (1993) for the measurement of the total antioxidant capacity of body fluids and drug solutions. It has since been improved upon and used for the determination of the antioxidant capacity of pure compounds (Miller & Rice-Evans, 1997; Rice-Evans *et al*., 1997) and food (Wang & Ballington, 2007; Vasoc *et al*., 2008). The improved assay makes use of a pre-formed, cationic radical named 2,2'-azino-bis-[3-ethylbenzothiazoline-6-sulfonic acid] ($ABTS^{+\bullet}$) (Re *et al*., 1999). $ABTS^{+\bullet}$ has peak absorbances at 417, 645, 734 and 815 nm, whilst $ABTS^{2-}$ itself is colourless at visible wavelengths (Miller, 1998). The blue/green $ABTS^{+\bullet}$ is formed by the oxidation of $ABTS^{2-}$ with potassium persulphate. This oxidation is fairly slow and the mixture is left to stand for 12-16 hours. Measurement of $ABTS^{+\bullet}$ at 734 nm reduces the possibility of interference from most other coloured substances that might be present (for example, carotenoids and anthocyanins) as well as reducing interference as a result of sample turbidity.

Provided an electron or hydrogen-donating agent is present in the reaction mixture, $ABTS^{+\bullet}$ is reduced. The extent of the decolourisation of $ABTS^{+\bullet}$ is a function of the antioxidant concentration and the duration of the decolourisation reaction. The change in the absorbance reading (ΔA) of the sample is plotted against antioxidant concentration to achieve a straight line. The antioxidant capacity of the substance in question is determined by comparing it to that of the Trolox standard (under the same conditions). Trolox is a water soluble vitamin E analogue and an antioxidant with a known antioxidant capacity (Pellegrini *et al*., 1999). The concentration of Trolox resulting in the same ΔA as a 1 mM solution of a pure compound is regarded as Trolox equivalent antioxidant capacity (TEAC).

This method is an improvement upon the original $ABTS^{+\bullet}$ assay (ferryl myoglobin/ABTS assay) since $ABTS^{+\bullet}$ is now directly generated (pre-formed, without the use of an intermediate) and can be applied to both hydrophilic and hydrophobic systems (Re *et al*., 1999).

The $TEAC_{ABTS}$ values for pure antioxidant compounds do not illustrate a correlation with the number of electrons that can be donated (Huang *et al*., 2005). The $TEAC_{ABTS}$ values for ascorbic acid (1.05), α-tocopherol (0.97), glutathione (1.28) and uric acid (1.01) are very similar, yet glutathione is capable of donating only a single electron (to form oxidised glutathione), whereas the other compounds donate two electrons (two electron reductants). Ferulic acid (1.90) and *p*-coumaric acid (2.00) have similar $TEAC_{ABTS}$ values, whereas caffeic acid (which is similar in structure to ferulic acid), only has a $TEAC_{ABTS}$ value of 1.00. Similarly, quercetin (3.00) and kaempferol (1.00) have similar structures (quercetin has one additional OH-group) but differ markedly in their $TEAC_{ABTS}$ values. The $ABTS^{+\bullet}$ assay does reflect reaction rate differences between antioxidants and oxidants as it is an end-point assay.

Prior *et al*. (2005) summarise the advantages of the $ABTS^{+\bullet}$ assay as follows:

(i) The assay is simple and is widely used in laboratories studying antioxidants.

(ii) There is a large database of TEAC values for many compounds.

(iii) $ABTS^{+\bullet}$ reacts rapidly with antioxidants (usually within 30 min).

(iv) The assay can be used over a wide range of pH values and can thus be used to evaluate the effect of pH on antioxidant activity.

(v) $ABTS^{+\bullet}$ is soluble in both aqueous and organic solvents and is not affected by ionic strength, meaning it can be used to determine the antioxidant capacity of lipophilic and hydrophilic fluids.

(vi) The $ABTS^{+\bullet}$ assay can be automated and adapted to microplates, flow injection and stopped flow.

The disadvantages of the $ABTS^{+\bullet}$ assay is as follows (Prior *et al*., 2005):

(i) The radical used in the $ABTS^{+\bullet}$ assay is non-physiological of origin.

(ii) Thermodynamically, any compound that has a redox potential of less than 0.68 V can reduce $ABTS^{\bullet+}$.

(iii) The $ABTS^{+\bullet}$ reaction is not necessarily the same for fast and slow reactions. In the latter, the endpoint may not be reached. A reaction time of 4 - 6 min may thus not deliver the same result as one of longer duration. Based on this fact, Van den Berg *et al*. (1999) concluded that "quantitative evaluation of the antioxidant capacity using the TEAC assay may be troublesome or even impossible, but it can be used to provide a ranking order of antioxidants."

The the FRAP assay is similar to the $ABTS^{+\bullet}$ assay, except for the fact that the FRAP assay is conducted under acidic conditions (pH 3.6, to maintain iron solubility) whilst the $ABTS^{+\bullet}$ assay is generally performed at a neutral pH (Huang *et al*. 2005). The FRAP assay may, however, also be performed under different pH conditions and in different media (Lemańska *et al*., 2001; Labrinea & Georgiou, 2004). Reaction at a low pH decreases the ionisation potential that drives electron transfer and increases the redox potential, causing a shift in the dominant reaction mechanism (Prior *et al*., 2005). For this reason, FRAP values often have a poor relationship to other antioxidant measures. Similar compounds, however, react in both the FRAP and the $ABTS^{+\bullet}$ assays, since the redox potential of $Fe^{3+}(TPTZ)_2$ is comparable to that of the ABTS radical (0.68 V). Table 2.3 compares the $ABTS^{+\bullet}$ and FRAP values of some antioxidant compounds.

With respect to Table 2.3, both the FRAP and $TEAC_{ABTS}$ values (and thus the antioxidant activity) of the flavanols decrease in the following order: epigallocatechin gallate; epicatechin gallate; epigallocatechin; epicatechin and catechin (Soobrattee *et al*., 2005). Both the decreasing solubility and number of OH groups could be responsible for this observation. The galloyl moiety, attached to the 3-position of the gallates (epigallocatechin gallate and epicatechin gallate), is responsible for the overall high antioxidant activity of these compounds.

5.4.4 The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH radical is a stable, commercially available organic nitrogen radical with an absorbance maximum at 515 nm (Brand-Williams *et al*., 1995). The colour of the $DPPH^{\bullet}$ solution fades upon reduction, allowing

for the monitoring of the reaction process with a spectrophotometer. The absorbance is measured until a stable reading is obtained. The percentage $DPPH^{\bullet}$ remaining (% $DPPH^{\bullet}_{rem}$) is calculated as the ratio between the remaining concentration of $DPPH^{\bullet}$ ($[DPPH^{\bullet}]_{rem}$) and that at time zero ($[DPPH^{\bullet}]_{T=0}$).

$$\% \, DPPH^{\bullet}_{rem} = 100 \times [DPPH^{\bullet}]_{rem}/[DPPH^{\bullet}]_{T=0} \quad [10]$$

The percentage $DPPH^{\bullet}$ remaining is proportional to the antioxidant concentration. The concentration that causes a 50% decrease in the initial $DPPH^{\bullet}$ concentration is defined as the effective concentration (EC_{50}). The time needed to reach the steady state with the EC_{50} is calculated from the kinetic curve and is defined as T_{50}. Antiradical efficiency (AE), which defines the antioxidant capacity of a certain antioxidant, is calculated as follows (Sanchez-Moreno *et al.*, 1998):

$$AE = (1/EC_{50})T_{EC50} \quad [11]$$

Prior *et al.* (2005) summarise the advantages and disadvantages of the $DPPH^{\bullet}$ assay as follows:

(i) The test is simple, rapid and requires only a UV-visible spectrophotometer.

(ii) Orange and red pigments such as carotenoids and anthocyanins may interfere with the interpretation of the results as they have absorbance spectra that overlap with that of $DPPH^{\bullet}$ (515 nm), although this may be corrected with sample blanks.

(iii) $DPPH^{\bullet}$ colour may be lost due to hydrogen atom transfer or single electron transfer mechanisms, as well as unrelated reactions.

(iv) Steric accessibility is a determinant in the reaction: smaller molecules that have better access to the radical site appear to have a higher antioxidant capacity using this assay.

(v) Although the $DPPH^{\bullet}$ assay is relatively simple, it differs mechanistically from the usual HAT reaction that occurs between antioxidants and peroxyl radicals. Furthermore, since $DPPH^{\bullet}$ is a "long-lived" nitrogen radical, it bears no similarity to the short-lived peroxyl radicals involved in lipid peroxidation. Antioxidants that react quickly with peroxyl radicals may react slowly or be inert to $DPPH^{\bullet}$.

The DPPH assay was originally believed to involve transfer of hydrogen atoms, although a recent paper by Foti *et al.* (2004) suggested that the reaction behaves like an electron transfer reaction. This observation was made for the reaction between phenols and $DPPH^{\bullet}$. According to the authors, the rate-determining step is a fast electron transfer from phenoxide anions to $DPPH^{\bullet}$. Hydrogen abstraction from the neutral aromatic nucleus occurs very slowly in strong hydrogen bond accepting solvents such as methanol and ethanol. Furthermore, the presence of acid influences the ionisation equilibrium of polyphenols and causes either a reduction or enhancement of the measured rate constant. These findings imply that the $DPPH^{\bullet}$ assay may not be a valid assay for measurement of antiradical capacity.

5.5 Correlation between total polyphenol content of plant extracts and their radical scavenging capacity

There are a significant number of research papers indicating that a strong correlation exists between the antioxidant capacity of a range of food and beverage products and their TP content. Some examples have been listed below:

(i) The antioxidant capacity of black tea as measured using the DPPH radical scavenging assay was found to be well correlated with its TP content (Mello *et al*., 2005).

(ii) A strong correlation between the TP content, $TEAC_{DPPH}$ and $TEAC_{FRAP}$ values of 25 edible tropical plants suggested that the polyphenols in the extracts were largely responsible for their antioxidant activity (Wong *et al*., 2006).

(iii) Kiselova *et al*. (2006) studied the antioxidant activity ($TEAC_{ABTS}$) and polyphenol content (Folin-Ciocalteu) of aqueous extracts of 23 Bulgarian medicinal plants and found that the antioxidant activity of the extracts was positively correlated (r = 0.92) with the polyphenol content. This lead the authors to conclude that the polyphenols present in the plant extracts were largely responsible for their antioxidant activity.

(iv) Von Gadow *et al*. (1997a) found that the DPPH radical scavenging capacity of rooibos decreased with increasing degree of fermentation. This finding was reflected in the TP content of the respective teas.

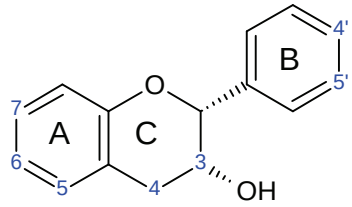
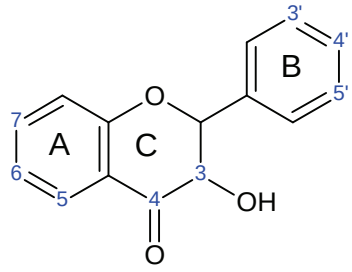
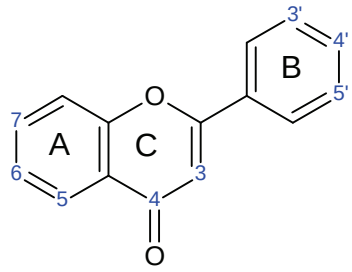
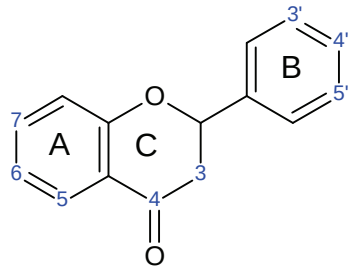
(v) The total antioxidant activity ($TEAC_{ABTS}$) of unfermented rooibos has been shown to be twice that of fermented rooibos (Bramati *et al*., 2003). The unfermented tea also contained more flavonoids than its fermented counterpart, indicating the contribution of the flavonoid content of the teas to their antioxidant activity.

(vi) Fermentation of honeybush tea lead to a significant reduction in extract yields, their TP content and individual polyphenol contents (Joubert *et al*., 2008a). Fermentation also lowered the antioxidant activity of all species of honeybush, assessed using the $TEAC_{ABTS}$ and FRAP assays.

(vii) Joubert *et al*. (2008b) found that the TP content of aqueous rooibos extracts correlated well (r = 0.99) with the total antioxidant activity as determined with the ABTS assay. In contrast, the TP content of honeybush (*Cyclopia genistoides*) aqueous extracts was not a good indication of their total antioxidant activity (r = 0.27). The aspalathin content of the rooibos extracts correlated well with the total antioxidant activity (r = 0.96) whilst the mangiferin content of honeybush extracts was only moderately correlated with the total antioxidant activity (r = 0.75).

Cases exist where the correlation between the TP content and radical scavenging capacity of a sample is poor. This usually occurs when the phenolic compounds in the sample are not the main

Table 2.3 Trolox equivalent antioxidant capacity (TEAC) and ferric reducing antioxidant potential (FRAP) of some antioxidant compounds (Soobrattee *et al*., 2005)

General structure	Compound	TEAC[a]	FRAP[b]
Flavanol 	(-)-Epigallocatechin gallate	4.39	8.04
	(-)-Epicatechin gallate	4.23	7.03
	(-)-Epigallocatechin	3.86	4.01
	(-)-Epicatechin	3.58	2.90
	(+)-Catechin	3.16	2.47
Flavonol 	Quercetin	3.68	7.39
	Myricetin	3.07	4.58
	Isoquercitrin	2.72	5.52
	Hyperoside	2.33	4.89
	Kaempferol	1.03	1.95
Flavone 	Luteolin	2.23	2.23
	Apigenin	2.01	2.01
	Orientin	1.05	2.81
	Vitexin	-	0.56
Flavanone 	Naringenin	0.13	0.44
Simple phenolic acids	Gallic acid	3.62	5.25
	Hydroxybenzoic acid	0.13	-
Hydroxycinnamic acids	Ferulic acid	0.98	1.33
	3-Coumaric acid	0.17	-
Synthetic antioxidants	Butylated hydroxytoluene	-	0.04
	Butylated hydroxyanisole	1.11	3.05
	Trolox	1.00	1.91

[a]Results expressed in units mmol Trolox/L, [b]values are expressed in units of mmol Fe (II)/L. These values can be converted to TEAC values by dividing by two.

contributors to antioxidant capacity (Dasgupta & De, 2007). Examples include leafy vegetables (Dasgupta & De, 2007) and carotenoid-rich plants (Hu *et al*., 2008).

6. ROOIBOS

Rooibos is a uniquely South African beverage, brewed from the leaves and stems of *Aspalathus linearis* (Reyneke *et al*., 1949; Coetzee *et al*., 1953). This shrub grows mainly in the Cederberg region of the Western Cape (South Africa) where the microclimate and soil conditions lend the area suitable for rooibos cultivation. The outstanding positive qualities of rooibos include its caffeine-free nature and relatively low tannin content, compared to black tea (Blommaert & Steenkamp, 1978). Caffeine has a variety of effects in the human body, including blocking the functioning of the adenine receptors which regulate functions such as myocardial oxygen consumption and blood flow (Bimms, 1995), as well as acting as a diuretic and a central nervous system stimulant. Persons wishing to avoid caffeine-containing beverages may thus find rooibos a suitable alternative to black tea. Since rooibos does not contain any compounds known to be detrimental to human health, it is regarded as a "health beverage". It possesses antioxidant (Von Gadow *et al*., 1997a) and antimutagenic activity (Sasaki *et al*. 1993). Many of the positive health effects of rooibos have been linked to this underlying antioxidant mechanism. The *in vitro* anticarcinogenic and antimutagenic properties of rooibos were first established by Sasaki *et al*. (1993) and Komatsu *et al*. (1994). Research indicating that this tea has antiviral activity (Shindo & Kato, 1991), reduces inflammation (Niwa *et al*., 1988; Shindo & Kato, 1991) and lowers serum lipid peroxide levels (Niwa *et al*., 1988) possibly add to the popularity of this beverage. Furthermore, rooibos is claimed to have antispasmodic qualities and a soothing effect on the nervous system. The antispasmodic action of rooibos is thought to result from the action of quercetin and luteolin (Snyckers & Salemi, 1974), two of the many flavonoids present in this beverage. Further anecdotal claims that have been linked to the consumption of rooibos include the relief of skin irritations such as eczema, as well as an ability to improve appetite, relieve insomnia, allergies and nervous complaints (Joubert *et al*., 2008c).

6.1 Processing

Rooibos shrubs are harvested between December (summer) and March (early autumn) when the climate lends itself to optimal tea production (Morton, 1983). No flowers should be present on the shrubs during harvesting, as these impart an unpleasant flavor to the tea (Joubert & Schulz, 2006). Harvested plant material generally undergoes "fermentation" (oxidation) for production of traditional rooibos. In order to initiate "fermentation", the plant material must be shredded, which releases endogenous enzymes that oxidise the plant material. The addition of water accelerates the process. Fermentation is responsible for the development of the characteristic red-brown colour and pleasant honey-like aroma of rooibos.

Other forms of rooibos processing include the production of aqueous extracts and extract powders from the fermented product (Joubert & Schulz, 2006). Although first developed in the 1980s, extracts of rooibos have only recently begun to find application in the food and beverage industry (Anon., 2005; Joubert

& Schulz, 2006). Examples include ready-to-drink beverages of which several brands are available on the South African market. Extracts with increased aspalathin content are made from green rooibos plant material and find use in the cosmetic and nutraceutical industry (Tiedtke & Marks, 2002; Otto *et al*., 2003).

6.2 Phenolic components of rooibos

Rooibos contains the unique dihydrochalcone C-C glucoside, aspalathin (Fig. 2.11), the structure of which was elucidated by Koeppen & Roux (1966). Almost as unique is the 4'-dehydroxy-dihydrochalcone C-C glucoside, nothofagin (Fig. 2.11) (Joubert, 1996). It contains a host of other compounds including the flavanones 2,3-dihydro-iso-orientin and 2,3-dihydro-orientin (Koeppen & Roux, 1965), the flavones chrysoeriol (Ferreira *et al*., 1995) and luteolin (Snyckers & Salemi, 1974), as well as the C-C linked flavone glucosides vitexin, iso-vitexin (Rabe *et al*., 1994), orientin and iso-orientin (Fig. 2.11) (Koeppen & Roux, 1965). The flavonols isoquercitrin, rutin (Koeppen *et al*., 1962) and quercetin (Snyckers & Salemi, 1974) also occur in the plant matter. Caffeic acid, *p*-hydroxy benzoic acid, protocatechuic acid, vanillic acid, *p*-coumaric acid, syringic acid and ferulic acid (Rabe *et al*., 1994) were isolated from fermented rooibos. Rooibos contains extremely low concentrations of (+)-catechin, procyanidin B3 and the profistinidin triflavanoid bis-fisetinidol-(4β,6:4β,8)-catechin, giving credibility to the claim that rooibos has a low tannin content (Ferreira *et al*., 1995). Recent analysis of fermented rooibos shows that it contains, amongst others, small amounts of the lignins secoisolariciresinol and vladinol F (Shimamura *et al*., 2006), the flavonols quercetin-3-galactoside (hyperoside) (Bramati *et al*., 2003), quercetin-3-robinobioside (Shimamura *et al*., 2006) and hemiphlorin (Shimamura *et al*., 2006), a flavanone derivative of nothofagin.

Approximately 1.9 g/100 g of the dry matter of green rooibos comprises dihydrochalcones (Joubert, 1996). Manley *et al*. (2006) reported values of 0.66-9.61 g/100 g (average = 4.21 g/100 g). This data set, however, consisted of a variety of samples, including those comprising only leaves, thus explaining the high aspalathin values. Table 2.4 distinguishes between the aspalathin and nothofagin (dihydrochalcone) content of unfermented rooibos reported by various researchers.

The abovementioned dihydrochalcone content of rooibos decreases drastically with fermentation (Joubert, 1996). Fermented plant material contains approximately 7% of the dihydrochalcones originally present in the green plant material. This reduction can be attributed to enzymatic and chemical oxidation. Based upon the analysis of a large number of samples, the aspalathin and nothofagin content of fermented rooibos was respectively 3.9 and 17.9% of that of unfermented rooibos (Joubert & Schulz, 2006). Oxidation of aspalathin results in the formation of 2,3-dihydro-iso-orientin and 2,3-dihydro-orientin (Koeppen & Roux, 1965), the flavanone precursors to iso-orientin and orientin.

The recent study by Krafczyk & Glomb (2008) shed more light on the oxidation of aspalathin in solution (0.2 M phosphate buffer, pH 7.4) (Fig. 2.12). They showed that the half-life of aspalathin is 8 h (compound 5 in Fig. 2.12), when incubated in phosphate buffer at 37ºC. After 48 h, all structures in the buffer were reported to have degraded to high molecular weight, uncharacterised material. (S)-eriodictyol-6-C-β-D-glucopyranoside and (R)-eriodictyol-6-C-β-D-glucopyranoside (dihydroiso-orientin, compounds 2 and 4 in Fig. 2.12) were the major products during the process with minor concentrations of (S)-eriodictyol-

8-C-β-D-glucopyranoside and (R)-eriodictyol-8-C-β-D-glucopyranoside (dihydro-orientin, compounds 1 and 3 in Fig. 2.12). Iso-orientin (6) and orientin (7) were also found. These occurred at a maximum concentration at 6 h. Orientin degraded slowly compared to iso-orientin, which was no longer detectable after 10 h. The degradation of aspalathin was explained by oxidation via an *o*-quinone (5a). This structure rearranges to a quinone methide (5b) which is converted to an equilibrium mixture of eriodictyols. (S)-eriodictyol-6-C-β-D-glucopyranoside and (R)-eriodictyol-6-C-β-D-glucopyranoside (1 and 3 in Fig. 2.12) form rapidly, in 1:1 ratio, whilst the formation of (S)-eriodictyol-8-C-β-D-glucopyranoside and (R)-eriodictyol-8-C-β-D-glucopyranoside (2 and 4, Fig. 2.12) was slow. The latter could be converted to (S)-eriodictyol-6-C-β-D-glucopyranoside and (R)-eriodictyol-6-C-β-D-glucopyranoside, followed by conversion to iso-orientin and subsequently, orientin. Conversion of (S)-eriodictyol-8-C-β-D-glucopyranoside and (R)-eriodictyol-8-C-β-D-glucopyranoside (2 and 4, Fig. 2.12) to orientin was not demonstrated. Iso-orientin was irreversibly converted to orientin, although this was only a minor product amongst uncharacterised brown material. Conversion was initiated by opening of the vinyl ester structure of iso-orientin via a chalcone intermediate (not formed via reduction since no eriodictyols were formed). Oxidation of iso-orientin (half-life 135 h) and orientin (half-life 208 h) resulted in the formation of brown products.

Despite an increasing interest in “wild” rooibos, the large phenolic variation (Van Heerden *et al*., 2003) and sometimes extremely low aspalathin content (Joubert & Schulz, 2006) of such bushes makes them less desirable for the production of rooibos extracts. However, due to the use of seedlings for propagation, and thus genetic variation, the aspalathin and nothofagin content of cultivated rooibos bushes also varies (Joubert & Schulz, 2006). Great variation thus exists within the plant itself and may be enhanced by processes such as “fermentation”.

6.2.1 Extrinsic factors that affect the flavonoid content of plants

Extrinsic factors such as variation in plant distribution, variety, season, light intensity/quality and climate may affect the polyphenol content of plant material (Aherne & O'Brien, 2002). The accumulation of plant flavonoids, for example, is enhanced in response to stress, i.e. increased light exposure, and in particular, UV-B rays (Stewart *et al*., 2000). Glass prevents UV light from reaching the surface of plants that grow in greenhouses, with the result that such plants have a reduced flavonoid content compared to those growing in the outdoors (Gliszczyńska-Świgło *et al*., 2007). Fertilisation (Toor *et al*., 2006), drought (De Abreu & Mazzafera, 2005) and insect infestation (Duncan & Linhoss, 2005) also have a varying effect.

6.3 Antioxidant activity of rooibos

Antioxidant activity has been demonstrated for an infusion of rooibos in several systems, ranging from *in vitro* scavenging of selected radicals to a variety of *in vivo* effects, e.g. suppression of lipid peroxidation in rat brain (Inanami *et al*., 1995).

Table 2.4 Aspalathin and nothofagin content (g/100 g) of unfermented rooibos plant material

Compound	Range	Average	Samples (n)	Reference
Aspalathin	-	4.99	n.d.[a]	Bramati *et al*., 2003
	3.84-9.66	6.62	97	Joubert & Schulz, 2006
	0.60-8.61	3.77	220	Manley *et al*., 2006
Nothofagin[b]	0.2-1.24	0.67	97	Joubert & Schulz, 2006
	0.07-1.09	0.45	220	Manley *et al*., 2006

[a]Not determined, [b]nothofagin content expressed as g aspalathin equivalents/100 g unfermented rooibos plant material.

Von Gadow *et al*. (1997a) were the first to investigate the effect of fermentation on the antioxidant activity of rooibos. It was established that the antioxidant activity of rooibos decreased with increasing degree of oxidation: unfermented > semifermented > fermented (Von Gadow *et al*., 1997a). The antioxidant potential of the individual phenolic compounds in rooibos was also investigated and compared to that of α-tocopherol and the synthetic antioxidants butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) (Von Gadow *et al*., 1997b). The rooibos compounds tested included aspalathin, vitexin, rutin, quercetin, luteolin, isoquercitrin, (+)-catechin, protocatechuic acid, caffeic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, ferulic acid, syringic acid and vanillic acid. Aspalathin, the major flavonoid present in unfermented rooibos, was found to be a potent DPPH radical scavenger; more effective than any of the commercial antioxidants.

In another study, Winterton (1999) evaluated the antioxidant activity of water extracts, crude polymeric fractions and ethyl acetate fractions of both fermented and unfermented rooibos in model lipid systems. The activity of these fractions was compared to the antioxidant activity of BHT, BHA, tertiary butyl hydroquinone (TBHQ) and Trolox. A crude aspalathin fraction and a tannin fraction from unfermented rooibos were also evaluated. The unfermented ethyl acetate and crude aspalathin fractions were found to have the greatest antioxidant activity in both sunflower oil-in-water (peroxide and conjugated dienes determined) and linoleic acid emulsions (conjugated dienes determined), as well as methyl linoleate micelles (oxidation initiated with Fenton's reagent and measured using thiobarbituric acid). This was ascribed to the presence of flavonoids with a high antioxidant activity in the fractions.

The radical scavenging activity of rooibos flavonoids differs according to the type of radical scavenged (Joubert *et al*., 2004). The $DPPH^{\bullet}$ scavenging ability of rooibos flavonoids decreases in the order: quercetin ≥ procyanidin B3 ≥ orientin ≥ luteolin ≥ aspalathin ≈ isoquercitrin > iso-orientin > catechin > rutin >> vitexin ≥ chrysoeriol. The order for $O_2^{-\bullet}$ scavenging ability is: quercetin ≈ aspalathin > orientin ≥ catechin ≥ rutin ≥ isoquercitrin > iso-orientin > luteolin > chrysoeriol > vitexin. Joubert *et al.* (2004) also investigated the radical scavenging activity of aqueous extracts, crude phenolic fractions and tannins of rooibos. The ethyl acetate soluble fraction of aqueous extracts of unfermented rooibos together with the crude aspalathin fraction had the highest radical scavenging capacity. This was attributed to the high TP content of the extracts. Rooibos tannin, having a low TP content, was the least effective radical scavenger. Table 2.5 is a comparison of the antioxidant activity of the aqueous soluble solids of unfermented (green) and fermented rooibos.

The previously mentioned fractions were also evaluated for pro-oxidant activity, using the deoxyribose assay (Joubert *et al*., 2005). The aqueous extracts and crude fractions of unfermented rooibos were found to be significantly more pro-oxidant than their fermented counterparts. In general, the greater the antioxidant activity ($O_2^{-\bullet}$ scavenging and H-donating) of the extracts, the more pro-oxidant they were found to be in the deoxyribose system. This pro-oxidant activity was found to be linear with respect to dihydrochalcone (aspalathin and nothofagin) content. Due to lowering of the dihydrochalcone content, oxidation of rooibos results in decreased pro-oxidant activity. These results suggest that, depending on the type of rooibos (fermented or unfermented), the quantities used in food or as supplements, are very

Table 2.5 Comparison of the antioxidant activity of the aqueous soluble solids of green and fermented rooibos (Joubert & Schulz, 2006)

Assay	Endpoint	Green Rooibos	Fermented Rooibos	Reference(s)
$DPPH^{\bullet}$	% Scavenging[a]	86.6	83.4	Von Gadow *et al*., 1997a
	% Scavenging[b]	87.3	83.0	Joubert *et al*., 2004
	EC_{50}[c]	2.33	3.62	adapted from Standley *et al*., 2001
	EC_{50}[c]	3.24	3.87	Joubert *et al*., 2004
	Rate of scavenging[d]	8.3×10^{-4}	7.35×10^{-4}	Winterton, 1999
$O_2^{-\bullet}$	IC_{50}[e]	44.4	60.5	adapted from Standley *et al*., 2001
	IC_{50}[e]	69.4	78.3	Joubert *et al*., 2004
Linoleic acid emulsion	% Inhibition (CD)[f]	28.6	28.0	Joubert *et al*., 2005
β-Carotene-linoleic acid oxidation	ACC[g]	557	607	Von Gadow *et al*., 1997a
Sunflower oil-in-water emulsion	% Inhibition (peroxides)[h]	90.0	80.9	Winterton, 1999
	Induction time (PV)[i]	35	31	
	% Inhibition (CD)[j]	58.1	54.5	
Methyl linoleate micelles	% Inhibition of TBARS[k]	22.8	30.3	Winterton, 1999

[a]Scavenging (%) of $DPPH^{\bullet}$ (6×10^{-5} M) after 2h, [b]scavenging (%) of $DPPH^{\bullet}$ (3.04×10^{-5} M) after 20 min, [c]effective concentration of soluble solids (mg) per ml reaction mixture to scavenge 50% of DPPH, [d]$DPPH^{\bullet}$ rate of scavenging (s^{-1}), calculated during unsteady state conditions (time 0 - 3 min), expressed as the change in the absorbance at 515 nm over time, [e]concentration of soluble solids (mg) per ml reaction mixture required to inhibit 50% of NBT reduction, [f]inhibition (%) of conjugated diene (CD) formation after 21 h incubation at 40°C, [g]antioxidant activity, [h]inhibition of peroxides after 35 days incubation at 30°C, [i]time required for oxidation to reach a peroxide value (PV) of 10 meq/kg oil with incubation at 30°C, [j]inhibition of CD formation after 31 days incubation at 30°C, [k]inhibition of the formation of thiobarbituric acid reactive substances (TBARS) after 16 h incubation at 37°C.

important. Smaller quantities of unfermented rooibos, compared to fermented rooibos, should be used (for antioxidant purposes) in order to prevent oxidative damage (Joubert *et al*., 2005).

7. CONCLUSION

Today, due to improved living standards and advances in health care, most people are living longer. This has seen a dramatic increase in the occurrence of diseases that generally occur later in life, for example, cancer and cardiovascular disease. Lately, researchers have established that a diet containing ample fruits and vegetables, rich in phytochemicals such as flavonoids, may reduce or even prevent the development of such diseases. The benefits of such diets are thought to arise from the free radical neutralising ability of compounds such as flavonoids, carotenoids and tocopherols.

Awareness of the role that diet can play in the prevention of such diseases is gradually permeating the minds of consumers and consequently, the demand for natural, antioxidant-rich products is on the increase. Unfortunately, the highly lucrative nature of this industry has lured many unscrupulous manufacturers. Due to inadequate regulation, the functional food market undoubtedly contains a mass of food and beverage products that do not contain any functional ingredients (or contain quantities that are too low to be effective). This may be due to processing losses as well as a host of other potential reactions. Apart from this major hurdle, scientific data concerning the bioavailability, toxicity and *in vivo* antioxidant functionality of the majority of these compounds is still lacking, leading to uncertainty over the future of the functional food industry. The way forward will depend on the persistence of proven *in vitro* disease modulating compounds in functional food products and the establishment of their *in vivo* functionality.

Rooibos has been shown to exhibit significant antioxidant activity. In the unfermented material, this has been ascribed to the presence of the dihydrochalcone glucoside, aspalathin. As a functional ingredient, however, data concerning the processing stability of this key ingredient is lacking. In order to promote usage of this South African shrub in upcoming functional food products, research in this area is required.

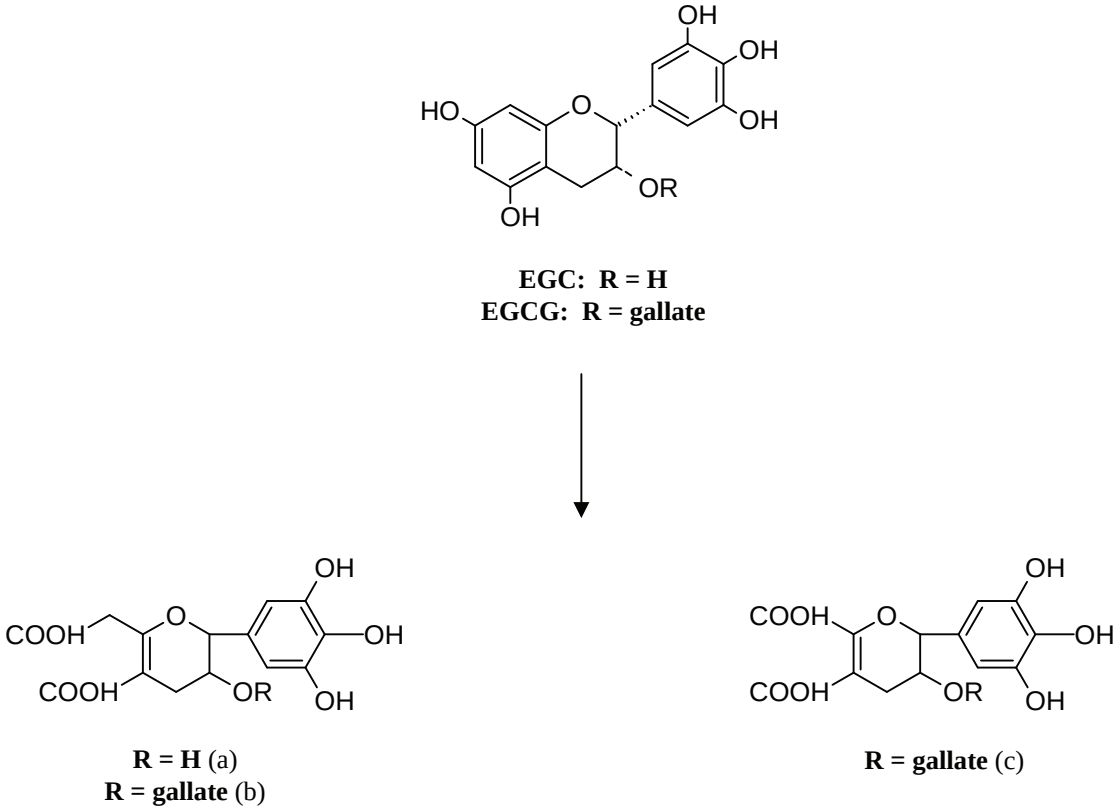


Figure 2.1 The hydrogen peroxide-induced oxidation products of (a) EGC and (b and c) EGCG (Zhu *et al*., 2000).

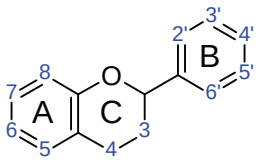


Figure 2.2 The general structure of flavonoids (Das & Pereira, 1990).

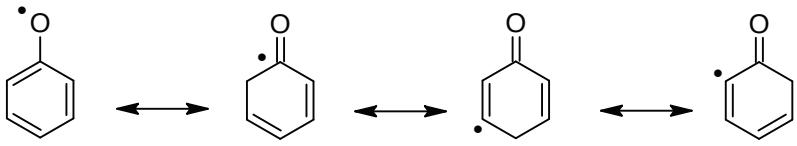
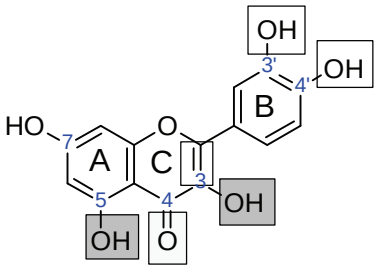
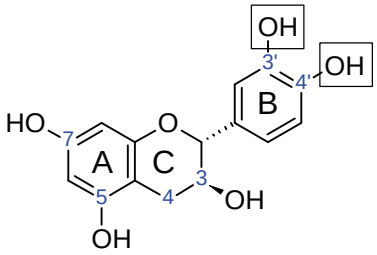


Figure 2.3 The stabilisation of a phenoxyl radical by via electron delocalisation (Shahidi & Wanasandara, 1992).

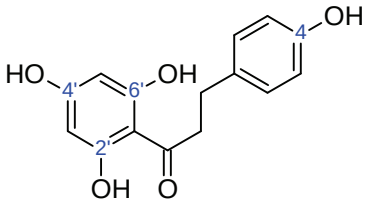


(a)

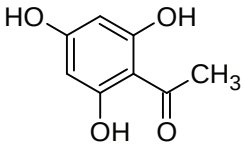


(b)

Figure 2.4 The structure-activity relationship of (a) quercetin and (b) catechin (Rice-Evans & Miller, 1998).



(a)



(b)

Figure 2.5 The structure of (a) phloretin and (b) 2,4,6 trihydroxyacetophenone (Rezk *et al.*, 2002).

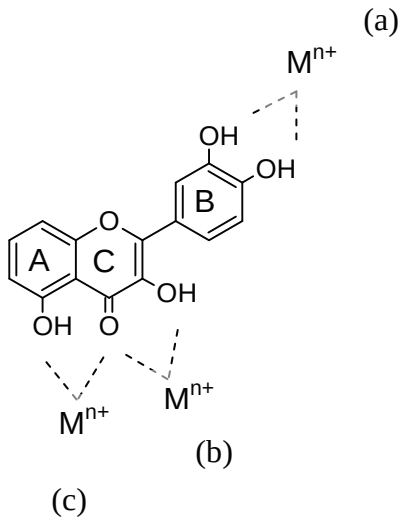


Figure 2.6 The binding sites of metal ions on flavonoids are (a) the 3',4'-*o*-diphenolic group in the B-ring, (b) the 4-keto and 3-OH groups in the C-ring or (c) the 4-keto (C-ring) and 5-OH group in the A-rings, respectively (Pietta, 2000).

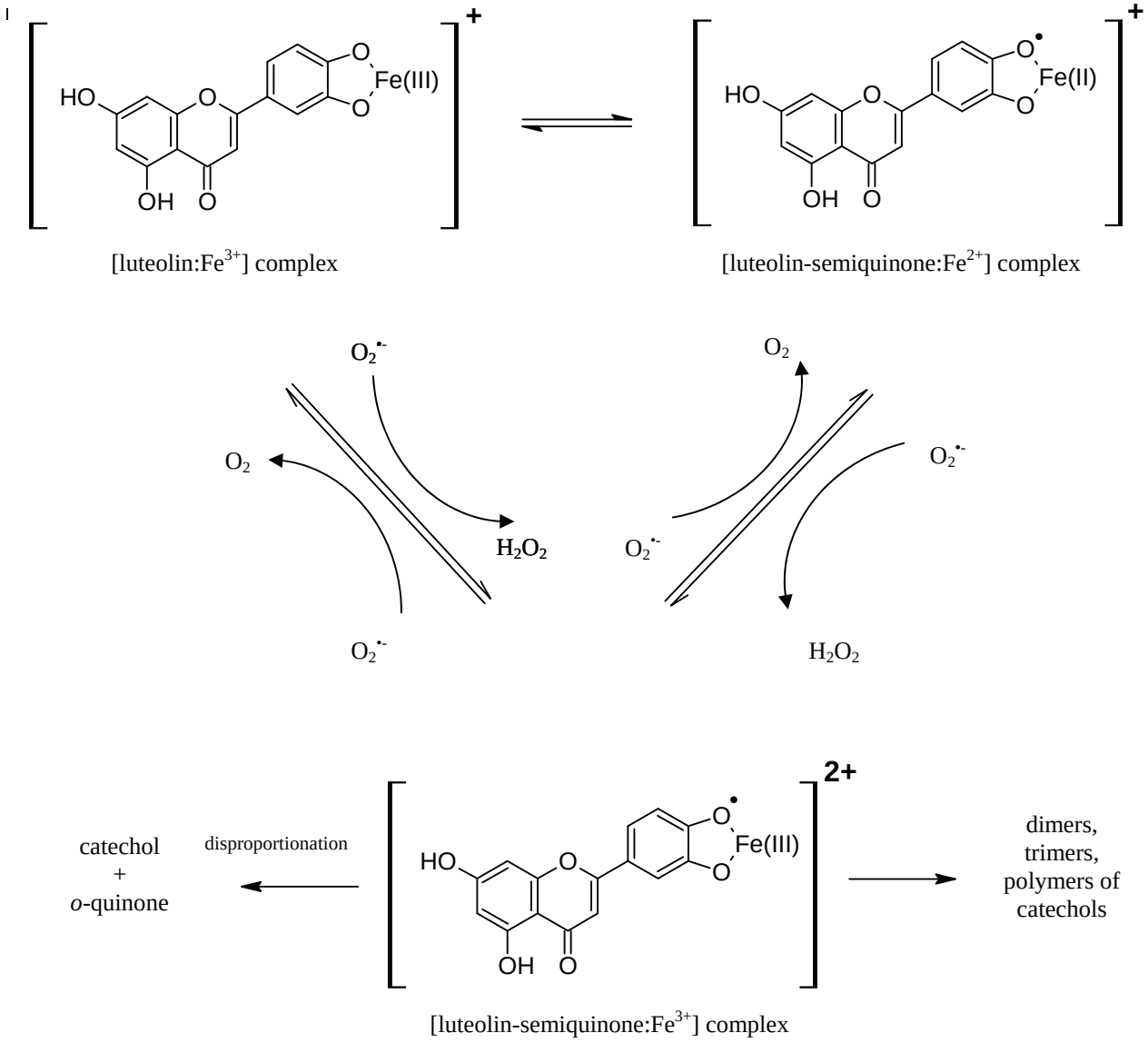


Figure 2.7 A schematic representation of the mechanism of superoxide radical scavenging by flavonoid:iron complexes (Moridani *et al*., 2003).

Low pH Higher pH

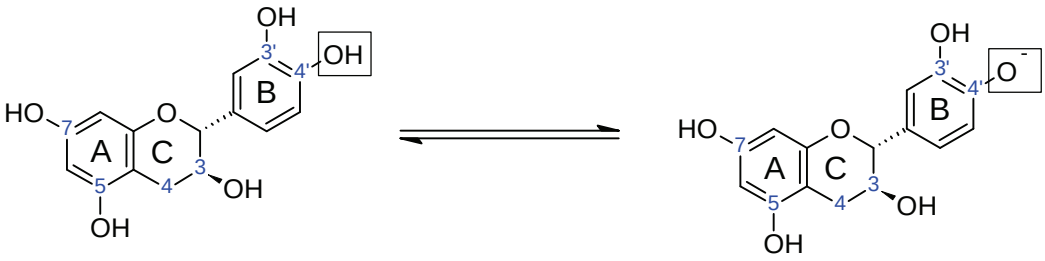
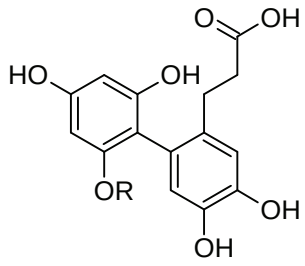
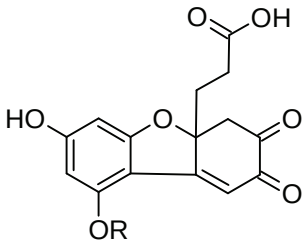


Figure 2.8 Deprotonation of quercetin (adapted from Lemanska *et al*., 2001).

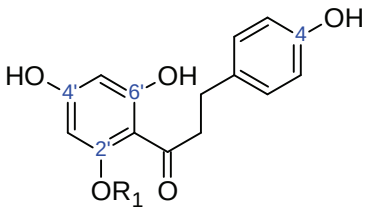


(a)

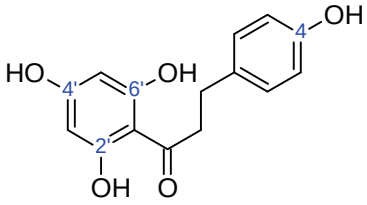


(b)

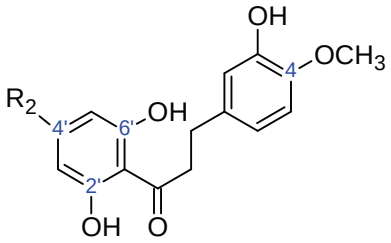
Figure 2.9 Oxidation products of phloridzin and polyphenol oxidase: (a) intermediate and (b) pigment (Guyot *et al*., 2007).



(a)



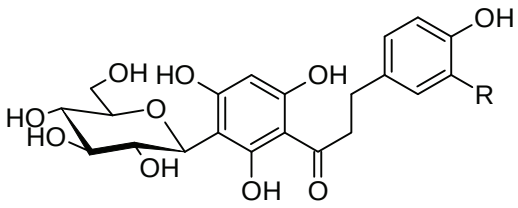
(b)



(c)

Figure 2.10 The structures of (a) phloridzin (R_1 = β-D-glucopyranosyl) (Rezk *et al*., 2002), (b) phloretin (Rezk *et al*., 2002) and (c) neohesperidin dihydrochalcone (R_2 = α-L-rhamnosyl-*p*-(1→2)-D-glucopyranosyl residue via β-*O*1 glycosidic linkage, often referred to as β-neohesperidosyl residue) (Malpezzi *et al*., 2004).

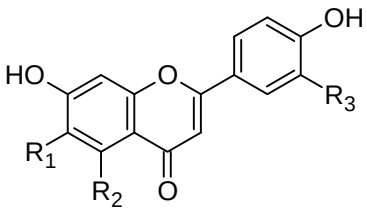
Dihydrochalcones



Aspalathin: R = OH

Nothofagin: R = H

Flavone C-glycosides



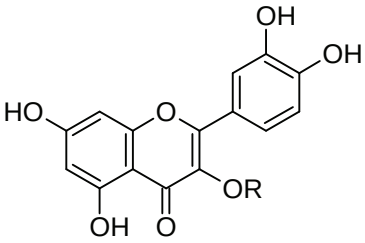
Orientin: R_1 = H, R_2 = glucosyl, R_3 = OH

Iso-orientin: R_1 = glucosyl, R_2 = H, R_3 = OH

Vitexin: R_1 = H, R_2 = glucosyl, R_3 = H

Iso-vitexin: R_1 = glucosyl, R_2 = H, R_3 = H

Flavonols

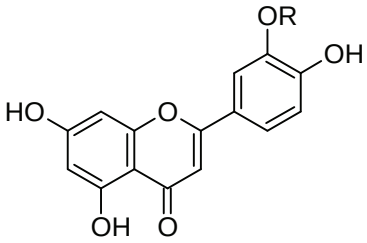


Quercetin: R = H

Isoquercitrin: R = glycosyl

Rutin: R = rutinosyl

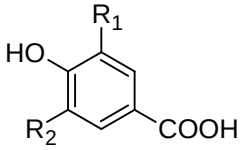
Flavone aglycons



Chrysoeriol: R = CH_3

Luteolin: R = H

Hydroxybenzoic acids



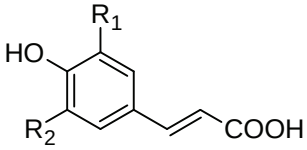
p-Hydroxybenzoic acid: R_1 = R_2 = H

Protocatechuic acid: R_1 = OH ; R_2 = H

Vanillic acid: R_1 = OCH_3; R_2 = H

Syringic acid: R_1 = R_2 = OCH_3

Hydroxycinnamic acids



p-Coumaric acid: R_1 = R_2 = H

Caffeic acid: R_1 = OH ; R_2 = H

Ferulic acid: R_1 = OCH_3; R_2 = H

Figure 2.11 The structures of some of the main flavonoids and phenolic acids present in rooibos (Rabe *et al*., 1994; Joubert & Ferreira, 1996).

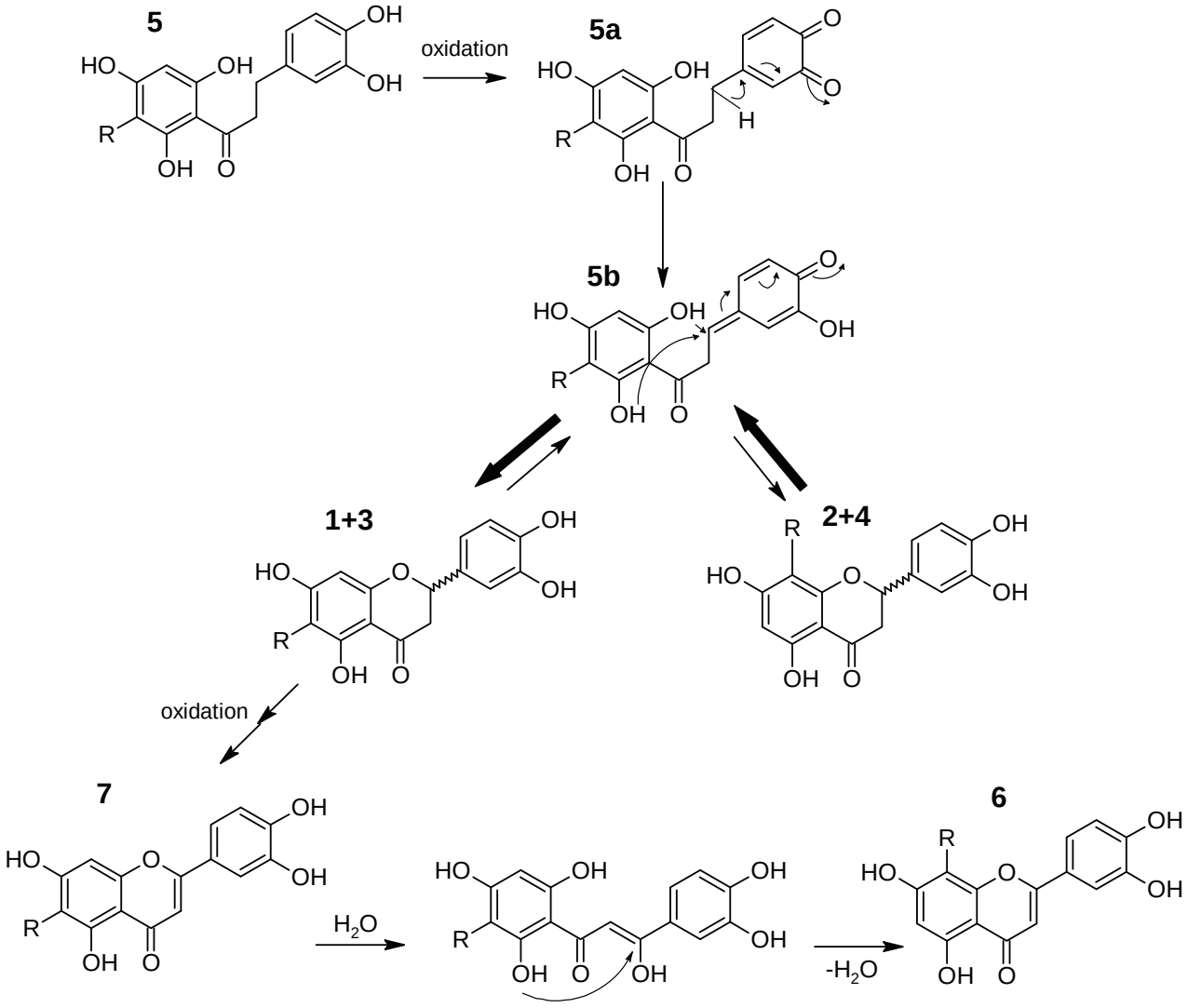


Figure 2.12 Oxidative degradation of (**5**) aspalathin to (**6**) orientin and (**7**) iso-orientin. Compounds **1-4**, eriodictyols, are key intermediates. R = glucose (Krafczyk & Glomb, 2008).

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CHAPTER 3

EFFECT OF THERMAL PROCESSING ON THE PHENOLIC COMPOSITION AND COLOUR OF ROOIBOS ICED TEA

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ABSTRACT

The change in the aspalathin, iso-orientin, orientin and total polyphenol (TP) content of a commercially produced fermented rooibos (FR) extract was monitored throughout production. The effect of spray-drying on both FR and unfermented rooibos (UR) extracts was given particular attention. The quality of commercial, South African rooibos iced teas was also investigated with respect to the aforementioned parameters. Changes in individual flavonoids were quantified using HPLC, while the Folin-Ciocalteu assay was used to assess the TP content of the extracts and iced teas. The impact of heating on experimental FR and UR iced teas as well as an nano emulsified unfermented rooibos (NEUR) iced tea was also investigated. Phenolic compounds and TP content were determined as before whilst browning of the teas was monitored spectrophotometrically (420 nm).

Aspalathin, iso-orientin and orientin were found to be present after all stages of the FR extract production process. Spray-drying did not significantly alter the aspalathin, iso-orientin, orientin or TP content of FR or UR extracts. The aspalathin, iso-orientin and orientin content of the commercial iced teas was extremely low or absent. The quality and quantity of the extracts used, and not the duration of storage, appeared to be the main factor influencing the phenolic quality of the commercial iced teas.

The aspalathin, iso-orientin and orientin content of both FR and UR experimental iced teas was significantly reduced ($P<0.05$) by normal temperature sterilisation (NTS) and high temperature sterilisation (HTS), but not necessarily by pasteurisation. The addition of citric and ascorbic acid proved beneficial for the retention of the aforementioned compounds. For example, loss of aspalathin in formulation B (base, extract in deionised water) of the FR iced teas was 76.0% and 78.1% after NTS and HTS, respectively. In contrast, losses were 9.7% and 13.6% in formulations containing base + citric acid (BC) and base + citric + ascorbic acid (BCA), respectively. This pattern persisted for the two UR iced teas. Overall, the NEUR iced teas proved most heat stable with aspalathin losses of 22.7% and 17.5% (HTS and NTS) in formulation NE (NEUR extract in deionised water). Apart from a reduction in aspalathin content, NTS and HTS also resulted in the formation of new flavonoid compounds, as detected at 288 nm. These peaks were observed in the FR and UR iced teas as well as the NEUR iced tea. Pasteurisation (30 min at 93ºC) significantly decreased ($P<0.05$) the aspalathin content of formulation B and significantly increased ($P<0.05$) the aspalathin content of formulation BCA of both the FR and UR iced teas. Formulation NE of the NEUR iced tea also experienced a significant decrease ($P<0.05$) in aspalathin content.

* Part of this work was presented at the 24th International Conference on Polyphenols, 8-11 July 2008, Salamanca, Spain.

Heat-induced losses of iso-orientin and orientin were lower than those for aspalathin in all three types of experimental iced tea. For example, NTS and HTS significantly reduced ($P<0.05$) the iso-orientin content of UR iced tea (formulation B) by 12.0 and 11.3% respectively, but did not significantly ($P\geq0.05$) reduce the orientin content. This was in contrast to aspalathin losses of 34.0 and 37.9% in the same tea formulation. The absorbance of most of the iced teas increased as a result of heating. The addition of citric and ascorbic acid minimised absorbance changes for FR and UR iced teas, reducing the absorbance of certain FR iced teas after HTS. The absorbance of the formulation BC (base + citric acid) and BCA decreased significantly ($P<0.05$) by 12.3% and 12.4%, respectively, after HTS. The addition of citric acid had the opposite effect on the absorbance of the NEUR iced tea, formulation NEC (NE + citric acid). In general, the TP content of the iced teas increased upon heating.

INTRODUCTION

The rooibos shrub, *Aspalathus linearis*, contains the unique dihydrochalcone C-C glucoside aspalathin (Koeppen & Roux, 1965a) that is known to possess significant antioxidant activity (Von Gadow *et al*., 1997a).

The oxidation step ("fermentation"), required for the production of fermented rooibos (FR) extracts typically reduces the aspalathin content of the plant material (Joubert, 1996). Consequently, the hot-water-soluble solids of unoxidised (green or unfermented) rooibos has a greater antioxidant capacity than traditional, oxidised rooibos (Von Gadow *et al*., 1997b). Rooibos contains a host of other compounds including flavones (Snyckers & Salemi, 1974; Ferreira *et al*., 1995), flavonols (Koeppen *et al*., 1962; Snyckers & Salemi, 1974) and the C-C linked flavone glycosides orientin and iso-orientin (Koeppen & Roux, 1965b), to mention but a few. Iso-orientin and orientin are major compounds in FR plant material and have been shown to form as a result of the oxidation of aspalathin (Krafczyk & Glomb, 2008). Koeppen & Roux (1965a; 1966) first proposed a mechanism by which aspalathin oxidation occurs, i.e. via the intermediary flavanones, dihydro-iso-orientin and dihydro-orientin. The presence of brown polymers was also noted.

Extracts of FR are popular as ingredients in the food and beverage industry, particularly in processed products such as ready-to-drink iced teas (Joubert & Schulz, 2006). Thermally processed products typically undergo chemical and physical changes during manufacture (Torregrosa *et al*., 2006). Chemical changes mostly constitute oxidative degradation since oxidation is enhanced at elevated temperatures. Losses of organoleptic properties such as colour and flavour (Torregrosa *et al*., 2006), as well as desirable antioxidants (Manzocco *et al*., 1998a) and nutrients (Ewald *et al*., 1999; Shi & LeMaguer, 2000; Shi *et al*., 2008) are common. For example, catechins have been shown to undergo epimerisation during the production of tea and tea beverages (Kiatgrajai *et al*., 1982; Komatsu *et al*., 1993; Wang & Helliwell, 2000) as well as during autoclaving (20 min at 121ºC, Chen *et al*., 2001). When heated for longer periods (7 h) and under less severe conditions (98ºC), however, degradation occurs (Chen *et al*., 2001).

Processing does not always result in food products with a reduced antioxidant capacity compared to their unprocessed counterparts (Nicoli *et al*., 1997a). The formation of new antioxidant compounds with novel antioxidant properties may maintain or enhance the antioxidant properties of the specific food (Nicoli *et al*., 1997a; Nicoli *et al*., 1997b; Takeoka *et al*., 2001; Dewanto *et al*., 2002; Gerard & Roberts, 2004; Jeong *et al*., 2004). Steam pasteurisation of rooibos leaves has been noted to increase their antioxidant activity (Standley *et al*., 2001). Similarly, the antioxidant activity and polyphenolic content of Shiitake mushrooms were found to increase proportionally to autoclave heating time and temperature (Choi *et al*., 2006). Miglio *et al*. (2008) reported similar findings for a selection of vegetables, namely carrots, courgettes and broccoli. In red wine, however, aging was associated with the progressive polymerisation of polyphenols to form brown macromolecular products and a decrease in antioxidant activity (Manzocco *et al*., 1998b).

Microencapsulation is a technique that can be used to improve the stability of compounds that are susceptible to oxidative changes during processing (Gouin, 2004; Lucas-Abellán *et al*., 2008). Spray-drying is regularly used to encapsulate chemically reactive compounds (McNamee *et al*., 1998). This allows for the inclusion of such ingredients in foods to which the ingredients could not previously be added (Gouin, 2004). In most encapsulated products, carriers act as barriers that stabilise the ingredients during processing. Carriers may also improve the handling or functionality of the encapsulated compound. Nanoencapsulation is the encapsulation of compounds or extracts in micelles of less than 100 nm in diameter (Weiss, 2006; Forssell *et al*., 2006). Due to their sub-cellular size, nano emulsification improves the bioavailability and effectivity of a range of substances (Chen *et al*., 2006; Weiss *et al*., 2006; Wang *et al*., 2008). Due to its processing stability, a nano emulsified unfermented rooibos (NEUR) extract was included in this investigation.

At present, it is not known how the processing of rooibos (as for the production of iced tea) affects its aspalathin, iso-orientin and orientin content. These compounds are important as antioxidants but also as indicators of product authenticity. Should these compounds survive processing, their presence could serve as an infallible method for the verification of the use of rooibos extract in so-called rooibos products. The aim of this study was to determine the change in total polyphenol (TP) content and product colour, as well as aspalathin, iso-orientin and orientin content of rooibos iced tea after different heat treatments i.e., simulating pasteurisation, normal temperature sterilisation (NTS) and high temperature sterilisation (HTS). Apart from the use of a NEUR extract (aspalathin-enriched), an aspalathin enriched unfermented rooibos (UR) extract and a fermented rooibos (FR) extract were subjected to stability testing. The effect of ascorbic acid and citric acid on the stability of aspalathin, iso-orientin and orientin in rooibos iced teas during heat treatment was also investigated.

MATERIALS AND METHODS

Chemicals and reagents

The chemicals required for the HPLC analysis of rooibos iced tea were 99.8% acetic acid (Fluka, Buchs, Switzerland), 99.9% LiChrosolv “Far UV” acetonitrile for gradient analysis (Merck, Darmstadt, Germany),

dimethyl sulfoxide (Sigma Chemical Co., St Louis, MO, USA) and ascorbic acid (Sigma Chemical Co.). The aspalathin, iso-orientin and orientin standards were supplied by the PROMEC Unit of the Medical Research Council (Parow, South Africa), Extrasynthese (Genay, France) and Roth (Karlsruhe, Germany), respectively. A mixture of flavanones isolated from rooibos (isolation by B.H. Koeppen; available from the compound library of the Agricultural Research Council (ARC)) was also used. Deionised water was prepared using a Modulab water purifier (Continental Water Systems Corporation, San Antonio). Further purification using a Milli-Q academic water purifier (Millipore, Bedford, MA, USA) was required to obtain HPLC grade water. The reagents required for the quantification of the TP content of the samples were Folin-Ciocalteu's phenol reagent (Merck), anhydrous sodium carbonate (Saarchem, Gauteng, South Africa) and gallic acid (Sigma Chemical Co.). Food grade ingredients for the production of rooibos iced tea comprised sucrose (Huletts, Rossburgh, Durban, South Africa) and citric acid (Warren Chem Specialities, Cape Town, South Africa). Analytical grade ascorbic acid (Sigma Chemical Co.) was used for iced tea preparation. Ethylene diamine tetraacetic acid di-sodium salt (EDTA, Saarchem) was required for soaking glassware to remove traces of metal ions.

Collection of industrial process samples

Samples were collected during processing at a commercial rooibos extract powder manufacturer. Fermented rooibos extract was collected directly after extraction and cooling to 20-24ºC with a heat exchanger (n = 10). Further samples were collected after microfiltration (0.2 µm filter, n = 9). The micro-filtered extracts were continuously fed into a holding tank, where a minimum of four batches were collected before reverse osmosis commenced. The subsequent semi-concentrated extract was sampled (n = 3) on-line after reverse osmosis, before collecting in another holding tank. Pooled (i.e. from more than one production lot), semi-concentrated extract was further concentrated under vacuum at 70-76ºC in a climbing film evaporator. Sampling (n = 7) took place on-line, directly after concentration. Several batches of concentrate were pooled in the holding tank before spray-drying commenced. Due to the large quantities required for operation of different units, necessitating pooling of batches, consecutive samples taken during production do not represent the previous sample in a more advanced processing stage.

During spray-drying, rooibos concentrate was exposed to an inlet temperature of 210ºC and outlet temperature of 95ºC. Spray-dried sample sets comprised (a) the concentrate fed to the spray-drier and (b) its corresponding spray-dried product. The spray-drying sampling procedure was repeated five and six times for FR and UR extracts, respectively.

The collected extracts and concentrates were frozen (-20ºC) and freeze-dried in an Edwards Modulyo freeze-drier (Edwards, Sussex, England). The freeze-dried soluble solids were pulverised for 15 s to ensure homogeneity, using a Retsch MM301 ball mill (Retsch GmbH, Haan, Germany) and then stored at room temperature in sealed transparent plastic tubes in a desiccator in the dark.

Commercial rooibos iced teas/fruit teas

Eight commercial brands of rooibos iced tea (A – H) were purchased from local supermarkets on three separate occasions. The ingredients of the iced teas are listed in Table 3.1. Two formulations of brand D were purchased: one containing sucrose (D_1) and the other an artificial sweetener (D_2). Except for brand G (four samples) and H (16 samples), three bottles of each brand of iced tea (with differing batch numbers) were purchased. Brand H represented a major brand of which the basic formulation was used as basis for preparation of the experimental rooibos iced teas, investigated in the present study.

Table 3.1 Ingredients of commercial rooibos iced teas/fruit teas[a]

Brand	Packaging	Ascorbic acid	Citric acid	Other ingredients
A	Tetrapak box	Yes	Yes	rooibos, apple juice, fructose, sour cherry juice, raspberry juice, black current juice, buchu extract and flavourants
B	Glass	Yes	No	cranberry juice, apple juice, pear juice, rooibos extract, beta carotene, vitamin E, vitamin B6, folic acid, vitamin B12 and sodium selenium anhydrous
C	Aluminium can	Yes	Yes	water, sugar, fructose, rooibos extract, red fruit tea extract and nature identical flavouring
D_1[b]	PET[c] bottle	Yes	Yes	water, brown sugar, sodium citrate, calcium phosphate, peach approved flavouring and rooibos extract
D_2	PET bottle	Yes	Yes (sodium citrate)	water, Advantage sweetener, malic acid, calcium phosphate, peach approved flavouring and rooibos extract
E	Aluminium can	No	No	water, sucrose, apple juice, malic acid, rooibos extract, flavourants
F	PET bottle	No	Yes	rooibos, apple/pear juice, sucrose, fructose, ginger extract, permitted flavours, caramel, pimaricin, sodium benzoate and potassium sorbate
G	Tetrapak box	No	Yes	hot brewed rooibos, fruit flavourants, water and sugar
H	PET bottle	Yes	Yes	water, sucrose, fructose, rooibos extract (hot brew process), nature identical flavouring, natural lemon flavour, sodium benzoate and pimaricin

[a]All brands listed rooibos extract/rooibos as an ingredient on the label, [b]two formulations of brand D were purchased: (D_1) containing sucrose and (D_2) containing an articial sweetener, [c]polyethylene terephthalate.

Extracts for preparation of experimental rooibos iced tea

For the production of rooibos iced tea three rooibos extracts were obtained from different sources: powdered UR extract enriched in aspalathin through a patented extraction process (Raps Foundation, Freising-Weihenstephan, Germany), powdered FR extract (Afriplex, Paarl, South Africa) and an aspalathin-enriched NEUR extract prepared by Aquanova (Darmstadt, Germany) and supplied by Raps Foundation. The NEUR extract comprised *ca*. 15% UR extract (Raps & Co), *ca*. 5% ascorbic acid and *ca*. 80% Tween 20 (an emulsifier).

Effect of heat and product formulation on the phenolic composition and colour of experimental rooibos iced teas

The three commercial rooibos extracts were used for the formulation of the iced teas. Three formulations of FR and UR iced teas were made: base (formulation B, rooibos extract in deionised water); base + citric acid (BC), and base + citric + ascorbic acid (BCA). In each case, deionised water was used. The NEUR extract inherently contains *ca*. 5% ascorbic acid as an antioxidant. The addition of further ascorbic acid (as in formulation BCA) seemed redundant. For this reason only two formulations of the NEUR iced tea were prepared: NE (NEUR extract in deionised water) and NEC (NE + citric acid). Since these two formulations (NE and NEC) both contained ascorbic acid, they differed from those of the FR and UR formulations. All iced teas contained 60 g/L sugar, formulations BC, BCA and NEC contained 1.2 g/L citric acid and formulation BCA contained an additional 0.2 g/L ascorbic acid. The exact mass of the ingredients used for the various formulations was recorded to a minimum of four decimal places (Precisa Instruments AG, Switzerland). Values in Table 3.2 were rounded to two decimal places as they are merely indicative of the desired mass. The heat treatments were replicated, independently, four times (i.e. tea of a specific formulation was heated on four separate occasions, directly after preparation).

Table 3.2 The mass (g) of the three types of rooibos extract[a] used for the various types and formulations of one liter of rooibos iced tea

Iced tea and extract	**Formulation**		
	B[b]/NE[c]	**BC[d]/NEC[e]**	**BCA[f]**
FR	1.75	1.75	1.75
UR	1.75	1.75	1.75
NEUR	14.00 (2.10)[g]	14.00 (2.10)[g]	-

[a]Fermented rooibos (FR); unfermented rooibos (UR) and nano emulsified unfermented rooibos (NEUR), [b]base (FR or UR extract in deionised water), [c]NEUR extract in deionised water, [d]base + citric acid, [e]NE + citric acid, [f]base + citric + ascorbic acid, [g]value in brackets represents the equivalent amount of unfermented rooibos extract.

The heat treatments in question were a pasteurisation-like heat treatment (to be referred to as pasteurisation), "normal" temperature sterilisation (NTS) and high temperature sterilisation (HTS). Pasterurisation was achieved, using a self-assembled laboratory-scale "pasteuriser" (built with the help of Dr C. Hansmann, Division Post-Harvest and Wine Technology, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa). The pasteuriser consisted of a 3 m long stainless steel gas chromatography coil (5 mm inner diameter), Tygon flexible plastic tubing (formulation R3606, Saint Gobain Verneret, i.d. = 4.76 mm, o.d. = 7.94 mm, wall = 1.59 mm), a water bath at 93°C (Labotec, South Africa), Watson Marlow 505U peristaltic pump (220 rpm and a flow rate of approximately 13.80 mL/s) (Watson Marlow Bredel Pumps Ltd., Cornwall, England) and a 1 L vacuum flask (to serve as a holding vessel). The exit temperature of 93°C was maintained by continuously re-circulating the iced tea that collected in the vacuum flask for 30 min. Upon completion of the heating step, the tea was collected in 25 mL glass bottles (Separations, Cape Town, South Africa) and held on ice for 30 min. Unheated samples (controls) were retained so as to evaluate the effect of the heat treatment on the iced tea.

A bench-top autoclave steriliser (Sturdy Industrial Co. Ltd., Taiwan) capable of temperatures of 135°C was used for NTS and HTS of the iced tea formulations. The iced tea samples were placed in new 20 mL gas chromatography headspace vials, sealed with aluminium caps containing PTFE/butyl rubber septa (Separations, Cape Town). Normal temperature sterilisation (NTS) comprised autoclaving at 121°C for 15 min, whereas the HTS treatment comprised autoclaving at 135°C for 4 min. Due to the duration of the heating and cooling process, samples were in the autoclave for approximately 45 min. Following removal from the autoclave, the samples were immediately placed on ice for 30 min. Unheated samples (controls) were retained so as to evaluate the effect of NTS and HTS on the iced tea.

All samples were divided, without delay, into 2 mL aliquots and frozen for analysis at a later stage.

Effect of heat and product formulation on the phenolic composition and colour of experimental rooibos iced teas – pasteurisation reinvestigated

A second pasteurisation heat treatment was carried out by heating the iced tea samples, in 20 mL Pyrex glass tubes (i.d. = 15 mm) with screw caps, in a water bath (93°C) for 5 min or 30 min. Samples achieved a temperature of 93°C after *ca*. 2 min. Unheated samples served as controls. The tubes and screw caps were soaked in 0.5% EDTA overnight (Farag *et al*., 1989), followed by rinsing in deionised water three times. This treatment was performed to prevent unintentional metal ion contamination from the previously used tubes. The heat treatment was replicated, independently, four times. All samples were divided without delay into 2 mL aliquots and frozen for analysis at a later stage.

Determination of the total soluble solids content of industrial aqueous extracts of fermented rooibos

The total soluble solids content (g/100 g extract) of rooibos extracts and concentrates collected during the industrial extract manufacturing process was determined gravimetrically, in duplicate, on 10 g aliquots prior to freeze drying of the remaining extract. The aliquots were evaporated to dryness in nickel moisture dishes on a

steam bath (Merck), followed by oven drying (100ºC for 2 h) (Term-O-Mat, Labotec). The samples were then allowed to cool in a desiccator before weighing.

Determination of moisture content of freeze-dried and spray-dried powdered industrial extracts of rooibos

The moisture content of the industrial samples was analysed with a HR73 Mettler halogen moisture analyser (Mettler, Switzerland). Approximately 3 g of FR extract was used for analysis.

Determination of the iron content of rooibos extracts

The iron content of the three rooibos extracts used for the production of the experimental iced teas was determined by Bemlab (Somerset West, South Africa) using an inductive coupled plasma spectrophotometer (ICPS).

pH determination of iced tea

A Crison GLP 21 pH meter (Crison Instruments SA, South Africa) was used to determine the pH of the respective iced tea formulations. The pH meter was standardised with Sigma buffer reference standards, i.e. pH 7 (pH 7 ± 0.01 at 25°C) and pH 4 (pH 4 ± 0.01 at 25°C).

Monitoring browning in experimental rooibos iced tea

Browning of the iced tea samples, occurring during heat treatment, was determined as an increase in absorbance at 420 nm (Pokorny, 1987; Cilliers & Singleton, 1990; Turker *et al*., 2004). Due to the formation of a precipitate, the samples were centrifuged before colour changes could be quantified spectrophotometrically. All samples were centrifuged at 18 000 rpm for 4 min in a Hettich Universal 16 centrifuge (Hettich, Tuttlingen, Germany), fitted with a 1612 rotor, after which 200 µL of each supernatant was pipetted in duplicate into a 96-well Greiner, round bottom plate (Lasec, Cape Town, South Africa). The absorbance of the samples was measured at 420 nm using a Biotek Synergy HT multiplate reader (Biotek Instruments, Winooski, USA). Gen5 Secure software was used to set-up a protocol for the analysis, manage the plate layout and capture data. The absorbance of the empty plate was measured prior to the addition of sample. The absorbance of the empty plate was subtracted from the final absorbance reading in order to obtain the absorbance reading of the sample alone. Absorbance readings of different samples were normalised based on the mass of extract in the iced tea originally weighed off.

Determination of the total polyphenol content of rooibos extracts and iced teas

The TP content of the iced tea samples was determined according to the method by Singleton & Rossi (1965), scaled-down for a microplate reader. Gallic acid served as a standard. Twenty microlitres (20 µL) of each of the standards (10 mg/L – 80 mg/L), samples and assay control (deionised H_2O) was pipetted in triplicate into a 96-

well, Greiner round bottom plate. A blank reading was taken of the empty plate prior to the addition of the control, standards or samples. Ten times diluted Folin-Ciocalteu reagent (100 µL) was then added to all wells using a manual Gilson multipipette. Without delay, 80 µL of the Na_2CO_3 (7.5 %) solution was added, after which the contents in the wells were mixed in a Biotek Synergy HT multiplate reader. This was followed by incubation at 30°C for 2 h in a forced draft oven. The absorbance readings of the control, standards and the samples were taken at 765 nm after the incubation period, using the Biotek Synergy HT multiplate reader. Results were expressed as g gallic acid equivalents/g tea extract (g GAE/g). In the case of the commercial iced teas (with unknown mass of tea extract), results were expressed as g GAE/100 ml iced tea.

HPLC quantification of the phenolic content of rooibos extracts and iced teas

Quantification of aspalathin, orientin and iso-orientin was performed by HPLC-DAD. The HPLC apparatus used was an Agilent 1200 system (Agilent, Santa Clara, CA, USA) comprising a quaternary pump (model G1311A), autosampler (model G1329A), in-line degasser (model G1322A), column thermostat (model G1316A) and diode-array detector (model G1315B). Chemstation software for LC 3D systems (Agilent, Rev. B.02.01-SR2[260]) was used to control the system and record data. Separation was performed at 38°C on a Zorbax Eclipse XDB-C18, 5 µm, 150×4.6 mm column (Agilent) protected with a RP/C18 guard column (Vici-AG International, Schenkon, Switzerland). The gradient profile for separation is shown in Table 3.3. Chromatograms were recorded at 288 nm for quantification of aspalathin and at 350 nm for quantification of orientin and iso-orientin.

Spray- and freeze-dried powdered samples were prepared for HPLC analysis by dissolving *ca.* 88 mg of extract in 50 mL deionised water containing 0.5% ascorbic acid. Iced tea samples (commercial and experimental) were prepared by mixing 1.5 mL iced tea with 100 µL of 10% ascorbic acid. All samples were filtered before HPLC analysis using 33 mm Millex-HV 0.45 µm syringe tip filters (Millipore). Stock solutions of the standards were prepared with dimethyl sulfoxide and frozen in multiple aliquots. An aliquot of each standard was defrosted at 30°C for 10 min before the preparation of the working standard mixtures for injection. Calibration curves were prepared using five concentrations of aspalathin (1.9 - 95.5 µg/mL), orientin (1.5 - 74.4 µg/mL) and iso-orientin (1.6 - 80.8 µg/mL). Injection volumes were adjusted according to the content of phenolic compounds and were as follows: 5 µL for UR iced tea or solubilised tea extracts; 50 µL for FR iced tea or solubilised tea extracts; 100 µL for commercial iced tea; 3 µL for iced tea prepared with the NEUR extract; and 20 µL for calibration standards.

Peaks were identified as aspalathin, iso-orientin or orientin according to retention time and spectral characteristics. A flavanone mixture, isolated from rooibos, was injected to confirm the retention time of these compounds (oxidation products of aspalathin). Peak area integration was performed by Chemstation software and the average peak area of duplicate injections was used calculate the aspalathin, iso-orientin and orientin content of the samples from the respective standard curves. The regression parameters and standard curve for aspalathin are shown in Table A1.1 and Figure A 1.1, respectively in ADDENDUM 1.

Table 3.3 The gradient program for HPLC analysis

Time (min.)	Acetonitrile (%)	2% acetic acid (%)
0	18	82
10	20	80
13	80	20
16	18	82
23	18	82

Typical retention time and peak area of aspalathin, iso-orientin and orientin in the three types of rooibos extracts at the respective injection volumes are given in Figure A2.1 (288 nm) and Figure A2.2 (350 nm) in ADDENDUM 2. The spectra (220-420 nm) of aspalathin, iso-orientin and orientin are shown in Figure A2.3 in ADDENDUM 2.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) using SAS version 9.3 (SAS Institute, Cary, NC, USA) and analysed for normality using the Shapiro-Wilk test for normality ($P \geq 0.05$). The data arising from FR, UR and NEUR iced teas were analysed separately due to their vastly different aspalathin contents. The Student t-test was used to ascertain whether there were significant differences between samples, within a particular extract type. The teas made from different extracts were analysed separately due to the large difference in phenolic make-up between the FR and UR extracts. Differences between formulations of the same extract type were lost when all the data were analysed together. Differences with a significance level of 5% ($P<0.05$) were considered significant (SAS, 2002). In the case of the second pasteurisation experiment, data were normalised (in terms of extract mass) so that results of the first and second experiment could be compared.

RESULTS

The phenolic content of rooibos extracts at various stages of the production process

As expected the soluble solid content of the rooibos extract gradually increased as production progressed (Table 3.4). Similarly, the TP content of the concentrated product was greater than that of the initial extract.

Table 3.4 Soluble solid and total polyphenol content of aqueous extracts of fermented rooibos taken at four stages of the commercial extract production process

	Soluble solids[a]		Total polyphenol content[b]	
	Range	Average ±SD	Range	Average ±SD
Extract	0.85-1.59	1.19±0.24	20.57-26.42	22.90±1.91
Microfiltration	0.44-0.81	0.68±0.14	21.63-24.80	23.28±1.11
Reverse osmosis	9.66-11.09	10.18±0.79	25.29-27.67	26.54±1.20
Concentration	21.52-25.24	23.45±1.59	24.59-29.80	26.77±1.60

[a]Results expressed as g/100 g extract, [b]results expressed as g GAE/100 g extract.

The aspalathin, iso-orientin and orientin content of the extracts at the various production stages are shown in Table 3.5. Direct comparison of the extract at the various stages (e.g. microfiltartion vs. reverse osmosis) was not possible due to pooling of production batches between processing steps. It is, however, clear that aspalathin, iso-orientin and orientin are present in the samples taken at all stages of production, and major losses of these compounds did not occur during processing.

Table 3.5 Phenolic content of freeze-dried aqueous extracts of fermented rooibos taken at four stages of the commercial extract production process

	Batches	Aspalathin		Iso-orientin		Orientin	
		Range	Ave[a] ±SD	Range	Ave ±SD	Range	Ave ±SD
Extract	10	0.07-0.37[b]	0.22±0.09	0.43-0.71	0.58±0.07	0.76-1.09	0.89±0.10
Microfiltration	9	0.14-0.35	0.24±0.08	0.54-0.74	0.66±0.08	0.81-1.21	1.10±0.13
Reverse osmosis	3	0.23-0.44	0.35±0.11	0.70-0.74	0.71±0.02	1.15-1.21	1.19±0.03
Concentration	7	0.18-0.38	0.32±0.09	0.68-0.74	0.71±0.03	1.16-1.20	1.18±0.01

[a]Average, [b]results expressed as g/100 g extract.

The difference in moisture content (Table 3.6) between the spray-dried and freeze-dried powder samples obtained from rooibos concentrates was taken into account when calculating the total polyphenol (Table 3.7) as well as aspalathin, iso-orientin and orientin content (Table 3.8). Spray-drying did not significantly ($P<0.05$) affect the composition of either of the two types (FR and UR) of extract, since the TP (Table 3.7), aspalathin, iso-orientin and orientin content (Table 3.8) of the freeze-dried concentrate did not differ significantly ($P\geq0.05$) from that of the spray-dried concentrate.

Table 3.6 Moisture content of freeze-dried and spray-dried powder samples obtained from concentrates of fermented and unfermented rooibos

Rooibos extract	Sample	Batches	Range	Average ±SD
Fermented	Freeze-dried	5	4.33-5.76[a]	5.01±0.67
	Spray-dried	5	2.95-3.73	3.32±0.32
Unfermented	Freeze-dried	6	3.28-4.33	3.85±0.34
	Spray-dried	6	2.42-3.34	2.97±0.34

[a]Results expressed as g/100 g extract.

Table 3.7 Total polyphenol content of freeze-dried and spray-dried powder samples obtained from concentrates of fermented and unfermented rooibos

Rooibos extract	Sample	Batches	Range	Average ±SD
Fermented	Freeze-dried	5	26.66-30.40[a]	28.38±1.46 a[b]
	Spray-dried	5	26.57-31.18	28.88±1.79 a
Unfermented	Freeze-dried	6	30.46-33.26	32.03±1.01 a
	Spray-dried	6	31.71-34.43	32.91±1.22 a

[a]Results expressed as g GAE/100 g extract, [b]averages in same column for the same type of extract (fermented or unfermented) with different alphabetical letters differ significantly at the 5% level of significance ($P<0.05$).

Table 3.8 Phenolic content of freeze-dried concentrate and spray-dried aqueous extracts of fermented and unfermented rooibos

	Drying	Batches	Aspalathin		Iso-orientin		Orientin	
			Range	Ave[a] ±SD	Range	Ave ±SD	Range	Ave ±SD
Fer[b]	Freeze	5	0.18-0.28[c]	0.23±0.04 a[d]	0.51-0.74	0.59±0.09 a	1.01-1.21	1.10±0.08 a
	Spray	5	0.21-0.34	0.26±0.05 a	0.56-0.79	0.64±0.09 a	1.04-1.26	1.16±0.08 a
Unf[e]	Freeze	6	5.81-9.19	7.10±1.25 a	1.72-2.55	2.19±0.32 a	1.56-1.99	1.74±0.18 a
	Spray	6	6.11-9.22	7.12±1.11 a	1.90-2.62	2.26±0.24 a	1.67-1.87	1.77±0.08 a

[a]Average, [b]fermented; [c]results expressed as g/100 g extract, [d]averages in same column for the same type of extract (fermented or unfermented) with different alphabetical letters differ significantly at the 5% level of significance ($P<0.05$), [e]unfermented.

Phenolic content of commercial fermented rooibos iced teas/fruit teas

All six samples of brand D tested negative for the presence of aspalathin, iso-orientin and orientin (Table 3.9), despite having higher TP contents than most of the other iced teas (Table 3.10). Four of the iced tea brands, i.e. A, E, G and H, contained aspalathin, iso-orientin and orientin in all of the analysed samples (Table 3.9). One of

the samples of brand B was lacking both aspalathin and iso-orientin (see Table 3.9). None of the samples of brands C and F contained aspalathin, and two samples of brand C also contained no iso-orientin. However, all samples of both brands contained orientin. Brand F had the lowest average TP content of the nine iced teas (Table 3.10).

Table 3.9 Aspalathin, iso-orientin and orientin content of nine commercial, fermented rooibos iced teas

Brand	Number of samples	Aspalathin		Iso-orientin		Orientin	
		Range	Ave[a] ± SD	Range	Ave ± SD	Range	Ave ± SD
A	3	0.11-0.15[b]	0.13±0.02	0.62-0.64	0.63±0.01	1.14-1.16	1.15±0.01
B	3	nd[c]-0.16	0.13±0.04	nd[d] -0.29	0.29±0.00	0.38-0.39	0.39±0.00
C	3	nd	-	nd[e]-0.29	-	0.41-0.42	0.41±0.01
D_1[f]	3	nd	-	nd	-	nd	-
D_2[f]	3	nd	-	nd	-	nd	-
E	3	0.29-0.34	0.32±0.03	0.03-0.86	0.54±0.44	1.26-1.41	1.36±0.09
F	3	nd	-	0.21-0.22	0.22±0.00	0.33-0.35	0.34±0.01
G	4	0.10-0.69	0.43±0.24	0.77-0.93	0.85±0.07	1.07-1.29	1.20±0.10
H	16	0.24-0.52	0.41±0.09	0.59-1.20	0.93±0.17	0.95-1.72	1.38±0.19

[a]Average, [b]results expressed in mg/100 mL iced tea, [c]not detected, [d]compound not detected in one of the three samples, [e]compound not detected in two of the three samples, [f]two formulations of brand D were purchased: (D_1) containing sucrose and (D_2) containing an articial sweetener.

Table 3.10 The total polyphenol content of nine commercial, fermented rooibos iced teas

Iced tea brand	Number of samples analysed	Range	Average ±SD
A	3	7.85-8.26[a]	8.12±0.23
B	3	7.81-8.18	8.01±0.19
C	3	3.88-4.04	3.95±0.08
D_1	3	6.24-8.10	7.24±0.94
D_2	3	7.58-11.39	9.08±2.03
E	3	4.92-5.28	5.13±0.19
F	3	2.19-2.28	2.24±0.04
G	4	4.25-4.65	4.51±0.23
H	16	4.19-6.69	5.78±0.78

[a]Results expressed as g GAE/100 mL rooibos iced tea.

Brand H was investigated more extensively than the other brands, i.e. sixteen bottles of varying production dates, were analysed for their aspalathin, iso-orientin and orientin content. No distinct pattern with respect to the production date could be observed for any of these compounds (Fig. 3.1). In some cases, bottles sampled after their expiry date (i.e. 92 days) had higher aspalathin, iso-orientin and orientin contents than bottles sampled after a shorter storage period (i.e. 37 and 44 days, not expired).

Composition of rooibos extracts used for the production of iced tea and pH of experimental rooibos iced teas

The average composition of the three types of rooibos extract utilised for the production of the experimental iced teas (heating experiment) is given in Table 3.11. The TP, aspalathin, iso-orientin and orientin content of the UR extract was the highest of the three extracts. The FR extract contained substantially less TPs and flavonoids, however, its iron content was higher than that of the UR extract. The TP content of the NEUR extract, containing UR extract, was not consistent with the expected value of *ca*. 5%, based on the values of UR extract, but was much higher.

The pH values of the UR and FR iced teas (before heating) are given in Table 3.12. The pH of the rooibos iced tea containing NEUR extract only (formulation NE) was 3.45±0.03, while that of the iced tea with added citric acid (formulation NEC) was 2.80±0.01. This was similar to the pH of formulation BC and BCA of the FR and UR iced tea (iced teas containg citric acid). Formulation B (FR and UR iced tea) and NE (NEUR iced tea) gave the highest pH values.

Effect of heat and product formulation on the phenolic composition and colour of experimental rooibos iced teas

The change (%) in absorbance as well as TP, aspalathin, iso-orientin and orientin content of the various iced teas and formulations due to heating can be found in Tables A3.1-A3.3 in ADDENDUM 3.

Browning

All three heat treatments led to a significant ($P<0.05$) increase in the absorbance (i.e. browning) of formulation B of the FR iced tea, as well as a significant ($P<0.05$) increase in the absorbance of pasteurised formulation BC (Fig. 3.2a). The absorbance of BC and BCA was unaffected by NTS, but significantly ($P<0.05$) decreased by HTS.

All heat treatment × formulation combinations resulted in a significant ($P<0.05$) increase in the absorbance of UR iced tea (Fig. 3.2b). For the respective formulations, NTS and HTS led to a greater increase in absorbance than pasteurisation. However, the absorbance of the NTS samples, irrespective of formulation, did not differ significantly ($P\geq0.05$) from that of the corresponding HTS samples.

The absorbance of all the rooibos iced tea samples containing NEUR extract increased significantly ($P<0.05$) upon heating, with the greatest increase being observed for the HTS samples (Fig. 3.2c). Formulations NE and NEC subjected to pasteurisation had the same absorbance, while the absorbance of formulation NEC of both NTS and HTS samples was significantly ($P<0.05$) higher than that of formulation NE.

Total polyphenol content

In general, heating resulted in a significant ($P<0.05$) increase in the TP content of the FR and UR iced teas (Figs. 3.3 a, b). Of all the different heat treatment × formulation combinations, the TP content of only a few of the FR iced teas (Pasteurised formulation B, NTS formulation BCA and HTS formulation BC) and one treatment × formulation combination of UR iced tea (Pasteurised formulation B) did not increase. In the case of the iced teas containing NEUR extract, the TP content of formulation NEC, subjected to pasteurisation and NTS, remained unchanged. The remaining treatment × formulation combinations experienced a significant ($P<0.05$) increase in TP content upon heating.

Qualitative phenolic profile

Figure 3.4 shows the chromatographic profile at (a) 288 nm and (b) 350 nm of formulation BCA of the UR iced tea before and after heating. HTS treatment resulted in a reduction in aspalathin content and the formation of new peaks: a peak at 2.5 min (compound 1, see Fig. A2.4c in ADDENDUM 2 for UV-vis spectrum), two peaks at *ca.* 7 - 7.5 min; two peaks at *ca.*10 - 11.5 min and an increase in the peak area of possible polymeric compounds with a retention time ranging from *ca.* 13.5 - 16.5 min. The formation of new peaks was also observed in formulation BC (UR iced tea), although slightly less of compound 1 was formed and aspalathin losses were slightly larger. These peak changes were not very obvious on the chromatogram of formulation B

(UR iced tea). The same peak changes were also observed for the FR iced teas (B, BC, BCA) as well as the NEUR iced teas (NE and NEC). Changes were, however, less noticeable in the FR iced teas than in the UR and NEUR iced teas. Normal temperature sterilisation (NTS), but not pasteurisation, of the rooibos iced teas produced the same new peaks in the chromatogram.

Table 3.11 Average (±SD) composition of the rooibos extracts used in the production of the experimental iced teas

Extract	Total polyphenols[a]	Iron[b]	Aspalathin[c]	Iso-orientin[c]	Orientin[c]
UR[d]	34.66±1.69	94.24	20.70±0.99	1.55±0.07	1.39±0.06
FR[e]	26.30±1.37	132.91	0.92±0.05	0.65±0.04	1.06±0.02
NEUR[f]	9.04±0.37	14.14	3.08±0.13	0.21±0.06	0.20±0.04

[a]Results expressed as GAE/100 g extract, [b]determined by Bemlab and result given in mg/kg (no SD provided), [c]results expressed as g/100 g extract, [d]unfermented rooibos, [e]fermented rooibos, [f]nano emulsified unfermented rooibos.

Table 3.12 The pH of the various types and formulations of rooibos iced tea before heating

Iced tea formulation	Extract		
	FR[a]	UR[b]	NEUR[c]
B[d]/NE[e]	5.05±0.02	4.40±0.01	3.45±0.03
BC[f]/NEC[g]	2.95±0.03	2.80±0.02	2.80±0.01
BCA[h]	2.95±0.02	2.80±0.03	-

[a]Fermented rooibos, [b]unfermented rooibos, [c]nano emulsified unfermented rooibos (NEUR), [d]base (FR or UR extract in deionised water), [e]NEUR extract in deionised water, [f]base + citric acid, [g]NE + citric acid, [h]base + citric + ascorbic acid. There is no NECA formulation for the NEUR extract as the extract inherently contained ascorbic acid.

Aspalathin content

The aspalathin content of all three formulations of FR iced tea, namely B, BC and BCA was significantly ($P<0.05$) reduced after NTS and HTS treatment (Fig. 3.5a). In both cases, a significant ($P<0.05$) reduction was observed for formulation B, i.e. 76.0% and 78.1% reduction for NTS and HTS treatments, respectively. Pasteurisation, on the other hand, led to a very small but significant ($P<0.05$) reduction in the aspalathin content of formulation B, while its content increased significantly ($P<0.05$) by 7.1% and 11.0% after pasteurisation in formulations BC and BCA, respectively.

Similar observations were made for UR iced teas (Fig. 3.5b). Both NTS and HTS resulted in a significant ($P<0.05$) reduction in aspalathin content, with this compound being least well preserved in formulation B. As with the FR iced tea, pasteurisation of UR iced tea resulted in significantly ($P<0.05$) increased aspalathin levels for BC and BCA. The aspalathin content of both formulations of the NEUR iced tea decreased significantly ($P<0.05$) as a result of NTS and HTS (Fig. 3.5c), with the greatest loss occurring in formulation NE subjected to HTS (22.7% loss). No change in aspalathin content was observed as a result of pasteurisation.

Iso-orientin content

The iso-orientin content of FR iced tea decreased significantly ($P<0.05$) with NTS and HTS (Fig. 3.6a). Within a specific heat treatment (i.e. NTS or HTS), the iso-orientin content of the three formulations either did not differ significantly ($P\geq0.05$) (e.g. formulation B and BCA of HTS) or differed very little (formulation B and BC of NTS). Overall, loss of this compound was less than that of aspalathin, irrespective of the heat treatment × formulation combination. Pasteurisation resulted in a significant ($P<0.05$) increase in the iso-orientin content of all formulations of FR iced tea.

The effect of NTS and HTS heating on the iso-orientin content of UR iced tea was similar compared to that observed for FR iced, although formulation had no effect on the iso-orientin content of the HTS samples (Fig. 3.6b). Of the pasteurised samples, formulation B and BC did not experience any significant ($P\geq0.05$) change in iso-orientin content whilst formulation BCA increased significantly ($P<0.05$).

The iso-orientin content of iced tea made with NEUR extract decreased significantly ($P<0.05$) for both formulations (NE and NEC) during NTS and HTS treatment, although formulation had no effect (Fig. 3.6c). No significant ($P\geq0.05$) change in the iso-orientin content of this iced tea was observed as a result of pasteurisation.

Orientin content

NTS and HTS treatment of formulations BC and BCA of both the FR (Fig. 3.7a) and UR (Fig. 3.7b) iced teas significantly ($P<0.05$) decreased their orientin content, while the orientin content of formulation B remained unaffected. Pasteurisation on the other hand, either had no effect (formulation BC of FR iced tea) or led to significantly ($P<0.05$) increased values (B and BCA of FR iced tea; B, BC and BCA of UR iced tea). The orientin content of the iced tea containing NEUR extract decreased significantly ($P<0.05$) as a result of NTS and HTS treatments, but pasteurisation had no effect (Fig. 3.7c).

Effect of heat and product formulation on the phenolic composition and colour of experimental rooibos iced teas – pasteurisation reinvestigated

This investigation was prompted due to the increases in the aspalathin, iso-orientin and orientin content of certain formulations of iced tea after pasteurisation (first heating experiment). In this experiment, a closed system was simulated by using sealed containers, thereby eliminating the possibility of evaporation. The pH

values of the formulations used in the pasteurisation experiment are given in Table 3.13. Values similar to that of the first pasteurisation experiment were obtained.

Table 3.13 The pH values of the various iced tea formulations, before heating in the second pasteurisation experiment

	Extract		
Iced tea formulation	**FR[a]**	**UR[b]**	**NEUR[c]**
B[d]/NE[e]	5.05±0.01	4.29±0.02	3.51±0.03
BC[f]/NEC[g]	2.98±0.03	2.72±0.06	2.80±0.01
BCA[h]	2.96±01	2.79±0.02	-

[a]Fermented rooibos, [b]unfermented rooibos, [c]nano emulsified unfermented rooibos (NEUR), [d]base (FR or UR extract in deionised water), [e]NEUR extract in deionised water, [f]base + citric acid, [g]NE + citric acid, [h]base + citric + ascorbic acid. There is no NECA formulation for the NEUR extract as the extract inherently contained ascorbic acid.

Browning

Similar trends were observed for both FR and UR rooibos iced teas. After the 5 min pasteurisation treatment, the absorbance of formulation B increased significantly ($P<0.05$), whilst BC and BCA remained unchanged (Fig. 3.8a, b). However, after the 30 min heat treatment, the absorbance of all formulations of these two iced teas was significantly ($P<0.05$) increased. Change in the absorbance of rooibos iced tea containing NEUR extract (Fig. 3.8c) was very slight and limited to the formulations subjected to pasteurisation for 30 min (4.5% and 5.8%).

Qualitative phenolic profile

Figs. 3.9 (a) 288 nm and (b) 350 nm show the chromatographic profiles of formulation B of the FR iced tea before (control) and after heating (30 min at 93ºC). The significant ($P<0.05$) reduction in aspalathin content (Fig. 3.10a) is clearly visible on the chromatogram (Fig. 3.9a, the offset aspalathin peak of the pasteurised sample is smaller in height compared to that of the control). The changes in iso-orientin and orientin [Figs. 3.11 (a) and 12 (a)] content were not significant ($P\geq0.05$) and were not clearly visible on the chromatogram (Fig. 3.9b).

Aspalathin content

The aspalathin content of FR iced tea decreased significantly ($P<0.05$) in formulation B after both 5 and 30 min pasteurisation (Fig. 3.10a), with 30 min pasteurisation having a greater effect (39.8% loss compared to 4.2%). The aspalathin content of formulation BCA increased significantly ($P<0.05$) after 30 min (4.6%) whilst BC remained unchanged. The aspalathin content of BC and BCA was unaffected after heating for 5 min.

Pasteurisation (30 min at 93ºC) had a significant ($P<0.05$) effect on the aspalathin content of UR iced tea. In the case of formulation B, a significant ($P<0.05$) 2.6% decrease in aspalathin content was observed whilst a significant ($P<0.05$) increase was observed for formulation BCA (2.5%). Once again the aspalathin content of BC (30 min heating) and all formulations subjected to 5 min heating were unaffected

The aspalathin content of rooibos iced tea containing NEUR extract (formulation NE) decreased significantly ($P<0.05$), although very slightly, after both the 5 and 30 min pasteurisation heat treatment (1.5% and 1.7%, respectively). No changes were observed for formulation NEC, irrespective of heating period (Fig. 3.10c).

Iso-orientin content

A significant ($P<0.05$) reduction in the iso-orientin content of formulation B, BC and BCA (FR iced tea) after the 5 min pasteurisation process was observed (Fig. 3.11a); it was however, 2% or less. After the 30 min treatment, the iso-orientin content of BC and BCA increased significantly ($P<0.05$) (1.6% and 1.9%), whilst formulation B was unchanged.

The iso-orientin content of UR iced tea was generally unaltered by 5 and 30 min pasteurisation heat treatments (Fig. 3.11b). The only significant ($P<0.05$) change in iso-orientin content, i.e. a decrease of 1.7%, was observed for formulation B after 30 min heating.

The iso-orientin content of rooibos iced tea containing NEUR extract was slightly, significantly ($P<0.05$) reduced after the 5 min pasteurisation treatment (Fig. 3.11c). A reduction of 1.0% and 1.1% for formulation NE and NEC, respectively, was observed. The iso-orientin content formulation NE decreased significantly ($P<0.05$) by 1% after the 30 min heat treatment, whilst formulation NEC exhibited no significant ($P\geq0.05$) change.

Orientin content

A significant ($P<0.05$) decrease in the orientin content was only observed for the three formulations of FR iced tea, subjected to a 5 min pasteurisation treatment (Fig. 3.12a). The orientin content of formulation B, BC and BCA decreased by 1.4%, 1.6% and 1.8 %, respectively. No significant ($P<0.05$) change was observed for any of the formulations after a 30 min heat treatment. The orientin content of none of the remaining iced teas was significantly ($P\geq0.05$) affected, irrespective of formulation or pasteurisation treatment (Figs. 3.12b & c).

DISCUSSION

Commercial rooibos iced tea and extracts

Several brands of FR iced tea are on the market in South Africa. No regulations exist that specify the quantity of rooibos extract in such beverages. Subsequently no tests are carried out by the regulatory authority to ensure that the products that reach the market (a) contain rooibos as indicated on the label and (b) specify minimum quantities present. Analysis of a number of the major branded products on the market showed that one brand (D)

did not to contain aspalathin, iso-orientin or orientin, while some of the other brands either lacked aspalathin and/or one of these flavones. Despite the fact that brand D contained none of these rooibos flavonoids, its TP content was relatively high compared to the remaining iced teas. This can most likely be ascribed to the addition of ascorbic acid to the beverage. Folin's reagent does not only react with phenols, but also reducing compounds such as ascorbic acid (Vinson *et al*., 2001). A preliminary experiment carried out during the present study also confirmed this (ADDENDUM 4). Thus, as a method to test for the presence of rooibos extract, the TP assay has no value.

Although brands A, G, E and H all contained rooibos flavonoids, their levels were extremely low and mostly less than would be found in a cup of rooibos. Typically, 100 ml rooibos beverage at "tea cup strength" may contain 0.70-2.06 mg aspalathin, 0.32-1.67 mg orientin and 0.11-1.39 mg iso-orientin (Joubert & Schulz, 2006). The use of a small amount of extract, containing low amounts of the flavonoids in question, is most likely to be blamed for the poor phenolic quality of these beverages. Despite containing both tea extract and fruit juice, the TP content of sample F was lower than that of the other iced teas. It is possible that very little rooibos and/or fruit juice were used in its formulation. Closer inspection of a number of production samples of brand H revealed that no pattern, with respect to flavonoid content, exists as a result of production date. Both higher and lower values were observed shortly after production (well before expiry), as well as shortly prior to expiry. This indicates that the quantity and phenolic quality of the extract used in the production of the beverage, rather than the storage duration to which the beverage was subjected, determined its aspalathin, iso-orientin and orientin content. Further investigation of this aspect is needed.

Since aspalathin is unique to rooibos, its presence in a product is thus an indication that the product contains measurable quantities of rooibos extract. However, its absence does not necessarily mean that the product contains no rooibos extract, since the stability of aspalathin is a deciding factor. Under the oxidative conditions prevailing during the manufacturing process, the aspalathin present in the plant material is very unstable. A small percentage does, however, remain in the plant material (Joubert, 1996). Analysis of a large number of samples of FR plant material indicated that aspalathin is always present, albeit in varying quantities (Joubert & Schulz, 2006). Furthermore, commercial powdered extracts of FR, reconstituted to "cup strength" gives *ca.* 0.7 mg aspalathin per 100 ml (*ca.* 0.35% of powdered extract) (Joubert & Schulz, 2006). Samples taken during the production of commercial powdered FR extract (after extraction, microfiltration, reverse osmosis, concentration and spray-drying) all contained aspalathin, confirming that this compound should be present in extracts used for the production of commercial rooibos iced tea, and thus in the iced tea itself.

Similar to aspalathin, variation in the orientin and iso-orientin content was also observed for samples taken during commercial, powdered rooibos extract production. The composition of the plant material, in general, is subject to high variability as a result of external factors, e.g., soil and climatic conditions (Areias *et al*., 2000), altitude (Yang *et al*., 2005), harvest time (Tan *et al*., 2008), seasonal variation (Høgedal & Mølgaard, 2000; Celiktas *et al*., 2007), sunlight (Rozema *et al*., 2002) and post-harvest processing (Aherne & O'Brien, 2002). Several studies on a number of herbal plants (Areias *et al*., 2000; Høgedal & Mølgaard, 2000; Celiktas *et*

al., 2007; Tan *et al*., 2008) supports this. Although the influence of all these factors has not been investigated for rooibos, harvesting date does play a role, as well as the fact that seeds are used for propagation (Prof. E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, personal communication, 2008).

Iso-orientin and orientin not only occur naturally in rooibos plant material (Koeppen & Roux, 1965b), but they are also oxidation products of aspalathin (Krafczyk & Glomb, 2008). Since they are more oxidised than aspalathin (dihydrochalcone versus flavone), they are more stable, and will thus be less prone to oxidation during processing, i.e. extract manufacture. Orientin and iso-orientin are not unique to rooibos, but they are not constituents of the plant extracts and fruit juices normally used for iced tea production. The use of these "normal" ingredients could thus not be the source of these specific flavones. Their presence would therefore indicate that rooibos extract was used in formulation. Literary sources did not indicate that the flavones iso-orientin and orientin occur in fruit, but their presence in medicinal plants from the species *Passiflora* (Antognoni *et al*., 2007) and *Cyrtomium* (Iwashina *et al*., 2006) as well as other plants such as bamboo (Zhang *et al*., 2008) has been indicated. With the increased availability of functional ingredients from a wide range of sources on the market, future use of plant extracts containing these flavones is not impossible.

The compositional characteristics of rooibos present the analyst with marker compounds that can be used for quality and regulatory control. However, before product specification, testing of authenticity and compliance with regulations can be considered, knowledge of their stability during the manufacturing process and storage of rooibos iced tea is required. Following the finding that aspalathin is mostly absent from commercial rooibos iced teas, despite the fact that poedered extracts contain this compound, the present study was conducted. The effect of the processes involved in iced tea production was investigated for their possible role in reducing the aspalathin content of commercial rooibos iced tea.

Effect of heat and product formulation on the phenolic composition and colour of experimental rooibos iced teas

Commercial rooibos iced tea products are formulated in such a manner as to give a beverage with pH suitable for pasteurisation (2 min at *ca*. 90ºC and pH < 4) (Dr Chris Hansmann, ARC-Infruitec-Nietvoorbij, Stellenbosch, personal communication, 2007). Higher temperatures are required to achieve microbial stability at higher pH values. Although the pH values of the experimental iced teas containing ascorbic and/or citric acid were low enough so that pasteurisation could be used, the samples were also subjected to extreme heating conditions. Not only is degradation of aspalathin, iso-orientin and orientin more likely under such extreme conditions, but their "survival" would give further confirmation that it would not be unreasonable to expect to detect these flavonoids commercial rooibos iced teas. Furthermore, different formulations were included in the study to investigate the role of ascorbic acid and/or citric acid, i.e. ingredients normally added to iced teas, on the stability of these flavonoids.

Stability of the experimental iced teas during processing was evaluated in terms of absorbance at 420 nm (brown colour) and TP content, in addition to the qualitative phenolic profile and the aspalathin, iso-orientin and

orientin content. Total polyphenol content also serves as an indication of antioxidant activity. Several studies showed that the antioxidant activity of a range of plant extracts (as measured with the DPPH or ABTS assays) correlates with their TP content (Mello *et al*., 2005; Kiselova *et al*., 2006) and that the Folin-Ciocalteu method could thus be used to indicate antioxidant activity (Prior *et al*., 2005; Stratil *et al*., 2007). Huang *et al*. (2005) recommended that the Folin-Ciocalteu assay, used for many years on a variety of plant extracts, should be used in preference to the other methods to measure antioxidant capacity due to its good reproducibility, simplicity and convenience.

The observed increase in the absorbance of the iced teas at 420 nm indicated increased conjugation of unsaturated bonds in phenolic compounds, especially polymers. This was supported by the HPLC profile of especially the HTS samples, indicating formation of polymeric substances. The greater the conjugation of the phenolic compounds in a sample, the greater the area for electron delocalisation, the lower the energy requirements for delocalisation and the greater the absorbance (Whitfield, 1969; Chen & Ho, 1997).

Although the formation of coloured products upon flavonoid oxidation has mostly been ascribed to the formation of polymeric compounds, Le Guernevé *et al*. (2004) showed that the main oxidation products of phloridzin (a dihydrochalcone glucoside) were monomers resulting from successive oxidation reactions and nucleophilic intramolecular additions. One of the oxidation products (monomer) was yellow in colour, and was theorised to contribute to the colour of apple-derived products. Formation of yellow compounds would contribute to an increase in absorbance at 420 nm. Flavones are less susceptible to oxidation than dihydrochalcones (Dziedzic *et al.*, 1985), although, upon heating, the stability of flavones may be reduced. For example, during pasteurisation (10 min at internal temperature of 74ºC) the luteolin (flavone) content of yellow banana peppers has been found to decrease by 45% (Lee & Howard, 1999).

Loss of aspalathin during processing of rooibos plant material is accompanied by extensive browning (Joubert, 1996). Koeppen & Roux (1965a; 1966) showed that conversion of aspalathin in solution at room temperature takes place very slowly and results in the formation of ill-defined brown products. Furthermore, in a complex mixture such as the rooibos extracts, which contains a large number of phenolic compounds, it is possible that aspalathin could react with other flavonoids, including orientin and iso-orientin, to form polymeric substances. The formation of polymeric compounds from a chalcone and an unidentified flavonoid has been demonstrated (Gujer *et al.*, 1986). No clear increase in polymeric compounds was, however, observed in the chromatograms of the iced tea samples. The ill-defined peak area appearing at *ca.* 13.5-16.5 min (at 288 nm) may be associated with the polymeric compounds of rooibos. [According to Peng *et al*. (2001), the broad UV-absorbing peak, eluting late in the chromatogram (reversed phase column) may be associated with polymeric polyphenolic compounds]. Since the UR iced teas contain greater quantities of aspalathin than the FR iced teas, they should have greater potential to form polymeric compounds and exhibit an increased absorbance at 420 nm. This was confirmed by the results of this study (Δ absorbance UR > Δ absorbance FR). Although the increases in absorbance were significant for the iced teas containing NEUR extract, the presence of ascorbic acid in the emulsion possibly prevented extensive oxidation and polymerisation (large Δ absorbance).

Krafczyk & Glomb (2008) also demonstrated the formation of uncharacterised, brown material after the incubation of aspalathin in a phosphate buffer solution (pH 7.4) for 48 h at 37˚C. Although no information is available on the respective brown polymers obtained by Koeppen & Roux (1966) and Krafczyk & Glomb (2008), the timeframe of their experiments suggests that the higher temperature used by Krafczyk & Glomb (2008) accelerated the polymerisation. Kim *et al*. (2007) noted that heating of green tea liquor, having a high flavanol content, resulted in the tea becoming deeper yellow in colour with increasing temperature from 85-120ºC.

The severity of the heat treatments, as well as the formulations and the type of extract used in the present study clearly affected the extent and even the direction of absorbance change. The UR iced tea and iced tea formulations containing NEUR extract gave significant increases in absorbance, irrespective of formulation, with pasteurisation having the lesser effect. This was attributed to the high aspalathin content of these extracts. Employing a shorter pasteurisation time protected formulation BC and BCA (UR iced tea) as well as formulation NE and NEC (NEUR iced tea) against measurable colour change, indicating the importance of heating time (and formulation – discussed later). Nano emulsification of the UR extract (NEUR) offered some protection against heat-induced changes in colour in comparison to the UR extract alone.

Except for formulation B, which also showed increased absorbance (browning) with heating, the FR iced tea followed a different trend compared to UR iced tea. The absorbance of formulations BC and BCA of the FR iced tea either remained unchanged (no browning) or decreased (HTS treatment). Not only was the aspalathin content of the FR iced teas much lower than that of the iced teas containing UR or NEUR extract (less effect on colour), but factors such as decolourisation likely played a role, as indicated by the decrease in absorbance.

Ascorbic acid is an antioxidant, capable of protecting many flavonoids from oxidation (Talcott *et al*., 2003; Yen *et al*., 2008). In addition, the pH reducing effect of citric acid possibly inhibited flavonoid deprotonation and oxidation to a certain extent (Lemańska *et al*., 2001; Janeiro *et al*., 2005). Decolourisation (as in the FR iced tea) may occur when previously deprotonated phenolic compounds become protonated due to the reduction in pH (Robertson, 1983; Gupta, 1989). Decolourisation could thus counter some of the browning resulting from heating.

In the case of formulation B, extensive polymerisation, that could lead to insolubility and precipitation of phenolic compounds, is a likely cause of the decreased absorbance of the HTS treated samples of FR iced tea (a precipitate would be removed during centrifugation, prior to absorbance reading). During the manufacture of apple concentrate, procyanidins can polymerise to form insoluble compounds which precipitate (Bengoechea *et al*., 1997; Oszmiański *et al*., 2008). In the case of formulation B, the absence of the reducing agent, ascorbic acid, as well as its higher pH (in relation to the remaining formulations) also possibly contributed to more rapid oxidation of flavonoids in this formulation.

Pasteurisation, under slightly different conditions (closed containers), showed that the 5 min heat treatment increased the absorbance of formulation B (FR iced tea) in accordance with findings of the previous experiment, but had no effect on formulations BC and BCA. However, by increasing the heating time to 30 min,

increased absorbance was also demonstrated for BC and BCA, suggesting that under more severe heating conditions (e.g. longer pasteurisation time) oxidation and polymerisation could be more extensive than may be countered by ascorbic acid and/or citric acid, leading to a net increase in absorbance.

ADDENDUM 4 indicates that sugar and citric acid do not have an appreciable effect on the TP content of FR, UR and NEUR iced tea. The addition of ascorbic acid resulted in an increase in the TP content of all the extract solutions (ADDENDUM 4, Table A4.1). However, after heating, even formulations that did not contain ascorbic acid exhibited an increase in TP content. A significant increase in the TP content of most of the formulations of FR and UR iced tea, as a result of heating, was demonstrated. This was in spite of changes indicating that oxidation and polymerisation of the phenolic compounds had taken place, an occurrence that could have been responsible for a reduced TP content (Gómez-Alonso *et al*., 2007). Polymerisation generally results in a reduction in the number of free OH groups capable of forming phenolate ions which react with the Folin-Ciocalteu reagent (Singleton *et al*., 1999). No clear trend regarding formulation was observed for the different iced teas. Generally, it was expected that formulation BCA would deliver the highest TP results as it contains ascorbic acid. This was, however, not observed for HTS treated FR iced tea, formulation BCA. The extreme heat possibly destroyed some of the ascorbic acid (Chua *et al*., 2000; Kabasakalis *et al*., 2000; Zerdin *et al*., 2003) in formulation BCA, lowering its apparent TP value. Furthermore, the interaction between the polyphenols in the presence of heat and acid (formulation BC) may have resulted in the formation of new compounds capable of reacting with the Folin reagent. For example, release of phenolic groups attached to sugars (Vinson *et al*., 2001) and the formation of macromolecular compounds with increased antioxidant activity (Pokorny, 1987; Turker *et al*., 2004; Yamada *et al*., 2007) are possible explanations. The HPLC chromatogram of the HTS samples of the UR iced tea clearly indicated the formation of new peaks on the chromatogram at 288 nm. Compound 1 had a retention time similar to that of flavones isolated from fermented rooibos (Figs. A3.4 a & b ADDENDUM 2), but the UV-vis spectra, as well as λ_{max} of the latter differed from that of compound 1. The shorter retention time of compound 1 implies that it is more polar than aspalathin and/or has a smaller molecular mass than aspalathin (Cheynier & Moutounet, 1992). Hurst & Harborne (1967) noted that cleavage of the dihydrochalcone phloretin yielded phenolic acids. Compound 1 may thus also be a phenolic acid. The greater formation of compound 1 in formulation BCA as opposed to BC may be attributed to the stabilising effect of ascorbic acid in this formulation.

A large decrease in the aspalathin, iso-orientin and orientin content of the FR and UR iced tea samples subjected to NTS and HTS was observed. This can most likely be attributed to the severity and duration of these heat treatments (Chen *et al*., 2001; Pellegrini *et al*., 2001; Brenes *et al*., 2002). In this study, smaller losses of iso-orientin and orientin, compared to aspalathin, were reported. This may, in part, be attributed to the difference in structure between these two types of compounds. The open C-3 chain of aspalathin would be susceptible to ring closure under oxidative conditions (Koeppen & Roux, 1966). Heat is expected to accelerate this conversion.

Pasteurisation did not influence the aspalathin, iso-orientin or orientin levels of rooibos iced tea as greatly as NTS or HTS. Dihydrochalcones appear to be relatively resistant to mild heat treatments. Montijano *et al*. (1996) showed that neohesperidin dihydrochalcone did not undergo hydrolysis in juice based drinks after heating for 1 h at 90ºC, nor after pasteurisation at temperatures ranging from 60ºC (4 h) to 100ºC (45 min). It is, however, important to remember that neohesperidin dihydrochalcone (Benavente-García *et al*., 2001) and aspalathin differ in structure (neohesperidin dihydrochalcone has two fewer OH groups compared to aspalathin and the type as well as position of the sugar molecules differ). These structural differences may be responsible for the differing heat stability of neohesperidin dihydrochalcone compared to aspalathin. Koeppen & Roux (1966) noted that under the oxidative conditions used to obtain dihydro-iso-orientin from aspalathin, no conversion of phloretin to naringenin took place. Phloretin thus possessed greater resistance to oxidative changes than aspalathin. In some cases (formulation BCA of FR and UR iced tea) the heat treatment even appeared to have a beneficial effect on phenolic composition, e.g. increased aspalathin content. In the case of aspalathin, this observation may possibly be attributed to the release of this compound from an association with other compounds (perhaps proteins) in the rooibos matrix upon heating. Currently, however, there is no evidence for this. Despite this uncertainty, it is clear that a 5 and 30 min pasteurisation procedure does not have a significant, negative effect on FR and UR iced tea containing citric or citric and ascorbic acid. The use of sealed vials in the second pasteurisation experiment precluded loss of water through evaporation, which could have otherwise been a factor contributing to an apparent increase in flavonoid content. According to these results it may thus be speculated that industrial pasteurisation would not have a negative effect on the phenolic composition of rooibos iced tea. This again emphasises the importance of using rooibos extracts of good phenolic quality to produce iced teas containing comparable (to a cup of brewed rooibos) amounts of flavonoids such as aspalathin.

Although the iced teas containing the NEUR extract also exhibited a decreased aspalathin, iso-orientin and orientin content upon heating, the percentage reduction was less than that observed for the UR or FR iced teas. The ascorbic acid inherently present in NEUR iced teas (as part of the NEUR extract) is most likely responsible for protecting the flavonoids from extensive oxidation. In the nano capsules, the UR extract is completely surrounded by a layer of ascorbic acid and emulsifier, thus acting as a barrier between the extract and e.g. oxygen (Anon., 2007). Pasteurisation had very little effect on the iced teas containing the NEUR extract, and no increases in aspalathin content were observed. This was contrary to the findings for both the FR and UR iced teas, possibly since the NEUR extract is already in its most soluble form. The NEUR extract is completely and irreversibly water soluble (Anon., 2007).

The heat stability of dihydrochalcones (and phenolic compounds in general) is greater at lower pH values (Canales *et al*., 1993; Coiffard, 1998). This may explain why formulation B (highest pH), of both the FR and UR iced teas, experienced the greatest loss of aspalathin: at less acidic pH values, the deprotonation and subsequent oxidation of flavonoids proceeds more easily (Lemańska *et al*., 2001; Janeiro *et al*., 2005). Similar findings have been reported for tea catechins (Chen *et al*., 2001). Furthermore, the presence of ascorbic acid (a

reducing agent) in formulation BCA may have limited oxidation. The same pattern was observed for all the heat treatments, the smallest losses occurring in the samples subjected to the least severe heat treatments. Iso-orientin behaved similarly to aspalathin, but orientin did not.

The absence of a decrease in the orientin content of formulation B after NTS and HTS for both the FR and UR iced teas was unexpected, as large decreases were noted for both aspalathin and iso-orientin. This lack of orientin loss may, in part, be as a result of the oxidation of aspalathin and the formation of 2,3-dihydro-iso-orientin (Koeppen & Roux, 1965a; 1966). Further oxidation of the latter yields iso-orientin and orientin (Krafczyk & Glomb, 2008). In support of the role of pH, it is worth mentioning that the greatest aspalathin losses occurred in formulation B. Based on the stability of formulation B, it may be speculated that the combination of high temperature and high pH destabilise orientin. Orientin and iso-orientin differ only with respect to the position of the sugar molecule. This difference is possibly responsible for reduced stability of orientin under the aforementioned conditions.

Based on percentage aspalathin loss, it would appear as though the UR iced tea is more heat stable than the FR type. Apart from the role of pH, this may, in part, also be explained by the greater aspalathin content of the UR extract compared to that of the FR extract. Furthermore, the presence of larger amounts of iron in the FR extract may also have played a role. The role of iron in the Fenton reaction and flavonoid oxidation will be disucussed in Chapter 5. The greater iron content of the FR extract compared to that of the UR extract may be attributed to the fermentation process, which reduces the soluble solid content of the plant material (Joubert *et al*., 2008), thereby increasing the ratio of iron:soluble solids.

CONCLUSIONS

The analysis of commercial rooibos iced teas indicated that the phenolic profile of none of these products is comparable to that of a typical cup of brewed rooibos. In fact, many contain negligible quantities of aspalathin, iso-orientin and orientin. In this study it was, however, shown that the processes involved in extract production do not significantly reduce the aspalathin, iso-orientin and orientin content of rooibos extract. Furthermore, it was shown that heating of rooibos iced tea containing citric and ascorbic acid does not reduce the aspalathin, iso-orientin and orientin content to such an extent that it would be unreasonable to expect a commercial product to contain measurable quantities of these phenolic compounds directly after production. It would thus appear as though extracts of inferior quality, or insufficient amounts of rooibos extract powder, were used to produce these commercial rooibos iced teas. The role of storage in the retention of the phenolic quality of commercial beverages remains to be investigated.

Although abusive heat treatments such as NTS and HTS resulted in significant reductions in the aspalathin, iso-orientin and orientin content of rooibos iced tea, their survival indicated greater heat stability than initially anticipated. Investigation with respect to the identity of the new compounds formed after NTS and HTS of rooibos iced tea may be considered in future.

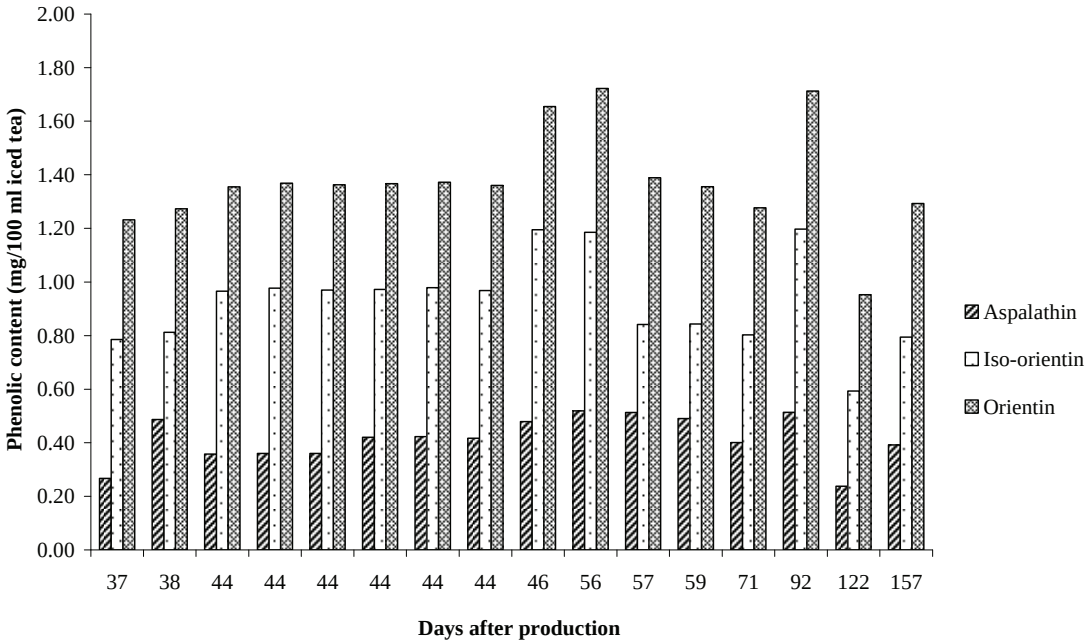
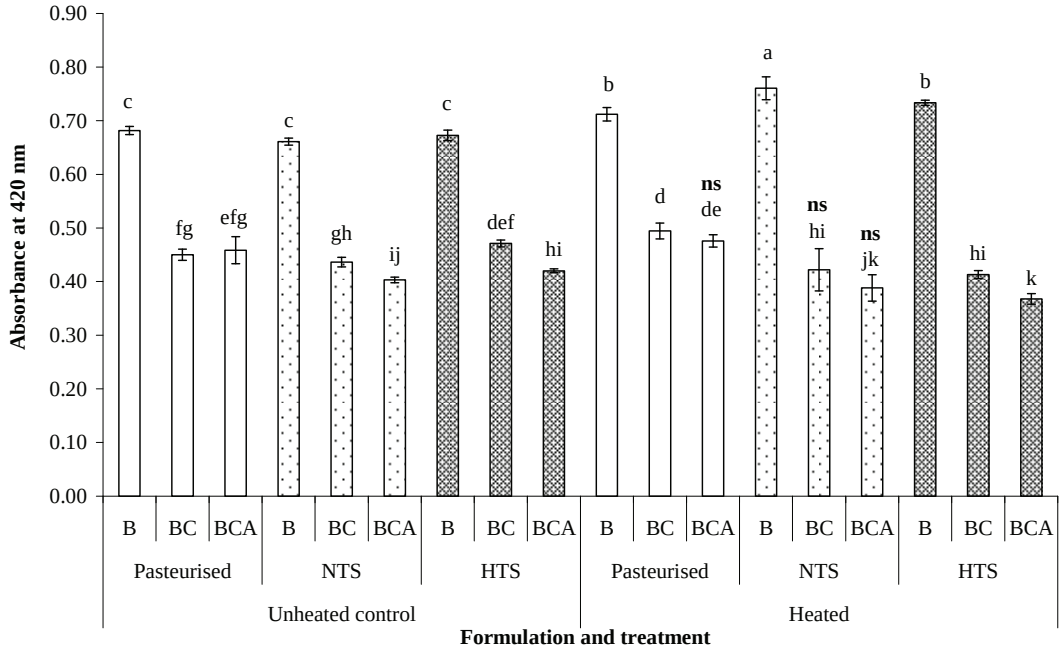
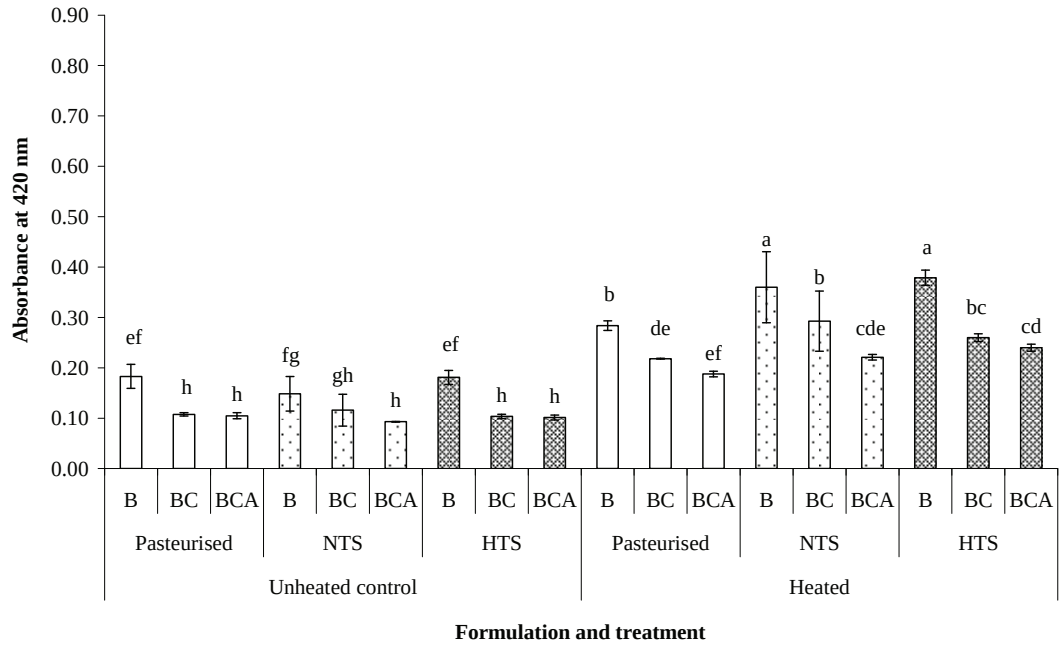


Figure 3.1 Aspalathin, iso-orientin and orientin content of commercial fermented rooibos iced tea (brand H) arranged by production date. Each bar represents a single iced tea sample.

a)


Absorbance at 420 nm
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
c
fg
efg
c
gh
ij
c
def
hi
b
d
ns
de
a
ns
hi
ns
jk
b
hi
k
B
BC
BCA
Pasteurised
NTS
HTS
Unheated control
Heated
Formulation and treatment

b)


Absorbance at 420 nm
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
ef
h
h
fg
gh
h
ef
h
h
b
de
ef
a
b
cde
a
bc
cd
B
BC
BCA
Pasteurised
NTS
HTS
Unheated control
Heated
Formulation and treatment

c)

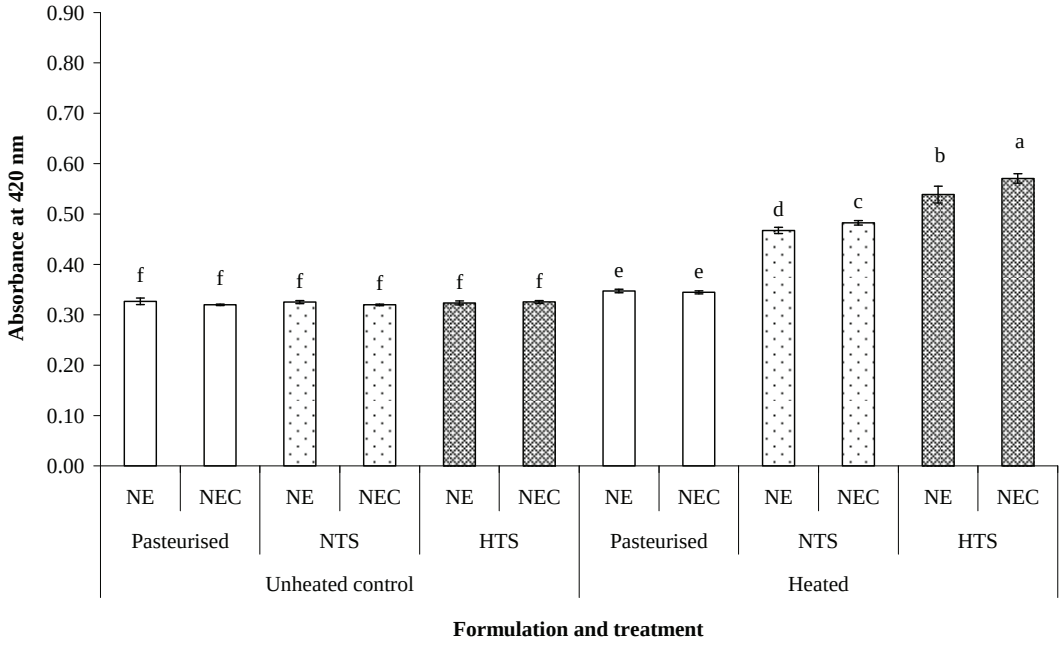
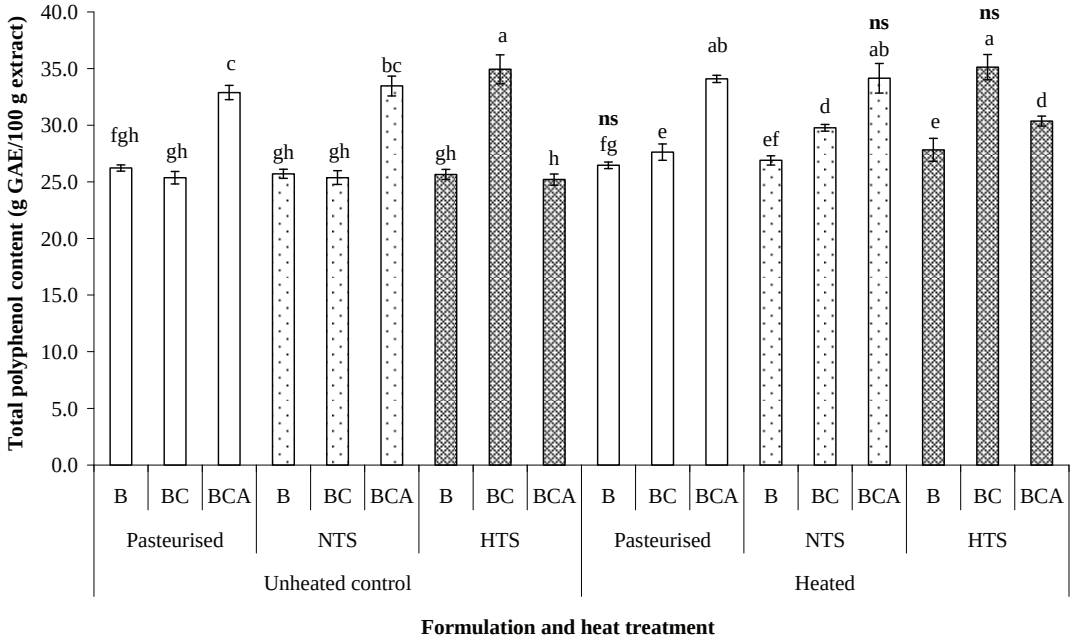
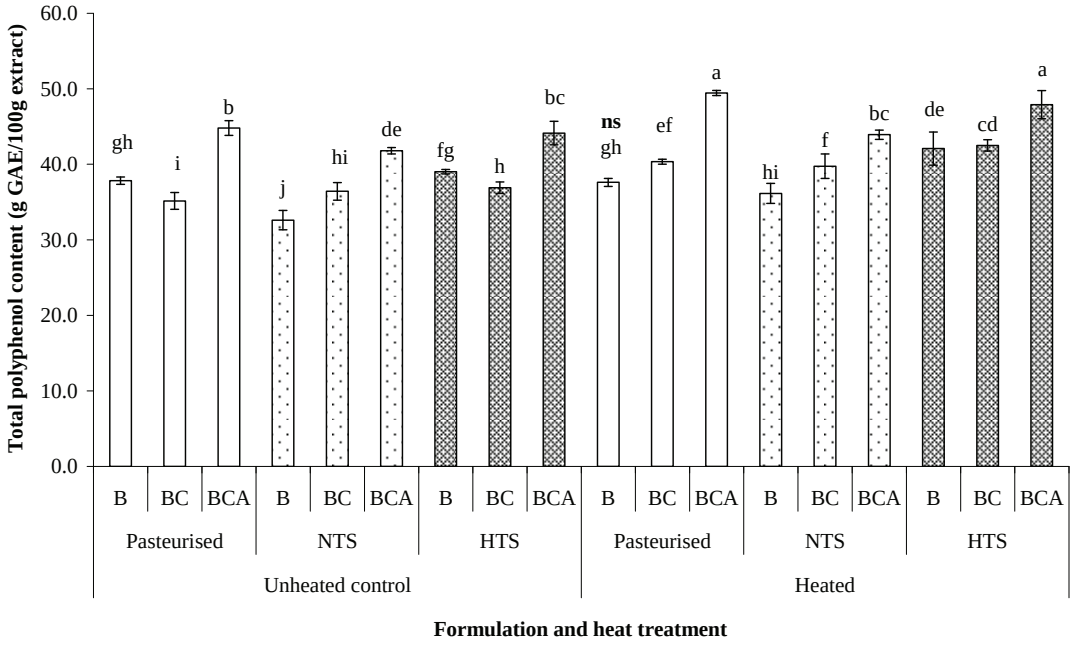


Figure 3.2 The effect of formulation and heat treatment on the absorbance (420 nm) of (a) fermented rooibos (b) unfermented rooibos and (c) nano emulsified unfermented rooibos iced tea. Formulation B = base (rooibos extract in deionised water), BC = base + citric acid, BCA = base + citric + ascorbic acid, NE = nano emulsified unfermented rooibos (NEUR) extract in deionised water, NEC = NE + citric acid, normal temperature sterilisation (NTS) and high temperature sterilisation (HTS). There is no NECA formulation for the NEUR extract as the extract inherently contained ascorbic acid. Means (±SD), represented by bars, labelled with different alphabetical letters differ significantly ($P<0.05$). Means additionally labelled with **ns** do not differ significantly ($P\geq0.05$) from their corresponding untreated control.

a)


Total polyphenol content (g GAE/100 g extract)
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
fgh
gh
c
gh
gh
bc
gh
a
h
ns
fg
e
ab
ef
d
ns
ab
e
ns
a
d
B
BC
BCA
Pasteurised
NTS
HTS
Unheated control
Heated
Formulation and heat treatment

b)


Total polyphenol content (g GAE/100g extract)
60.0
50.0
40.0
30.0
20.0
10.0
0.0
gh
i
b
j
hi
de
fg
h
bc
ns
gh
ef
a
hi
f
bc
de
cd
a
B
BC
BCA
Pasteurised
NTS
HTS
Unheated control
Heated
Formulation and heat treatment

c)

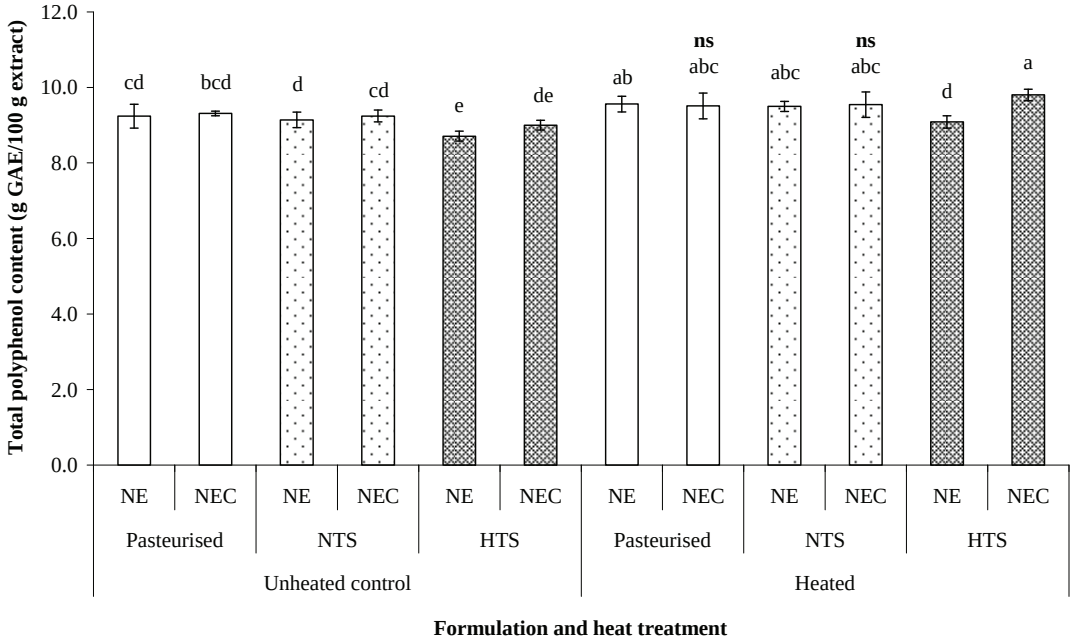
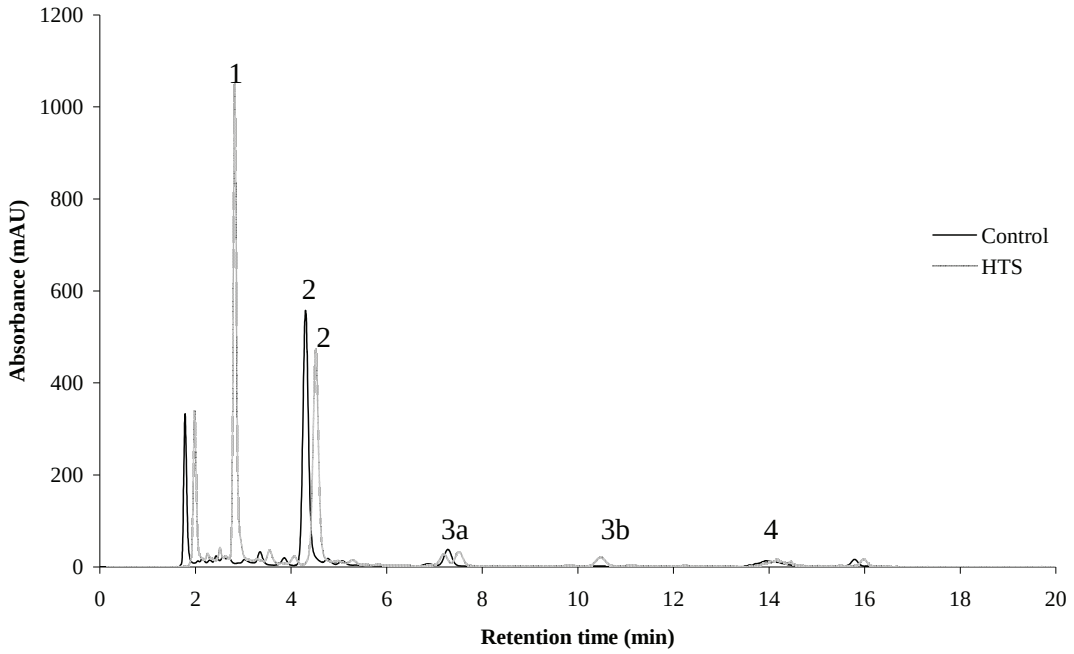


Figure 3.3 The effect of formulation and heat treatment on the total polyphenol content of (a) fermented, (b) unfermented and (c) nano emulsified unfermented rooibos iced tea. Formulation B = base (rooibos extract in deionised water), BC = base + citric acid, BCA = base + citric + ascorbic acid, NE = nano emulsified unfermented rooibos (NEUR) extract in deionised water, NEC = NE + citric acid, GAE = gallic acid equivalents, normal temperature sterilisation (NTS) and high temperature sterilisation (HTS). There is no NECA formulation for the NEUR extract as the extract inherently contained ascorbic acid. Bars labelled with different alphabetical letters differ significantly ($P<0.05$). Means (±SD), represented by bars, labelled with different alphabetical letters differ significantly ($P<0.05$). Means additionally labelled with **ns** do not differ significantly ($P\geq0.05$) from their corresponding untreated control.

a)



b)

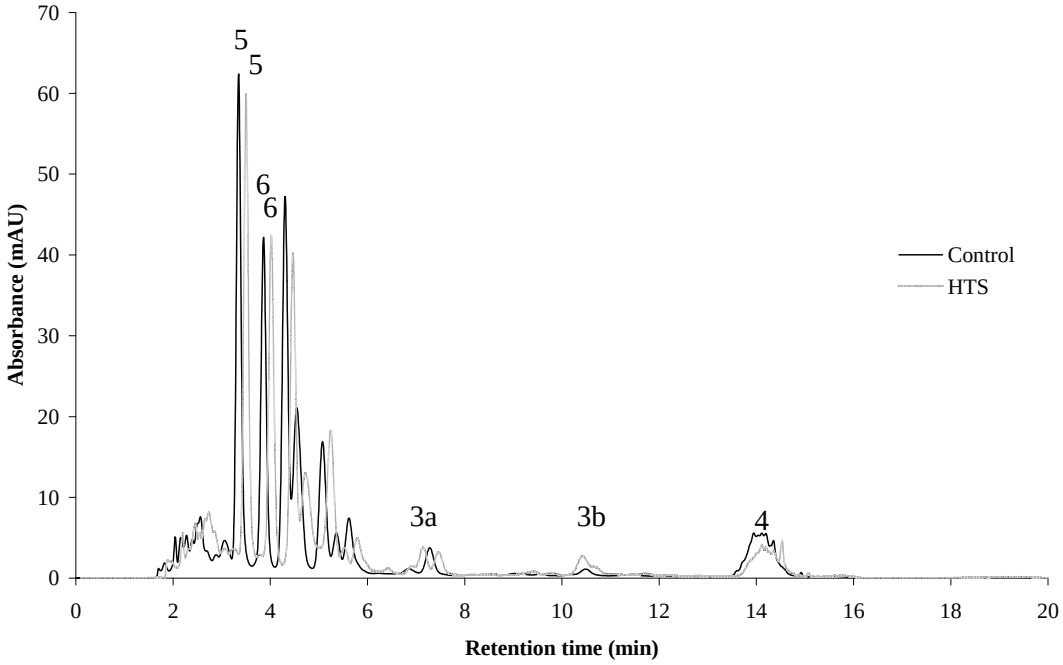
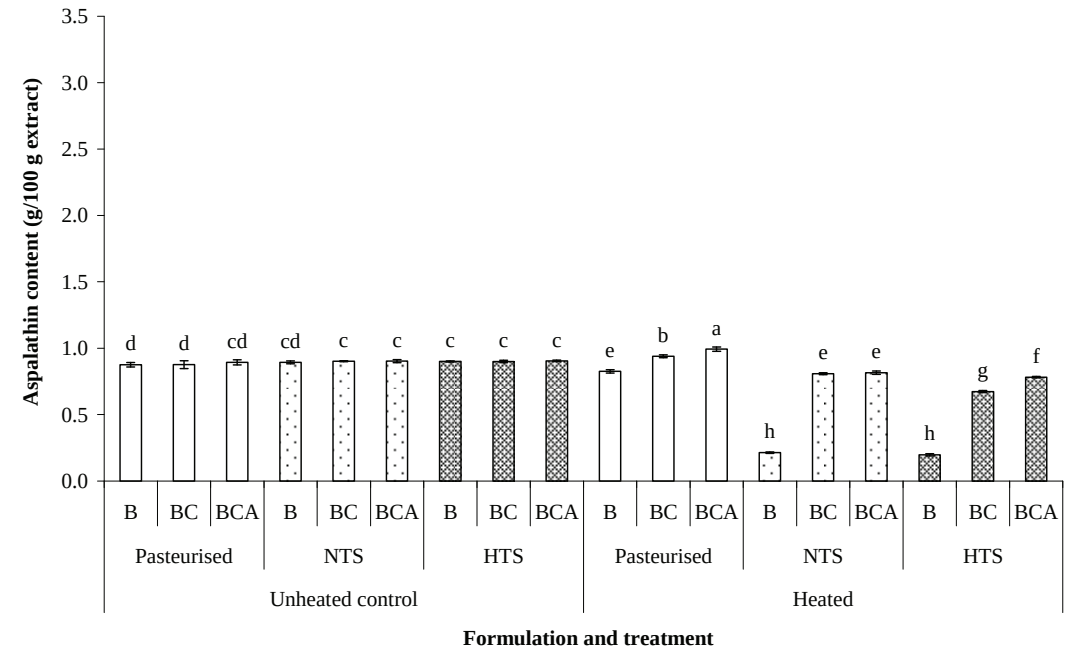
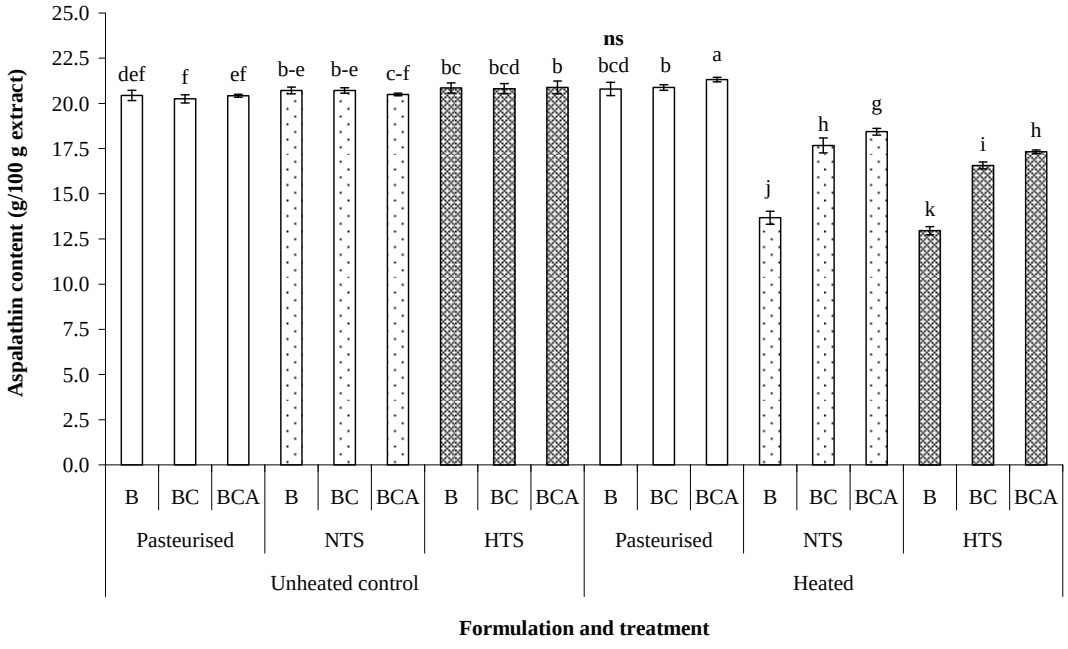


Figure 3.4 Chromatograms of unfermented rooibos iced tea, formulation BCA, before (control) and after HTS at (a) 288 nm and (b) 350 nm. Injection volume was 5 µL. Indicated are (1) compound 1, (2) aspalathin, (3a and 3b) new compounds, (4) polymeric compounds, (5) iso-orientin and (6) orientin. The x-axis of the HTS chromatogram was slightly offset to enable visual comparison with the control.

a)


Aspalathin content (g/100 g extract)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
d d cd cd c c c c c e b a h e e h g f
B BC BCA B BC BCA B BC BCA B BC BCA B BC BCA B BC BCA
Pasteurised NTS HTS Pasteurised NTS HTS
Unheated control Heated
Formulation and treatment

b)


Aspalathin content (g/100 g extract)
25.0
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
def f ef b-e b-e c-f bc bcd b ns bcd b a j h g k i h
B BC BCA B BC BCA B BC BCA B BC BCA B BC BCA B BC BCA
Pasteurised NTS HTS Pasteurised NTS HTS
Unheated control Heated
Formulation and treatment

c)

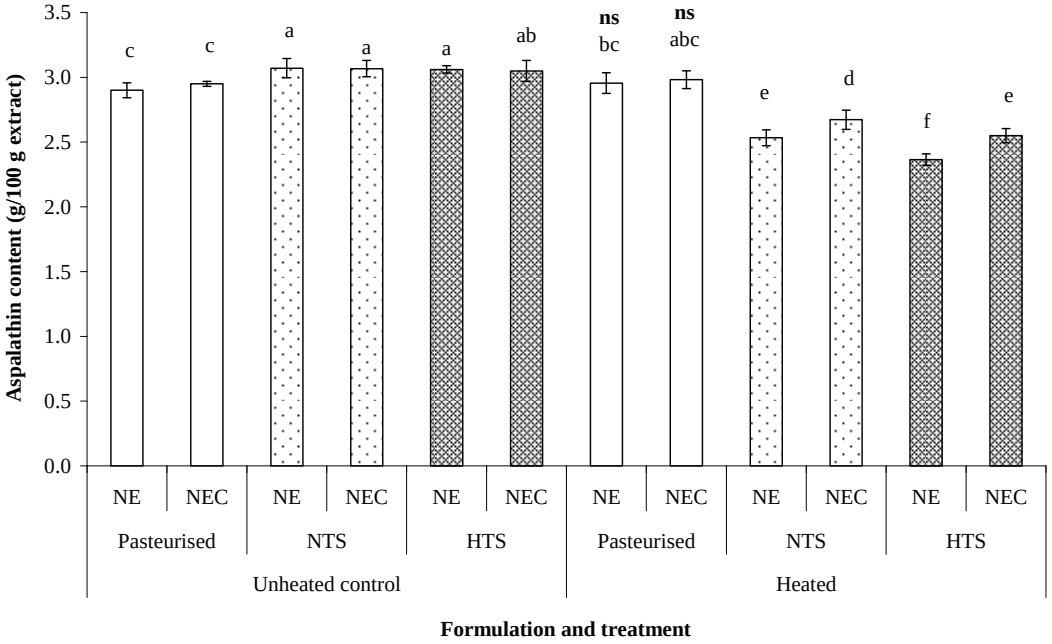
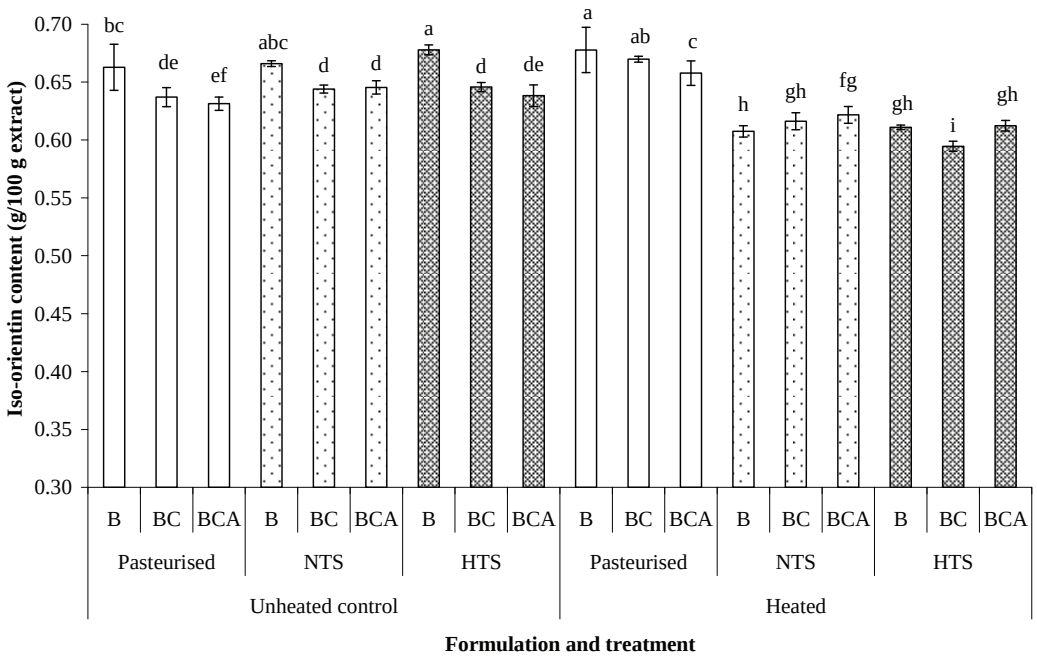
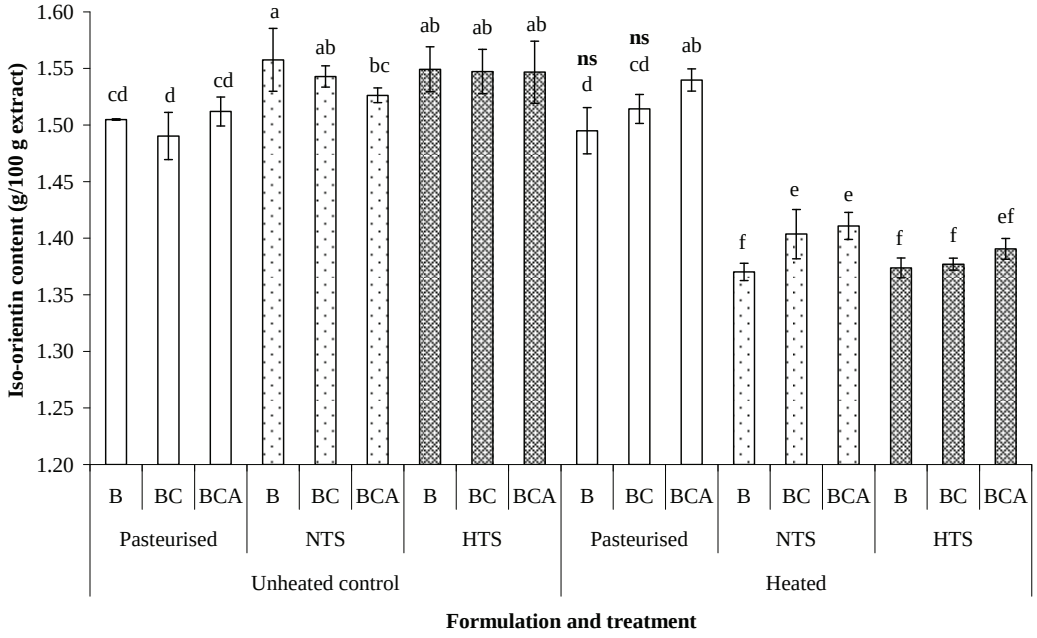


Figure 3.5 The effect of formulation and heat treatment on the aspalathin content of (a) fermented rooibos (b) unfermented rooibos and (c) nano emulsified unfermented rooibos iced tea. Formulation B = base (rooibos extract in deionised water), BC = base + citric acid, BCA = base + citric + ascorbic acid, NE = nano emulsified unfermented rooibos (NEUR) extract in deionised water, NEC = NE + citric acid, normal temperature sterilisation (NTS) and high temperature sterilisation (HTS). There is no NECA formulation for the NEUR extract as the extract inherently contained ascorbic acid. Means (±SD), represented by bars, labelled with different alphabetical letters differ significantly ($P<0.05$). Means additionally labelled with **ns** do not differ significantly ($P\geq0.05$) from their corresponding untreated control.

a)


Iso-orientin content (g/100 g extract)
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
bc de ef abc d d a d de a ab c h gh fg gh i gh
B BC BCA B BC BCA B BC BCA B BC BCA B BC BCA B BC BCA
Pasteurised NTS HTS Pasteurised NTS HTS
Unheated control Heated
Formulation and treatment

b)


Iso-orientin content (g/100 g extract)
1.60
1.55
1.50
1.45
1.40
1.35
1.30
1.25
1.20
cd d cd a ab bc ab ab ab ns d ns cd ab f e e f f ef
B BC BCA B BC BCA B BC BCA B BC BCA B BC BCA B BC BCA
Pasteurised NTS HTS Pasteurised NTS HTS
Unheated control Heated
Formulation and treatment

c)

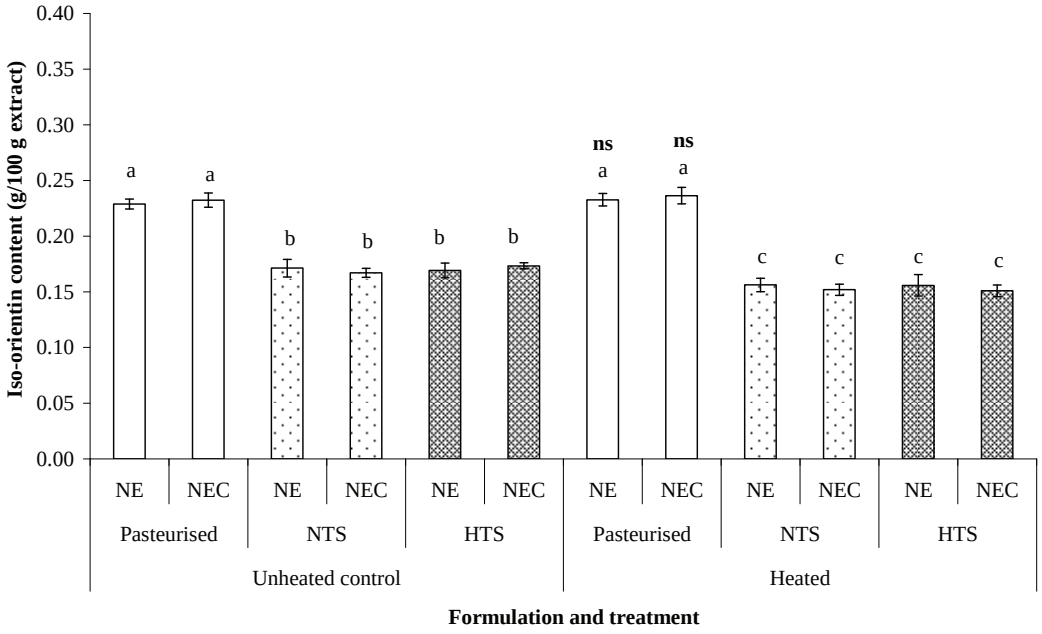
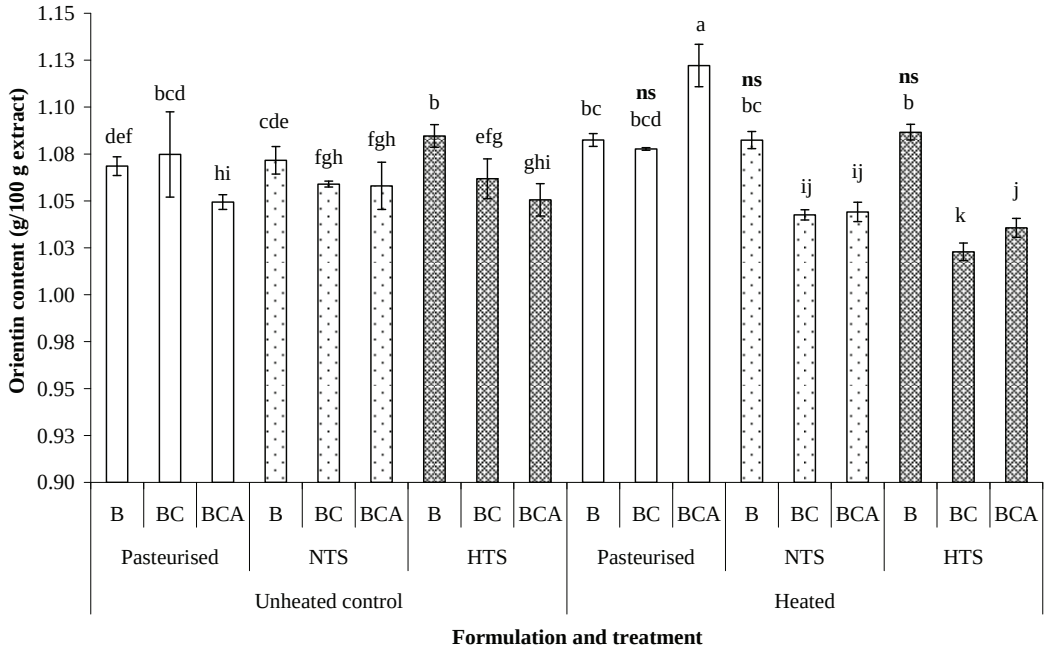
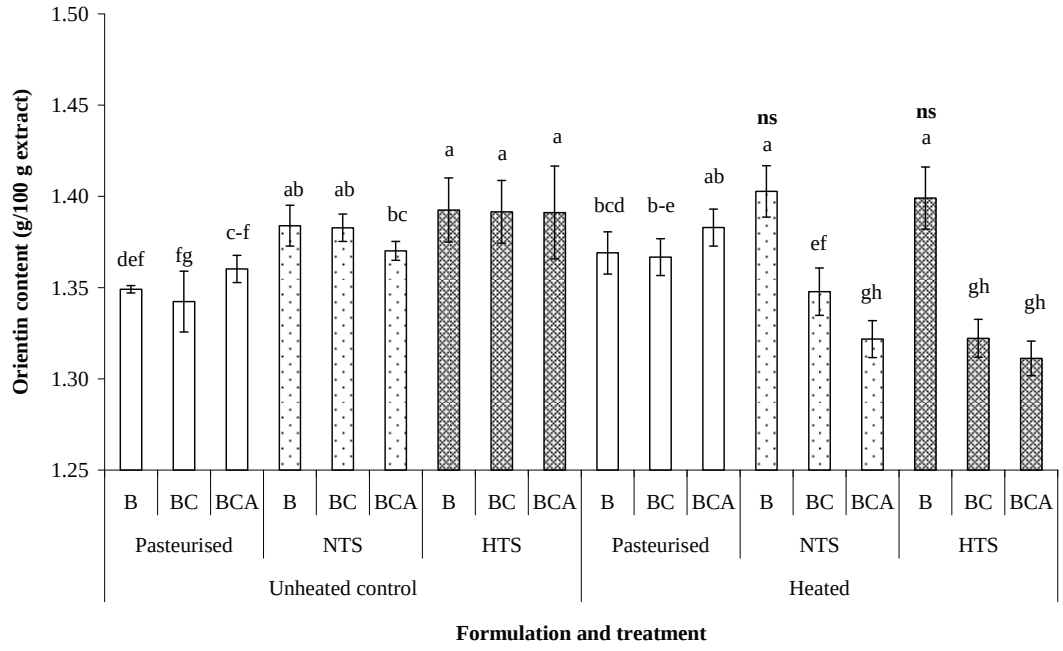


Figure 3.6 The effect of formulation and heat treatment on the iso-orientin content of (a) fermented rooibos (b) unfermented rooibos and (c) nano emulsified unfermented rooibos iced tea. Formulation B = base (rooibos extract in deionised water), BC = base + citric acid, BCA = base + citric + ascorbic acid, NE = nano emulsified unfermented rooibos (NEUR) extract in deionised water, NEC = NE + citric acid, normal temperature sterilisation (NTS) and high temperature sterilisation (HTS). There is no NECA formulation for the NEUR extract as the extract inherently contained ascorbic acid. Means (±SD), represented by bars, labelled with different alphabetical letters differ significantly ($P<0.05$). Means additionally labelled with **ns** do not differ significantly ($P\geq0.05$) from their corresponding untreated control.

a)


Orientin content (g/100 g extract)
1.15
1.13
1.10
1.08
1.05
1.03
1.00
0.98
0.95
0.93
0.90
def
bcd
hi
cde
fgh
fgh
b
efg
ghi
bc
ns
bcd
a
ns
bc
ij
ij
ns
b
k
j
B
BC
BCA
Pasteurised
NTS
HTS
Unheated control
Heated
Formulation and treatment

b)


Orientin content (g/100 g extract)
1.50
1.45
1.40
1.35
1.30
1.25
def
fg
c-f
ab
ab
bc
a
a
a
bcd
b-e
ab
ns
a
ef
gh
ns
a
gh
gh
B
BC
BCA
Pasteurised
NTS
HTS
Unheated control
Heated
Formulation and treatment

c)

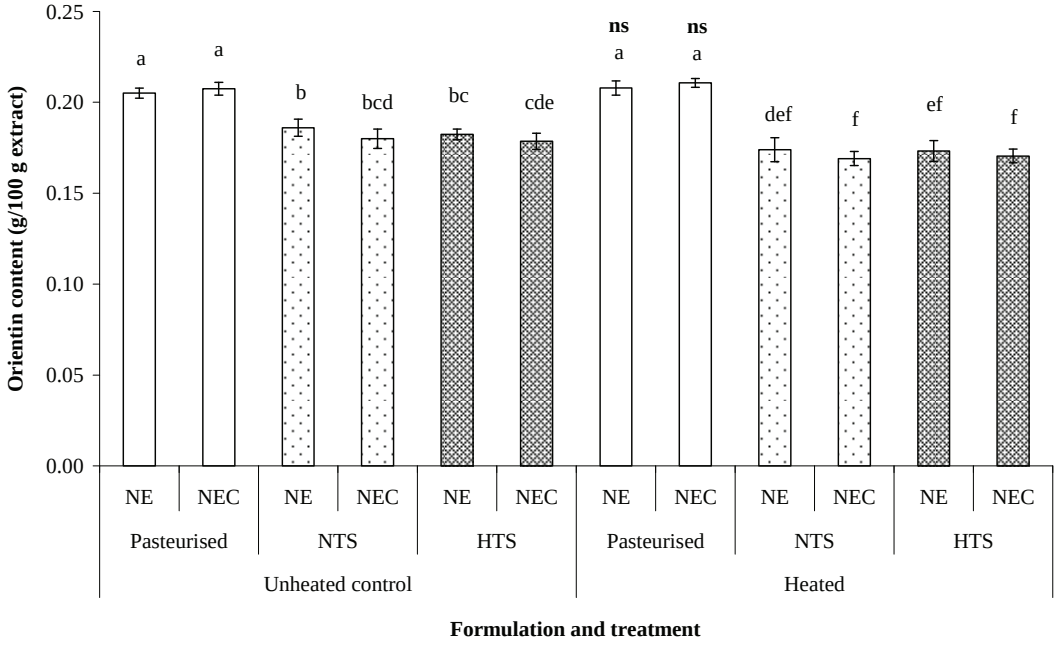
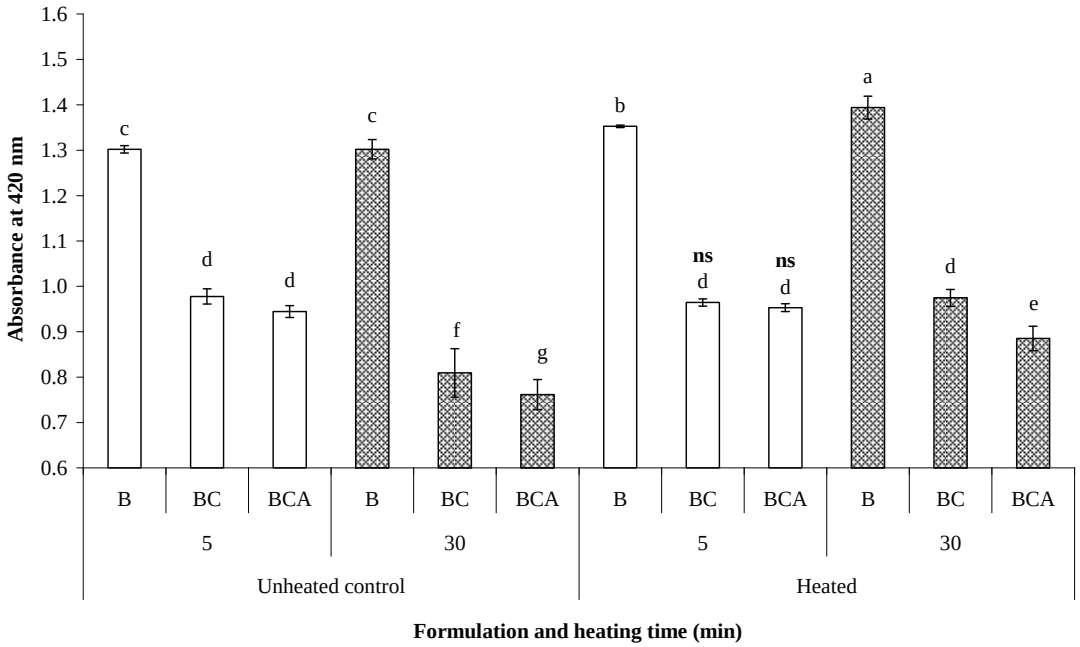
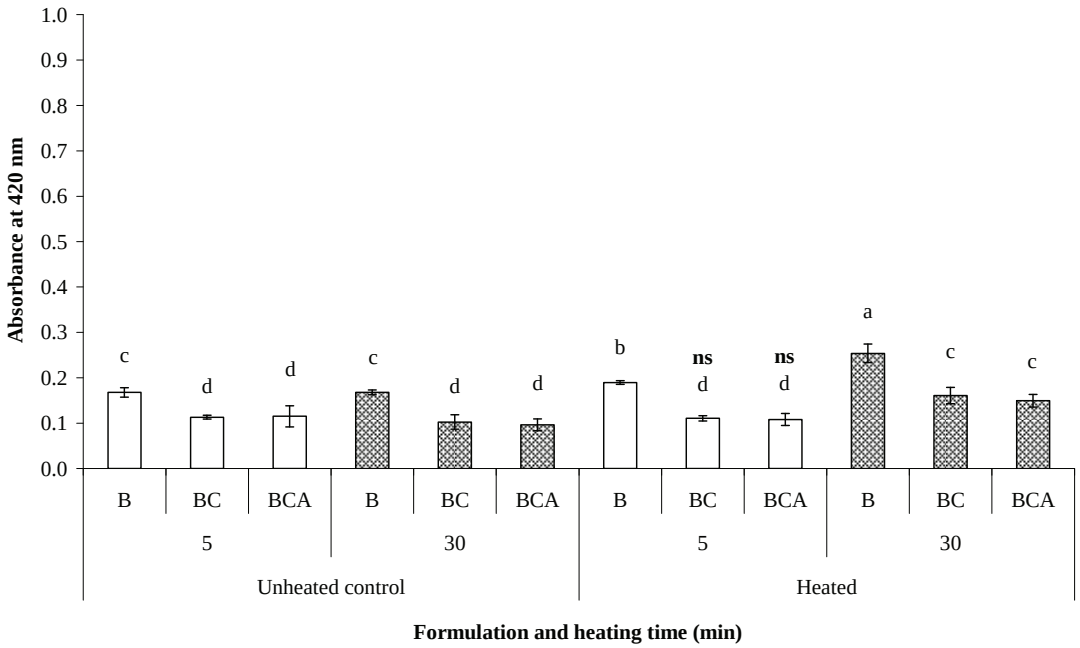


Figure 3.7 The effect of formulation and heat treatment on the orientin content of (a) fermented rooibos (b) unfermented rooibos and (c) nano emulsified unfermented rooibos iced tea. Formulation B = base (rooibos extract in deionised water), BC = base + citric acid, BCA = base + citric + ascorbic acid, NE = nano emulsified unfermented rooibos (NEUR) extract in deionised water, NEC = NE + citric acid, normal temperature sterilisation (NTS) and high temperature sterilisation (HTS). There is no NECA formulation for the NEUR extract as the extract inherently contained ascorbic acid. Means (±SD), represented by bars, labelled with different alphabetical letters differ significantly ($P<0.05$). Means additionally labelled with **ns** do not differ significantly ($P\geq0.05$) from their corresponding untreated control.

a)


Absorbance at 420 nm
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
c
d
d
c
f
g
b
ns
d
ns
d
a
d
e
B
BC
BCA
B
BC
BCA
B
BC
BCA
B
BC
BCA
5
30
5
30
Unheated control
Heated
Formulation and heating time (min)

b)


Absorbance at 420 nm
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
c
d
d
c
d
d
b
ns
d
ns
d
a
c
c
B
BC
BCA
B
BC
BCA
B
BC
BCA
B
BC
BCA
5
30
5
30
Unheated control
Heated
Formulation and heating time (min)

c)

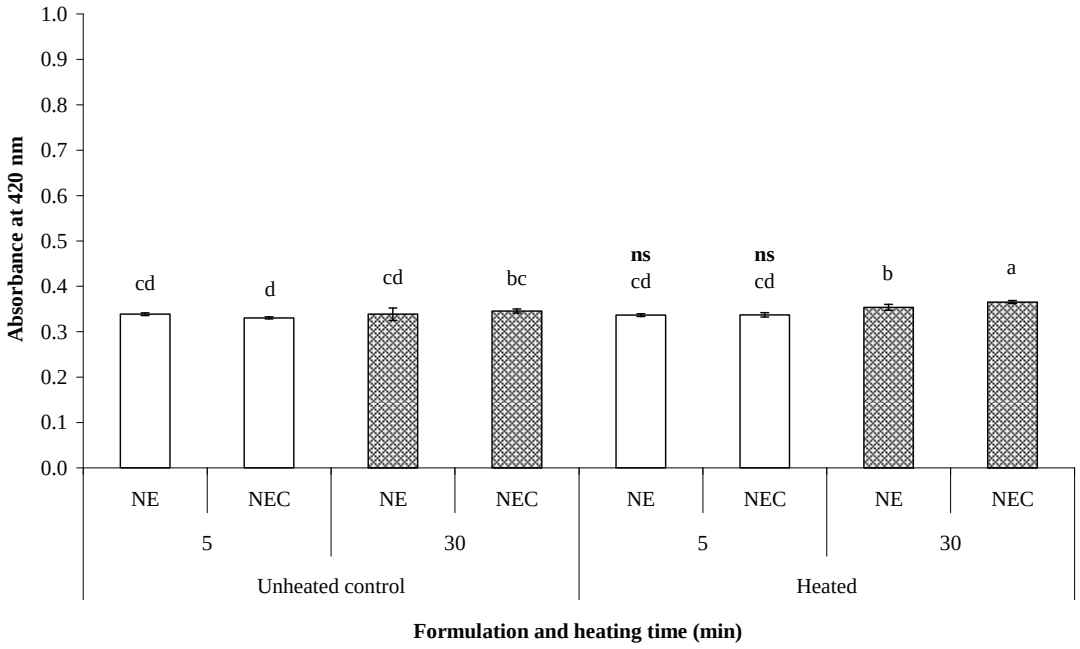
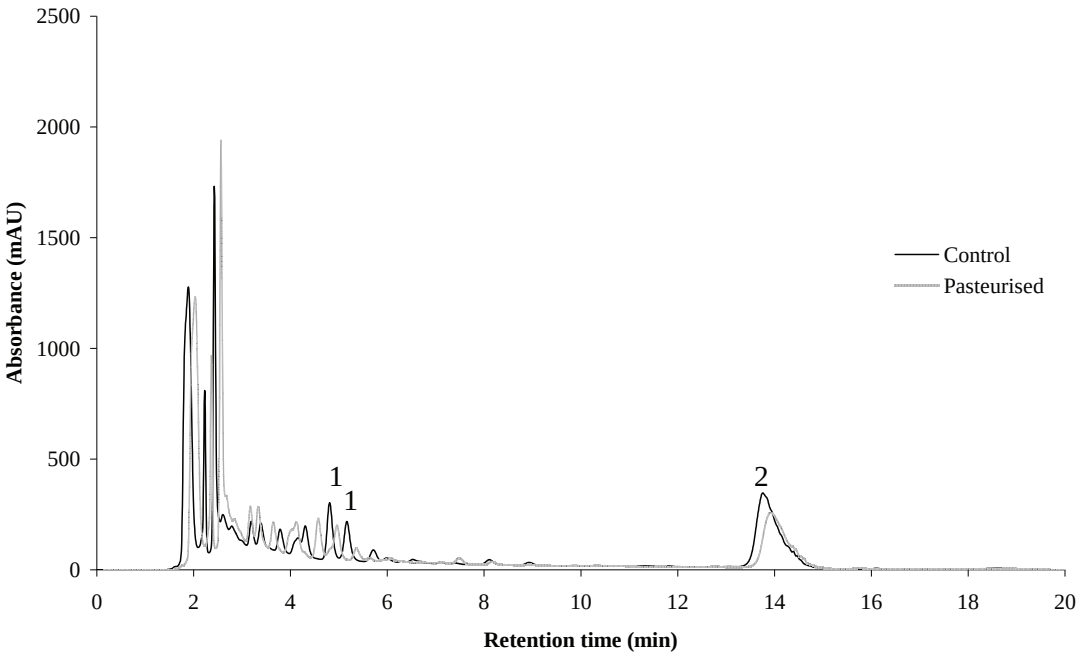


Figure 3.8 The effect of formulation and pasteurisation on the absorbance (420 nm) of (a) fermented, (b) unfermented and (c) nano emulsified unfermented rooibos iced tea after a five and 30 min pasteurisation-like heat treatment. Formulation B = base (rooibos extract in deionised water), BC = base + citric acid, BCA = base + citric + ascorbic acid, NE = nano emulsified unfermented rooibos (NEUR) extract in deionised water, NEC = NE + citric acid. There is no NECA formulation for the NEUR extract as the extract inherently contained ascorbic acid. Means (±SD), represented by bars, labelled with different alphabetical letters differ significantly ($P<0.05$). Means additionally labelled with **ns** do not differ significantly ($P\geq0.05$) from their corresponding untreated control.

a)



b)

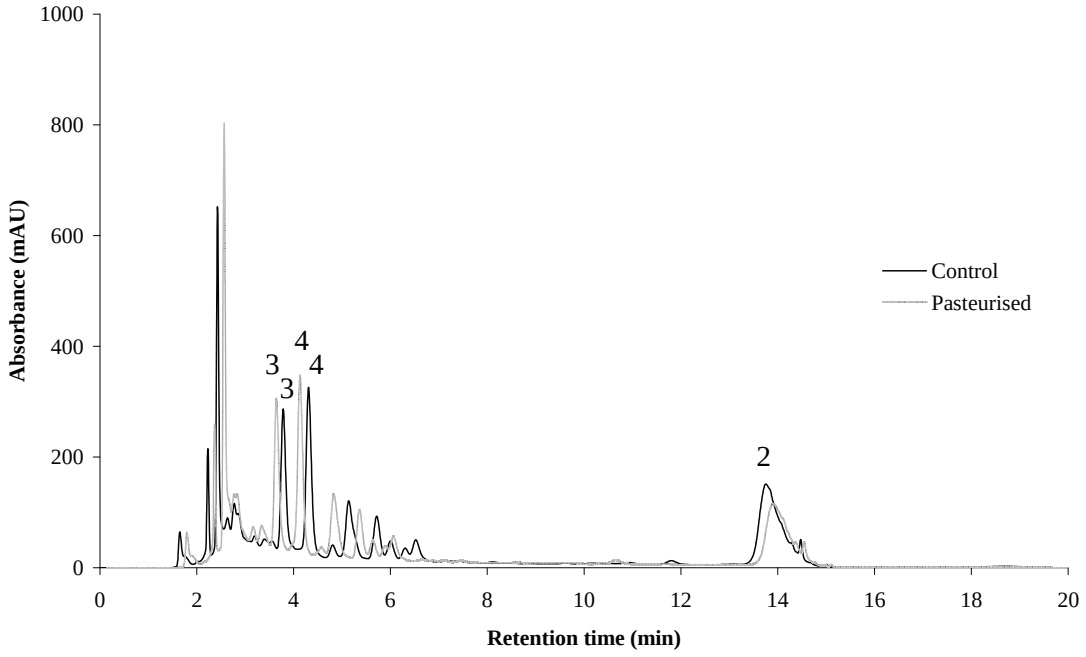
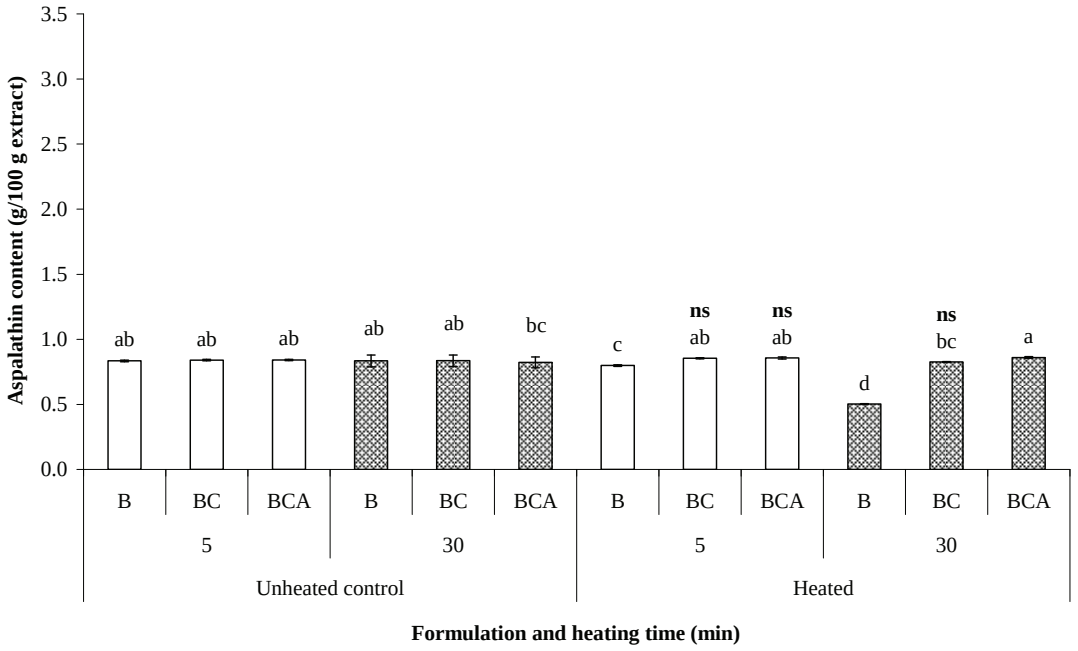
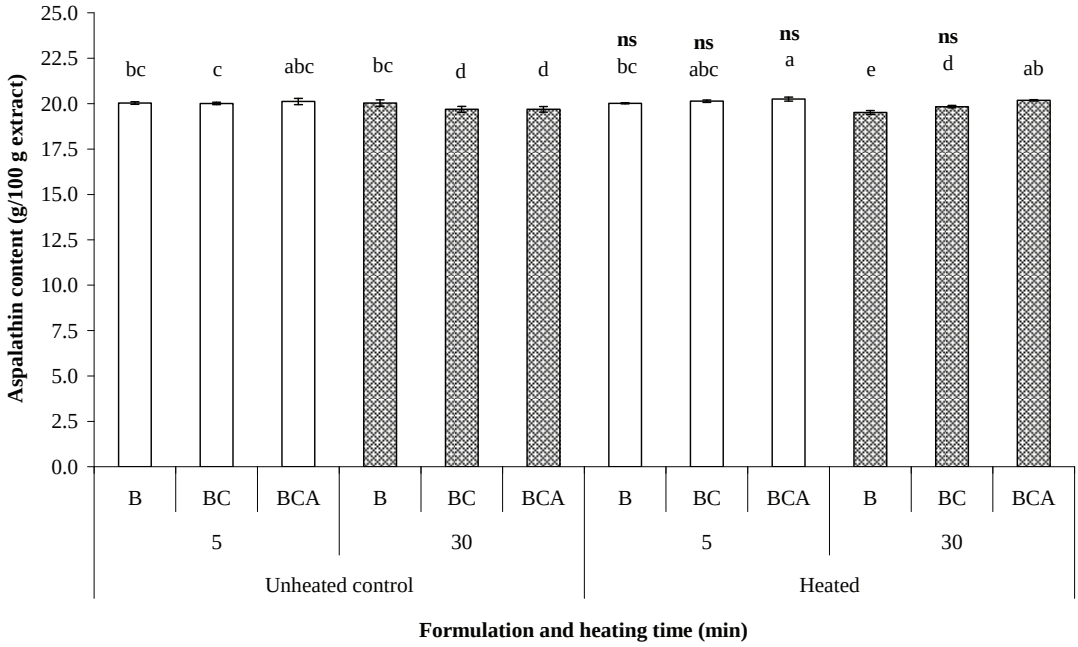


Figure 3.9 Chromatograms (second heating experiment) of fermented rooibos iced tea, formulation B, before (control) and after pasteurisation at (a) 288 nm and (b) 350 nm. Injection volume was 50 μL. Indicated on the applicable chromatograms are (1) aspalathin, (2) polymeric compounds, (3) iso-orientin and (4) orientin. The x-axis of the "pasteurised" chromatogram was slightly offset to enable visual comparison with the control.

a)


Aspalathin content (g/100 g extract)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ab ab ab ab ab bc c ns ab ns ab d ns bc a
B BC BCA B BC BCA B BC BCA B BC BCA
5 30 5 30
Unheated control
Heated
Formulation and heating time (min)

b)


Aspalathin content (g/100 g extract)
25.0
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
bc c abc bc d d ns bc ns abc ns a e ns d ab
B BC BCA B BC BCA B BC BCA B BC BCA
5 30 5 30
Unheated control
Heated
Formulation and heating time (min)

c)

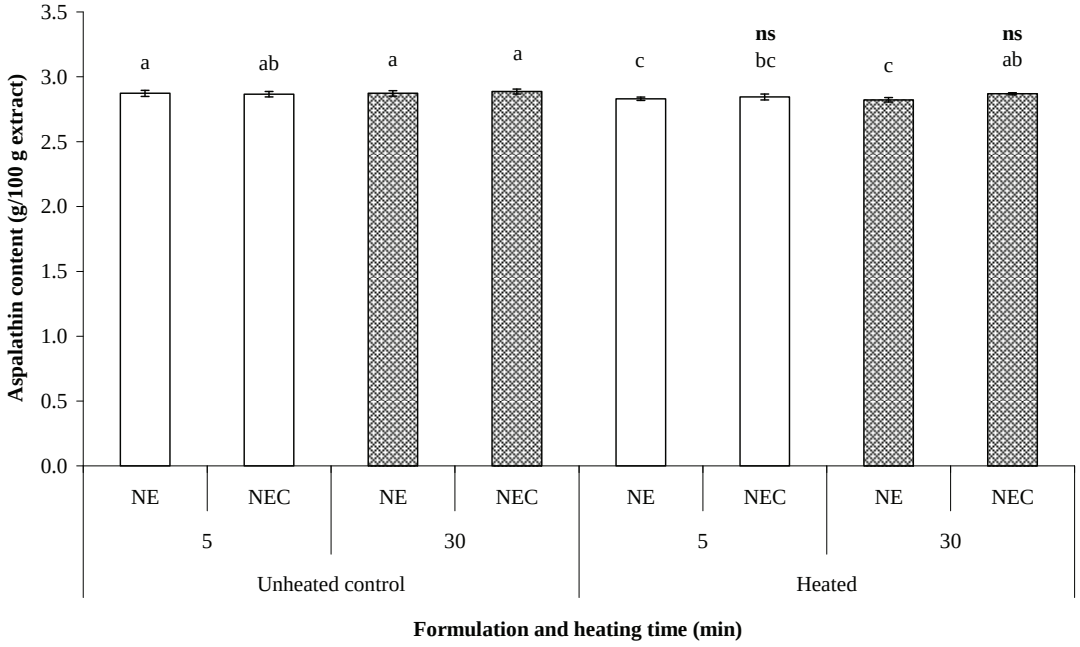
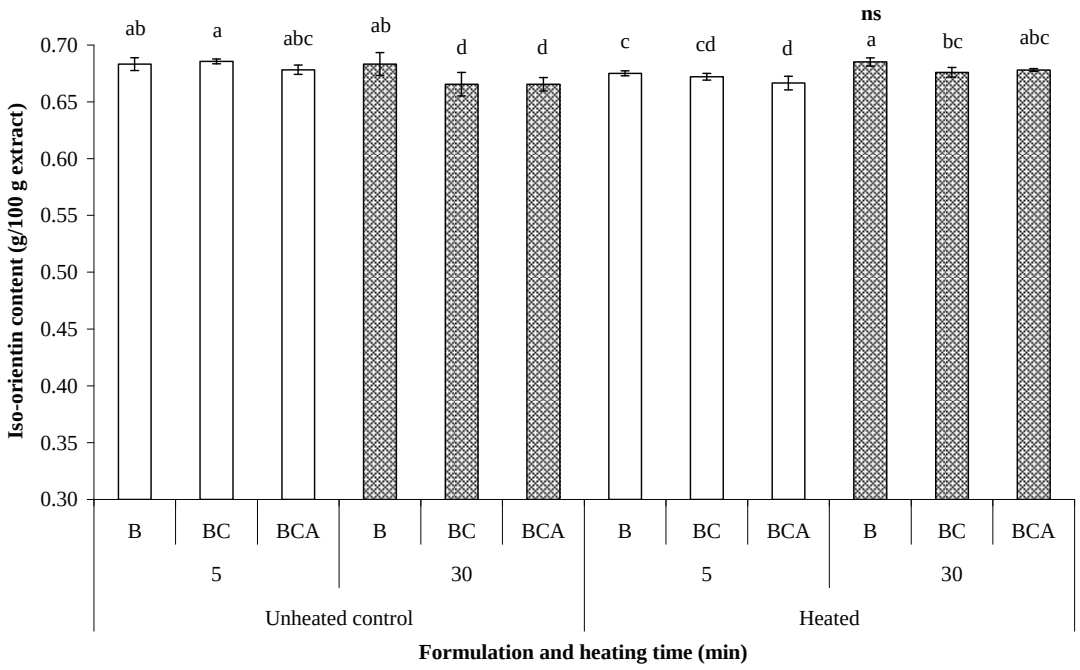
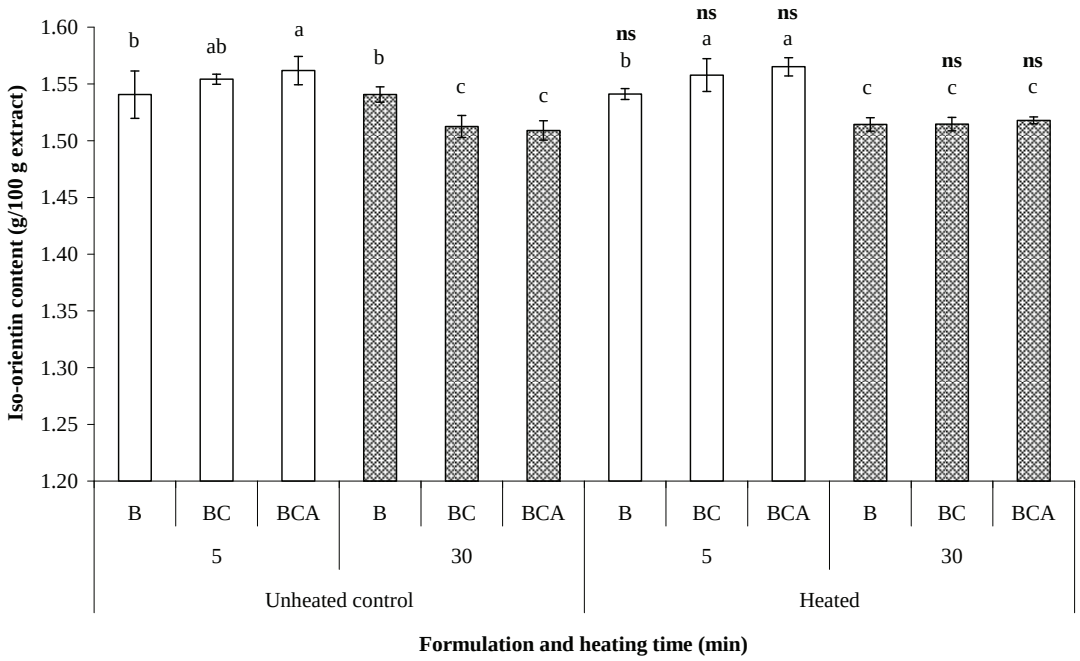


Figure 3.10 The effect of formulation and pasteurisation on the aspalathin content of (a) fermented, (b) unfermented and (c) nano emulsified unfermented rooibos iced tea after a five and 30 min pasteurisation-like heat treatment. Formulation B = base (rooibos extract in deionised water), BC = base + citric acid, BCA = base + citric + ascorbic acid, NE = nano emulsified unfermented rooibos (NEUR) extract in deionised water, NEC = NE + citric acid. There is no NECA formulation for the NEUR extract as the extract inherently contained ascorbic acid. Means (±SD), represented by bars, labelled with different alphabetical letters differ significantly ($P<0.05$). Means additionally labelled with **ns** do not differ significantly ($P\geq0.05$) from their corresponding untreated control.

a)


Iso-orientin content (g/100 g extract)
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
ab a abc ab d d c cd d ns a bc abc
B BC BCA B BC BCA B BC BCA B BC BCA
5 30 5 30
Unheated control
Heated
Formulation and heating time (min)

b)


Iso-orientin content (g/100 g extract)
1.60
1.55
1.50
1.45
1.40
1.35
1.30
1.25
1.20
b ab a b c c ns b ns a ns a c ns c ns c
B BC BCA B BC BCA B BC BCA B BC BCA
5 30 5 30
Unheated control
Heated
Formulation and heating time (min)

c)

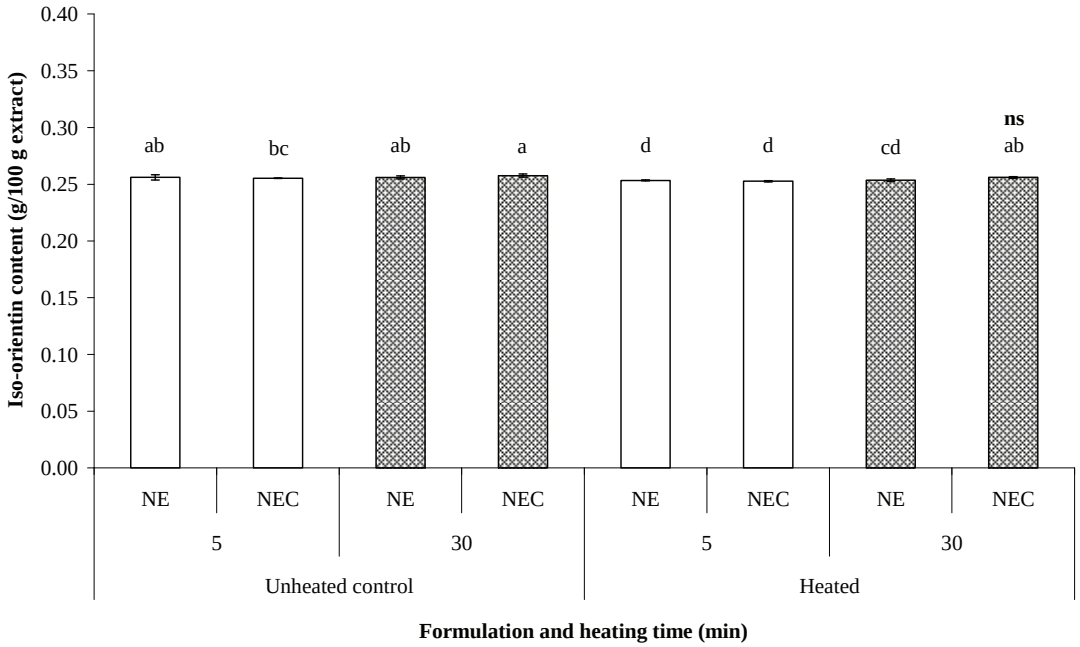
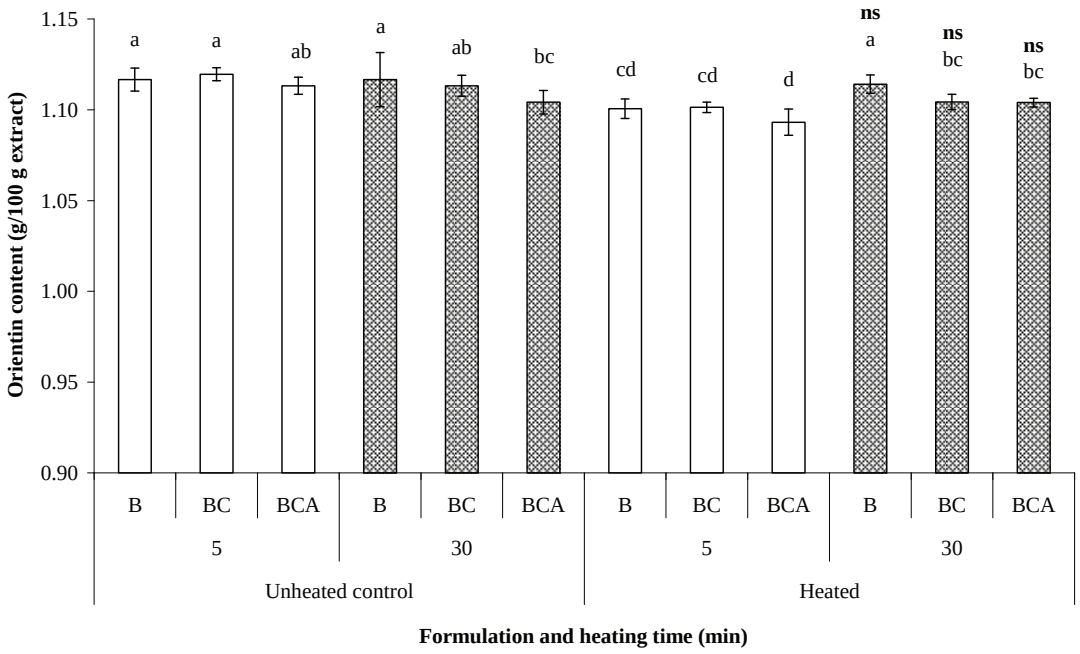
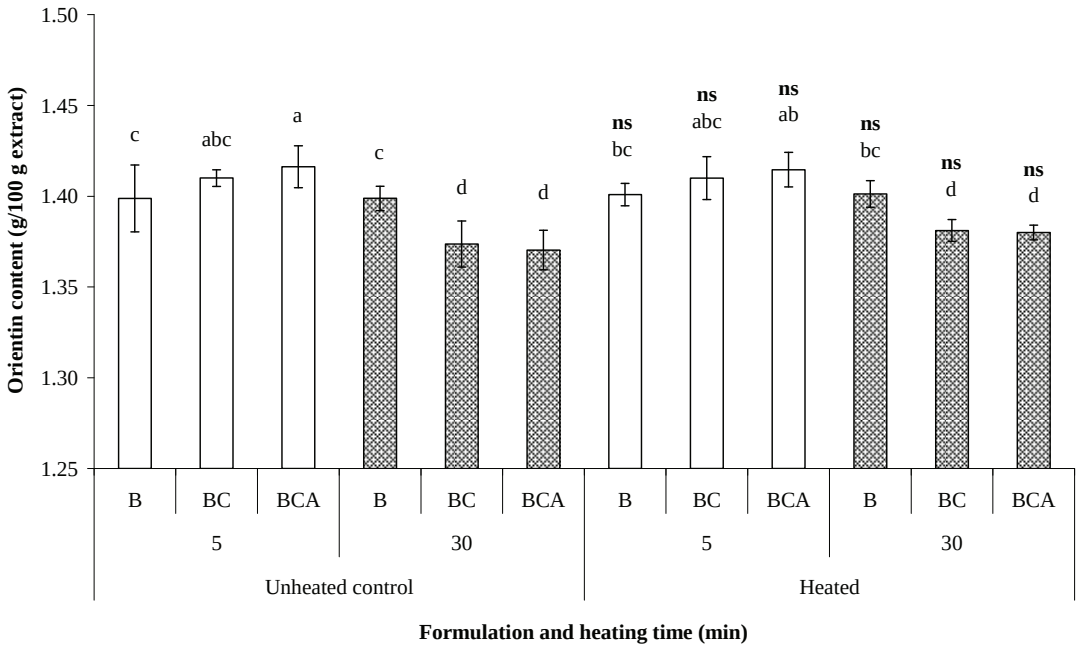


Figure 3.11 The effect of formulation and pasteurisation on the iso-orientin content of (a) fermented, (b) unfermented and (c) nano emulsified unfermented rooibos iced tea after a five and 30 min pasteurisation-like heat treatment. Formulation B = base (rooibos extract in deionised water), BC = base + citric acid, BCA = base + citric + ascorbic acid, NE = nano emulsified unfermented rooibos (NEUR) extract in deionised water, NEC = NE + citric acid. There is no NECA formulation for the NEUR extract as the extract inherently contained ascorbic acid. Means (±SD), represented by bars, labelled with different alphabetical letters differ significantly ($P<0.05$). Means additionally labelled with **ns** do not differ significantly ($P\geq0.05$) from their corresponding untreated control.

a)


Orientin content (g/100 g extract)
1.15
1.10
1.05
1.00
0.95
0.90
a a ab a ab bc cd cd d ns a ns bc ns bc
B BC BCA B BC BCA B BC BCA B BC BCA
5 30 5 30
Unheated control
Heated
Formulation and heating time (min)

b)


Orientin content (g/100 g extract)
1.50
1.45
1.40
1.35
1.30
1.25
c abc a c d d ns bc ns abc ns ab ns bc ns d ns d
B BC BCA B BC BCA B BC BCA B BC BCA
5 30 5 30
Unheated control
Heated
Formulation and heating time (min)

c)

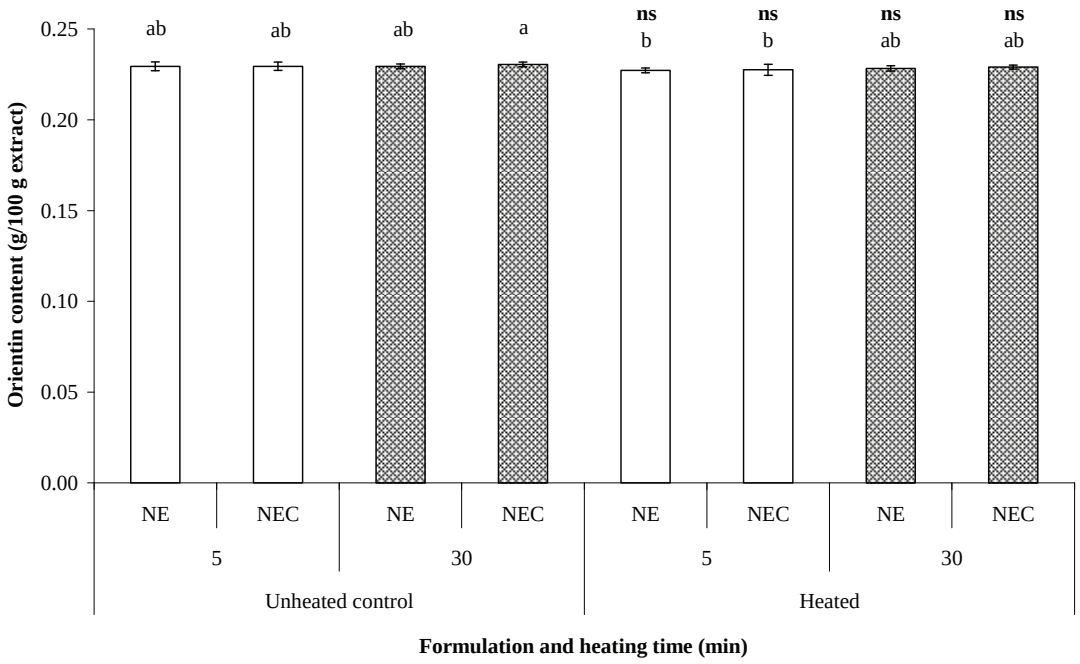


Figure 3.12 The effect of formulation and pasteurisation on the orientin content of (a) fermented, (b) unfermented and (c) nano emulsified unfermented rooibos iced tea after a five and 30 min pasteurisation-like heat treatment. Formulation B = base (rooibos extract in deionised water), BC = base + citric acid, BCA = base + citric + ascorbic acid, NE = nano emulsified unfermented rooibos (NEUR) extract in deionised water, NEC = NE + citric acid. There is no NECA formulation for the NEUR extract as the extract inherently contained ascorbic acid. Means (±SD), represented by bars, labelled with different alphabetical letters differ significantly ($P<0.05$). Means additionally with **ns** do not differ significantly ($P\geq0.05$) from their corresponding untreated control.

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CHAPTER 4

EFFECT OF STORAGE ON THE PHENOLIC COMPOSITION AND COLOUR OF ROOIBOS ICED TEA

CHAPTER 4

EFFECT OF STORAGE ON THE PHENOLIC COMPOSITION AND COLOUR OF ROOIBOS ICED TEA*

ABSTRACT

In this study, the effect of storage (25ºC) on different formulations of experimental fermented rooibos (FR) and unfermented rooibos (UR) iced teas as well as nano emulsified unfermented rooibos (NEUR) iced tea was investigated. Browning of the teas was monitored spectrophotometrically (420 nm) whilst the Folin-Ciocalteu assay was used to assess the total polyphenol (TP) content. Changes in individual flavonoids were quantified using HPLC.

Storage significantly ($P<0.05$) reduced the aspalathin, iso-orientin, orientin and TP content of all types of rooibos iced tea. The addition of citric and ascorbic acid proved beneficial for the preservation of these flavonoid compounds in FR iced tea, whilst the addition of citric acid appeared to have a negative effect on the long-term preservation of aspalathin in UR iced tea. The aspalathin content of formulation B (base: rooibos extract in deionised water) of the FR iced tea was reduced to undetectable levels between the fourth and eighth week of storage, whilst formulation BC (base + citric acid) and BCA (base + citric + ascorbic acid) underwent significant ($P<0.05$) reductions of 38.6% and 25.1%, respectively, over the 12 week period. Losses were similar or less for the UR iced teas and lower still for the NEUR iced teas. In the case of the latter, formulation NE (NEUR extract in deionised water) and NEC (NE + citric acid) underwent losses of 12.7% and 11.9%, respectively.

Storage-induced losses of the flavones iso-orientin and orientin were lower than those for aspalathin for all three types of iced tea. The iso-orientin content of UR iced tea decreased by 12.0% (BC), 10.6% (B) and 8.8% (BCA) whilst no significant ($P\geq0.05$) changes in orientin content were detected. The iced teas containing the NEUR extract showed the smallest losses of these compounds. The iso-orientin and orientin content of most of the iced tea (irrespective of extract identity and formulation) was found to increase significantly ($P<0.05$) between one and two weeks of storage, due to aspalathin conversion. The absorbance of most of the iced teas (irrespective of extract identity and formulation) increased significantly ($P<0.05$) during storage, indicating the formation of oxidation products. The TP content generally decreased, irrespective of the presence or absence of ascorbic acid, indicating that a true loss of polyphenols occurred.

* Part of this work was presented at the 24th International Conference on Polyphenols, 8-11 July 2008, Salamanca, Spain.

INTRODUCTION

Storage may alter the chemical, physical and antioxidant properties of food products. Storage of apple juice concentrates (9 months at 25ºC) typically results in a 60% loss of phloretin glycosides (Spanos *et al*., 1990). In orange juice, loss of polyphenols, vitamin C and antioxidant capacity has been reported (Klimczak *et al*., 2007). Similarly, Patthamakanokporn *et al*. (2008) noted a continuous decrease in the total polyphenol (TP) content of homogenised guava pulp during storage (three months at -20ºC). Naithani *et al*. (2006) reported that the storage of Indian herbal tea leaves resulted in a decline in antioxidant capacity as a result of oxidative changes. In honey, storage has been associated with browning and the oxidation of polyphenols (Gonzales *et al*., 1999).

In certain instances, storage has been associated with the improvement of certain characteristics of food and beverage products. For example, the chain breaking antioxidant activity of red wine has been shown to increase upon extended air exposure (Manzocco *et al*., 1998) and the TP content of bottled tomato pulp was shown to increase after 180 days of storage at 20ºC (Ordóñez-Santos *et al*., 2009). In other instances, storage induced changes have been shown to balance one another. Pinotage wine, matured in oak barrels for 15 months, demonstrated a gradual loss of monomeric flavonoid compounds combined with an increase in the concentration of gallic acid and the formation of new oligomeric compounds (De Beer *et al*., 2008). This could not be attributed to storage alone, but also to the extraction of phenolic compounds from the wood (oak). As a result of these changes, the total antioxidant capacity (TAC) of the wine was not significantly altered by storage. This was in contrast to findings for bottle-stored wines, the TAC of which was shown to decrease as a result of storage (De Beer *et al*., 2005).

With respect to rooibos, the storage of ethanolic and aqueous solutions of aspalathin has been shown to almost exclusively yield (S)- and (R)-eriodictyol-6-C-β-D-glucopyranoside, also known as dihydro-iso-orientin (Koeppen & Roux, 1965; Koeppen & Roux, 1966; Krafczyk & Glomb, 2008). Dihydro-orientin [(S)- and (R)-eriodictyol-8-C-β-D-glucopyranoside] was formed to a lesser extent. Oxidation of dihydro-iso-orientin resulted in the formation of iso-orientin, which was then further, irreversibly converted to orientin (Krafczyk & Glomb, 2008). Orientin was, however, only a minor product amongst uncharacterised brown material. Although iso-orientin was shown to form as a result of the oxidation of dihydro-iso-orientin, orientin does not form from dihydro-orientin. Conversion of iso-orientin into orientin occurs via opening of the vinyl ester structure of iso-orientin to form a chalcone intermediate. Furthermore, orientin, with a half-life of 208 h, was shown to be more resistant to oxidation than iso-orientin (half-life 135 h) (Krafczyk & Glomb, 2008).

The storage stability of phenolic compounds may be modulated by the addition of compounds generally applied during food processing. For example, ascorbic acid has been shown to both protect and accelerate the loss of tea catechins during storage (preservation of green tea catechins for one month, after which degradation was accelerated), whilst the addition of citric acid was found to accelerate the loss of tea catechins during storage (Chen *et al*., 2001). No information is available on the effect of a combination of citric and ascorbic acid on the preservation of the tea catechins.

The popularity of rooibos extracts for application in the food and beverage industry in South Africa was briefly mentioned in Chapter 3. In the latter chapter, the effect of heating and extract production was investigated. Rooibos extracts were found to contain aspalathin, iso-orientin and orientin at all stages of production, and the effect of heat was found not to result in the complete destruction of these compounds in rooibos iced tea. Despite these facts, the phenolic quality of commercial rooibos iced teas was extremely poor.

Commercial rooibos iced teas can be expected to undergo a storage period post processing. This introduces another potential source of flavonoid loss before the final product is consumed. The aim of this study was thus to investigate whether storage may be responsible for a significant loss of phenolic quality of rooibos iced tea. Changes in TP content and product colour, as well as aspalathin, iso-orientin and orientin content of the iced tea were investigated. These changes were determined at specific time points during a three month storage period (25ºC). Fermented rooibos (FR), unfermented rooibos (UR) and nano emulsified unfermented rooibos (NEUR) iced teas, as well as combination iced teas, consisting of FR and either UR or NEUR were subjected to stability testing. The latter teas were included to establish whether the FR extract (containing more iron) would accelerate the degradation of the phenolic compounds. The effect of ascorbic acid and citric acid on the stability of the different iced teas was also investigated.

MATERIALS AND METHODS

Chemicals and reagents

The chemicals and reagents required for the present study were the same as those listed in Chapter 3, with the exception of ethylene diamine tetraacetic acid di-sodium salt (EDTA), which was not required.

Extracts for preparation of rooibos iced tea

The rooibos extracts described and characterised in Chapter 3 (Table 3.11) were used.

Effect of storage time and product formulation on the phenolic composition and colour of rooibos iced teas

In this study the stability of rooibos iced tea prepared from the UR, FR and NEUR extract was investigated. The mass of rooibos extract used in each formulation is given in Chapter 3 (Table 3.2). In addition, two combination iced teas were investigated (a combination of FR and UR, as well as a combination of FR and NEUR). The mass of extract used in the two iced tea combinations (FR/UR and FR/NEUR) is indicated in Table 4.1. All iced tea combination×formulations contained 60.0 g/L sugar, formulations BC, BCA and NEC contained 1.2 g/L citric acid and formulation BCA contained an additional 0.2 g/L ascorbic acid.

Aliquots of the rooibos iced teas were sealed in new 20 mL gas chromatography headspace vials with aluminium crimp top caps, containing polytetrafluoroethylene/butyl (PTFE/butyl) rubber septa. These aliquots were heated (121°C for 2 min) in a gas operated laboratory-scale pressure cooker (Filery Health cooker, Filery,

England) in order to ensure microbial stability during storage. Due to the heating and cooling process, samples were in the pressure cooker for approximately 45 min. Samples were placed on ice directly after removal from the pressure cooker. Untreated samples were retained so as to evaluate the effect of the heat treatment on the iced tea. The heat treatment was replicated, independently, four times (i.e. tea of a specific formulation was heated on four separate occasions with fresh formulations prepared on each occasion). Aliquots were stored at -20ºC until analysis.

Storage procedure

The heat-treated iced tea samples were stored for three months. This is the shelf-life of the commercial product upon which the basic formulation of the experimental iced teas was based. The samples were stored at 25°C, in the dark. One sealed sample vial per formulation × replication removed from storage at six time intervals: week 0 (day of production); 1; 2; 4; 8 and 12. After removal, the samples were divided into sub-aliquots of 2 mL, which were stored at -20°C until analysis.

Table 4.1 The mass (g) of the three types of rooibos extract[a] used for the various formulations of one liter of the two combination rooibos iced teas

		Iced tea formulation		
Combination iced tea	**Extract**	**B[b]/NE[c]**	**BC[d]/NEC[e]**	**BCA[f]**
FR/UR	FR	0.875	0.875	0.875
	UR	0.875	0.875	0.875
FR/NEUR	FR	0.875	0.875	0.875
	NEUR	7.000 (1.050)[g]	7.000 (1.050)	-

[a]Fermented rooibos (FR); unfermented rooibos (UR) and nano emulsified unfermented rooibos (NEUR), [b]base (FR/UR extract in water), [c]NEUR extract in water, [d]base + citric acid, [e]NE + citric acid, [f]base + citric + ascorbic acid, [g]equivalent amount of UR extract in the added amount of NEUR extract. There is no NECA formulation for the NEUR iced tea as the extract inherently contains ascorbic acid.

pH determination of rooibos iced tea

The pH of the various iced tea solutions was determined as described in Chapter 3.

Monitoring browning of rooibos iced tea

Browning of the rooibos iced tea samples, as result of storage, was determined as an increase in absorbance at 420 nm, relative to the control. In each case, the control was represented by the samples directly after heating, but before storage. The procedure was as described in Chapter 3.

Determination of the total polyphenol content of rooibos iced tea

The polyphenol content of the iced tea samples was determined using the method by Singleton & Rossi (1965), scaled-down for a microplate reader. Gallic acid served as a standard. The procedure was described in Chapter 3.

HPLC quantification of the phenolic content of rooibos iced tea

Quantification of aspalathin, orientin and iso-orientin was performed by HPLC-DAD. The HPLC apparatus, column, gradient profile for separation, software, standards and sample preparation procedure were as described in Chapter 3.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) using SAS version 9.3 (SAS Institute, Cary, NC, USA) and analysed for normality using the Shapiro-Wilk test for normality ($P>0.05$). The Student t-test was used to ascertain whether there were significant differences between samples, within a particular extract type. The teas made from different extracts were analysed separately due to the large difference in phenolic make-up between the FR and UR extracts. Differences between formulations of the same extract type were lost when all the data were analysed together. Differences with a significance level of 5% ($P\leq0.05$) were considered significant (SAS, 2002).

RESULTS

Effect of storage time and product formulation on the phenolic composition and colour of rooibos iced teas

Prior to storage, the iced teas underwent a heat treatment, which resulted in changes in aspalathin, iso-orientin and orientin content. The absorbance of the iced teas as well as the TP content was also affected. The samples were analysed to confirm that the addition of citric and ascorbic acid, to rooibos iced tea, had a protective effect against changes occurring during heating, as was observed in Chapter 3. The changes for the respective teas (FR, UR and NEUR) and formulations (B, BC and BCA), as a result of heating, are summarised in ADDENDUM 5 (Tables A5.1-A5.3). As the effect of heating was discussed in detail in Chapter 3, no further attention will be given to this particular data in this chapter. The pH values of the iced teas, determined before heating and storage, are given in Table 4.2. The pH of the formulations containing citric acid (BC and BCA) was the lowest.

Storage induced marked changes in the absorbance as well as the TP, aspalathin, iso-orientin and orientin content of the iced teas. Actual changes are reported below, whilst the percentage loss in each case is summarised in ADDENDUM 6 (Tables A6.1 and A6.2).

Table 4.2 The pH values of the iced tea formulations before heating and storage

Formulation	Extract		
	FR[a]	UR[b]	NEUR[c]
B[d]/NE[e]	5.05±0.03	4.34±0.04	3.40±0.02
BC[f]/NEC[g]	2.94±0.02	2.80±0.02	2.80±0.02
BCA[h]	2.92±0.03	2.78±0.01	-

[a]Fermented rooibos, [b]unfermented rooibos, [c]nano emulsified unfermented rooibos, [d]base (FR/UR extract in deionised water), [e]NEUR extract in water, [f]base + citric acid, [g]NE + citric acid, [h]base + citric + ascorbic acid. There is no NECA formulation for the NEUR iced tea as the extract inherently contained ascorbic acid.

Browning

A small, yet significant ($P<0.05$), increase in brown colour (absorbance at 420 nm), over the 12 week storage period, was noted for formulation B of both FR and UR rooibos iced teas (Figs. 4.1a & b), although the increase observed for UR was less (Δ_{420} = 0.09 for UR and Δ_{420} = 0.17 for FR). On the other hand, the absorbance change of formulation BC (FR) was not significant over the same period, despite the fact that after two and four weeks the absorbance was significantly ($P<0.05$) increased. A gradual increase in the absorbance of UR iced tea, formulation BC was noted. The absorbance of formulation BCA of both FR and UR iced teas remained unchanged from weeks 4 to 12. Both showed an initial significant ($P<0.05$) decrease after one week, although in the case of UR iced tea the absorbance after one week was not significantly ($P\geq0.05$) different from that after two weeks.

The absorbance of both formulations of the NEUR iced tea increased slightly, but significantly ($P<0.05$) over the 12 week period (Figure 4.1c). In the case of formulation NE, the absorbance only reached significantly ($P<0.05$) higher values than the control after eight weeks. After two weeks, the NEC formulation had a significantly ($P<0.05$) higher absorbance than its control, remaining so for the remainder of the storage period. No significant ($P\geq0.05$) difference between NE and NEC was observed after 12 weeks of storage, although the absorbance of NEC was significantly ($P<0.05$) higher than that of NE at week 1, 2, 4 and 8.

Total polyphenols

The TP content of the FR iced teas generally changed little during storage, although significant ($P<0.05$) for reductions were observed for formulations BCA and B at weeks 2 and 12, respectively (Fig. 4.2a). Only the TP content of BCA of UR iced teas was affected by storage. After an initial significant ($P<0.05$) decrease at week 1, it remained unchanged until week 12 (Fig. 4.2b). In contrast, the TP content of both formulations of NEUR iced tea (NE and NEC) decreased significantly ($P<0.05$) during storage (Fig. 4.2c). Formulation NE, however, showed a significant ($P<0.05$) increase in TP content after two weeks, before again decreasing significantly ($P<0.05$).

Qualitative phenolic profile

Most notably, irrespective of formulation, there was a decrease in area of the peaks representing aspalathin, orientin and iso-orientin on chromatograms of FR, UR and NEUR iced teas. These changes are shown on the chromatogram of UR iced tea, formulation B, at 288 (Fig. 4.3a) and 350 nm (Fig. 4.3b). Other discrete peaks on the chromatograms of FR and UR iced teas were also smaller and the ill-defined area representing polymeric substances (2 on chromatogram, Figs. 4.3a, b) was unexpectedly lower after storage. The decrease was not as apparent for NEUR iced teas (ADDENDUM 6, Figs. A6.1a & b). In the case of formulation BC and BCA (FR and UR iced teas), as well as formulation NE and NEC (NEUR iced tea), an additional observation was made, namely the area of the peak with a retention time of ~2.8 min (288 nm) enlarged as a result of storage. The latter change is indicated on the chromatogram of UR iced tea, formulation BCA (ADDENDUM 6, Fig. A6.2a). The chromatogram at 350 nm is also shown (ADDENDUM 6, Fig. A6.2b).

Aspalathin

The change in the aspalathin content of FR iced tea, as a result of storage, was significant ($P<0.05$) for formulation B, BC and BCA (Fig. 4.4a). After eight weeks of storage, no aspalathin was detected in formulation B, whilst its content in BC and BCA was significantly ($P<0.05$) reduced by 39% and 25%, respectively, over the 12 week storage period. The rate of change in aspalathin content (%/week) was also greatest for formulation B (ADDENDUM 6, Table A6.4). Formulation BCA thus retained aspalathin the best during storage.

The UR iced teas also experienced a significant ($P<0.05$) decrease in aspalathin content as a result of storage (Fig. 4.4b). Formulation BC underwent the largest overall change, losing 33.4% of its initial aspalathin content. The rate of decrease was also highest in this formulation (ADDENDUM 6, Table A6.4). Aspalathin loss due to storage was 23.3% and 22.1% for formulations B and BCA, respectively.

The iced tea containing NEUR extract showed very good retention of aspalathin during storage (Fig. 4.4c). Formulation (i.e. citric acid) had no effect, with NE and NEC respectively losing *ca*. 13% and 12% of their aspalathin content during the entire storage period. The rate of aspalathin degradation was also similar in these two formulations (ADDENDUM 6, Table A6.4).

Iso-orientin

The iso-orientin content of FR iced tea was significantly ($P<0.05$) reduced during the 12 week storage period (Fig. 4.5a). Formulation B experienced a significant ($P<0.05$) 20.1% loss whilst formulations BC and BCA were also significantly ($P<0.05$) reduced by 14.5% and 15.7%, respectively. The iso-orientin content of the UR iced tea showed a distinct decrease after one week of storage (although not significant, $P\geq0.05$) followed by a sharp, significant ($P<0.05$) increase, with values of each formulation (at weeks 2 and 4) not differing significantly ($P<0.05$) (Fig. 4.5b). Formulations B and BC had similar, but lower orientin contents than formulation BCA after 12 weeks of storage.

The lowest iso-orientin losses were observed for iced tea containing NEUR extract (Fig. 4.5c). Overall the iso-orientin content decreased as a result of storage, but this decrease was not significant ($P \geq 0.05$) in the case of formulation NEC. Significant ($P < 0.05$) increases in the iso-orientin content of both formulations was noted after weeks 2 and 4, as well as after 12 weeks (compared to week 8).

Orientin

The orientin content of all three formulations of FR iced tea was significantly ($P < 0.05$) lower after the 12 week storage period (Fig. 4.6a), leading to overall losses of 3.1%, 3.3% and 5.6% in formulations B, BC and BCA. Both BC and BCA had the same orientin content after 12 weeks, although the content of BCA at week 0 was higher than that of BC. A significant ($P < 0.05$) increase in orientin content was noted for all formulations after two weeks. The increase from week 1 to week 2 was, however, only significant ($P < 0.05$) in the case of formulation BC. The orientin content of formulation B did, however, increase significantly ($P < 0.05$) from week 4 to week 8.

There were no significant ($P \geq 0.05$) losses of orientin in any of the formulations of the UR (Fig. 4.6b) or NEUR iced teas over the 12 week storage period (week 0 vs. week 12) (Fig. 4.6c). However, during this period, significant ($P < 0.05$) changes occurred that were more pronounced in the three formulations of UR iced tea. The trends observed for iso-orientin in UR and NEUR iced teas were also observed for orientin. For all three formulations of UR iced tea as well as the two NEUR iced teas, the increase in orientin content from weeks 8 to 12 was significant ($P < 0.05$).

Effect of storage time and product formulation on the phenolic composition and colour of rooibos iced tea combinations

Prior to storage, the iced teas underwent a heat treatment, which resulted in changes in aspalathin, iso-orientin and orientin content. The absorbance (420 nm) of the teas was also affected. The changes for the respective iced teas (FR/UR and FR/NEUR) and formulations (B, BC, BCA and NE, NEC), as a result of the heat treatment prior to storage, are summarised in ADDENDUM 5 (Tables A5.4 and A5.5).

The chromatograms for FR/UR and FR/NEUR iced teas exhibited losses of aspalathin (ADDENDUM 6, Figs. 4.3a and 4.4a, respectively), iso-orientin and orientin (ADDENDUM 6, Figs. A6.3b and A6.4b, respectively). Unlike the “pure” iced tea formulations, however, no large reduction in the area of the polymeric compounds was evident after the 12 week storage period. An increase in the area of the compound eluting at ~2.8 min was also observed. The increase was more easily visible for FR/UR than FR/NEUR iced teas (ADDENDUM 6, Fig. A6.3).

The changes (%) in absorbance (420 nm), aspalathin, iso-orientin and orientin content, occurring as a result of storage, are given in ADDENDUM 6 (Table A6.3). The pH values of the various formulations, before heat treatment, are given in Table 4.3. The aspalathin, iso-orientin and orientin contribution of the respective extracts (FR, UR and NEUR extract) to the overall aspalathin, iso-orientin and orientin content of the

combination iced teas is given in Table 4.4. Based on the values in Table 3.11 (Chapter 3), the iron content of the FR/UR iced tea was 113.575 mg/kg combined extract whilst the FR/NEUR iced tea was 73.525 mg/kg combined extract. These values were greater than those for UR and NEUR, respectively (Chapter 3, Table 3.11).

Table 4.3 The pH values of the combination iced tea formulations that were stored at 25°C

	Combination iced tea	
Formulation	**FR[a]/UR[b]**	**FR/NEUR[c]**
B[d]/NE[e]	4.56±0.02	3.61±0.05
BC[f]/NEC[g]	2.82±0.01	2.84±0.03
BCA[h]	2.83±0.02	-

[a]Fermented rooibos, [b]unfermented rooibos, [c]nano emulsified unfermented rooibos, [d]base (FR and UR extract in water), [e]FR and NEUR extract in deionised water, [f]base + citric acid, [g]NE + citric acid, [h]base + citric + ascorbic acid. There is no NECA formulation since the NEUR extract inherently contained ascorbic acid.

Table 4.4 Contribution (%) of the UR[a], FR[b] and NEUR[c] extracts to the aspalathin, iso-orientin, orientin and iron content of the iced tea combinations stored at 25°C

Extract	**Mass**	**Contribution[d] to FR/UR iced tea**				**Contribution to FR/NEUR iced tea**			
	(g/L)	Asp[e]	Iso[f]	Ori[g]	Iron[h]	Asp	Iso	Ori	Iron
UR	0.875	95.79[i]	70.74	56.48	41.49	-	-	-	-
FR	0.875	4.21	29.26	43.52	58.58	3.56	32.65	43.33	90.38
NEUR	7.000	-	-	-	-	96.44	67.35	56.67	9.62

[a]Fermented rooibos, [b]unfermented rooibos, [c]nano emulsified unfermented rooibos, [d]contribution based on values for aspalathin, iso-orientin and orientin of the extracts characterised in Chapter 3 (Table 3.11), [e]aspalathin, [f]iso-orientin, [g]orientin, [h]based on values of the extracts characterised in Chapter 3, [i]results reported as %.

Browning

A slight, yet significant ($P<0.05$) increase in absorbance was observed for formulation B and BC of the FR/UR combination iced teas (Fig. 4.7a), whilst BCA decreased. The absorbance of all three formulations of FR/UR iced teas increased significantly ($P<0.05$) over the 12 week storage period, with the absorbance of formulation B remaining the highest, throughout. A very small, but significant ($P<0.05$) increase in absorbance of both formulations of the FR/NEUR combination iced tea was observed after the 12 week storage period (Fig. 4.7b). The absorbance of formulation NE and NEC were similar between week 0 and 4, with the absorbance of NE being greater than that of NEC between weeks 8 and 12.

Aspalathin

A significant ($P<0.05$) decrease in the aspalathin content of all formulations of the FR/UR combination iced teas was observed (Fig. 4.8a). Losses were 51.4%, 35.1% and 20.2% (ADDENDUM 6, Table A6.3) for formulation B, BC and BCA, respectively. The rate of aspalathin degradation was more rapid in formulation B, compared to BC and BCA (ADDENDUM 6, Table A6.5), and it was also faster than that of formulation B of the UR iced tea (ADDENDUM 6, Table A6.4)

After 12 weeks of storage, the aspalathin content of both formulations of the FR/NEUR combination iced teas had decreased significantly ($P<0.05$) (Fig. 4.8b). Aspalathin losses were 19.4% and 15.0% for formulation NE and NEC, respectively.

Iso-orientin

The changes in iso-orientin content of the combination iced teas, FR/UR and FR/NEUR, were different from those observed in the "pure" iced teas: UR and NEUR, respectively. A sharp, significant ($P<0.05$) decrease in iso-orientin content was observed for all formulations of the FR/UR combination iced teas (Fig. 4.9a), reaching the lowest values at week 4 of storage, after which the iso-orientin content increased slightly, but significantly ($P<0.05$). The iso-orientin content of all three formulations of the FR/UR combination iced teas was, however, significantly ($P<0.05$) reduced by the storage treatment (compared to content at week 0). The iso-orientin content of both formulations of FR/NEUR iced tea (Fig. 4.9b) was lowest at weeks 2 and 4 of storage, after which it increased significantly ($P<0.05$) (until week 12). The overall iso-orientin content of both these formulations was significantly ($P<0.05$) lower at the end of storage (after 12 weeks) compared to the initial content.

Orientin

Similar trends compared to iso-orientin were observed (Fig. 4.10). Formulation affected the change in orientin content of the FR/UR combination iced teas during the first four weeks of storage (Fig. 4.10a). Iso-orientin was progressively less stable in formulations B, BC and BCA during this period. The orientin content of formulation B remained stable for two weeks, followed by sharp, significant ($P<0.05$) decrease between weeks 2 and 4. Formulation BC remained stable for week 1, followed by a slight, but significant ($P<0.05$) decrease between weeks 1 and 2, and a sharp, significant ($P<0.05$) decrease between weeks 2 and 4. Formulation BCA also remained stable only for week 1, but then deceased significantly ($P<0.05$) between weeks 1 and 2, followed by a more gradual decrease between weeks 2 and 4. From week 4 onwards (until week 12), the orientin content of all three formulations increased significantly ($P<0.05$). The final (week 12) orientin content of all three formulations was, however, significantly ($P<0.05$) lower than that of the control. Only the orientin content of the NEC formulation of the FR/NEUR iced tea increased significantly ($P<0.05$) between weeks 0 and 1 (Fig. 4.10b). For both formulations NE and NEC, the orientin content decreased significantly ($P<0.05$) between

weeks 1 and 2 and then increased significantly ($P<0.05$) from week 2 onwards (with the exception of formulation NEC between weeks 2 and 4).

DISCUSSION

In the normal course of processing, iced tea is stored and losses of phenolic compounds can be expected, in addition to those losses resulting from heat treatments such as pasteurisation and sterilisation. In Chapter 3, the latter aspect was investigated. Prior to storage, the iced teas were subjected to sterilisation, to ensure microbiological stability over the three month storage period. This was especially important in the case of formulation B, due to its pH (pH 5 for FR iced tea and pH 4.3 for UR iced tea). By subjecting the samples to heat treatment before storage, degradation of the phenolic compounds was initiated, which continued throughout storage. The storage stability of the experimental iced teas was evaluated using the same parameters as in Chapter 3: absorbance at 420 nm (brown colour); TP content; qualitative phenolic profile and the aspalathin, iso-orientin and orientin content.

In general, increased browning (absorbance) of the iced teas was observed with increased storage duration. Oxidation of phenolic compounds such as aspalathin is thought to be responsible for this observation since Koeppen & Roux (1965; 1966), as well as Krafczyk & Glomb (2008), noted that the oxidation of aspalathin eventually resulted in the formation of uncharacterized, brown material. Joubert (1996) also linked browning of rooibos plant material with the oxidation/loss of aspalathin. The oxidation and polymerisation of flavonoids typically results in increased absorbance at 420 nm (Cilliers & Singleton, 1990; Cheynier & Moutounet, 1992; Li *et al*., 2008). The largest storage-induced absorbance increases were observed for formulation B of both the FR and UR iced teas. In support of these changes occurring as a result of aspalathin oxidation, it is worth mentioning that the increased absorbance observed between week 1 and 2 coincided with maximal aspalathin losses during the same period. Furthermore, the formation of iso-orientin and orientin, oxidation products of aspalathin (Krafczyk & Glomb, 2008), was observed during the same period. Although the absorbance of formulation BC (FR iced tea) was not significantly different after 12 weeks of storage (week 0 vs. week 12), a sharp increase in the absorbance of this iced tea was also observed between week 1 and 2. This corresponded to the period of maximal aspalathin degradation.

In the case of formulation BCA (FR iced tea), absorbance behaviour was contrary to expectation. Although the aspalathin content decreased (less than for B and BC), absorbance also decreased, suggesting that other factors than those prevalent for formulation B and BC were of importance. The lower pH, having a decolourising effect (Robertson, 1983; Gupta, 1989) and/or the reduction of quinones in the tea to *o*-dihydroxyphenols by ascorbic acid could be contributing factors. Ascorbic acid would have reversed some of the effects of the oxidation of rooibos and prevented or slowed down further oxidation as well as polymerisation of phenolic compounds in the tea. A similar argument applies to formulation BCA of the UR iced, where no significant change in absorbance was observed over the 12 week period.

When combined with FR extract, the absorbance increase in the UR iced tea (i.e. FR/UR) was smaller. This is most likely as a result of the lower aspalathin content of FR/UR iced tea compared to UR iced tea. A reduced number of oxidation products can be expected to form as a result of the reduced aspalathin content.

As with the FR and UR iced teas, the overall increase in absorbance of both formulations of the NEUR iced tea can be linked to aspalathin degradation. Although the increase (%) in absorbance for both formulations over the 12 week storage period was low, the increase (%) for formulation NE, containing no citric acid, was slightly higher. This was accompanied by aspalathin degradation. Since the changes in iso-orientin and orientin were very small, their contribution would be slight. The higher pH value of formulation NE compared to NEC could be a factor (Guyot *et al*., 2007).

When combined with FR extract, absorbance increases for formulation NEC were smaller compared to those for "pure" NEUR iced tea. Again, the lower aspalathin content of the FR extract, compared to that of the UR extract, is thought to play a role in the smaller absorbance changes observed for FR/NEUR compared to NEUR alone. In the case of both FR/UR and FR/NEUR iced teas, the smaller absorbance changes were also linked to smaller changes in the area of the polymeric material on the chromatograms.

Oxidation (of compounds like aspalathin) and the formation of large, insoluble polymers during storage can be expected to be accompanied by a gradual reduction in the polymers remaining in solution in the iced tea samples. This complicates observations in terms of type of extract × formulation combinations as large polymers are expected to precipitate (Oszmiański *et al*., 2008), and to be removed from the solution (centrifuged or filtered prior to colour measurement and HPLC analysis, respectively). Such an observation was indeed made, which manifested as a decrease in the area of the polymeric compounds (13.5-16.5 min) on the chromatograms of FR and UR iced tea samples. According to Peng *et al*. (2001), the broad UV-absorbing peak, eluting late in the chromatogram (reversed phase column) may be associated with polymeric polyphenolic compounds. The fact that the absorbance, with the exception of formulation BCA of the FR iced tea, did not decrease concommitantly may be explained by the presence and formation of smaller polymers (Le Guernevé *et al*., 2004). Small polymers (such as oligomers), formed as a result of the oxidation of flavonoids, will contribute to increased absorbance at 420 nm but not to the polymer peak on the HPLC chromatogram. Polymeric compounds have been shown to form from chalcones and other (unidentified) flavonoids (Gujer *et al.*, 1986).

In the case of the NEUR iced teas, the lack of large changes in the area of the polymeric material may be attributed to the nature of the NEUR extract. The ascorbic acid present in this extract most likely protected the rooibos flavonoids from extensive oxidation, and thus polymerisation. In the nano capsules, the tea extract is completely surrounded by a layer of ascorbic acid and emulsifier, thus acting as a barrier between the tea extract and e.g. oxygen (Anon., 2007).

Considering the combination iced teas, a smaller decrease in the area of the polymeric compounds was observed compared to the "pure" iced teas. In the case of FR/UR, this may be explained by the smaller amount of phenolics (mostly aspalathin) available for oxidation (and the formation of "extra" large polymers), compared

to UR iced teas. In the case of FR/NEUR iced teas, however, the addition of the NEUR extract to FR iced tea is most likely responsible, since the NEUR iced teas showed very little change in polymeric area.

The increase in the area of the compound with a retention time of ~2.8 min (288 nm), in most iced teas, could most likely be attributed to oxidative changes, although the identity of the compound is not known. Considering the losses of aspalathin, the prevalent rooibos flavonoid in the UR iced teas, it may thus be speculated that this compound is an oxidation product of aspalathin. Support for this postulation may be found in the more apparent increase in the peak area of this compound observed for the UR and NEUR iced teas. Oszmiański & Lee (1991) and Guyot *et al*. (2007) have shown that storage of phloridzin results in the formation of oxidation products. In the study by Guyot *et al*. (2007), the concentration of a yellow oxidation product of phloridzin was shown to increase between 5 and 31 h of storage at 30ºC.

Based on the findings by Koeppen & Roux (1965) and Krafczyk & Glomb (2008), a decrease in the aspalathin, iso-orientin and orientin content (to a lesser extent) of the iced teas, as a result of storage, was expected. [The observations made by Krafczyk & Glomb (2008), however, were made at high pH values, dissimilar to the relatively low pH conditions of the iced teas.] Similarly, the phloridzin (a dihydrochalcone, like aspalathin) content of apple juice concentrates has also been reported to decrease by up to 60% during storage (Spanos *et al*., 1990; Oszmiański *et al*., 2008). On the other hand, Tomás-Barberán *et al*. (1995) showed that neohesperidin dihydrochalcone, used as a sweetener in blackcurrant jams (pH 3.08), did not undergo significant degradation after 18 months of storage at room temperature. Montijano *et al*. (1997) detected no change in the neohesperidin dihydrochalcone levels of a lemonade beverage (pH 3.3) stored for one year at room temperature (or for three months at 40ºC). These findings would suggest that the use of a low pH may be beneficial for preservation of dihydrochalcones during storage. Due to structural differences between aspalathin and the aforementioned dihydrochalcones, however, differences in storage stability may be expected. For example, loss of other phenolic compounds such as quercetin (Häkkinnen *et al*., 2000) and quercetin-3-glucoside (Zafrilla *et al*., 2001) are common during storage.

The role of pH was not very clear in the case of the UR iced teas, since unexpectedly, aspalathin was less stable in formulation BC than in B, with the former having a lower pH. Similar findings with respect to the effect of citric acid on green tea catechins have been reported (Chen *et al*., 2001). This is contrary to many reports indicating that flavonoid stability is greater under more acidic pH conditions (Canales *et al*., 1993; Coiffard *et al*., 1998; Friedman & Jürgens, 2000; Lemańska *et al*., 2001). In the case of formulations B and BC of FR iced tea, however, lower pH supported greater stability of aspalathin.

Ascorbic acid has been shown to assist in the retention of phenolic compounds in general (Chen *et al*., 1998; Chen *et al*., 2001), as well as during storage (Aoshima & Ayabe, 2007; Yen *et al*., 2008). Over the 12 week storage period, aspalathin loss in both the FR and UR iced teas was lowest in formulation BCA, indicating that the addition of ascorbic acid is beneficial for the preservation of this phenolic compound in iced tea. Oszmiański *et al*. (2008) reported similar findings for the preservation of phloridzin in apple pulp. The initial heat treatment to which the iced teas were exposed, reduced the aspalathin content of formulation B further than

that of BC or BCA. However, the abscence of aspalathin in formulation B of the FR iced tea, from week 8 onwards, clearly illustrates the importance of ingredients such as citric and ascorbic acid. Without these ingredients, FR iced teas may not achieve a 12 week shelf life in terms of aspalathin content (if same quality and quantity of ingredients are used). On the other hand, the initial reduction in aspalathin content, as a result of heat treatment, is likely to be significantly less pronounced in commercial products.

Overall, the loss (%) of iso-orientin and orientin was less than that of aspalathin in all the iced teas (FR, UR and NEUR). Flavones are less susceptible to oxidation than dihydrochalcones (Dziedzic *et al*, 1985). Significant increases in the iso-orientin and orientin content of FR and UR iced teas (weeks 1 and 2 as well as weeks 8 and 12), were accompanied by large aspalathin losses, as well as increased absorbance at 420 nm. The latter findings may be explained in terms of the findings of Krafczyk & Glomb (2008), showing that iso-orientin can form as a result of the oxidation of aspalathin and that orientin may subsequently form from iso-orientin. This work was, however, conducted on a pure aspalathin solution, over a much shorter period of time (48 h), at pH 7.4.

Iso-orientin appeared to be more stable in UR iced teas compared to the FR iced teas, as overall losses (%) of this compound were higher in the latter. Since the UR iced teas contain more aspalathin than the FR iced teas, it is possible that more extensive iso-orientin supplementation, via the oxidation of aspalathin, occurred in the former. Overall, orientin losses in FR and UR iced teas were generally lower than those for iso-orientin, indicating greater stability of the former. The apparent increased stability of orientin may also, in part, be as a result of the formation of the latter from iso-orientin. This process has been shown to occur in alcoholic solution (Koeppen *et al*., 1962), as well as in phosphate buffered solution (Krafczyk & Glomb, 2008). Throughout storage, the orientin content of formulation B remained highest. Since aspalathin loss was greatest in this formulation, leading to the formation of iso-orientin and subsequently orientin, it may explain the apparent stability of orientin.

The storage stability of the NEUR iced teas was superior compared to FR and UR iced teas, as losses of aspalathin, iso-orientin and orientin were generally lower in the former. The protective effect of both micro- (Gouin, 2004; Forssell *et al*., 2006; Lucas-Abellán *et al*., 2008) and nano encapsulation (Morris, 2006; Weiss *et al*., 2006) has been demonstrated for numerous compounds. In the NEUR iced teas, the high (*ca*. 5%) ascorbic acid content of the extract also likely played a role, acting as a barrier which protected the UR tea flavonoids from oxidation. As with the FR and UR iced teas, aspalathin degradation did occur and could be linked to the formation of iso-orientin and orientin between week 1 and 2 as well as between week 8 and 12, although absorbance changes in these regions were not significant. This may be attributed to the overall smaller losses of aspalathin (greater stability) in NEUR iced tea.

Combining the FR and UR extracts (i.e. FR/UR iced tea) appears to accelerate loss of phenolic compounds. This may be due to the increased iron content of the combination iced teas compared to their UR counterparts. (The FR extract served as an additional source of iron in the combination iced teas). Iron plays an important role in hydroxyl radical generation and prooxidant activity, via the Fenton reaction (Gutteridge &

Halliwell, 1994). Such prooxidant activity has been linked to the loss of flavonoids (Valcic *et al*., 2000; Sang *et al*., 2003). Interestingly, the combination iced tea did not exhibit the same increase in iso-orientin and orientin content between week 1 and 2 as the "pure" FR and UR iced teas. Instead, a dramatic decline in these compounds was observed over this period. The iso-orientin and orientin content of the FR/UR combination iced teas did, however, increase between weeks 4 and 12 of storage. The initial rate of iso-orientin and orientin degradation in the combination iced teas presumably exceeded that at which aspalathin oxidation could supplement it, until week 4. Generally, changes in FR/NEUR iced tea were small, and similar to those observed in the "pure" NEUR iced teas.

The TP content of the iced teas was considered indicative of the antioxidant activity (Prior *et al*., 2005; Stratil *et al*., 2007). Numerous studies link the antioxidant activity (DPPH or ABTS assays) of a range of plant extracts with their TP content (Mello *et al*., 2005; Kiselova *et al*., 2006). With the exception of formulation BCA (FR and UR iced teas), the TP content of the iced teas generally remained unchanged during storage. This was contrary to expectation since, during storage, a decrease in the TP content of other polyphenol containing foods and beverages has been observed (Morelló *et al*., 2004; Recamales *et al*., 2006; Gómez-Alonso, *et al*., 2007; Kevers *et al*., 2007). The decrease in aspalathin, iso-orientin and orientin may have been counteracted by increases in oxidation products of these phenolics, such as polymers, which will also react in the TP assay. The decrease in the TP content of formulation BCA of both the FR and UR iced teas after the 12 week storage period can most likely be ascribed to a loss of ascorbic acid (Shui *et al*., 2004; Aaby *et al*., 2007; Kabasakalis *et al*., 2000). Ascorbic acid is known to contribute to the apparent "TP" content of food and beverage products (Vinson *et al*., 2001). The total polyphenol content of orange juice has been found to decrease upon storage due to a loss of ascorbic acid (Klimczak *et al*., 2007). The reduced TP content of both formulations of the nano emulsified iced tea is thus likely to have similar origins, as it contains a significant amount of ascorbic acid. The absence of ascorbic acid in formulation BC of FR and UR ice teas would explain the stability of its TP content.

Oxidation of phenolics and possibly the formation of large polymeric compounds with lesser reducing capacity (Pokorny, 1987; Manzocco *et al*., 1998; Turker *et al*., 2004; Yamada *et al*., 2007) are most likely responsible for the reduced TP content of formulation B of the FR iced tea, after week 8. Furthermore, precipitation (removal from test solution) of extremely large polymers (Bengoechea *et al*., 1997; Oszmiański *et al*., 2008) could also have reduced the TP content of the iced tea.

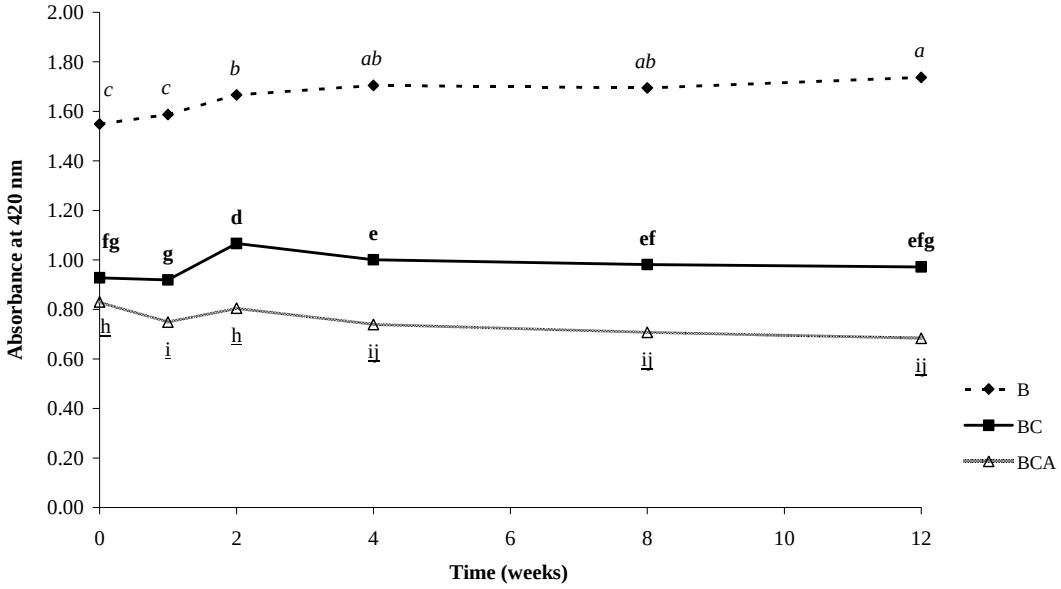
CONCLUSION

In this study, it was shown that although storage plays an important role in the final phenolic quality of rooibos iced tea, commercial products containing both citric and ascorbic acid and stored for three months could be expected to retain *ca*. 75-88% of their aspalathin content, depending upon the type of rooibos extract used. Even greater stability can be expected for iso-orientin and orientin. Products containing citric acid alone could be expected to perform marginally less well.

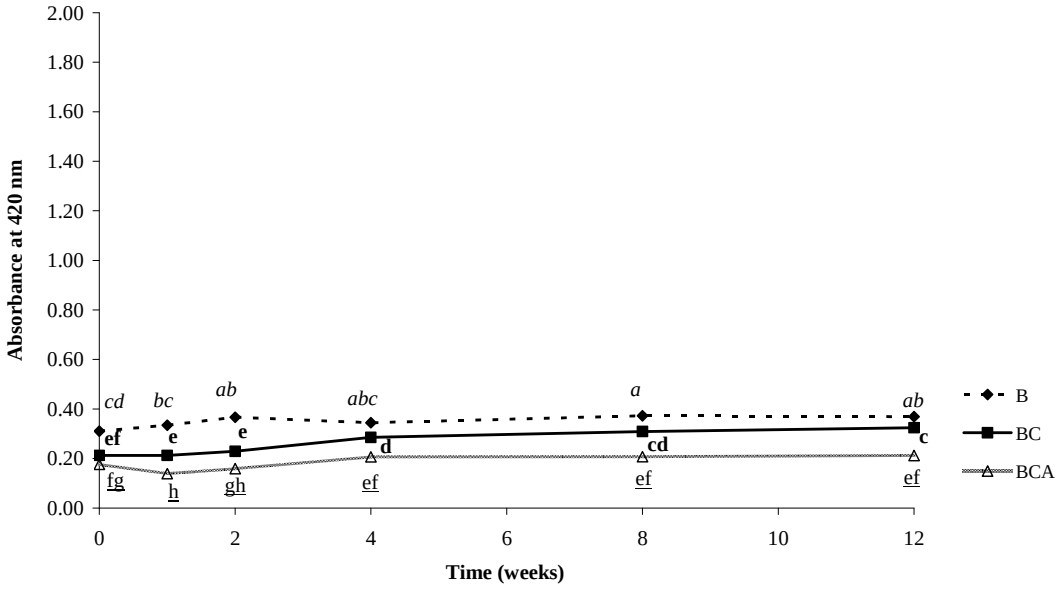
The results in this study confirm that it would not be unreasonable to expect commercial rooibos iced teas, sold before their expiry date (3 month shelf life) to contain aspalathin, iso-orientin and orientin. Furthermore, these results indicate that that the apparent poor phenolic quality (not equivalent to a cup of brewed rooibos) of the tested commercial rooibos iced teas is most likely attributable to the use of insufficient quantities of rooibos extract or the use of poor quality extracts.

The data in this study indicate that iso-orientin and orientin may indeed form as a result of aspalathin degradation, not only in pure solution, but also in rooibos products. Further research with respect to the formation and conversion of the intermediate products of aspalathin oxidation (flavanones such as dihydro-iso-orientin and dihydro-orientin) during storage is required.

a)


Absorbance at 420 nm
Time (weeks)
B
BC
BCA
c c b ab ab a
fg g d e ef efg
h i h ij ij ij

b)


Absorbance at 420 nm
Time (weeks)
B
BC
BCA
cd bc ab abc a ab
ef e e d cd c
fg h gh ef ef ef

c)

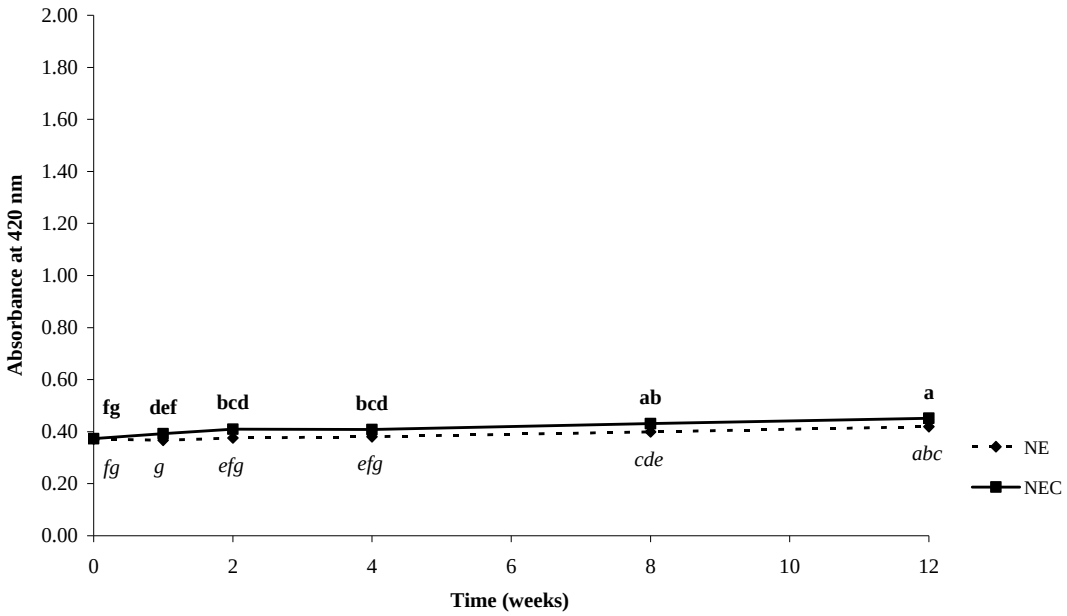
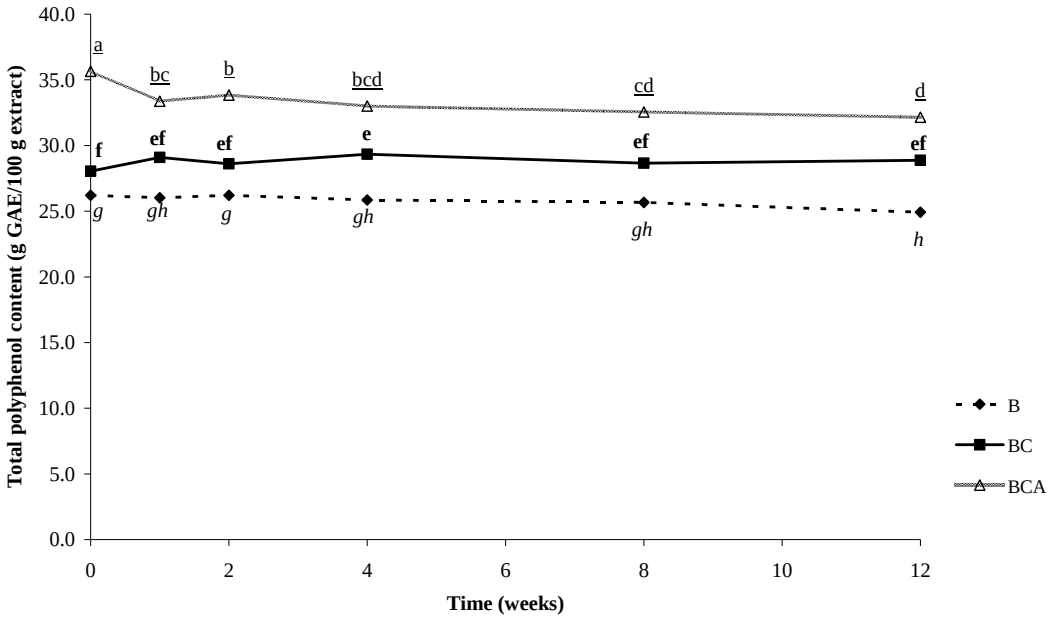
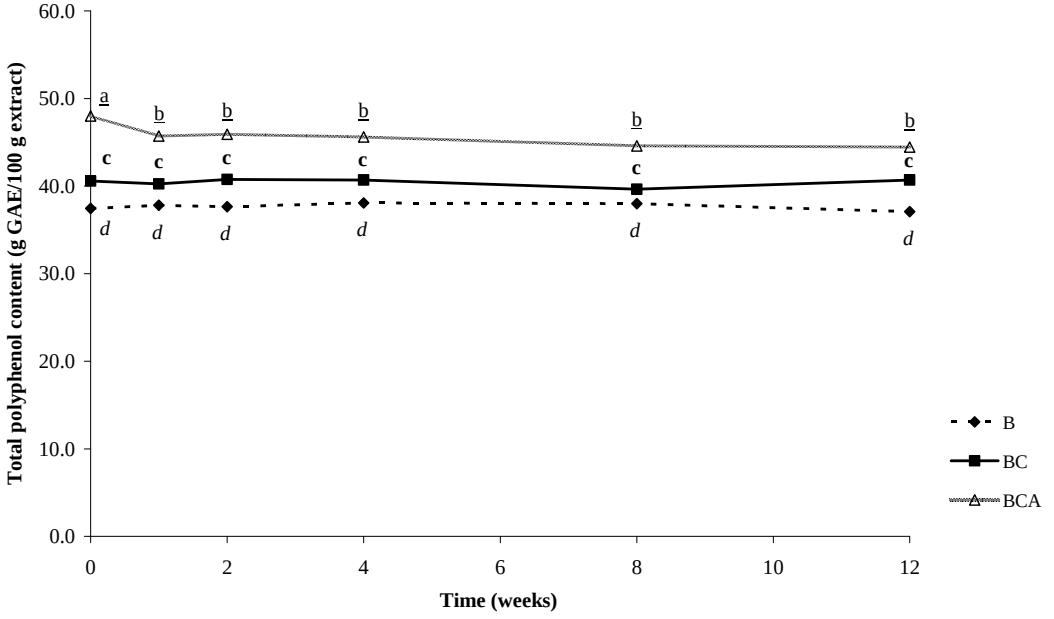


Figure 4.1 The effect of formulation and temperature controlled storage on the absorbance (420 nm) of (a) fermented rooibos (b) unfermented rooibos and (c) nano emulsified unfermented rooibos iced tea. Formulation B = base (rooibos extract in deionised water), BC = base + citric acid, BCA = base + citric + ascorbic acid, NE = nano emulsified unfermented rooibos (NEUR) extract in deionised water, NEC = NE + citric acid. There is no NECA formulation for the NEUR extract as the extract inherently contained ascorbic acid. Means, represented by points, labelled with different alphabetical letters differ significantly ($P<0.05$). The data labels for the base formulation (also NE) are shown in italics whilst those for base + citric acid (also NEC) are shown in bold. The data labels for the base + citric + ascorbic acid formulation are underlined.

a)


Total polyphenol content (g GAE/100 g extract)
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
0
2
4
6
8
10
12
Time (weeks)
a
bc
b
bcd
cd
d
f
ef
ef
e
ef
ef
g
gh
g
gh
gh
h
B
BC
BCA

b)


Total polyphenol content (g GAE/100 g extract)
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0
2
4
6
8
10
12
Time (weeks)
a
b
b
b
b
b
c
c
c
c
c
c
d
d
d
d
d
d
B
BC
BCA

c)

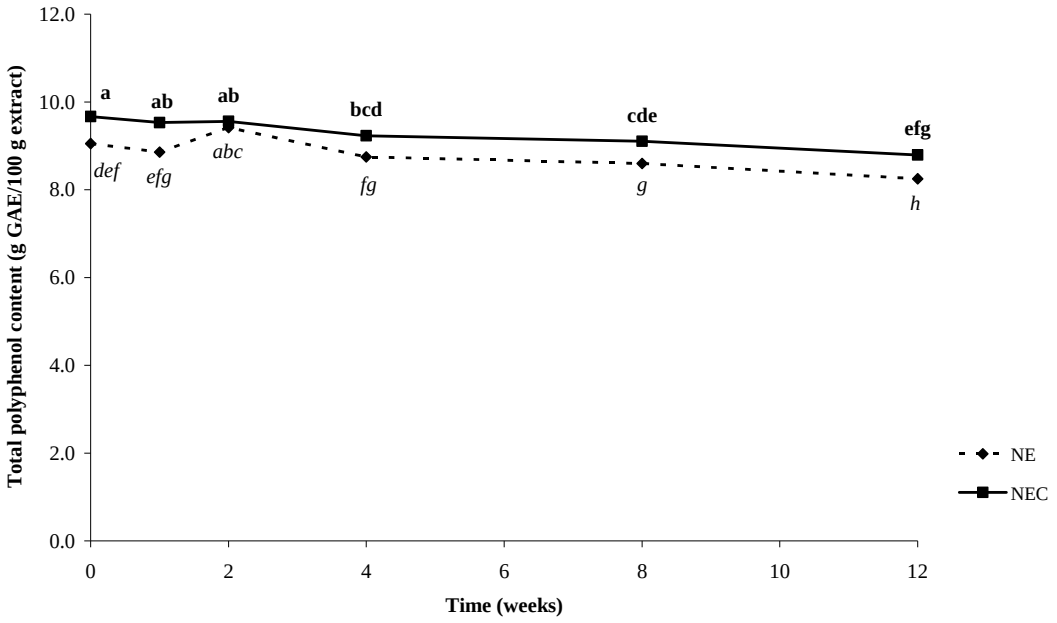
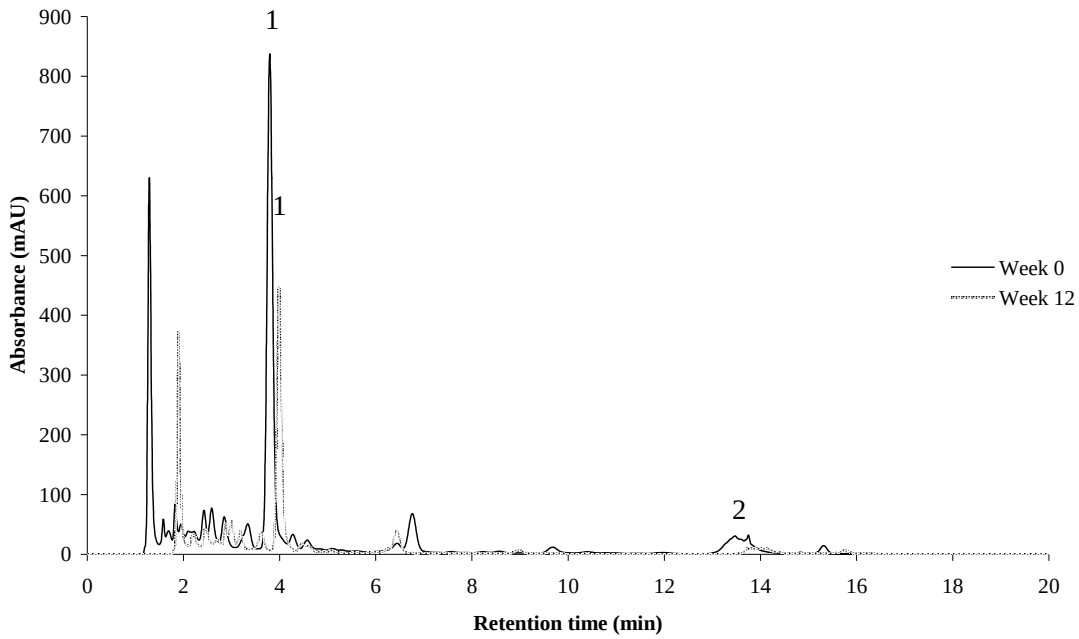


Figure 4.2 The effect of formulation and temperature controlled storage on the total polyphenol content of (a) fermented, (b) unfermented and (c) nano emulsified unfermented rooibos iced tea. Formulation B = base (rooibos extract in deionised water), BC = base + citric acid, BCA = base + citric + ascorbic acid, NE = nano emulsified unfermented rooibos (NEUR) extract in deionised water, NEC = NE + citric acid. There is no NECA formulation for the NEUR extract as the extract inherently contained ascorbic acid. Means, represented by points, labelled with different alphabetical letters differ significantly ($P<0.05$). Total polyphenol content measured in GAE = gallic acid equivalents. The data labels for the base formulation (also NE) are shown in italics whilst those for base + citric acid (also NEC) are shown in bold. The data labels for the base + citric + ascorbic acid formulation are underlined.

a)



b)

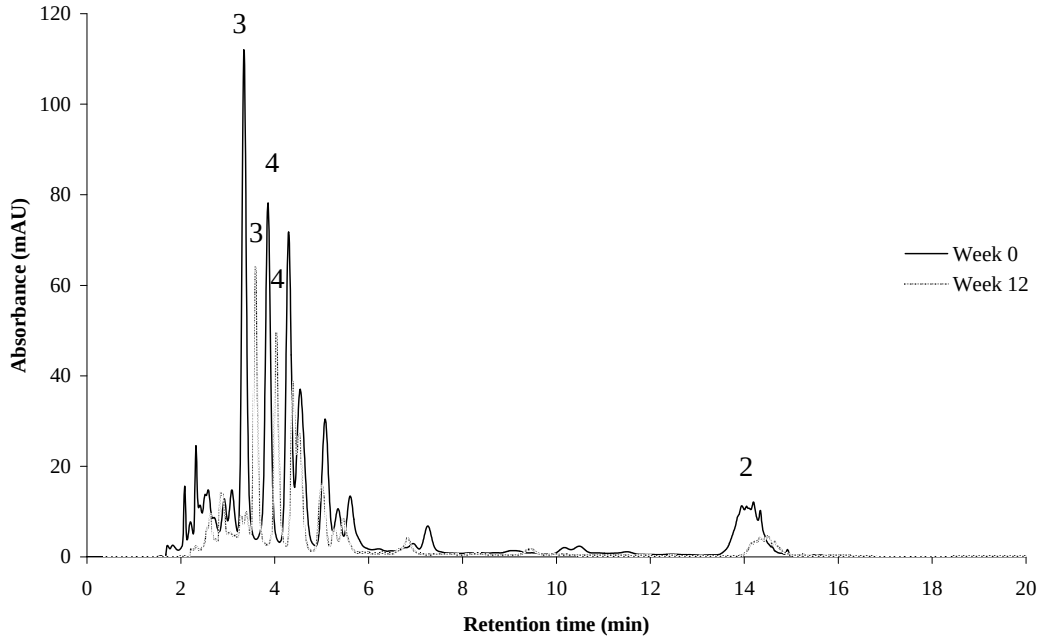
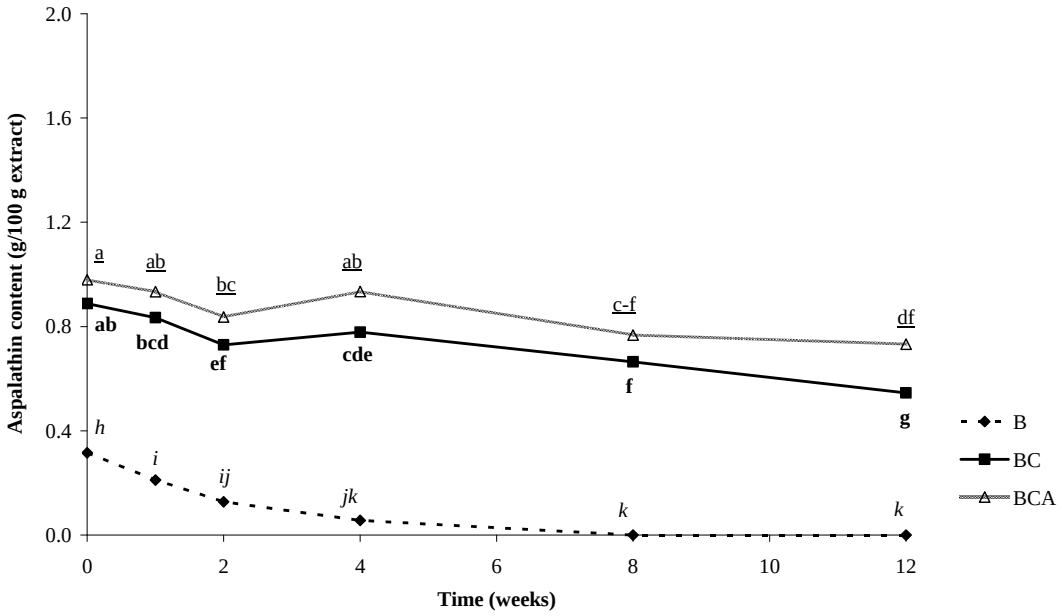
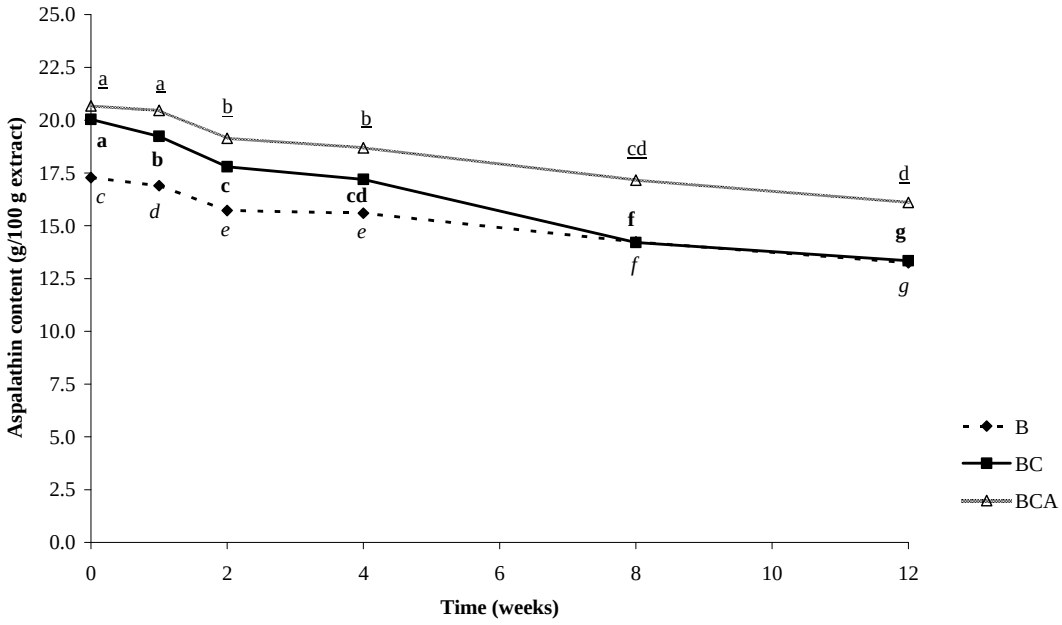


Figure 4.3 Chromatograms of unfermented rooibos iced tea (formulation B) at (a) 288 nm and (b) 350 nm. The change in the chromatographic profile from week 0 to week 12 is illustrated. Injection volume was 5 µL. Indicated on the relevant chromatograms are (1) aspalathin, (2) polymeric material, (3) iso-orientin and (4) orientin. The x-axis of the week 12 chromatogram was slightly offset to enable visual comparison with the control.

a)


Aspalathin content (g/100 g extract)
Time (weeks)
B
BC
BCA

b)


Aspalathin content (g/100 g extract)
Time (weeks)
B
BC
BCA

c)

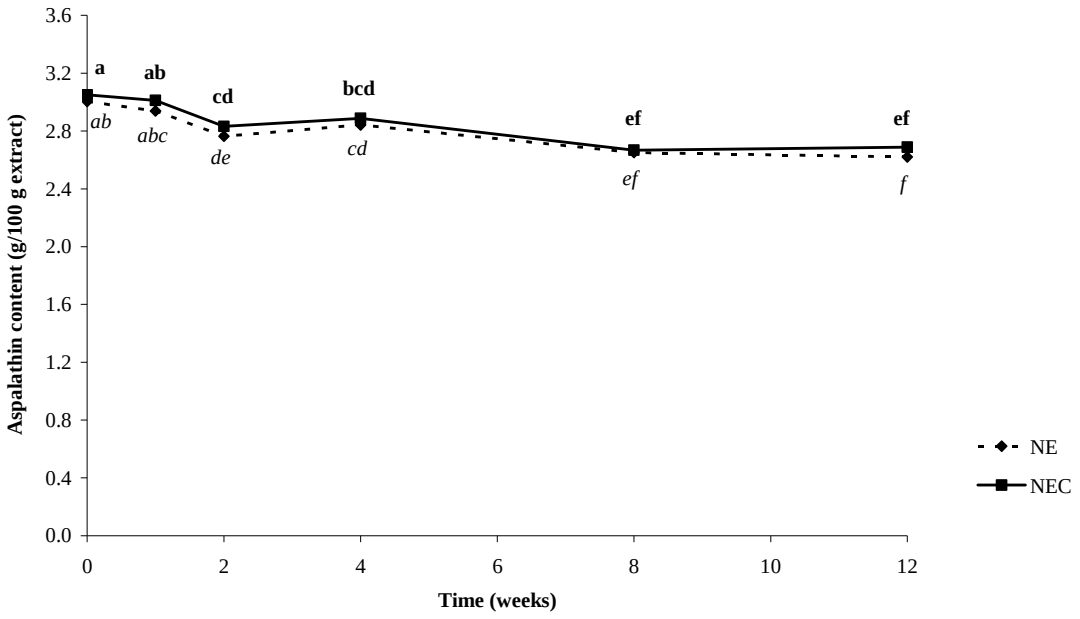
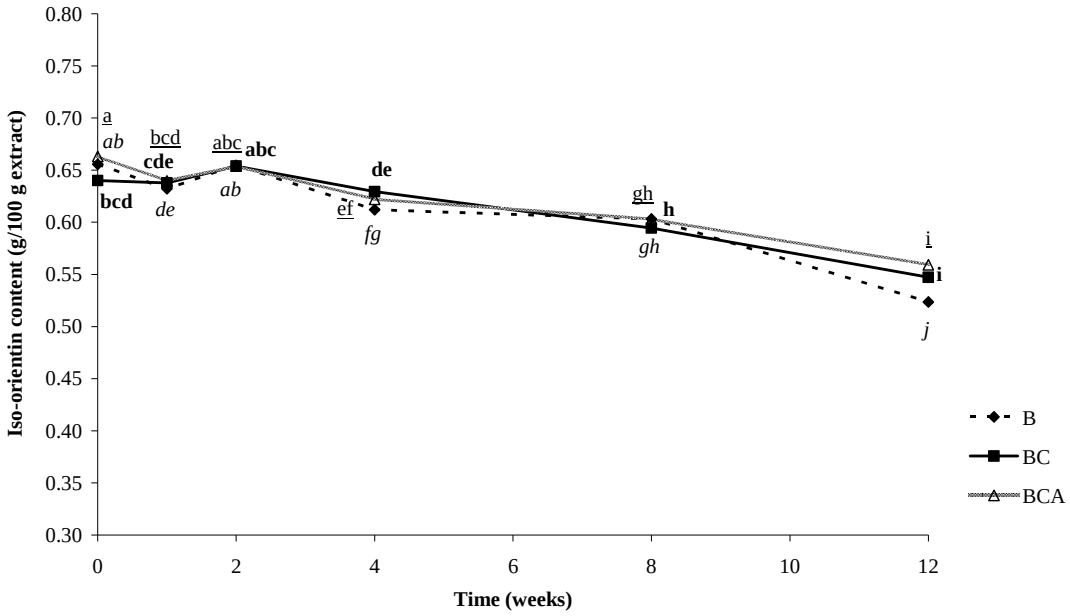
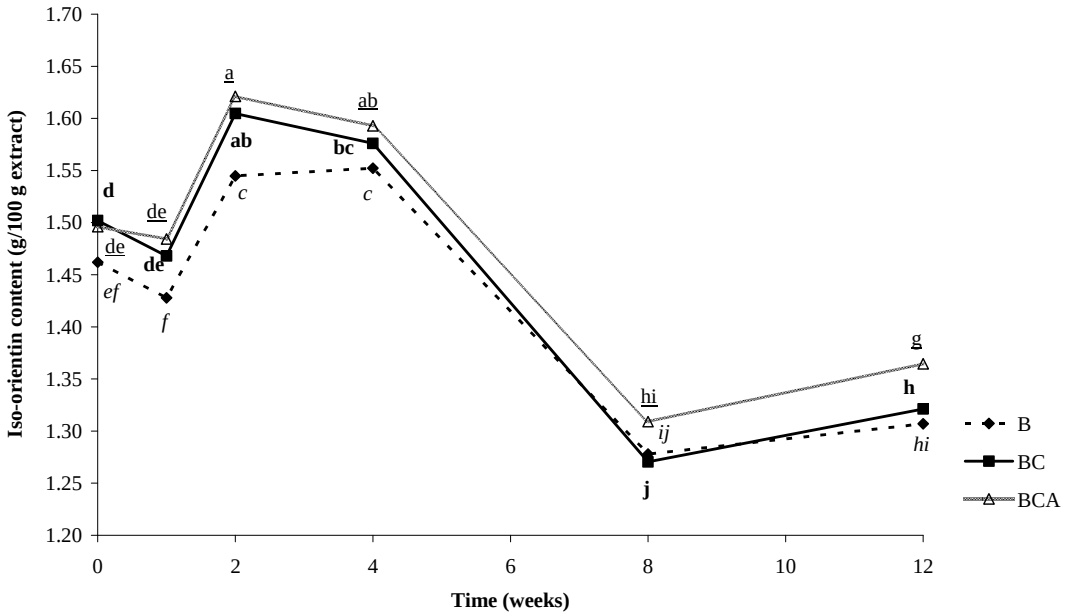


Figure 4.4 The effect of formulation and temperature controlled storage on the aspalathin content of (a) fermented rooibos (b) unfermented rooibos and (c) nano emulsified unfermented rooibos iced tea. Formulation B = base (rooibos extract in deionised water), BC = base + citric acid, BCA = base + citric + ascorbic acid, NE = nano emulsified unfermented rooibos (NEUR) extract in deionised water, NEC = NE + citric acid. There is no NECA formulation for the NEUR extract as the extract inherently contained ascorbic acid. Means, represented by points, labelled with different alphabetical letters differ significantly ($P<0.05$). The data labels for the base formulation (also NE) are shown in italics whilst those for base + citric acid (also NEC) are shown in bold. The data labels for the base + citric + ascorbic acid formulation are underlined.

a)


Iso-orientin content (g/100 g extract)
Time (weeks)
B
BC
BCA

b)


Iso-orientin content (g/100 g extract)
Time (weeks)
B
BC
BCA

c)

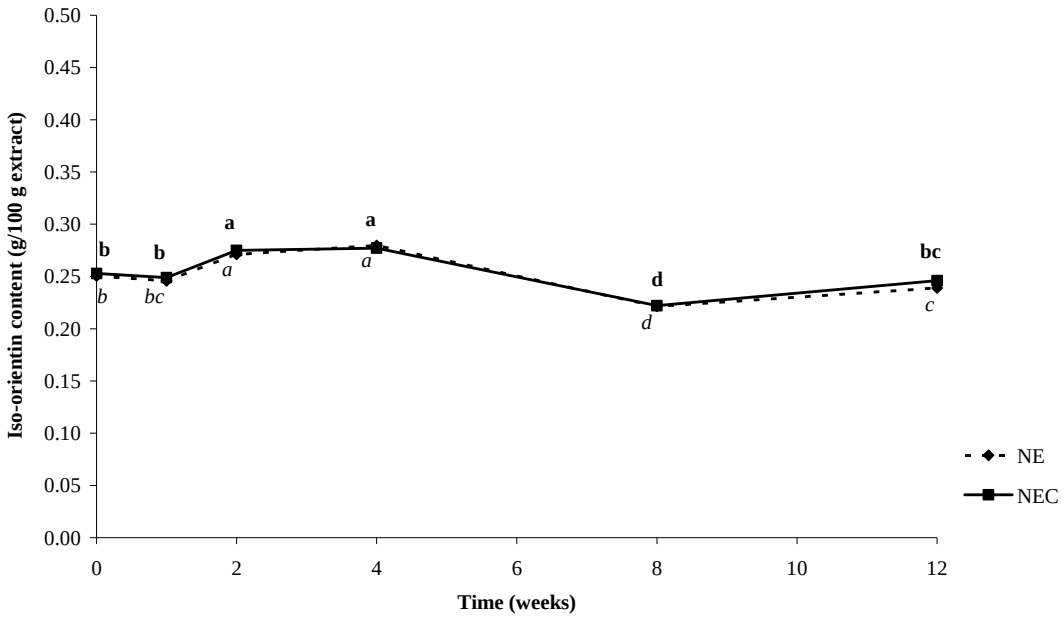
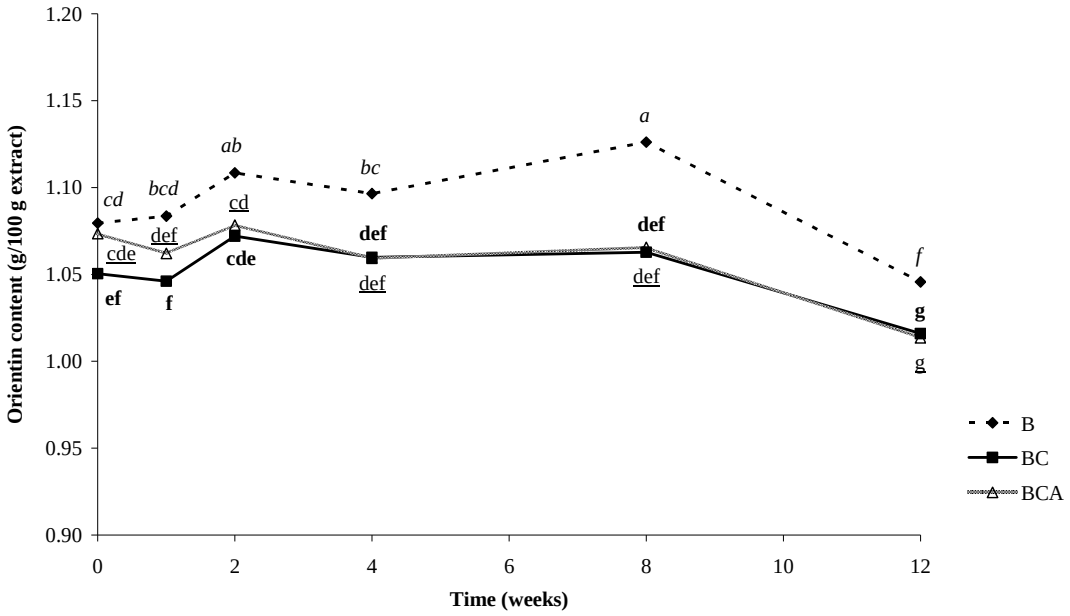
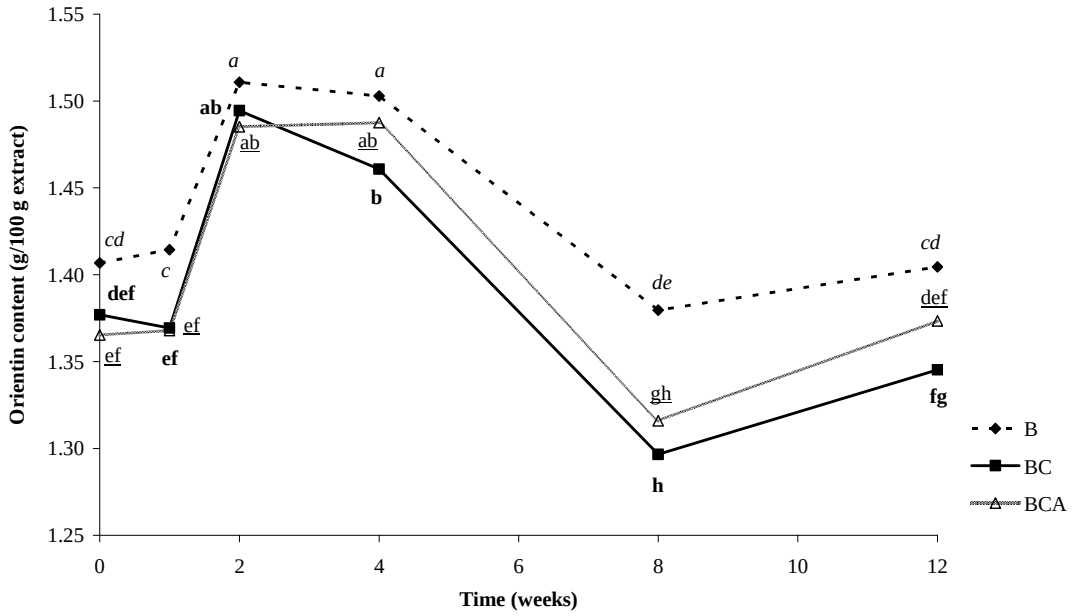


Figure 4.5 The effect of formulation and temperature controlled storage on the iso-orientin content of (a) fermented rooibos (b) unfermented rooibos and (c) nano emulsified unfermented rooibos iced tea. Formulation B = base (rooibos extract in deionised water), BC = base + citric acid, BCA = base + citric + ascorbic acid, NE = nano emulsified unfermented rooibos (NEUR) extract in deionised water, NEC = NE + citric acid. There is no NECA formulation for the NEUR extract as the extract inherently contained ascorbic acid. Means, represented by points, labelled with different alphabetical letters differ significantly ($P<0.05$). The data labels for the base formulation (also NE) are shown in italics whilst those for base + citric acid (also NEC) are shown in bold. The data labels for the base + citric + ascorbic acid formulation are underlined.

a)


Orientin content (g/100 g extract)
1.20
1.15
1.10
1.05
1.00
0.95
0.90
0
2
4
6
8
10
12
Time (weeks)
B
BC
BCA

b)


Orientin content (g/100 g extract)
1.55
1.50
1.45
1.40
1.35
1.30
1.25
0
2
4
6
8
10
12
Time (weeks)
B
BC
BCA

c)

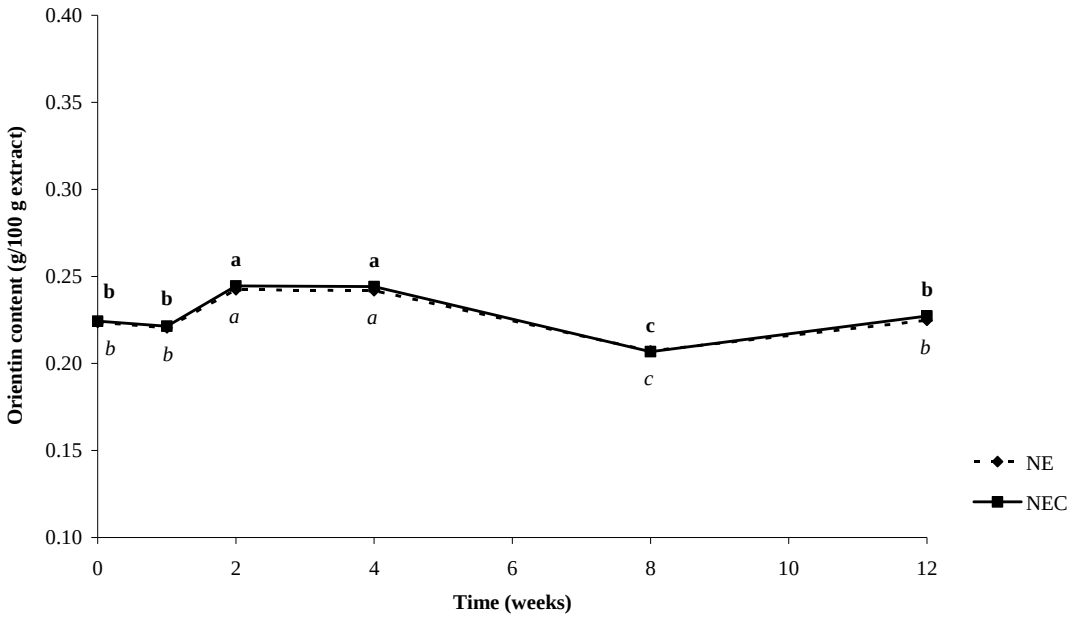
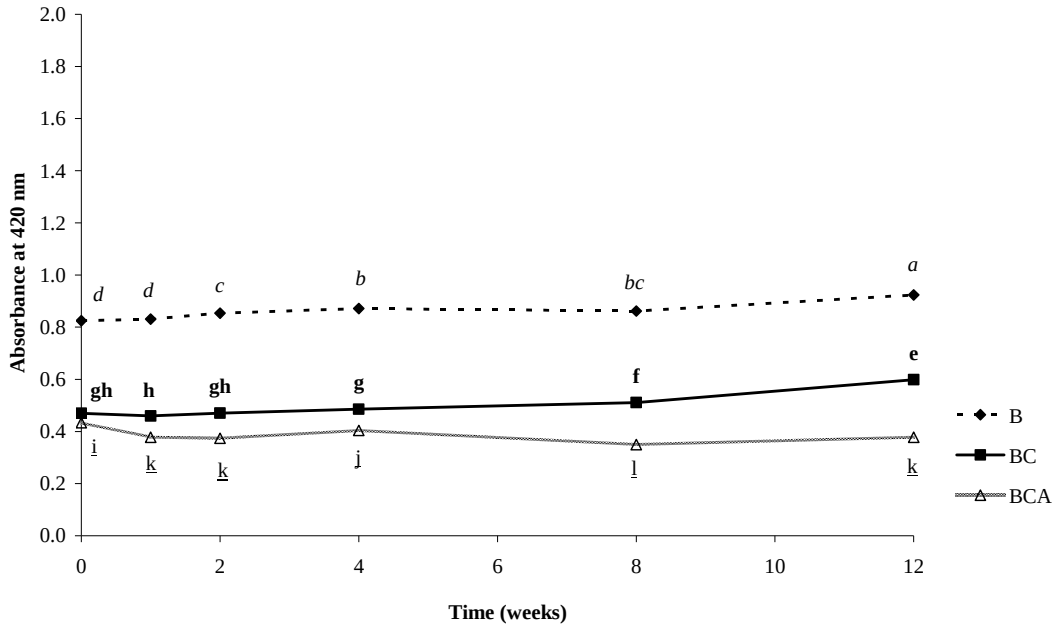


Figure 4.6 The effect of formulation and temperature controlled storage on the orientin content of (a) fermented rooibos (b) unfermented rooibos and (c) nano emulsified unfermented rooibos iced tea. Formulation B = base (rooibos extract in deionised water), BC = base + citric acid, BCA = base + citric + ascorbic acid, NE = nano emulsified unfermented rooibos (NEUR) extract in deionised water, NEC = NE + citric acid. There is no NECA formulation for the NEUR extract as the extract inherently contained ascorbic acid. Means, represented by points, labelled with different alphabetical letters differ significantly ($P<0.05$). The data labels for the base formulation (also NE) are shown in italics whilst those for base + citric acid (also NEC) are shown in bold. The data labels for the base + citric + ascorbic acid formulation are underlined.

a)



b)

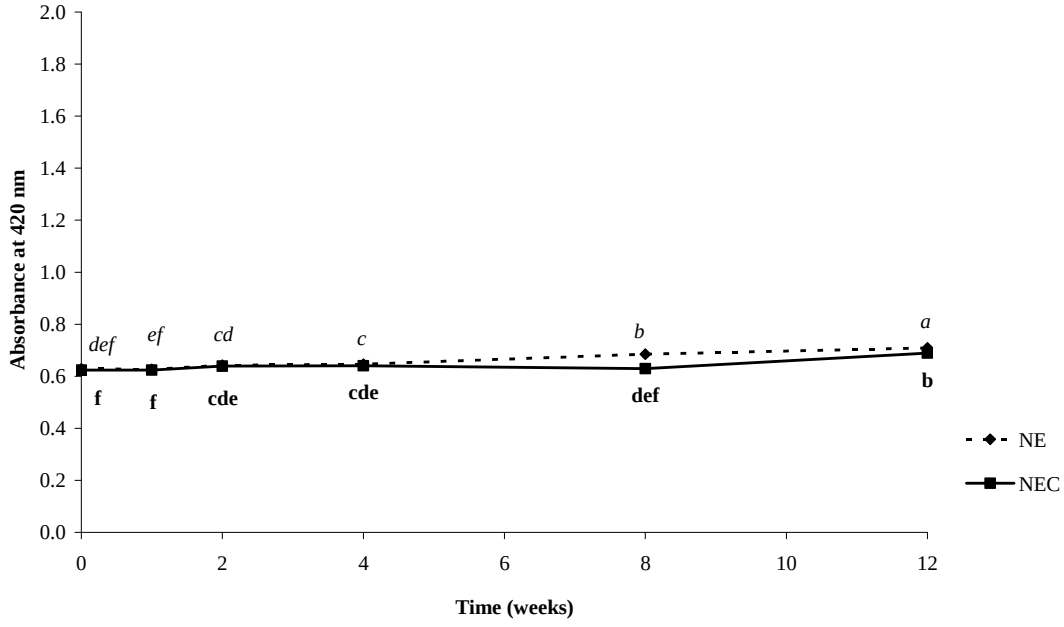
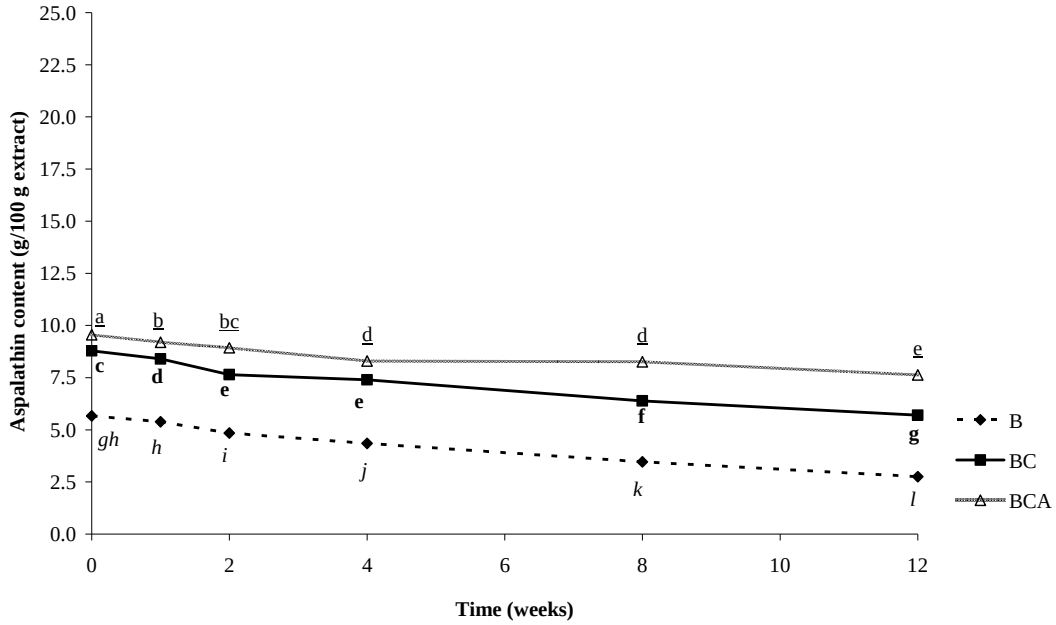


Figure 4.7 The effect of controlled temperature storage on the absorbance (420 nm) of (a) a combination of fermented and unfermented rooibos and (b) a combination of fermented rooibos and nano emulsified unfermented rooibos iced tea. The abbreviations for the iced tea formulations were explained in the text. Means, represented by points, labelled with different alphabetical letters differ significantly ($P<0.05$). The data labels for the base formulation (also NE) are shown in italics whilst those for base + citric acid (also NEC) are shown in bold. The data labels for the base + citric + ascorbic acid formulation are underlined.

a)



b)

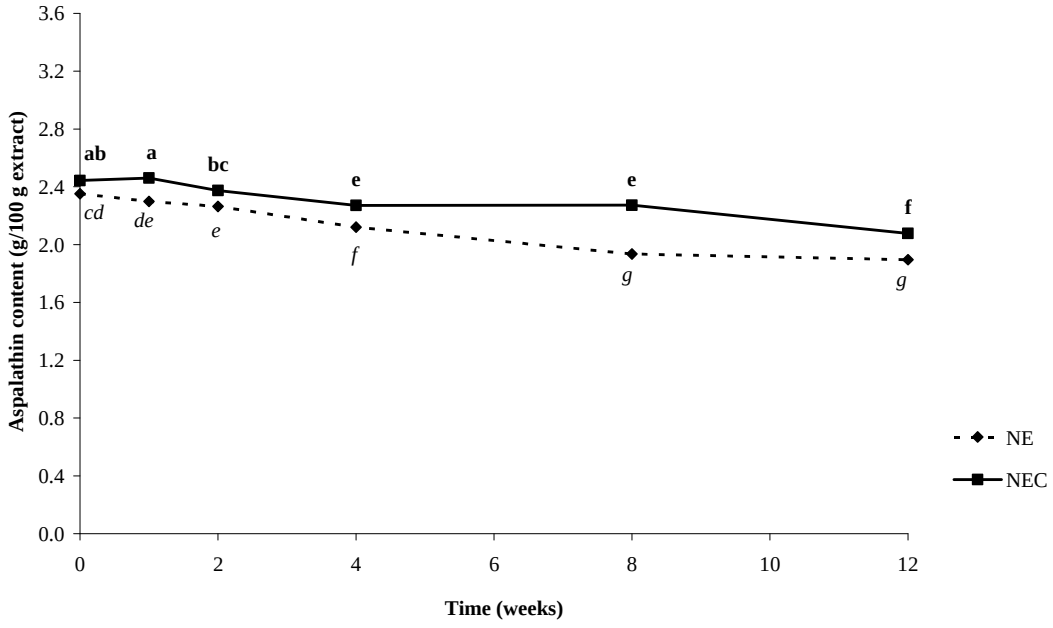
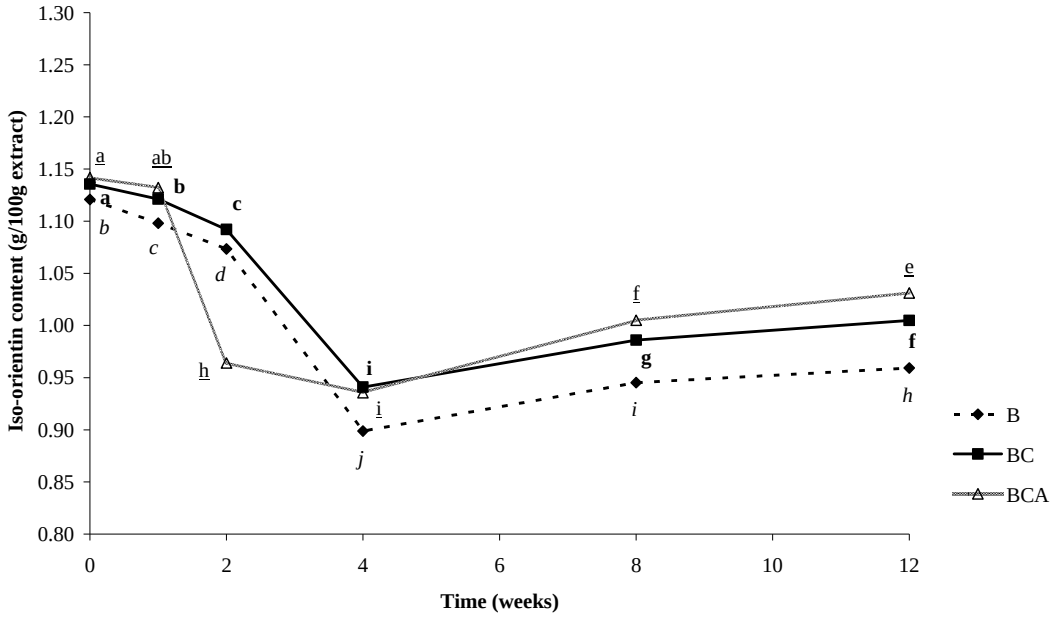


Figure 4.8 The effect of formulation and controlled temperature storage on the aspalathin content of (a) a combination of fermented and unfermented rooibos and (b) a combination of fermented rooibos and nano emulsified unfermented rooibos iced tea. The abbreviations for the iced tea formulations were explained in the text. Means, represented by points, labelled with different alphabetical letters differ significantly ($P<0.05$). The data labels for the base formulation (also NE) are shown in italics whilst those for base + citric acid (also NEC) are shown in bold. The data labels for the base + citric + ascorbic acid formulation are underlined.

a)



b)

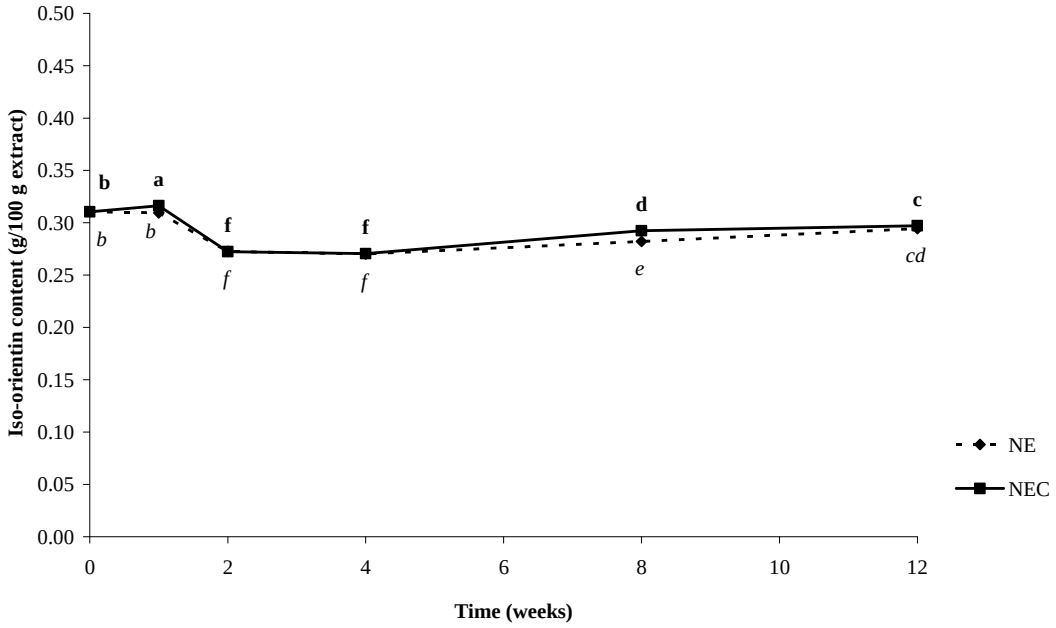
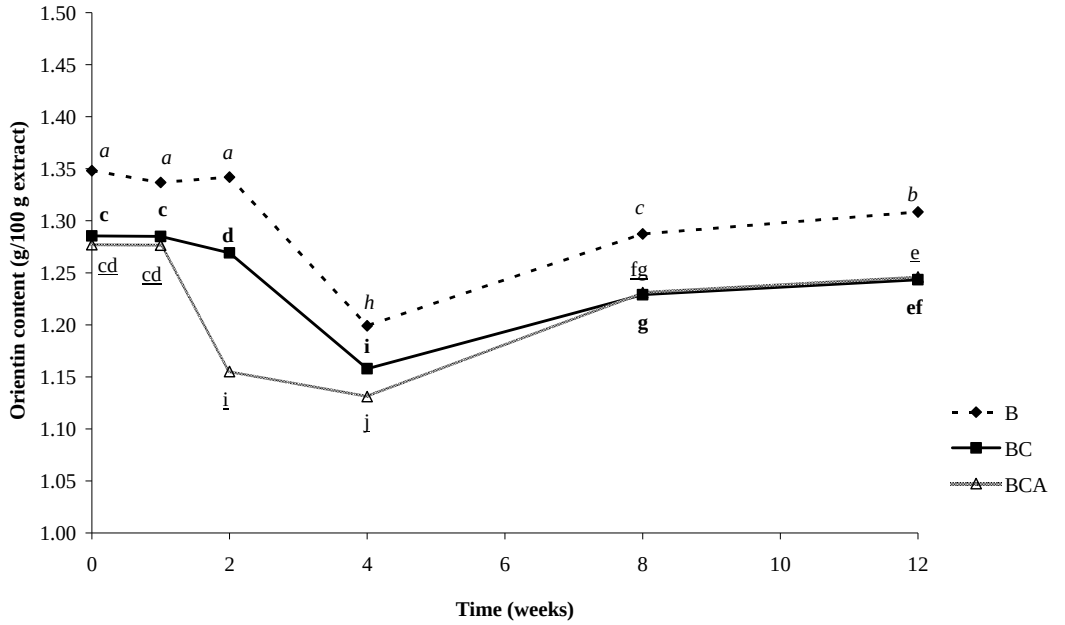


Figure 4.9 The effect of controlled temperature storage on the iso-orientin content of (a) a combination of fermented and unfermented rooibos and (b) a combination of fermented rooibos and nano emulsified unfermented rooibos iced tea. The abbreviations for the iced tea formulations were explained in the text. Means, represented by points, labelled with different alphabetical letters differ significantly ($P<0.05$). The data labels for the base formulation (also NE) are shown in italics whilst those for base + citric acid (also NEC) are shown in bold. The data labels for the base + citric + ascorbic acid formulation are underlined.

a)



b)

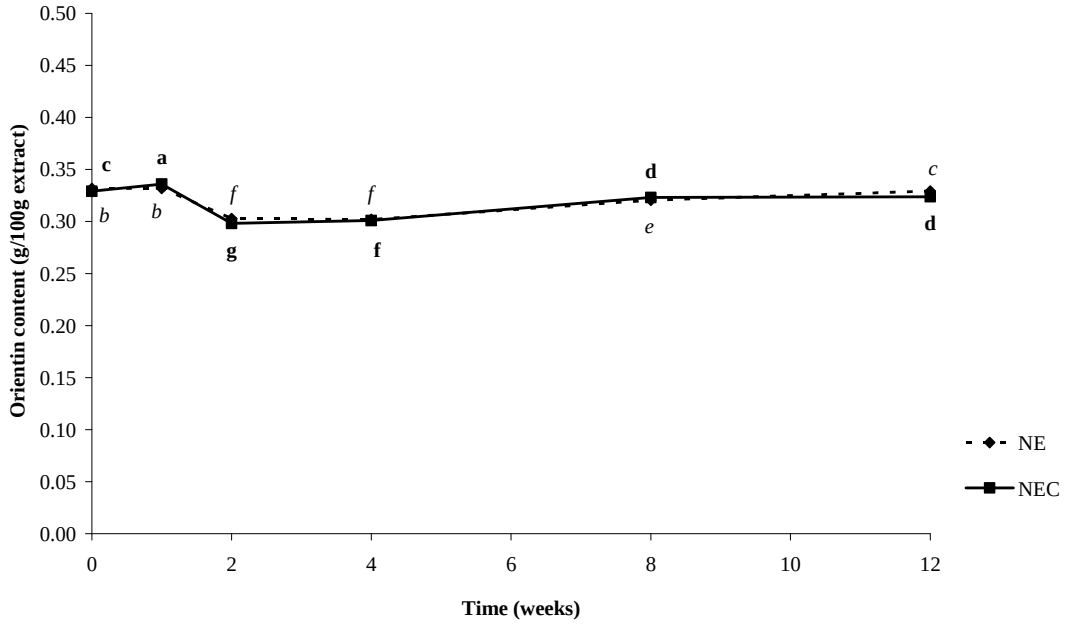


Figure 4.10 The effect of controlled temperature storage on the orientin content of (a) a combination of fermented and unfermented rooibos and (b) a combination of fermented rooibos and nano emulsified unfermented rooibos iced tea. The abbreviations for the iced tea formulations were explained in the text. Means, represented by points, labelled with different alphabetical letters differ significantly ($P<0.05$). The data labels for the base formulation (also NE) are shown in italics whilst those for base + citric acid (also NEC) are shown in bold. The data labels for the base + citric + ascorbic acid formulation are underlined.

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CHAPTER 5

EFFECT OF PH AND HYDROGEN PEROXIDE ON THE PHENOLIC COMPOSITION AND COLOUR OF VARIOUS ROOIBOS EXTRACT FORMULATIONS

CHAPTER 5

EFFECT OF PH AND HYDROGEN PEROXIDE ON THE PHENOLIC COMPOSITION AND COLOUR OF VARIOUS ROOIBOS EXTRACT FORMULATIONS

ABSTRACT

The stability of aspalathin, iso-orientin and orientin in fermented rooibos (FR), unfermented rooibos (UR) and nano emulsified unfermented rooibos (NEUR) extracts, at different pH values (pH 3-7) and storage temperatures (5, 30 and 40ºC), was investigated. Browning of the solutions was measured spectrophotometrically at 420 nm and changes in the aspalathin, iso-orientin and orientin content of the samples was quantified using HPLC analysis. The effect of H_2O_2 (660 mg/L) on different UR extract formulations was investigated. Additional factors that could influence the extent of oxidation under these conditions, such as the addition of ascorbic or citric acid (pH) and short term storage (0-7 days) were investigated. The effect of a low concentration (0.5 mg/L) of H_2O_2, equivalent to the maximal residual levels permitted in food, on FR, UR and NEUR extract formulations was also investigated. Losses of individual flavonoids and browning were determined by means of HPLC analysis and absorbance measurement (420 nm), respectively.

Both the FR and UR extract formulations exhibited increasing losses of phenolic compounds with increasing pH (pH 3-7) and storage temperature (5-40ºC). The absorbance (420 nm) of the solutions increased with increasing values for the latter two parameters. The FR extract formulation exhibited a significant ($P<0.05$) increase in aspalathin content at pH 3 (30ºC), but it decreased significantly ($P<0.05$) with increasing pH from pH 4 onwards. No aspalathin survived storage at pH 7. The UR extract formulation exhibited a significant ($P<0.05$) decrease in aspalathin content at pH 6 and pH 7, the greatest loss of aspalathin being detected at pH 7 (40ºC) (76.4%). Iso-orientin and orientin were generally less susceptible to changes in pH than aspalathin. No significant ($P\geq0.05$) losses of these two compounds were detected for either the FR or UR extract formulation at pH 3, 4 or 5. Between pH 5 and 7, the stability of aspalathin was superior in the NEUR extract formulation, compared to FR and UR extracts. The NEUR extract formulations also greatly resisted absorbance changes at pH 3 and 4, despite the oxidation of aspalathin.

The addition of H_2O_2 (660 mg/L) to UR extract formulations resulted in an immediate and significant ($P<0.05$) decrease in the aspalathin, iso-orientin and orientin content. Generally, no significant ($P\geq0.05$) change in absorbance was detected, suggesting that the oxidation products formed due to a reaction with H_2O_2 differed from those formed as a result of slow chemical oxidation. Formulation BA (base + ascorbic acid) underwent the largest (%) decrease in the aforementioned flavonoids. Storage of H_2O_2-containing formulations (30ºC for one week) resulted in additional aspalathin loss in all formulations, except formulation BA. Losses of iso-orientin and orientin were only detected in formulation BC (base + citric acid, where base is UR extract in deionised water). The absorbance of all the stored formulations increased significantly ($P<0.05$) compared to the controls.

The addition of H_2O_2 to three types of rooibos extract formulations at the legal residual level for aseptically packaged beverages (0.5 mg/L) resulted in a small, but significant ($P<0.05$) decrease in the aspalathin (0.7%), iso-orientin (1.1%) and orientin (0.7%) content of FR extract formulations containing citric and ascorbic acid. No concomitant change in absorbance was noted. The UR and NEUR extract formulations remained unchanged by the treatment.

INTRODUCTION

The stability of polyphenols such as catechins is said to be greater at lower pH values (Chen *et al*., 1998). Guyot *et al*. (2007) found that the oxidation of phloridzin, a dihydrochalcone glycoside specifically found in apple, was more pronounced at higher pH values. The colour of the phloridzin-derivative also changed according to the pH of the medium: the compound had a bright yellow colour at pH 3 but became darker/orange at higher pH values (pH 5). Similarly, the oxidation of tea catechins with peroxyl radicals has been found to deliver non-polymeric, yellow products (Sang *et al*., 2003).

Numerous browning reactions in food have been attributed to changes in pH (Cilliers & Singleton, 1990; Yeo & Shibamoto, 1991; García *et al*., 1992), as well as processing parameters such as heating duration and temperature (Reyes *et al*., 1982; Friedman & Molnar-Perl, 1990). As a result of autoxidation, phenolic polymerisation and the development of brown-coloured pigments may occur (Talcott & Howard, 1999). In caffeic acid solutions, browning has been shown to be pH dependent (Cilliers & Singleton, 1989; Cilliers & Singleton, 1990). Cilliers & Singleton (1989) found that the extent of caffeic acid oxidation increased with increasing pH and that the generation of a brown pigment at 420 nm correlated well with caffeic acid consumption at all pH values. The presence and concentration of the phenolate ion, which decreases with decreasing pH, has been shown to be the limiting factor in phenolic autoxidation (Cilliers & Singleton, 1989). The phenolate ion is a highly reactive species that catalyses oxidative reactions in phenolic acid systems (Bucheli & Robinson, 1994).

The pH of a medium also has an important effect on the progression of the Fenton reaction (Kremer, 2003; Jeong & Yoon, 2005; Rivas *et al*., 2005). In 1934, Haber and Weiss proposed that $^{\bullet}OH$, a radical capable of significant oxidative damage, could be formed from $O_2^{-\bullet}$ and H_2O_2 (Morris *et al*., 1995). The rate constant for this reaction, however, is low and it will not proceed at a significant rate *in vivo* (Halliwell & Gutteridge, 1982). In the presence of iron, a modified version of the Haber-Weiss reaction, the Fenton reaction, takes place at a higher rate (Halliwell & Gutteridge, 1984; Namiki, 1990; Morris *et al*., 1995). The reaction follows:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + {}^{-}OH \qquad [1]$$

The rate of the Fenton reaction can be greatly accelerated by reducing agents such as ascorbic acid (Aruoma, 1994), a common ingredient in iced tea (Chen *et al*., 1998). Ascorbic acid has an iron "recycling"

ability: reducing Fe^{3+} to Fe^{2+} and thus increasing the rate of hydroxyl radical formation (Morris *et al*., 1995). Ascorbic acid, however, is not the only compound capable of increasing the rate of the Fenton reaction. Laughton *et al*. (1989) and Puppo (1992) have shown that flavonoids accelerate hydroxyl radical generation in Fenton-type reactions. As with ascorbic acid, the mechanism involved is the reduction of Fe^{3+}, with consequent oxidation of the flavonoid.

Beverages such as black tea and rooibos naturally contain both iron (Reyneke *et al*., 1949; Gallaher *et al*., 2006) and flavonoids (Koeppen & Roux, 1965; Tijburg *et al*., 1997; Wiseman *et al*., 1997). Today, an increasing number of beverage products are aseptically packaged as the storage costs of such products are lower than refrigerated products, the shelf-life is extended and the product is generally free from additives (Ansari & Datta, 2003). Packaging material for aseptic applications is sterilised with a 35% H_2O_2 solution (70ºC) (B. Vienings, quality assurance manager, Tetra Pak, South Africa, personal communication, 2007). In the filling machine, rollers and an air knife (125ºC) reduce the H_2O_2 concentration on the packaging material to less than 1 mg/L in the final food products. A Food and Drug Administration (FDA) regulation currently limits residual H_2O_2 to 0.5 mg/L, leached into distilled water, in finished food packages (Anon., 2000). Hydroxyl radical formation via a Fenton-type reaction may thus be possible in aseptically packaged iced tea.

There are many contradictory reports with respect to the true pH requirements of the Fenton reaction (Zepp, 1992; Burbano *et al*., 2005; Jeong & Yoon, 2005; Rivas *et al*., 2005; Li *et al*., 2007). Jeong & Yoon (2005) reported that the pH requirement of the Fenton reaction is dependent on the availability of H_2O_2. In the absence of externally supplied H_2O_2, degradation of organic compounds is enhanced with increasing pH. In the presence of excess H_2O_2, however, the degradation of organic compounds is reduced with increasing pH, thus indicating the requirement for a low pH environment under the latter conditions. The low pH is required to prevent $Fe(OH)_3$ precipitation (Li *et al*., 2007).

Citric acid may form a range of complexes with metal ions, depending on the pH, oxidation state of the metal and the metal:citric acid ratio (Francis *et al*., 1992; Dodge & Francis, 2002). Citric acid is a common additive in beverages such as iced tea (Zhu *et al*., 1997; Lambert & Stratford, 1999; Battey & Schaffner, 2001; Battey *et al*., 2002). In the presence of H_2O_2, Fe^{2+} and its citrate complexes efficiently react to produce $^{\bullet}OH$ in water at pH values ranging from 3-8 (Zepp, 1992). Complex formation with citric acid thus does not inhibit radical formation.

The stability of flavonoids is largely determined by the pH of the solution. The effect of changes in pH on the stability of rooibos flavonoids has not yet been determined. In Chapter 3 and 4, it was postulated that pH played a role in the differing stability of aspalathin, iso-orientin and orientin in the various rooibos iced tea formulations. The aim of this study was thus to evaluate the effect of changes in pH on the aspalathin, iso-orientin and orientin content as well as absorbance (420 nm, brown colour) of rooibos extract formulations produced with fermented rooibos (FR), unfermented rooibos (UR) and nano emulsified unfermented rooibos (NEUR) extracts. The addition of H_2O_2, at both high (UR only) and low concentrations (FR, UR and NEUR), on the aspalathin, iso-orientin and orientin content, as well as absorbance (420 nm) of various aqueous extract

formulations of rooibos was also investigated. This was done in order to evaluate the potential effect (if any) of H_2O_2 on the flavonoid compounds present in aseptically packaged rooibos iced tea products.

MATERIALS AND METHODS

Chemicals and reagents

The chemicals and reagents required were listed in Chapter 3. Additionaly, dipotassium hydrogen phosphate (Merck, Darmstadt, Germany), potassium dihydrogen phosphate (Merck), potassium hydroxide (Merck), hydrochloric acid (Merck) and 30% hydrogen peroxide (Saarchem) were acquired.

Extracts for preparation of aqueous rooibos extract formulations

The rooibos extracts described and characterised in Chapter 3 (Table 3.11) were used.

Effect of pH and temperature on the phenolic composition and colour of rooibos extract formulations

Five 10 mM buffers with pH 3, 4, 5, 6 and 7 were prepared using dipotassium hydrogen phosphate, potassium dihydrogen phosphate and distilled, deionised water. Adjustments to the required pH were made using 0.1 M potassium hydroxide and 0.1 M hydrochloric acid. The pH of the buffers was determined using a Crison GLP 21 pH meter (Crison Instruments SA, South Africa). A magnetic stirrer was used to ensure the homogeneity of the solutions during pH measurement. The pH meter was standardised with Sigma buffer reference standards pH 7 (pH 7 ± 0.01 at 25°C) and pH 4 (pH 4 ± 0.01 at 25°C).

The effect of pH on the stability of aspalathin, iso-orientin and orientin in FR, UR and NEUR extracts was investigated as follows: 0.44 g of each of the two rooibos extracts and 3.50 g of the NEUR extract were weighed off into a 250 mL volumetric flask. Each extract was weighed off five times (for the five pH solutions), and the mass recorded to 5 decimal places. Buffer of the appropriate pH was then added to each flask and the mixture sonicated for 5 min in a 50/60 Hz Branson ultrasonic bath (Branson Cleaning Equipment Company, Connecticut, USA) to aid solubilisation. Each of the duplicate pH solutions was then decanted into four separate 25 mL glass bottles. Three bottles were stored at three different temperatures, namely 5, 30 and 40°C for 48 hours whilst the remaining bottle was retained as a control (not stored). The aspalathin, iso-orientin and orientin content of the stored samples and controls was determined by means of HPLC analysis and the absorbance measured at 420 nm. The experiment was replicated twice, independently (i.e. on two separate occasions, five buffer solutions of each extract, namely FR, UR and NEUR were prepared).

Effect of hydrogen peroxide on the phenolic composition and colour of rooibos extract formulations

Effect of 660 mg/L hydrogen peroxide on the phenolic composition and colour of unfermented rooibos extract formulations

The effect of H_2O_2 addition was investigated on four formulations of UR extract: base (UR extract in deionised water), base + citric acid, base + ascorbic acid, base + citric + ascorbic acid. The UR extract was specifically used due to its high aspalathin content i.e. any changes would be apparent. The mass of the required ingredients (recorded to five decimals) is given in Table 5.1. Distilled, deionised water was used to solubilise the extract formulations. Hydrogen peroxide (30%) was added to the four formulations (660 mg/L). Formulations without added H_2O_2 were considered controls. The experiment was replicated, independently, three times (i.e. four formulations of UR extract were freshly prepared on three separate occasions). The aspalathin, iso-orientin and orientin content of the formulations was quantified before and directly after the addition of H_2O_2 as well as after one week of storage. Quantification of the flavonoids was performed by means of HPLC analysis and the absorbance measured at 420 nm.

Table 5.1 The mass (g) of dry ingredients required for 1 L of the four UR[a] extract formulations

Ingredient	Base[b]	Base + ascorbic acid	Base + citric acid	Base + citric + ascorbic acid
UR extract	1.75	1.75	1.75	1.75
Citric acid	-	-	1.20	1.20
Ascorbic acid	-	0.20	-	0.20

[a]Unfermented rooibos, [b]UR extract in deionised water.

Effect of 0.5 mg/L hydrogen peroxide on the phenolic composition and colour of fermented, unfermented and nano emulsified unfermented rooibos extract formulations

Two formulations of the respective FR and UR extracts were prepared: BA and BCA. For the NEUR extract, two formulations were prepared: NE (NEUR extract in deionised water) and NEC (NE + citric acid). The NEUR extract inherently contained ascorbic acid, thus the formulations differed from those of FR and UR. The mentioned extract formulations (BA, BCA, NE and NEC) were chosen for investigation due to their ascorbic acid content. Ascorbic acid accelerates the rate of the Fenton reaction by reducing Fe^{3+} to Fe^{2+} (Aruoma, 1994). The amount of the reagent required for each formulation is given in Table 5.2. The mass of the ingredients was recorded to five decimals. Distilled, deionised water was used to solubilise the extract formulations. Hydrogen peroxide (30%) was added to each of the two formulations (for all three extracts) so that the final H_2O_2 concentration in the sample was 0.5 mg/L (legal H_2O_2 residue limit, Anon., 2000). These formulations were considered "treated". Formulations without added H_2O_2 were considered controls. The experiment was replicated, independently, four times (i.e. two formulations each of UR, FR and NEUR extract were freshly

prepared on four separate occasions). The samples were left standing at room temperature for *ca.* 1 h, after which aliquots were frozen for later analysis.

Table 5.2 The mass (g) of dry ingredients required for the two formulations of rooibos extract solution (1 L)

Ingredient	**BA**[a]	**BCA**[b]	**NE**[c]	**NEC**[d]
FR[e] extract	1.75	1.75	-	-
UR[f] extract	1.75	1.75	-	-
NEUR[g] extract	-	-	14.00 (2.10)[h]	14.00 (2.10)[h]
Citric acid	-	1.20	-	1.20
Ascorbic acid	0.20	0.20	-	-

[a]Base (extract in deionised water) + ascorbic acid, [b]base + citric + ascorbic acid, [c]nano emulsified unfermented rooibos extract in deionised water, [d]NE + citric acid, [e]fermented rooibos, [f]unfermented rooibos, [g]nano emulsified unfermented rooibos, [h]equivalent amount of UR extract.

Monitoring of browning of rooibos extract formulations

Browning of the rooibos extract formulations was determined as an increase in absorbance at 420 nm, relative to the control. In the case of the pH experiment, there were five controls: at the relevant pH value, the sample not subjected to a 48 h storage period. For the H_2O_2 experiment, the samples without this reagent were considered controls. The spectrophotometric procedure used, was as described in Chapter 3.

HPLC quantification of the phenolic content of rooibos extract formulations

Quantification of aspalathin, orientin and iso-orientin was performed by HPLC-DAD. The HPLC apparatus, column, gradient profile for separation, software, standards and sample preparation procedure was as described in Chapter 3.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) using SAS version 9.3 (SAS Institute, Cary, NC, USA) and analysed for normality using the Shapiro-Wilk test for normality ($P>0.05$). The Student t-test was used to ascertain whether there were significant differences between samples. Differences with a significance level of 5% ($P\leq0.05$) were considered significant (SAS, 2002). The teas made from different extracts were analysed separately due to the large difference in phenolic make-up between the FR and UR extracts. Differences between formulations of the same extract type were lost when all the data were analysed together.

RESULTS

The change (%) in the absorbance, as well as aspalathin, iso-orientin and orientin content of the extract formulations can be found in ADDENDUM 7 (Tables A7.1-A7.4).

Effect of pH and temperature on the phenolic composition and colour of rooibos extract formulations

Browning

A pattern of increasing absorbance with increasing pH and temperature was generally observed for the FR extract formulations (Fig. 5.1a). There were, however, exceptions. At pH 4, the absorbance of the 5ºC sample did not differ significantly ($P \geq 0.05$) from that of the control and the absorbance at 30ºC did not differ significantly ($P \geq 0.05$) from that at 40ºC. At pH 7, the absorbance of the 30ºC sample was greater than that of the 40ºC sample.

The same pattern of increasing absorbance with increasing pH and temperature was observed for the UR extract formulation samples (Fig. 5.1b). At pH 7, for example, the absorbance of the 5, 30 and 40ºC samples increased by 2.9, 30.6 and 40.6% compared to that of the control.

The absorbance of the nano emulsion was stable between pH 3 and 6 (Fig. 5.1c). At pH 7, however, a very slight, but significant ($P < 0.05$) decrease in absorbance (compared to the control) was observed. The decrease was only significant ($P < 0.05$) for the samples stored at 5 and 30ºC, although these did not differ significantly ($P \geq 0.05$) from the sample stored at 40ºC.

Aspalathin

The aspalathin content of all the FR extract formulation samples generally decreased with increasing pH and temperature, i.e. the largest decrease for each pH was observed for the sample stored at 40ºC. The exception was that the aspalathin content of the samples stored at pH 3 (30ºC) increased significantly ($P < 0.05$) with respect to the control (Fig. 5.2a). After two days (48 h) of storage, no aspalathin remained in samples stored at pH 6 (30 and 40ºC) or pH 7 (all storage temperatures).

There was no significant ($P \geq 0.05$) change in the aspalathin content, compared to the control, of the UR extract formulations at any of the three temperatures investigated at pH 3, 4 or 5 (Fig. 5.2b). At pH 6 (30 and 40ºC) and pH 7 (all temperatures), however, significant ($P < 0.05$) decreases were observed. The samples stored at 5, 30 and 40ºC (pH 7) decreased by 6.8, 44.8 and 76.4%, respectively.

Changes in the phenolic profile (288 nm) of the UR extract formulation, between pH 3 (control) and pH 7 (40ºC), are depicted in Figure 5.3a. Despite the decreases in aspalathin content with increasing pH, no real changes in the area of the polymeric compounds (peak 2 in Fig. 5.3a) was visible on the chromatogram.

At pH 3, the NEUR extract formulation stored at 30ºC had a significantly ($P < 0.05$) lower aspalathin content compared to its control. The same was true for the samples at pH 4, stored at 30 and 40ºC. The stability of aspalathin in NEUR extract formulations was generally greatest between pH 5 and 6. At pH 7, a significant

($P<0.05$) decrease in aspalathin content was observed for samples stored at 5, 30 and 40ºC, although the loss (%) was less than that observed for the UR extract formulation (45.0 vs. 76.4% at 40ºC, respectively).

Iso-orientin

The iso-orientin content of FR extract formulations was generally less affected by changes in pH and storage temperature than aspalathin content. No significant ($P\geq0.05$) decrease in iso-orientin content was observed for the FR extract formulation at pH 3, 4 or 5, irrespective of the storage temperature (Fig. 5.4a). At pH 6, however, there was a significant ($P<0.05$) decrease in iso-orientin content at 40ºC. Not only were the decreases more pronounced at pH 7 (40ºC), but the iso-orientin content of the sample at 30ºC was also significantly ($P<0.05$) lower than that of the control (pH 7).

The iso-orientin content of the UR extract formulations was unchanged at pH 3 and 4, but increased significantly ($P<0.05$) at pH 5 for samples stored at 40ºC (Fig. 5.4b). At pH 6, however, a significant ($P<0.05$) reduction in iso-orientin content was observed for the samples stored at 40ºC. More extensive losses were observed at pH 7 (30 and 40ºC).

The iso-orientin content of all the NEUR extract formulations decreased significantly ($P<0.05$), irrespective of temperature and pH (Fig. 5.4c). Furthermore, the iso-orientin content of the stored samples (5, 30 and 40°C), at a specific pH value, did not differ significantly ($P\geq0.05$) from one another, except for the sample at pH 4 (40ºC). It had a significantly ($P<0.05$) lower iso-orientin content than the samples stored at 5 and 30ºC (pH 4).

Orientin

As with iso-orientin, the orientin content of the FR rooibos extract formulations was not as greatly influence by changes in pH and temperature as the aspalathin content. No significant ($P\geq0.05$) change in the orientin content of FR extract formulations was observed between pH 3 and 5 (Fig. 5.5a). At pH 6 (30ºC) and pH 7 (30 and 40ºC), however, the orientin content of the samples decreased significantly ($P<0.05$).

The orientin content of UR extract formulations remained mostly unchanged at all pH values. At pH 5 and 7 (40ºC), significant ($P<0.05$) increases of 4.9 and 3.2% were observed. Apart from changes in iso-orientin and orientin content, no major changes could be observed on the chromatograms (Fig. 5.3b).

A significant ($P<0.05$) decrease in orientin content was observed for all pH-combinations of the NEUR extract formulations (Fig. 5.5c), compared to the control. Temperature did not have an effect, irrespective of pH, except for the samples stored at pH 4 (40ºC). The orientin content of the latter was significantly ($P<0.05$) lower than that of samples stored at 5 and 30ºC.

Effect of hydrogen peroxide on the phenolic composition and colour of rooibos extract formulations

Effect of 660 mg/L hydrogen peroxide on the phenolic composition and colour of unfermented rooibos extract formulations

Since pH plays an important role in the progression of Fenton reaction (Kremer, 2003; Rivas *et al*., 2005; Jeong & Yoon, 2005), the pH values of the solutions investigated in this experiment were recorded. The results are shown in Table 5.3. Two prominent comparisons were drawn in this experiment, i.e. the immediate effect of H_2O_2 addition (A vs. C, Fig. 5.6) and the effect of storage on H_2O_2-treated samples (C vs. D, Fig. 5.6). The change (%) in the absorbance as well as aspalathin, iso-orientin and orientin content of the UR extract formulations, as a result of H_2O_2 addition, can be found in ADDENDUM 7 (Table A7.5).

In addition to the abovementioned comparisons, three more comparisons could be made: the effect of storage on the controls (A vs. B; indirectly also covered in Chapter 4), comparison of H_2O_2-treated samples and control samples, both subjected to 7 days storage (B vs. D) and the combined effect of storage and H_2O_2-addition (A vs. D). For sake of completeness, these additional comparisons are noted in ADDENDUM 7 (Table A7.6), although, they will not be discussed.

Table 5.3 The average (±SD) pH values of the unfermented rooibos extract formulations used for the H_2O_2 experiment

Formulation	**B**[a]	**BA**[b]	**BC**[c]	**BCA**[d]
pH	4.37±0.01	3.74±0.02	2.78±0.1	2.78±0.01

[a]Base (unfermented rooibos extract in deionised water); [b]B + ascorbic acid; [c]B + citric acid; [d]B + citric + ascorbic acid.

Browning

Generally, the addition of H_2O_2 had no "immediate" effect on the absorbance of the various UR extract formulations, except for formulation BC, which exhibited a significant ($P<0.05$) decrease in absorbance (Fig. 5.6a; A vs. C). Storage of the H_2O_2-treated samples caused an increase in absorbance at 420 nm (Fig. 5.6a; C vs. D). The largest increase was observed for formulation BC (84.4%). A significant ($P<0.05$) increase in absorbance, as a result of storage, also occurred in the control samples.

Aspalathin

The addition of H_2O_2 to UR extract formulations resulted in an immediate, significant ($P<0.05$) decrease in the aspalathin content of all formulations (Fig. 5.6b; A vs. C). Formulation BA underwent the largest decrease (25.3%) in aspalathin content, followed by formulations BCA (20.8%), BC (13.5%) and B (12.0%). Changes due to the addition of H_2O_2 were clearly visible on the chromatograms of the samples recorded at 288 nm. Figure 5.7 shows the chromatogram of formulation B: before H_2O_2 addition (control); after H_2O_2 addition (H_2O_2); after H_2O_2 addition and one week of storage [H_2O_2 (stored)]. The decrease in aspalathin content and

increase in the area of the polymeric compounds (retention time of 13-17 min) after H_2O_2 addition, is clearly visible. The insert (Fig. 5.7) shows compounds eluting at *ca*. 14 min (350 nm) decreased, whilst an ill-defined area developed between *ca*. 15 and 17 min (Fig. 5.7). Figure 5.8 illustrates the immediate effect of H_2O_2 addition on the area of the polymeric compounds in the four formulations. A significant ($P<0.05$) increase in the area of the polymeric compounds was observed for all formulations. The increase (%) in polymer area for the various formulations, as a result of H_2O_2 addition, can be found in ADDENDUM 7 (Table A7.7). The largest and smallest increase in polymer area occurred in samples B and BCA, respectively. However, in terms of increased polymer area, formulations BCA and BA did not differ significantly ($P\geq0.05$).

Storage of the H_2O_2-treated samples led to a significant ($P<0.05$) decrease in the aspalathin content of all formulations of UR extract (Fig. 5.6b; C vs. D). Overall these samples had the lowest aspalathin content and the greatest loss was observed for formulation BCA (29.5%; A vs. D). A decrease in aspalathin content with storage also occurred in the control samples, with the greatest loss observed for formulation BCA (12.0%; A vs. B).

Iso-orientin

The iso-orientin content of all four formulations of UR extract experienced a significant ($P<0.05$) decrease directly after the addition of H_2O_2 (Fig. 5.6c; A vs. C). The decrease (%) was similar compared to that for aspalathin (ADDENDUM 7, Table A7.5). Formulations BA and BCA underwent the largest decrease in iso-orientin content, namely 21.4 and 21.8% (iso-orientin content not significantly different, $P\geq0.05$).

Orientin

The addition of H_2O_2 to UR extract formulations resulted in a significant ($P<0.05$) decrease in the orientin content of all four formulations (Fig. 5.6d; A vs. C). The magnitude of the decrease in orientin content for the various formulations was similar to that observed for iso-orientin and aspalathin. Once again the greatest loss was observed for formulations BA (24.2%) and BCA (23.0%), the iso-orientin content of which did not differ significantly ($P\geq0.05$). The iso-orientin content of the H_2O_2-treated samples remained stable during storage, except for a slight, but significant ($P<0.05$) decrease in formulation BC (5.7%) (C vs. D). Similar to iso-orientin, the orientin content of formulation B increased significantly ($P<0.05$) (6.3%), whilst it decreased significantly ($P<0.05$) (6.5%) in formulation BCA.

Effect of 0.5 mg/L hydrogen peroxide on the phenolic composition and colour of fermented, unfermented and nano emulsified unfermented rooibos extract formulations

The change (%) in the absorbance as well as aspalathin, iso-orientin and orientin content of the various extract formulations, as a result of H_2O_2 addition, can be found in ADDENDUM 7 (Table A7.8).

The pH values of the solutions to which H_2O_2 was added are shown in Table 5.4. No significant ($P\geq0.05$) change in the absorbance of either of the two formulations of the three types of rooibos extract was noted after the addition of 0.5 mg/L H_2O_2 (Table 5.5). A very small (0.7%) but significant ($P<0.05$) decrease in

the aspalathin, iso-orientin and orientin content of formulation BCA of the FR extract was, however, noted after the addition of 0.5 mg/L H_2O_2 (Table 5.5). At the same time, no significant ($P \geq 0.05$) change in the content of these flavonoids in either of the two types of unfermented rooibos extract (UR and NEUR) was noted (Table 5.5).

Table 5.4 The pH values of the extract formulations used for the investigation into the effect of 0.5 mg/L H_2O_2 on the phenolic content and colour of rooibos

Formulation	**FR[a]**	**UR[b]**	**NEUR[c]**
BA[d]/NE[e]	4.09±0.02	3.77±0.01	3.55±0.02
BCA[f]/NEC[g]	3.00±0.02	2.83±0.02	2.65±0.05

[a]Fermented rooibos, [b]unfermented rooibos, [c]nano emulsified unfermented rooibos, [d]base (rooibos extract in deionised water) + ascorbic acid, [e]NEUR extract in deionised water, [f]base + citric + ascorbic acid, [g]NE + citric acid.

Table 5.5 The effect of 0.5 mg/L H_2O_2 on fermented, unfermented and nano emulsified unfermented rooibos extract formulations

Extract	**Treat[a]**	**Form[b]**	**Aspalathin**	**Iso-orientin** (g/100 g extract)	**Orientin**	**Absorbance** (420 nm)
FR	Control[c]	BA[d]	0.835±0.002[e] a[f]	0.657±0.002 a	1.052±0.002 a	1.072±0.020 a
	HP[g]	BA	0.831±0.004 ab	0.654±0.006 a	1.046±0.007 a	1.070±0.011 a
	Control	BCA[h]	0.834±0.005 a	0.620±0.003 b	1.026±0.004 b	0.652±0.006 b
	HP	BCA	0.828±0.004 b	0.612±0.001 c	1.018±0.001 c	0.655±0.007 b
UR	Control	BA	19.340±0.030 a	1.494±0.003 a	1.384±0.002 ab	0.089±0.009 a
	HP	BA	19.349±0.045 a	1.492±0.007 a	1.382±0.005 b	0.087±0.002 a
	Control	BCA	19.305±0.021 a	1.498±0.010 a	1.384±0.002 ab	0.069±0.003 b
	HP	BCA	19.335±0.014 a	1.499±0.002 a	1.388±0.001 a	0.077±0.006 b
NEUR	Control	NE[i]	2.723±0.009 b	0.250±0.001 b	0.227±0.001 b	0.333±0.003 a
	HP	NE	2.723±0.005 b	0.249±0.001 b	0.228±0.001 b	0.336±0.002 a
	Control	NEC[j]	2.717±0.002 b	0.252±0.001 a	0.230±0.000 a	0.325±0.001 b
	HP	NEC	2.721±0.014 b	0.253±0.001 a	0.232±0.001 a	0.327±0.004 b

[a]Treatment, [b]formulation, [c]sample without added H_2O_2, [d]base (extract in deionised water) + ascorbic acid, [e]mean value ± standard deviation, [f]means in same column with different alphabetical letters (for the same type of extract) differ significantly at the 5% level of significance ($P<0.05$), [g]sample containing added H_2O_2, [h]B + citric + ascorbic acid, [i]nano emulsified unfermented rooibos extract in deionised water, [j]NE + citric acid. There was no formulation NECA as the NEUR extract inherently contained ascorbic acid (no further addition done).

DISCUSSION

Effect of pH and temperature on the phenolic composition and colour of rooibos extract formulations

Apart from a few minor deviations, the absorbance of both the FR and UR extract formulations increased with increasing pH. This was expected since the rate of polyphenol oxidation in aqueous solutions has been reported to increase with increasing pH (Cilliers & Singleton, 1990; Yeo & Shibamoto, 1991; García *et al*., 1992; Bucheli & Robinson, 1994) and colour changes usually accompany the pH-induced oxidation of polyphenols, i.e. polyphenol solutions generally become darker with increasing pH (Guyot *et al*., 1995). Furthermore, within each pH category, the change in absorbance of both the FR and UR extract formulations was generally found to increase with increasing temperature. This was expected as temperature is known to increase the rate of chemical reactions (Göğüş & Eren, 1998). The role of phenolic oxidation (Cilliers & Singleton, 1989), polymerisation (Cilliers & Singleton, 1989; Talcott & Howard, 1999) and electron delocalisation in absorbance changes and browning was discussed in detail in Chapter 3.

The general decrease in aspalathin, iso-orientin and orientin content of the rooibos extract formulations with increasing pH and temperature was expected as increased absorbance at 420 nm (browning) of the rooibos extracts (iced tea) was linked to flavonoid oxidation in previous chapters. Since 100% aspalathin loss was recorded for FR extract formulations at pH 6 and 7 after two days of storage, it may be concluded that aspalathin is highly susceptible to oxidation at these pH values.

The UR extract formulations exhibited greater resistance pH-induced oxidative changes than the FR extract formulations, as significant decreases in aspalathin content in the latter were only noted from pH 6 (30ºC) onwards. The greater resistance of the UR extract formulation to pH-induced oxidation may be related to its unfermented nature. The abundance of unoxidised phenolic compounds in this extract (relative to that in the FR extract) may have had an aspalathin-sparing effect. Furthermore, differences in the rate of the Fenton reaction within the various extract formulations may also have played a role. The reaction between H_2O_2 and Fe^{2+} results in the formation of hydroxyl radicals and is known as the Fenton reaction (Morris *et al*., 1995). The iron content of the FR extract is greater than that of the UR extract. The rate of the Fenton reaction is enhanced with increased iron content (Morris *et al*., 1995), although hydrogen peroxide must also be present. The hydroxyl radicals formed as a result of the progression of the Fenton reaction are capable of oxidising most organic compounds (Jeong & Yoon, 2005), including flavonoids (Zhu *et al*., 2000), such as aspalathin. Oxidation of catechins in tea (Hathway & Seakins, 1957a; Hathway & Seakins, 1957b; Chai *et al*., 2003; Aoshima & Ayabe, 2007) and mixtures of tea and herbal tea (Aoshima *et al*., 2007) has been noted to result in the formation of polymeric compounds (oxidised phenolic compounds) and H_2O_2. Flavonoid oxidation and the formation of H_2O_2 are also favoured at higher pH values, e.g. pH 7 (Aoshima & Ayabe, 2007). Due to the elevated iron content of the FR extract, compared to the UR extract, conditions in the FR extract formulations may have been

more favourable for the formation of H_2O_2, accounting for the reduced stability of rooibos flavonoids in the FR extract as a result of increased pH.

An interesting observation was, however, made for the FR extract formulation at pH 3, i.e. the aspalathin content increased significantly, compared to the control, when stored at 30ºC. Under similar pH conditions, the aspalathin content of the FR extract formulation was also noted to increase during a pasteurisation-like treatment (Chapter 3). It would thus appear that a mild temperature, in combination with a low pH, induces changes within the FR extract matrix, resulting in the increased "availability" of aspalathin. The release of aspalathin from a loose association with polymeric phenolic compounds, matrix proteins or carbohydrates (which would otherwise be filtered out prior to HPLC analysis), may be operational under these conditions. Pengilly *et al*. (2008) demonstrated that improved extraction of aspalathin is obtained after treatment of rooibos plant material with hydrolysing enzymes. Further investigation will, however, be required to clarify the matter.

The formation of polymeric compounds in flavonoid-containing solutions is not the only source of browning upon oxidation. Le Guernevé *et al*. (2004) showed that the main oxidation products of phloridzin were monomers (one of which was yellow), and not polymers. With respect to the UR extract formulation, the unexpectedly lower absorbance of the sample at 40ºC (pH 7) compared to that at 30ºC may be attributed to the quantity and/or physical size of the oxidation products formed. It could be postulated that more and/or larger polymers (coloured compounds) may have formed at 40ºC compared to 30ºC, with subsequent precipitation and removal during centrifugation (prior to absorbance reading). Their removal from the 40ºC sample may explain the reduced absorbance of this sample compared to that of the sample stored at 30ºC.

Polymeric compounds such as that arising from the oxidation of flavonoids, e.g. procyanidins has been described as "*ill defined"* (Lazarus *et al*., 1999). In particular, analysis of oligomers (≥ tetramers) is complicated by the increasing number of isomers that occur upon an increasing degree of polymerisation. Procyanidins with differing degrees of polymerisation often co-elute as one large, unresolved peak when using reverse-phase HPLC. The fact that the area of the polymeric compounds on the chromatogram of UR extract formulations does not show great variation in size between pH 3 and 7 is perhaps a further indication that large polymers may have been removed from the solution by centrifugation.

Almost no significant change in the absorbance of the NEUR extract formulation was detected between pH 3 and 6, possibly indicating minimal flavonoid oxidation in this pH range. The oxidation of compounds such as aspalathin and the formation of yellow or brown monomers and polymers were clearly restricted in this extract formulation, as confirmed by the aspalathin data. The ascorbic acid content of the NEUR extract formulation most likely played a role, as the former is a well-known antioxidant. Furthermore, in the NEUR extract, ascorbic acid and an emulsifier enclose the rooibos extract in a micelle (Anon., 2007). This micelle will undoubtedly protect the UR extract, in the NEUR formulation, from changes in pH (and hydrogen ion concentration). The decreased absorbance at pH 7, however, was unexpected, as the aspalathin content decreased significantly. A loss in aspalathin content was previously linked to increased absorbance at 420 nm. It may be speculated that oxidation (a) only proceeded to the formation of colourless intermediate oxidation

product(s) or (b) proceeded rapidly to a stage where highly polymerised compounds formed. The latter compounds would have been removed from solution by centrifugation or filtering, prior to absorbance measurement and HPLC analysis, respectively. Similarly as for the rooibos flavonoids, the stability of Trolox and α-tocopherol in Tween 20 (the emulsifier used in the NEUR extract) has been shown to be greater at pH 3 compared to pH 7 (Huang *et al*., 1996). No information regarding the stability of Tween 20, as affected by changes in pH, could be found. Further investigation into the matter is required.

The pH resistance of iso-orientin and orientin was greater than that of aspalathin in both the FR and UR extracts; no decreases in iso-orientin or orientin content were detected between pH 3 and 5. In the first instance, this may be due to the difference in oxidation state between these two compounds (flavones) compared to aspalathin (dihydrochalcone). Dihydrochalcones are more effective antioxidants than their corresponding flavanones (Dziedzic & Hudson, 1983; Dziedzic *et al*., 1985; Pratt & Hudson, 1990; Shahidi & Wanasundara, 1992; Nakamura *et al*., 2003) and flavones (Dziedzic *et al*, 1985) due to their less oxidised nature. Based on the above, iso-orientin and orientin should be less easily oxidised than the aspalathin. This has been confirmed for aspalathin and its corresponding flavones in selected antioxidant systems (Joubert *et al*., 2004).

In the second instance, the apparent greater pH resistance of iso-orientin and orientin, compared to aspalathin, may be as a result of the formation of these compounds from aspalathin (Koeppen & Roux, 1966; Krafczyk & Glomb, 2008). During fermentation, aspalathin is oxidised primarily to dihydro-iso-orientin (Koeppen & Roux, 1965; Koeppen & Roux, 1966; Marias *et al*., 2000), a flavanone that may be converted to iso-orientin via oxidation (Krafczyk & Glomb, 2008). Iso-orientin may then undergo further conversion to orientin (Krafczyk & Glomb, 2008). The formation of iso-orientin and orientin in this manner may thus partially account for the overall reduced losses of iso-orientin and orientin compared to aspalathin.

Iso-orientin and orientin exhibited greater resistance to pH-induced oxidation in the UR extract formulation compared to the FR extract formulation. The iso-orientin and orientin content of the samples at pH 5 (40°C) increased significantly, despite a lack of significant losses in aspalathin content. This would suggest that the precursor to iso-orientin (and indirectly, orientin), namely dihydro-iso-orientin, was already present in the extract formulation (Krafczyk & Glomb, 2008). Another possibility is that under the aforementioned conditions (pH 5, 40°C) aspalathin is more available in the extract matrix (greater availability would negate losses occurring as a result of oxidation). At pH 7, a significant loss of iso-orientin was detected in the UR extract. Despite this fact, the orientin content was unchanged at 30ºC and increased significantly at 40ºC. The conversion of iso-orientin into orientin seems feasible. Reaction rates for the degradation and formation of these compounds at different temperatures are required to clarify these differences.

In the NEUR extract formulation, iso-orientin and orientin were generally more susceptible to changes in pH than aspalathin, with the exception of pH 7. This was contrary to expectation as aspalathin stability was shown to be superior in this extract compared to FR or UR extracts. Reduced formation of iso-orientin and orientin from aspalathin, between pH 3 and 6, in the NEUR extract (compared to the FR and UR extract) may explain this observation. Temperature did not seemed to play a role in the stability of iso-orientin and orientin in

the NEUR extract formulation, since at a specific pH value, the iso-orientin and orientin content of the samples stored at the three different temperatures generally did not differ significantly from one another.

Effect of hydrogen peroxide on the phenolic composition and colour of rooibos extract formulations

The addition of H_2O_2 to UR extract formulations generally had no immediate effect on their absorbance, despite observed decreases in aspalathin, iso-orientin and orientin content, as well as an increase in the area of polymeric compounds. This was contrary to expectation since, in previous chapters, these changes in composition were linked to increased absorbance at 420 nm. These results can only be explained if it is assumed that the addition of H_2O_2 led to the formation of dimeric or trimeric oxidation products that were not highly coloured, (e.g. lack electron delocalisation between the monomer units).

Subsequent storage of the H_2O_2-treated samples resulted in browning (increased absorbance at 420 nm) of all the formulations. This was accompanied by further decreases in the aspalathin content of formulations B, BC and BCA. Loss of iso-orientin and orientin was only noted in formulation BC. The loss of the latter compounds in fewer formulations than aspalathin may be attributed to the greater stability of flavones against oxidation, compared to their corresponding dihydrochalcones (Dziedzic & Hudson, 1983; Dziedzic *et al*., 1985). Storage of the control samples (H_2O_2-free), however, also resulted in increases in absorbance and changes in the flavonoid content of the UR extract formulations. This finding suggests that the colour and compositional changes observed for the H_2O_2-containing formulation upon storage is not specifically linked to H_2O_2-induced oxidation.

Flavonoids are known to scavenge hydroxyl radicals (Husain *et al*., 1987), resulting in oxidation and degradation. They are also known to complex metal ions (Lopes *et al*., 1999; Andreu *et al*., 2005; Andrade *et al*., 2006), a process which may promote oxidation, as radical formation (after interaction with H_2O_2) occurs at the site of the flavonoid (Lopes *et al*., 1999). The decrease (%) in the aspalathin, iso-orientin and orientin content within a specific UR extract formulation was similar, suggesting that the structural differences between these compounds did not play a significant role during H_2O_2-induced oxidation.

The presence of ascorbic acid had a negative effect on phenolic retention upon the addition of H_2O_2, with the loss of the aforementioned compounds being more pronounced in formulations BA and BCA. This is in contrast to findings in Chapter 3 and 4, where flavonoid retention was usually greatest in formulation BCA. With respect to the Fenton reaction, however, ascorbic acid is known to increase the rate of hydroxyl radical generation via the reduction of Fe^{3+} to Fe^{2+} (Aruoma, 1994). Interestingly, the aspalathin content of BCA decreased significantly less than that of BA. Formulation BCA contains citric acid, a compound known to form a range of complexes with metal ions, depending on the pH, oxidation state of the metal ion and the metal ion:citric acid ratio (Francis *et al*., 1992; Dodge & Francis, 2002). Despite the formation of these complexes, Zepp (1992) reported that Fe^{2+} and its citrate complexes efficiently react with H_2O_2 to produce $^{\bullet}OH$ in aqueous solutions ranging in pH from pH 3 to 8. Although the addition of citric acid clearly did not inhibit radical

formation, it possibly reduced the rate of hydroxyl radical production by causing a portion of the metal ions in the solution to be less accessible for reaction with H_2O_2 (Li *et al*., 2007).

The reason for the storage-induced reduction in the iso-orientin and orientin content of formulation BC after H_2O_2 addtion, is unclear. Chen *et al*. (2001) have shown that citric acid is capable of accelerating the degradation of green tea catechins during storage, but no explanation was offered. Although the low pH of formulation BC can be expected to promote the rate Fenton reaction in the presence of excess H_2O_2 (Jeong & Yoon, 2005), the absence of further decreases in the case of formulation BCA (also containing citric acid) is interesting. The ascorbic acid in this formulation possibly initiated maximal aspalathin degradation immediately after H_2O_2 addition and a concomitant formation of iso-orientin and orientin, leading to the observed higher "stability" of these compounds in formulation BCA.

A Food and Drug Administration (FDA) regulation currently limits residual H_2O_2 to 0.5 mg/L, leached into distilled water, in finished food packages (Anon., 2000). This level of H_2O_2 was found to have no negative effect on UR or NEUR extract formulations, however, a small, but significant decrease in the aspalathin, iso-orientin and orientin content of formulation BCA of the FR extract formulation was noted. The rate of the Fenton reaction is determined by, amongst others, the availability of iron (Halliwell & Gutteridge, 1984; Namiki, 1990; Morris *et al*., 1995). The fact that the iron content of the FR extract is greater than that of either of the two unfermented extracts (UR and NEUR), possibly explains the greater occurrence of this reaction in the FR extract formulation. Furthermore, with respect to the FR extract formulation, the reduction in the aspalathin, iso-orientin and orientin content of formulation BCA, but not BA, may possibly be attributed to the difference in pH between these two formulations. Reducing agents such as ascorbic acid are known to increase the rate of the Fenton reaction (Aruoma, 1994), but both formulation BA and BCA contain this compound. The pH of formulation BCA was, however, lower than that of BA. A lower pH has been shown to benefit the progression of the Fenton reaction in the presence of externally supplied H_2O_2 (Jeong & Yoon, 2005).

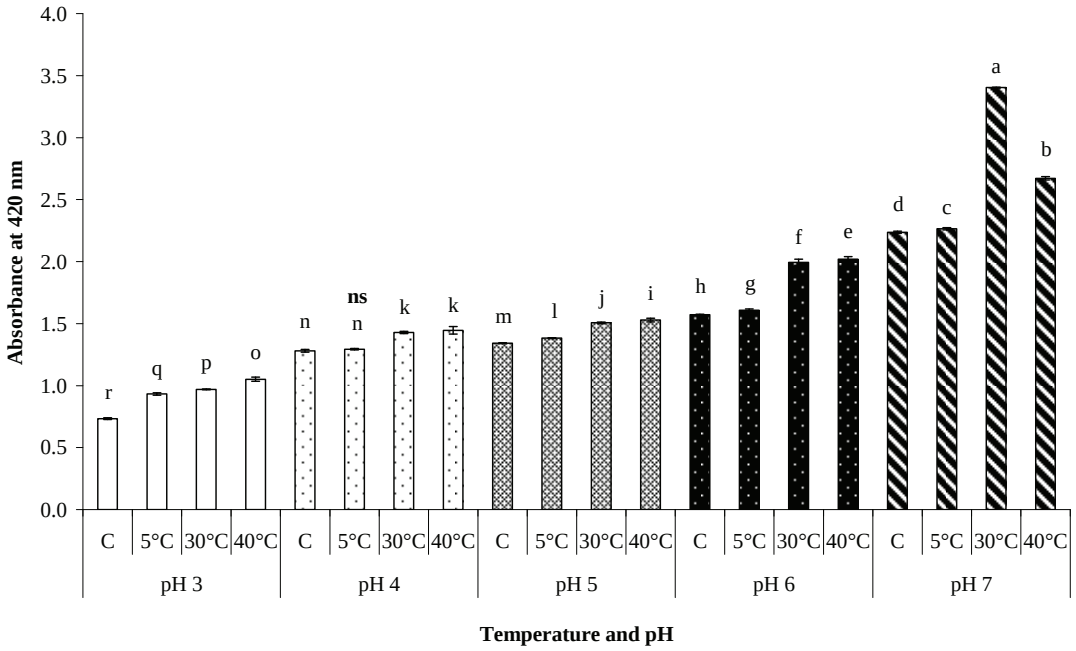
Despite the abovementioned loss/oxidation of aspalathin, as well as the decrease in iso-orientin and orientin content in FR extract formulation BCA, the absorbance was found to remain stable. Similarly, the absorbance of the UR and NEUR extract formulations (formulation BCA and formulation NEC, respectively) also remained unchanged after H_2O_2 addition. This could once again be ascribed to the formation of colourless intermediary oxidation products and/or the pH lowering effect of citric acid (Battey & Schaffner, 2001; Battey *et al*., 2002). The latter will influence the ionisation state of the flavonoids present in the solutions (Lemańska *et al*., 2001): protonation of deprotonated phenolic compounds would occur in the reduced-pH environment (Robertson, 1983; Gupta, 1989). In Chapter 3, the addition of this ingredient to rooibos iced teas was shown to result in reduced absorbance.

CONCLUSIONS

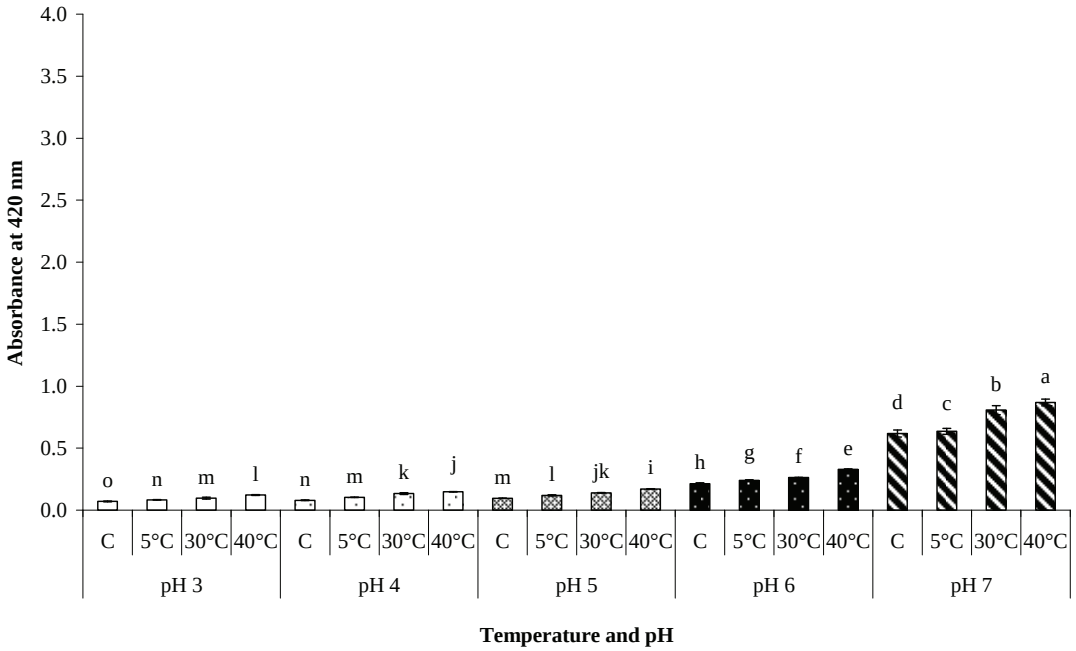
This study confirmed that the aspalathin, iso-orientin and orientin content of FR and UR extract formulations decreases with increasing pH and storage temperature. A concomitant increase in absorbance (420 nm) was observed with increasing pH and temperature, indicating that flavonoid oxidation and polymerisation occurred. The stability of aspalathin, iso-orientin and orientin to changes in pH (pH 5-7) was generally greater in the NEUR extract formulations compared to the FR and UR extract formulations. At low pH values (pH 3-4), however, the stability of the aforementioned flavonoids was superior in the UR extract formulations. Furthermore, this study confirms that the stability of the phenolic compounds present in rooibos extracts are dependent upon their structure, dihydrochalcones such as aspalathin being more susceptible to oxididation than flavones (iso-orientin and orientin). Further studies are required to confirm the mechanism of flavonoid loss in rooibos. Future studies may include investigation of the pH-induced degradation of pure rooibos flavonoids such as aspalathin: monitoring the formation of, and identifying, the oxidation product(s) formed.

The addition of H_2O_2 (660 mg/L) to UR extract formulations resulted in an immediate, significant reduction in the aspalathin, iso-orientin and orientin content, with the loss of the aforementioned compounds being more pronounced in formulations containing ascorbic acid. The progression of the Fenton reaction was implicated for the changes, with the latter effect of ascorbic acid being in accordance with that which is documented in literature. Although there were no immediate changes in the absorbance of the samples, an increase in the area of polymeric compounds was noted, confirming the occurrence of phenolic oxidation, but suggesting formation of intermediate colourless products. The loss of aspalathin in formulations containing citric acid was evident, despite the pH-lowering and metal ion-chelating ability of this compound. Storage of H_2O_2-treated solutions led to further decreases in aspalathin, iso-orientin and orientin content as well as an accompanying increase in absorbance, although this could not be ascribed to Fenton chemistry alone. Storage itself is detrimental to the retention of these compounds, as shown in Chapter 4. Furthermore, it was shown that the highest permissible levels of H_2O_2 that may be present on packaging material will likely have a minimal effect on the phenolic quality of aseptically packaged rooibos products. The loss of phenolic quality in rooibos beverages will, however, be dependent upon the iron and ascorbic acid content of the beverage, as well as the pH.

a)


Absorbance at 420 nm
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
r q p o n ns n k k m l j i h g f e d c a b
C 5°C 30°C 40°C C 5°C 30°C 40°C C 5°C 30°C 40°C C 5°C 30°C 40°C C 5°C 30°C 40°C
pH 3 pH 4 pH 5 pH 6 pH 7
Temperature and pH

b)


Absorbance at 420 nm
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
o n m l n m k j m l jk i h g f e d c b a
C 5°C 30°C 40°C C 5°C 30°C 40°C C 5°C 30°C 40°C C 5°C 30°C 40°C C 5°C 30°C 40°C
pH 3 pH 4 pH 5 pH 6 pH 7
Temperature and pH

c)

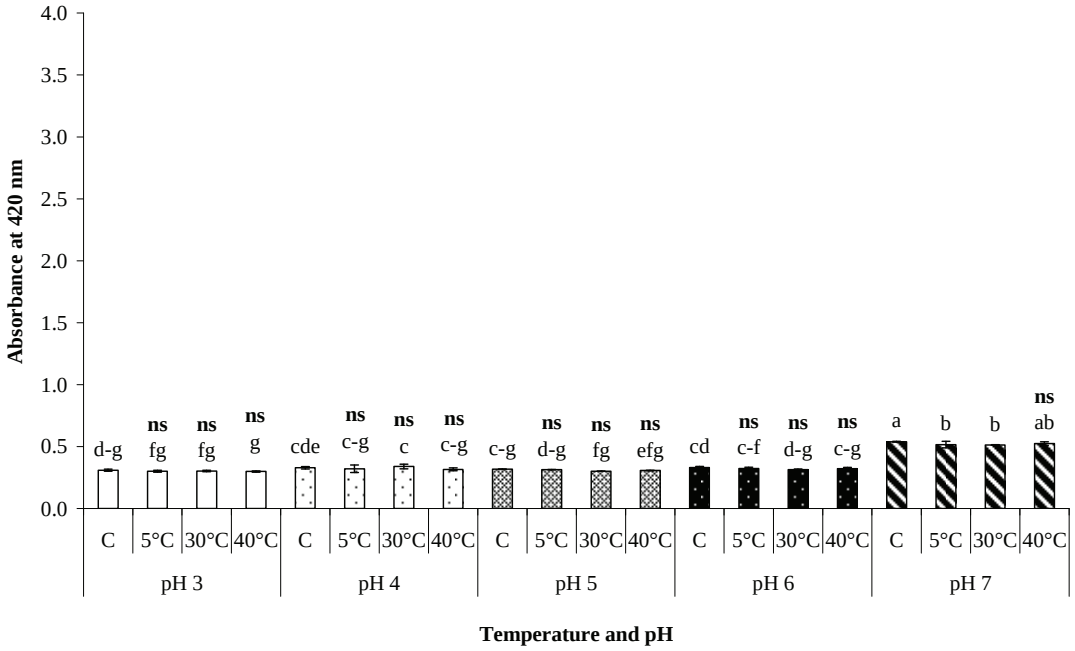
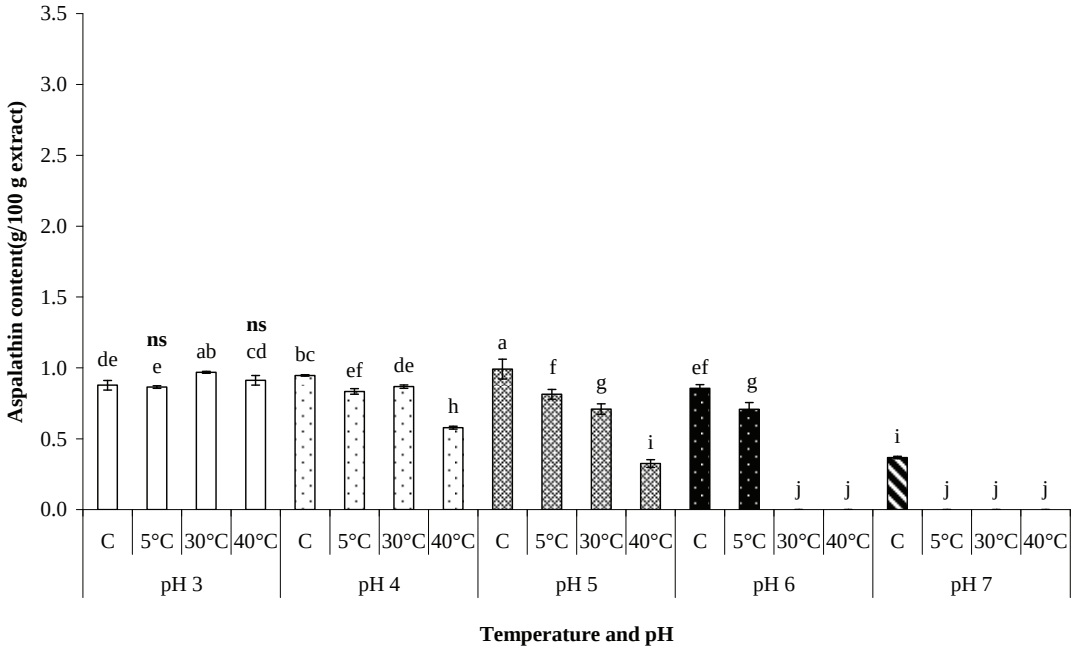
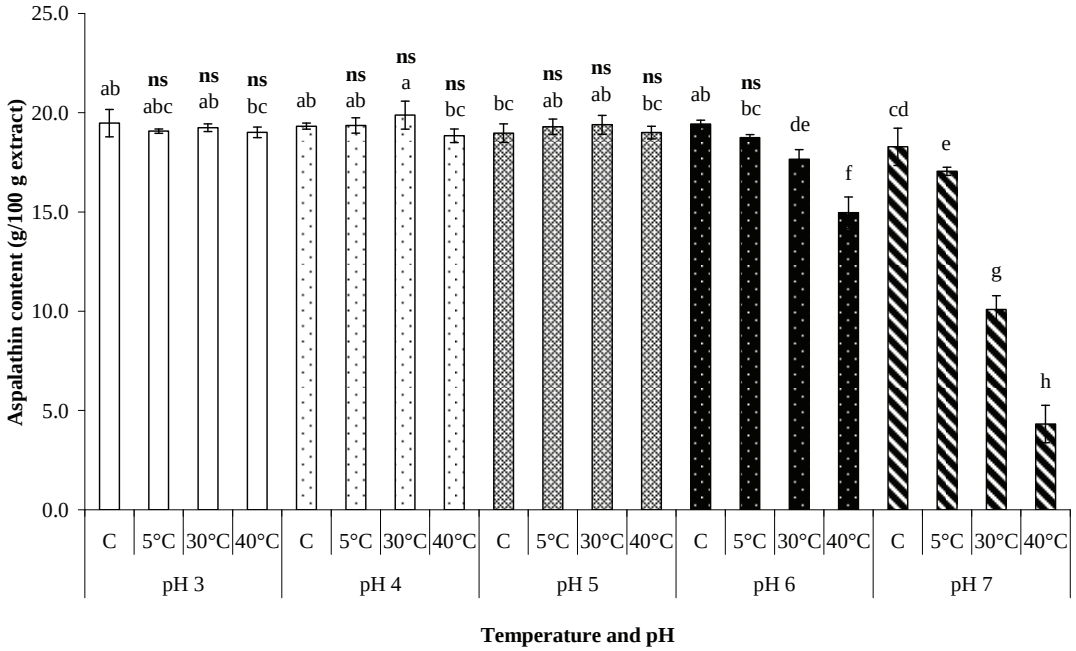


Figure 5.1 Effect of storage temperature and pH on the absorbance (420 nm) of (a) fermented (b) unfermented and (c) nano emulsified unfermented rooibos extract. Samples were stored for 48 h. Means (±SD), represented by bars, labelled with different alphabetical letters differ significantly ($P<0.05$). Means labelled with "**ns**" do not differ significantly from the control (C).

a)


Aspalathin content(g/100 g extract)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
de
ns
e
ab
ns
cd
bc
ef
de
h
a
f
g
i
ef
g
j
j
i
j
j
j
C
5°C
30°C
40°C
pH 3
pH 4
pH 5
pH 6
pH 7
Temperature and pH

b)


Aspalathin content (g/100 g extract)
25.0
20.0
15.0
10.0
5.0
0.0
ab
ns
abc
ns
ab
ns
bc
ab
ns
ab
ns
a
ns
bc
bc
ns
ab
ns
ab
ns
bc
ab
ns
bc
de
f
cd
e
g
h
C
5°C
30°C
40°C
pH 3
pH 4
pH 5
pH 6
pH 7
Temperature and pH

c)

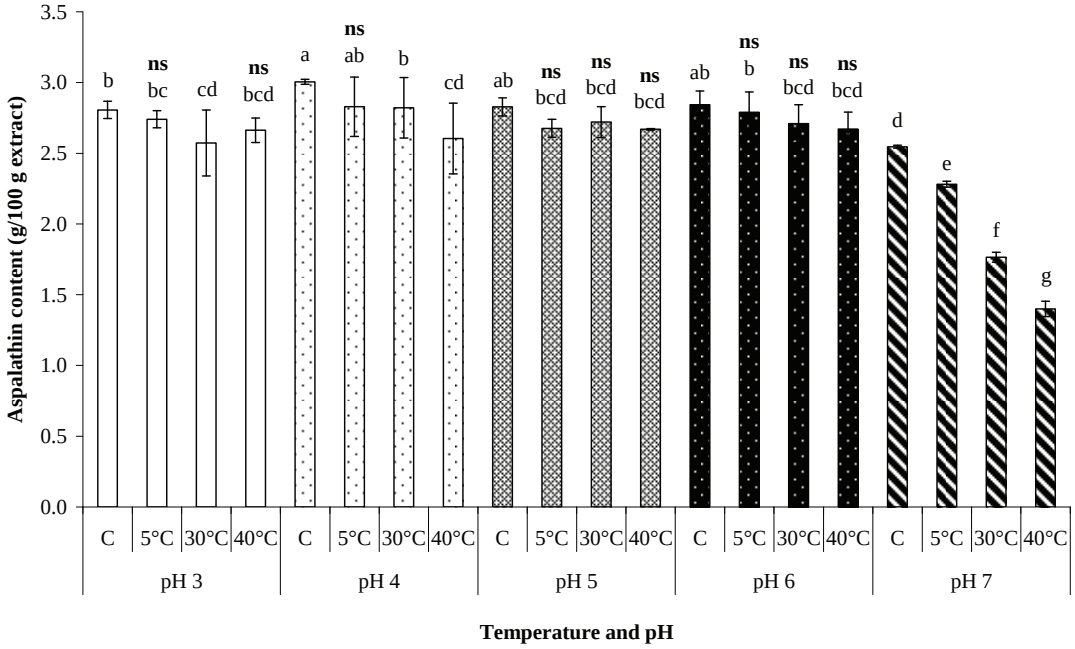
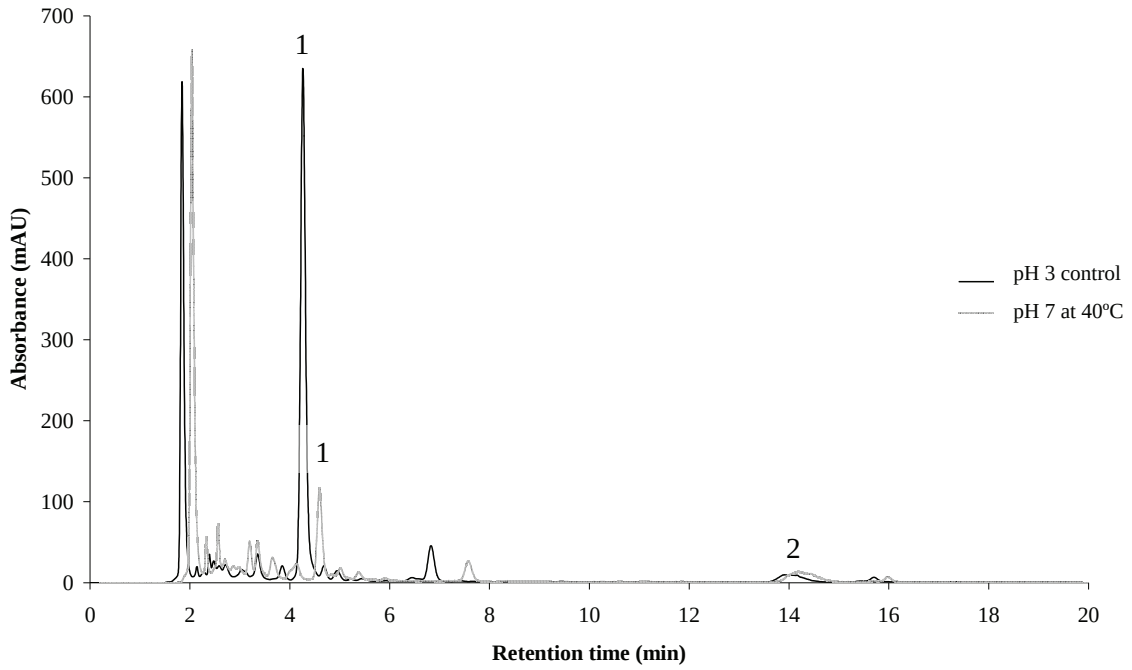


Figure 5.2 Effect of storage temperature and pH on the aspalathin content of (a) fermented (b) unfermented and (c) nano emulsified unfermented rooibos extract. Samples were stored for 48 h. Means (±SD), represented by bars, labelled with different alphabetical letters differ significantly ($P<0.05$). Means labelled with "**ns**" do not differ significantly from the control (C).

a)



b)

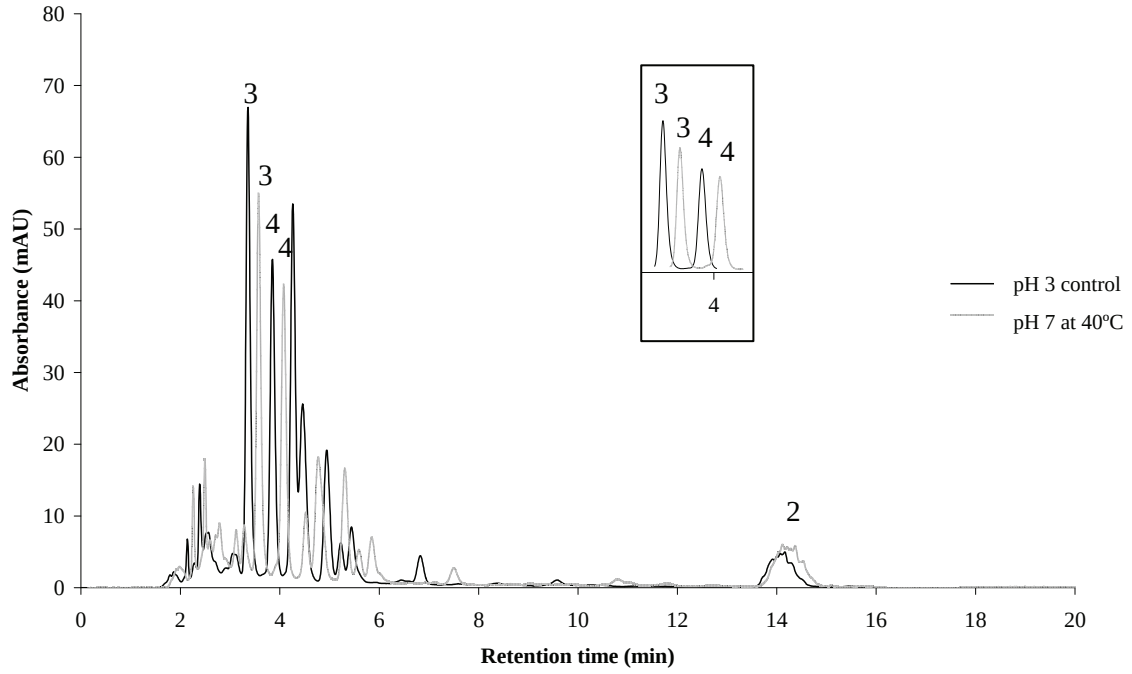
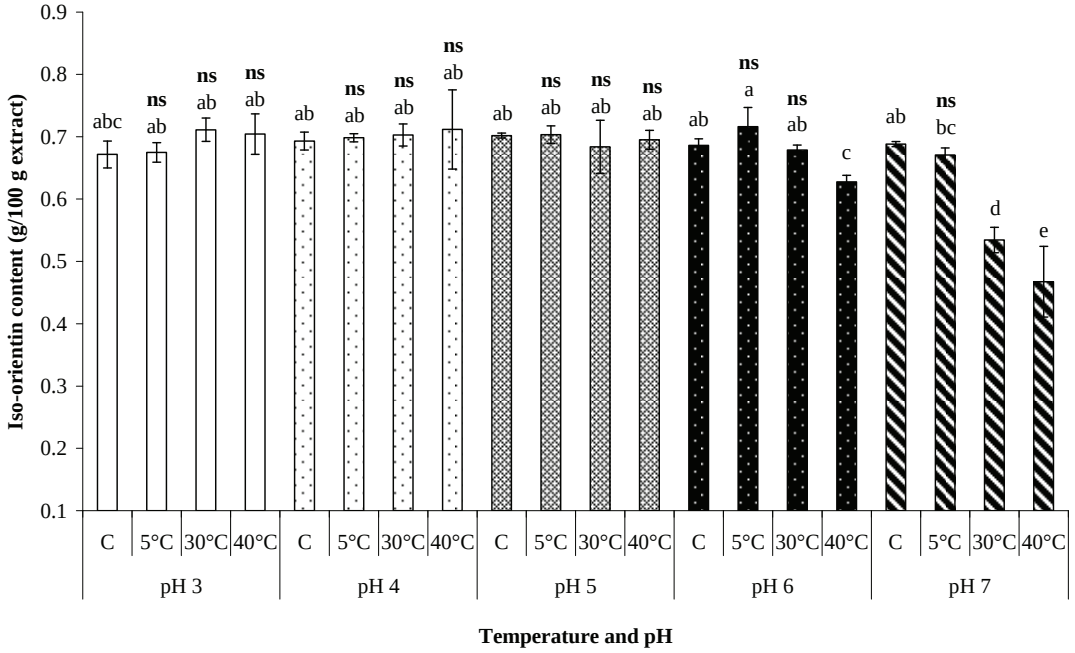
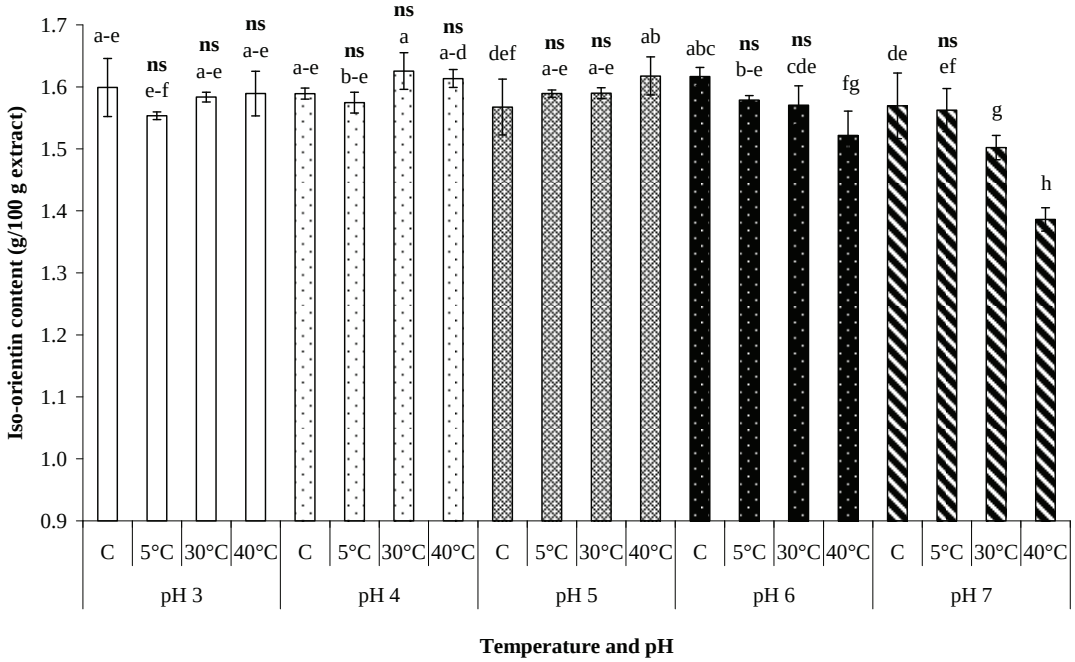


Figure 5.3 Effect of storage temperature and pH on the aspalathin content of unfermented rooibos (a) at 288 nm and (b) 350 nm. Indicated are (1) aspalathin, (2) polymeric compounds, (3) iso-orientin and (4) orientin. The x-axis of the pH 7 chromatogram was slightly offset to enable visual comparison with the control.

a)


Iso-orientin content (g/100 g extract)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
abc ns ab ns ab ns ab ns ab ns ab ns ab ns ab ns a ns ab c ns ab ns bc d e
C 5°C 30°C 40°C C 5°C 30°C 40°C C 5°C 30°C 40°C C 5°C 30°C 40°C C 5°C 30°C 40°C
pH 3 pH 4 pH 5 pH 6 pH 7
Temperature and pH

b)


Iso-orientin content (g/100 g extract)
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
a-e ns e-f ns a-e ns a-e a-e ns b-e ns a ns a-d def ns a-e ns a-e ab abc ns b-e ns cde fg de ns ef g h
C 5°C 30°C 40°C C 5°C 30°C 40°C C 5°C 30°C 40°C C 5°C 30°C 40°C C 5°C 30°C 40°C
pH 3 pH 4 pH 5 pH 6 pH 7
Temperature and pH

c)

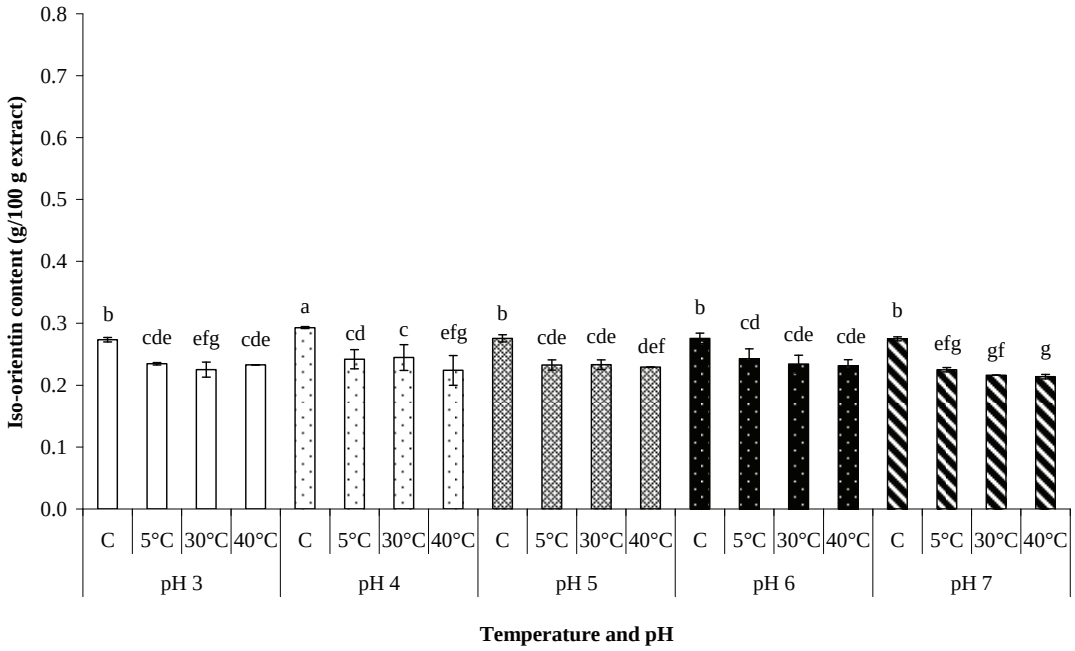
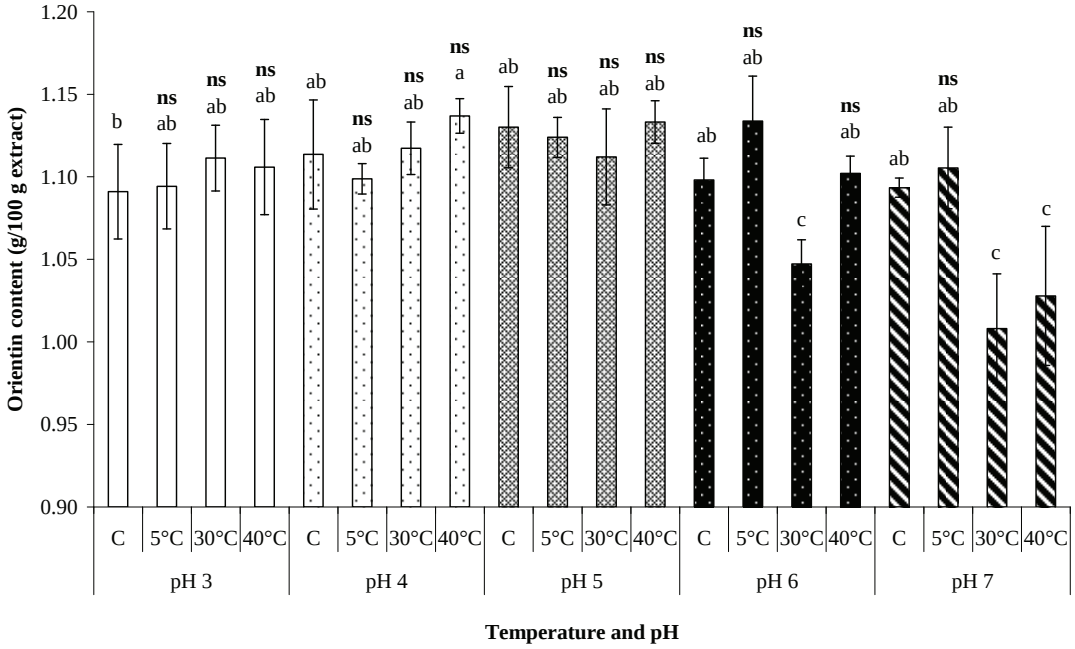
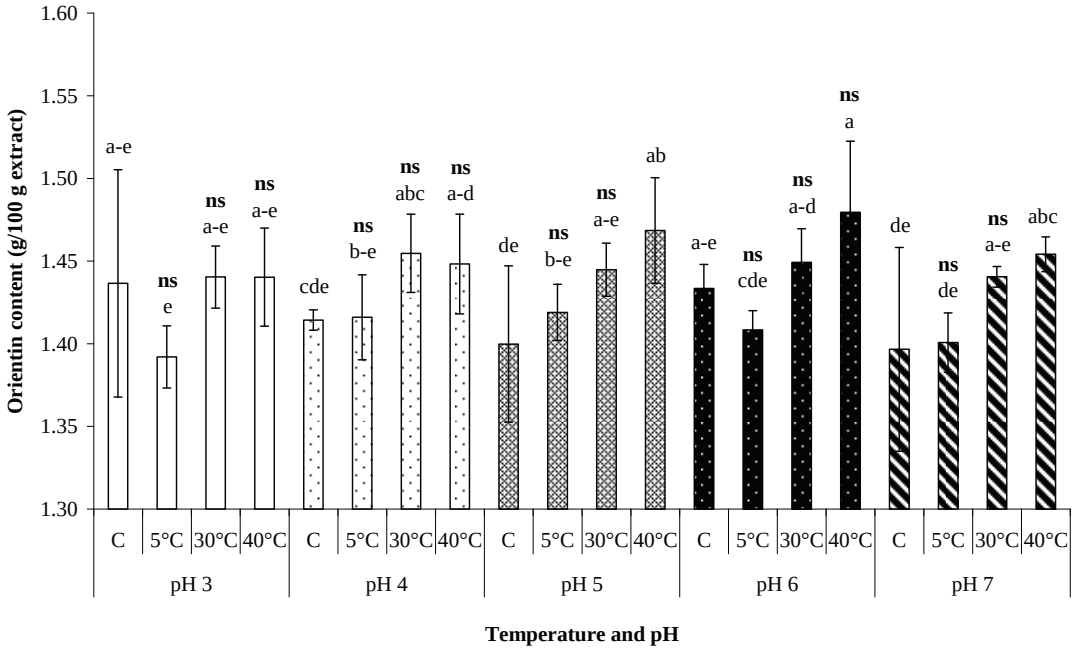


Figure 5.4 The effect of temperature and pH on the iso-orientin content of (a) fermented (b) unfermented and (c) nano emulsified unfermented rooibos extract. Samples were stored for 48 h. Means (±SD), represented by bars, labelled with different alphabetical letters differ significantly ($P<0.05$). Means labelled with "**ns**" do not differ significantly from the control (C).

a)


Orientin content (g/100 g extract)
1.20
1.15
1.10
1.05
1.00
0.95
0.90
b
ns ab
ns ab
ns ab
ab
ns ab
ns ab
ns a
ab
ns ab
ns ab
ns ab
ab
ns ab
c
ns ab
ab
ns ab
c
c
C
5°C
30°C
40°C
pH 3
pH 4
pH 5
pH 6
pH 7
Temperature and pH

b)


Orientin content (g/100 g extract)
1.60
1.55
1.50
1.45
1.40
1.35
1.30
a-e
ns e
ns a-e
ns a-e
cde
ns b-e
ns abc
ns a-d
de
ns b-e
ns a-e
ab
a-e
ns cde
ns a-d
ns a
de
ns de
ns a-e
abc
C
5°C
30°C
40°C
pH 3
pH 4
pH 5
pH 6
pH 7
Temperature and pH

c)

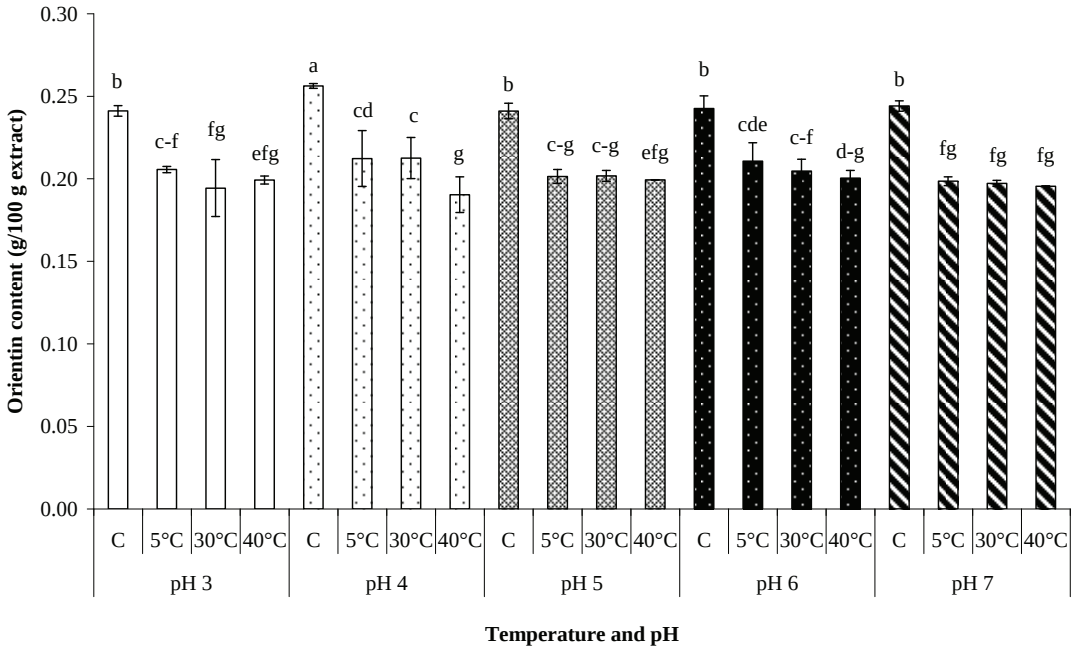
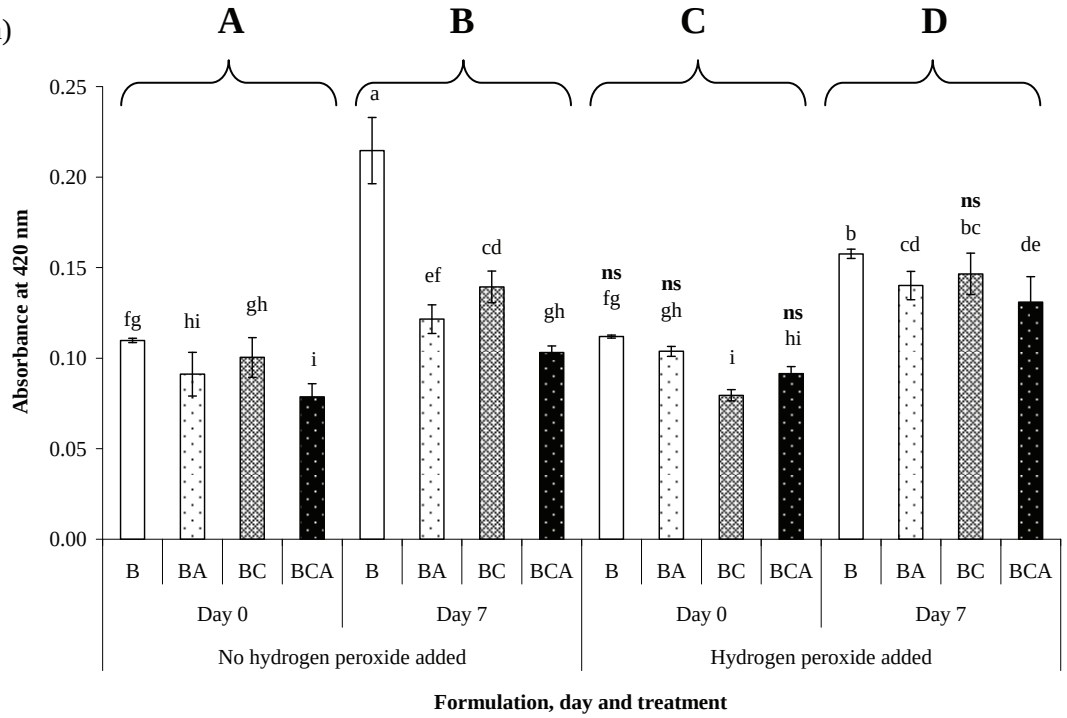
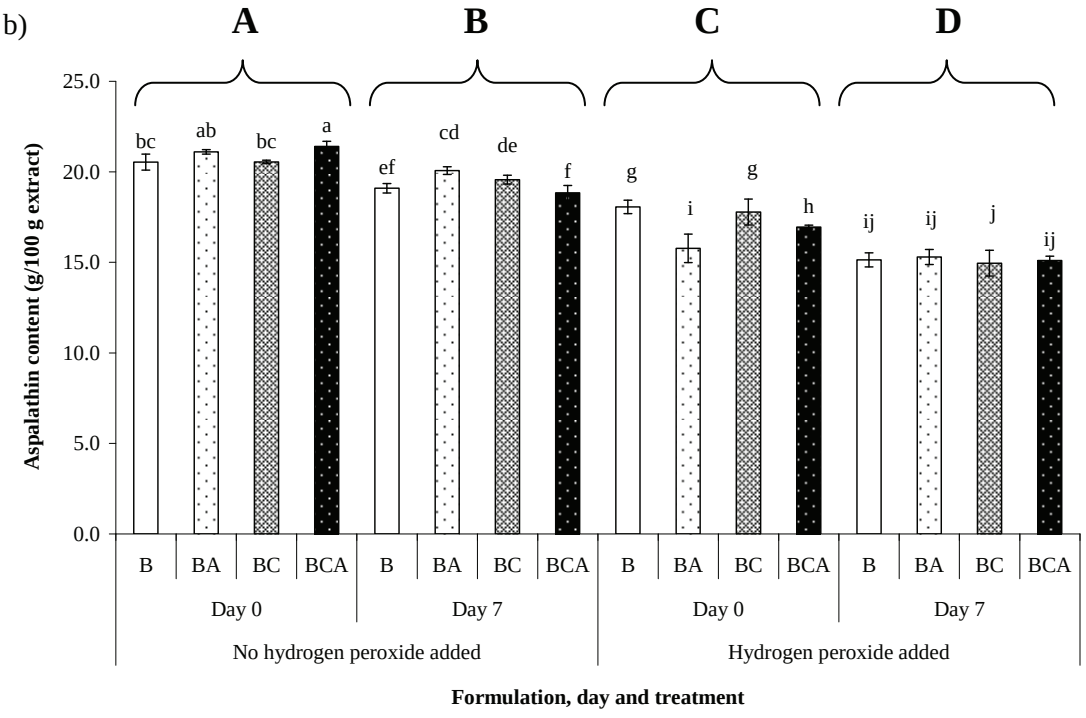


Figure 5.5 The effect of temperature and pH on the orientin content of (a) fermented (b) unfermented and (c) nano emulsified unfermented rooibos extract. Samples were stored for 48 h. Means (±SD), represented by bars, labelled with different alphabetical letters differ significantly ($P<0.05$). Means labelled with "**ns**" do not differ significantly from the control (C).

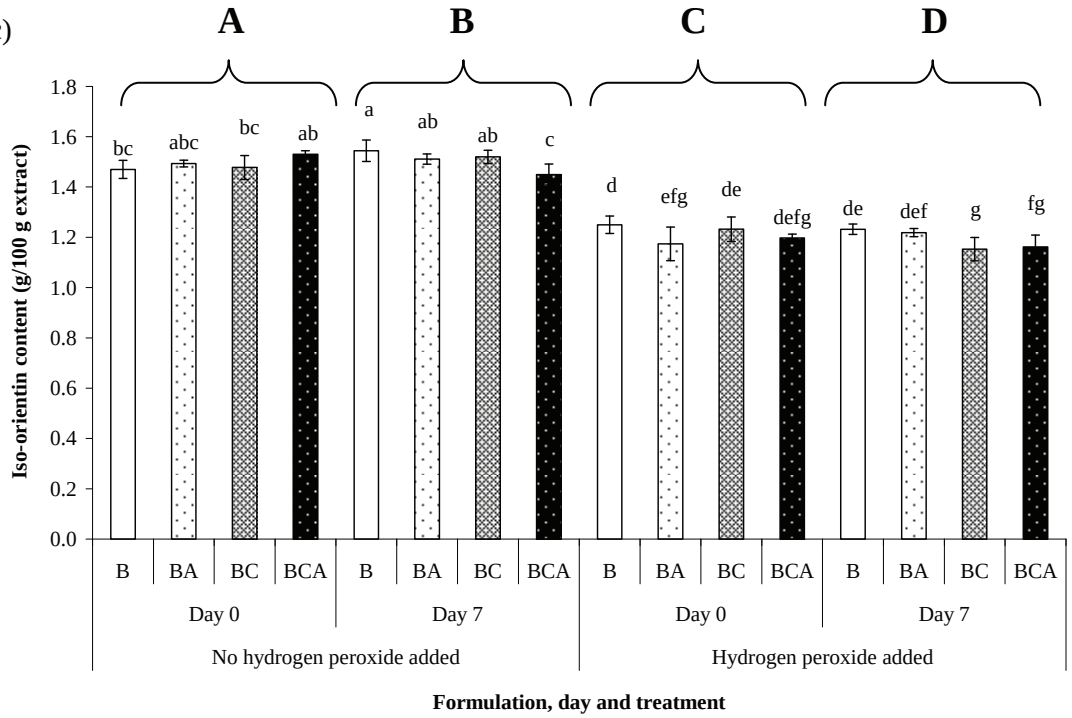
a)


A
B
C
D
Absorbance at 420 nm
0.25
0.20
0.15
0.10
0.05
0.00
fg
hi
gh
i
a
ef
cd
gh
ns
fg
ns
gh
i
ns
hi
b
cd
ns
bc
de
B
BA
BC
BCA
Day 0
Day 7
No hydrogen peroxide added
Hydrogen peroxide added
Formulation, day and treatment

b)


A
B
C
D
Aspalathin content (g/100 g extract)
25.0
20.0
15.0
10.0
5.0
0.0
bc
ab
bc
a
ef
cd
de
f
g
i
g
h
ij
ij
j
ij
B
BA
BC
BCA
Day 0
Day 7
No hydrogen peroxide added
Hydrogen peroxide added
Formulation, day and treatment

c)



d)

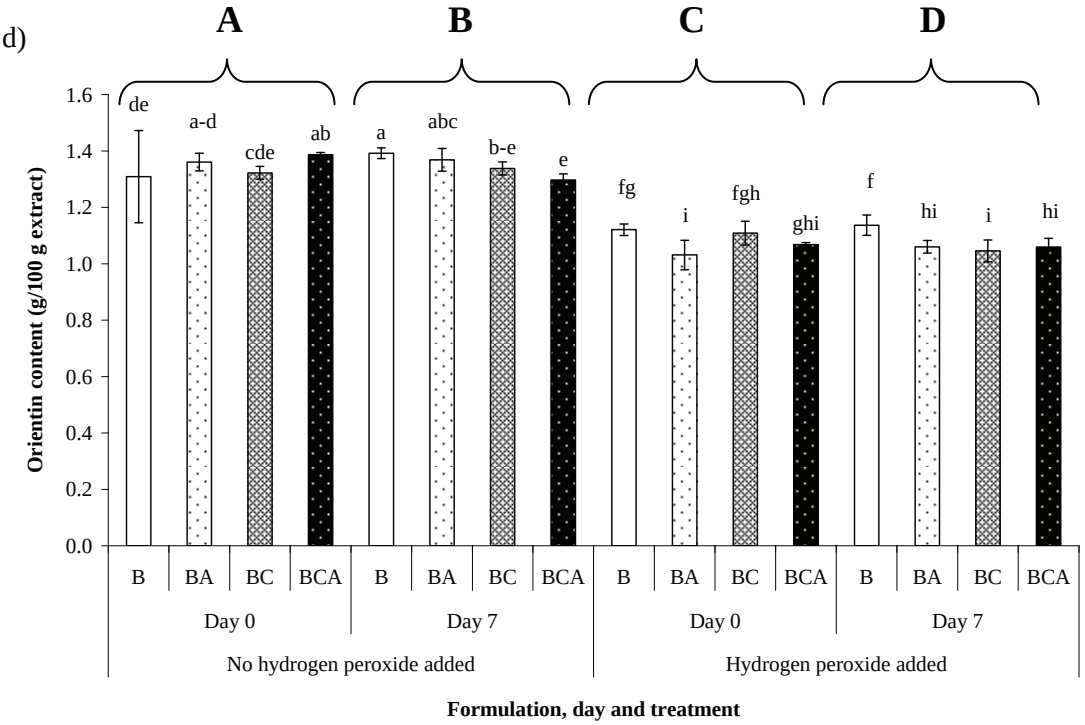


Figure 5.6 Effect of formulation, H_2O_2 addition and storage on the (a) absorbance at 420 nm, (b) aspalathin content, (c) iso-orientin content and (d) orientin content of unfermented rooibos extract. Formulation B = base, BA = base + ascorbic acid, BC = base + citric acid and BCA = base + citric + ascorbic acid. Means (±SD), represented by bars, labelled with different alphabetical letters differ significantly ($P<0.05$). Means labelled with "**ns**" do not differ significantly from the control (no H_2O_2 added). The bars bracketed with A, B, C and D are included for ease of comparison and are referred to in the text.

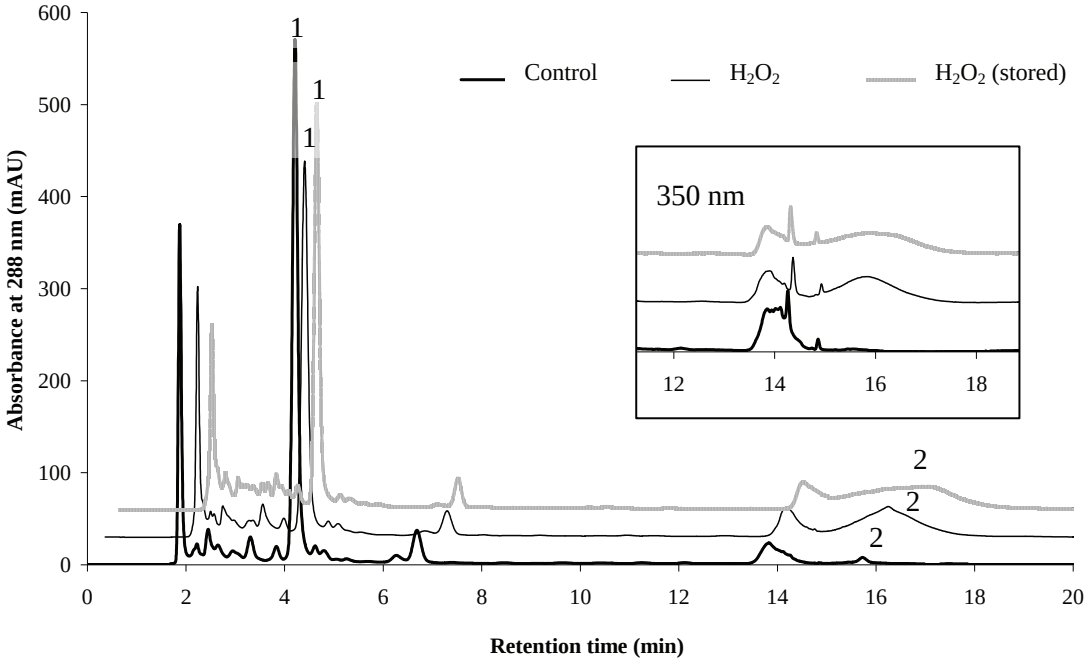


Figure 5.7 Chromatogram of unfermented rooibos extract (formulation B) at 288 nm before H_2O_2 addition (control), after H_2O_2 addition and after one week of storage at 30ºC (H_2O_2 stored). The x- and y-axes of the latter two chromatograms were slightly offset to enable visual comparison (of the polymeric compounds) with the control. Injection volume of the sample was 5 µL. The box indicates the change in the polymeric compounds of the sample at 350 nm. Indicated are (1) aspalathin and (2) polymeric compounds.

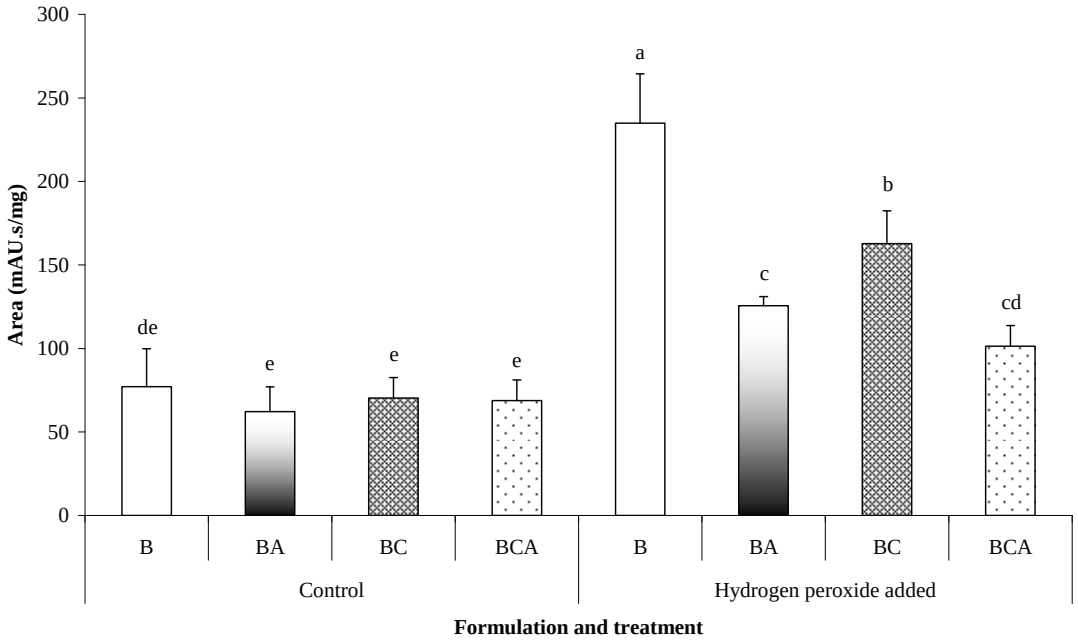


Figure 5.8 Area of the polymeric compounds at 288 nm before (control) and after H_2O_2 addition. Means (±SD), represented by bars, labelled with different alphabetical letters differ significantly ($P<0.05$). B = base (extract in water), BA = base + ascorbic acid, BC = base + citric acid, BCA = base + citric + ascorbic acid.

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CHAPTER 6

SENSORY ATTRIBUTES OF ROOIBOS ICED TEA AND THE DEGREE OF LIKING OF FLAVOURED ICED TEA SAMPLES

CHAPTER 6

SENSORY ATTRIBUTES OF ROOIBOS ICED TEA AND THE DEGREE OF LIKING OF FLAVOURED ICED TEA SAMPLES

ABSTRACT

Eight rooibos iced tea samples were analysed for their sensory attributes with the help of descriptive sensory analysis. The eight iced teas were: fermented rooibos (F); lemon flavoured fermented rooibos (F/LEMON); nano emulsified unfermented rooibos (N); lemon flavoured nano emulsified unfermented rooibos (N/LEMON); nano emulsified unfermented rooibos and fermented rooibos (NF); lemon flavoured nano emulsified unfermented rooibos and fermented rooibos (NF/LEMON); unfermented rooibos (UF) and lemon flavoured unfermented rooibos (UF/LEMON). Four flavour descriptors were used in the analysis, i.e. plant-like; hay-like; rooibos; and lemon. Astringency was also evaluated. The degree of liking of the four flavoured variants of rooibos iced tea was evaluated by a consumer panel consisting of iced tea drinkers.

Two iced tea formulation exhibited a plant-like character, i.e. UF and UF/LEMON. The addition of lemon flavour significantly ($P<0.05$) reduced this characteristic. Four of the iced tea samples were found to have a hay-like flavour, i.e. N; N/LEMON; NF and NF/LEMON. The hay-like flavour of NF was significantly ($P<0.05$) lower than that of N and the addition of lemon flavour significantly reduced ($P<0.05$) the hay-like character of both these teas. Almost no hay-like flavour was detected in the remaining four iced teas.

The iced tea samples containing FR extract all exhibited a prominent rooibos flavour. The perceived intensity of this characteristic decreased in the order F>F/LEMON>NF>NF/LEMON. Lastly, the trained panel could distinguish between the iced tea samples containing lemon flavour and those without. Lemon flavour was perceived to be greater in F/LEMON and N/LEMON than NF/LEMON or UF/LEMON, despite the four teas containing exactly the same amount of lemon flavour.

All the samples exhibited a measure of astringency. The astringency of F was the greatest and the addition of lemon flavour significantly ($P<0.05$) reduced the astringency of this iced tea. Iced teas made with the unfermented rooibos (UR) extract (UF and UF/LEMON) were judged to be significantly ($P<0.05$) less astringent than those made with fermented rooibos (FR) extract (F and F/LEMON). The addition of lemon flavour did not reduce the astringency of UF and NF, but did significantly ($P<0.05$) reduce the astringency of N. Overall, the astringency of N and NF, as well as their flavoured counterparts, was low (<10%).

Principal component analysis (PCA) indicated that the iced teas made with UR extract (UF and UF/LEMON) were correlated with plant-like flavour and that a hay-like flavour correlated well with iced tea formulations containing the nano emulsified unfermented rooibos (NEUR) extract (i.e. N and NF, as well as their flavoured variants). The iced teas containing FR extract, namely F and F/LEMON, were both highly

correlated with rooibos flavour. Apart from NF/LEMON, none of the iced teas correlated strongly with lemon flavour. Plant-like flavour was negatively correlated with hay-like, rooibos and lemon flavour.

The four flavoured iced teas were evaluated by a consumer panel, 77% of whom indicated that they drink iced tea at least once a month. The panel indicated a preference for F/LEMON and NF/LEMON above N/LEMON. The consumers disliked UF/LEMON. Preference mapping indicated that the presence of rooibos flavour and the absence of a plant-like flavour most likely drive preference of rooibos iced tea. The presence of a slight hay-like character (as in NF/LEMON) did not significantly ($P \geq 0.05$) reduce the liking of rooibos iced teas.

Despite the comparatively higher total flavonoid and aspalathin content of UF/LEMON compared to F/LEMON, consumers disliked the product. The sensory data indicated that the development of a rooibos iced tea with the abovementioned phenolic characteristics is only likely to be successful if the flavour profile of the tea corresponds to that of the fermented beverage.

INTRODUCTION

Natural antioxidant compounds, such as flavonoids (Bors *et al*., 1990; Das, & Pereira, 1990; Hanasaki *et al*., 1994), carotenoids (Gey *et al*., 1993; Pellegrini *et al*., 1999) and tocopherols (Raskin *et al*., 2002) have recently been receiving increased attention as food ingredients. This follows reports linking the consumption of a diet containing plenty of antioxidant-rich fruits and vegetables (Hertog *et al*., 1992; Hertog *et al*., 1993) with reduced disease incidence (Hertog *et al*., 1995). As a result, food and beverage manufacturers are suddenly experiencing an increase in the demand for natural, healthy, antioxidant-containing food and beverage products, i.e. products with benefits beyond basic nutrition (Bech-Larsen & Scholderer, 2007). Rooibos falls in this category, and consumption of this beverage, as well as ready-to-drink alternatives such as rooibos iced tea, has increased significantly in South Africa (Anon., 2006).

Rooibos has been marketed as a caffeine-free (Blommaert & Steenkamp, 1978), low tannin alternative to black or green tea. It is brewed from the leaves of *Aspalathus linearis* and has an agreeable, sweet, honey-like aroma (Joubert & Ferreira, 1996). Apart from its natural appeal, rooibos is the only source of aspalathin (Koeppen & Roux, 1965; Joubert, 1996), a dihydrochalcone with significant antioxidant (Von Gadow *et al*., 1997a; Joubert *et al*., 2004; Joubert *et al*., 2005) and antimutagenic activity (Standley *et al*., 2001; Marnewick *et al*., 2005; Van der Merwe *et al*., 2006).

In order to produce traditional rooibos, the plant material undergoes a “fermentation” (oxidation) step (Joubert & De Villiers, 1997). This process significantly reduces its antioxidant capacity (Von Gadow *et al*., 1997a). Despite the popularity of traditional rooibos, the production of iced tea from unfermented rooibos would better suit current consumer demands for antioxidant-rich food and beverage products. As such, no unfermented rooibos iced tea has yet emerged on the South African or global markets.

Sensory analysis is a powerful tool for food scientists as it provides important information concerning the sensory characteristics of new and existing food products and beverages (Lawless & Heymann, 1998a). Two of the sensory techniques frequently employed for the analysis of food products are

descriptive sensory analysis and testing the degree of liking. *Descriptive sensory analysis* is the internationally accepted means of profiling food products (Lawless & Heymann, 1998b). This technique makes use of a trained panel of judges to determine the *sensory attributes* of the food and beverage products in question. The *nine-point hedonic scale* is an internationally recognized scale, found to be extremely useful in the hedonic assessment of food and beverage products (Lawless & Heymann, 1998c). Consumers are asked to indicate which one of nine terms ranging from (9) *like extremely* to (1) *dislike extremely* best describes his/her attitude towards the product being tasted. The test makes use of unbranded (unlabelled) products and gives an indication of their preference as well as acceptance (Lawless & Heymann, 1998c). An indication of purchase intent is not given. This test also does not ask consumers to motivate their choices as consumers may vary with respect to their interpretation of a product attribute (McEwan *et al*., 1998). For this reason, a means by which the sensory quality attributes of a product may be related to consumer liking will be useful in the understanding of product preference, i.e. which attributes most likely drive preference for a particular product (McEwan *et al*., 1998). Preference mapping is such a tool and makes use of multivariate statistical methods to link sensory and consumer data (McEwan *et al*., 1998). Unlike univariate analysis, product scores are not averaged over consumers but rather direction of individual preference is indicated. There are two types of preference mapping, namely internal and external preference mapping. Internal preference mapping is a combined map of the products' and the consumers' preference ratings. External reference mapping relates consumer preference to a product map obtained from other product data, such as sensory, physical or chemical product information. The strength of preference mapping lies in the fact that it allows for individual preference analysis (McEwan *et al*., 1998). Consumers with contrasting preferences may be observed with preference mapping, whereas such data would be lost in univariate analysis.

The aim of this study was to compare the sensory characteristics of a traditional ("fermented") rooibos iced tea to that of two unfermented counterparts and a fermented-unfermented mixture. It was aimed to make a prediction concerning the consumer liking of unfermented rooibos iced tea in comparison to a "regular" fermented product. Eight iced teas were tested for a spectrum of sensory attributes using a trained panel and the technique of descriptive sensory analysis, while four of the iced teas were then tested for their degree of liking. Preference mapping was used to relate the iced teas to consumer preference and establish the identity of the sensory attributes driving preference of rooibos iced tea. High pressure liquid chromatography (HPLC) analysis was used to determine the aspalathin, iso-orientin and orientin content of the teas whilst the Folin-Ciocalteu assay was used to determine their total phenolic content, allowing for comparison of the iced teas on a non-sensory basis.

MATERIALS AND METHODS

Chemicals and reagents

The chemicals and reagents required for HPLC analysis and total polyphenol content determination are as listed in Chapter 3.

Rooibos extracts and rooibos iced tea formulations

The three rooibos extracts characterised and used in Chapter 3, namely fermented rooibos (FR), unfermented rooibos (UR) and nano emulsified unfermented rooibos (NEUR) were used for iced tea formulation in this study. New codes (abbreviations) were used to define the iced teas in this study as the extract concentration differed from that used in the previous studies. In this study, the extract quantities were adapted to produce acceptable iced teas. In total, eight iced teas were formulated and analysed (Table 6.1). The eight iced teas were: fermented rooibos (F); lemon flavoured fermented rooibos (F/LEMON); nano emulsified unfermented rooibos (N); lemon flavoured nano emulsified unfermented rooibos (N/LEMON); nano emulsified unfermented rooibos and fermented rooibos (NF); lemon flavoured nano emulsified unfermented rooibos and fermented rooibos (NF/LEMON); unfermented rooibos (UF) and lemon flavoured unfermented rooibos (UF/LEMON). The iced teas all contained 60 g/L sugar, 1.2 g/L citric acid and 0.2 g/L ascorbic acid (suppliers listed in Chapter 3). Lemon flavour (E225043) was obtained from Mane (Kenilworth, South Africa).

Table 6.1 The composition of the eight iced tea samples

	Extract (g/L)			**Lemon flavour (ml/L)**
Iced tea sample	**FR[a]**	**UR[b]**	**NEUR[c]**	
N[d]	-	-	2.200	-
UF[e]	-	0.625	-	-
F[f]	1.250	-	-	-
NF[g]	0.625	-	1.100	-
N/LEMON	-	-	2.200	3.250
UF/LEMON	-	0.625	-	3.250
F/LEMON	1.250	-	-	3.250
NF/LEMON	0.625	-	1.100	3.250

[a]Fermented rooibos, [b]unfermented rooibos, [c]nano emulsified unfermented rooibos, [d]nano emulsified unfermented rooibos iced tea, [e]unfermented rooibos iced tea, [f]fermented rooibos iced tea, [g]nano emulsified unfermented rooibos and fermented rooibos iced tea. For each of the aforementioned formulations, a lemon variant was prepared.

pH determination of rooibos iced tea

The pH of the various iced tea solutions was determined as described in Chapter 3.

Determination of the total polyphenol content of rooibos iced tea

The polyphenol content of the iced tea samples was determined using the method by Singleton & Rossi (1965), scaled-down for a microplate reader. Gallic acid served as a standard. The procedure was described in Chapter 3.

HPLC quantification of the phenolic content of rooibos iced tea

Quantification of aspalathin, orientin and iso-orientin was performed by HPLC-DAD. The HPLC apparatus, column, gradient profile for separation, software, standards and sample preparation procedure were as described in Chapter 3.

Trained panel

The technique of descriptive sensory analysis was used to analyse the attributes of the iced tea samples. The panel of trained judges consisted of twenty-six (n = 26) individuals, trained according to the consensus method as described by Lawless & Heymann (1998b). A 100 mm unstructured line scale was used for attribute intensity analysis. The left side of the scale corresponded to the lowest intensity (zero) and the right side corresponded to the highest intensity (100). The eight iced teas (Table 6.1) were analysed on three separate occasions, for five sensory attributes, as agreed upon according to the consensus method. The attributes were plant-like flavour, hay-like flavour, rooibos flavour, lemon flavour and astringency. The scale used to analyse the various sensory attributes of rooibos iced tea is given in Table 6.2. An example of the questionnaire used for the analysis is included in ADDENDUM 8. It was decided not to analyse the samples for aroma in addition to flavour as the latter attributes gave similar results to that of aroma.

Table 6.2 Descriptors for the sensory attributes of rooibos iced tea

Sensory attributes		Description	Scale
Flavour	Plant-like	Green, plant-like flavour associated with the unfermented rooibos extract	0 = none, 100 = Prominent plant-like flavour
	Hay-like	Hay-like flavour associated with the nano emulsified unfermented rooibos extract	0 = none, 100 = Prominent hay-like flavour
	Rooibos	Typical of a fermented rooibos extract	0 = none, 100 = Prominent rooibos flavour
	Lemon	Typical of lemon flavour used in commercial rooibos iced teas	0 = none, 100 = Prominent lemon flavour
Astringency			0 = none, 100 = Prominent astringent mouth feel

rooibos flavour was described as that which is typical of a fermented rooibos extract.

The samples were presented in a randomised order. The sample size was 30 mL and the treatments were served at room temperature (±21°C) in standard wine tasting glasses, closed with clear, polystyrene Petri dish lids (Kimix, Cape Town). Each sample was coded with a three-digit random code. The judges

used still mineral water and unsalted, fat free biscuits (Water Biscuits, Woolworths) to cleanse their palates. Analyses were conducted in a light and temperature controlled room (±21°C).

Consumer panel

The degree of liking of four variants of lemon flavoured iced tea, namely F/LEMON, N/LEMON, NF/LEMON and UF/LEMON was tested using a consumer panel. The unflavoured samples were not analysed as commercial iced teas are mostly flavoured. For this project, the questionnaire was completed by 97 target consumers (regular iced tea consumers) above the age of 18. Approximately 28% of the consumers were male (n = 27) and 72% were female (n = 70). The gender distribution of this panel closely represents that of the iced tea target market in South Africa (G. Pistorius, R&D Manager of beverages, Parmalat, South Africa, personal communication, 2007).

The consumers were asked to complete one questionnaire, comparing the overall degree of liking of the four samples in front of them (ADDENDUM 9). The consumers scored the products using the nine point hedonic scale: (9) *Like extremely*, (8) *Like very much*, (7) *Like moderately*, (6) *Like slightly*, (5) *Neither like nor dislike*, (4) *Dislike slightly*, (3) *Dislike moderately*, (2) *Dislike very much*, (1) *Dislike extremely* (Lawless & Heymann, 1998c). The samples were presented in a randomised order. Samples (30 mL) were served directly from the refrigerator (*ca*. 10°C) in clean plastic glasses, coded with a 3-digit random code. Analyses were conducted in a light and temperature controlled room (*ca*. 21°C).

Statistical analysis

The trained panel of judges was tested for consistency (judge × replicate and judge × product interaction) after which the 15 most reliable judges were selected. The selected judges analysed the iced tea samples according to the sensory attributes and the data were subjected to statistical analysis. The consumer sensory data comprised data collected from 97 target individuals. Both the trained panel and consumer sensory data were subjected to analysis of variance (ANOVA) and t-tests to ascertain whether significant differences existed between samples. Differences with a significance level of 5% are considered significant (Ott, 1998; SAS, 2002). Data were tested for normality using the Shapiro-Wilk test for normality. The P-value for the Shapiro-Wilk test for normality should be larger than 0.05 if the data is normally distributed (Shapiro & Wilk, 1965). Preference mapping was performed using XLstat (Version 2007). Internal preference mapping was performed using MDPREF (multidimensional analysis of preference data). This method is based on principal component analysis (PCA) performed on the preference data with the products as observations (scores) and the consumers as variables (loadings) (Anon., 2008). The map obtained is a bi-plot of the observations and the variables. External preference mapping was performed in two steps, namely by (a) mapping the iced tea products on the basis of their sensory characteristics, using PCA and then (b) applying the PREFMAP method. The latter involves regressing of the consumer data (y space) onto the trained panel data (x space) using a partial least squared (PLS) regression.

RESULTS

Phenolic content

Iced tea for the trained panel

The pH, total polyphenol and specific phenolic content (aspalathin, iso-orientin and orientin) of the iced teas is given in Table 6.3. The sum of the latter three flavonoids was also reported as total flavonoids (TF). The pH of the iced teas (flavoured and unflavoured together) decreased in the order: F>NF>N>UF. The total polyphenol and orientin content of the iced teas (flavoured and unflavoured) decreased in the following order: F>UF>NF>N. The aspalathin and total flavonoid content of the iced teas decreased in the order: UF; N; NF and F, whilst iso-orientin content exhibited a different order (UF>F>NF>N).

Table 6.3 The pH, total polyphenol (mg GAE/L iced tea), specific flavonoid content (mg/L iced tea), total flavonoid content and total "polymeric" content of the eight rooibos iced teas analysed by the trained sensory panel.

Iced tea	pH	TP[a]	Asp[b]	Iso[c]	Ori[d]	TF[e]	Polymer[f]
N	2.76	338.74	64.72	5.92	3.73	74.37	264.37
N/LEMON	2.77	338.05	64.79	5.95	3.75	74.48	263.57
NF	2.84	407.41	37.71	7.78	6.40	51.90	341.86
NF/LEMON	2.83	393.74	37.70	7.78	6.40	51.88	355.51
F	2.92	497.12	12.79	9.78	9.78	32.36	464.76
F/LEMON	2.93	508.76	12.66	9.77	9.65	32.08	476.68
UF	2.73	420.90	137.99	13.94	8.07	159.99	260.91
UF/LEMON	2.74	415.41	137.64	13.94	8.06	159.64	255.77

[a]Total polyphenol content, [b]aspalathin, [c]iso-orientin, [d]orientin, [e]total flavonoid content (aspalathin, iso-orientin and orientin), [f]total polymeric content (total polyphenol content – total flavonoid content).

Iced tea for the consumer panel

The pH of the lemon flavoured iced teas tested by the consumer panel decreased in the order: F>NF>N>UF (Table 6.4) whereas the total polyphenol and orientin content decreased in the order: F; UF; NF and N. The aspalathin and total flavonoid content of the iced teas decreased in the order: UF; N; NF and F, whilst the iso-orientin content decreased in the order: UF>F>NF>N.

Table 6.4 The pH, total polyphenol (mg GAE/L iced tea), specific flavonoid content (mg/L iced tea), total flavonoid content and total "polymeric" content of the four lemon flavoured rooibos iced teas analysed by the consumer panel.

Iced tea	pH	TP[a]	Asp[b]	Iso[c]	Ori[d]	TF[e]	Polymer[f]
N/LEMON	2.77	331.96	65.24	5.95	3.75	74.94	257.02
NF/LEMON	2.83	403.90	37.63	7.77	6.39	51.78	352.12
F/LEMON	2.94	494.31	13.02	9.89	9.76	32.67	461.64
UF/LEMON	2.73	429.30	138.18	13.69	7.94	159.81	269.49

[a]Total polyphenol content, [b]aspalathin, [c]iso-orientin, [d]orientin, [e]total flavonoid content (aspalathin, iso-orientin and orientin), [f]total polymeric content (total polyphenol content – total flavonoid content).

Sensory attributes

Figure 6.1 illustrates the difference between the sensory attributes of the eight iced tea samples. These results indicate that the iced teas differ significantly ($P<0.05$) with respect to astringency, plant-like flavour, hay-like flavour, rooibos flavour and lemon flavour. According to Figure 6.1, the astringency of F was the greatest of all the samples. It was significantly ($P<0.05$) more astringent than F/LEMON, UF and UF/LEMON, which did not differ significantly ($P>0.05$). The astringency of N, NF and NF/LEMON was lowest and the teas did not differ significantly ($P>0.05$). The N/LEMON iced tea was judged least astringent.

Only two of the iced tea samples, UF and UF/LEMON, possessed a plant-like character (Fig. 6.1). The plant-like character of UF was significantly ($P<0.05$) greater than that of UF/LEMON. Four iced teas possess a hay-like character (Fig. 6.1). The hay-like character of N dominated, being significantly ($P<0.05$) greater than that of N/LEMON and NF, which were significantly ($P<0.05$) greater than NF/LEMON. The rooibos flavour of F dominated amongst the eight iced tea samples, having a significantly ($P<0.05$) greater rooibos flavour than its flavoured counterpart (F/LEMON, Fig. 6.1). The rooibos flavour of NF was significantly ($P<0.05$) less than that of F/LEMON but significantly ($P<0.05$) greater than its flavoured counterpart (NF/LEMON). The remaining samples did not possess any notable rooibos tea flavour. Lastly, the flavoured samples were judged to have a notable lemon flavour (Fig. 6.1). Of the flavoured samples, F/LEMON and N/LEMON possessed the greatest lemon flavour. The lemon flavour of NF/LEMON was significantly ($P<0.05$) less than the latter two samples, but exceeded that of UF/LEMON.

In Figure 6.2, the relationship between the astringency of the eight iced tea samples and (a) total polyphenol content, (b) total flavonoid content, (c) total "polymeric" material content (total polyphenol - total flavonoid content) and (d) pH can be seen. From Figure 6.2, it is apparent that a significant ($P<0.05$) positive correlation ($r = 0.887$) exists between the astringency of the iced teas and their total polyphenol content (Fig. 6.2a). There does not appear to be any correlation between the astringency of the iced teas and the remaining three parameters (Fig. 6.2b-d).

Figure 6.3 shows PCA bi-plot of the eight rooibos iced tea samples and the five sensory descriptors. The sensory descriptors of the iced tea samples (scores) and the eight iced tea samples (loadings) are shown.

As can be seen in Figure 6.3, 73.8% of the variance could be explained by the first two principal components. Figure 6.3 indicates that an association exists between certain sensory descriptors and iced tea samples: F and F/LEMON are characterized by a rooibos flavour; UF and UF/LEMON by a plant-like flavour; N, NF and N/LEMON by a hay-like flavour and NF/LEMON by a lemon flavour. Astringency is mostly associated with F and F/LEMON, as well as UF and UF/LEMON.

Degree of liking

The consumer group consisted of 97 individuals, 72% female and 28% male consumers. Ninety percent (90%) of the consumers were between the ages of 18 and 27 whilst the remaining 10% were older than 28. With regard to consumption, 42% of the consumers indicated that they consume iced tea at least once a week, whilst 35% indicated that they drink the beverage once a month. The remaining 23% of the consumers indicated that they drink iced tea 2-3 times per year. Seventy-seven percent (77%) of the consumer panel thus indicated that they consume iced tea at least once a month.

Neither the association between gender by consumption (χ^2 = 8.1031), nor age group by consumption (χ^2 = 10.0773) was significant (P = 0.0879 and 0.8626, respectively). The association between gender and liking for F/LEMON, N/LEMON, NF/LEMON and UF/LEMON was as follows: χ^2 = 8.0128, 9.1802, 2.2173 and 10.9464. None of these respective values were significant: P = 0.3315, 0.2400, 0.8987 and 0.2048. The association between product and liking (χ^2 = 144.5098) for the total group was highly significant ($P<0.0001$).

Preference

Figure 6.4 illustrates the preference for the rooibos iced teas. The total group of consumers, as well as the female consumers, significantly preferred ($P<0.05$) the overall flavour of F/LEMON and NF/LEMON above other iced teas. Male consumers did not distinguish between F/LEMON and NF/LEMON and N/LEMON. Although not significant ($P\geq0.05$), the mean value of F/LEMON was slightly higher than that of NF/LEMON for all three consumer groups.

Acceptability

The distribution of preference of the four iced tea samples analysed by the consumer panel (total group) is shown in Figure 6.5. The hedonic score (1-9) gives an indication as to which part of the scale most of the consumers used for each of the four products, i.e. the distribution of scores across the nine classes of the hedonic scale. The latter is an indication of the acceptability of the products (Lawless & Heymann, 1998c).

In Figure 6.5, it can be seen that the total group consider F/LEMON and NF/LEMON reasonably acceptable. Seventy-two percent (72%) and 77% of the consumers gave these products a positive rating (scores of between six and nine on the hedonic scale: *Like slightly, Like moderately, Like very much and Like extremely*).

Preference mapping

Internal preference mapping was performed to establish the direction of preference for the iced tea by the consumers. Figure 6.6 is the preference map indicating the position of the consumers in relation to the iced tea samples. The four iced tea samples (scores) and the 97 consumers (loadings) are shown. As can be seen in Figure 6.6, 85.6% of the variance could be explained by the first two principal components. Figure 6.6 illustrates that consumer preference was directed towards NF/LEMON, F/LEMON and N/LEMON. Preference was directed away from UF/LEMON iced tea.

The external preference map in Figure 6.7, used to establish the identity of the sensory attributes driving preference of rooibos iced tea, indicates that the consumer preference was directed towards NF/LEMON, F/LEMON and N/LEMON and that the sensory attributes likely driving preference were lemon and rooibos flavour. Preference was directed away from plant-like flavour, which was associated with UF/LEMON.

DISCUSSION

Each of the eight iced tea samples possessed a distinctive flavour profile which may be characterised by one or more of the following flavour descriptors: plant-like; hay-like; rooibos and lemon. Astringency, as a taste sensation, was also analysed.

The sensation of astringency results from the interaction between tannins and salivary proteins (Kallithraka *et al*., 1998). Aggregation of the formed tannin-protein complexes reduces the lubricating property of saliva (Green, 1993; Prinz & Lucas, 2000). As a result, a dry, puckering sensation is experienced throughout the mouth (Kallithraka *et al*., 1998). Astringency is one of the more complicated sensory attributes to analyse, since factors such as salivary flow rate as well as saliva composition (variation in the type and amount of protein) play a role (Lesschaeve & Noble, 2005).

All the iced teas exhibited a measure of astringency, although the astringency of F was judged to be the greatest. Considering that F contained the highest TP content, it could be assumed that TP content would contribute to the perceived astringency. The trend observed between TP content and astringency would further support this assumption. Unfermented rooibos iced tea (UF) had a lower TP content than F and was perceived less astringent. On the other hand, UF and NF had very similar TP contents, yet their astringency differed significantly, with NF having the lower astringency. If total flavonoid (TF) content is taken into account, then UF and NF differ greatly, with NF having a substantially lower TF content than UF, indicating that monomers also play a role in the astringency of the iced teas.

The nature (size and structure) of polyphenols affects the way they bind to proteins, such as those in saliva (Zhu *et al*., 1997). Polyphenols, having a molecular mass (MM) of 500 of higher are per definition considered to be tannins (Naish *et al*., 1993). The individual flavonoids quantified for this study have MMs slightly lower than 500. Naish *et al*. (1993) have shown that despite not classifying as a tannin, 5-*O*-caffeoylquinic acid (MM of 354 g/mol), is similarly astringent compared to tannic acid and grape-seed tannin, which are accepted astringents.

Differences in the tannin structure of F and UF could be responsible for the differences in their astringency. Oxidation is known to promote the polymerisation of polyphenols to form large tannin-like compounds (Cilliers & Singleton, 1989; Talcott & Howard, 1999). In rooibos, "fermentation" (oxidation) has been shown to result in the formation of brown polymers (Koeppen & Roux, 1965) and discolouration of the plant material from green to red-brown (Joubert & De Villiers, 1997). Since fermented rooibos undergoes oxidation and unfermented not, fermented rooibos can be expected to contain more condensed tannin-like compounds than the latter. As much as 50% of the water-soluble extract of fermented rooibos are complex tannin-like structures (procyanidin type), having at least 5 monomer units consisting of the flavanols, (+)-catechin and (-)-epicatechin. A methanol extract of unfermented rooibos contains 14% tannins (Joubert *et al*., 2008). The degree of polymerisation (DP) is important, with the highest astringency for larger proanthocyanindins with a DP between 5 and 9 (Lea & Arnold, 1978). The addition of an anthocyanin fraction (containing derived tannins) to a model wine system has been shown to increase astringency (Vidal *et al*., 2004). Peleg *et al*. (1999) found that the astringency of the phenolic compounds in tea (*Camellia sinensis*) increased upon increased DP. The astringency of flavanol monomers was found to be lower than that of dimers or trimers. The bond linking the flavanol monomers, as well as the identity of the monomeric units within dimers, affected astringency: the astringency of catechin-(4→8)-catechin was lower than that of both catechin-(4→6)-catechin and catechin-(4→8)-epicatechin. However, they found no significant differences among the polymers investigated (Peleg *et al*., 1999). Other researchers have also shown that changes in phenolic composition of tea (*C. sinensis*), as a result of fermentation, change its sensory characteristics (Owuor & Obanda, 1993; Owuor & Obanda, 1998; Obanda *et al*., 2001). A reduction in the monomer content of defatted, ground cocoa beans has been associated with a concommitant decrease in astringency (Misnawi *et al*., 2003). More work is, required with respect to the flavonoid compounds and polyphenols affecting the astringency of rooibos, as the composition of the latter differs markedly from that of black tea (*C. sinensis*).

pH is known to affect astringency (Guinard, *et al*., 1986; Kallithraka *et al*., 1997; Peleg & Noble, 1999). In cranberry juice, decreasing the pH from 3 to 2.65 resulted in increased astringency (Peleg & Noble, 1999). Similarly, in model wine solutions decreasing pH resulted in increased astringency (Guinard, *et al*., 1986; Kallithraka *et al*., 1997). The lower pH of UF compared to F may also explain why the astringency of UF was relatively high, considering its substantially lower polymeric content than F.

The pH values of the iced teas, however, did not explain their astringency as F and UF had respectively the highest and lowest pH values, yet F and it lemon flavoured variant were the most astringent, followed by UF and it lemon flavoured variant.

The addition of lemon flavour was shown to significantly affect astringency, i.e. it reduced the perceived astringency of both F and N. It may be speculated that the use of lemon flavour prepared consumers for a measure of astringency, as most fruits are associated with astringency (Joslyn & Goldstein, 1964). This association of lemon with astringency may have been responsible for the perceived reduced intensity of this attribute or the level was perceived as more acceptable. In soy yoghurts, the use of fruit flavours has been shown to reduce the perceived astringency of the product (Drake *et al*., 2001). The aroma

compounds were speculated to have a masking effect on astringency. Despite the observation made for F and N (containing unfermented rooibos extract), the addition of lemon flavour did not have the same effect on iced tea made with the unfermented rooibos extract (UF and UF/LEMON). This observation requires further investigation.

Contrary to expectation, the addition of lemon flavour did not reduce the perceived astringency of NF, as it comprises N and F in which flavour addition was shown to have an effect. In combination, the mechanisms applicable to N and F separately, clearly do not apply.

The astringency of both N and NF were low compared to the other iced teas (less than 10%). The structure of the nano emulsion and the fact that the unfermented rooibos extract is trapped within the nano particle may play a role in this case. Microencapsulation of polyphenol compositions has been shown to significantly reduce their astringency and protects the compositions from oxidation and interactions with other ingredients (Ludwig *et al*., 2007). Plant-like flavour appeared to be characteristic of the unfermented extract as none of the remaining iced teas were found to possess this characteristic. This association was confirmed in the PCA bi-plot: the loadings for the unfermented iced teas (UF and UF/LEMON) lay in the direction of the "plant-like" attribute. For black tea, it was shown that the presence of chlorophyll is negatively associated with tea quality, as it imparts a grassy taste and other negative properties (Bokuchava & Skobeleva, 1969). The presence of greater quantities of chlorophyll in the unfermented extract compared to the fermented extract (Prof. E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, personal communication, 2008) is thus likely responsible for the plant-like character of UF. The addition of lemon flavour reduced the plant-like character of UF iced tea. This may be attributed to masking of this sensory attribute by the lemon flavour. It is well known that spices (generally used to add flavour to food) can be used to mask off odours/flavours (Takahashi *et al*., 2004; Bett *et al*., 2007).

A hay-like flavour was associated with iced teas containing nano emulsified unfermented rooibos (NEUR) extract, as illustrated on the PCA-biplot (Fig. 6.3). Three of the four iced teas containing NEUR extract lay in the direction of the hay-like flavour. Almost no hay-like flavour was detected in the remaining four iced teas. This would suggest that nano emulsification of the unfermented rooibos extract contributed to the hay-like flavour. Despite containing equal amounts of NEUR extract, N/LEMON was judged to be significantly less hay-like than N. Once again, a masking effect was observed with the addition of lemon flavour, as the hay-like flavour of N was reduced with addition of the latter. NF contained lower concentrations of NEUR extract than N and consequently, the perceived hay-like flavour of NF was significantly lower than that of N. The addition of lemon flavour to NF significantly reduced its hay-like character, but the intensity of latter characteristic could not be reduced in N. The higher concentrations of NEUR extract (and thus greater intensity of hay-like flavour) in N, compared to NF, was likely responsible. Once again the inclusion of the unfermented rooibos extract in the nano particle may play a role. Rooibos volatile compounds such as 5,6-epoxy-β-ionone and dihydroactinidilode, imparting a hay-like flavour (Joubert & De Villiers, 1997), may be better retained in the NEUR extract than in powdered unfermented or fermented extract.

The PCA-biplot (Fig. 6.3) indicated that a connection existed between the fermented rooibos extract and rooibos flavour, with the iced teas containing fermented extract, namely F and F/LEMON, both highly correlated with this attribute. Despite containing the same quantities of fermented rooibos extract, the intensity of rooibos flavour in F/LEMON and NF/LEMON was lower than that in F and NF, respectively. As was noted for other iced tea descriptors, the use of lemon flavour reduced the intensity of rooibos flavour.

The samples containing lemon flavour (F/LEMON, N/LEMON, NF/LEMON and UF/LEMON), exhibited a significant lemon flavour compared to the non-flavoured iced teas, although its intensity was perceived to be greater in F/LEMON and N/LEMON than NF/LEMON or UF/LEMON. Differences in the phenolic composition (Owuor & Obanda, 1995; Lesschaeve & Noble, 2005) and sensory attributes of these iced teas likely played a role, since these iced teas containing the same amount of lemon flavour. The difference in the intensity of lemon flavour in the various iced teas has an important implication for industrial production: certain formulations of iced tea would require greater quantities of flavouring than others to obtain a similar level of perceived lemon flavour.

Seventy-seven percent (77%) of the consumer panel indicated that they consume iced tea at least once a month. Neither the association of age group by consumption, nor gender by consumption was significant, indicating that consumption frequency was independent of the age and gender of the consumer. This latter (gender) finding was contrary to expectation as the consumers of iced tea in South Africa (target market) are mostly female (G. Pistorius, R&D Manager of beverages, Parmalat, South Africa, personal communication, 2007). The association between gender and liking was also not significant for any of the four iced teas (F/LEMON, N/LEMON, NF/LEMON and UF/LEMON), indicating that liking of iced tea was independent of gender.

The highly significant association between product and liking for the total group, indicated that liking was dependent on the product analysed. This was expected as the sensory attributes of the products differed markedly. The consumer panel, consisting of 72% female and 28% male consumers, indicated a preference for F/LEMON and NF/LEMON above N/LEMON. This finding was confirmed in Fig. 6.6. The consumers disliked UF/LEMON (average hedonic score of between 3 and 4: *Dislike moderately* and *Dislike slightly*). NF/LEMON and N/LEMON contain respectively 1.6 (3.0) and 2.3 (5.1) times the amount of TF (aspalathin) than F/LEMON, giving an iced tea with enhanced functional status. Respectively, 72% and 77% of the consumers gave F/LEMON and NF/LEMON a positive rating (a score of six or more on the hedonic scale). Based on the Fig 6.4, as well as well as external preference mapping (Fig. 6.7), it may be deduced that rooibos flavour is the main sensory attribute driving consumer acceptance of rooibos iced tea. Furthermore, the absence of plant-like flavour also appears to be a deciding factor as this attribute exhibited a strong negative correlation with consumer preference (as seen on the external preference map). In a study carried out in Turkey, Dos *et al*. (2005) found that the addition of vanilla flavour did not significantly increase the acceptability of brewed rooibos. In the present study, however, the data would suggest that the addition of flavour (lemon) can be used to improve the acceptability of unfermented rooibos iced tea, as it has a masking effect on plant-like flavour (perceived as negative by the consumer).

Consumer preference for iced tea with a rooibos flavour, associated with the fermented rooibos tea extract, may also be linked to familiarity with the product (Distel *et al*., 1999). Currently, the rooibos iced teas available on the South African market are produced with this type of extract. Furthermore, the difference in colour between fermented and unfermented rooibos likely also played a role in the acceptability of the iced teas. Although the colour of the iced teas was not analysed by the panel, unfermented rooibos extract was a light yellow-orange whereas the traditional, fermented type, was the characteristic red-brown. As the consumers may not have been expecting the uncharacteristic coloured rooibos iced tea, it may have affected their preference. Serving under red or green lights was considered. The difference in colour between the beverages was, however, too great to disguise using this technique. Commercial rooibos iced teas are generally light red-brown, thus consumer preference for iced tea with a red-brown colour is likely as it may seem more familiar (Distel *et al*., 1999). Colour has an important effect on flavour perception. The use of atypical colours has been shown to induce incorrect flavour responses to non-carbonated beverages (flavour responses associated with the atypical colour and not the flavour) (DuBose *et al*., 2006). In addition, it was found that the colour level of the beverages has significant effects on their overall acceptability, acceptability of the flavour, as well as on flavour intensity (DuBose *et al*., 2006).

The iced tea with the highest TF and aspalathin content, namely unfermented rooibos iced tea, was disliked by consumers. The high aspalathin content of the beverage lends it greater functional food status, in view of the antioxidant potency of aspalathin (Von Gadow *et al*., 1997b; Joubert *et al*., 2004; Snijman, 2007) but future developments for the South African market should include the production of an iced tea with sensory qualities closely resembling that of the fermented beverage due to their familiarity with fermented rooibos. As was illustrated in this study, there is potential for such a combination and additional experimentation with colour and flavour may also prove beneficial. This study does not, however, take the preferences of other markets into consideration. The Japanese have enjoyed green tea for centuries (Fujiki *et al*., 2002) and may even exhibit a preference for unfermented rooibos above its fermented counterpart. The United States market is also worth mentioning, as here, rooibos is gaining popularity (Anon., 2005). In future, however, the preference of the South African market may even change, due to recent increased popularity of green tea from *Camellia sinensis* (Anon., 2007).

CONCLUSION

The results of this study links each of the three sensory descriptors: *plant-like; hay-like* and *rooibos* to a specific type of rooibos extract/iced tea. The unfermented extract contributed *a plant-like* character to iced teas, the NEUR extract a *hay-like* character and the fermented extract a distinct *rooibos* character. Astringency was noted in all the iced teas, being most pronounced in iced tea produced with fermented rooibos extract. The addition of lemon flavouring generally reduced the intensity of these characteristics in rooibos iced teas and contributed an additional sensory attribute, namely lemon flavour.

From this research, it appears as though South African consumers would not greatly support the release of an unfermented rooibos iced tea product at this point in time. Changes in the market may, however, occur in future. The significantly different flavour profile of unfermented rooibos iced tea, compared to fermented rooibos iced tea, is likely responsible for this result. Preference for rooibos iced teas was directed towards products containing fermented rooibos tea extract with an unmistakable rooibos flavour. Although the addition of NEUR extract to fermented rooibos tea did not reduce consumer acceptance or preference for this product, it increased the aspalathin content of the beverage and thus enhancing its functional status. Future development work may thus be focused upon the inclusion of small quantities of unfermented rooibos extract in fermented iced teas. This will allow for the production of a beverage with an improved phenolic quality and the desired flavour profile.

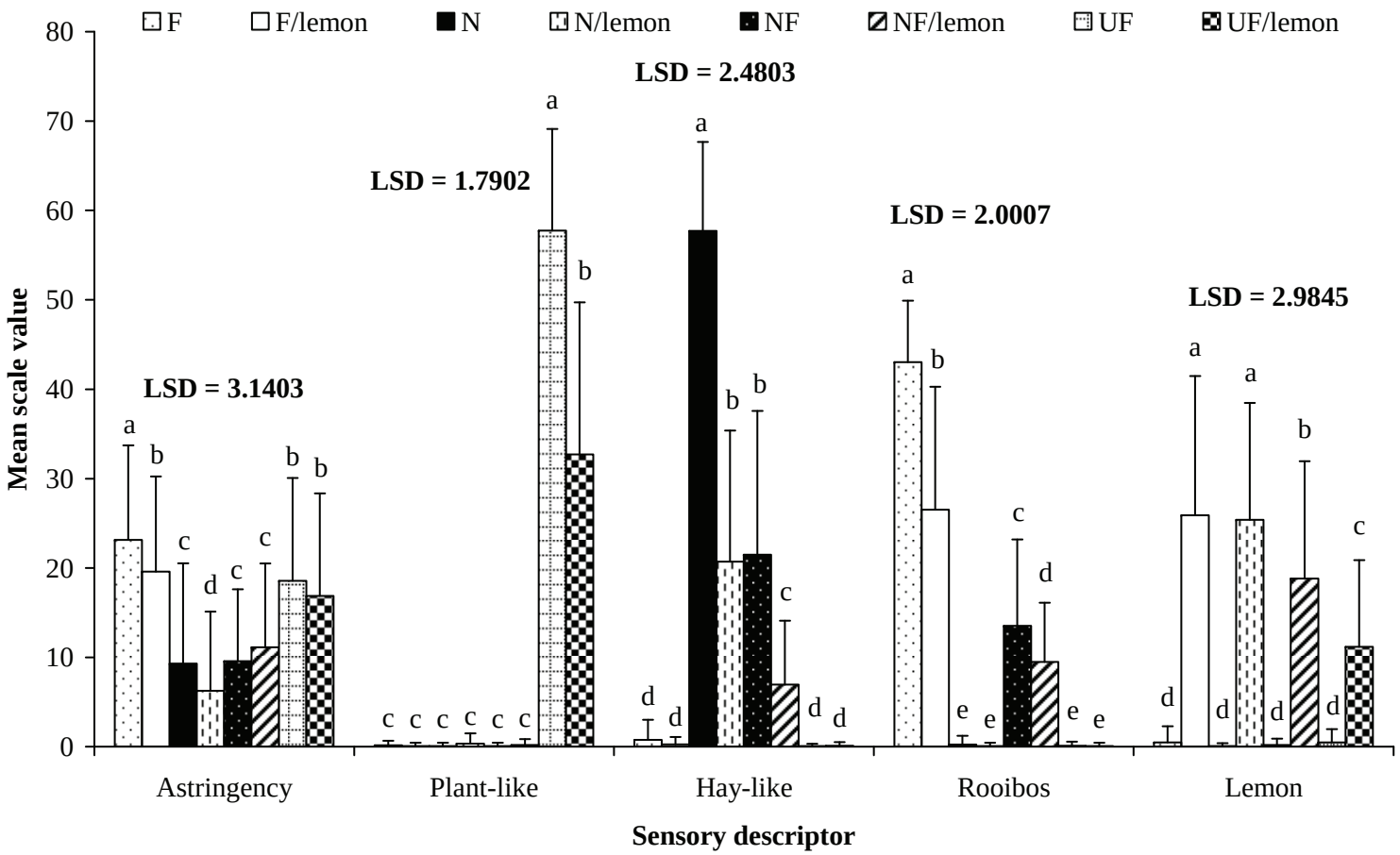
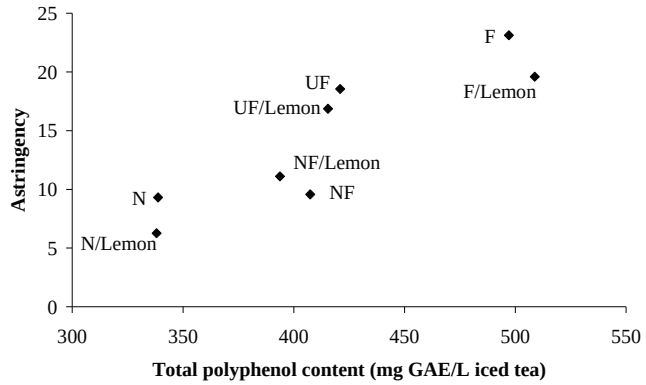


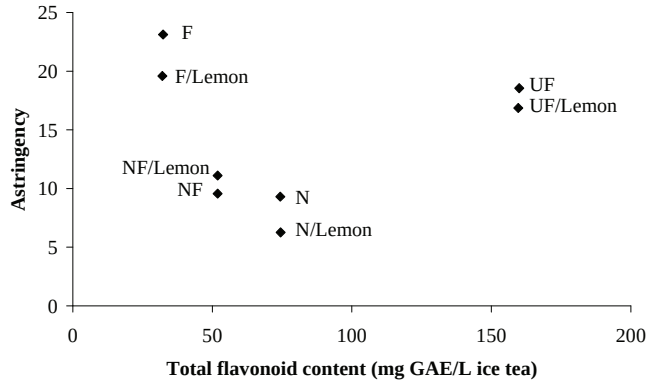
Figure 6.1 The five sensory attributes (astringency, plant-like flavour, hay-like flavour, rooibos flavour and lemon flavour) of the eight iced tea samples: fermented rooibos (F); lemon flavoured fermented rooibos (F/LEMON); nano emulsified unfermented rooibos (N); lemon flavoured nano emulsified unfermented rooibos (N/LEMON); nano emulsified unfermented rooibos and fermented rooibos (NF); lemon flavoured nano emulsified unfermented rooibos and fermented rooibos (NF/LEMON); unfermented rooibos (UF) and lemon flavoured unfermented rooibos (UF/LEMON). Means (+SD) with different alphabetical letters, within a sensory attribute, differ significantly ($P \leq 0.05$). The least significant difference (LSD) for each sensory attribute is indicated at the 5% level of significance.

a)



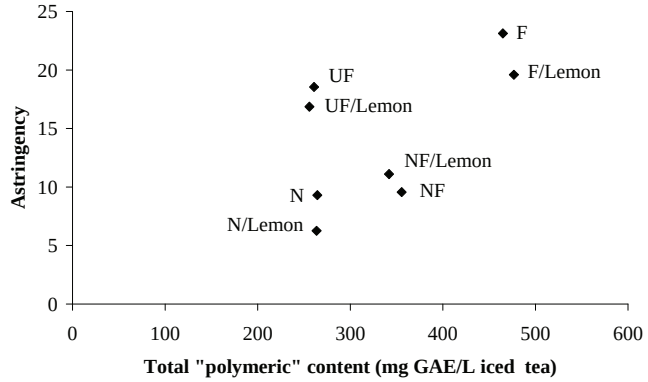
r = 0.887, p = 0.003

b)



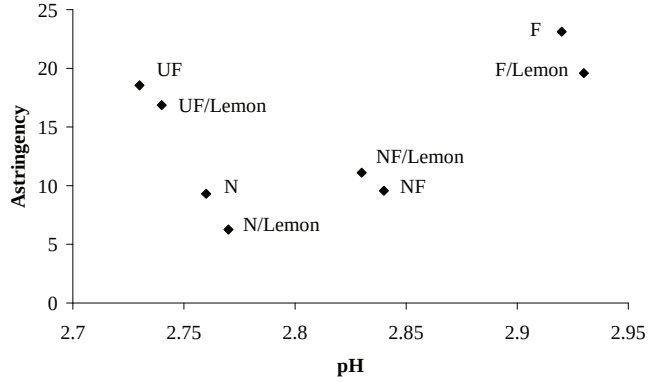
r = 0.075, p = 0.859

c)



r = 0.565, p = 0.145

d)



r = 0.075, p = 0.859

Figure 6.2 The relationship between astringency and (a) total polyphenol content, (b) total flavonoid content, (c) total "polymeric" content (total polyphenol - total flavonoid content) and (d) pH of the eight iced teas. See Fig. 6.1 for iced tea abbreviations.

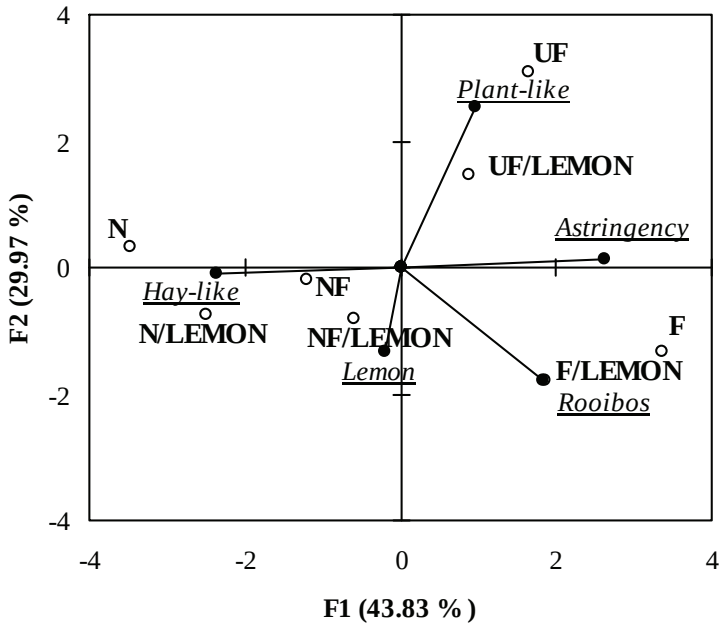


Figure 6.3 Principal component analysis bi-plot indicating the position of the sensory attributes (loadings) in relation to the iced tea samples (scores). The iced teas, indicated in capitals/bold were: fermented rooibos (F); lemon flavoured fermented rooibos (F/LEMON); nano emulsified unfermented rooibos (N); lemon flavoured nano emulsified unfermented rooibos (N/LEMON); nano emulsified unfermented rooibos and fermented rooibos (NF); lemon flavoured nano emulsified unfermented rooibos and fermented rooibos (NF/LEMON); unfermented rooibos (UF) and lemon flavoured unfermented rooibos (UF/LEMON). The five sensory descriptors, namely astringency, plant-like flavour, hay-like flavour, rooibos flavour and lemon flavour are indicated in italics. The first two principal components explained 73.8% of the variance.

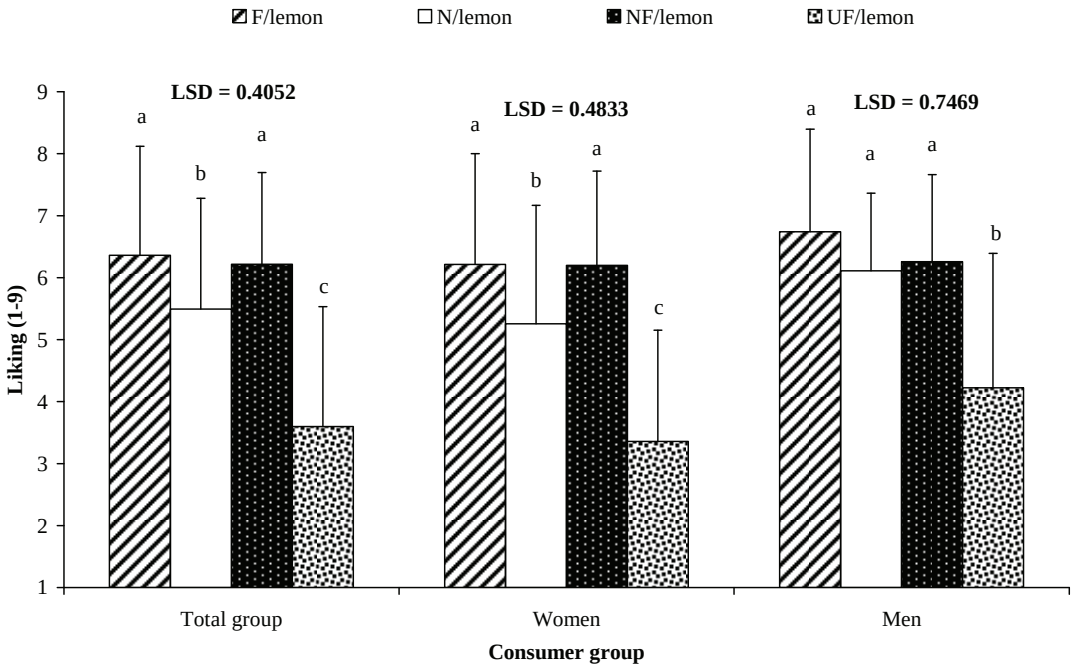


Figure 6.4 The overall preference for the four iced teas by the total, female and male consumers. The iced teas were: lemon flavoured fermented rooibos (F/LEMON); lemon flavoured nano emulsified unfermented rooibos (N/LEMON); lemon flavoured nano emulsified unfermented rooibos and fermented rooibos (NF/LEMON) and lemon flavoured unfermented rooibos (UF/LEMON). Means (+SD) with different alphabetical letters differ significantly. The least significant difference (LSD) for each group is indicated at the 5% level of significance.

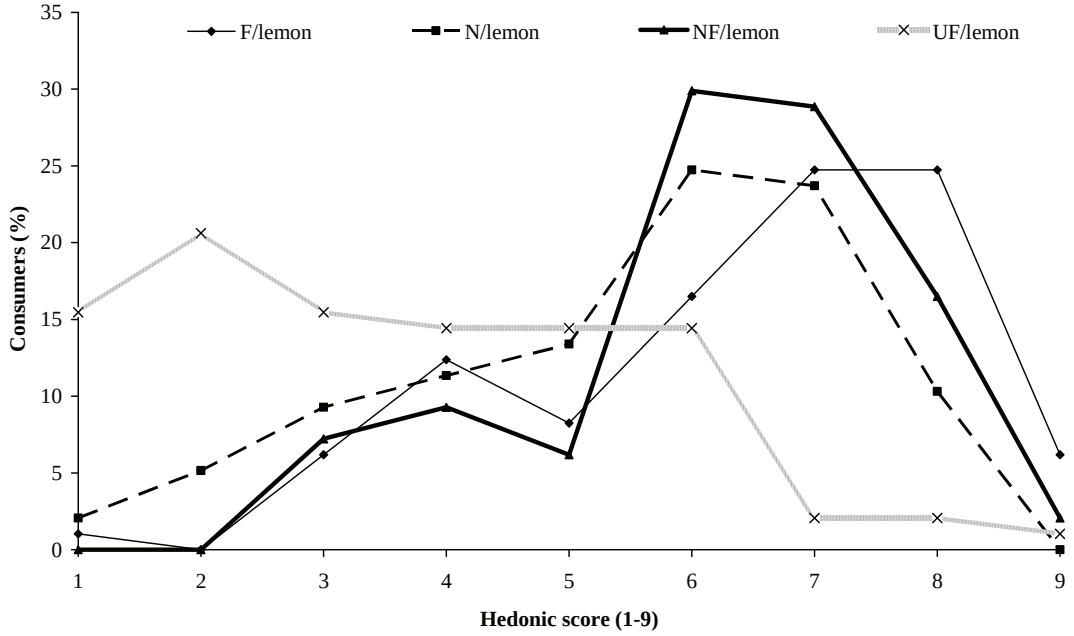


Figure 6.5 The distribution of preference of the four iced tea samples for the total consumer group. The iced teas were: lemon flavoured fermented rooibos (F/LEMON); lemon flavoured nano emulsified unfermented rooibos (N/LEMON); lemon flavoured nano emulsified unfermented rooibos and fermented rooibos (NF/LEMON) and lemon flavoured unfermented rooibos (UF/LEMON). The association between product and liking (χ^2 = 144.5098) for the total group was highly significant (P<0.0001). The values on the x-axis refer to the nine classes of hedonic scale, with nine indicating "like extremely".

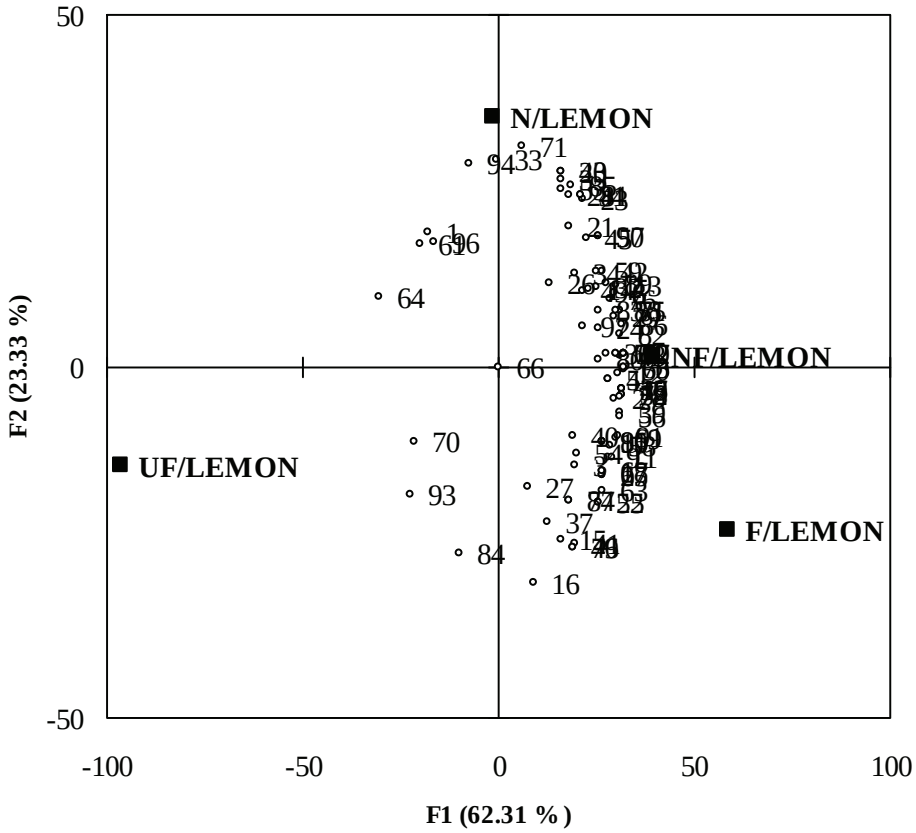


Figure 6.6 Principal component analysis bi-plot indicating the position of the consumers (loadings) in relation to the iced tea samples (scores). The iced tea samples were: lemon flavoured fermented rooibos (F/LEMON); lemon flavoured nano emulsified unfermented rooibos (N/LEMON); lemon flavoured nano emulsified unfermented rooibos and fermented rooibos (NF/LEMON) and lemon flavoured unfermented rooibos (UF/LEMON) and are indicated in bold whilst the consumers are represented by non-filled circles. The first two principal components explained 85.64% of the variance.

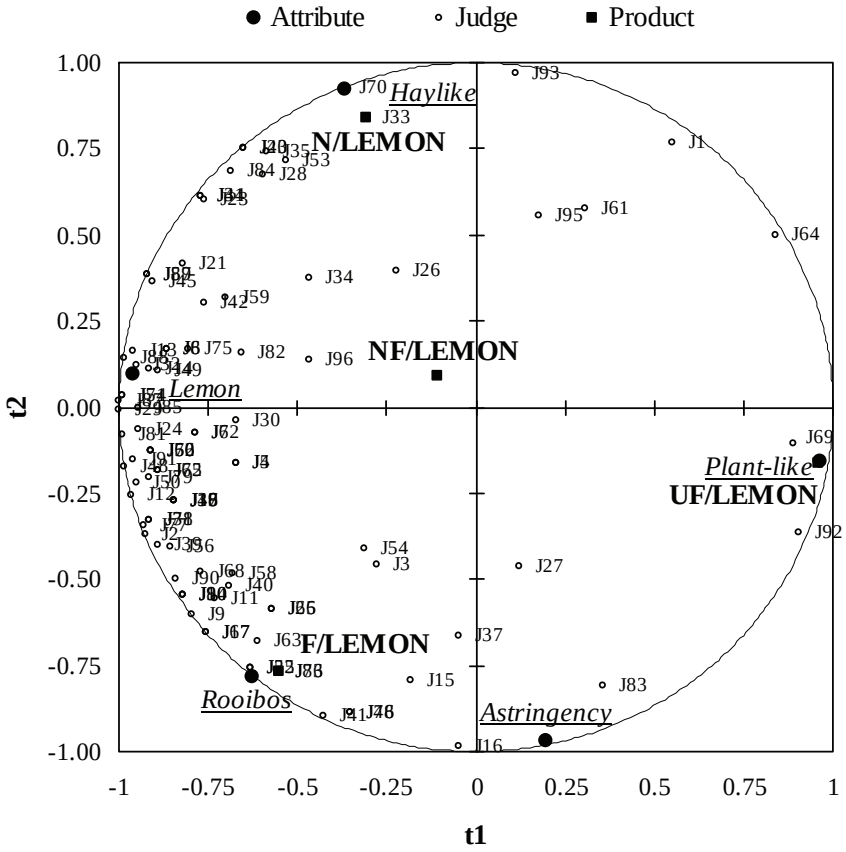


Figure 6.7 External preference map indicating the position of the judges (consumers) in relation to the four iced tea samples (capital letters/bold) and the five sensory attributes (italics). The iced tea samples were: lemon flavoured fermented rooibos (F/LEMON); lemon flavoured nano emulsified unfermented rooibos (N/LEMON); lemon flavoured nano emulsified unfermented rooibos and fermented rooibos (NF/LEMON) and lemon flavoured unfermented rooibos (UF/LEMON). The map was obtained using a partial least square regression, where the consumer data (y space) was regressed onto the trained panel data (x space).

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

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Research linking the consumption of plenty of antioxidant-rich fruits and vegetables (Hertog *et al*., 1992; Hertog *et al*., 1993) with reduced disease incidence (Hertog *et al*., 1995) has led to an increased demand for antioxidant-containing food products and beverages over the past decade (Bech-Larsen & Scholderer, 2007). A similar connection exists between tea consumption and health (Dufresne & Farnworth, 2001), resulting in increased consumption of ready-to-drink tea beverages, such as iced tea, produced from both *Camellia sinensis* and *Aspalathus linearis* (Anon., 2006). Presently, research is being done on the health effects (Anon., 2008) and bioavailability (Van der Merwe *et al*., 2008) of the rooibos flavonoid, aspalathin.

The abovementioned trends have resulted in many food/beverage manufacturers becoming eager to produce antioxidant/functional products. Since the levels of the functional ingredients are generally not regulated, little thought is given to the stability of the functional ingredients within the products during and after processing (Katan & De Roos, 2004). No data exists with respect to the levels of aspalathin and its corresponding flavones, iso-orientin and orientin, in ready-to-drink rooibos iced tea. The question thus arises: are consumers obtaining the same levels of rooibos flavonoids from these ready-to-drink alternatives compared to a cup of brewed rooibos? Information with respect to the stability of such compounds may be of importance in future, for ethical as well as regulatory reasons.

Brewed, fermented rooibos (2.5 g teabag/150 ml, brewed for 10 min) typically contains 10.3 mg/500 mL aspalathin, 6.9 mg/500 mL iso-orientin and 8.4 mg/500 mL orientin (Joubert & Schulz, 2006). The phenolic content of commercial rooibos iced teas analysed during this study did not compare well to the phenolic "strength" and composition of a typical cup of brewed rooibos. Four of nine brands tested negative for aspalathin and the highest level detected was 2.1 mg aspalathin/500 mL beverage. More brands contained iso-orientin and orientin compared to aspalathin, but the highest levels were still lower than those of brewed rooibos. One brand contained none of the compounds, indicating that extremely low quantities of rooibos extract or no extract was used in its production. In this study it was shown that the processes involved in extract production, and specifically spray-drying do not impact the aspalathin, iso-orientin and orientin content of FR and UR extract to such an extent that no trace of these compounds will be found in the iced tea product. Due to the low and often negligible quantities of these compounds detected in the tested commercial rooibos iced tea products, the aim of this study was to investigate the factors which could be responsible for reducing the flavonoid content of such beverages, and clarify whether it would be unreasonable to expect their presence in commercially available rooibos iced tea products.

The effect of processing and storage on the stability of aspalathin in fermented rooibos (FR), unfermented rooibos (UR) and nano emulsified unfermented rooibos (NEUR) iced teas was investigated, despite the fact that currently, only FR iced teas are available on the South African market. This was done in

anticipation of future developments for an UR iced tea, which is in accordance with the current trend towards increased consumption of antioxidant-rich functional foods and beverages. Encapsulation of rooibos flavonoids in nano particles could not only potentially offer higher product stability (Weiss *et al*., 2006), but also improved bioavailability (Zang & Kosaraju, 2007; Hu *et al*., 2008). The production of functional foods and ingredients with "improved" bioavailability, however, may make current knowledge with respect to their toxicity obsolete and necessitates re-evaluation of their safety (Weiss *et al*., 2006). The usefulness of citric and ascorbic acid, ingredients normally used in iced tea formulation, in preventing processing and storage induced losses of aspalathin, iso-orientin and orientin was also investigated. Furthermore, the expected processing stability and shelf-life of these flavonoids were compared in different types of rooibos iced tea: iced tea made with UR extract and NEUR extract was investigated in comparison to that of iced tea made with FR extract. With this work it was aimed to provide guidelines for the formulation of rooibos iced teas in which the phenolic quality (in terms of aspalathin content) will be optimally maintained during and after processing.

Heating of experimental FR, UR and NEUR rooibos iced teas containing citric and ascorbic acid resulted in losses of aspalathin, iso-orientin and orientin, but in no case was this content reduced to undetectable levels. The higher levels of retention of aspalathin, iso-orientin and orientin in UR and NEUR iced teas were attributed to the overall greater initial aspalathin content and lower iron content (relevant in the Fenton reaction) of these iced teas compared to that of the FR iced teas. In the case of the NEUR iced teas, the protection of the UR extract in nano-sized micelles containing ascorbic acid was also thought to play a role. Iced tea formulations containing UR and NEUR extracts had a lower pH compared to FR iced tea, despite the iced teas containing the same amount of citric acid (where applicable). The lower pH was thought to play a role in the overall increased stability of aspalathin, iso-orientin and orientin in the iced tea formulations containing UR and NEUR extracts. Flavonoids resist oxidation to a greater extent when the pH is reduced (Lemańska *et al*., 2001).

Losses of aspalathin, iso-orientin and orientin as a result of sterilisation heat treatments [(normal temperature sterilisation (NTS) and high temperature sterilisation (HTS)] were generally more pronounced than those observed for pasteurisation, which could be linked to the severity and duration of the heat treatment (Chen *et al*., 2001; Pellegrini *et al*., 2001; Brenes *et al*., 2002). In the case of pasteurised, FR iced tea (formulation BCA), an increased aspalathin content was observed. This was ascribed to release of aspalathin from compounds within the rooibos extract matrix, possibly polymeric material. The higher level of aspalathin in formulation BCA can further be attributed to the combined effect of citric and ascorbic acid. The more acidic environment would not only create conditions suitable for hydrolysis, but also suppress the ionisation of flavonoids whilst the antioxidant action of ascorbic acid would offer further protection. Hydrolysis of rooibos plant material with enzymes has been shown to lead to increased extraction of compounds such as aspalathin (Pengilly *et al*., 2008). The increased availability of aspalathin in FR iced tea after pasteurisation (in the presence of citric and ascorbic acid), however, requires more attention in future.

During heating, losses of the flavones, iso-orientin and orientin, were less pronounced than those for aspalathin. This was ascribed to structural differences between the compounds (Dziedzic *et al*., 1985),

aspalathin being the better antioxidant compared to the flavones (Joubert *et al*., 2005) and thus more susceptible to oxidation. Furthermore, the formation of iso-orientin from aspalathin and orientin from iso-orientin has been demonstrated (Koeppen & Roux, 1966; Krafczyk & Glomb, 2008). The formation of iso-orientin and orientin in such a manner was thus also thought to be responsible for the apparent "enhanced" stability of these compounds, compared to aspalathin. Overall, orientin exhibited the greatest heat stability, which could be linked to its formation from iso-orientin under oxidative conditions (Krafczyk & Glomb, 2008). This finding was indirectly confirmed by the fact that most of the commercial teas contained orientin, whereas fewer contained iso-orientin and aspalathin. This finding also suggests that, in future, orientin could be used as an indicator compound in claimed rooibos products, and could thus serve as a marker for quality.

In most of the iced tea formulations, heat-induced losses of aspalathin were associated with browning. This was attributed to the oxidation of aspalathin as the latter reaction has been connected to the formation of uncharacterised brown material (Koeppen & Roux, 1966; Krafczyk & Glomb, 2008). There were, however, exceptions: perhaps most notably the decreased absorbance of FR iced tea (formulation BCA) after HTS (Chapter 3). This was ascribed to (a) the antioxidant action of ascorbic acid and (b) protonation of deprotonated flavonoids (Robertson, 1983; Gupta, 1989) due to the pH-reducing effect of citric acid. The latter results in reduced electron delocalisation (Whitfield, 1969) and thus less absorbance.

The total polyphenol (TP) content of the rooibos iced teas was considered a measure of their antioxidant activity since a good correlation between TP content and radical scavenging activity has been found for rooibos (Bramati *et al*., 2003; Joubert *et al*., 2008a; Joubert *et al*., 2008b). The TP content of most of the FR and UR iced teas increased significantly after heat treatment, in spite of changes indicating that oxidation and polymerisation of phenolic compounds had taken place (browning of the iced teas and reduced aspalathin content). The latter occurrences were expected to lower the TP content of the iced teas (Gómez-Alonso *et al*., 2007). The increases observed for the TP content of many formulations was ascribed to the formation of new compounds (as seen on the chromatograms) with improved reactivity towards the Folin reagent, compared to their precursor compounds (Pokorný & Schmidt, 2001; Vinson *et al*., 2001; Turker *et al*., 2004; Yamada *et al*., 2007). A few exceptions were observed: most notably, the absence of change of the TP content of FR iced tea (formulation BCA). The loss of ascorbic acid (Chua *et al*., 2000) combined with the formation of new compounds (more reactive to the Folin reagent) was implicated for the observation.

Although the identity of the new compounds was not established, one of the new compounds had a retention time similar to that of dihydro-iso-orientin or dihydro-orientin (oxidative products of aspalathin). The UV-vis spectra, as well as λ_{max} of this compound, however, differed from that of the flavanones. Based on its elution before aspalathin, the new compound is more polar than aspalathin and could be a phenolic acid. The cleavage of the dihydrochalcone phloretin has been shown to yield phenolic acids (Hurst & Harborne, 1967). Due to its ascorbic acid (antioxidant) content, the formation and/or preservation of this unknown compound was greatest in formulation BCA. In future, investigation with respect to the identity of the new compounds formed

after NTS and HTS of rooibos iced tea may be considered to provide a fuller picture of the phenolic compounds in rooibos extracts, subjected to heat treatment.

The present study established that the Folin-Ciocalteu assay is not a good indicator of changes occurring in the phenolic profile of rooibos iced tea during heating, as the TP content generally increased despite decreased levels of aspalathin, iso-orientin and orientin. This means that the latter assay, despite its ease of use and application to high through-put analysis, would not be a good measure of loss of phenolic quality of iced teas in industrial applications. It is thus recommended that future work include the development of kinetic models for the prediction of aspalathin loss in beverages, as a function of temperature and heat treatment duration. The effect of citric and ascorbic acid, separately as well as in combination (at the levels usually applied in industry) may also be considered. The same may be done for iso-orientin and orientin.

Heating was shown to account for some flavonoid losses (as can be expected under normal production circumstances: pasteurisation at a low pH value), yet it is unlikely that a 100% loss of rooibos flavonoids will occur. Storage was thus postulated to have the greatest effect on the retention of flavonoids in commercial rooibos iced tea and was consequently investigated.

Although storage was shown to play a role in the loss of rooibos flavonoids in experimental iced teas, this study indicated that a rooibos iced tea, containing both citric and ascorbic acid and stored for three months, can be expected to retain *ca*. 75-88% of its aspalathin content, depending upon the type of rooibos extract used. As observed for heating, storage of rooibos iced tea was associated with the oxidation of aspalathin and was accompanied by browning. This was generally reduced in formulations containing citric acid and ascorbic acid, (due to their pH-reducing and antioxidant effects, respectively), as seen for heating.

In the case of UR iced tea, the presence of citric acid alone (formulation BC) appeared to accelerate the degradation of aspalathin during storage. It may explain the low aspalathin content of the commercial FR iced tea products, as many contain citric acid alone (no ascorbic acid). The poor stability of aspalathin under these conditions was contrary to expectation, since the susceptibility of flavonoids to oxidation is generally reduced at low pH (Canales *et al*., 1993; Coiffard *et al*., 1998; Friedman & Jürgens, 2000; Lemańska *et al*., 2001). Prolonged storage was not investigated by the latter researchers, possibly accounting for the apparent contradiction. Chen *et al*. (2001), however, reported accelerated loss of green tea catechins during storage, in the presence of citric acid, but no explanation was offered. The detrimental role of citric acid on the stability of aspalathin in FR iced tea thus remains to be clarified.

Overall, the beneficial effect of citric and ascorbic acid in UR iced tea appears to be greater during heating than storage. Apart from the accelerated loss of aspalathin observed in formulation BC during storage, there was very little difference in aspalathin loss (%) between formulation B and BCA. The difference in the stability of FR and UR iced teas was speculated to result from the overall lower levels of aspalathin in the FR extract, but also to the difference in pH (comparing formulation B) and perhaps even iron content (iron content of FR extract higher than UR or URNE extracts). The role of iron relates to its catalytic function in hydroxyl radical generation via the Fenton reaction (Morris *et al*., 1995). The lower stability of aspalathin, iso-orientin

and orientin in the iced teas containing a combination of the FR extract with UR or NEUR extract (compared to UR or NEUR iced teas), was again attributed to the increased iron content of the combination iced teas, compared to the "pure" counterparts, and its catalytic role in the Fenton reaction.

Greater storage stability was demonstrated for iso-orientin and orientin (flavones) compared to aspalathin (dihydrochalcone), as was the case for heat treatment. Structural differences (resulting in altered oxidative stability) as well as the formation of iso-orientin and orientin from aspalathin oxidation, were implicated. This was especially so during the first two weeks of storage when the iso-orientin and orientin content of the iced teas was shown to increase significantly with a concomitant decrease in aspalathin content and browning. The results of the storage experiment thus indicated that iso-orientin and orientin may indeed form as a result of aspalathin degradation, not only in pure solution (Koeppen & Roux, 1966; Krafczyk & Glomb, 2008), but also in rooibos products such as iced tea. In future, research with respect to the rate of formation, identity and conversion of the intermediate products of aspalathin oxidation (flavanones such as dihydro-iso-orientin and dihydro-orientin as well as polymeric compounds) could be considered.

During storage, an increase in polymeric compounds was expected, as aspalathin conversion has been shown to lead to the formation of such compounds (Koeppen & Roux, 1966; Krafczyk & Glomb, 2008). The unexpected decrease in area of the polymeric material on the chromatograms of the iced tea samples was thus associated with their removal from solution, during filtering, prior to HPLC analysis. However, the fact that browning of the iced tea samples was recorded, despite the loss of polymeric compounds, indicated the presence of smaller coloured compounds, not large enough to be removed during filtering. Analysis of the iced tea samples with normal phase HPLC, capable of distinguishing between polymers of different mass (Spranger *et al*., 2008), would be particularly useful and could provide some insights as to the evolution (change in size) of the oxidation products formed at various stages during the storage of rooibos iced tea. This could aid in the explanation of the browning (or lack thereof) observed during storage.

Again, the TP assay was shown not to be a good measure of specific flavonoid changes occurring in the iced teas during storage, as the TP content was generally unchanged whilst losses of aspalathin, iso-orientin and orientin occurred. This was attributed to an equalising effect between (a) the formation of new antioxidant compounds (more reactive to Folin-Cioacalteu reagent) and (b) the loss of individual flavonoids and polymeric material. In addition, the samples analysed with HPLC were filtered prior to analysis whilst the TP samples were not. The inclusion of large, newly formed polymers (reactive towards the Folin reagent) in the samples subjected to the TP assay could also account for the result.

Based on the heating and storage data of experimental iced teas, it would not be unreasonable to expect commercial FR iced teas, sold before their expiry date (3 month shelf life), to contain aspalathin, iso-orientin and orientin. These compounds are still present in FR iced tea, after storage, provided citric acid and ascorbic acid are present. These results suggested that the poor phenolic quality (not equivalent to the phenolic "strength" and composition of a cup of brewed rooibos) of the commercial, FR rooibos iced teas tested was most likely

attributable to the use of insufficient quantities of rooibos extract or the use of extract of unusually low phenolic content.

During heating and storage, the difference in pH between the iced tea formulations, as well as types of iced tea (FR, UR and NEUR) was postulated to affect the stability of the rooibos flavonoids. In a subsequent follow-up investigation, the aspalathin, iso-orientin and orientin content of FR, UR and NEUR extract formulations (no sugar, citric acid or ascorbic acid was added) was indeed shown to decrease with increasing pH and storage temperature. A concomitant increase in absorbance at 420 nm (browning) was observed, which was attributed to flavonoid oxidation and polymerisation. The NEUR extract displayed greater resistance to pH-induced changes (pH 5-7) than the FR and UR extracts, which was ascribed to its inherent *ca*. 5% ascorbic acid content as well as protection of the UR extract (from oxidation) within nano micelles. The greater stability of the UR extract formulation, in comparison to the FR extract formulation, at low pH (pH 3-4), was attributed to its increased aspalathin content and the presence of smaller amounts of iron (implicated in the Fenton reaction). The relatively high pH levels (pH 6 and 7) investigated may not be relevant for an iced tea, but provide an insight into the stability of these rooibos compounds in food products of low acidity, such as milk drinks. For example, there have already been investigations into the stability of green tea catechins in yoghurt (pH 4.5-4.6) (Jaziri *et al*., 2009). Here, however, other factors such as protein-polyphenol interaction need to be considered (Arts *et al*., 2001). The effect of pH on the stability of rooibos compounds even has some relevance in terms of the pH conditions the compounds will encounter in the human gastrointestinal system, where pH values as high as 7.4 and 7.7 are prevalent in the jejunum and ileum, respectively (Ekmekcioglu, 2002).

pH-induced oxidation of rooibos flavonoids, such as aspalathin, would most probably have proceeded via hydroxyl moiety deprotonation (Lemańska *et al*., 2001). Krafczyk & Glomb (2008) have suggested a model for aspalathin oxidation, which includes the formation of iso-orientin and then orientin from aspalathin. This mechanism most likely accounts for the apparent enhanced stability of the flavones, and especially orientin above that of aspalathin. The mechanism was, however only investigated at pH 7.4. Further studies are required to confirm its applicability at low pH values, and to identify the oxidation product(s) formed. Such work will be useful and provide clues into the observed increase in the iso-orientin and orientin content of the UR extract formulation (pH 5 and 40ºC), despite no significant decrease in aspalathin content. In this case it was presumed to result from the conversion of precursor compounds, already present in the extract, into iso-orientin and orientin. The mechanism behind the increased availability of aspalathin in the FR extract (pH 3) also remains to be investigated. It also remains to be determined whether the mechanism involved here, is the release of aspalathin from matrix compounds such as polymeric material and whether the same mechanism is at work during pasteurisation of FR iced tea (formulation BCA). Furthermore, the involvement of heat and a low pH in the occurrence must be elucidated.

Since the Fenton reaction was implicated for some of the previous results and small amounts of H_2O_2 may be present in aseptically packaged beverage products (Stefanovic & Dickerson, 1986), the addition of H_2O_2 to various rooibos extract formulations was investigated. The addition of a relatively high concentration of H_2O_2

(600 mg/L) to UR extract formulations resulted in an immediate, significant reduction in the aspalathin, iso-orientin and orientin content, with the loss of the aforementioned compounds being more pronounced in formulations containing ascorbic acid. This was in accordance with the expected catalytic role of ascorbic acid in the Fenton reaction (Halliwell *et al*., 1987). Despite the higher stability of iso-orientin and orientin to that of aspalathin during processing (heating and storage) as well as at neutral pH (pH 7), the same trend was not observed with the addition of H_2O_2. The nature of the oxidation process induced by hydrogen peroxide possibly differs compared to that occurring as a result of heating, storage or changes in pH. Based on the observations made during heating and storage, the loss of aspalathin, iso-orientin and orientin (due to H_2O_2) was expected to be accompanied by increased absorbance (browning). There were, however, no immediate changes in the absorbance of the samples, although an increase in the area of the polymeric compounds (on the chromatogram) was noted, confirming the occurrence of phenolic oxidation (Cilliers & Singleton, 1990; Cheynier & Moutounet, 1992; Li *et al*., 2008). The lack of absorbance change was attributed to a difference in the oxidation products formed as a result of slow chemical oxidation (heating, storage and pH) compared to rapid H_2O_2-induced oxidation. Different oxidation products have been shown to form after the oxidation of green tea catechins with H_2O_2 (Zhu *et al*., 2000) and peroxyl radicals (Valcic *et al*., 2000; Sang *et al*., 2003).

Storage of H_2O_2-containing solutions lead to further decreases in the aspalathin, iso-orientin and orientin content, as well as an accompanying increase in absorbance. The losses were mostly ascribed to the effect of storage and not H_2O_2 since no accelerated decrease in these compounds were observed, when compared to the control samples receiving no H_2O_2. In Chapter 4, storage was shown to have a detrimental effect on the retention of aspalathin, iso-orientin and orientin in rooibos iced tea.

To bring the effects of H_2O_2 in context, the effect of H_2O_2 at the highest permissible residual level in aseptically packaged beverage products (0.5 mg/L) was tested. At this level, H_2O_2-addition was found to have a minimal effect on the phenolic quality of rooibos extract formulations. Losses of aspalathin, iso-orientin and orientin were only associated with FR iced tea containing citric and ascorbic acid. This was linked to the increased iron content of the FR extract compared to that of the UR or NEUR extracts. The loss of phenolic quality (via a Fenton type reaction) in rooibos beverages will, however, be dependent upon the iron and ascorbic acid content (Morris *et al*., 1995) of the beverage, as well its pH (Jeong & Yoon, 2005). A change in any one of these parameters can, thus alter the effect of H_2O_2. This work highlights the contrasting effect of ascorbic acid during heating and storage, compared to its effect in the presence of H_2O_2. The use of ascorbic acid in FR iced tea should thus be carefully considered in aseptic applications. Ascorbic acid is, however, not the only compound capable of increasing the rate of hydroxyl radical generation via the Fenton reaction. Laughton *et al*. (1989) and Puppo (1992) have both shown that flavonoids accelerate hydroxyl radical generation in Fenton-type reactions, by reducing Fe^{3+} to Fe^{2+}. Joubert *et al*. (2005) showed that pro-oxidant activity of rooibos extracts is linked to aspalathin content. At low concentrations, in the absence of ascorbic acid, flavonoids may act as pro-oxidants, whilst at high concentrations they scavenge H_2O_2, thus acting as antioxidants (Joubert *et al*., 2005). The pro-oxidant activity of these compounds at a low concentration may be attributed to Fe^{3+} reduction (Aruoma

et al., 1997). The difference in the aspalathin content (principal rooibos antioxidant) between the three types of rooibos extract could thus also be responsible for the difference in susceptibility of the extracts to the addition of low levels of H_2O_2.

In future, it is recommended that research be directed towards the identification of the oxidation products formed as a result of the reaction between H_2O_2 and (a) pure aspalathin as well as (b) various rooibos extracts. Investigation of the effect of high and low levels of H_2O_2, as well as different iron, ascorbic acid and citric acid levels (alone and in combination) will also be useful in understanding the effect of the Fenton reaction in rooibos. Specific attention should be paid to the identification of the different oxidation products, and perhaps polymeric material, formed directly after H_2O_2 addition. As suggested previously, normal phase HPLC may specifically be applied for the analysis of the polymeric material (Spranger *et al*., 2008).

Sensory analysis of rooibos iced teas was included in the present study to obtain information on the sensory drivers that will be important with respect to the development of an UR iced tea for the South African market. For the sensory analysis, different concentrations of rooibos tea extract were used, compared to the processing experiments. The FR iced teas comprised less FR extract than used in the processing experiment (1.25 g/L instead of 1.75 g/L), but this quantity of FR extract was exactly equivalent to that used by a local iced tea manufacturer, one of the few brands of iced tea consistently found to contain aspalathin. Some product development, in terms of extract formulation, was required for the UR and NEUR iced teas, in order to improve sensory properties. The UR iced tea was perceived as excessively astringent at the levels used for the heating and storage experiments. Consequently the amount of extract used for the sensory evaluation was reduced to half of that of the FR extract (i.e. 0.625 g/L). Bitterness was a particular problem in the case of the NEUR extract. This negative taste attribute was associated with the emulsifier used in the NEUR extract, namely Tween 20 (Dr B. Weinreich, R & D Manager, Raps Foundation, Germany, personal communication, 2007). Reduction of the levels of NEUR extract used for the production of iced tea, however, eliminated the problem and consequently bitterness was not evaluated. Furthermore, the World Health Organization has recommended an acceptable daily intake of 0-25 mg of polyoxyethylene sorbitan esters (such as Tween 20) per kilogram body weight. This implies that an adult, of average body mass 70 kg, may consume 1 L of NEUR iced tea/day without exceeding this limit (WHO, 1974).

The results of the sensory study linked each of the three sensory descriptors: plant-like; hay-like and rooibos to a specific type of rooibos extract/iced tea. No lexicon existed for either fermented or unfermented rooibos and the creation of lexicons to describe the flavour characteristics of the respective rooibos iced teas was a problem, due to the intricate flavour of the extracts. The UR iced tea was perceived as plant-like, and this was attributed to the presence of chlorophyll, as indicated by the greenish-tan colour of the dry extract powder. Iced teas made with the NEUR extract were perceived as hay-like. This was presumably due to the improved retention of volatile compounds associated with hay flavour, as a result of nano emulsification. The FR iced teas were described as having a distinct rooibos flavour. The panel indicated that their familiarity with fermented rooibos justified the use of the word “rooibos” as a descriptor for these iced teas specifically.

The addition of lemon flavouring generally reduced the intensity of the plant-like, hay-like and rooibos flavour of the various iced teas. In the case of the UR iced tea, this effect was considered positive as plant-like flavour was shown to be negatively correlated with consumer preference. Consumer preference for iced teas with a rooibos flavour was linked to familiarity with the product, as only this type of iced tea is currently available in South Africa.

Despite information indicating that South African iced tea consumers are primarily female, no association between gender and consumption, nor gender and liking, was obtained. The data perhaps suggested that a change is occurring in the market. Possibly a greater number of male consumers are now also focussing their beverage choices towards perceived "healthier" products. This idea is perhaps supported by a marketing campaign by the international beverage manufacturer, Pepsi, who targeted the launch of Pepsi Max (sugar-free Pepsi alternative with maximum flavour) at health conscious male consumers (Plaskitt, 2008).

From this research, it appears as though future developments towards an UR iced tea should be directed towards a product with a flavour profile resembling that of the FR product. This was partially achieved with the formulation of iced tea comprising FR and NEUR extract. Although the addition of NEUR extract to FR iced tea did not reduce consumer acceptance or preference for this product, it increased the aspalathin content of the beverage and thus enhanced its functional beverage status. Further improvement of the iced tea comprising FR and NEUR extract, with flavours, sugar and pH adjustment is possible and could result in a successful product in future. The same applies to the UR iced tea, which consumers disliked. From a functional food point-of-view, this iced tea has much to offer, but much work is required to adapt the flavour to that which is acceptable for the consumer.

Another area for future research is the effect of heating on the sensory properties of rooibos iced tea. The samples analysed in this study were not heated prior to consumption as the large (in laboratory terms) volumes of iced tea required for the analysis could not be heated to the required temperature in a time equivalent to that used for commercial rooibos iced tea products. The effect of heating on laboratory scale in less than ideal conditions was thought to have a greater detrimental effect on the beverage than no heat at all. Future work should include the analysis of heat treated samples, as heating has been shown to affect the flavour profile of products such as green tea (Kim *et al*., 2007).

In this study it was shown that, with an adequate amount of FR extract and ingredients such as citric and ascorbic acid, it is possible to produce a rooibos iced tea (heated and stored for three months) with a phenolic quality similar to that of brewed tea. Improvement of the phenolic profile of FR iced tea with NEUR extract is also possible, without compromising consumer acceptability. This is positive in the light of the recent increased demand for functional food products. Although research is still being conducted on the contrasting effect of antioxidants in the human body, depending on nutritional status, health status (cancer), iron levels, age, and more (Salganik, 2001), there is no direct competitor product against iced tea made from green tea (*Camellia sinensis*). For the same reasons people consume rooibos in preference to green tea, green/unfermented rooibos may find approval amongst certain individuals. In this study, however, it was shown that the flavour profile of

unfermented rooibos is not desirable to the South African consumer, despite its more favourable phenolic profile. Since this research was based in South Africa, it does not take the preference of consumers elsewhere, especially in Japan where green tea is part of the normal diet, into consideration. There, the product could be highly acceptable.

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ADDENDA

ADDENDUM 1

Table A1.1 The regression parameters for calibration curves of aspalathin, iso-orientin and orientin used for HPLC analysis of rooibos iced teas and extract formulations

	Aspalathin	**Iso-orientin**	**Orientin**
Intercept	-28.1	-44.3	-26.6
Slope	2756.5	3256.7	2646.6
R^2	0.9999	0.9998	0.9999

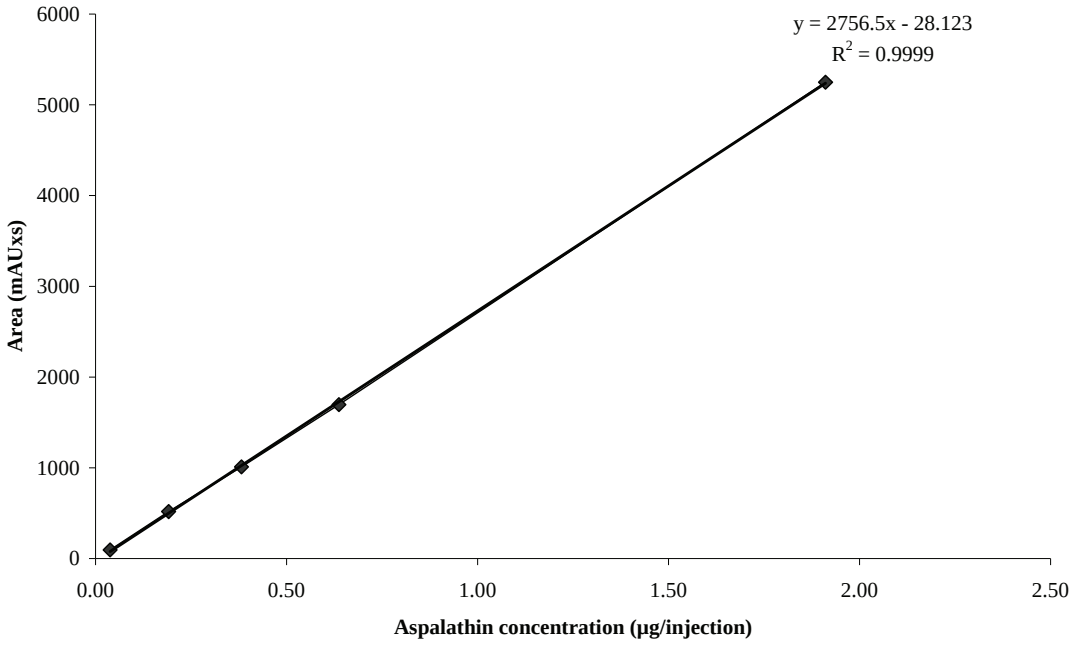
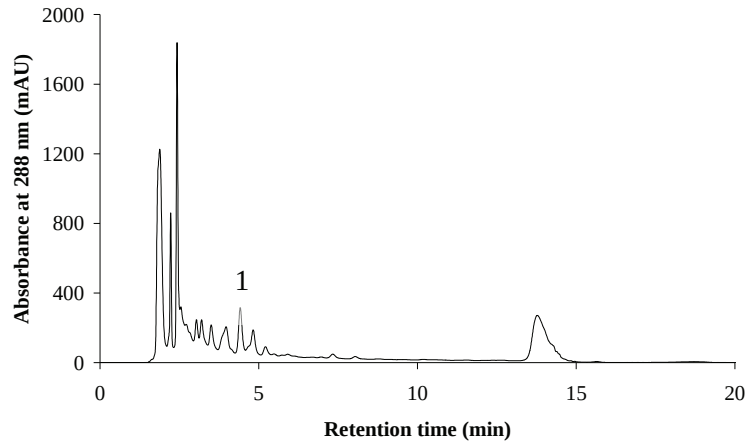


Figure A1.1 A typical standard curve for aspalathin (injection volume 20 µL) used for HPLC analysis of rooibos iced teas and extract formulations.

ADDENDUM 2

a)



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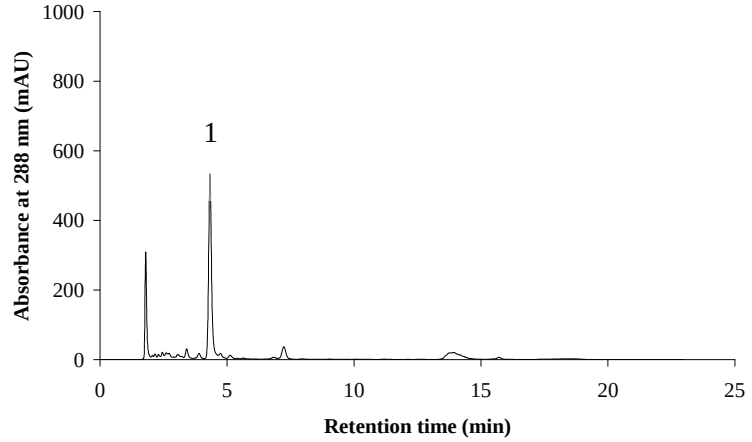

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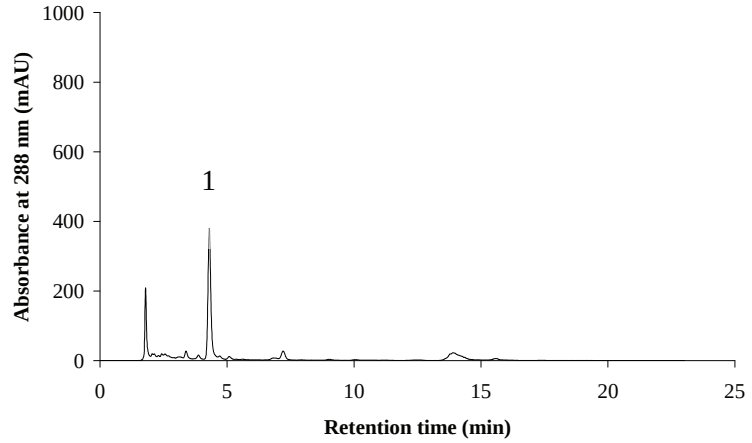
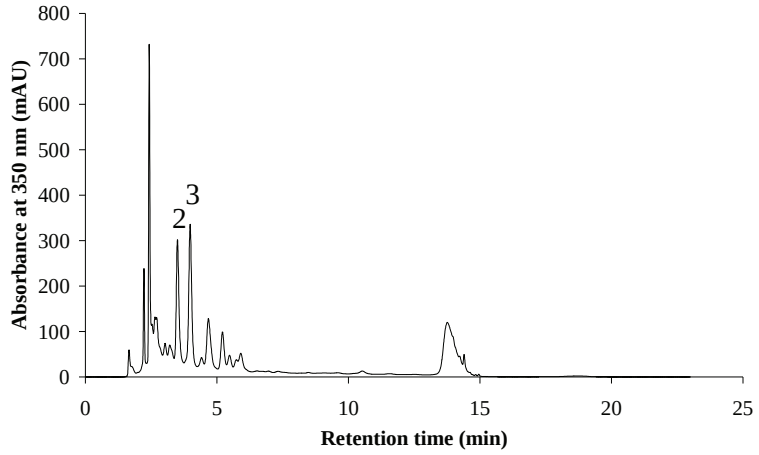


Figure A2.1 HPLC chromatograms of (a) fermented rooibos extract (1.75 g/L, injection volume of 50 µL), (b) unfermented rooibos extract (1.75 g/L, injection volume of 5 µL) and (c) nano emulsified unfermented rooibos extract (14 g/L, injection volume of 3 µL) at 288 nm. The retention time of (1) aspalathin was approximately 4.5 min.

a)



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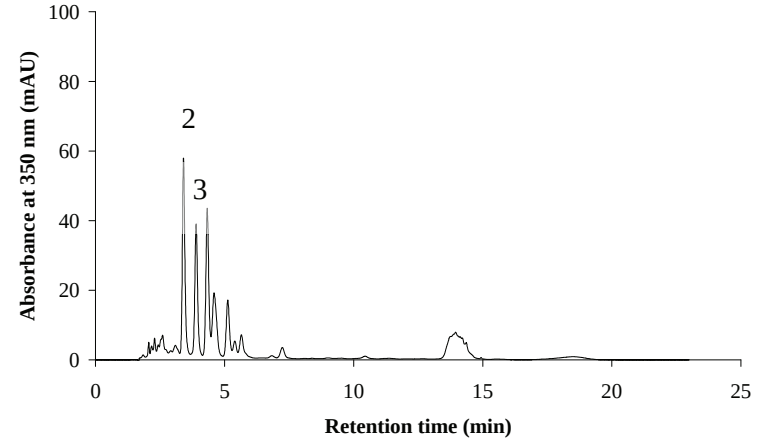

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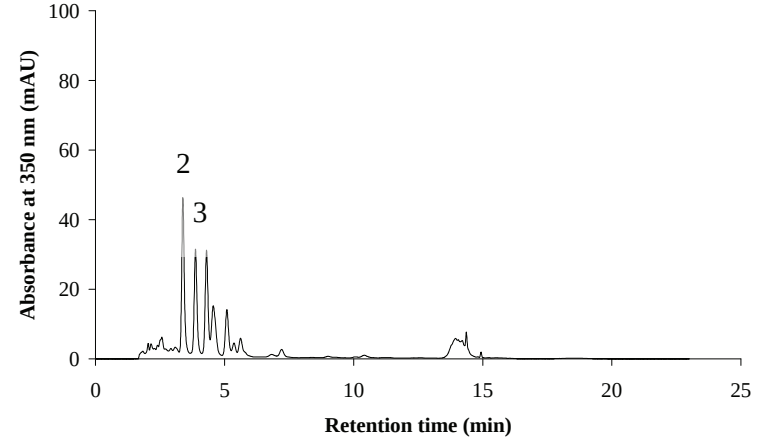
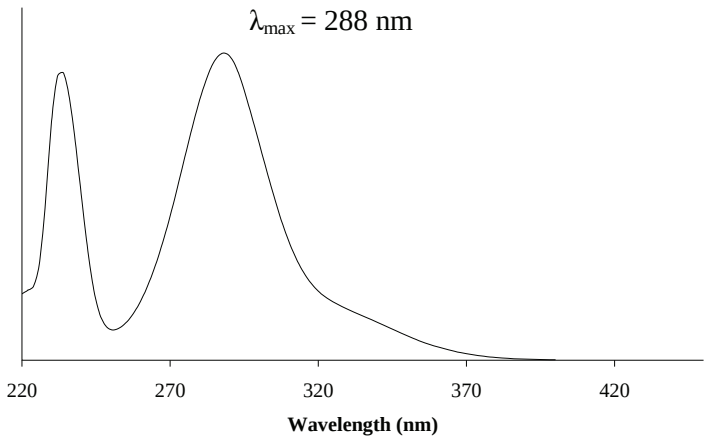


Figure A2.2 HPLC chromatograms of (a) fermented rooibos extract (1.75 g/L, injection volume of 50 µL), (b) unfermented rooibos extract (1.75 g/L, injection volume of 5 µL) and (c) nano emulsified unfermented rooibos extract (14 g/L, injected at 3 µL) at 350 nm. The retention times of (2) iso-orientin and (3) orientin were approximately 3.8 and 4.3 min.

a)



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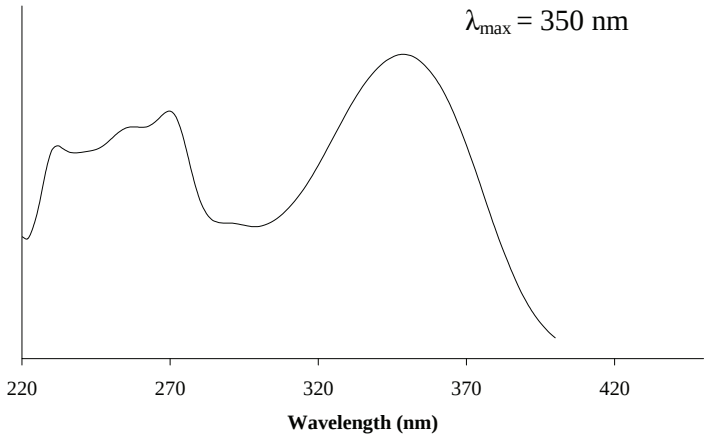


c)

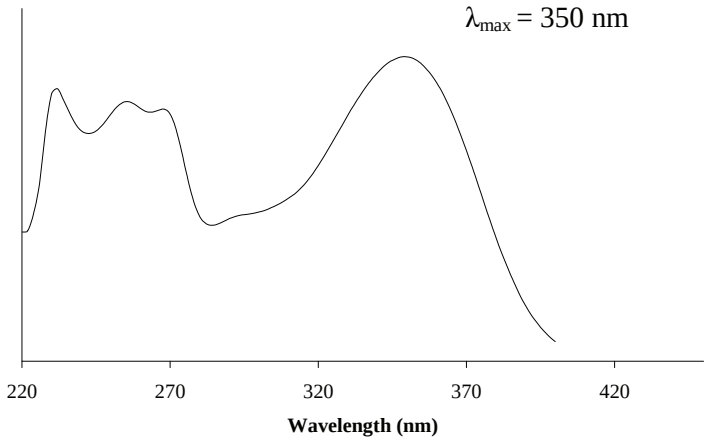
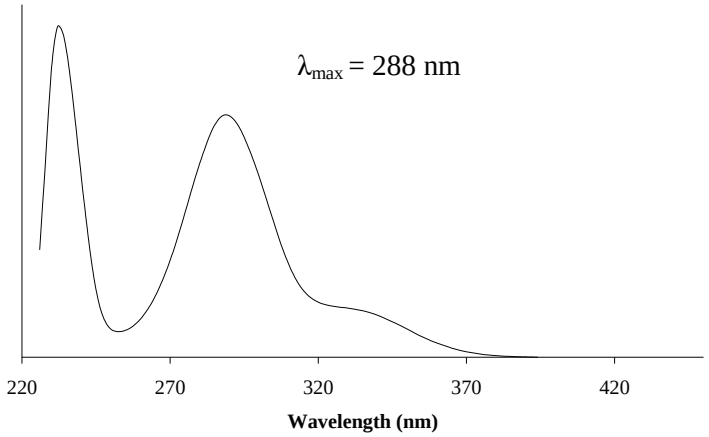
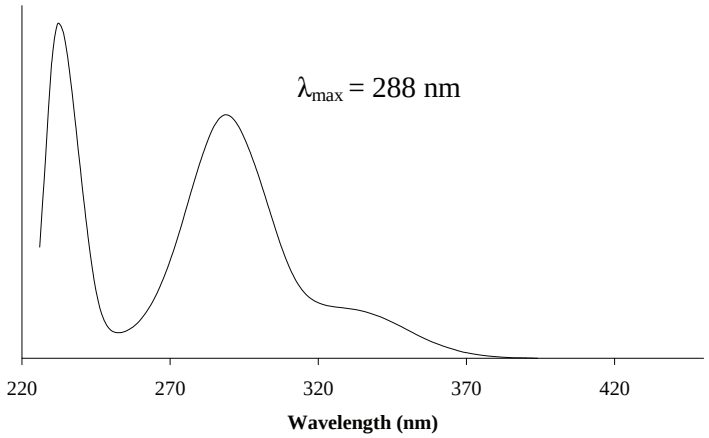


Figure A2.3 The UV spectra of (a) aspalathin, (b) iso-orientin and (c) orientin.

a)



b)



c)

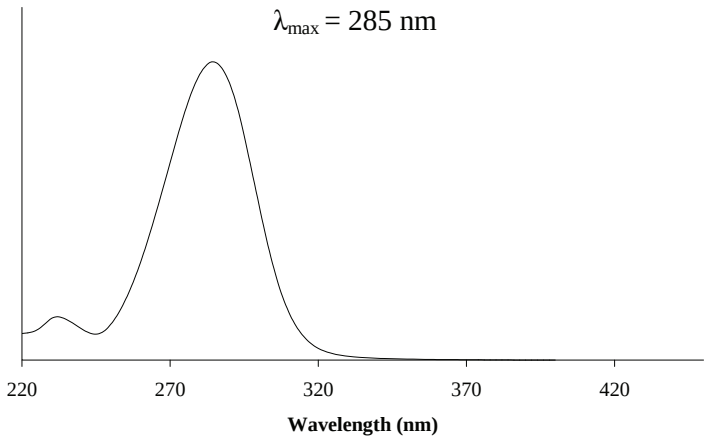


Figure A2.4 The UV spectra of (a and b) the major flavanones of fermented rooibos and (c) unknown compound 1.

ADDENDUM 3

Table A3.1 The change (%) in the aspalathin, iso-orientin and orientin content as well as absorbance (420 nm) of rooibos iced tea due to different types of heating

Extract	Heat	Formulation	Absorbance	Aspalathin	Iso-orientin	Orientin
Fermented	NTS[a]	B[b]	+15.1	-76.0	-8.8	+1.0
rooibos		BC[c]	-3.3	-10.4	-4.3	-1.6
		BCA[d]	-3.7	-9.7	-3.7	-1.3
	Pasteurised	B	+4.5	-5.7	+2.3	+1.3
		BC	+9.8	+7.1	+5.1	+0.3
		BCA	+3.8	+11.0	+4.2	+6.9
	HTS[e]	B	+9.0	-78.1	-9.9	+0.2
		BC	-12.3	-25.1	-7.9	-3.7
		BCA	-12.4	-13.6	-4.1	-1.4
Unfermented	NTS	B	+142.4	-34.0	-12.0	+1.4
rooibos		BC	+152.7	-14.7	-9.0	-2.5
		BCA	+138.0	-10.1	-7.6	-3.5
	Pasteurised	B	+55.0	+1.7	-0.7	+1.5
		BC	+102.7	+3.1	+1.6	+1.8
		BCA	+79.4	+4.4	+1.8	+1.7
	HTS	B	+109.3	-37.9	-11.3	+0.5
		BC	+150.8	-20.4	-11.0	-5.0
		BCA	+136.9	-17.0	-10.1	-5.8
Nano	NTS	NE[f]	+43.7	-17.5	-8.8	-6.5
emulsified		NEC[g]	+51.0	-12.8	-9.1	-6.1
unfermented	Pasteurised	NE	+6.2	+1.9	+1.7	+1.4
rooibos		NEC	+7.8	+1.1	+1.7	+1.5
	HTS	NE	+66.6	-22.7	-7.9	-5.0
		NEC	+75.4	-16.4	-12.9	-4.5

[a]Normal temperature sterilisation, [b]base (fermented or unfermented rooibos extract in deionised water), [c]base + citric acid, [d]base + citric + ascorbic acid, [e]high temperature sterilisation, [f]nano emulsified unfermented rooibos (NEUR) extract in deionised water, [g]NE + citric acid. There is no NECA formulation as the NEUR extract inherently contained ascorbic acid.

Table A3.2 The change (%) in the total polyphenol content of rooibos iced tea due to heating

		Extract		
Heat treatment	**Formulation**	**FR[a]**	**UR[b]**	**NEUR[c]**
NTS[d]	B[e]/NE[f]	+4.6	+10.9	+3.9
	BC[g]/NEC[h]	+17.3	+9.1	+3.3
	BCA[i]	+2.1	+5.1	
Pasteurised	B/NE	+0.9	-0.6	+3.5
	BC/NEC	+8.9	+14.8	+2.1
	BCA	+3.7	+10.4	
HTS[j]	B/NE	+8.5	+7.9	+4.3
	BC/NEC	+0.5	+15.2	+8.9
	BCA	+20.5	+8.5	

[a]Fermented rooibos, [b]unfermented rooibos, [c]nano emulsified unfermented rooibos, [d]normal temperature sterilisation, [e]base (FR or UR extract in deionised water), [f]NEUR extract in deionised water, [g]base + citric acid, [h]NE + citric acid, [i]base + citric + ascorbic acid, [j]high temperature sterilisation. There is no NECA formulation as the NEUR extract inherently contained ascorbic acid.

Table A3.3 The change (%) in the aspalathin, iso-orientin and orientin content as well as absorbance of three formulations of rooibos iced tea after a 5 and 30 min pasteurisation-like heat treatment

Extract	Formulation	Heating (min)	Absorbance	Aspalathin	Iso-orientin	Orientin
Fermented rooibos	B[a]	5	+3.9	-4.2	-1.2	-1.4
	BC[b]		-1.4	+1.6	-2.0	-1.6
	BCA[c]		+0.9	+1.9	-1.7	-1.8
	B	30	+7.0	-39.8	+0.3	-0.2
	BC		+20.4	-1.2	+1.6	-0.8
	BCA		+16.2	+4.6	+1.9	-0.0
Unfermented rooibos	B	5	+12.9	-0.1	+0.0	+0.2
	BC		-1.9	+0.6	+0.2	-0.0
	BCA		-6.4	+0.6	+0.2	-0.1
	B	30	+51.2	-2.6	-1.7	+0.2
	BC		+56.7	+0.8	+0.1	+0.6
	BCA		+54.8	+2.5	+0.6	+0.7
Nano emulsified unfermented rooibos	NE[d]	5	-0.6	-1.5	-1.0	-1.0
	NEC[e]		+2.0	-0.7	-1.1	-0.9
	NE	30	+4.5	-1.7	-1.0	-0.5
	NEC		+5.8	-0.6	-0.6	-0.6

[a]Base (fermented or unfermented rooibos extract in deionised water), [b]base + citric acid, [c]base + citric + ascorbic acid, [d]nano emulsified unfermented rooibos (NEUR) extract in deionised water, [e]NE + citric acid. There is no NECA formulation as the NEUR extract inherently contained ascorbic acid.

ADDENDUM 4

Aim

A number of substances e.g. sugars, aromatic amines, sulphur dioxide, ascorbic acid, enediols and reductones, organic acids and Fe^{2+} may interfere with the total polyphenol assay (Singleton *et al*., 1999; Prior *et al*., 2005). The aim of this experiment was to establish whether the amounts of sugar, citric and ascorbic acid used in the rooibos iced tea formulations have an impact on the total polyphenol content of the beverage.

Materials and method

The total polyphenol content of the iced tea formulations was determined as described in Chapter 3.

Results

The total polyphenol content of the various formulations of rooibos iced tea is given in Table 1.

Table A4.1 The total polyphenol content (g GAE/100 g extract) of three formulations of iced tea developed with three rooibos extracts

Extract type	Extract	Extract + sugar	Extract + citric	Extract + ascorbic	Extract + citric and ascorbic
FR[a]	26.60	26.78	25.33	33.88	34.21
UR[b]	35.85	36.24	34.56	43.68	45.27
NEUR[c]	9.09	9.16	8.86		

[a]Fermented rooibos, [b]unfermented rooibos, [c]nano emulsified unfermented rooibos.

Discussion

Formulations containing ascorbic acid (Extract + ascorbic acid and Extract + citric + ascorbic acid) exhibited an increased total polyphenol content compared to formulations not containing this ingredient. In all cases, the total polyphenol values for Extract were similar to those for Extract + sugar and Extract + citric, indicating that these ingredients did not alter the total polyphenol content of the beverage. Citric acid was anticipated to significantly alter the total polyphenol value as it lowers the pH of medium to which it is added and the pH requirement of the total polyphenol reaction is alkaline (Singleton *et al*., 1999). The amount of citric acid added was clearly not sufficient to have an appreciable impact on the pH of the reaction medium.

The total polyphenol content of the nano emulsion was higher than expected: most likely due to its significant ascorbic acid content (Vinson *et al*., 2001). The increased total polyphenol value of the extract, citric acid and ascorbic acid combination is thus ascribed to the presence of ascorbic acid. Iced teas containing

ascorbic acid can be expected to have greater total polyphenol contents than iced tea formulations not containing this ingredient.

Conclusion

Sugar and citric acid (in the proportions used in the iced tea formulations) do not appreciably affect the total polyphenol content of the beverages, while ascorbic acid has a measurable effect.

References

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ADDENDUM 5

Table A5.1 The change in the absorbance (420 nm) as well as the total polyphenol, aspalathin, iso-orientin and orientin content of fermented rooibos iced tea as a result of heating prior to storage

	Formulation		
	B[a]	**BC[b]**	**BCA[c]**
Absorbance at 420 nm			
Before heating	1.26±0.11	0.83±0.10	0.82±0.05
After heating	1.55±0.03	0.93±0.02	0.83±0.05
Percentage change	+23.0	+12.0	+1.2
Total polyphenol content (g GAE/100 g soluble solids)			
Before heating	26.45±0.78	25.35±0.60	34.81±1.00
After heating	26.21±0.86	28.04±1.09	35.66±1.40
Percentage change	-0.9	+10.6	+2.4
Aspalathin (g/100 g extract)			
Before heating	0.94±0.03	0.90±0.00	0.90±0.01
After heating	0.32±0.04	0.89±0.01	0.98±0.01
Percentage change	-66.0	-1.1	+8.9
Iso-orientin (g/100 g extract)			
Before heating	0.67±0.00	0.63±0.00	0.64±0.01
After heating	0.66±0.01	0.64±0.01	0.66±0.00
Percentage change	-1.5	+1.6	+3.1
Orientin (g/100 g extract)			
Before heating	1.08±0.00	1.04±0.00	1.05±0.01
After heating	1.08±0.00	1.05±0.00	1.07±0.01
Percentage change	0.0	+1.0	+1.9

[a]Base (fermented rooibos extract in deionised water), [b]base + citric acid, [c]base + citric + ascorbic acid.

Table A5.2 The change in the absorbance (420 nm) as well as the total polyphenol, aspalathin, iso-orientin and orientin content of unfermented rooibos iced tea as a result of heating prior to storage

	Formulation		
	B[a]	**BC[b]**	**BCA[c]**
Absorbance at 420 nm			
Before heating	0.15±0.01	0.08±0.01	0.09±0.03
After heating	0.31±0.02	0.21±0.02	0.18±0.01
Percentage change	+106.7	+162.5	+100.0
Total polyphenol content (g GAE/100 g soluble solids)			
Before heating	37.93±0.57	37.39±0.98	47.45±1.07
After heating	38.16±1.67	40.56±1.40	48.01±1.27
Percentage change	+0.6	+8.5	+1.2
Aspalathin (g/100 g extract)			
Before heating	20.59±0.05	20.75±0.24	20.75±0.05
After heating	17.83±1.19	20.04±0.52	20.67±0.14
Percentage change	-13.4	-3.4	0.4
Iso-orientin (g/100 g extract)			
Before heating	1.53±0.01	1.54±0.02	1.54±0.01
After heating	1.46±0.04	1.50±0.03	1.50±0.01
Percentage change	-4.6	-2.6	-2.6
Orientin (g/100 g extract)			
Before heating	1.37±0.00	1.39±0.02	1.38±0.01
After heating	1.41±0.01	1.38±0.01	1.37±0.00
Percentage change	+2.9	-0.7	-0.7

[a]Unfermented rooibos extract in deionised water, [b]base + citric acid, [c]base + citric + ascorbic acid.

Table A5.3 The change in the absorbance (420 nm) as well as the total polyphenol, aspalathin, iso-orientin and orientin content of nano emulsified unfermented rooibos iced tea as a result of heating prior to storage

	Formulation	
	NE[a]	**NEC[b]**
Absorbance at 420 nm		
Before heating	0.31±0.03	0.31±0.01
After heating	0.37±0.01	0.37±0.03
Percentage change	+19.4	+19.4
Total polyphenol content (g GAE/100 g soluble solids)		
Before heating	9.07±0.11	9.22±0.32
After heating	9.05±0.18	9.67±0.22
Percentage change	-0.2	+4.9
Aspalathin (g/100 g extract)		
Before heating	3.05±0.00	3.09±0.04
After heating	3.00±0.10	3.05±0.07
Percentage change	-1.6	-1.3
Iso-orientin (g/100 g extract)		
Before heating	0.25±0.00	0.26±0.00
After heating	0.25±0.01	0.25±0.01
Percentage change	0.0	-3.8
Orientin (g/100 g extract)		
Before heating	0.22±0.00	0.23±0.00
After heating	0.22±0.01	0.22±0.00
Percentage change	0.0	-4.3

[a]Nano emulsified unfermented rooibos (NEUR) extract in deionised water, [b]NE + citric acid. There is no NECA formulation as the NEUR extract inherently contained ascorbic acid.

Table A5.4 The change in the absorbance (420 nm) as well as aspalathin, iso-orientin and orientin content of a FR[a]/UR[b] iced tea as a result of heating prior to storage

	Formulation		
	B[c]	**BC[d]**	**BCA[e]**
Absorbance at 420 nm			
Before heating	0.72±0.02	0.63±0.01	0.67±0.05
After heating	0.82±0.01	0.47±0.02	0.43±0.01
Percentage change	+13.9	-25.4	-35.8
Aspalathin (g/100 g extract)			
Before heating	11.24±0.52	11.59±0.18	10.54±0.90
After heating	5.66±0.08	8.78±0.11	9.56±0.06
Percentage change	-49.6	-24.2	-9.3
Iso-orientin (g/100 g extract)			
Before heating	1.41±0.03	1.43±0.01	1.22±0.04
After heating	1.12±0.00	1.14±0.01	1.14±0.00
Percentage change	-20.6	-20.3	-6.6
Orientin (g/100 g extract)			
Before heating	1.49±0.01	1.50±0.01	1.31±0.02
After heating	1.35±0.00	1.29±0.01	1.28±0.01
Percentage change	-9.4	-14.0	-2.3

[a]Fermented rooibos, [b]unfermented rooibos, [c]base (FR and UR extract in deionised water), [d]base + citric acid, [e]base + citric + ascorbic acid.

Table A5.5 The change in the absorbance (420 nm) as well as aspalathin, iso-orientin and orientin content of FR[a]/NEUR[b] iced tea as a result of heating prior to storage

	Formulation	
	NE[c]	**NEC[d]**
Absorbance at 420 nm		
Before heating	0.52±0.01	0.51±0.01
After heating	0.64±0.02	0.62±0.01
Percentage change	+23.1	+21.6
Aspalathin (g/100 g extract)		
Before heating	2.25±0.05	2.25±0.05
After heating	2.35±0.05	2.44±0.01
Percentage change	+4.4	+8.4
Iso-orientin (g/100 g extract)		
Before heating	0.26±0.00	0.27±0.00
After heating	0.31±0.00	0.31±0.00
Percentage change	+19.2	+14.8
Orientin (g/100 g extract)		
Before heating	0.29±0.01	0.29±0.00
After heating	0.33±0.00	0.33±0.00
Percentage change	+13.8	+13.8

[a]Fermented rooibos, [b]nano emulsified unfermented rooibos, FR and NEUR extract in deionised water, [b]NE + citric acid. There is no NECA formulation as the NEUR extract inherently contained ascorbic acid.

ADDENDUM 6

Table A6.1 The change (%) in the absorbance as well as the aspalathin, iso-orientin and orientin content of three types[a] of rooibos iced tea as a result of storage at 25°C

Extract	Formulation	Absorbance[b]	Aspalathin	Iso-orientin	Orientin
FR	B[c]	+12.1	-100.0	-20.1	-3.1
	BC[d]	+4.7	-38.6	-14.5	-3.3
	BCA[e]	-17.6	-25.2	-15.7	-5.6
UR	B	+18.8	-23.3	-10.6	-0.2
	BC	+53.0	-33.4	-12.0	-2.3
	BCA	+20.2	-22.1	-8.8	+0.6
NEUR	NE[f]	+12.8	-12.7	-4.5	-0.5
	NEC[g]	+20.9	-11.9	-2.8	-1.4

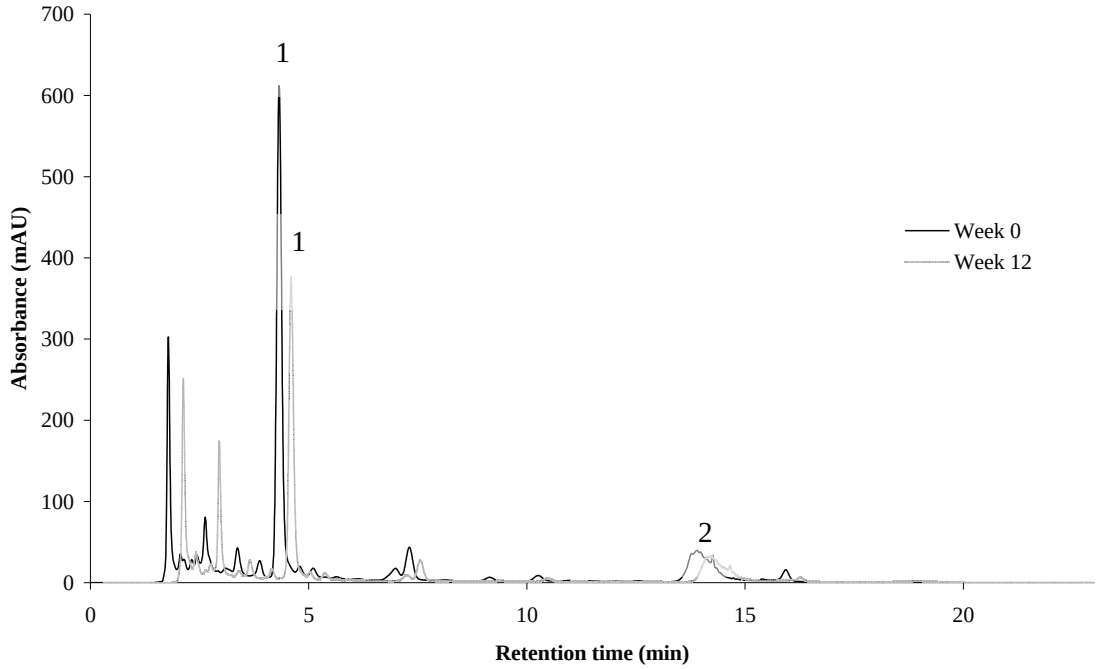
[a]Fermented rooibos (FR), unfermented rooibos (UR) or nano emulsified unfermented rooibos (NEUR) [b]determined at 420 nm, [c]base (FR or UR extract in deionised water), [d]base + citric acid, [e]base + citric + ascorbic acid, [f]NEUR extract extract in deionised water, [g]NE + citric acid. There is no NECA formulation as the NEUR extract inherently contained ascorbic acid.

Table A6.2 The change (%) in the total polyphenol content of three types rooibos iced tea as a result of storage at 25°C

Formulation	**FR[a]**	**UR[b]**	**NEUR[c]**
B[d]	-4.9	-1.0	-8.9
BC[e]/NE[f]	+3.0	+0.3	-9.1
BCA[g]/NEC[h]	-9.8	-7.4	

[a]Fermented rooibos, [b]Unfermented rooibos, [c]nano emulsified unfermented rooibos, [d]Base (Fr or UR extract in deionised water), [e]base + citric acid, [f]NEUR extract in deionised water, [g]base + citric + ascorbic acid, [h]NE + citric acid. There is no NECA formulation as the NEUR extract inherently contained ascorbic acid.

a)



b)

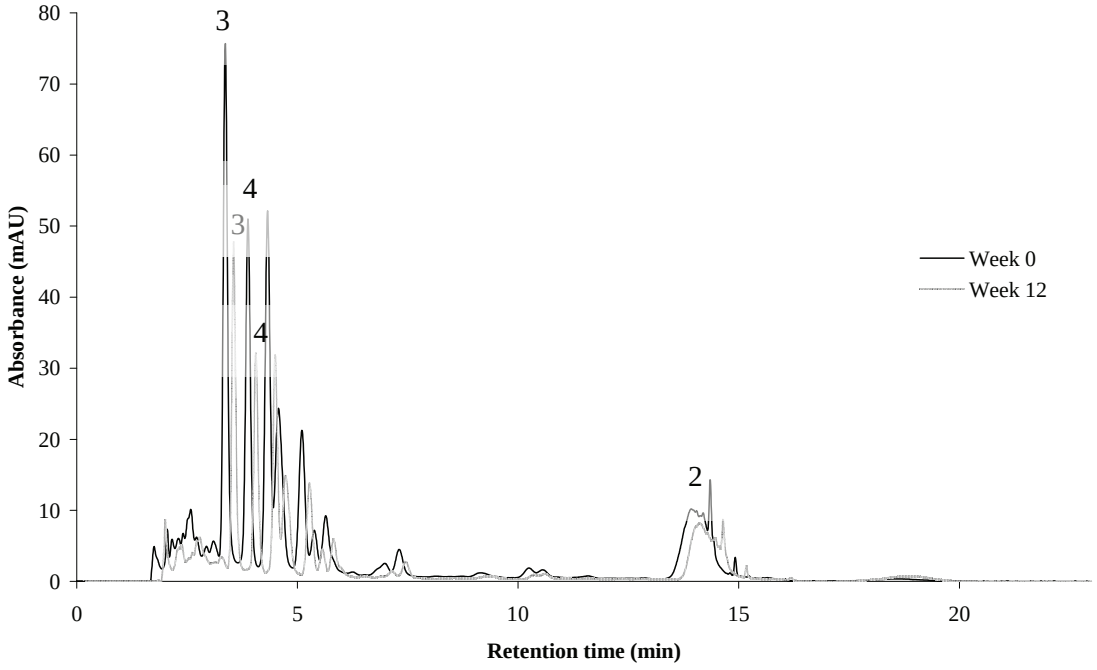
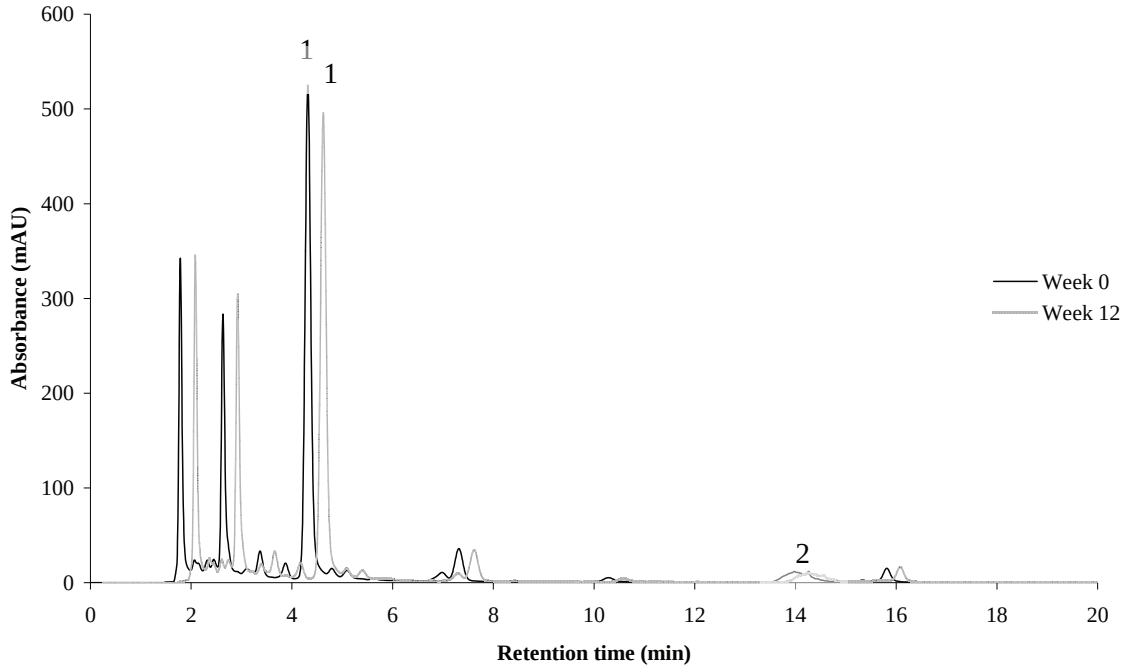


Figure A6.1 Chromatograms of nano emulsified unfermented rooibos iced tea (formulation NE) at (a) 288 nm and (b) 350 nm. The change in the chromatographic profile from week 0 to week 12 is illustrated. Injection volume was 3 µL. Indicated on the relevant chromatograms are (1) aspalathin, (2) polymeric compounds, (3) iso-orientin and (4) orientin. The x-axis of the week 12 chromatogram was slightly offset to enable visual comparison with the stored sample.

a)



b)

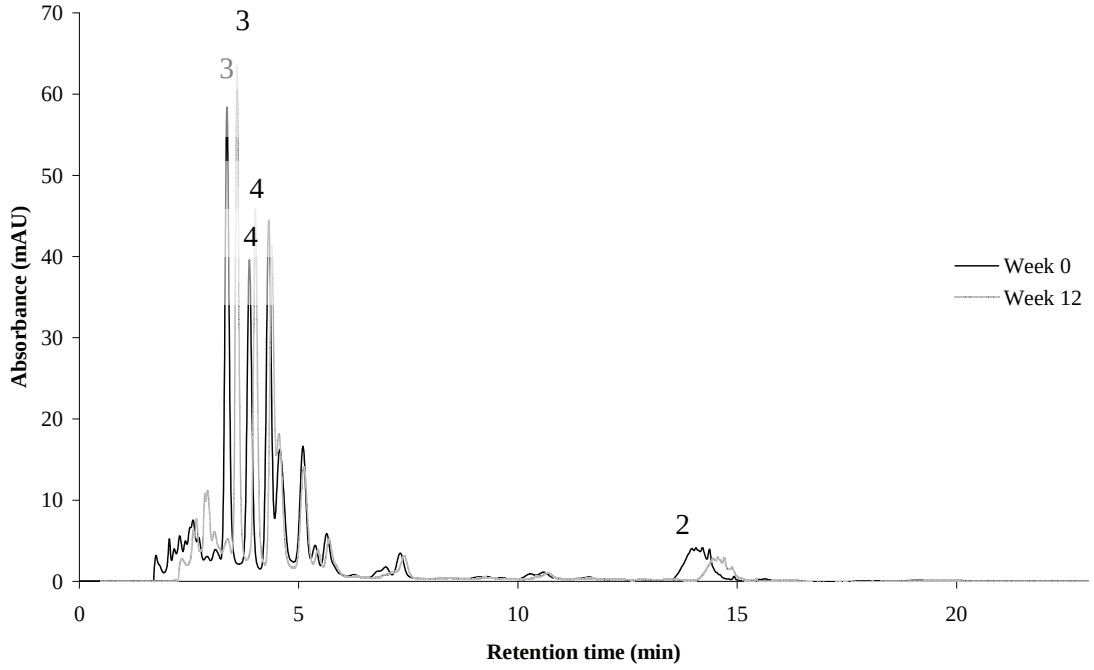
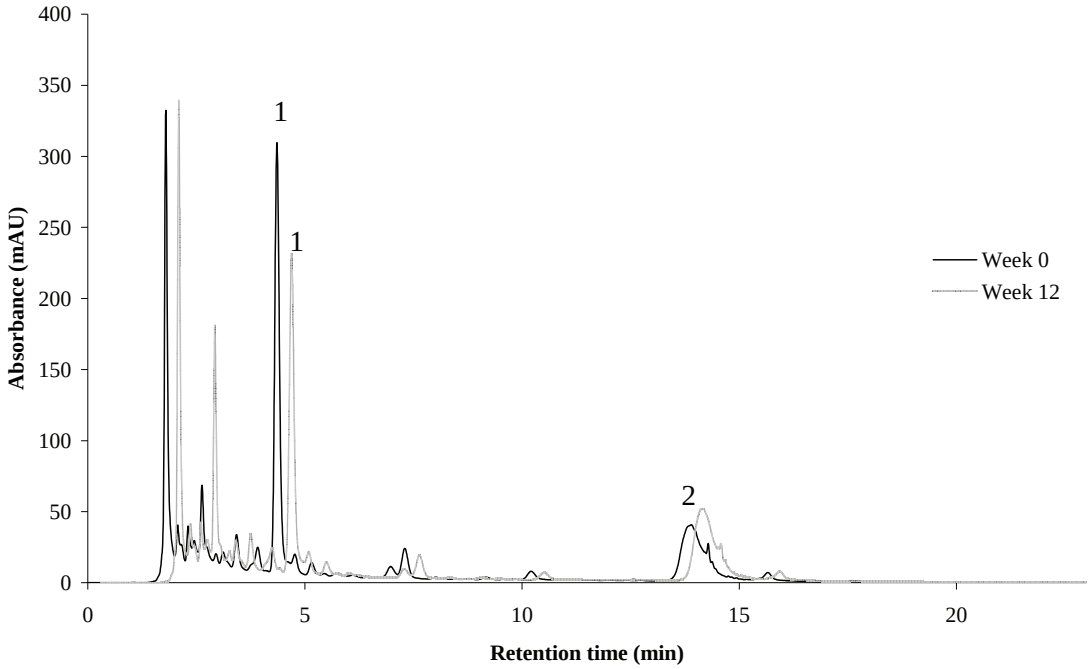


Figure A6.2 Chromatograms of unfermented rooibos iced tea (formulation BCA) at (a) 288 nm and (b) 350 nm. The change in the chromatographic profile from week 0 to week 12 is illustrated. Injection volume was 5 µL. Indicated on the relevant chromatograms are (1) aspalathin, (2) polymeric compounds, (3) iso-orientin and (4) orientin. The x-axis of the week 12 chromatogram was slightly offset to enable visual comparison with the stored sample.

a)



b)

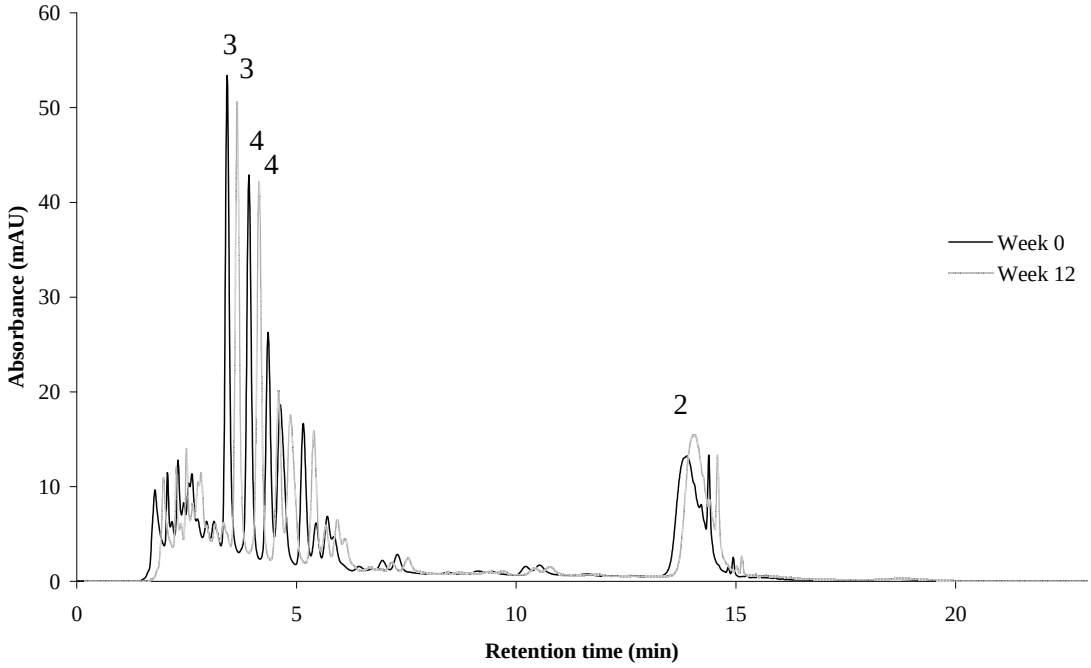
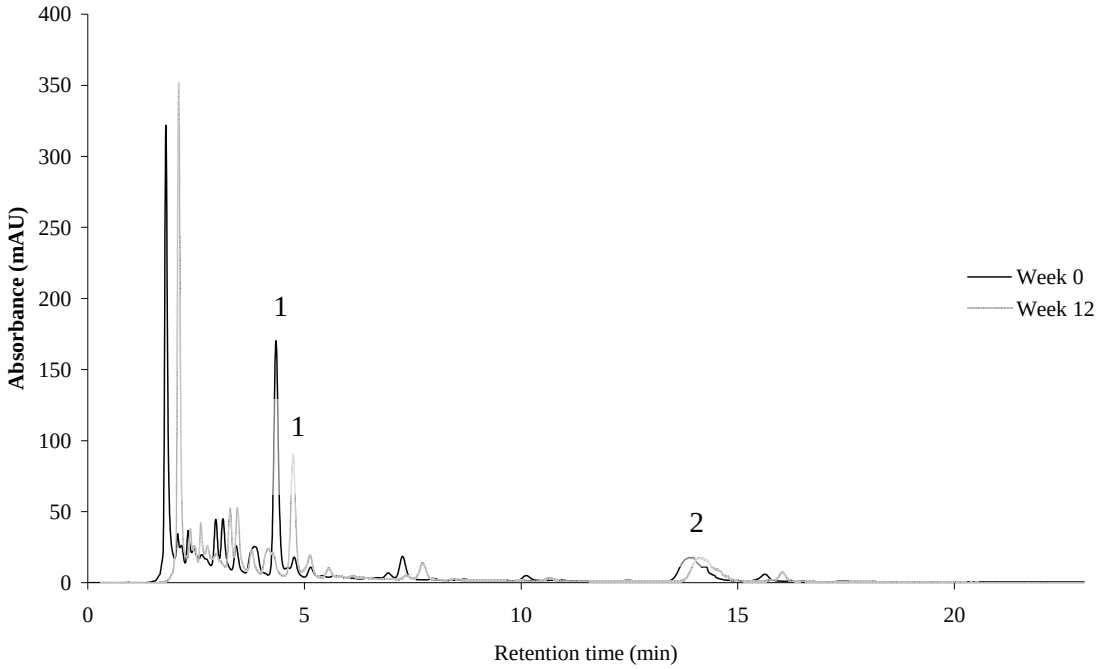


Figure A6.3 Chromatograms of FR/UR iced tea (formulation B) at (a) 288 nm and (b) 350 nm. The change in the chromatographic profile from week 0 to week 12 is illustrated. Injection volume was 5 μL. Indicated on the relevant chromatograms are (1) aspalathin, (2) polymeric compounds, (3) iso-orientin and (4) orientin. The x-axis of the week 12 chromatogram was slightly offset to enable visual comparison with the control.

a)



b)

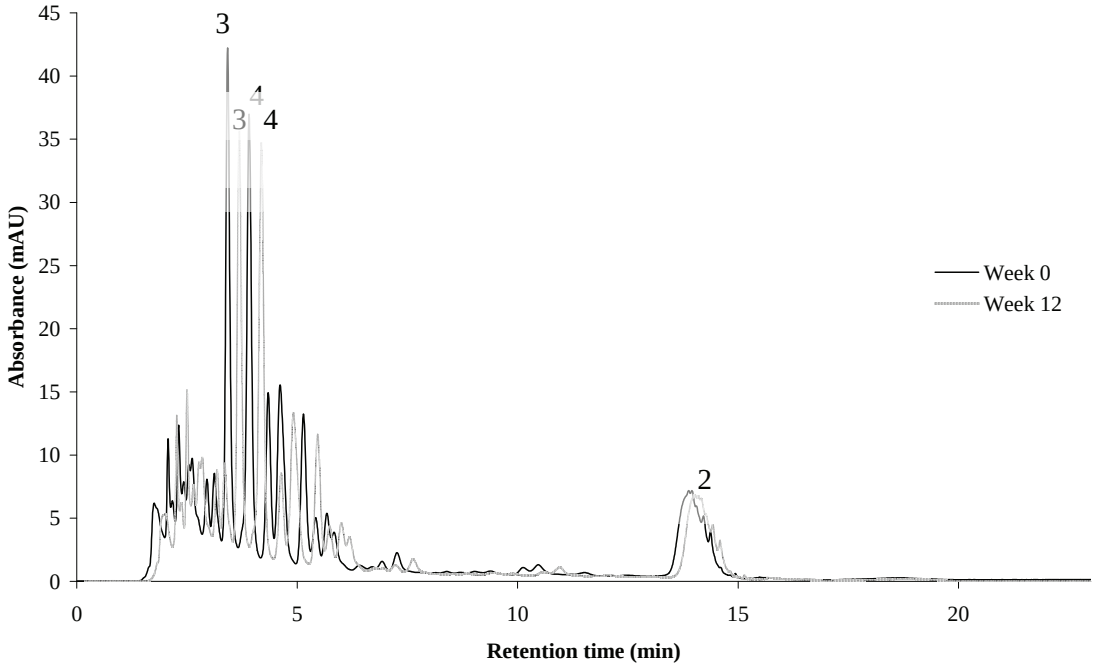


Figure A6.4 Chromatograms of FR/NEUR iced tea (formulation B) at (a) 288 nm and (b) 350 nm. The change in the chromatographic profile from week 0 to week 12 is illustrated. Injection volume was 5 μL. Indicated on the relevant chromatograms are (1) aspalathin, (2) polymeric compounds, (3) iso-orientin and (4) orientin. The x-axis of the week 12 chromatogram was slightly offset to enable visual comparison with the control.

Table A6.3 The change (%) in the absorbance as well as the aspalathin, iso-orientin and orientin content of two combination rooibos iced teas as a result of storage at 25°C

Extract	Formulation	Absorbance at 420 nm	Aspalathin	Iso-orientin	Orientin
FR[a]/UR[b]	B[c]	+12.0	-51.4	-14.4	-2.9
	BC[d]	+27.6	-35.1	-11.5	-3.3
	BCA[e]	-12.8	-20.2	-9.7	-2.4
FR/NEUR[f]	NE[g]	+12.8	-19.4	-5.2	-0.7
	NEC[h]	+10.5	-15.0	-4.3	-1.6

[a]Fermented rooibos, [b]unfermented rooibos, [c]base (FR and UR extract in deionised water), [d]base + citric acid, [e]base + citric + ascorbic acid, [f]nano emulsified unfermented rooibos, [g]NEUR extract in deionised water, [h]NE + citric acid. There is no NECA formulation as the NEUR extract inherently contained ascorbic acid.

Table A6.4 The rate of change (%/week) of the absorbance (420 nm) as well as the aspalathin, iso-orientin and orientin content of three types of rooibos iced tea during storage at 25°C

	FR[a] iced tea			UR[b] iced tea			NEUR[c] iced tea	
	B[d]	BC[e]	BCA[f]	B	BC	BCA	NE[g]	NEC[h]
Absorbance (determined at 420 nm)								
W[i] 0-1	2.46	-0.95	-0.08	7.78	0.32	-0.04	-1.31	5.12
W 1-2	4.98	16.06	0.06	9.63	7.80	0.02	2.41	4.33
W 2-4	1.16	-3.09	-0.03	-3.08	12.22	0.02	0.70	-0.14
W 4-8	-0.15	-0.48	-0.01	2.05	2.06	0.00	1.20	1.41
W 8-12	0.63	-0.24	-0.01	-0.25	1.25	0.00	1.26	1.17
Total polyphenol content								
W 0-1	-0.75	3.73	-6.41	0.97	-0.79	-4.79	-2.14	-1.39
W 1-2	0.75	-1.67	1.39	-0.40	1.28	0.42	6.33	0.30
W 2-4	-0.68	1.29	-1.22	0.56	-0.08	-0.33	-3.55	-1.72
W 4-8	-0.18	-0.58	-0.35	-0.05	-0.64	-0.55	-0.44	-0.34
W 8-12	-0.72	0.19	-0.31	-0.61	0.66	-0.08	-1.01	-0.86
Aspalathin								
W 0-1	-32.94	-6.11	-4.67	-2.16	-4.04	-1.02	-2.17	-1.23
W 1-2	-39.81	-12.50	-10.34	-7.00	-7.45	-6.46	-5.93	-5.97
W 2-4	-27.93	3.32	5.80	-0.39	-1.70	-1.17	1.39	0.98
W 4-8	-25.00	-3.65	-4.46	-2.19	-4.35	-2.05	-1.67	-1.91
W 8-12	No rate	-4.50	-1.13	-1.73	-1.52	-1.55	-0.29	0.20
Iso-orientin								
W 0-1	-3.57	-0.34	-3.55	-2.34	-2.26	-0.80	-1.82	-1.60
W 1-2	3.54	2.52	2.22	8.20	9.32	9.22	10.27	10.48
W 2-4	-3.25	-1.88	-2.44	0.24	-0.89	-0.87	1.59	0.40
W 4-8	-0.37	-1.39	-0.77	-4.41	-4.85	-4.45	-5.22	-4.96
W 8-12	-3.29	-1.99	-1.81	0.57	1.00	1.06	2.00	2.68
Orientin								
W 0-1	0.37	-0.41	-1.07	0.54	-0.55	0.19	-1.42	-1.29
W1-2	2.31	2.48	1.54	6.82	9.13	8.58	9.97	10.47
W 2-4	-0.54	-0.57	-0.89	-0.26	-1.12	0.08	-0.11	-0.08
W 4-8	0.68	0.07	0.15	-2.05	-2.81	-2.88	-3.59	-3.83
W 8-12	-1.79	-1.10	-1.22	0.45	0.94	1.09	2.13	2.48

[a]Fermented rooibos, [b]unfermented rooibos, [c]nano emulsified unfermented rooibos, [d]base (FR or UR extract in deionised water), [e]base + citric acid, [f]base + citric + ascorbic acid, [g]NEUR extract in deionised water, [h]NE + citric acid, [i]week. There is no NECA formulation as the nano emulsified unfermented rooibos extract inherently contained ascorbic acid.

Table A6.5 The rate of change (%/week) of the aspalathin, iso-orientin and orientin content as well as absorbance (420 nm) of two combination rooibos iced teas during storage at 25°C. Five formulations of rooibos iced tea were made with the soluble solids of three types of rooibos tea extract

	FR[a]/UR[b] iced tea			FR/NEUR[c] iced tea	
	B[d]	BC[e]	BCA[f]	NE[g]	NEC[h]
Absorbance (determined at 420 nm)					
W[i] 0-1	0.82	-2.18	-12.92	-0.32	-0.04
W 1-2	2.69	2.42	-1.06	2.51	2.46
W 2-4	1.05	1.65	4.10	0.35	0.07
W 4-8	-0.28	1.29	-3.37	1.47	-0.41
W 8-12	1.79	4.30	2.03	0.89	2.37
Aspalathin					
W 0-1	-4.93	-4.40	-3.75	-2.26	0.68
W 1-2	-10.14	-8.97	-2.91	-1.50	-3.53
W 2-4	-5.15	-1.62	-3.55	-3.17	-2.15
W 4-8	-5.07	-3.42	-0.09	-2.19	0.01
W 8-12	-5.11	-2.68	-1.92	-0.50	-2.14
Iso-orientin					
W 0-1	-2.04	-1.26	-0.84	-0.35	1.88
W 1-2	-2.25	-2.60	-14.86	-11.89	-13.90
W 2-4	-8.14	-6.92	-1.48	-0.45	-0.33
W 4-8	1.30	1.20	1.85	1.10	2.02
W 8-12	0.37	0.48	0.65	1.09	0.40
Orientin					
W 0-1	-0.84	-0.04	-0.05	0.08	2.08
W 1-2	0.39	-1.23	-9.51	-8.77	-11.26
W 2-4	-5.33	-4.39	-1.04	-0.14	0.48
W 4-8	1.84	1.54	2.21	1.53	1.84
W 8-12	0.41	0.29	0.30	0.69	0.06

[a]Fermented rooibos, [b]unfermented rooibos, [c]nano emulsified unfermented rooibos, [d]base (FR and UR extract in deionised water), [e]base + citric acid, [f]base + citric + ascorbic acid, [g]FR and NEUR extract in deionised water, [h]NE + citric acid, [i]week. There is no NECA formulation as the NEUR extract inherently contained ascorbic acid.

ADDENDUM 7

Table A7.1 The effect of pH and temperature (ºC) on the change (%) in absorbance (420 nm) of three rooibos extract formulations

pH	Temperature	FR[a] extract	UR[b] extract	NEUR[c] extract
pH 3	5	+27.2	+17.3	-2.6
	30	+32.2	+37.3	-2.1
	40	+43.3	+73.8	-3.2
pH 4	5	+1.0	+28.7	-2.4
	30	+11.6	+67.3	+3.2
	40	+12.9	+86.2	-3.7
pH 5	5	+3.0	+24.5	-0.8
	30	+12.2	+47.2	-5.1
	40	+13.9	+78.9	-3.0
pH 6	5	+2.4	+12.6	-2.3
	30	+26.9	+23.5	-5.0
	40	+28.5	+54.6	-3.1
pH 7	5	+1.2	+2.9	-4.4
	30	+52.2	+30.6	-4.9
	40	+19.4	+40.6	-2.8

[a]Fermented rooibos, [b]unfermented rooibos, [c]nano emulsified unfermented rooibos.

Table A7.2 The effect of pH and temperature on the change (%) in the aspalathin content of three rooibos extract formulations

pH	Temperature	FR[a] extract	UR[b] extract	NEUR[c] extract
pH 3	5	-1.4	-2.1	-2.3
	30	+10.4	-1.2	-8.3
	40	+4.0	-2.4	-5.1
pH 4	5	-11.9	+0.2	-5.8
	30	-8.3	+2.9	-6.1
	40	-38.9	-2.5	-13.4
pH 5	5	-18.0	+1.7	-5.4
	30	-28.4	+2.2	-3.8
	40	-67.2	+0.1	-5.6
pH 6	5	-17.3	-3.6	-1.9
	30	-100.0	-9.1	-4.7
	40	-100.0	-23.0	-6.1
pH 7	5	-100.0	-6.8	-10.4
	30	-100.0	-44.8	-30.7
	40	-100.0	-76.4	-45.0

[a]Fermented rooibos, [b]unfermented rooibos, [c]nano emulsified unfermented rooibos.

Table A7.3 The effect of pH and temperature on the change (%) in the iso-orientin content of three rooibos extract formulations

pH	Temperature	FR[a] extract	UR[b] extract	NEUR[c] extract
pH 3	5	+0.5	-2.9	-14.2
	30	+5.9	-1.0	-17.7
	40	+4.9	-0.6	-14.9
pH 4	5	+0.7	-0.9	-17.4
	30	+1.4	+2.3	-16.5
	40	+2.7	+1.5	-23.6
pH 5	5	+0.3	+1.4	-15.6
	30	-2.6	+1.4	-15.5
	40	-0.9	+3.2	-16.8
pH 6	5	+4.4	-2.4	-12.0
	30	-1.1	-2.9	-15.1
	40	-8.6	-5.9	-16.0
pH 7	5	-2.5	-0.4	-18.2
	30	-22.4	-4.3	-21.5
	40	-32.1	-11.7	-22.2

[a]Fermented rooibos, [b]unfermented rooibos, [c]nano emulsified unfermented rooibos.

Table A7.4 The effect of pH and temperature on the change (%) in the orientin content of three rooibos extract formulations

pH	Temperature	FR[a] extract	UR[b] extract	NEUR[c] extract
pH 3	5	+0.3	-3.1	-14.7
	30	+1.9	+0.3	-19.4
	40	+1.4	+0.3	-17.4
pH 4	5	-1.3	+0.1	-17.2
	30	+0.3	+2.9	-17.1
	40	+2.1	+2.4	-25.7
pH 5	5	-0.6	+1.4	-16.5
	30	-1.6	+3.2	-16.3
	40	+0.3	+4.9	-17.3
pH 6	5	+3.3	-1.7	-13.2
	30	-4.6	+1.1	-15.6
	40	+0.4	+3.2	-17.4
pH 7	5	+1.1	+0.3	-18.7
	30	-7.8	+3.1	-19.2
	40	-6.0	+4.1	-19.9

[a]Fermented rooibos, [b]unfermented rooibos, [c]nano emulsified unfermented rooibos.

Table A7.5 The main effects of hydrogen peroxide addition (660 mg/L) and storage (30ºC) on the change (%) in the absorbance as well as the aspalathin, iso-orientin and orientin content of four unfermented rooibos extract formulations

Compound or Absorbance	**Formulation**	**A vs. C[a]**	**C vs.D[b]**
Absorbance (at 420 nm)	B[c]	+2.0	+40.9
	BA[d]	+13.8	+35.0
	BC[e]	-20.9	+84.4
	BCA[f]	+16.4	+43.1
Aspalathin	B	-12.0	-16.2
	BA	-25.3	-3.0
	BC	-13.5	-15.9
	BCA	-20.8	-10.9
Iso-orientin	B	-15.0	-1.5
	BA	-21.4	+3.8
	BC	-16.6	-6.4
	BCA	-21.8	-3.0
Orientin	B	-14.4	+1.4
	BA	-24.2	+2.8
	BC	-16.2	-5.7
	BCA	-23.0	-0.8

[a]Day 0 without H_2O_2 vs. day 0 with H_2O_2, [b]day 0 with H_2O_2 vs. day 7 with H_2O_2, [c]base (unfermented rooibos extract in deionised water), [d]base + ascorbic acid, [e]base + citric acid, [f]base + citric + ascorbic acid.

Table A7.6 The change (%) in the absorbance as well as the aspalathin, iso-orientin and orientin content of four unfermented rooibos extract formulations as a result of hydrogen peroxide addition (660 mg/L) and storage at 30ºC

Absorbance or compound	**Formulation**	**A vs. B[a]**	**B vs. D[b]**	**A vs. D[c]**
Absorbance (at 420 nm)	B[d]	+95.6	-26.6	+43.6
	BA[e]	+33.4	+15.2	+53.7
	BC[f]	+38.8	+5.1	+45.9
	BCA[g]	+31.1	+27.0	+66.5
Aspalathin	B	-7.0	-20.7	-26.3
	BA	-4.9	-23.8	-27.5
	BC	-4.6	-23.6	-27.2
	BCA	-12.0	-19.8	-29.5
Iso-orientin	B	+5.1	-20.2	-16.2
	BA	+1.2	-19.4	-18.4
	BC	+2.8	-24.1	-22.01
	BCA	-5.3	-19.9	-24. 1
Orientin	B	+6.3	-18.3	-13.2
	BA	+0.5	-22.5	-22.1
	BC	+1.2	-21.9	-20.9
	BCA	-6.5	-18.3	-23.6

[a]Day 0 without H_2O_2 vs. day 7 without H_2O_2, [b]day 7 without H_2O_2 vs. day 7 with H_2O_2, [c]day 0 without H_2O_2 vs. day 7 with H_2O_2, [d]base (unfermented rooibos extract in deionised water), [e]base + ascorbic acid, [f]base + citric acid, [g]base + citric + ascorbic acid.

Table A7.7 The increase (%) in polymer area of four unfermented rooibos extract formulations upon the addition of hydrogen peroxide (660 mg/L)

Formulation	**Increase in polymer area**
B[a]	+204.38
BA[b]	+102.17
BC[c]	+131.47
BCA[d]	+47.26

[a]Base (unfermented rooibos extract in water), [b]base + ascorbic acid, [c]base + citric acid, [d]base + citric + ascorbic acid.

Table A7.8 The change (%) in the absorbance as well as the aspalathin, iso-orientin and orientin content of three rooibos extract formulations as a result of the addition of 0.5 mg/L hydrogen peroxide

Extract	Formulation	Absorbance[a]	Aspalathin	Iso-orientin	Orientin
FR[b]	BA[c]	-0.2	-0.5	-0.5	-0.5
	BCA[d]	+0.5	-0.7	-1.1	-0.7
UR[e]	BA	-2.4	+0.1	-0.2	-0.1
	BCA	+11.9	+0.2	+0.0	+0.3
NEUR[f]	NE[g]	+0.9	+0.0	-0.1	+0.4
	NEC[h]	+0.6	+0.9	+0.4	+0.7

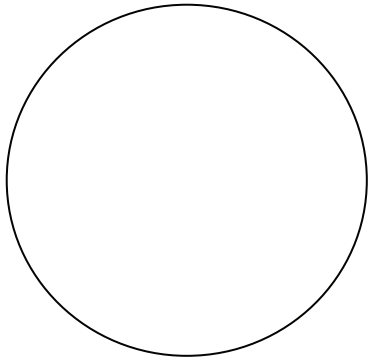
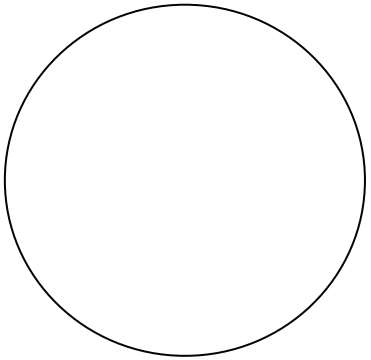
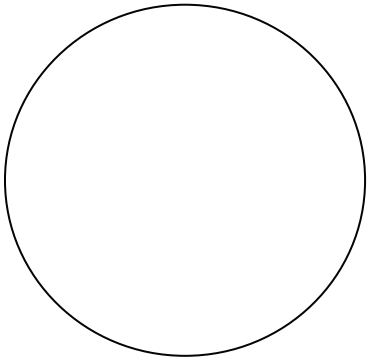
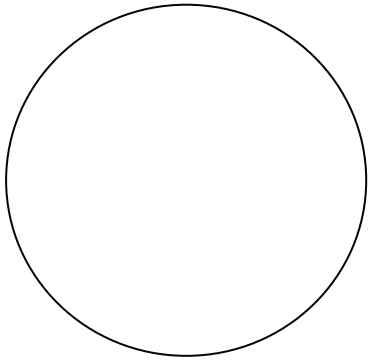
[a]Determined at 420 nm, [b]fermented rooibos, [c]base (FR or UR extract in deionised water) + ascorbic acid, [d]base + citric + ascorbic acid, [e]unfermented rooibos, [f]nano emulsified unfermented rooibos, [g]NEUR extract in deionised water, [h]NE + citric acid. There is no NECA formulation as the NEUR extract inherently contained ascorbic acid.

ADDENDUM 8

Addendum 8 is an example of the questionnaire for the descriptive sensory analysis of rooibos iced tea. The questionnaire for the consumer panel is shown in Addendum 9.

REPLICATE	JUDGE NO	NAME OF JUDGE:
MOUTH-FEEL Evaluate the mouthfeel of each sample by swirling a mouthfull	None 0----100 Prominent ***ASTRIN-GENCY***	_____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100
Refresh your palate between each attribute - first with biscuit and then with water		
FLAVOUR Evaluate the flavour of each sample by swirling a mouthfull	None 0-----100 Prominent ***PLANT-LIKE*** flavour	_____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100
	None 0 ---100 Prominent ***HAY-LIKE*** **flavour**	_____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100
	None 0 ---100 Prominent ***ROOIBOS*** **flavour**	_____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100
	None 0----100 Prominent ***LEMON*** **flavour**	_____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100 _____ 0|_____|__100

For each attribute, please check that you have analysed all the samples

ACCEPTABILITY OF ICED TEA

NAME OF JUDGE_____		JUDGE NO_____
PLEASE CIRCLE WHICHEVER IS APPLICABLE		
GENDER Male / Female	AGE 18-22 / 23 - 27 / 28 – 32 / 33 - 40 / 41+	*CONSUMPTION OF ICED TEA* 2-3 x per week / 1 x per week / 1 x per month / 2-3 x per year / NEVER

ADDENDUM 9

INSTRUCTIONS

- PLEASE **TASTE** THE 4 SAMPLES IN THE ORDER PRESENTED, I.E. FROM **LEFT TO RIGHT**.
- RINSE YOUR MOUTH WITH **WATER** BEFORE BEGINNING AND BETWEEN SAMPLES.
- TAKE A **GENEROUS SIP** FROM EACH SAMPLE.
- RANK THE SAMPLES ON THE NINE-POINT SCALE.

IN EACH CASE, CIRCLE THE NUMBER NEXT TO THE PREFERRED DEGREE OF LIKING.

CODE		CODE		CODE		CODE	
9	Like extremely	9	Like extremely	9	Like extremely	9	Like extremely
8	Like very much	8	Like very much	8	Like very much	8	Like very much
7	Like moderately	7	Like moderately	7	Like moderately	7	Like moderately
6	Like slightly	6	Like slightly	6	Like slightly	6	Like slightly
5	Neither like nor dislike	5	Neither like nor dislike	5	Neither like nor dislike	5	Neither like nor dislike
4	Dislike slightly	4	Dislike slightly	4	Dislike slightly	4	Dislike slightly
3	Dislike moderately	3	Dislike moderately	3	Dislike moderately	3	Dislike moderately
2	Dislike very much	2	Dislike very much	2	Dislike very much	2	Dislike very much
1	Dislike extremely	1	Dislike extremely	1	Dislike extremely	1	Dislike extremely

THANK YOU VERY MUCH FOR YOUR ASSISTANCE, PLEASE COLLECT A SMALL "GIFT" AFTER YOU HAVE TASTED THE SAMPLES