

Evaluation of capillary electrophoresis for the analysis of indigenous tea phenolics

by
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DECLARATION

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SUMMARY

The endemic South African plant species, *Aspalathus linearis* (rooibos) and *Cyclopia* spp. (honeybush) are consumed worldwide as popular herbal teas. In addition, recent trends in the consumption of these products as ready-to-drink commodities and the use of extracts as ingredients in food, beverage, cosmetic and nutraceutical products are partially ascribed to their potential health-promoting effects. Both rooibos and honeybush teas contain relatively high levels of rare anti-oxidative phenolic compounds. The health properties of these plants are largely associated with their content of phenolic compounds. The analysis of phenolics in these herbal teas is therefore important, not only in support of fundamental research, but also for quality control and marketing purposes.

High performance liquid chromatography (HPLC) is the method of choice for the analysis of phenolic compounds in natural products, due to the proven performance of the technique in terms of robustness and sensitivity. However, HPLC also suffers from some drawbacks such as relatively high solvent consumption, long analyses and challenges to obtain sufficient resolution for highly complex samples such as these. Capillary electrophoresis (CE) is a potentially promising alternative technique for the separation of phenolics. CE offers cheap routine analyses, high speed and high efficiencies and an alternative separation mechanism compared to HPLC. The aim of this study was to evaluate CE as an alternative to HPLC for the analysis of rooibos and honeybush tea phenolics.

Following extensive method optimisation, two capillary zone electrophoresis (CZE) methods which provided efficient separation of the principal rooibos (15) and honeybush (8) tea phenolics, were successfully developed for the first time. Experimental parameters evaluated and optimised include the background electrolyte (BGE) composition and pH, capillary dimensions, analysis temperature, applied voltage and injection volumes. A borate buffer was found to be essential due to the capability of this BGE to form anionic complexes with phenolics containing vicinal diols, thereby affecting the selectivity of the separation. The optimised BGE pHs for the rooibos and honeybush methods were 8.80 and 9.25, respectively, resulting in total analysis times (including conditioning of capillary) of 31 (honeybush) and 42 minutes (rooibos). Analysis times were slightly shorter than the routine HPLC methods. The optimised CZE methods were characterised by alternative selectivity compared to HPLC, and higher resolution of critical compound pairs was obtained. The methods were validated in terms of repeatability of electrophoretic mobilities, linearity of calibration curves and sensitivity (limits of detection (LODs) and limits of quantification (LOQs)). Sensitivity and repeatability of the CZE methods were however not comparable with HPLC. Finally, the developed methods were applied to the analysis of fermented and unfermented rooibos (*Aspalathus linearis*) and honeybush tea (*C. subternata* and *C. maculata* samples). Quantitative data obtained for 10 samples of fermented and unfermented rooibos and *C. subternata* and 9 of *C. maculata* each were compared to those obtained by routine HPLC methods, and were found to be statistically comparable for the majority of

compounds, with a few exceptions. The two quantitative CZE methods demonstrated their utility for the routine quantitative analysis of phenolics in rooibos and honeybush teas, respectively, thereby confirming the potential of CE as an alternative to HPLC for the routine analysis of these samples.

OPSOMMING

Die endemiese Suid-Afrikaanse plantspesies, *Aspalathus linearis* (rooibos) en die *Cyclopia* spesies (heuningbos) word wêreldwyd verbruik as gewilde kruietees. Verder word onlangse tendense ook gevolg met die verbruik van hierdie produkte as drink-gereed kommoditeite, asook die gebruik van ekstrakte daarvan as bestandele in voedsel, drank en kosmetiese produkte wat gedeeltelik toegeskryf kan word aan hul potensiële gesondheidsvoordele. Beide rooibos en heuningbos tee bevat relatief hoë vlakke van skaars anti-oksidante, sogenaamde fenoliese verbindings. Die gesondheidseienskappe van hierdie plante hou tot 'n groot mate verband met hul inhoud van fenoliese verbindings. Die analiese van fenoliese komponente in hierdie kruietees is dus belangrik, nie net ter ondersteuning van fundamentele navorsing nie, maar ook vir gehaltebeheer en bemarkings doeleindes.

Hoë-druk vloeistof chromatografie (HDVC) is die mees algemene metode wat aangewend word vir die analise van fenoliese verbindings in natuurlike produkte as gevolg van die doeltreffendheid van die tegniek in terme van herhaalbaarheid en sensitiwiteit. Maar HDVC word egter ook gekenmerk deur 'n paar nadele soos relatief hoë oplosmiddel verbruik, lang analises en die uitdaging om voldoende resolusie vir baie komplekse monsters soos natuurlike produkte daar te stel. Kapillêre elektroforese (KE) is 'n belowende potensiële alternatiewe tegniek vir die skeiding van fenole. KE is goedkoop vir roetine analise, is vinnig en bied hoe effektiwiteit en 'n alternatiewe skeidingsmeganisme in vergelyking met HDVC. Die doel van hierdie studie was om KE te ondersoek as 'n alternatief vir HDVC vir die analiese van rooibos en heuningbos tee fenole.

Na intensieve metode optimisering is twee kapillêre zone elektroforese (KZE) metodes wat effektiewe skeiding van die hoof rooibos (15) en heuningbos (8) tee fenole verskaf het vir die eerste keer suksesvol ontwikkel. Veranderlikes wat eksperimenteel geëvalueerd en geoptomiseer is, sluit die samestelling en pH van agtergrond elektrolyet (AE) in, kapillêre dimensies, analise temperatuur, aangewende hoogspanning en volume wat ingespuit word. Die gebruik van 'n boraat buffer was noodsaaklik, as gevolg van sy vermoë om anioniese komplekse te vorm met fenole met visinale diole en dit affekteer sodoende die selektiwiteit van die skeiding. Die optimale AE pH vir die rooibos en heuningbos metodes was 8.80 en 9.25 onderskeidelik, en die totale analise tyd (insluitend kondisionering van kapillêre) van 31 (heuningbos) en 42 minute (rooibos), onderskeidelik. Analise tyd was effens korter as die roetine HDVC metodes. Die optimale KZE metodes is gekenmerk deur alternatiewe selektiwiteit in vergelyking met HDVC, en hoér resolusie van kritiese pareverbindingen is bewerkstellig. Die metodes is gevalideer in terme van herhaalbaarheid van elektroforetiese mobiliteit, lineariteit van kalibrasie kurwes en sensitiwiteit (limiete van deteksie (LVDs) en limiete van kwantifisering (LVKs)). Sensitiwiteit en herhaalbaarheid van die KZE metodes was nie vergelykbaar met die van HDVC nie. Ten slotte is die ontwikkelde metodes toegepas vir die analise van beide gefermenteerde en ongefermenteerde rooibos (*Aspalathus linearis*) en heuningbos tee (*C.*

subternata en *C. maculata*) monsters. Kwantitatiewe data is verkry vir 10 monsters van gefermenteerde en ongefermenteerde rooibos en *C. subternata* en 9 van *C. maculata*. In vergelyking met data wat verkry is deur roetine HDVC was die resultate statisties vergelykbaar vir die meerderheid van komponente, met enkele uitsonderings. Die twee kwantitatiewe KZE metodes demonstreer hul nut vir die roetine kwantitatiewe analise van fenole in rooibos en heuningbos tee, onderskeidelik, en sodoende illustreer die potensiaal van KE as 'n alternatief vir HDVC vir roetine analise van hierdie monsters.

DEDICATION

and

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LIST OF ABBREVIATIONS

1D – One dimensional

2D – Two dimensional

ACN – Acetonitrile

AD - Amperometric detection

ANOVA – Analysis of variance

ASEI - Anion-selective exhaustive injection

BGE - Background electrolyte

C - Catechin

CCD - Contactless conductivity detection

CD - Cyclodextrin

CE – Capillary electrophoresis

CEC - Capillary electrochromatography

CG – Catechin gallate

CGE - Capillary gel electrophoresis

CIEF - Capillary isoelectric focusing

CITP - Capillary isotachophoresis

CMC - Critical micelle concentration

CTAB - Cetyltrimethylammonium bromide

CZE - Capillary zone electrophoresis

DAD - Diode array detection

DC - Direct current

DI – Deionised

DMSO - Dimethylsulfoxide

DNA - Deoxyribonucleic acid

EC – Epicatechin

ECG – Epicatechin gallate

EGC – Epigallo catechin

EGCG - Epigallocatechingallate

EOF – Electroosmotic flow

ESI – Electro spray ionisation

FASS - Field amplified sample stacking

FASI - Field amplified sample injection

FD - Fluorescence detection

GC - Gallocatechin

GCG – Gallocatechin gallate

GLM - General linear models

HCS - High conductivity solutions

HP - Hydroxypropyl

HPLC – High performance liquid chromatography

i.d. – Internal diameter

ITP – Isotachophoretic

LC-MS – Liquid chromatography-mass spectrometry

LC-MS/MS – Liquid chromatography tandem mass spectrometry

LIFD - Laser induced fluorescence detection

LOD – Limit of detection

LOQ – Limit of quantification

MEEKC - Micellar emulsion electrokinetic chromatography

MEKC - Micellar elektrokinetic chromatography

MeOH - Methanol

MSS - Micelle to solvent stacking

NACE - Non-aqueous capillary electrophoresis

NACZE – Non-aqueous capillary zone electrophoresis

NAMEKC – Non-aqueous micellar electrokinetic chromatography

NMR – Nuclear magnetic resonance

PPAG - Phenylpyruvic acid-2-*O*-glucoside

PVDF - polyvinylidene fluoride

REPSM - Reversed electrode polarity stacking mode

RP – Reversed phase

SC - Sodium cholate

SDS - Sodium dodecyl sulfate

S/N – Signal-to-noise

SI - Supporting information

s-β-CD - sulfated-β-cyclodextrin

THF - Tetrahydrofuran

TMS – Trimethylsiloxane

UHPLC – Ultra high performance liquid chromatography

UV-vis - Ultraviolet-visible

CHAPTER 1

GENERAL INTRODUCTION

1.1. Introduction

Produced from the respective native South African plant species, *Aspalathus linearis* and *Cyclopia* spp., rooibos and honeybush are globally enjoyed as popular herbal teas. There are two forms of the teas: fermented (“oxidised”) and unfermented (“green”), of which the former is mainly marketed. Organic and green rooibos and honeybush teas are also produced, but the demand for these products are less. The rooibos tea industry is well established, unlike the honeybush tea industry, and contributes to the local economy of the Western Cape province, where rooibos occurs naturally. Production per annum of both teas is increasing, with growing exports especially for honeybush during the last few years. The leading export destinations for both products include European countries (Germany, The Netherlands and United Kingdom (UK)), Japan and United States of America (USA). In addition to the consumption of rooibos and honeybush as popular herbal teas, alternative uses of aqueous extracts of these products as ingredients in food, beverage, pharmaceutical and nutraceutical industries are increasing [1,2].

In recent years, interest in the consumption of rooibos and honeybush teas as health beverages has increased due to the potential health benefits ascribed to their consumption [3]. Both these teas are caffeine free, low in tannins and contain high levels of phenolic compounds. Several of the purported health-promoting properties of rooibos and honeybush have been attributed to the presence of the phenolic compounds [3,4]. These are anti-oxidants, which are known to reduce the risk of cardiovascular and degradative diseases in humans [5]. In addition, rooibos and honeybush exhibit potential anti-diabetic, anti-obesity, cardiovascular and chemoprotective properties, associated with the presence of compounds such as aspalathin, phenylpyruvic acid-2-*O*-glucoside, orientin and isoorientin in rooibos and hesperidin, mangiferin, isomangiferin and iriflophenone-3-*C*- β -glucoside-4-*O*- β -glucoside in honeybush [6-15]. Isomangiferin and iriflophenone-3-*C*- β -glucoside, both major honeybush phenolic constituents, are strong anti-oxidants and show potential in the treatment of rheumatoid arthritis [16,17]. Detailed knowledge on the phenolic composition of these teas and their derived commodities is critically important for the industry in support of manufacturing, quality control, marketing and research purposes. This requires the availability of accurate, robust and sensitive analytical methods for phenolic determination.

Reversed phase liquid chromatography (RP-LC) is most often used for the analysis of phenolics, and has also found widespread application in the analysis of tea phenolics, including herbal teas [18,19]. The technique is sensitive, reproducible and robust, and therefore well suited to the routine analysis of teas. Furthermore, hyphenation to mass spectrometry (MS) allows tentative identification of novel phenolic constituents at low levels [20-22]. However, RP-LC also suffers from some disadvantages, including the use of relatively large volumes of solvent and relatively long analysis times for the separation of complex mixtures of phenolics such as encountered in tea. Routine RP-LC methods for

the analysis of rooibos and honeybush tea phenolics currently employed at the Agricultural Research Council (ARC), while suitable for routine analysis of selected compounds, suffer from incomplete separation of some minor and/or unidentified constituents in the case of rooibos tea [20] and the requirement of multiple species-specific methods for honeybush tea [21]. The development of alternative separation methods for the analysis of the phenolic composition of these herbal teas is therefore of interest in the context of overcoming some of these limitations.

Capillary electrophoresis (CE) is an alternative separation technique which has been used with some success in the analysis of tea phenolics [19]. CE offers a different separation mechanism compared to high performance liquid chromatography (HPLC), is fast, cost-effective, highly efficient and uses no or very little solvent, although the technique is generally less sensitive and reproducible compared to HPLC.

1.2. Aim and objectives

In the context of the above, the aim of this study was to evaluate CE as an alternative to HPLC for the analysis of the principal rooibos and honeybush tea phenolics. To attain this aim, the following objectives were set for this study:

- Optimisation of experimental parameters (buffer pH and concentration, temperature, voltage, capillary dimensions and injection volume) to attain efficient separation of the principal rooibos and honeybush phenolics by CE. For honeybush tea analyses, standard compounds will be selected to represent the composition of several *Cyclopia* species.
- To validate the optimised CE methods, one each for rooibos and honeybush phenolics, in terms of linearity, sensitivity, specificity and repeatability.
- To perform quantitative analysis of unfermented and fermented rooibos and honeybush tea samples; in the case of honeybush tea this will be done for both *C. subternata* and *C. maculata* samples.
- To compare the quantitative data for the analysed samples with those obtained by HPLC as reference method to assess accuracy, and to compare the overall performance of the CE methods to the HPLC reference methods currently in use.

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CHAPTER 2

LITERATURE REVIEW

2.1. Indigenous South African herbal teas

Rooibos (*Aspalathus linearis*), honeybush (*Cyclopia* spp) and bush tea (*Athrixia phylicoides*) are 3 well-known endemic plants to South Africa. Rooibos and honeybush form part of the fynbos biome and are enjoyed as herbal tea drinks, whereas bush tea is more popular for its traditional medicinal uses (treatment of boils, acne, infected wounds and throat conditions) and is distributed along the mountain regions of Mpumalanga, Limpopo, the northern parts of the Eastern Cape Province, KwaZulu-Natal and Swaziland. Bioactivity research focussing on rooibos and honeybush is increasing, owing to their purported health properties, which may lead to production of recognised medicinal products (for example in the treatment of *diabetes mellitus*). The following discussion provides some general background on rooibos and honeybush teas.

2.2. Historical background and distribution of Rooibos (*Aspalathus linearis*) and Honeybush (*Cyclopia* spp.)

Rooibos (*Aspalathus linearis*), belonging to (Burm.f.) Dahlg. (Family Fabaceae, Tribe Crotalarieae), is found in the fynbos biome of South Africa and is popular worldwide as a herbal tea. Naturally grown rooibos tea is distributed, cultivated and harvested in the greater Cederberg area in the Western Cape province of South Africa. Production primarily occurs in the region of Clanwilliam (**Figure 2.1.**) [1]. Rooibos is listed as a South African medicinal plant and was first used by the Khoi as a fermented beverage [2]. Previously rooibos tea was produced from different *Aspalathus* species from the Cederberg mountain area [3], referred as “rooi tee” (“red tea”), “vaal tee” (“grey”), “swart tee” (“black”) and “rooibruiin tee” (“red-brown”). Only the “rooi tee” from the Pakhuis Pass area is commercialised today and can be divided into two types: Nortier (cultivated) and Cederberg (wild-growing) tea [2].

In the early 1900’s marketing of rooibos was pioneered by Benjamin Ginsberg; the product was produced by chopping, crushing and “sweating” of the shredded plant material, followed by sun drying. In 1930 a medical practitioner, Dr. P. le Frais Nortier, and his friends, Oloff Bergh and William Riordan, were the first to recognise the agricultural value of rooibos. The rooibos tea industry was and still is in competition with oriental (black) tea, but it was not until the outbreak of World War II, when oriental tea markets decreased, that rooibos exports started increasing significantly. However, the industry declined shortly thereafter, owing to the poor quality grades of the product [2].

In 1968, Annetjie Theron fed her restless and crying baby daughter, Lorinda Theron, a warm bottle of rooibos tea; Lorinda’s symptoms of chronic restlessness, vomiting and stomach cramps were eased [2]. Since then, rooibos became popular as a health beverage, and extensive research on the health properties and bioactivity of rooibos has been performed [4-22].

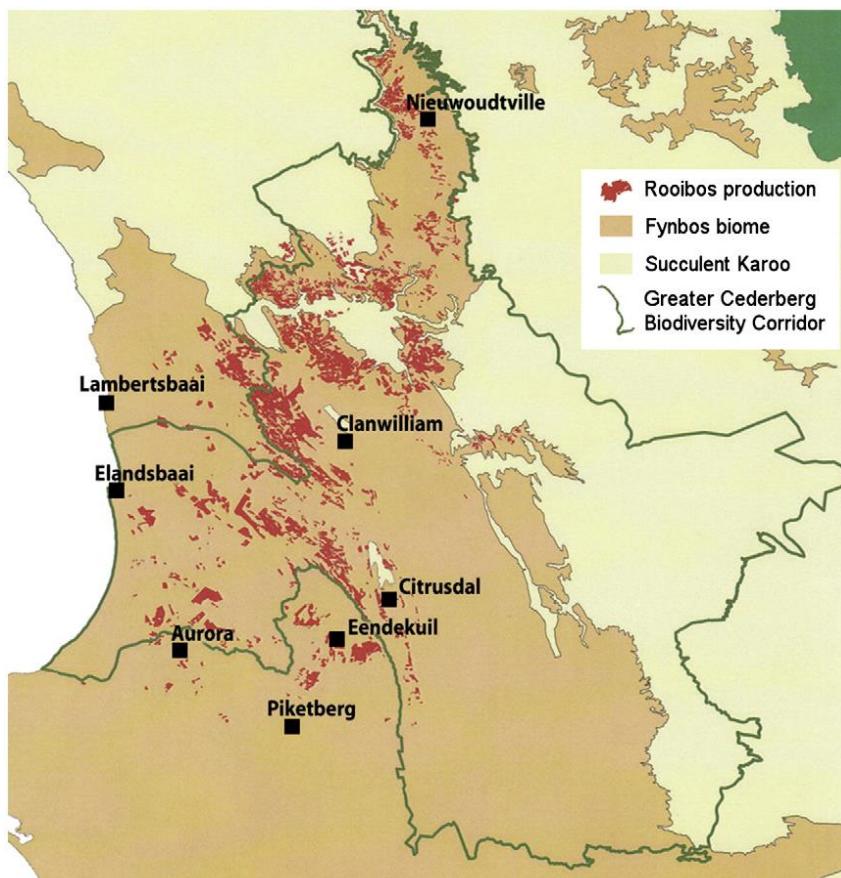


Figure 2.1. Distribution and production areas of rooibos tea (reproduced from [3]).

Honeybush (*Cyclopia* Vent species, family Fabaceae; tribe Podalyrieae), is another indigenous South African plant endemic to the Cape fynbos biome. Honeybush is, like rooibos, enjoyed worldwide as a herbal tea. Unlike rooibos, honeybush includes several species. More than 20 species have been described to date, of which only three, *Cyclopia genistoides*, *Cyclopia intermedia* and *Cyclopia subternata*, are of commercial importance for tea production. Currently, a further three species, *Cyclopia longifolia*, *Cyclopia sessiliflora* and *Cyclopia maculata*, are being considered for commercial production to meet the growing demand for the product [23-27].

The name honeybush is derived from ‘honingtee’ (Dutch, meaning ‘honey tea’) or ‘heuning- of heuningbostee’ (Afrikaans), terms likely used to describe the sweet honey-like scent of the plant’s flowers when in blossom. The first documented consumption of honeybush tea was when it was served to C. Latrobe in 1815 by Langkloof inhabitants as ‘tea-water’ [28]. *C. genistoides* was the first species mentioned to be used as a tea [6] and blends were commonly used by locals for medicinal purposes such as treating chronic catarrh and pulmonary tuberculosis [29]. *C. longifolia* and *C. subternata* were also historically used as tea. Specific species were consumed as tea mostly around the area of their natural occurrence. For example, *C. genistoides* was popular in the Cape Peninsula and *C. Subternata* in the Overberg and George areas. Honeybush grows naturally in the Western and Eastern Cape provinces of Southern Africa, from the Malmesbury-Darling area, across the Cape

Peninsula and Overberg areas (*C. genistoides*) and along the coastal mountains of the Langeberg (*C. sessiliflora*, *C. subternata* and *C. intermedia*). *C. subternata* and *C. intermedia* are found in the Outeniqua and Tsitsikama regions, and *C. intermedia* and *C. longifolia* in the mountains near Port Elizabeth. *C. maculata* occurs in the south-western and southern regions of the Western Cape (**Figure 2.2.**) [23,30].

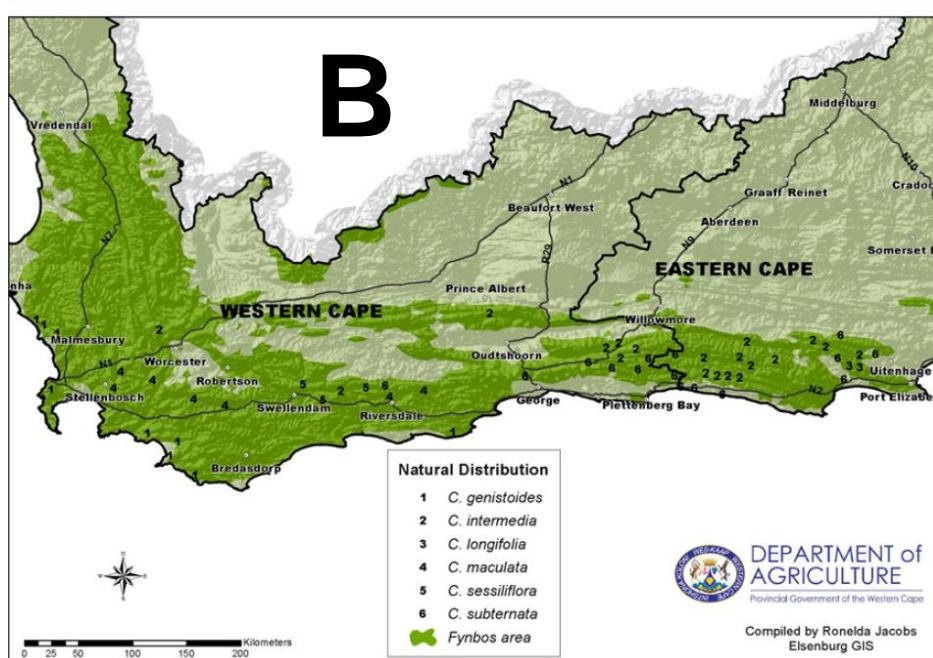
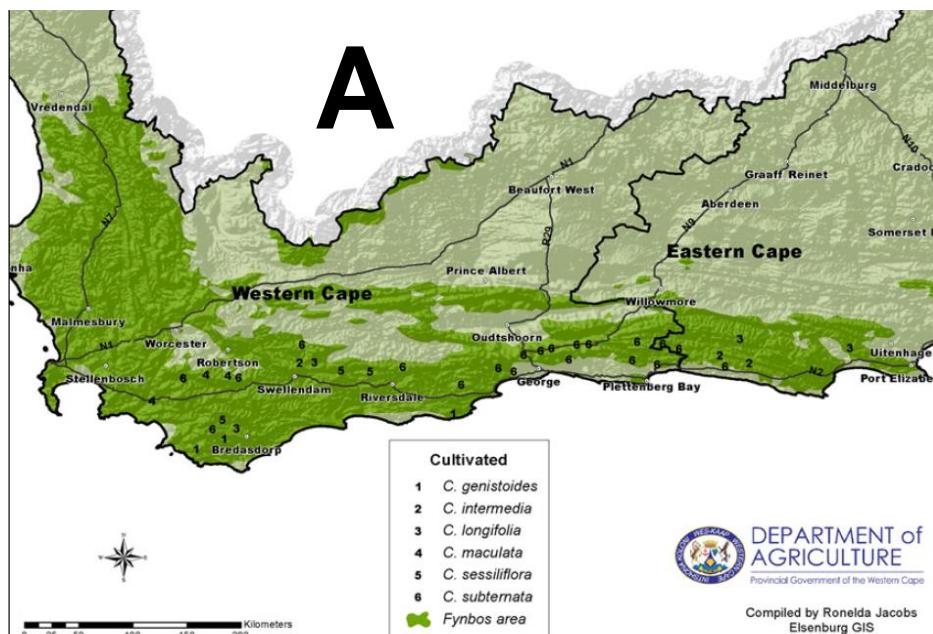


Figure 2.2. Cultivation (A) and natural distribution (B) areas of *Cyclopia* spp. in the Western and Eastern Cape provinces of South Africa (reproduced from [23]).

2.3. Production of the rooibos and honeybush herbal teas

Harvesting of rooibos tea takes place during the summer months (January-April). The production process for fermented rooibos involves cutting of the plant material, wetting, bruising, oxidation (“fermentation”), drying and sieving. Enzymatic oxidation occurs as soon as the plants are cut and the colour of the shredded plant material changes quickly from green to brown. Wetting and bruising enhance the “fermentation” process, resulting in the release of phenolic compounds and formation of an even, red-brown colour. The heaps are turned over manually a few times to ensure thorough aeration for oxidation. Oxidation occurs at ambient temperature for 12-14 hours before the material is spread out to dry in the sun. After this, the fermented tea is ready to be sieved and packaged [1]. Unfermented (“green”) rooibos is produced by spreading a thin layer of shredded plant material, followed by rapid drying, then sieving and packaging [3].

In the production of fermented honeybush, rotary fermentation is performed by blowing hot air through drums containing the shredded plant material at 80-85°C, followed by drying to a humidity of 8% and sieving [31]. Unfermented honeybush tea is produced by immediate steaming and drying (sun-dried) of shredded plant material to maintain better colour (green) retention and assure quality products [1].

2.4. Industry and value addition

A decline in the rooibos tea market after World War II led to the establishment of the Rooibos Tea Control Board of South Africa. New marketing strategies were devised, and the quality of tea was improved and production increased [1,3]. Domestic production (estimated to be 12500 tons in 2014) is increasing in part due to the variety of rooibos products being marketed, including organic and green rooibos. The most important export markets include Germany (1058 tons in 2013), the United Kingdom (UK, 276 tons), Japan (207 tons), The Netherlands (164 tons) and United States of America (USA, 67 tons). Japan and Germany are the most important export destinations for organic and green rooibos tea [31].

Nowadays, aqueous extracts of rooibos are also used in the food, beverage, pharmaceutical and nutraceutical industries. The food and beverage industries, which use rooibos extracts (mostly in fermented form) as ingredients, are the most important customers of the rooibos industry. Current food and beverage products on the market include: yogurt, drinking yogurt, ready-to-drink iced teas, jam and ‘instant rooibos cappuccino’. The volatile fraction of rooibos was first used as a flavour ingredient in yogurt by the then Van Riebeeck Diaries in 1984 [32]. Annique (Annetjie) Theron

launched the first cosmetic care product range containing rooibos extracts in 1971. The use of rooibos extracts in alterative industries is growing [1,3].

Commercialisation of honeybush tea was uncommon until the 1930's, when *C. intermedia* was sold in the Langkloof area. Similar to rooibos, increased demand for honeybush occurred during World War II, and prices per kg roughly doubled during this period. The first branded honeybush product appeared in the 1960's on the South African market as '*Caspa Cyclopia Tea*' due to efforts of rooibos tea marketer, Benjamin Ginsberg. Highlights from the period of 1992-2010 to establish honeybush tea as a popular product driven by sustainability, marketing and research are summerised by Joubert *et al.* [23]. The honeybush industry remains relatively small compared to rooibos, comprising in annual production of approximately 300 tons [33]. In 2012 the main export destinations for the product included Germany (188 tons), followed by The Netherlands (57 tons), UK (56.6 tons), USA (19.8 tons) and Japan (17.6). Export of organic and green honeybush tea was mainly to Japan (19.7 tons) and Germany (5.9 tons) [33].

Honeybush is still predominantly marketed as a herbal tea product, since the value-adding potential of the product has not been clearly established, although work in this area is on-going. Except for tea, limited alternative use of powdered honeybush extracts include in the food, beverage and cosmetic industries [3,23]. Potential opportunities for use of honeybush extracts exist in the neutraceutical industry and the production of medicinal extracts for specific purposes [3,33].

2.5. Health promoting properties of phenolics

Both rooibos and honeybush teas contain high levels of phenolics, low tannin levels and are caffeine free [2]. Both herbal teas became popular as healthy beverages, and their potential health benefits have been linked to their phenolic content. As a result, research focussing on the phenolic composition, which might contribute to reducing risks of degradative diseases in humans, has grown significantly.

Extensive research has been performed on the contribution of specific phenolic compounds in rooibos towards health benefits ascribed to the product (see section on phenolic composition of rooibos tea (**Section 2.6.2.1.**)). Joubert and co-workers comprehensively reviewed the *in vitro*, *ex vivo* and *in vivo* health benefits of fermented and unfermented rooibos extracts [1]. Rooibos has been shown to have anti-diabetic [34,35], anti-cancer [36], anti-mutagenic (chemoprotective) [37], anti-bacterial [38], anti-cardiovascular [13], anti-spasmodic [39], anti-ageing [8], hepatoprotective [40] and phyto-oestrogenic properties [11]. Specific compounds have been identified to be responsible for particular health benefits. Aspalathin, a potent anti-oxidant [41], and the phenylpropanoid, phenylpyruvic acid-

2-O-glucoside (PPAG) exhibit anti-diabetic properties [18-20,35,42,43], whilst the former shows moderate phyto-oestrogenic [11] and antimutagenic properties [44]. Nothofagin, another major dihydrochalcone present in rooibos, has been shown to have moderate antimutagenic properties [21], but high phyto-oestrogenic activity [11]. Isoorientin has hypoglycemic [45], anti-inflammatory [46], hepatoprotective [47] and moderate antimutagenic (like orientin) properties [21]. Vitexin exhibited properties that may prevent or reduce the risk of cardiovascular diseases in humans [22] and strong antispasmodic effects [39]. Shimamura and co-workers studied the phyto-oestrogenic activity of methanolic extracts of rooibos. They found that isovitexin and luteolin-7-O-glucoside showed moderate oestrogenicity, whilst eriodictyol showed less activity [11]. Luteolin exhibits antimutagenic properties [44], but less phyto-oestrogenic acitivity [11]. Researchers have also shown that chrysoeriol may be used in the treatment of vascular diseases (i.e. arteriosclerosis) [22], lowers blood pressure [22] and has antispasmodic [39] and antimutagenic properties [44]. Quercetin, a potent anti-oxidant [48], showed low oestrogenicity [11]. To date no compound has been specifically linked to the possible anti-cancer properties of rooibos tea.

Owing to the anti-oxidant, anti-inflammatory and antimicrobial properties of rooibos, extracts have been used in hair and skin cosmetic products [49]. Chuarienthong and co-workers studied the anti-wrinkle effect of a formulation containing rooibos and *Camellia sinensis*. The results were promising, but they concluded that the efficacy of rooibos as an anti-wrinkle agent could not be claimed, because the formulation was a mixture of two teas [50].

Similar to rooibos, significant research has been performed on the potential health benefits of honeybush phenolics [1,51-53] (refer to **Section 2.6.2.2.** for details on honeybush phenolics). Honeybush has also been shown to have anti-mutagenic [54], anti-cancer [55] and phytoestrogenic properties [56,57]. Recent studies have demonstrated anti-diabetic and anti-obesity qualities of honeybush, which have been ascribed to the high xanthone and benzophenone (and their glycosylated derivatives) content of hot water extracts of various fermented *Cyclopia* spp. [58-62].

2.6. Phenolic compounds

2.6.1. Classification of phenolic compounds

Compounds containing an aromatic ring and one or more hydroxyl groups are classified as phenolic compounds or polyphenols [63]. Phenolic compounds are secondary plant metabolites [64-66] and are responsible for the colour and taste of various fruits, vegetables, plants and plant-derived food and beverages [67,68]. They can be divided into two main classes, flavonoids and non-flavonoids, which differ in their basic chemical structures, properties and functions. Flavonoids have a C₆-C₃-C₆ skeleton

and are divided into various sub-classes depending on the oxidation state of the central heterocyclic ring [69,70]. The total number of identified flavonoids by 2005 was more than 7000, with extensive research focusing on these compounds [69]. The main flavonoid classes include the flavones, isoflavones, flavanones, flavanols (catechins and tannins), flavonols, flavanonols, anthocyanidins, chalcones and dihydrochalcones (**Table 2.1.**).

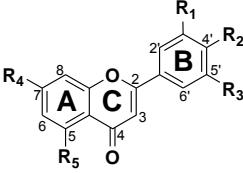
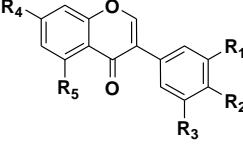
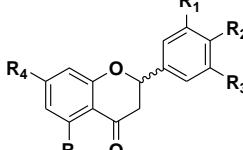
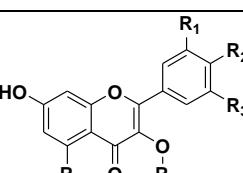
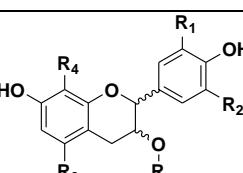
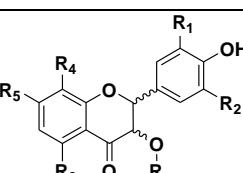
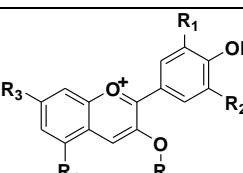
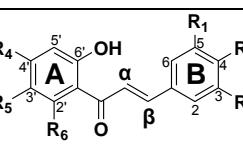
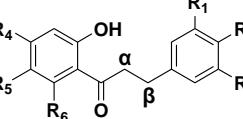
Chalcones and dihydrochalcones are comprised of a C₆-C₃-C₆ skeleton in an open-chain form. Chalcones contain a double bond between the α and β carbons of the C₃-linkage and occur naturally as *cis*-isomers. The *cis*-form of 2'-hydroxy chalcones rapidly undergoes isomerisation with ring closure to form the corresponding flavanones, and is therefore unlikely to exist in nature. Dihydrochalcones are formed via reduction of the α-β double bond of the chalcones [71].

Tannins are oligomeric and polymeric phenolics, which can be divided into two types: hydrolysable and non-hydrolysable (condensed) tannins. Hydrolysable tannins are esters formed via hydrolysis of phenolic acids and sugars or their derivatives [72,73]. The sugar moiety can either be glucose or a polysaccharide and the phenolic acid, gallic acid (gallotannins) or ellagic acid (ellagitannins). Condensed tannins (proanthocyanidins) are oligomeric flavan-3-ols species, so named since they form coloured anthocyanidins following acid hydrolysis [73].

Non-flavonoids include simple phenols, phenolic acids (hydroxybenzoic and hydroxycinnamic acids), stilbenes, lignans, lignins, coumarins and xanthones (**Table 2.2.**).

Interest in phenolic compounds continues to grow owing to their anti-oxidant properties, which are linked to potential health benefits in humans [74]. Various studies focusing on the bioactivity of phenolics have shown that these compounds have anti-inflammatory [75], antimutagenic [37], anticancer [76], antidiabetic [77], hepatoprotective [78], phyto-oestrogenic [11,56], antispasmodic [77,79], anti-ageing [77], antimicrobial and antiviral [77,80] and anti-allergic properties [80,81]. In addition to their health-promoting properties, phenolic compounds also play a role in determining the quality and sensory properties of plant-derived food products. For example, phenolics determine chemical and colour stability [82,83], astringency and bitterness [68], and they play a role in plant growth and defence [84] and in attracting insects [85].

Table 2.1. Chemical structures of the principal classes of flavonoids.

General structure	Phenolic class
	Flavones
	Isoflavones ^a
	Flavanones ^a
	Flavonols ^{a,b}
	Flavanols ^{a,b}
	Flavanonols ^{a,b}
	Anthocyanidins ^{a,b}
	Chalcones
	Dihydrochalcones ^c

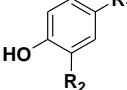
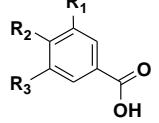
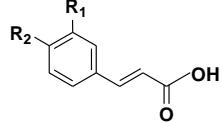
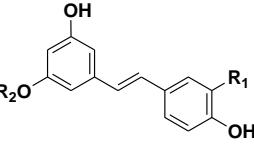
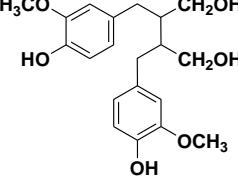
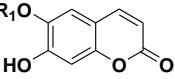
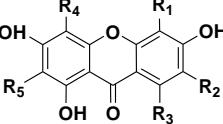
^aR₁₋₆ may be -H, -OH, -OCH₃ or mono- to polysaccharides (O- and/or C-saccharides).

^bsame numbering as flavones.

^bR may be H or mono- to polysaccharides (O-saccharides).

^csame numbering as chalcones.

Table 2.2. Chemical structures of the principal classes of non-flavonoids.

General structure	Phenolic class
	Simple phenols ^a
	Hydroxybenzoic acids ^a
	Hydroxycinnamic acids ^a
	Stilbenes ^a
	Lignans
	Coumarins ^b
	Xanthones

^aR₁₋₅ may be -H, -OH, -OCH₃, or mono- to polysaccharides (O- and/or C-saccharides).

^aR₁₋₃ = H, OH, OCH₃.

^bR₁ = H, CH₃, -glucoside.

2.6.2. Tea phenolics

Tea, produced from the leaves of the plant *Camellia sinensis* (*C. sinensis*), is one of the most popular beverages consumed by humans [86-91]. There are 3 main types of tea, namely black, oolong and green tea. Black tea is produced by fermentation of the green leaves of *C. sinensis*, whilst oolong (yellow) and green tea are semi-fermented and unfermented, respectively. Fresh tea leaves comprise approximately 36% (of the dry weight) of phenolics [87]. Partly as a consequence of this high phenolic content, consumption of tea is linked to potential health benefits in humans [87-95]. The phenolic composition varies between different types of tea, with the main difference being the absence of theaflavins and thearubigins in green tea; these compounds are formed during the

manufacturing (oxidation) of oolong and black tea [96]. Green tea also contains more flavanols than black tea [87,96].

In the next sections, more detailed information on the phenolic composition of rooibos and honeybush teas will be presented, as this aspects is pertinent to the main objective of this study.

2.6.2.1. Phenolic composition of rooibos tea

Rooibos tea contains a wide range of phenolic compounds (**Table 2.3.**), including dihydrochalcones (aspalathin and nothofagin), flavones (isoorientin (luteolin-6-C-glucoside), orientin (luteolin-8-C-glucoside), vitexin (apigenin-8-C-glucoside), isovitexin (apigenin-6-C-glucoside), luteolin, luteolin-7-O-glucoside and chrysoeriol (3'-methoxyapigenin)), flavonols (quercetin, isoquercitrin (quercetin-3-O-glucoside), hyperoside (quercetin-3-O-galactoside) and rutin (quercetin-3-O-rutinoside)) and hydroxycinnamic acids (ferulic acid) as major compounds.

Aspalathin occurs naturally only in rooibos, and was first isolated, characterised and identified by nuclear magnetic resonance (NMR) by Koeppen and Roux [97,98]. The concentration of aspalathin in unfermented rooibos is much higher than its corresponding flavone analogues, orientin and isoorientin [99-102], whereas in fermented extracts their levels are more or less the same [99,100,103]. Other dihydrochalcones found in rooibos include nothofagin [104], a rare natural compound (previously identified in *Nothofagus fusca*) [105] and the cyclic dihydrochalcone, aspalalinin. Nothofagin is structurally similar to aspalathin, but lacks a hydroxyl group attached to the B-ring. Aspalalinin was isolated and characterised from fermented rooibos by Shimamaru and co-workers [11]. Recently, Beelders *et al.* [99] tentatively identified a novel C-5'-hexosyl derivative of aspalathin in a fermented rooibos sample by liquid chromatography tandem mass spectrometry (LC-MS/MS).

Aside from the dihydrochalcones, the flavones isoorientin and orientin are also major constituents of both unfermented and fermented rooibos plant material. Other flavones present in rooibos include vitexin, isovitexin, luteolin, luteolin-7-O-glucoside and chrysoeriol. Luteolin and chrysoeriol are present in low levels in both fermented and unfermented rooibos, whereas luteolin-7-O-glucoside is present at trace levels and is often not detected. Low concentration levels of vitexin and isovitexin are present in unfermented rooibos samples. Other flavones tentatively identified in rooibos teas include luteolin-6,8-di-C-hexoside, apigenin-6,8-di-C-hexoside (vicenin-2, also present in the seeds of *Aspalathus* [106]), luteolin-6-C-glucoside-8-C-arabinoside, luteolin-6-C-arabinoside-8-C-glucoside, apigenin-6-C-arabinoside-8-C-glucoside [107], patuletin-7-glucoside and scoparin [106]. Isocarlinoside and/or neocarlinoside isomers were tentatively identified in rooibos by LC-MS(/MS) [99,108].

Ferulic acid, a hydroxycinnamic acid, was identified in rooibos by NMR [109,110]. This compound displays anti-oxidative properties which may be beneficial in prevention of cardiovascular and degradative diseases in humans [111].

A flavanone analogue of isovitexin, hemiphlorin, was detected in a methanol extract of rooibos and showed phyto-oestrogenic activity [11]. Naringenin-C-glucoside diastereoisomers (tentatively identified by LC-MS/MS) were detected in fermented rooibos tea at trace levels. These compounds are possibly formed from nothofagin during oxidation [100]. Eriodictyol-5',3'-di-*O*-glucoside was tentatively identified by LC-MS/MS and detected in trace amounts in an ethanolic fermented rooibos extract by Iswaldi and co-workers [108].

Flavonols detected in rooibos include quercetin, isoquercitrin, hyperoside, rutin and quercetin-3-*O*- β -D-robinobioside. Quercetin, isoquercitrin and hyperoside are present at trace levels in rooibos samples [99-102,112-114], whereas concentrations of quercetin-3-*O*- β -D-robinoside are relatively high [99] and levels of rutin are low to moderate [99-102,112-114]. Another flavonol glucoside, quercetin-3-*O*-arabinoglucoside, was tentatively identified in an ethanolic extract of fermented rooibos by LC-MS and MS/MS [108].

The flavan-3-ols identified in rooibos are the monomeric flavan-3-ol (+)-catechin [109,115] and oligomeric flavan-3-ols procyanidin B3 and bis-fisetinidol-(4 β ,6:4 β ,8)-catechin [115].

Although not a phenolic compound, PPAG is a major constituent of unfermented and fermented rooibos. Muller and co-workers [19] showed that PPAG has potential anti-diabetic properties that may contribute to the observed antidiabetic effects of rooibos tea.

Other compounds identified in fermented rooibos material include glycol derivatives (*p*-hydroxyphenylglycol and vanylglycol), the aldehyde syringin [11] and a chalcone derivative, saffloomin A [108] (**Table 2.3.**). Shimamura and co-workers identified the lignans secoisolariciresinol, secoisolariciresinol-*O*-glucoside, vladinol F and 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxy-1-(*E*)-propenyl)-2,6-dimethoxyphenoxy]propyl- β -D-glucopyranoside in fermented rooibos plant material [11]. The coumarins esculetin and esculin were identified by Shimamura *et al.* [11] and Iswaldi *et al.* [108]. Esculetin was isolated and characterised by NMR, and esculin was identified by LC-MS/MS.

The “fermentation” process used in the production of rooibos tea is a spontaneous enzymatic oxidation process occurring when the tea is wetted. This alters the phenolic composition and concentrations of individual constituents. During this process the concentration of aspalathin decreases markedly, with a corresponding increase in the levels of isoorientin and orientin [1]. Krafczyk and Glomb proposed a mechanism for the oxidative degradation of aspalathin to produce

isoorientin and orientin via the diastereomeric flavanones dihydro-isoorientin and dihydro-orientin, (*R*)- and (*S*)-eriodictyol-6-C-glucoside and (*R*)- and (*S*)-eriodictyol-8-C-glucoside, respectively, as intermediates (**Figure 2.3.**). Isoorientin is a degradative product of (*R*)- and (*S*)-eriodictyol-6-C-glucoside, whereas orientin is irreversibly formed via ring-opening of isoorientin to produce a chalcone intermediate. This is followed by bond rotation and ring-closure (recyclization, the Wessely-Moser rearrangement) and loss of a water molecule [109]. Further oxidative degradation products of aspalathin formed during fermentation include colourless aspalathin dimers [116], 5,7-dihydroxy-6-C- β -D-glucopyranosyl-chromone [115], yellow dibenzofurans and unknown polymeric brown material [116]. The chromone is formed by further oxidation of eriodictyol-6-C- β -D-glucopyranoside during fermentation [115]. All the listed degradation products, including the diastereomeric intermediates of dihydro-isoorientin and dihydro-orientin [109], have been detected in fermented and unfermented rooibos samples [107].

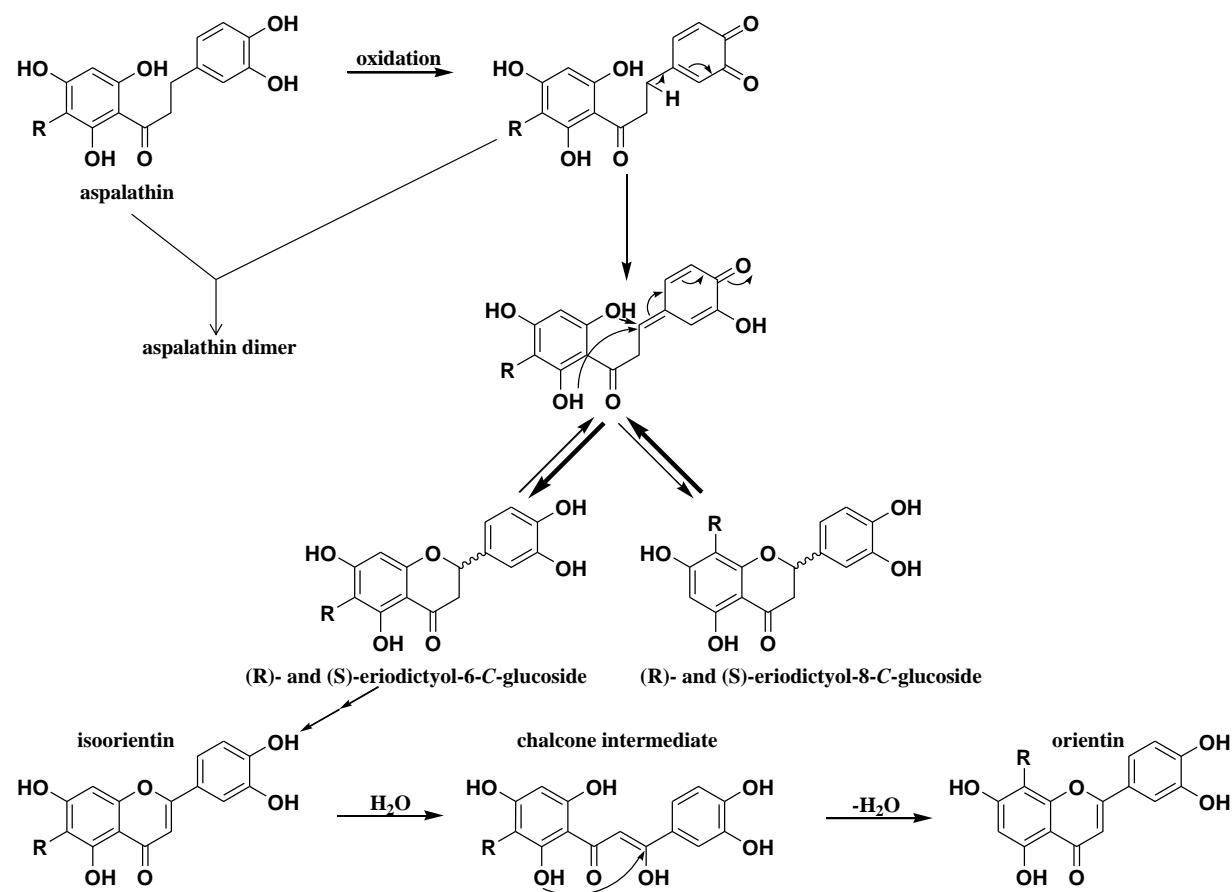


Figure 2.3. Mechanism of aspalathin oxidation during rooibos tea fermentation, leading to the formation of isoorientin and orientin (adapted from [3]).

Table 2.3. Phenolic compounds identified in fermented rooibos (*Aspalathus linearis*) plant material (adapted and updated from [1]).

General structure	Phenolic class, names and substituents
	Dihydrochalcones Aspalathin ^{a,b,c,d,e,f,g} : R ₁ = C-β-glucosyl, R ₂ = OH Nothofagin ^{e,f,g,h} : R ₁ = C-β-glucosyl, R ₂ = H
	Cyclic dihydrochalcone Aspalalilin ^f : R = C-β-glucosyl
	Flavones Orientin ^{a,c,e,f,g,i,j} : R ₁ = C-β-D-glucosyl, R ₂ = R ₅ = OH, R ₃ = R ₄ = H Iso-orientin ^{c,e,g,i,j} : R ₁ = C-β-D-glucosyl, R ₂ = R ₅ = OH, R ₃ = R ₄ = H Vitexin ^{a,c,e,f,g} : R ₁ = C-β-D-glucosyl, R ₂ = OH, R ₃ = R ₄ = R ₅ = H Isovitexin ^{c,e,f,g} : R ₁ = R ₄ = R ₅ = H, R ₂ = OH, R ₃ = C-β-D-glucosyl Apigenin-6,8-di-C-hexoside ^{k,l,m,n} : R ₁ = R ₃ = C-β-D-hexosyl, R ₂ = OH, R ₄ = R ₅ = H Apigenin-6-C-arabinoside-8-C-glucoside ^{l,m} : R ₁ = C-β-D-glucosyl, R ₂ = OH, R ₃ = C-β-D-arabinosyl, R ₄ = R ₅ = H Luteolin ^{c,e,f,g} : R ₁ = R ₃ = R ₄ = H, R ₂ = R ₅ = OH Luteolin-7-O-glucoside ^d : R ₁ = R ₃ = R ₄ = H, R ₂ = O-β-D-glucosyl, R ₅ = OH Luteolin-6,8-di-C-hexoside ^{l,m,n} : R ₁ = R ₃ = C-β-D-hexosyl, R ₂ = R ₅ = OH, R ₄ = H Luteolin-6-C-glucoside-8-C-arabinoside ^{l,m} : R ₁ = C-β-D-pentosyl, R ₂ = R ₅ = OH, R ₃ = C-β-D-hexosyl, R ₄ = H Luteolin-6-C-arabinoside-8-C-glucoside ^{l,m} : R ₁ = C-β-D-hexosyl, R ₂ = R ₅ = OH, R ₃ = C-β-D-pentosyl, R ₄ = H Chrysoeriol ^{c,e,g} : R ₁ = R ₃ = R ₄ = H, R ₂ = OH, R ₅ = OCH ₃ Patuletin-7-O-glucoside ^k : R ₁ = R ₄ = H, R ₂ = O-β-D-glucosyl, R ₃ = OCH ₃ , R ₅ = OH Scoparin ^{k,m} : R ₁ = C-β-D-glucosyl, R ₂ = OH, R ₃ = R ₅ = H, R ₄ = OCH ₃
	Flavanones Dihydro-orientin [(R)/(S)-eriodictyol-8-glucoside] ^{e,f,g} : R ₁ = C-β-D-glucosyl, R ₂ = R ₄ = H, R ₃ = R ₅ = OH Dihydro-iso-orientin [(R)/(S)-eriodictyol-6-glucoside] ^{e,f,g} : R ₁ = R ₄ = H, R ₂ = C-β-D-glucosyl, R ₃ = R ₅ = OH Hemiphlorin ^f : R ₁ = C-β-D-glucosyl, R ₂ = R ₄ = R ₅ = H, R ₃ = OH Naringenin-C-glucoside diastereomeric isomers ^o : R ₁ or R ₂ = C-β-D-glucosyl, R ₃ = OH, R ₄ = R ₅ = H, Eriodictyol-3',5-di-O-glucoside ^k : R ₁ = R ₂ = R ₅ = H, R ₃ = R ₄ = O-glucosyl

Table 2.3. (Continued)

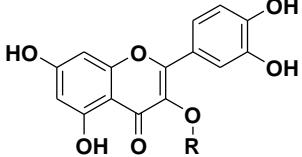
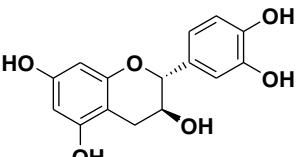
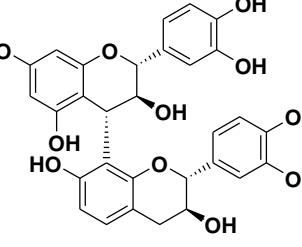
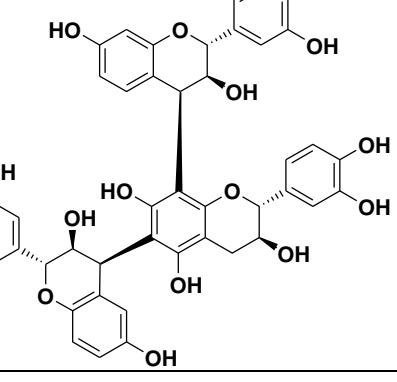
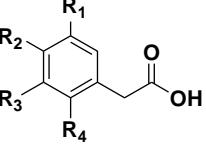
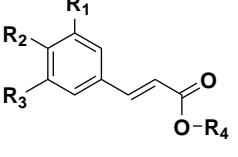
General structure	Phenolic class, names and substituents
	Flavonols
	Quercetin ^{c,e,f,g} : R = H Isoquercitrin ^{c,e,f,g,j} : R = O-β-glucosyl Hyperoside ^{e,f,g} : R = O-β-galactosyl Rutin ^{e,g,j} : R = O-β-rutinosyl Quercetin-3-O-β-robinobioside ^f : R = O-robinosyl Quercetin-3-O-β-arabinoglucoside ^{k,m} : R = O-arabinoglucoside
	Flavan-3-ols
	Monomeric flavan-3-ol: (+)-Catechin^{d,g}
	Oligomeric flavan-3-ol: Procyanolidin B3^d
	Oligomeric flavan-3-ol: Bis-fisetinidol-(4β,6:4β,8)-catechin^d
	Phenolic carboxylic acids
	<i>p</i> -Hydroxybenzoic acid ^{c,f,g} : R ₁ = R ₃ = R ₄ = H, R ₂ = OH Protocatechuic acid ^{c,g} : R ₁ = R ₂ = OH, R ₃ = R ₄ = OH 3,5-Dihydroxybenzoic acid ^g : R ₁ = R ₃ = H, R ₂ = R ₄ = OH Gentisic acid ^g : R ₁ = R ₃ = OH, R ₂ = R ₄ = H Salicylic acid ^g : R ₁ = R ₂ = R ₃ = H, R ₄ = OH Gallic acid ^g : R ₁ = R ₂ = OH, R ₃ = OH, R ₄ = H Vanillic acid ^{c,g} : R ₁ = OCH ₃ , R ₂ = OH, R ₃ = R ₄ = H Syringic acid ^{d,g} : R ₁ = R ₃ = OCH ₃ , R ₂ = OH, R ₄ = H
	Hydroxycinnamic acids
	3,4,5-Trihydroxycinnamic acid ^c : R ₁ = R ₂ = R ₃ = OH, R ₄ = H <i>p</i> -Coumaric acid ^{c,f,g} : R ₁ = R ₃ = H, R ₂ = OH, R ₄ = H Caffeic acid ^{c,g} : R ₁ = R ₂ = OH, R ₃ = R ₄ = H Ferulic acid ^{c,g} : R ₁ = OCH ₃ , R ₂ = OH, R ₃ = R ₄ = H Sinapic acid ^c : R ₁ = R ₃ = OCH ₃ , R ₂ = OH, R ₄ = H Chlorogenic acid ^g : R ₁ = R ₂ = OH, R ₃ = H, R ₄ = quinic acid

Table 2.3. (Continued)

General structure	Phenolic class, names and substituents
	Lignans Secoisolariciresinol^f: R = OH Secoisolariciresinol-O-glucoside^f: R = O-glucosyl
	Vladinol F^f
	3-Hydroxy-1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxy-1-(E)-propenyl)-2,6-dimethoxyphenoxy]propyl-beta-D-glucopyranoside^f: R = O-β-D-glucosyl
	Coumarins Esculetin^f: R = H Esculin^k: R = O-glucosyl
	Chromone 5,7-Dihydroxy-6-C-glucosyl-chromone^d: R = C-β-glucosyl
	Glycol derivatives p-Hydroxyphenylglycol^f: R ₁ = R ₂ = H Vanillyl glycol^g: R ₁ = OH, R ₂ = OCH ₃
	Phenylpropanoid derivative 3-Phenyl-2-glucopyranosyloxypropenoic acid^p: R = O-glucosyl
	Aldehyde Syringin^f: R = O-glucosyl
	Chalcone derivative Safflomin A^k: R ₁ = R ₂ = C-β-D-glucosyl

^a Identified by NMR [97]^b Identified by NMR [98]^c Identified by NMR [110]^d Identified by NMR [115]^e Identified by LC-MS [103]^f Identified by NMR [11]^g Identified by NMR [109]^h Identified by co-elution with pure standard [104]ⁱ Identified by NMR [117]^j Identified by NMR [118]^k Tentatively identified by LC-MS [108]^l Tentatively identified by LC-MS [101]^m Tentatively identified by LC-MS [107]ⁿ Tentatively identified by LC-MS [99]^o Tentatively identified by LC-MS [100]^p Identified by NMR [119]

2.6.2.2. Phenolic composition of honeybush tea

Honeybush, unlike rooibos, comprises several species. Information on the phenolic composition of each of these species, in both unfermented and fermented forms, is of importance for commercial and scientific reasons.

The main honeybush phenolics include xanthones (mangiferin and isomangiferin), flavanones (hesperidin (hesperetin-7-rutinoside) and eriocitrin (eriodictyol-7-O-rutinoside), the flavone scolymoside (luteolin-7-O-rhamnoside) and the dihydrochalcone, phloretin-3',5'-di-C-glucoside. Mangiferin, isomangiferin and hesperidin are present in all six commercial species analysed to date [1,23,26,27]. Other major phenolics include the flavone vicenin-2 (apigenin-6,8-di-C-hexoside) [52], also present in trace levels in the seeds of most of *Cyclopia* spp. [106], the flavanones eriodictyol, eriodictyol-5-O-glucoside and eriodictyol-7-O-glucoside [1] and the dihydrochalcone 3-hydroxyphloretin-3'5'-di-C-hexoside [120] (**Table 2.4.**).

In addition to these major constituents, a range of phenolic compounds have also been identified, occurring either at lower levels, or in specific species. These include the flavanones hesperitin, narirutin (naringenin-3-O-rutinoside), naringenin and naringenin-5-O-rutinoside. De Beer and co-workers also tentatively identified (R)- and (S)-eriodictyol-di-C-hexoside in unfermented and fermented *C. subternata* samples using LC-MS/MS [120].

Additional flavones identified in honeybush include luteolin, diosmetin (5,7,3'-trihydroxy-4'-methoxyflavone) [51,52] and 5-deoxyluteolin [53] and isorhoifolin (apigenin-7-O-rutinoside) [121], while the isoflavones formononetin (7-hydroxy-4'-methoxyisoflavone) [51], formononetin diglucoside (4'-methoxyisoflavone-7-O- α -apiofuranosyl-(1" \rightarrow 6")- β -D-glucopyranosyl) [52], afromosin (7-hydroxy-6,4'-dimethoxyisoflavone), calcosin (3',7-dihydroxy-4'-methoxyisoflavone-7-O- β -glucoside) [51], wistin (6,4'-dimethoxyisoflavone-7-O-glucoside) [52] and orobol (isoluteolin, 3',4',5,7-tetrahydroxyisoflavone) [53] have also been reported in honeybush. The methylinedioxyisoflavone derivatives pseudobaptigenin (7-hydroxy-3',4'-methylenedioxyisoflavone) and fujikinetin (7-hydroxy-6-methoxy-3',4'-methylenedioxyisoflavone) [51] have been detected in *Cyclopia* plant material. Kokotkiewicz and co-workers isolated and characterised three isoflavone glucosides, namely calcosin-7-O- β -glucoside (3',7-dihydroxy-4'-methoxyisoflavone-7-O- β -glucoside), rothindin (7-hydroxy-3',4'-methylenedioxyisoflavone-7-O- β -glucoside) and ononin (7-hydroxy-4'-methoxyisoflavone-7-O- β -glucoside) in unfermented *C. subternata* [121].

The flavonol glucosides kaempferol-5-O-, -6-C-, -8-C- β - and -8-C- α -glucoside and a methylinedioxyflavonol derivative (3',4'-methylenedioxyflavonol-O-apiofuranosyl-(1" \rightarrow 6")- β -D-glucopyranosyl) were detected in fermented and unfermented *C. intermedia* and *C. subternata*, respectively [53].

Three coumestans, medicagol (3-hydroxy-8,9-methylenedioxy coumestan), flemichapparin (3-methoxy-8,9-methylenedioxy coumestan) and sophoracoumestan (3-hydroxy-4-methoxy-8,9-methylenedioxy coumestan) were identified by NMR in fermented *C. intermedia* by Ferreira and co-workers [51]. An intense bright blue fluorescence on thin layer chromatography (TLC) under ultra-violet (UV) light confirmed the presence of coumestans [122]. Coumestans, like isoflavones, have phytoestrogenic properties [123].

The flavan-3-ol (-)-epigallocatechin gallate was isolated from unfermented *C. subternata* by Kamara and co-workers and characterised by NMR. This catechin galloyl ester is characterised by high anti-oxidant activity [124] and has been reported to have anti-cancer properties [87].

The benzophenones iriflophenone-3-C- β -glucoside-4-O- β -glucoside and iriflophenone-3-C-glucoside, although not phenolics, are additional major constituents of honeybush species. In 2013 Kokotkiewicz and co-workers isolated and characterised the benzophenone derivative maclurin-3-C- β -glucoside from unfermented *C. genistoides* [124]. This compound was also tentatively identified in unfermented and fermented samples of *C. subternata* [120], *C. maculata* [26] and recently *C. longifolia* and *C. genistoides* [27]. A new benzophenone derivative, iriflophenone-3-C- β -glucoside-4-O- β -glucoside, was isolated from *C. genistoides* and characterised [60]. This compound was previously tentatively identified as an iriflophenone-di-O,C-hexoside in *C. genistoides* [125] and *C. subternata* [120].

Recently, Beelders and co-workers studied the phenolic profile of both fermented and unfermented *C. genistoides* in detail. Novel xanthone and benzophenone derivatives were tentatively identified based on LC-MS data. Benzophenone derivatives identified include maclurin-di-O,C-hexoside, iriflophenone-di-C-hexoside and iriflophenone-di-O-C-hexoside isomers. In addition, new xanthone derivatives were identified, including tetrahydroxyxanthone-C-hexoside dimers, aspalathin and nothofagin derivatives of (iso)mangiferin, and the tetrahydroxyxanthone derivatives of mangiferin and isomangiferin. Other phenolic compounds tentatively identified were the flavone diosmin and several glycosylated phenolic acids [125]. Some of these compounds were also tentatively identified in fermented and unfermented *C. longifolia* (refer to the Supporting Information of Schulze *et al.* [27]).

Additional phenolic compounds reported in *Cyclopia* species include several tyrosol derivatives identified in fermented *C. intermedia* extracts [52]. Tyrosol, an anti-oxidant, showed anti-microbial activity [126] and has been reported to have cardioprotective properties [127].

Table 2.4. Phenolic compounds identified in honeybush (*Cyclopia* species) plant material (adapted and updated from [1]).

General structure	Phenolic class, names and substituents
	Xanthones Mangiferin ^{1a,2a,3,4,5,6} : R ₁ = C-β-D-glucosyl, R ₂ = H Hydroxymangiferin ⁵ : R ₁ = C-β-D-glucosyl, R ₂ = OH Isomangiferin ^{1a,2b,3,4,5,6} : R ₁ = H, R ₂ = C-β-D-glucosyl Hydroxyisomangiferin ⁵ : R ₁ = OH, R ₂ = C-β-D-glucosyl
	Benzophenones Iriflophenone-3-C-β-D-glucoside ^{5,6,7a,7b,9} : R ₁ = R ₃ = R ₄ = H, R ₂ = C-β-D-glucosyl Iriflophenone-di-O-C-hexoside ^{5,6,8,9} : R ₁ or R ₃ = hexosyl, R ₂ = hexosyl, R ₄ = H Maclurin-3-C-β-glucoside ^{5,6b,10a,10b} : R ₁ = R ₃ = H, R ₂ = C-β-D-glucosyl, R ₄ = OH
	Flavones Luteolin ^{1a, 2a, 3, 4} : R ₁ = R ₃ = H, R ₂ = R ₄ = R ₅ = R ₆ = OH Diosmetin ^{1a} : R ₁ = R ₃ = H, R ₂ = R ₄ = R ₆ = OH, R ₅ = OCH ₃ 5-Deoxyluteolin ^{2a} : R ₁ = R ₃ = R ₄ = H, R ₂ = R ₅ = R ₆ = OH Scolymoside ^{2a,5,6b} : R ₁ = R ₃ = H, R ₂ = O-rutinosyl, R ₄ = R ₅ = R ₆ = OH Isorhoifolin ^{6b,11} : R ₁ = R ₃ = R ₆ = H, R ₂ = O-rutinosyl, R ₄ = R ₅ = OH Apigenin-6,8-di-C-glucoside (Vicenin-2) ^{5,6b,8,10a} : R ₁ = R ₃ = C-β-D-glucosyl, R ₂ = R ₄ = R ₅ = OH, R ₆ = H
	Flavanones Hesperidin ^{1a, 2a, 3, 4, 5, 6, 9} : R ₁ = R ₃ = H, R ₂ = O-rutinosyl, R ₄ = R ₆ = OH, R ₅ = OCH ₃ Hesperetin ^{1a} : R ₁ = R ₃ = H, R ₂ = R ₄ = R ₆ = OH, R ₅ = OCH ₃ Eriocitrin ^{1b, 2a, 4, 5, 6, 10a} : R ₁ = R ₃ = H, R ₂ = O-rutinosyl, R ₄ = R ₅ = R ₆ = OH Eriodictyol ^{1a} : R ₁ = R ₃ = H, R ₂ = R ₄ = R ₅ = R ₆ = OH Narirutin ^{1b, 2a, 3, 4, 6} : R ₁ = R ₃ = R ₆ = H, R ₂ = O-rutinosyl, R ₄ = R ₅ = OH Naringenin ^{1a} : R ₁ = R ₃ = R ₆ = H, R ₂ = R ₄ = R ₅ = OH Prunin ^{1a} : R ₁ = R ₃ = R ₆ = H, R ₂ = O-glucoside, R ₄ = R ₅ = OH Naringenin-5-O-rutinoside ^{1a} : R ₁ = R ₃ = R ₆ = H, R ₂ = R ₅ = OH, R ₄ = O-rutinosyl Eriodictyol-5-O-glucoside ^{1a,8} : R ₁ = R ₃ = H, R ₂ = R ₅ = R ₆ = OH, R ₄ = C-β-D-glucosyl Eriodictyol-7-O-glucoside ^{1a,8} : R ₁ = R ₃ = H, R ₂ = C-β-D-glucosyl, R ₄ = R ₅ = R ₆ = OH (S)- and (R) Eriodictyol-di-C-hexoside ⁸ : R ₁ = R ₃ = hexosyl, R ₂ = R ₄ = R ₅ = R ₆ = OH
	Dihydrochalcones 3-Hydroxyphloretin-3',5'-di-C-hexoside ^{5,6,8,10a} : R ₁ = R ₂ = C-β-D-glucosyl, R ₃ = OH Phloretin-3',5'-di-C-glucoside ^{5,6,8,10a,11} : R ₁ = R ₂ = C-β-D-glucosyl, R ₃ = H

Table 2.4. (Continued)

General structure	Phenolic class, names and substituents
	<p>Isoflavones</p> <p>Formononetin^{1a}: R₁ = OH, R₂ = R₃ = R₄ = H, R₅ = OCH₃</p> <p>Formononetin-di-O-glucoside^{1a}:</p> <p>R₁ = O-α-apiofuranosyl-(1" → 6")-β-D-glucopyranosyl, R₂ = R₃ = R₄ = H, R₅ = OCH₃</p> <p>Afromosin^{1a}: R₁ = OH, R₂ = R₅ = OCH₃, R₃ = R₄ = H</p> <p>Calycosin^{1a}: R₁ = R₄ = OH, R₂ = R₃ = H, R₅ = OCH₃</p> <p>Calycosin-7-O-glucoside¹¹: R₁ = O-β-D-glucosyl, R₂ = R₃ = H, R₄ = OH, R₅ = OCH₃</p> <p>Afromosin-7-O-glucoside (wistin)¹²:</p> <p>R₁ = C-β-D-glucosyl, R₂ = R₅ = OCH₃, R₃ = R₄ = H</p> <p>Isoluteolin (orobol)^{2a}: R₁ = R₃ = R₄ = R₅ = OH, R₂ = H</p> <p>Formononetin-7-O-glucoside (ononin)¹¹:</p> <p>R₁ = O-β-D-glucosyl, R₂ = R₃ = R₄ = H, R₅ = OCH₃</p>
	<p>Methylinedioxyisoflavone derivatives</p> <p>Pseudobaptigenin^{1a}: R₁ = OH, R₂ = H</p> <p>Fujikinetin^{1a}: R₁ = OH, R₂ = OCH₃</p> <p>Pseudobaptigenin-7-O-glucoside (rothindin)¹¹:</p> <p>R₁ = C-β-D-glucosyl, R₂ = H</p>
	<p>Phenolic carboxylic acid</p> <p>p-Coumaric acid^{1a}</p>
	<p>Organic acid</p> <p>(±)-Shikimic acid^{2a}</p>
	<p>Flavonols</p> <p>Kaempferol-5-O-glucoside¹²: R₁ = R₂ = H, R₃ = O-β-D-glucosyl</p> <p>Kaempferol-6-C-glucoside^{2a,12}: R₁ = H, R₂ = C-β-D-glucosyl, R₃ = OH</p> <p>Kaempferol-8-C-glucoside¹²: R₁ = C-β-D-glucosyl, R₂ = H, R₃ = OH</p> <p>Kaempferol-8-C-glucoside¹²: R₁ = C-α-D-glucosyl, R₂ = H, R₃ = OH</p>
	<p>Methylinedioxyflavonol derivatives</p> <p>3,4,-Methylineoxyflavonol di-O-glucoside¹²:</p> <p>R = O-α-apiofuranosyl-(1" → 6")-β-D-glucopyranosyl</p>
	<p>Flavan-3-ols</p> <p>(-)-Epigallocatechin gallate^{2a}</p>

Table 2.4. (Continued)

General structure	Phenolic class, names and substituents
	Coumestans Medicagol ^{1a} : R ₁ = H, R ₂ = OH Flemichapparin ^{1a} : R ₁ = H, R ₂ = OCH ₃ Sophoracoumestan B ^{1a} : R ₁ = OCH ₃ , R ₂ = OH
	Phenylethanol derivatives Tyrosol ¹² : R ₁ = H, R ₂ = OH 3-Methoxy-tyrosol ¹² : R ₁ = OCH ₃ , R ₂ = OH 4-Glucosyltyrosol ^{2a} : R ₁ = H, R ₂ = O-β-D-glucosyl

(1a) identified in fermented *C. intermedia* using NMR [51]; (1b) identified in *C. intermedia* using LC-MS [128]; (2a) identified in *C. subternata* using NMR [52]; (2b) identified in *C. subternata* using LC-MS [128]; (3) identified in *C. genistoides* using LC-MS [128]; (4) identified in *C. sessiliflora* using LC-MS [128]. (5) identified in unfermented and fermented *C. maculata* using LC-MS/MS [26,129]; (6a) identified in *C. genistoides* using NMR [60] (6b) identified in unfermented and fermented *C. longifolia* using LC-MS/MS [27] (7a) identified in unfermented *C. subternata* using NMR [121]; (7b) identified in unfermented *C. subternata* using LC-MS [121]; (8) identified in unfermented and fermented *C. subternata* using LC-MS/MS [53]; (9) identified in *C. genistoides* using NMR and LC-MS [124]; (10a) identified in *C. genistoides* using LC-MS/MS [125]. (10b) identified in *C. genistoides* using NMR [60]. (11) identified in callus of unfermented *C. subternata* using NMR and LC-MS [121]. (12) identified in fermented *C. intermedia* using NMR [52]. Identity was confirmed by NMR as iriflophenone-3-C-β-glucoside-4-O-β-glucoside [60].

2.7. Analysis of tea phenolics

2.7.1. High performance liquid chromatography (HPLC) analysis of rooibos and honeybush tea phenolics

The analysis of tea phenolics is important for many reasons, including research aimed at improved production and due to their bioactivity as antioxidants, which is linked to potential health benefits in humans [90,130,131-133]. HPLC, and in particular reversed-phase (RP)-HPLC is most often used for the analysis of tea phenolics [134]. In the following section, a brief overview of RP-HPLC methods for the analysis of rooibos and honeybush phenolics is presented.

2.7.1.1. Reversed phase (RP)-HPLC analysis of rooibos tea phenolics

RP-HPLC methods reported for the analysis of rooibos and honeybush tea phenolics are summarised in **Tables 2.5.** and **2.6.**, respectively. In 1996, Joubert was the first to report an HPLC method for the quantitative analysis of the dihydrochalcones aspalathin and nothofagin in rooibos tea [104]. The developed method was used to determine the quantities of above-mentioned compounds as affected by processing. Quantitative data of aspalathin and nothofagin present in unfermented and fermented tea samples produced under controlled processing conditions were compared. Separation was attained on a C18 (LiChrospher, 250 × 4.0 mm, 5 µm) column using 2% aqueous formic acid and methanol (MeOH) as mobile phases and a 125 min gradient. In the same standard mixture, phenolic acids (protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, *p*-coumaric acid and ferulic

acid) and other flavonoids (orientin, isoorientin, vitexin, rutin and isoquercitrin) were also analysed. Incomplete separation of vanillic and caffeic acid and co-elution of orientin and ferulic acid, and rutin and isoquercitrin was observed. Analytes present in samples were identified by retention time comparison with authentic standards, spiking of tea samples with standards and UV-absorption comparison at 280 and 320 nm [104].

Bramati and co-workers achieved separation and quantification of some of the major rooibos flavonoids in a standard mixture [103] as well as unfermented [102] and fermented tea infusions [103]. Separation of 10 flavonoids, i.e. a dihydrochalcone (aspalathin), flavones (isoorientin, orientin, vitexin, isovitexin, luteolin and chrysoeriol) and flavonols (rutin, isoquercitrin and quercetin) in a standard mixture was attained in 30 min on a C18 (Symmetry Shield, 250 × 4.6 mm, 5 µm) column with 0.1% aqueous acetic acid and acetonitrile (ACN) as mobile phases. Incomplete separation of isoorientin and orientin and co-elution of isoquercitrin and hyperoside were observed for aqueous tea infusions. Identification of analytes present in samples was confirmed by comparison of retention time, UV- and MS-spectra with those of authentic standards. LC-MS analyses were carried out in negative and positive ionisation modes and 3 compounds were tentatively identified: dihydro-isoorientin, dihydro-orientin and nothofagin [103]. An interesting experiment was carried out by collecting the co-eluting peaks (isoquercitrin and hyperoside) 5 times and analysing these fractions using a micellar electrokinetic chromatography (MEKC) method described by Pietta and co-workers [135]. MEKC provided separation of these two compounds, which could not be achieved by RP-LC. The complementary selectivity offered by MEKC for this application may be due to slight differences in degree of ionisation, borate complexation and/or partitioning into the micelles based on differences in the sugar moieties (-glucoside vs. galactoside, respectively) between these compounds.

The quantitative LC-UV-MS method developed by Schulz and co-workers [136] in 2003 was faster and more sensitive than the previous method described by Joubert [104]. The aim of the study was to address the lack of quantitative data on aspalathin and nothofagin. Samples for this study were obtained from a large number of production areas. Separation of the dihydrochalcones aspalathin and nothofagin, flavones isoorientin and orientin and flavonols rutin and isoquercitrin was attained on a C18 column (Zorbax, 150 × 3.0 mm, 3.5 µm) at 35°C, using 1% aqueous formic acid (v/v) and ACN as mobile phases with a 40 min gradient. Incomplete separation of an unknown peak and isoorientin, isoorientin and orientin, rutin and isoquercitrin and nothofagin and an unknown peak was observed. Only quantitative data for aspalathin and nothofagin was presented, although quantities of the other flavonoids were also determined in both unfermented and fermented rooibos tea samples. Analytes were identified by comparing retention times, UV- and MS-spectra to those of authentic standards [136]. UV-spectra were recorded from 190-950 nm with detection performed at 288 nm [136].

An LC-MS/MS method was reported by Kazuno *et al.* [137] for the identification and quantification of 11 glycosylated flavonoids in unfermented rooibos tea, namely 2 dihydrochalcones (aspalathin and nothofagin), 5 flavones (isoorientin, orientin, vitexin, isovitexin, and luteolin-7-O-glucoside) and 4 flavonols (rutin “isomer”, rutin, hyperoside and isoquercitrin). Optimum separation was achieved on a C18 column (Develosil, 150 × 2.0 mm, 5 µm) at 35°C with a 50 min gradient. This was the first time a rutin “isomer” present in rooibos tea was identified and quantified [137].

Joubert and co-workers [138] developed a method for the rapid quantification of aspalathin, isoorientin and orientin in fermented rooibos iced tea samples. A Zorbax Eclipse XDB-C18 column (150 × 4.6 mm, 5 µm) was used at 38°C with a total analysis time of 23 min. All 3 compounds were completely separated. Aspalathin was quantified at 288 nm and isoorientin and orientin at 350 nm [138]. The method has been applied in studies pertaining to ready-to-drink rooibos iced teas, where LC-MS and MS/MS were used for additional identification [139,140].

Three studies [100,101,141] on the bioavailability of rooibos flavonoids following the ingestion of rooibos teas reported metabolite quantities in urine [100,101,141] and plasma [101] for the first time. First, Stalmach and co-workers achieved separation in 35 and 75 min for urine and tea samples, respectively on a C12 80Å column (Synergi, 250 × 4.6 mm, 4 µm). Co-elution or partial co-elution of several compounds was observed in the fermented tea sample, while (partial) co-elution of several phenolics metabolites was observed in the urine sample after ingestion of 500 mL fermented rooibos tea. Quantitative data of for 16 phenolic compounds were presented. Flavonoids (in tea samples) and metabolites (in urine samples) were identified and quantified using LC-MS in selective ion monitoring (SIM) mode on an ion trap instrument. Traces of naringenin-C-glucoside isomers, which are potential oxidation products of nothofagin, were detected in the fermented rooibos tea sample and tentatively identified by LC-MS/MS [100]. Courts and Williamson [141] conducted a study on the bioavailability of aspalathin in urine following ingestion of a hot water extract of unfermented rooibos tea leaves (14 g/L). A fast (15 min) HPLC-DAD method on a Zorbax Eclipse Plus C18 column (100 × 2.1 mm, 1.8 µm) was used to quantify aspalathin. LC-MS/MS analysis of urine samples was performed in multiple reaction monitoring (MRM) mode using a longer gradient. Two isomeric metabolites (3-O- and 4-O-methylaspalathin) were identified and quantified [141]. Breiter *et al.* [101] studied the bioavailability and anti-oxidant potential of rooibos flavonoids in humans following the consumption of hot water unfermented rooibos infusions (20 g/L). An LC-MS/MS method was developed on a phenyl-hexyl column (Luna, 250 × 4.6 mm, 5 µm) with a 88 min gradient. Incomplete separation between isoorientin and apigenin-6-C-arabinoside-8-C-glucoside (isoschaftoside), tentatively identified by LC-MS/MS, and rutin and a rutin “isomer” were observed. Quantitative data for the major flavonoids in an unfermented rooibos tea extract were presented. Isoorientin, orientin, isovitexin, vitexin, aspalathin and rutin were also quantified in the plasma following ingestion of the teas. Flavonoids were identified using authentic standards. Luteolin-6,8-di-C-hexoside, apigenin-6,8-

di-C-hexoside (vicenin-2), luteolin-6-C-hexoside-8-C-pentoside and apigenin-6-C-arabinoside-8-C-glucoside (isoschaftoside) were tentatively identified [101].

Cabooter and co-workers developed an ultra high performance liquid chromatography (UHPLC) method for rooibos phenolic analysis using a step-wise variable column length strategy based on an automated column coupler [107]. Several UHPLC columns were evaluated, with optimal separation being attained on a 200 mm long BEH Shield RP-18 (2.1 mm, 1.7 μm) column operated at 30°C and maximum pressure ($P_{\max} = 940$ bar). Complete separation of the flavonoids in the standard mixture and samples were observed. No quantitative data were presented [107].

Beelders *et al.* [99] developed and validated a quantitative HPLC method on conventional instrumentation for the separation of the 15 principal rooibos phenolics. Optimum separation was achieved on a 1.8 μm Zorbax SB-C18 column (100 \times 4.6 mm) for an analysis time of 50 min [99]. The method was applied to evaluate the differences in phenolic content and anti-oxidant activity of fermented rooibos tea infusions and the role of production season and quality grade on rooibos phenolic content [142]. Quantitative data for unfermented [99] and fermented [99,142] rooibos teas have been obtained using this method. Compounds were identified using authentic standards and UV- and MS data, while additional compounds were tentatively identified based on MS/MS data [99,142].

Recently de Beer and co-workers [143] developed a rapid HPLC method for the separation of the major flavonoids, the dihydrochalcones (aspalathin and nothofagin) and flavones (isoorientin and orientin) within 16 min. The method used a superficially porous C18 column (Poroshell SB-C18, 50 \times 4.6 mm, 2.7 μm), and was validated for the quantification of the four major flavonoids in a polyphenol-enriched fraction of unfermented rooibos tea leaves [143].

Table 2.5. Overview of RP-HPLC methods for the analysis of rooibos (*Aspalathus linearis*) flavonoids, phenolic acids and some metabolites.

Sample (s)	Analytes	Column (dimensions), flow rate, temperature, analysis times	Mobile phase (s)	Detector (s)	General remarks	Reference (s)
Standards, unfermented and fermented aqueous freeze-dried rooibos	Protocatechuic acid, <i>p</i> -hydroxybenzoic acid, vanillic acid and caffeic acid, <i>p</i> -coumaric acid, aspalathin, orientin and ferulic acid, isoorientin, vitexin, nothofagin and rutin and isoquercitrin.	LiChrospher 100 RP-18 (250 × 4.0 mm, 5 µm d_p), 0.4 – 1.2 mL/min, 38°C, 125 min	A: 2% formic acid in H ₂ O (v/v) B: MeOH	UV	Incomplete separation of vanillic and caffeic acid and co-elution of orientin and ferulic acid and rutin and isoquercitrin in a standard mixture. Quantification of aspalathin and nothofagin in unfermented and fermented aqueous freeze-dried samples.	[104]
Standards, unfermented (aqueous) and fermented (aqueous and methanolic) infusions	Isoorientin, orientin, aspalathin, vitexin, rutin, isovitexin, isoquercitrin and hyperoside, luteolin, quercetin and chrysoeriol.	Symmetry Shield C18 (250 × 4.6 mm, 5 µm d_p), 0.8 mL/min, room temperature, 30 min	A: 0.1% acetic acid in H ₂ O (v/v) B: ACN	UV and MS	Incomplete separation of isoorientin and orientin. Co-elution of isoquercitrin and hyperoside in unfermented and fermented rooibos aqueous infusions. Quantification of analytes, but isoquercitrin and hyperoside were quantified together.	[102] and [103]
Unfermented and fermented aqueous extracts	Isoorientin, orientin, aspalathin, rutin, isoquercitrin and nothofagin.	Zorbax SB-C18 (150 × 3.0 mm, 3.5 µm d_p) 0.5 – 0.7 mL/min, 35°C, 40 min	A: 1% formic acid in H ₂ O (v/v) B: ACN	UV and MS	Incomplete separation between an unknown peak and isoorientin, rutin and isoquercitrin and an unknown peak and nothofagin. Quantification of only a few (6) flavonoids in unfermented and fermented aqueous extracts.	[136]
Unfermented aqueous extract and commercially available green rooibos	Isoorientin, orientin, aspalathin, rutin "isomer", vitexin, rutin, isovitexin, hyperoside, isoquercitrin, luteolin-7-O-glucoside, nothofagin.	Develosil ODS UG-5 (150 × 2.0 mm, 5 µm d_p) 0.2 mL/min, 35°C, 50 min	A: 0.1% formic acid in H ₂ O (v/v) B: 0.1% formic acid in 80% ACN (v/v)	UV, MS and MS/MS	Separation and quantification of 11 glycosyl rooibos flavonoids using mass spectrometric methods in neutral scan mode and SRM.	[137]
Fermented rooibos iced teas. Green rooibos extracts and ready-to-drink product formulations. Hot aqueous fermented and organic-solvent based green rooibos extracts	Aspalathin, orientin and isoorientin.	Zorbax Eclipse XDB-C18 (150 × 4.6 mm, 5 µm d_p) 0.8 mL/min, 38°C, 23 min	A: 2% acetic acid in H ₂ O (v/v) B: ACN	UV UV, MS and MS/MS UV, MS and MS/MS	Rapid quantification of aspalathin, isoorientin and orientin in green rooibos extracts and ready-to-drink iced teas. After isolation, identification of unknown compound 1 was confirmed as 5-hydroxymethylfurfural using nuclear magnetic resonance (NMR) and gas chromatography-mass spectrometry (GC-MS). Citric and ascorbic acid stabilise major rooibos constituents in iced-tea formulations during storage.	[138] [139] [140]
Unfermented and fermented ready-to-drink rooibos teas, plasma and urine	Aspalathin, nothofagin, eriodictyol-C-glucosides (4), orientin, isoorientin, vitexin, isovitexin, hyperoside, isoquercitrin, rutin, rutin isomer, luteolin, quercetin. Aspalathin and eriodictyol metabolites in urine.	Synergi (C12) RP-MAX 80Å (250 × 4.6 mm, 4 µm d_p) 1.0 mL/min, 40°C, 35 min (urine), 75 min (teas)	A: 0.1% formic acid in H ₂ O (v/v) B: ACN	UV, MS and MS/MS	Co-elution of rutin isomer and vitexin, isovitexin and rutin and quercetin and luteolin. Quantification of analytes in teas and aspalathin and eriodictyol metabolites in urine, using SIM mode. Co-eluted compounds were quantified using SIM mode.	[100]

Table 2.5. (Continued)

Sample (s)	Analytes	Column (dimensions), flow rate, temperature, analysis times	Mobile phase (s)	Detector (s)	General remarks	Reference (s)
Standards and urine	Aspalathin, 3-O- and 4-O-methylaspalathin	Zorbax Eclipse PlusC18 (100 × 2.1 mm, 1.8 µm d_p) 0.25 mL/min, 35°C, 14.75 min (aspalathin), 21 min (urine)	A: 0.2% formic acid in H ₂ O (v/v) B: MeOH	UV, MS and MS/MS	Quantification of aspalathin by DAD. Identification and quantification of metabolites (3-O- and 4-O-methylaspalathin) in urine by LC-MS/MS (MRM mode).	[141]
Unfermented rooibos hot water extracts, urine and plasma	Aspalathin, nothofagin, isoorientin, orientin, rutin, hyperoside, isoquercitrin, vitexin, isovitexin, luteolin- <i>O</i> -galactoside in an aqueous extract of green rooibos and aspalathin, isoorientin and orientin in plasma	Luna Phenyl-Hexyl (250 × 4.6 mm, 5 µm d_p) 0.5 mL/min, r.t., 88 min	A: 2% acetic acid in H ₂ O (v/v) B: ACN	UV, MS and MS/MS	Incomplete separation of isoorientin and isoshaftoside (tentatively identified by LC-MS/MS) and rutin and rutin isomer. Quantification of major flavonoids (see analytes) in an aqueous unfermented rooibos extract; also unchanged flavonoids in plasma after ingestion of rooibos tea or active fraction isolated from rooibos tea. Tentative identification of metabolites in urine by LC-MS/MS.	[101]
Unfermented and fermented rooibos tea extracts	PPAG, orientin, isoorientin, aspalathin, vitexin, ferulic acid, isovitexin, luteolin-7- <i>O</i> -glucoside, nothofagin, rutin, hyperoside, isoquercitrin, luteolin, quercetin, chrysoeriol	Acuity BEH Shield RP-18 (200 × 2.1 mm, 1.7 µm d_p) 0.3 mL/min, 30°C, 31 min	A: 2% acetic acid in H ₂ O (v/v) B: ACN	UV and MS	Complete separation of 15 principal rooibos phenolics in a standard mixture, unfermented and fermented tea samples using the variable-column length method development strategy operating at maximum column pressure. No quantitative data were reported.	[107]
Standards, unfermented and fermented aqueous rooibos infusions	PPAG, isoorientin, orientin, aspalathin, vitexin, ferulic acid, isovitexin, luteolin-7- <i>O</i> -glucoside, nothofagin, rutin, hyperoside, isoquercitrin, luteolin, quercetin, chrysoeriol	Zorbax SB-C18 (100 × 4.6 mm, 1.8 µm d_p) 1.0 mL/min, 37°C, 50 min	A: 2% acetic acid in H ₂ O (v/v) B: ACN	UV, MS and MS/MS UV	Incomplete baseline separation between aspalathin and ferulic acid, hyperoside and rutin, rutin and isovitexin and quercetin and luteolin in the standard mixture, but complete separation is observed in the fermented samples. Incomplete separation of quercetin-3-O-robinobioside (tentatively identified by LC-MS/MS) and vitexin and rutin and isovitexin (in fermented sample) presented in reference 21. Quantitative data of analytes are presented.	[99] [142]
Standards and a polyphenol-enriched fraction (liquid-liquid fractionation with butanol from a hot water extract of dried, milled unfermented rooibos leaves)	Aspalathin, isoorientin, orientin and nothofagin	Poroshell SB-C18 (50 × 4.6 mm, 2.7 µm d_p superficially porous) 1.0 mL/min, 30°C, 16 min	A: 0.1% formic acid in H ₂ O B: ACN	UV, MS and MS/MS	Rapid separation and quantification of 4 major flavonoids in a polyphenol-enriched fraction.	[143]

2.7.1.2. HPLC analysis of honeybush tea phenolics

The first RP-HPLC method for the analysis of honeybush phenolics was reported in 2003 by Joubert and co-workers. Three columns of different dimensions and stationary phases, as well as mobile phases comprising 2% aqueous acetic acid, water (pH = 4, adjusted with citrate buffer), ACN and tetrahydrofuran (THF) and two gradients were evaluated. Severe peak tailing for all compounds as well as co-elution of mangiferin and isomangiferin was observed on a Multisphere C18 column. Modification of the mobile phase (solvent A) by adding 5% THF resolved the 2 isomeric xanthones, but did not reduce tailing. Co-elution of luteolin and eriodictyol was observed on a Synergy Polar RP column. Optimal separation of the standards (see **Table 2.6.**) was achieved on the Synergy MAX-RP C12 column using 2% aqueous acetic acid and ACN as mobile phases. The total analysis time was 29 min. The method was applied to the quantitative analysis of the xanthones mangiferin and isomangiferin and the flavanone hesperidin in unfermented methanolic extracts of *C. genistoides*, *C. intermedia*, *C. maculata* and *C. sessiliflora*. The developed method was used to study the effect of harvesting date on the phenolic composition of *C. genistoides*. The target analytes were identified by comparing retention times and UV-spectra with those of authentic standards [144].

The same method (column temperature 30°C) was applied for the determination of the phenolic profiles of unfermented aqueous and methanolic extracts and fermented aqueous extracts of *C. genistoides*, *C. intermedia*, *C. subternata* and *C. sessiliflora*. The phenolic profiles of three different harvests of all species were investigated. Known phenolic compounds present in the samples were also evaluated for phyto-oestrogenic activity. The compounds investigated include the flavanonones hesperidin, hesperitin, eriocitrin, eriodictyol, narirutin and naringenin, a flavone (luteolin), isoflavones (formononetin and genistein) and a xanthone, mangiferin. Quantitative data for mangiferin, hesperidin, eriocitrin, hesperitin and narirutin (both present in trace quantities) were presented. Luteolin, formononetin, naringenin and eriodictyol were not detected in any of the samples [56].

Joubert and co-workers further studied the variation in phenolic composition as a function *Cyclopia* species and processing [128] using the previously reported method [144]. Chromatograms for unfermented and fermented samples of *C. genistoides*, *C. intermedia*, *C. subternata* and *C. sessiliflora* presented a “hump”, which was more pronounced for fermented samples. This phenomenon, possibly due to polymeric phenolic material present in the samples, complicated integration of peaks eluting on the “hump” (unknown 2, eriocitrin, narirutin and hesperidin). Poor resolution of several peak pairs (mangiferin and an unidentified compound, isomangiferin and an unknown peak and an unknown peak and eriocitrin) was observed for *C. subternata* samples. Only known major phenolics, identified using authentic standards, were quantified. Hesperitin and luteolin were detected in trace amounts [128].

Extensive HPLC method development for the analysis of major honeybush tea phenolics was performed by de Beer and Joubert in 2010 [145]. Eight RP 150×4.6 mm columns were tested using the same solvent gradient described by Joubert and co-workers [144] to improve resolution and separation of co-eluting and/or partially co-eluting compounds. The evaluated columns included C12, polar-embedded C18, ether-linked phenyl, polar endcapped C18, hybrid C18-bonded silica, phenyl-hexyl, phenyl-ethyl extra-dense and double endcapped C18 phases. The initial mobile phases comprised 2% aqueous acetic acid (A) and ACN (B), but solvent A was later changed to 0.1% formic acid. Column performance in terms of peak symmetry for mangiferin and resolution between eridictyol and luteolin and eriocitrin and unidentified peaks was evaluated. The most efficient separation was attained on the extra-dense C18 phase (Zorbax Eclipse XDB-C18). The optimised method was validated and applied to the quantitative analysis of major honeybush tea phenolics and five unidentified compounds present in *C. genistoides*, *C. intermedia*, *C. subternata* and *C. sessiliflora*. Analytes were identified by comparing retention times, UV- and mass spectra with those of reference standards. Limitations of the method include 1) the “hump” observed in chromatograms of all samples, complicating integration for eriocitrin and several unidentified compounds, 2) co-elution of narirutin, mangiferin, isomangiferin and eriocitrin with unidentified UV-active compounds in some extracts, and 3) for the quantification of fermented samples, 2% acetic acid instead of 0.1% formic acid was preferred [145].

Kokotkiewicz and his group performed isolation, characterisation and identification of phenolics in unfermented *C. subternata* [121] and *C. genistoides* plant material [124]. Semi-preparative HPLC purification was performed using two serially coupled monolithic RP-18 columns and phenolic constituents were identified by 1D- and 2D-NMR and LC-DAD-MS. Separation of the isolated phenolic fractions was attained on a C18 column (Supelcosil, 150×4.5 mm, $3 \mu\text{m } d_p$) using a 82 min method. In general poor resolution was observed for the analysis of an ethyl acetate fraction of *C. subternata*; a “hump” was still evident [121]. Incomplete separation between mangiferin, an unknown peak and isomangiferin was observed in several different extracts of *C. genistoides*. A less evident “hump” was observed in the non-aqueous extract and fractions of *C. genistoides*. Quantitative data for mangiferin, isomangiferin, hesperidin, maclurin-3-*C*- β -glucoside and iriflophenone-3-*C*- β -glucoside were presented [124]. A study performed by the same group on micro-propagation of *C. genistoides* used the same LC-MS method to analyse and quantify 6 selected phenolics (mangiferin, isomangiferin, calycosin-7-*O*-glucoside, pseudobaptigenin-7-*O*-glucoside and formononetin-7-*O*-glucoside) present in methanolic extracts of the plant material. No “hump” was observed, although incomplete separation between mangiferin and isomangiferin was observed [146].

Two species-specific HPLC methods for the analysis of phenolic constituents in *C. subternata* and *C. maculata* were developed [26,120,129] by adapting the method described by de Beer and Joubert [145]. Four columns, including porous and superficially porous RP phases, as well as several mobile

phases, analysis temperatures and gradient times were evaluated. The optimised methods for both species differed only in terms of gradient conditions, and were attained on a Gemini-NX C18 column at 30°C, using 2% aqueous acetic acid and ACN as mobile phases. The method developed for *C. subternata* overcame several limitations of the previous method [145]: resolution was improved for mangiferin and isomangiferin, and the use of a shallow gradient (10-40% ACN) spread out the “hump”, whilst maintaining efficient separation between compounds [120]. The method developed for *C. maculata* was applied to the chemometric analysis of the chromatographic fingerprints [26].

Recently, Beelders *et al.* developed a validated quantitative HPLC-DAD-MS species-specific method for the comprehensive phenolic profiling of *C. genistoides*. The method was adapted from those reported by de Beer *et al.* [120] and Schulze *et al.* [129] to overcome the limitations of the generic method developed by de Beer and Joubert [145]. Again several columns, temperatures and mobile phases were evaluated. Optimal separation conditions were attained on the Kinetix C18 core shell column, thermostatted at 30°C with mobile phases A, B and C being 1% aqueous formic acid (v/v), MeOH and ACN, respectively. A relatively simple gradient (65 min) was used. Several novel constituents in *C. genistoides* were tentatively identified by LC-MS and MS/MS in this study (refer to **Section 2.6.2.2.** for further details) [125].

More recently another species-specific method for the analysis of phenolic constituents in *C. longifolia* was developed and validated by Schulze and co-workers [27]. The method of Beelders *et al.* [125] was used as starting point due to the high levels of xanthones (mangiferin and isomangiferin) present in the samples of this species. Gradient parameters were systematically optimised, resulted in a total run time of 59 min. Optimum separation of phenolic constituents in aqueous extracts of unfermented and fermented *C. longifolia* was attained on the Kinetix C18 core shell column (150 × 4.6 mm, 100 Å, 2.6 µm d_p) at 30°C, using 0.1% formic acid (v/v) and ACN as mobile phases. Luteolin-7-O-glucoside and a glycosylated phenolic acid, hydroxybenzene propanoic acid-2-O-hexoside, were tentatively identified by LC-MS and MS/MS for first time in *C. longifolia* and *Cyclopia* spp. Other compounds tentatively identified by LC-MS and MS/MS included amino acids and xanthone derivatives (also previously reported [125], **Section 2.6.2.2.**). The method was applied to the analysis of total anti-oxidant capacity (TAC) and statistical studies for the evaluation of the consumption of *C. longifolia* as herbal tea that might contribute to health-promoting effects in humans [27]. All the species-specific methods listed above [26,27,120,125,129] are currently in use by ARC (Infruitec-Nietvoorbij, Stellenbosch) for routine analysis of honeybush phenolics.

Table 2.6. Overview of RP-HPLC methods for the analysis of *Cyclopia* spp. phenolics.

Sample (s) and specie (s)	Analytes	Column (dimensions), flow rate, temperature, analysis times	Mobile phase (s)	Detector (s)	General remarks	Reference (s)
Standards and unfermented methanolic extracts of <i>C. genistoides</i> , <i>C. intermedia</i> , <i>C. maculata</i> and <i>C. sessiliflora</i>	Mangiferin, hesperidin, eriodictyol, luteolin, hesperitin, formononetin, isomangiferin.	Synergy MAX-RP C12 80A (150 × 4.6 mm, 4 $\mu\text{m } d_p$), 1 mL/min, r.t., 29 min	A: 2% acetic acid in H ₂ O (v/v) B: ACN	UV	Quantification of mangiferin, isomangiferin and hesperidin in unfermented methanolic extracts. The effect of harvesting date on above mentioned compounds in 2 types of <i>C. genistoides</i> (Overberg and West Coast) was also studied.	[144]
Standards, unfermented and fermented aqueous extracts of <i>C. intermedia</i> , <i>C. subternata</i> , <i>C. genistoides</i> and <i>C. sessiliflora</i>	Mangiferin, isomangiferin, eriocitrin, narirutin, hesperidin, hesperitin and luteolin.	Synergy MAX-RP C12 (80 Å (150 × 4.5 mm, 4 $\mu\text{m } d_p$), 1 mL/min, 30°C, 29 min	A: 2% acetic acid in H ₂ O (v/v) B: ACN	UV and MS	Incomplete separation of unknown 2 and eriocitrin in unfermented and fermented <i>C. subternata</i> as well as mangiferin, an unknown peak and isomangiferin in unfermented and fermented <i>C. genistoides</i> and unfermented <i>C. sessiliflora</i> . Quantitative data of analytes are presented. In vitro anti-oxidant activity of extracts of <i>Cyclopia</i> spp. was studied, including unfermented and fermented <i>Aspalathus linearis</i> (rooibos) and <i>Camellia sinensis</i> (green, oolong and black) as reference samples. A "hump", indicating possible polymeric substances, was present in all chromatograms.	[128]
Standards, unfermented and fermented aqueous extracts of <i>C. subternata</i> , <i>C. intermedia</i> , <i>C. genistoides</i> and <i>C. sessiliflora</i>	Mangiferin, isomangiferin, eriocitrin, narirutin, hesperidin, eriodictyol and luteolin.	Zorbax Eclipse XDB-C18 80 Å (150 × 4.6 mm, 5 $\mu\text{m } d_p$) 1 mL/min, 30°C, 29 min	A: 0.1% formic acid in H ₂ O (v/v) B: ACN	UV, MS and MS/MS	A quantitative HPLC method for the analysis of <i>Cyclopia</i> spp. phenolics was developed and validated. Quantitative data of mangiferin, isomangiferin, eriocitrin, hesperidin and 5 unidentified compounds present in unfermented and fermented samples are presented. A "hump" (possible polymeric material) is present in all chromatograms. Co-elution of mangiferin and isomangiferin in fermented <i>C. subternata</i> and <i>C. intermedia</i> , eriocitrin with unidentified compounds in fermented <i>C. sessiliflora</i> and narirutin with unidentified compounds in most samples. Eriocitrin and compounds 9-12 elute on the "hump", which complicates integration. Co-elution of these compounds with possible polymeric material ("hump") result in overestimation of amounts.	[145]
Unfermented <i>C. subternata</i> intact plant, callus ethyl acetate extracts, MeOH, ethyl acetate and 25% (v/v) aqueous ACN extracts of unfermented <i>C. genistoides</i> .	<i>C. subternata</i> extracts: Mangiferin, scolymoside, hesperidin, narirutin, iriflophenone-3-C-glucoside, phloretin-3',5'-di-C-glucoside, isorhoifolin, calycosin-7-O-glucoside, rothindin, ononin, isomangiferin and eriocitrin. <i>C. genistoides</i> extracts: Mangiferin, isomangiferin, iriflophenone-3-C-glucoside, hesperidin, luteolin and an unknown.	Supelcosil LC-18 (150 × 4.5 mm, 3 $\mu\text{m } d_p$), 0.6 mL/min, 30°C, 82 min	A: 0.1% formic acid in H ₂ O (v/v) B: 50% ACN and 50% 0.1% formic acid in H ₂ O	UV and MS	The semi-preparative HPLC method was used for isolation and characterisation of phenolics from <i>C. subternata</i> plant and callus extracts. No quantitative data were presented. Iriflophenone-3-C-glucoside, phloretin-3',5'-di-C-glucoside and isorhoifolin were identified for the first time in the plant extract of <i>C. subternata</i> and calycosin-7-O-glucoside, rothindin and ononin were reported for the first time in <i>Cyclopia</i> plants and were identified in callus. LC-MS, 1D- and 2D-NMR were used for the identification of compounds. Generally poor resolution of most compounds, except iriflophenone-3-C-glucoside. Incomplete baseline separation of mangiferin and isomangiferin in <i>C. genistoides</i> extracts and poor resolution of hesperidin with an unknown peak in the aqueous ACN extract.	[121] and [124]

Table 2.6. (Continued)

Sample (s) and specie (s)	Analytes	Column (dimensions), flow rate, temperature, analysis times	Mobile phase (s)	Detector (s)	General remarks	Reference (s)
Standards, unfermented and fermented aqueous extracts of <i>C. subternata</i>	Major analytes: Iriflophenone-3-C-glucoside, iriflophenone-3-C-glucoside-4-O-glucoside, vicenin-2, mangiferin, isomangiferin, 3-hydroxyphloretin-3',5'-di-C-hexoside, eriocitrin, scolymoside, phloretin-3',5'-di-C-glucoside and hesperidin.	Gemini-NX C18, 110 Å (150 × 4.6 mm, 3 µm d_p), 1 mL/min, 30°C, 40 min	A: 2% acetic acid in H ₂ O (v/v) B: ACN	UV, MS and MS/MS	Incomplete separation of (<i>R</i>)-Eriodictyol-di-C-hexoside (tentatively identified by LC-MS/MS) and iriflophenone-3-C-glucoside. Quantification of analytes, except aspalathin and luteolin, which were used to quantify the dihydrochalcones (3-hydroxyphloretin-3',5'-di-C-glucoside and phloretin-3',5'-di-C-glucoside) and scolymoside, respectively. Iriflophenone-3-C-glucoside-4-O-glucoside, (<i>R</i>)- and (<i>S</i>)-eriodictyol-di-C-hexoside, vicenin-2 and 3-hydroxyphloretin-3',5'-di-C-glucoside were tentatively identified in <i>C. subternata</i> for the first time. The variation in phenolic composition and anti-oxidant capacity were also studied.	[120]
Standards, unfermented and fermented aqueous extracts of <i>C. maculata</i>	Major analytes: Maclurin-3-C-glucoside, iriflophenone-3-C-glucoside, mangiferin, isomangiferin, 3-hydroxyphloretin-3',5'-di-C-glucoside, eriocitrin, scolymoside, phloretin-3',5'-di-C-glucoside, hesperidin and vicenin-2.	Gemini-NX C18, 110 Å (150 × 4.6 mm, 3 µm d_p), 1 mL/min, 30°C, 44 min	A: 2% acetic acid in H ₂ O (v/v) B: ACN	UV, MS and MS/MS	The quantitative and validated species-specific method for major phenolics in <i>C. maculata</i> aqueous extracts differs from a previously method reported [30] in a longer gradient (total analysis time 44 min). Incomplete separation of hydroxmangiferin and isomangiferin and vicenin-2 and eriodictyol-O-hexoside in the unfermented sample and also iriflophenone-3-C-glucoside with unknown minor peaks, co-elution of hydroxymangiferin and isomangiferin and a distorted peak of eriocitrin in the fermented sample was observed.	[26]
Standards, unfermented and fermented aqueous extracts of <i>C. genistoides</i>	Major analytes: Maclurin-3-C-glucoside, iriflophenone-3-C-glucoside, mangiferin, isomangiferin, 3-hydroxyphloretin-3',5'-di-C-glucoside, eriocitrin, scolymoside, phloretin-3',5'-di-C-glucoside, hesperidin, vicenin-2, narirutin, tyrosine, phenylalanine, phenyllactic acid-2-O-hexoside and maclurin-di-O,C-hexoside.	Kinetex C18 core shell (150 × 4.6 mm, 2.6 µm d_p), 1 mL/min, 30°C , 65 min	A: 1% formic acid in H ₂ O (v/v) B: MeOH (100%) C: ACN (100%)	UV, MS and MS/MS	A quantitative species-specific method developed and validated for the comprehensive profiling of phenolic constituents in hot water extracts of <i>C. genistoides</i> . Incomplete baseline separation of mangiferin and isomangiferin, dihydroxybenzoic acid-O-pentoside and phenyllactic acid 2-O-hexose (tentatively identified) and a tetrahydroxyxanthone-di-O,C-hexoside and coumaric acid-O-(pentosyl)hexoside (tentatively identified) was observed in an unfermented hot water extract. The method can be used to evaluate bioactivity of major and minor phenolic constituents present in <i>C. genistoides</i> .	[125]
Standards, unfermented and fermented aqueous extracts of <i>C. longifolia</i>	Major analytes are: Maclurin-3-C-glucoside, iriflophenone-3-C-glucoside, riflophenone-3-C-glucoside-4-O-glucoside, mangiferin, isomangiferin, scolymoside, eriocitrin, hesperidin and vicenin-2, tyrosine, phenylalanine.	Kinetex C18 core shell (150 × 4.6 mm, 2.6 µm d_p), 1 mL/min, 30°C , 59 min	A: 0.1% formic acid in H ₂ O (v/v) B: can	UV, MS and MS/MS	A quantitative species-specific method developed and validated for the analysis of phenolics in unfermented and fermented aqueous <i>C. longifolia</i> extracts. Co-elution of a tetrahydroxyxanthone-C-hexoside isomer (tentatively identified) and an unidentified compound and an eriodictyol-O-hexose-O-deoxyhexose isomer (tentatively identified) and isomangiferin and incomplete baseline of phloretin-3',5'-di-C-glucoside and an unknown peak was observed in the analysis of an unfermented hot water extract. The method was applied to total anti-oxidant capacity (TAC) studies to evaluate potential health effects of anti-oxidants in the diet.	[27]

2.7.2. CE analysis of phenolics

RP-HPLC is the most used separation technique for the analysis of phenolics [134]. The technique is sensitive, reproducible and selective, but requires high large amounts of organic solvents, and is therefore relatively expensive. CE is an attractive alternative to HPLC for the analysis of phenolics in natural products [147]. CE is fast, requires much less solvent (most of the times none), is cost effective and very efficient, but is less sensitive and reproducible compared to HPLC [148,149].

CE has been applied to the analysis of phenolics in various natural and plant derived products [150-156]. These include wine [157,158], chocolate and cocoa beans [159,160], lentils, almonds, beans [161], propolis [162], traditional Chinese medicines [163], herbal teas [164,165] and black, green and oolong teas [166]. The most widely CE modes used for the analysis of phenolics are capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), non-aqueous CZE (NACZE) and non-aqueous MEKC (NAMEKC). Typically uncoated fused-silica capillaries are used together with borate, borate-phosphate and sodium dodecyl sulfate (SDS) as background electrolyte (BGE) at moderately to high pHs and UV-detection between 194-280 nm. The following two sections will focus on the analysis of green, oolong and black tea phenolics by CZE and MEKC, since the CE analysis of rooibos and honeybush tea phenolics has not been reported to date. Parameters and experimental conditions of the methods discussed below are summarised in **Table 2.7..** For more detailed background on the principles of each of these modes of CE the reader is referred to the next section of this chapter.

2.7.2.1. CZE analysis of green and black tea phenolics

Only a few CZE methods for the analysis of green and black tea phenolics have been reported [167,168]. MEKC is mostly used, as this mode provides higher efficiency and resolution, while other modes such as non-aqueous capillary electrophoresis (NACE) [169,170] and MEEKC [171-174] have also been applied for analysis of green and black tea phenolics.

The first report on the CE analysis of tea phenolics involved CZE method developed for the analysis of green tea catechins by Horie and co-workers [167]. The method was applied to a Sencha tea and canned green tea infusions. Experimental conditions of the method are given in **Table 2.7..** (-)-Epigallocatechin (EGC), (-)-epicatechin (EC) and (+)-catechin (C) were not resolved, whilst the latter and (-)-epicatechin gallate (ECG) showed severe peak tailing. Baseline separation was not achieved between (-)-epigallocatechin gallate (EGCG) and ECG. The total analysis time (including rinsing and conditioning of the capillary) was 19 min. Caffeine, theanine and ascorbic acid were separated from the catechins. Quantitative data for all analytes were presented. The method overcame some of the

limitations of HPLC methods: it was faster and enabled simultaneous separation of the polar compounds theanine and ascorbic acid from caffeine and catechins [167]. Arce and co-workers developed a CZE method for the analysis of five catechins, caffeine, adenine, theophylline, gallic acid, quercetin and caffeic acid in green tea within 20 min. The group designed a flow injection interface to perform on-line sample extraction, filtration and dilution. Resolution of analytes was overall poor. Quantitative data for 8 commercial green teas were presented [168].

Two NACE methods for the analysis of black, green and oolong tea phenolics [169] and black tea theaflavins [170] have been reported. Lee and Ong described a NACE method for which the quantitative data were compared to those obtained by HPLC. Analytes (refer to **Table 2.7.** for details) were better resolved by HPLC, but the analysis time was much longer [169]. A high efficiency and rapid NACE method for the analysis of black tea theaflavins (4 analytes) was developed by Wright and co-workers. The BGE (90 mM ammonium acetate) was modified with an ACN/MeOH/acetic acid (71/25/4 v/v) solvent system. The method was validated and quantitative data for three grades of black teas were presented [170].

2.7.2.2. MEKC analysis of green and black tea phenolics

Eight reported MEKC methods from 1998 to 2000 [166,175-181] for the analysis of green, oolong and black tea phenolics are outlined below. The methods differ in terms of resolution, efficiency, speed, BGE composition and pH and focussed mostly on the analysis of catechins (C, EC, EGC, EGCG, ECG).

Horie and Kohata reported an MEKC method using a borate and SDS containing BGE to estimate the quality of black, oolong and especially green teas. Poor resolution of four catechins (EGC, EC, EGCG and ECG) and peak tailing of EC and ECG were observed. Other compounds that were separated and quantified include theanine, caffeine and ascorbic acid [166].

A qualitative NAMEKC method reported by Larger and co-workers involved a phosphate-borate-SDS buffer and 10% ACN as organic modifier. Optimisation of the method involved studying the effects of various parameters (pH, borate and SDS concentrations and % ACN) systematically. The overall separation of green and black tea phenolics proved to be unsatisfactory. However, interesting observations were made regarding the differences in phenolic composition of different solvent extracts (ethyl acetate, MeOH and water) obtained for black tea. Theobromine and caffeine were extracted by all three solvents, whilst brown pigments, quercetin-3-glucoside, chlorogenic acids, 3-galloylquinic acid and diverse flavonoids were extracted using MeOH and water. The catechins

(EGC, C, EC, EGCG and EGC), a bisflavonol and other flavonoids could be detected in the ethyl acetate extract. Most of the peaks in the electropherograms were not identified [175].

Three validated quantitative MEKC methods [177,178,180] for the analysis of green and black tea catechins reported in literature were found to differ widely in BGE composition and pH, speed of analysis, resolution and the number of analytes separated. Watanabe and co-workers developed a fast and relatively efficient MEKC method for the analysis of seven catechins (i.e. C, EC, EGC, ECG, EGCG, (-)-catechin gallate (CG) and (-)-gallocatechin gallate (GCG)), caffeine and ascorbic acid. The method was compared to HPLC and CZE methods in terms of speed and resolution, and found to be faster than the former and providing improved resolution compared to the latter. Quantitative data for canned green and black tea analytes were generally comparable with those obtained by HPLC [177]. The method developed by Barroso and van de Werken [178] comprised the same BGE composition and pH (although with different ionic strength). Fewer analytes were separated (C, EGC, EGCG, EC, ECG and caffeine), but the method provided better peak shapes and longer analysis times than reported by Watanabe and co-workers [177]. Quantitative data for green and black teas were presented and the stability of catechin was also studied [231]. A NAMEKC method was developed for the analysis of six catechins (CG, ECG, EC, EGCG, EGC and C), caffeine and 4-amino-2-hydroxybenzoic acid (internal standard) by Wörth and co-workers [180]. The phosphate-SDS BGE (pH 2.5) was adjusted with 10% MeOH (v/v) and all analytes eluted within 17.5 min. Good separation of peaks was observed for the standard solution, but EC and caffeine were not baseline separated from unknown peaks in real tea samples. The peak shape of EGC was, due to possible co-migration with an unknown analyte in the black tea, distorted. A broad peak was observed for catechin in the samples, but not in the standard solution [180].

The MEKC method developed by Kartsova and Ganzha [182] showed poor peak shapes for the gallated catechins (EGCG, GCG and ECG). Nevertheless, separation of 9 compounds (caffeine, EGC, EGCG, EC, gallic acid, GCG, ECG and benzoic acid) in a standard solution was attained in under 10 min. The BGE comprised phosphate, SDS, urea and β -cyclodextrin (β -CD). No quantitative data were presented [182].

Aucamp and co-workers validated a quantitative NAMEKC method for the analysis of black tea catechins (C, EGC, EGCG, EC and ECG) [179]. Theanine, caffeine, gallic acid, ascorbic acid and *p*-nitrophenol (internal standard) were all separated from the catechins within 13 min. The BGE was phosphate-SDS containing 6% MeOH (v/v) (5% for bottled tea samples). Pronounced peak tailing and fronting was seen respectively for EGCG and ECG. Distorted peak shapes of EGC, gallic acid, ascorbic acid and ECG were also seen when ascorbic acid was added, while incomplete separation of EGC was evident for the analysis of an undiluted canned tea sample [179].

An efficient and validated MEKC method for the separation of eight catechins, (-)-C, (+)-C, (-)-CG, (-)-EGCG, (-)-ECG, (-)-EGC, (+)-EC and (-)-EC, and caffeine was proposed by Kodama and co-workers [181]. The separation of *racemic* catechin, (-)-C and (+)-C, and epicatechin, (-)-EC and (+)-EC in tea was also achieved by 6-*O*- α -D-glucosyl- β -cyclodextrin-modified enantioselective MEKC [181]. An in-depth method development strategy was followed, optimising factors affecting chiral separation such as the type of CD and buffer pH and concentration. Resolution and binding constants of enantiomers were calculated and compared. Quantitative data for real tea samples were presented and separation of all analytes was attained in less than 24 min [181].

Efficiencies, resolution of critical pairs as well as analysis times of conventional (moderate BGE pH and positive polarity), reversed (low BGE pH and negative polarity) MEKC and MEEKC methods were compared by Kartsova and co-workers [174]. The efficiency and resolution obtained with the MEEKC method were the highest, but resulted in a long analysis time of about 42 min. The reversed MEKC method was chosen as method of choice and was applied to the analysis of green, oolong and black teas, and quantitative data were presented. All analytes eluted within 14 min, although EGCG and EC were not resolved and broad peaks of the target analytes (especially GCG and ECG) were observed [174].

Nelson and his group developed a NAMEKC method for the analysis of caffeine, 6 green tea catechins (EGC, C, EC, GCG, EGCG and ECG) and L-tryptophan (the internal standard) [176]. As starting point, CZE analysis of the 6 catechins was carried out using a 20 mM tetraborate BGE at pH 8. Poor resolution of EGC, C and EC (group A, the catechins) and GCG, EGCG and ECG (group B, the gallates) and co-migration of GCG and EGCG were observed. A BGE of 20 mM tetraborate (pH 8.2) and 80 mM SDS was initially used for MEKC method development. The effect of micelle charge (anionic SDS and cationic tetradecyltrimethylammonium bromide, TTAB), surfactant type (SDS and sodium cholate (SC)), organic modifier and volume fraction (v/v) (MeOH, ACN and dimethylformamide (DMF), 0-20%), surfactant concentration (60-115 mM), buffer pH (6-9) and cyclodextrin concentration (0.1, 0.5 and 1.0 mM) were evaluated stepwise. An in-depth discussion on how each parameter affects the separation of the catechins was presented. All analytes were baseline resolved, although peak tailing for the gallates was observed under optimal conditions (**Table 2.7.**). Separation was attained within 30 min and quantitative data for three different green tea types were presented [176].

A rapid, simple and quantitative MEKC method for the analysis of 7 green tea catechins and gallic acid was developed and validated by Bonoli and co-workers to compare performance and quantitative results with HPLC. The MEKC method was faster, more efficient, showed higher resolution and was more sensitive than HPLC. The latter observation could be due to the use of a different detection wavelength for the HPLC method. The HPLC method was more reproducible, but quantitative data

were generally comparable between MEKC and HPLC. GC and gallic acid co-eluted in HPLC, and most peaks showed slight tailing [183].

Gotti and co-workers developed and validated a fast quantitative chiral cyclodextrin micellar electrokinetic chromatography (CD-MEKC) method for the analysis of green tea catechins, syringic acid and methylxanthines (theophylline, theobromine and caffeine) using hydroxypropyl- β -CD as chiral selector [237]. Relatively broad peaks for later migrating compounds (theophylline, racemic C, GCG, theobromine and racemic GC) were observed. Separation and quantification of racemic GC were reported for the first time in green tea in this study. The method was applied to study the epimerisation of catechins using statistical methods. Quantitative results of 24 different types of green teas were presented [184].

A rapid and highly efficient low pH MEKC method using a significantly different BGE composition was reported by Peres and co-workers for the quantitative analysis of 5 major green tea catechins [185]. The group evaluated different chiral selectors, α , β , γ and sulfated- β -cyclodextrin (s- β -CD); s- β -CD was selected as optimal. The method proved useful for high-throughput routine analysis of green tea catechins and was applied to 6 Brazilian green teas [185].

The effect of several alcohols as cosurfactants on the MEEKC separation of green tea catechins (ECG, EGCG, EC, C, EGC and GC), caffeine and theophylline was studied in detail by Pomponio and co-workers. Cyclohexanol and 2-hexanol showed the most promising results. Cosurfactants play an important role in the selectivity of phenolic compounds and both altered the selectivity of the method for the green tea catechins. Optimum separation of 8 analytes was attained within 10 min (**Table 2.7.**). The quantitative MEEKC method was validated and applied to 3 types of green tea [171].

Qualitative and quantitative NAMEKC and MEEKC methods for the analysis of 7 phenolic acids (*p*-coumaric acid, vanillic acid, caffeic acid, 4-hydroxybenzoic acid, syringic acid, 3,4-dihydroxybenzoic acid and gallic acid), 6 catechins (ECG, C, EGCG, EC, EGC and GC) and 2 methylxanthines (caffeine and theophylline) were developed by Huang and co-workers following a systematic method optimisation process [172]. Resolution, efficiency, sensitivity and speed of the two methods were compared. The NAMEKC method was more sensitive and faster, but higher efficiencies were obtained by MEEKC. Only EGCG and EC and EGCG, EC and EGC were identified in real samples and no quantitative data were presented [172].

The MEEKC method of Huang and co-workers [172] was adapted and optimised for anion-selective exhaustive injection (ASEI) sweeping to detect trace levels of catechins in food products (including a tea). The influence of high conductivity solutions (HCSs) on ASEI-sweeping was evaluated. Optimal sensitivity was attained with 50 mM phosphate, owing to a deterioration of sweeping efficiency when using basic HCSs. The effect of microemulsion composition, especially oil type and cosurfactants on

stacking was also studied and optimised (refer to **Table 2.7.** for other optimal conditions). The efficiency and sensitivity of the conventional MEEKC and ASEI-sweeping MEEKC methods were compared. Higher efficiencies (width at half height method) and lower limits of detection (LODs) (extrapolation to S/N = 3) were attained by the sweeping method. Peak areas of catechins were 120-715-fold higher, without sacrificing resolution. The method was also applied to a tea sample (direct injection, 1:50 dilution with 55 mM borate-boric acid buffer, pH 8.0) and clear differences from the electropherograms were observed (no peaks were detected by conventional MEEKC) [173].

In general, it is clear that MEKC is the most used mode of CE for the separation of tea phenolics, especially flavanol-derivatives, followed by CZE. MEKC generally offers good performance, and a wide range of parameters to affect selectivity (including the option of MEEKC). CZE methods are as rule simpler, although for the specific case of green and black tea phenolics, often do not provide equivalent efficiency or resolution compared to MEKC methods. The ultimate choice of separation mode does however depend on the target analytes, such that it is recommended to initially evaluate both modes for a given application.

Table 2.7. A summary of CE methods reported for the analysis of major tea flavonoids and metabolites.

Samples and tea type	Analytes	CE mode	BGE and pH	Capillary dimensions (i.d., l and L), temperature, voltage, injection and analysis time	Detection and wavelength (s)	General remarks	Reference
Standards and green teas	Caffeine, theanine, EGC, EC, C, EGCG, ECG and ascorbic acid.	CZE	20 mM borate pH 8.0	(50 µm, 70 cm, 77 cm), 23°C, 30 kV, hydrodynamic for 5s; 18 min	UV, 200 nm	Poor resolution of EGC, EC and C. The method was validated and quantitative data of 4 types of green tea are presented.	[167]
Standards and green teas	Caffeine, adenine, theophylline, EGC, EC, C, EGCG, ECG, quercetin, gallic acid and caffeic acid.	CZE	150 mM boric acid pH 8.5	(75 µm, 57 cm), 20°C, 20 kV, hydrodynamic injection 10s; 20 min.	UV, 210 nm	A flow injection system was designed for online sample extraction, filtration and dilution. Poor resolution between EGC, EC and C and EGCG and ECG and co-elution of EOF marker and caffeine in standard solution. The validated quantitative method was applied to eight different types of green tea. A polymeric "hump" was observed in the electropherogram of a real sample (Afka).	[168]
Standards, green, oolong and black teas	Caffeine, adenine, theophylline, CG, EGC, EC, C, EGCG, ECG, quercetin, gallic acid, caffeic acid, theaflavin, theaflavin-3-monogallate, theaflavin-3'-monogallate and theaflavin-3,3'-gallate.	NACZE	200 mM boric acid (pH 7.2), 10 mM phosphate (pH 4.2), 4.5 mM β-CD, 27.5% ACN (v/v)	Extended light path capillary (50 µm, 40 cm), 30°C, 25 kV, hydrodynamic injection at 75 mbar.sec; 10 min.	DAD, 205 nm	A validated quantitative NACE method was compared to HPLC. The results of the major catechins and theaflavins in eight different types of tea were comparable. Overall poor resolution of the analytes was observed and surprisingly theaflavins were detected in green tea. A polymeric "hump" was observed in the electropherogram of the black tea and all analytes eluted in less than 10 min.	[169]
Standards and black teas	Theaflavin, theaflavin-3-monogallate, theaflavin-3'-monogallate and theaflavin-3,3'-gallate.	NACZE	90 mM ammonium acetate and ACN/MeOH/acetic acid (71/25/4) (v/v) and pH 6.2	(50 µm, 32 cm, 40 cm), 18.5°C, 27.5 kV, hydrodynamic injection at 250 mbar.sec; 10 min.	UV, 380 nm	A rapid validated quantitative NACE method for the analysis of black tea theaflavins was developed and compared to an aqueous CZE method. The CZE method resulted in poor peak shapes and the effect and composition of organic modifiers on electrophoretic mobilities and separation was evaluated. High resolution and efficiency of the theaflavins was observed and quantitative data of three grades of black teas were presented and compared.	[170]

Table 2.7. (Continued)

Samples and tea type	Analytes	CE mode	BGE and pH	Capillary dimensions (i.d., l and L), temperature, voltage, injection and analysis time	Detection and wavelength (s)	General remarks	Reference
Standards, green, oolong and black teas	Caffeine, theanine, EGC, EC, EGCG, ECG, ascorbic acid and <i>p</i> -hydroxybenzoic acid.	MEKC	20 mM sodium tetraborate, 80 mM boric acid, 50 mM SDS, pH 8.4	(75 µm, 70 cm, 77 cm), 30°C, 25 kV, by N ₂ pressure for 5s and about 18 mins	DAD, 194 and 270 nm	Incomplete separation of EGC and EC, and EGCG and ECG. Strong peak tailing for EC and ECG was observed. The method was used to estimate tea quality	[166]
Standards, green and black teas. Ethyl acetate, MeOH and aqueous fractions of black tea.	Theobromine, caffeine, EGC, C, EC, EGCG, ECG, chlorogenic acid, kaempferol derivative, bisflavanols, brown pigments, 3-galloylquinic acid, isoquercitrin, diverse flavonoids and their glucosides.	NAMEKC	50 mM sodium tetraborate, 50 mM phosphate, 20 mM SDS, 10% ACN (v/v), pH 6.0.	Bubble cell capillary (50 µm, 56 cm, 64.5 cm), 25°C, 30 kV hydrodynamic 750 mbar.sec and about 45 min.	UV, 278 nm	A low resolution qualitative method for the analysis of phenolics in green, black tea and black tea fractions. The phenolic profiles of the different black tea fractions differed, indicating extraction of diverse phenolics varies with solvent type. Polymeric "humps" were observed in all electropherograms.	[175]
Standards, canned green and black teas	Caffeine, C, EGC, EC, EGCG, GCG, ECG, CG and ascorbic acid.	MEKC	50 mM borate, 25 mM phosphate, 25 mM SDS, pH 7.0.	(50 µm, 32 cm, 36 cm), 20°C, 15 kV, hydrodynamic 350 mbar.sec and about 15 min.	UV, 280 nm	A quantitative MEKC method was developed and compared to previously published HPLC and CZE methods. Incomplete baseline separation of C and EGC, EGCG and GCG and ECG and CG was observed. All analytes were well resolved by HPLC, but analysis time was longer. MEKC provided better resolution than the CZE method. Quantitative results of canned green and black tea samples compared well with those obtained by HPLC.	[177]
Standards, green and black teas	Caffeine, C, EGC, EGCG, EC and ECG.	MEKC	20 mM borate, 20 mM phosphate, 25 mM SDS, pH 7.0	(50 µm, 70 cm, 85 cm), 21°C, 30 kV, 25 nL and 15 min.	UV, 200 nm	A highly efficient, simple and cost-effective quantitative MEKC method for the analysis of caffeine and major catechins was developed and validated. Peaks were well resolved in a standard mixture and green and black tea samples. A polymeric "hump" was less evident in the electropherogram of the green than the black tea sample. Quantitative data are presented.	[178]

Table 2.7. (Continued)

Samples and tea type	Analytes	CE mode	BGE and pH	Capillary dimensions (i.d., l and L), temperature, voltage, injection and analysis time	Detection and wavelength (s)	General remarks	Reference
Standards, green and black teas	CG, ECG, EC, EGCG, 4-amino-2-hydroxybenzoic acid, EGC, C and caffeine.	NAMEKC	20 mM sodium phosphate (pH 2.5), 100 mM SDS, 10% MeOH (v/v)	(50 µm, 45.4 cm, 50 cm), 25°C, 20 kV, hydrodynamic at 689.5 mbar.s within 20 min.	UV, 195 nm	A highly efficient quantitative low pH NAMEKC method for the analysis of major catechins and caffeine was developed. Incomplete separation of caffeine and an unknown peak in black and green tea samples was observed. Quantitative results presented for black and green teas. The method was used to evaluate catechins and caffeine content under different preparation procedures.	[180]
Standards, green and black teas.	Caffeine, C, EGC, EGCG, EC, gallic acid, GCG, ECG and benzoic acid.	MEKC	25 mM phosphate (pH 7.0), 20 mM SDS and 10 mM urea.	(75 µm, 50 cm, 60 cm), 20°C, 25 kV hydrodynamic 600 mbar.sec and within 10 min.	UV, 200 nm	Distorted peaks for most analytes and broad peaks for the gallates were observed. No quantitative data were presented.	[182]
Standards, diluted dried fresh leaf and black tea samples and undiluted canned black tea.	Theanine, C, caffeine, EGC, EGCG, gallic acid, EC, p-nitrophenol and ECG.	NAMEKC	25 mM phosphate, 100 mM SDS, 6 and 5% MeOH (v/v), pH 7.0	(50 µm, 50 cm, 57 cm), 25°C, 14 kV, hydrodynamic 69 mbar.sec and about 13 min.	UV, 200 nm	A fast validated quantitative NAMEKC method for the analysis of five catechins, theanine, caffeine and gallic acid was developed. 2 BGEs, differing in MeOH percentage, were used. Peak tailing and fronting of EGCG and ECG, respectively, were observed in 6% MeOH (v/v). Distorted peaks of gallic acid, ascorbic acid and ECG were observed in 5% MeOH (v/v). Quantitative data are presented.	[179]
Standards, green, oolong and black teas and commercial tea beverages.	(-)C, (+)-C, (-)-CG, (-)-EGCG, (-)-ECG, (-)-EGC, (+)-EC and (-)-EC) and caffeine.	MEKC	200 mM borate, 20 mM phosphate, 240 mM SDS 25 mM of 6-O- α -D-glucosyl- β -cyclodextrin (6G- β -CD), pH 6.4.	Bubble cell capillary (50 µm, 56 cm, 64.5 cm), 20°C, 25 kV, hydrodynamic 100 mbar.s and within 24 min.	UV, 210 nm	A cyclodextrin-modified quantitative MEKC method for the enantioseparation of racemic catechin and epicatechin was developed and validated. The simultaneously separation of (-)-C, (+)-C, (-)-EC and (+)-EC was reported for the first time. Resolution and efficiency was good overall, but broader peaks for (-)-EC and (+)-EC were observed.	[181]

Table 2.7. (Continued)

Samples and tea type	Analytes	CE mode	BGE and pH	Capillary dimensions (i.d., l and L), temperature, voltage, injection and analysis time	Detection and wavelength (s)	General remarks	Reference
Standards, green and black teas.	C, EGC, EGCG, EC, GCG, ECG and gallic acid.	MEKC	25 mM sodium phosphate (pH 7), 15 mM SDS	(50 µm, 50 cm, 60 cm), 20°C, 25 kV, injection not indicated, within 13 min.	UV, 200 nm	A qualitative and relatively low resolution MEKC method (high pH) was compared to low pH MEKC and MEEKC methods in terms of efficiency and resolution. Unstable baselines were observed for the analysis of real samples. The MEEKC method was the most efficient, followed by the low pH MEKC method.	[174]
Standards, commercial white tea (Lipton), green tea, oolong tea and various types of black tea	C, EGC, EGCG, EC, GCG, ECG and gallic acid.	MEKC	10 mM sodium acetate, 150 mM citric acid (pH 2), 120 mM SDS.	(50 µm, 50 cm, 60 cm), 20°C, 25 kV, injection not indicated, within 11 min.	UV, 200 nm	The MEKC (low pH) method resulted in baseline separation of analytes. Unstable baselines (worse than the high pH MEKC method) were observed in the electropherograms of real samples. Quantitative data are presented. Caffeine content quantified by the MEKC (low pH) and HPLC were comparable.	[174]
Standards and green tea	Caffeine, EGC, C, EC, L-tryptophan (IS), GCG, EGCG and ECG.	NAMEKC	20 mM tetraborate, 110 mM SDS, 14% MeOH (v/v), 1.5 M urea, 1 mmol/L β-CD, pH 8.0	(50 µm, 60 cm, 67 cm), 20°C, 20 kV, hydrodynamic 138 mbar.sec and about 30 min.	UV, 280 nm	An in-depth method development and evaluation strategy was followed. The NAMEKC method resolved all analytes resulted in a relatively long analysis (30 min). Slight tailing of the gallate peaks in the standard mixture was observed. Quantitative data for three different green teas are presented and an unknown peak was detected in the standard mixture.	[176]
Standards and green tea	GC, C, EGC, EGCG, GCG, ECG, EC and gallic acid.	MEKC	50 mM sodium tetraborate, 20 mM potassium dihydrogen phosphate, 200 mM SDS, pH 7.0.	(50 µm, 40 cm, 47 cm), 29°C, 30 kV, hydrodynamic 34.5 mbar.sec and within 4.5 min.	UV, 200 nm	A simple fast, efficient and quantitative MEKC method was developed and validated. A comparative study between HPLC and MEKC analysis of gallic acid and seven major green tea catechins was carried out. Most peaks tailed slightly, except gallic acid. Quantitative results were generally comparable.	[183]

Table 2.7. (Continued)

Samples and tea type	Analytes	CE mode	BGE and pH	Capillary dimensions (i.d., l and L), temperature, voltage, injection and analysis time	Detection and wavelength (s)	General remarks	Reference
Standards and green tea	EC, syringic acid, ECG, EGC, caffeine, EGCG, theophylline, (+)-C, (-)-C, GCG, (+)-GC and (-)-GC.	MEKC	25 mM borate-phosphate (pH 2.5), 90 mM SDS and 25 mM hydroxypropyl-β-CD (HP-βCD).	(50 µm, 21.5 cm, 30 cm), 25°C, 15 kV, hydrodynamic 50 mbar.s and within 9 min.	DAD, 200 nm	A fast, efficient and high resolution CD-MEKC method was developed and validated for the analysis of 13 analytes in less than 9 min. Slightly tailing peaks were observed for EC, syringic acid, EGC and caffeine in the standard solution. Relatively broader peaks were observed for the later migrating compounds (theophylline, (+)-C, (-)-C, GCG, (+)-GC and (-)-GC). The method was applied to study the epimerisation of catechins and quantitative data for 24 green tea samples are presented.	[184]
Standards and green tea	ECG, EGCG, EC, C, and EGC	MEKC	0.2% triethylamine (v/v), 50 mM SDS, 0.8% sulphated-β-CD (s-β-CD) (w/v), pH 2.9	(50 µm, 40 cm, 50.2 cm), 25°C, -30 kV, hydrodynamic 207 mbar.s and within 4 min.	DAD, 210 nm	A rapid, quantitative and highly efficient reduced flow (RF)-MEKC (low pH and negative voltage) method, using a sulphated-CD chiral selector developed and validated for the determination of five major green tea catechins in six types of Brazilian green teas.	[185]
Standards and green teas	ECG, EGCG, EC, C, EGC, GC, caffeine and theophylline	MEEKC	50 mM phosphate (pH 2.5), 2.89% SDS (w/v), 1.36% n-heptane (w/v), 7.66% cyclohexanol (w/v)	(50 µm, 19.5 cm, 24 cm), 40°C, -10 kV, hydrodynamic 68.9 mbar.s and within 10 min.	DAD, 230 nm	A fast and efficient quantitative MEEKC method developed and validated for the determination of six major catechins, caffeine and theophylline. The study focussed on the effect of cosurfactants (nine different alcohols) on the separation efficiency and selectivity. Cyclohexanol was selected as cosurfactant at optimal conditions. All analytes were baseline resolved and moderate peak broadening of late migrated compounds (EGC, GC and caffeine) was observed. Quantitative data were presented.	[171]

Table 2.7. (Continued)

Samples and tea type	Analytes	CE mode	BGE and pH	Capillary dimensions (i.d., / and L), temperature, voltage, injection and analysis time	Detection and wavelength (s)	General remarks	Reference
Standards, tea leaves and commercial tea beverages	<i>p</i> -Coumaric acid, ECG, C, EGCG, 4-hydroxybenzoic acid, vanillic acid, syringic acid, EC, caffeic acid, 3,4-dihydroxybenzoic acid, EGC, GC, caffeine, gallic acid and theophylline.	NAMEKC	25 mM phosphate (pH 2.0), 2.89% SDS (w/v), 2.0% MeOH (w/v)	(50 µm, 40 cm, 48.5 cm), 30°C, -27 kV, hydrodynamic 150 mbar.s and within 12 min.	UV, 200 nm	A qualitative high resolution NAMEKC method was developed and compared to a MEEKC method. The methods were compared in terms of efficiency, resolution, speed and sensitivity. The NAMEKC was faster and more sensitive. Peak tailing of EGCG and EC was observed in the analysis of a tea beverage and tea leave sample, respectively. No quantitative data were presented.	[172]
Standards, tea leaves and commercial tea beverages	<i>p</i> -Coumaric acid, ECG, C, EGCG, 4-hydroxybenzoic acid, vanillic acid, syringic acid, EC, caffeic acid, 3,4-dihydroxybenzoic acid, EGC, GC, caffeine, gallic acid and theophylline.	MEEKC	25 mM phosphate (pH 2.0), 2.89% SDS (w/v), 1.36% heptane (w/v), 7.66% cyclohexanol (w/v), 2.0% ACN (w/v)	(50 µm, 40 cm, 48.5 cm), 30°C, -27 kV, hydrodynamic 150 mbar.s and within 14 min.	UV, 200 nm	A highly efficient and qualitative MEEKC method was developed and compared to a NAMEKC method. The MEEKC method was more efficient, but less sensitive. Peak tailing of EGCG and EC was observed. Peaks of late migrating compounds (caffeine, gallic acid and theophylline) were relatively broad.	[172]
Standards and a tea beverage	ECG, EGCG, EC, C, EGC and GC.	MEEKC	50 mM phosphate (pH 2.0), 2.89% SDS (w/v), 1.36% cyclohexane (w/v), 7.66% cyclohexanol (w/v)	(50 µm, 40 cm, 48.5 cm), 30°C, -20 kV, electrokinetic (-10 kV for 300 s) and within 16 min.	UV, 200 nm	A highly efficient and sensitive anion-selective exhaustive injection (ASEI)-sweeping MEEKC method was developed for the analysis of six catechins. The sensitivity of the method was compared to a previously reported MEEKC [T6] method. The latter was less sensitive.	[173]
Standards	ECG, GCG, EGCG, EC and EGC	MEEKC	10 mM sodium acetate and 50 mM citric acid buffer (pH 2.0), 3.5% SDS, 1..36% heptane (w/v), 9.72% 1-butanol (w/v)	(50 µm, 50 cm, 60 cm), 25°C, 25 kV, injection not indicated, within 42 min.	UV, 200 nm	A very slow MEEKC method was developed and compared in terms of resolution, efficiency and analysis time to a low (2.0) and high (7.0) pH MEKC methods. Separation coefficients (R_s) and efficiencies obtained by the MEEKC method were the highest, but resulted in the longest analyses. Unstable baselines for all electropherograms were observed.	[174]

2.8. Capillary electrophoresis (CE)

2.8.1. History and background

Electrophoresis refers to the differential movement of charged ions in the presence of an external electrical field. Ions can either be attracted or repelled, depending on the charge and the polarity of the applied electric field. Electrophoresis was introduced by Tiselius as a separation technique in 1937 using a U-tube apparatus. However, wide tubes cause more thermal convection and this has a detrimental effect on the efficiency of separations [186]. Anti-convective media such as polyacrylamide or agarose gels on glass plates are therefore used to reduce thermal convection during electrophoretic analyses. It is for this reason that slab gel electrophoresis is often used in biochemical applications to separate bio-macromolecules like proteins and deoxyribonucleic acid (DNA), but the technique suffers from several drawbacks such as long analysis times, low efficiencies, difficulties in detection, lack of automation [187] and quantification difficulties [188].

Open-tubular electrophoresis was first performed by Hjertén [189], and the use of narrow-bore fused silica capillaries ($75\text{ }\mu\text{m}$) was introduced by Jorgenson and Lukacs in 1981 [190]. They also developed the theoretical aspects and demonstrated the practical application of capillary electrophoresis using narrow bore fused silica capillaries, thereby laying the foundation for the modern separation technique referred to as capillary electrophoresis (CE).

2.8.2. Theory and principles of capillary electrophoresis (CE)

2.8.2.1. Background theory of electrophoresis

In CE, solutes are separated based on differences in their velocities in the presence of an external electrical field. The following equation describes the relationship between velocity and field strength:

$$v = \mu_e E \quad (2.1.)$$

where v = the velocity of the charged species, μ_e the electrophoretic mobility of the species and E is the applied external electrical field. The electrical field (E) can be expressed as the voltage (V) being applied over a given length of a capillary (L):

$$E = \frac{V}{L} \quad (2.2.)$$

The electrophoretic mobility (μ_e) of an ion is a constant for that ion in a given medium. The mobility of an ion in free solution is determined by two forces acting on the ion: an electrical force (F_e) and a counter-balanced frictional force (F_f).

The former is the product of the charge of an ion (q) and the electrical field (E):

$$F_e = qE \quad (2.3.)$$

F_f for a spherical ion can be expressed by Stoke's Law:

$$F_f = -6\pi\eta rv \quad (2.4.)$$

where η is the viscosity of the solution, r is the hydrated radius of a spherical ion and v is the ion velocity. When the electrical field is applied, the ions experiences a steady state and above-mentioned forces are equal, but opposite, thus:

$$F_e = -F_f \quad (2.5.)$$

and therefore,

$$qE = 6\pi\eta rv \quad (2.6.)$$

Solving for v in equation 2.6. and substitution into equation 2.1. provides the relationship between the electrophoretic mobility and physicochemical parameters:

$$\mu_e = \frac{q}{6\pi\eta r} \quad (2.7.)$$

It is evident from equation 2.7. that separation of charged species in CE is based on their charge-to-size ratio. Small, highly charged species have higher mobilities than larger and less charged species, and therefore migrate faster in an electric field [186-188].

2.8.2.2. Electroosmotic flow (EOF)

The second fundamental aspect of CE in fused silica capillaries is the electroosmotic flow (EOF). It is defined as the drag of the bulk solution in presence of an external electrical field. The generation of the EOF can be described as follows:

- 1) The inside wall of uncoated fused silica capillaries comprise silanol (SiOH) functional groups. At a pH above 4, partial to full deprotonation of the silanol groups occur, resulting in negatively charged SiO⁻ ions (**Figure 2.4. (I)**).
- 2) Charge balance is attained in a buffer medium by the build-up of positively charged counter-ions close to the capillary wall. These counter-ions form a diffuse double layer at the capillary wall (**Figure 2.4. (II)**). A potential difference very close to the wall is thereby created, which is known as the zeta potential (ζ).

- 3) When an external electrical field is applied, the solvated positive ions will migrate to the cathode, dragging the bulk solution in this direction ((**Figure 2.4. (III)**)).

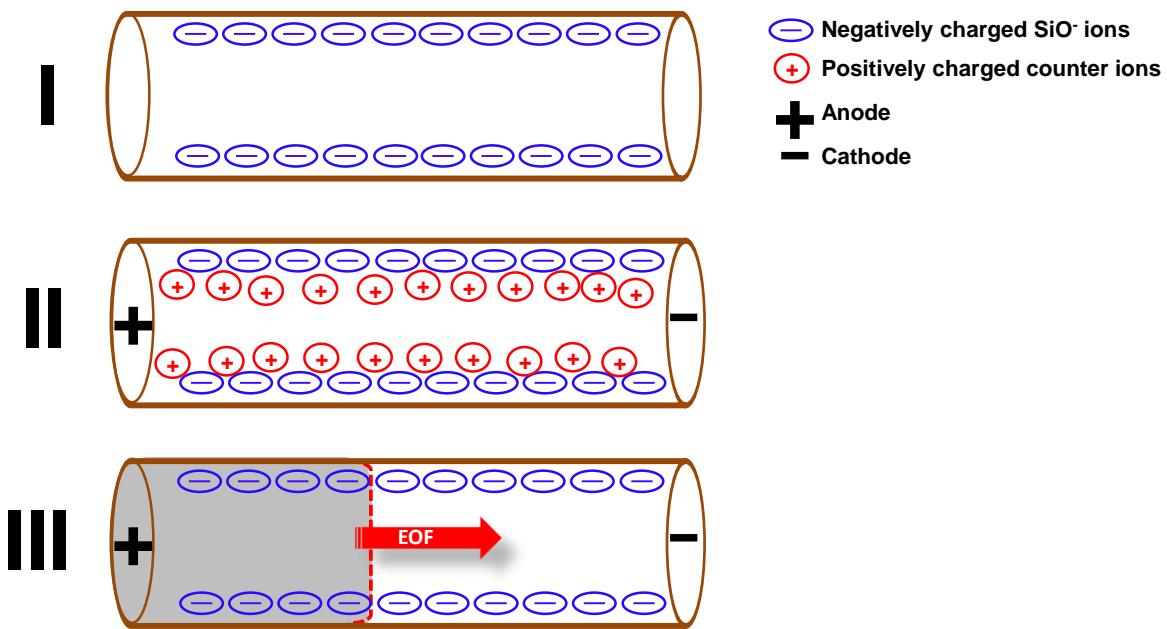


Figure 2.4. Schematic representation of the generation of the EOF in an uncoated fused silica capillary filled with a BGE of $\text{pH} \geq 4$.

The magnitude of the EOF can be expressed in either velocity (v_{eof}) or mobility (μ_{eof}) terms, according to the following equations:

$$v_{eof} = E \left(\frac{\varepsilon \zeta}{\eta} \right) \quad (2.8.)$$

$$\mu_{eof} = \left(\frac{\varepsilon \zeta}{\eta} \right) \quad (2.9.)$$

where ε is the dielectric constant of the solution, ζ the zeta potential and η the viscosity. The zeta potential is determined by the surface charge at the capillary wall. The surface charge is predominantly affected by pH, thus the magnitude of the EOF can be altered by changing the buffer pH. An increase in pH favours deprotonation of the silanol groups, thereby increasing the zeta potential and resulting in a higher EOF [191]. Buffer concentration (ionic strength) also affects the EOF: an increase in ionic strength leads to compression of the double layer at the capillary wall, decreasing the zeta potential and reducing the EOF [192].

The EOF can be controlled by other parameters such as voltage, temperature, organic modifiers, additives (e.g. surfactants), neutral hydrophilic polymers (in capillary gel electrophoresis (CGE)) and by coating the capillary wall. An increase in temperature reduces the viscosity of the buffer medium, thus resulting in an increased EOF. Similarly, the addition of organic modifiers affect the viscosity,

effective pH and dielectric constant of the buffer, which in turn alter ion solvation, the electrical double layer and zeta potential at the capillary wall and thereby the magnitude EOF. Surfactants can be used change the direction and magnitude of the EOF. Anionic surfactants such as sodium dodecyl sulfate (SDS) and cationic surfactants like cetyltrimethylammonium bromide (CTAB) typically increase and reduce the EOF, respectively. Neutral hydrophilic polymers increase the viscosity of the running buffer, thus reducing the EOF. In the case of coated capillaries, changes in the EOF depend on the charge and polarity of the coating.

The mobility of a solute can be determined experimentally from the electropherogram. The migration time (t_m) is directly proportional to the effective length (the capillary length from the inlet to the detector, l) and indirectly proportional to the applied field strength:

$$t_m = \frac{l}{\mu_a E} \quad (2.10.)$$

where μ_a is the apparent mobility and E the electrical strength.

Substitution of **2.2.** into **2.10.** results in the following relationship:

$$t_m = \frac{L}{\mu_a V} \quad (2.11.)$$

where L is the total length of the capillary and V the applied voltage. In the presence of the EOF, the apparent mobility of a given analyte is calculated from experimental parameters using the following equation:

$$\mu_a = \frac{L}{t_m V} \quad (2.12.)$$

The electroosmotic mobility, μ_{eof} , can be determined in the same manner using an EOF marker (a neutral marker which migrates with the EOF, such as mesityl oxide), according to:

$$\mu_{eof} = \frac{L}{t_{eof} V} \quad (2.13.)$$

The apparent mobility is then the sum of the electrophoretic (effective, μ_e) and electroosmotic (μ_{eof}) mobilities:

$$\mu_a = \mu_e + \mu_{eof} \quad (2.14.)$$

The effective mobility of charged species is:

$$\mu_e = \mu_a - \mu_{eof} \quad (2.15.)$$

where $\mu_e < 0$ for anionic species and > 0 for cationic species. Neutral molecules in capillary zone electrophoresis (CZE) migrate with the EOF as their electrophoretic mobilities are equal to zero, therefore their mobilities will be equal to μ_{eof} . The mobility of the EOF can be greater than μ_e of cationic and anionic species, and as a consequence cations, anions and neutrals can be analysed at the same time in CZE (although neutral species will not be separated). The effective mobility, which is a characteristic of a given ion under fixed conditions, can be used to identify the ion under specific experimental conditions, because its measurement takes into account possible small shifts in the EOF [186,193].

2.8.2.3. Efficiency in CE

CE is an electro-driven separation technique and the EOF creates a flat velocity profile across the capillary diameter (**Figure 2.5.A**). A uniform velocity is mostly maintained throughout an analysis. In contrast, pressure-driven techniques, like high performance liquid chromatography (HPLC), produce a laminar flow with a parabolic velocity flow profile, which results in band broadening and therefore relatively lower efficiencies (**Figure 2.5.B**) compared to electrodriven techniques.

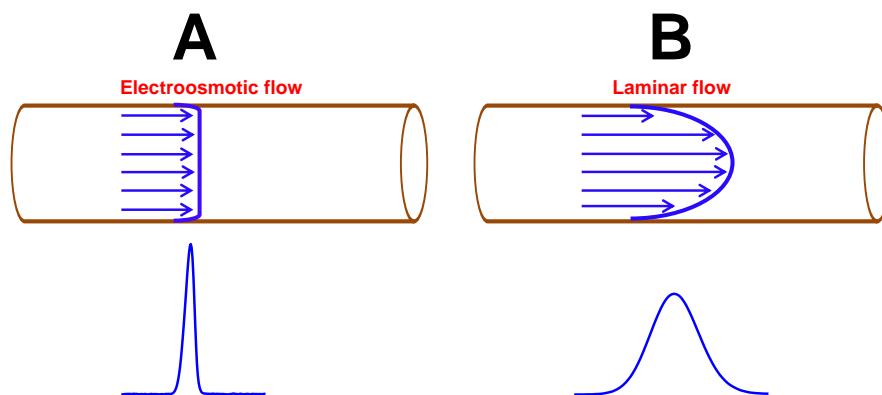


Figure 2.5. Flow velocity profiles produced by (A) electro-driven (CE) and (B) pressure driven (HPLC) separation techniques. The EOF (A) creates a flat flow profile and laminar flow (B) creates a parabolic flow profile, which negatively impacts on efficiency.

The van Deemter equation (2.16.) may be used to determine H , the theoretical plate height, which is a measure of zone broadening:

$$H = A + \frac{B}{u_0} + Cu_0 \quad (2.16.)$$

The A , B and C -terms are ascribed to the eddy diffusion, longitudinal diffusion and resistance to mass transfer contributions to band broadening, respectively. The mobile phase velocity is denoted u_0 . Although the van Deemter model is strictly applicable to chromatographic systems (i.e. where a two-

phase system is used), it can be used as an approximation in CE also. In CE (except in capillary electrochromatography (CEC)), under ideal conditions (i.e. in the absence of Joule heating and adsorption reactions) only longitudinal diffusion affects zone broadening due to the absence of a stationary phase. This is because both eddy diffusion and resistance to mass transfer effects are a result of the presence of a stationary phase. The mobile phase velocity is replaced by the electrophoretic mobility, μ_e . The theoretical plate height equation for CE therefore only comprises the B -term:

$$H = \frac{\sigma^2}{l} = \frac{2Dt_m}{l} \quad (2.17.)$$

where σ^2 is the variance of the peak and D is the diffusion coefficient of the analyte. Substitution of **2.10.** into **2.17.** with μ_e replacing μ_a results in:

$$H = \frac{2D}{\mu_{eE}} \quad (2.18.)$$

The plate number in CE is determined by:

$$N = \left(\frac{l^2}{\sigma^2} \right) \quad (2.19.)$$

where N is the number of theoretical plates, where:

$$N = \frac{l}{H} \quad (2.20.)$$

Substituting **2.18.** into **2.20.** results in:

$$N = \frac{l\mu_{eE}}{2D} \quad (2.21.)$$

It is evident from equation **2.21.** that N is directly proportional to the applied voltage: higher voltages result in high efficiencies. This is a consequence of the fact that higher applied voltages reduce analyte migration times for co-EOF separations, thereby minimising longitudinal diffusion [187,193]. Also, large molecules with low diffusion constants provide higher plate numbers than small ones. Plate numbers can be calculated experimentally from an electropherogram using the same equations as in chromatography, where t_r (the retention time) is replaced with t_m (migration time in CE) and $w_{1/2}$ is the width at half height of a given peak:

$$N = 5.54 \left(\frac{t_m^2}{w_{1/2}^2} \right) \quad (2.22.)$$

Note that this equation assumes Gaussian distributions for analyte peaks, which is rarely the case for CE, and therefore represents somewhat of an approximation. Higher voltages may result in the production of high currents and thereby cause Joule heating. The latter is defined as the heat generated

by the passage of an electrical current through a conductive medium. Joule heat causes zone broadening by generating parabolic flow profiles caused by radial temperature gradients. Decreasing the capillary diameter is an effective means of lowering such temperature gradients, thus decreasing viscosity differences and zone deformation. Reducing the buffer concentration and field strengths are all means to minimise Joule heating [194].

Evaluation of Joule heat is experimentally done by plotting the measured current against the applied voltage (Ohm's law). A non-linear response indicates the occurrence of Joule heating. The choice of applied voltage therefore entails a trade-off between potential Joule heating and lower analysis times [194].

2.8.2.4. Resolution in CE

Resolution is one of the most important performance characteristics in separation science. Resolution defines the degree of separation between any two peaks. In CE, as in chromatography, resolution can be determined experimentally according to:

$$R_s = \frac{t_{m,2} - t_{m,1}}{2(\sigma_1 + \sigma_2)} \quad (2.23.)$$

where R_s is the resolution between peaks 1 and 2, $t_{m,1}$ and $t_{m,2}$ and σ_1 and σ_2 is the migration times and baseline peak widths of the earlier (peak 1) and later (peak 2) migrating peaks, respectively. Separation in CE relies more on efficiency than selectivity, and resolution can also be expressed in terms of efficiency and apparent analyte mobilities:

$$R_s = \frac{1}{4} \left(\frac{\Delta\mu_a}{\bar{\mu}_a} \right) \sqrt{N} \quad (2.24.)$$

where $\Delta\mu_a = \mu_{a2} - \mu_{a1}$ is the difference of apparent mobilities between peaks 1 and 2, $\bar{\mu}_a = \frac{\mu_{a1} + \mu_{a2}}{2}$ is the average apparent mobility and \bar{N} is the average plate number. Substituting 2.2. into 2.21. and then into 2.24., resolution can be related to the applied voltage (V), capillary lengths (l , effective length and L , total length) and analyte diffusion coefficient as follows:

$$R_s = \frac{1}{4\sqrt{2}} (\Delta\mu_a) \left(\frac{V}{D(\bar{\mu}_a + \mu_{eof})} \right)^{1/2} \quad (2.25.)$$

Note that whereas the efficiency is directly related to the applied voltage, resolution is proportional to the square root of the applied voltage [193,195].

2.8.3. Operational modes in CE

Various modes of CE are available to separate analytes based on a range of mechanisms; this makes CE a very versatile separation technique. The most important modes include capillary zone electrophoresis (CZE), micellar elektrokinetic chromatography (MEKC), capillary gel electrophoresis (CGE), CEC, capillary isoelectric focusing (CIEF), capillary isotachophoresis (CITP) and non-aqueous capillary electrophoresis (NACE). The main differences between the modes involve the composition of the BGE and/or capillary, resulting in different separation mechanisms. CZE and MEKC are mostly used for the analysis of phenolic compounds in natural products (see **Section 2.7.2.**) and are discussed below in more detail.

2.8.3.1. Capillary zone electrophoresis (CZE)

Separation in capillary zone electrophoresis (CZE) is governed by the charge-to-size ratio of the solutes. In order to separate analytes in a mixture, they need to be charged; neutral molecules cannot be separated by CZE and will co-migrate with the EOF. Cations migrate towards the cathode and anions towards the anode; although the net migration of the latter in the presence of a positive EOF of relatively large magnitude may also be towards the cathode (see **Section 2.8.2.2.**). Solutes are separated according to differences in their mobilities (and thereby velocities). When the capillary is filled with BGE and the sample is introduced, then, when the external electrical field is applied, the solutes will migrate at different velocities in separate zones. The general migration order in CZE with detection at the cathode and in the presence of a large positive EOF is thus as follows: small highly charged cations, larger or less charged cations, neutrals, large or weakly charged anions and finally small and/or highly charged anions.

The EOF can be controlled by various experimental parameters such as BGE pH, ionic strength, BGE additives such as surfactants or organic modifiers, voltage and temperature. During method development each of these parameters, in addition to capillary dimensions and injection volume should be optimised. The most important parameter affecting the separation in CZE is the BGE pH. At low pH (< 3), the SiOH groups at the inner wall of uncoated fused silica capillaries largely remain protonated, and as a consequence the zeta potential and magnitude of the EOF are low. Under these conditions, the net migration of positively charged species is towards the cathode and for the majority of anionic species towards the anode. Under basic conditions, the magnitude of the EOF is significant and the net migration of most charged species is with the EOF towards the cathode.

For instance, in the case of moderate to high pH (pH 5-12, basic conditions) CZE analysis of anionic species, smaller, more negatively charged species will migrate faster against the EOF and therefore

show longer migration times. The opposite occurs for large, partially charged anions, which will elute earlier (closer to the EOF). Phenolic compounds such as phenolic acids and flavonoids are typically analysed under such conditions by CZE [196-205].

Under alkaline conditions phenolic acids ($pK_a \sim 4-12$) are fully deprotonated, whilst flavonoids ($pK_a \sim 7-12$) are only partially deprotonated. The former migrate faster against the EOF (towards the anode), resulting in longer migration times. Borate buffers ($pK_a \sim 9.25$) are often used for the CZE analysis of phenolic compounds at high pH due to the capability of borate ions to form anionic complexes with species containing vicinal diol functional groups [206-217]. This affects the electrophoretic mobilities of especially flavonoids, and thereby the selectivity of the separation.

2.8.3.2. Micellar electrokinetic chromatography (MEKC)

Micellar electrokinetic chromatography (MEKC) combines the principles of electrophoresis and chromatography, and was introduced by Terabe and co-workers in 1984 [218]. Neutral, cationic and anionic species can be separated by MEKC, which is not possible by CZE. Separation of neutral molecules by MEKC is made possible by the addition of surfactants to the BGE. Micelles are formed above the critical micelle concentration (CMC), unique to a specific surfactant. Neutrals are separated via a distribution (“partitioning”) process between the BGE (“mobile phase”) and hydrophobic micelles, which act as a moving stationary phase and therefore as a “pseudo stationary phase”. Both anionic and cationic surfactants may be used for this purpose, for example SDS and CTAB, respectively. SDS is most often used in MEKC (also for the analysis of flavonoids) [219-221]. For SDS, migration of micelles, and the neutral species partitioning into them, is towards the anode, although the net migration is typically towards the cathode due to the larger positive EOF. Neutrals are separated according to their interaction and distribution into the micelles. Strong hydrophobic interactions, such as occur for apolar neutral compounds, result in more significant partitioning into the micelles and therefore longer migration times.

Analogous to chromatographic separations, the capacity factor, k' , can be expressed as:

$$k' = \frac{(t_r - t_0)}{t_0(1 - \frac{t_r}{t_m})} = K \left(\frac{V_s}{V_M} \right) \quad (2.26.)$$

where t_r is migration time of the analyte, t_0 the migration time of the EOF, t_m the migration time of the micelles, K the distribution coefficient, V_s the volume of the micellar phase and V_M the volume of BGE. Selectivity in MEKC can be altered by changing the BGE and/or surfactant, their composition, concentration, pH, voltage, temperature and by the addition of additives such as bile salts, organic modifiers, chiral selectors and metal ions [222].

Micellar emulsion electrokinetic chromatography (MEEKC) is a modified version of MEKC where micro-emulsions are formed by the addition of co-surfactants such as alcohols (e.g. 1-butanol, *tert*-butanol, pentanol, cyclopentanol, hexanol, etc) and/or, apolar solvents (e.g. diethyl ether, heptane, cyclohexane, etc) with surfactants to the aqueous BGE to alter selectivity. The addition of such additives to surfactant-containing BGEs affects the partitioning of analytes into the modified micelles and therefore their effective mobilities. Co-surfactants stabilise micelles by generation of an interfacial film between the constituent surfactants. Under basic BGE conditions the interaction between apolar analytes and such negatively charged hydrophobic oil-droplets are strong, and therefore their effective migration times will be increased. Under acidic conditions using reversed polarity (detection at the anode), the EOF is greatly suppressed and hydrophobic analytes would elute first when using anionic micro-emulsions [223].

Care should be taken when optimising the temperature during MEKC and MEEKC method development. Higher temperatures increase CMCs and decrease the number of micelles, thereby affecting k' , selectivity and resolution.

2.8.4. CE instrumental and operating aspects

The basic components of a CE instrument are shown schematically in **Figure 2.6.** A photograph of the HP ^{3D}CE system used in this study is presented in **Figure 2.7.** The major components of a modern CE system comprise the following (below follows a brief discussion of the most important components):

- Coated or uncoated narrow bore (25-75 μm) fused silica capillaries of different dimensions coated on the outside with polyimide are typically used in CE. The capillaries can maintain high field strengths up to 500 V/cm.
- The BGE or sample is placed in vials at the inlet and outlet ends of the capillary. The capillary and electrodes must be immersed in the BGE filled vials to ensure conductivity during separation. BGEs can differ in type, pH, concentration and composition (see above), and should be replaced often to avoid depletion.
- Two electrodes, an anode (positive) and cathode (negative), are positioned in the inlet and outlet vials, normally with the capillary ends entering the vials through the electrodes.
- The heart of the CE instrument is the high voltage direct current (DC) supply. High voltages and currents up to 30 kV and 300 μA , respectively, can be applied using commercial instrumentation. The polarity of the applied potential difference can be switched to reverse the direction of the EOF, depending on the experimental conditions used. Field programming is possible as a function of voltages, current or output power.

- The instrument also comprises a pump to generate high (± 900 mbar) and low (± 50 mbar) pressures for flushing and hydrodynamic injection, respectively.
- Hydrodynamic or electrokinetic injection is possible with automated instruments. Injection programming can be performed using the software with the sample vial placed at the inlet.
- Temperature plays an important role in CE. Basic automated instruments have built-in thermostats that can be controlled via the software. Air or liquid thermostating can be used with the latter being better, but more complicated.
- On-column detection is generally performed in CE. Various modes of detection can be used in CE, including ultraviolet-visible (UV-vis), diode array detection (DAD), fluorescence detection (FD), laser induced fluorescence detection (LIFD), amperometric detection (AD), contactless conductivity detection (CCD), indirect UV and MS. In the case of UV-vis detection, the most common detection system for CE, a detection window must be created by removing the polyimide coating at the detection point. DADs can also be used, where UV-vis spectra are recorded between ~ 200 -700 nm. Data acquisition rates should be high (typically in the order of 10-20 Hz) to accommodate the narrow peak widths commonly encountered in CE.
- Electropherograms (in the case of UV-vis detection, plotted on an absorbance vs. time axis) are generated by the software and displayed on the computer for interpretation of results and data analysis, including integration, calibration, etc.

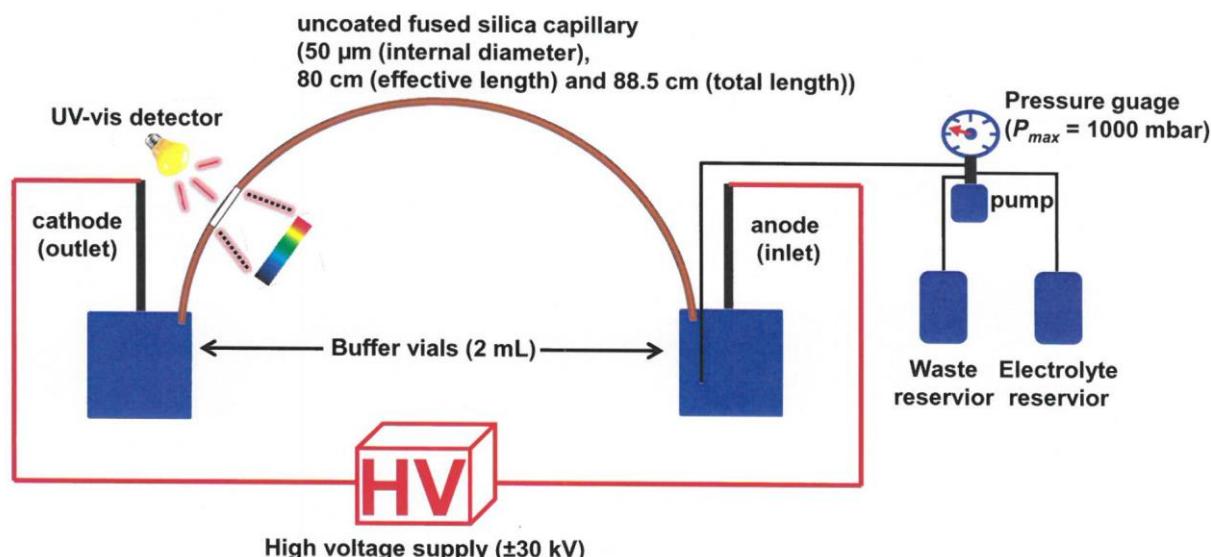


Figure 2.6.: Schematic representation of the basic components of a CE instrument.



Figure 2.7.: A photograph of the HP ^{3D}CE system used in this study.

2.8.4.1. Capillaries

CE capillaries vary in terms of their dimensions and coatings. The most common capillaries used are uncoated fused silica capillaries. Internal diameters vary in a range between 10-100 μm , and typical lengths in the range 5-100 cm.

Permanent coatings are chemically bound to the fused-silica capillary wall and thereby alter the EOF and may enhance reproducibility [224]. A range of different coated capillaries are commercially available, or can be prepared. Permanent chemical coatings that can be used for this purpose include alkylpolysiloxanes (hydrophobic) and polyvinylalcohol, poly(ethylene glycols), hydroxyalkyl celluloses, dextrans and polyacrylamide (all hydrophilic). The coatings are required to be stable over a wide range of pH; rinsing with specific solutions is often required to maintain long-term stability.

2.8.4.2. Injection in CE

Sample introduction is important in any analytical instrumental technique. There are two injection modes in CE: hydrodynamic (by pressure) and electrokinetic (by voltage). Both methods are commonly used in CE applications.

2.8.4.2.1. Hydrodynamic injection

Injection in CE occurs at the inlet vial - the injection end of the capillary on the opposite side of the detector. Hydrodynamic injection in CE can be accomplished in one of three ways: application of pressure at the inlet end of the capillary, vacuum at the outlet end of the capillary and by siphoning (gravity flow). The latter is achieved by elevation of the inlet vial relative to the outlet vial. Hydrodynamic injection, unlike electrokinetic injection, is non-discriminating and is often more reproducible than electrokinetic injection [187]. Automated hydrodynamic injection systems minimise operational errors. In the case of pressure injection, the most common form of injection in CE, the injection time and pressure (in s and mbar, respectively) as well as capillary dimensions can be used to determine the injected volume, which is typically in the nL range. Injection volume (V_{inj}) can be determined with the following equation:

$$V_{inj} = \frac{\Delta P d^4 \pi t_{inj}}{128 \eta L} \quad (2.27.)$$

where ΔP is the pressure difference across the capillary, d capillary diameter, t_{inj} injection time, η buffer viscosity and L the total capillary length. Injection plug lengths in CE should be minimised to prevent excessive injection band broadening, which will affect resolution and efficiency. If it is assumed that the sample plug length enters the capillary in rectangular form, the injection contribution to the total variance (σ^2_{inj}) is:

$$\sigma^2_{inj} = \frac{w_i^2}{12} \quad (2.28.)$$

where w_i^2 is the injection plug length. Injection of plug lengths of up to 1% of the total length of the capillary is a good practical guide to avoid injection band broadening [172].

2.8.4.2.2. Electrokinetic injection

In electrokinetic injection, sample analytes are introduced by the application of a potential difference. Before electrokinetic injection, the capillary should be filled with BGE in order to conduct current when the voltage is applied. Injection is then performed by placing the sample vial at the inlet and applying a potential difference. The applied voltage during injection is normally 3-5 times lower than the separation voltage, with injection times varying from 10-30 seconds. The sample enters the capillary by electromigration, resulting in mobility differences between charged species. This inevitably results in discrimination between solutes of different charges: analytes with higher effective mobilities in the direction of the detector are preferentially injected (this depends on the specific operating conditions such as sample/BGE pH and voltage polarity). This can be either beneficial or detrimental. Differences between BGE and sample solution matrices affect electrokinetic injection, which may therefore be less reproducible than hydrodynamic injection. Despite these potential

drawbacks, electrokinetic injection may be beneficially exploited for sample stacking, where conductivity differences between BGE and sample solution are exploited to provide narrow and concentrated sample zones to increase sensitivity and efficiency [187].

2.8.4.3. Detection in CE

Different detection modes are used in CE, with UV or DAD being the most common. Other potential detection modes include: FD, LIFD, AD, CCD, indirect UV and MS. UV-vis or DAD detection are most commonly used for the analysis of phenolics and are discussed further below.

2.8.4.3.1. Ultraviolet-visible (UV/vis) detection

UV-vis detection is a semi-universal mode of detection. The spectral information collected when using DAD detection, coupled with the relatively inexpensive nature of these detectors, make them some of the most used detectors in CE.

For UV/vis detection, on-capillary detection is typically performed to ensure maximum efficiency (i.e. minimise detection band broadening); this is done by removal of a small part of the outside polyimide coating to create a detection window. Small axial detection path lengths relative to the width of solute zones entering the detection window are required for high resolution.

The absorbance (A) of an analyte passing through the detection window in on-capillary CE-UV/vis is directly proportional to optical path length (b), the analyte concentration (c) and molar absorptivity (ε) as defined by the Beer-Lambert law [187,226]:

$$A = \varepsilon b c \quad (2.29.)$$

Low sensitivity is a well-known limitation of CE and is in fact a consequence of the small path length used in on-capillary detection. To partially overcome the sensitivity limitations of CE, extended path length flow cells such as bubble cells or Z-cells may be used. Furthermore, on-capillary sample concentration techniques, referred to as sample stacking methods, are also used. A few examples of sample stacking methods include field amplified sample stacking (FASS), field amplified sample injection (FASI), isotachophoretic (ITP) sample stacking, high-salt stacking, reversed electrode polarity stacking mode (REPSM), micelle to solvent stacking (MSS), sweeping and reverse migrating micelles. The use of extended path length flow cells and sample stacking methods to enhance sensitivity is beyond the scope of this study and the reader is referred to [187,227-235] for detailed discussions on this subject.

2.9. Conclusions

Phenolic compounds are important constituents of herbal teas to which potential health benefits in humans are attributed. Accurate analysis of these compounds is therefore required, but this is often rather challenging. HPLC remains the method of choice for phenolic determination, but requires extensive method optimisation and often re-development for different samples. This is illustrated by the number of HPLC methods reported for rooibos and honeybush phenolic in literature. CE has successfully been used for the analysis of green, oolong and black tea phenolics. The most popular mode for these applications is MEKC. The performance of CE methods for tea analysis varies, but exhaustive method optimisation is also often required. No CE methods on the analysis of rooibos and honeybush teas have been reported to date. These herbal teas contain unique and diverse phenolic compounds, and alternative separation methods such as CE might prove valuable for their analysis.

2.10. References

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CHAPTER 3

EVALUATION OF CAPILLARY ELECTROPHORESIS FOR THE ANALYSIS OF ROOIBOS AND HONEYBUSH TEA PHENOLICS*

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Abstract

Rooibos and honeybush are popular herbal teas produced from the shrubs of *Aspalathus linearis* and *Cyclopia* spp., respectively, which are indigenous to South Africa. Both herbal teas are rich in polyphenols and their consumption is associated with several health benefits, partly ascribed to their phenolic constituents. Quantification of phenolics in extracts and teas for quality control and research purposes is generally performed using HPLC, although dedicated and often species-specific methods are required. CE offers an attractive alternative to HPLC for the analysis of phenolics, with potential benefits in terms of efficiency, speed and operating costs. In this contribution, we report quantitative CZE methods for the analysis of the principal honeybush and rooibos phenolics. Optimal separation for honeybush and rooibos phenolics was achieved in 21 and 32 minutes, respectively, with good linearity and repeatability. Quantitative data for extracts of “unfermented” and “fermented” rooibos and two honeybush species were statistically comparable with those obtained by HPLC for the majority of compounds. The developed methods demonstrated their utility for the comparison of phenolic contents between different species and as a function of manufacturing processes, thus offering cost-effective, although less sensitive and robust, alternatives to HPLC analysis.

3.1. Introduction

Increased health awareness globally has led to the growing interest in purportedly healthy natural foods and beverages. Among these, herbal teas such as rooibos and honeybush have become an increasingly popular part of the diet of health-conscious populations, partly because of their caffeine-free status, but also due to their health-promoting effects [1]. Rooibos and honeybush teas are produced from the indigenous South African plant species, *Aspalathus linearis* and several *Cyclopia* species, respectively [2,3]. Both herbal teas are marketed in one of two forms: “unfermented” (green) and “fermented” (oxidised). The characteristic flavour and aroma of the respective fermented teas are produced during the “fermentation” process. Their aqueous extracts are also increasingly being used in food, beverage, cosmetic and nutraceutical products [2,3].

Both rooibos and honeybush contain polyphenols either novel or relatively rare, and are of interest in studying the value of these products as health-promoting beverages [2-4]. For example, compounds with anti-diabetic potential, such as aspalathin and phenylpyruvic acid-2-O-glucoside (PPAG) in rooibos [4-6] and xanthones and benzophenone glucosides in honeybush [7,8] are driving product development. The accurate determination of the levels of phenolics in these herbal teas is therefore essential for manufacturing and marketing purposes, but also in support of research into their chemical composition and the alteration thereof during fermentation and production.

HPLC coupled with ultraviolet-visible (UV-vis) and/or MS detection is mostly used for the analysis of phenolic compounds [9]. The technique is sensitive and reproducible, but suffers from a few limitations, including relatively low efficiencies (compared to techniques such as GC and CE), long analysis times and high solvent consumption, rendering it relatively expensive for routine operation. Several RP-LC methods for the analysis of rooibos phenolics have been described [10-16]. These methods differ in terms of the compounds separated and quantified; incremental improvements have been attained following extensive method development [15,16], and improvement of existing methods is ongoing. Due to the variation in phenolic composition between *Cyclopia* species, species-specific HPLC methods are required [17-23], again implying extensive method development.

In light of the above, cheaper, faster and more generic separation methods are of interest for the analysis of herbal tea phenolics. CE is a promising alternative for the analysis of phenolics [24,25], as it offers fast, cost-effective and efficient operation with low solvent consumption. Furthermore, CE offers an alternative separation mechanism compared to HPLC. The alternative selectivity of CE is potentially beneficial for the separation of closely related phenolic compounds. However, the technique also typically suffers from lower sensitivity and reproducibility compared to HPLC [26]. CE has been successfully applied for the analysis of phenolic compounds in wine [27], medicinal plants [28], Chinese herbal tea [29], green tea [30,31], oolong tea [31] and black tea [32]. Especially in light of the demonstrated capabilities of CE for the analysis of tea phenolics, the goal of this

communication was to evaluate CZE as an alternative to HPLC for the quantitative analysis of rooibos and honeybush tea phenolics.

3.2. Materials and methods

3.2.1. Chemicals and reagents

Sodium tetraborate was obtained from Holpro Analytics (Krugersdorp, South Africa (SA)). NaOH, HPLC-grade methanol (MeOH) and DMSO were obtained from Sigma-Aldrich (Johannesburg, SA), HCl from Merck (Darmstadt, Germany) and ascorbic acid and mesityl oxide from Hopkin & Williams (Johannesburg, SA) and Fluka (Sigma-Aldrich), respectively. Most certified authentic standards (**Table 3.1.**) were purchased from Sigma-Aldrich (**Asp, FA, Quer, Mang, Hd** and **Iri-glc**), Extrasynthese (Genay, France, **Lut-7-glc, Isovitt, Isoorient, Orient, Hyp, Chrys** and **Lut**) and Roth (Karlsruhe, Germany, **Isoquer** and **Vit**). **PPAG, Scoly, Phlor-di-glc** and **Iri-di-glc** were isolated (purity > 95%) and supplied by the Agricultural Research Council (ARC, Stellenbosch, SA). **Isomang, Rut** and **Eriot** were supplied by CHEMOS GmbH (Regenstauf, Germany), TransMIT GmbH PlantMetaChem (Gießen, Germany) and PhytoLab GmbH (Vestenbergsgreuth, Germany), respectively. **Not** was isolated (purity > 95%) and supplied by the PROMEC Unit of the Medical Research Council (Cape Town, SA).

3.2.2. Preparation of buffer and standard solutions

An appropriate amount of sodium tetraborate was dissolved in deionised (DI) water (Milli-Q, Millipore, Milford, MA, USA) and used as BGE (ionic strengths reported are before pH adjustment). The BGE pH was adjusted with 0.1 or 1 M NaOH or HCl. Individual standard stock solutions were prepared in MeOH (**Isovitt, Rut, Isoorient, Isoquer, Asp, FA, Quer, Scoly, Eriot, Phlor-di-glc** and **Iri-glc**), DMSO (**PPAG, Not, Hd, Mang** and **Isomang**), DMSO:MeOH, (**Lut-7-glc** (7:20), **Vit** (1:5), **Orient** (1:5), **Hyp** (3:20), **Chrys** (9:20) and **Lut** (3:10)) and water (**Iri-di-glc**). Rooibos and honeybush standard solutions were preserved with 10 and 0.5 mg/mL ascorbic acid, respectively, while **Asp** solutions contained 20 mg/mL ascorbic acid. All standard solutions, buffers and samples were sonicated and filtered through 0.45 µm hydrophilic polyvinlidene fluoride (PVDF) membrane filters (Millipore) before use.

3.2.3. Sample preparation

Aqueous extracts of rooibos (10 unfermented and 10 fermented) were prepared as described by Beelders *et al.* [16]. Aqueous extracts of honeybush, prepared according to de Beer *et al.* [19], com-

Table 3.1. Structures and abbreviations of the principal rooibos and honeybush tea phenolics and a phenylpropanoid used as standards for method development and quantification.

General structure	Phenolic class, compound names and structures
	<p>Flavones</p> <p>Luteolin-7-O-glucoside (Lut-7-glc): $R_1 = R_3 = H$, $R_2 = O-\beta-D\text{-glucosyl}$, $R_4 = OH$</p> <p>Isovitexin (Isovitz): $R_1 = R_4 = H$, $R_2 = OH$, $R_3 = O-\beta-D\text{-glucosyl}$</p> <p>Isoorientin (Isoorient): $R_1 = H$, $R_2 = R_4 = OH$, $R_3 = C-\beta-D\text{-glucosyl}$</p> <p>Vitexin (Vit): $R_1 = C-\beta-D\text{-glucosyl}$, $R_2 = OH$, $R_3 = R_4 = H$</p> <p>Orientin (Orient): $R_1 = C-\beta-D\text{-glucosyl}$, $R_2 = R_4 = OH$, $R_3 = H$</p> <p>Chrysoeriol (Chrys): $R_1 = R_3 = H$, $R_2 = OH$, $R_4 = OCH_3$</p> <p>Luteolin (Lut): $R_1 = R_3 = H$, $R_2 = R_4 = OH$</p> <p>Scolymoside (Scoly): $R_1 = R_3 = H$, $R_2 = O-\beta-D\text{-rutinosyl}$, $R_4 = OH$</p>
	<p>Flavanones</p> <p>Hesperidin (Hd): $R_1 = O-\beta-D\text{-rutinosyl}$, $R_2 = OCH_3$</p> <p>Eriocitrin (Eriot): $R_1 = O-\beta-D\text{-rutinosyl}$, $R_2 = OH$</p>
	<p>Flavonols</p> <p>Quercetin (Quer): $R = H$</p> <p>Isoquercitrin (Isoquer): $R = O-\beta-D\text{-glucosyl}$</p> <p>Hyperoside (Hyp): $R = O-\beta-D\text{-galactosyl}$</p> <p>Rutin (Rut): $R = O-\beta-D\text{-rutinosyl}$</p>
	<p>Dihydrochalcones</p> <p>Nothofagin (Not): $R_1 = R_3 = H$, $R_2 = C-\beta-D\text{-glucosyl}$</p> <p>Aspalathin (Asp): $R_1 = H$, $R_2 = C-\beta-D\text{-glucosyl}$, $R_3 = OH$</p> <p>Phloretin-3',5'-di-C-glucoside (Phlor-di-glc): $R_1 = R_2 = C-\beta-D\text{-glucosyl}$, $R_3 = H$</p>
	<p>Phenylpyruvic acid-2-O-glucoside (PPAG): $R = O-\beta-D\text{-glucosyl}$</p>
	<p>Hydroxycinnamic acid</p> <p>Ferulic acid (FA)</p>
	<p>Benzophenones</p> <p>Iriflophenone-3-C-glucoside-4-O-glucoside (Iri-di-glc): $R_1 = O-\beta-D\text{-glucosyl}$, $R_2 = C-\beta-D\text{-glucosyl}$</p> <p>Iriflphenone-3-C-glucoside (Iri-glc): $R_1 = OH$, $R_2 = C-\beta-D\text{-glucosyl}$</p>
	<p>Xanthones</p> <p>Mangiferin (Mang): $R_1 = H$, $R_2 = C-\beta-D\text{-glucosyl}$</p> <p>Isomangiferin (Isomang): $R_1 = C-\beta-D\text{-glucosyl}$, $R_2 = H$</p>

prised 19 unfermented and 19 fermented samples (10 each of *C. subternata* and 9 of *C. maculata*). The freeze-dried extracts were dissolved in DI water (6 mg/mL) containing 100 and 500 µg/mL mesityl oxide and ascorbic acid, respectively, and stored at -20°C.

3.2.4. Instrumentation and methods

Experiments were performed on an HP^{3D}CE system (Agilent Technologies, Palo Alto, CA, USA) equipped with a DAD. Uncoated fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of i.d. 50 µm and various lengths were used. New capillaries were conditioned with 1 M NaOH (5 min), followed by DI water (5 min) and BGE (30 min). Daily conditioning was as follows: 1 M NaOH (2.5 min), DI water (5 min) and BGE (10 min). Capillaries were flushed between runs with BGE (10 min). Injection was performed hydrodynamically at a pressure of 50 mbar. All separations were carried out in positive polarity mode (detection at the cathode). Electropherograms were recorded at 244, 283, 330 and 384 nm using acquisition rates of 20 and 10 Hz for the rooibos and honeybush methods, respectively, and UV-vis spectra were recorded between 190-450 nm.

The optimised methods for both rooibos and honeybush phenolics employed a capillary of 80 cm effective length (*l*) and 88.5 cm total length (*L*), an applied voltage of 30 kV and injection at 500 mbar.sec. For rooibos phenolics, the BGE was 200 mM borate (pH 8.80) and the capillary temperature 20°C, whereas for honeybush phenolics the BGE was 200 mM borate (pH 9.25) and the temperature 30°C.

3.2.5. CZE quantification and method validation

Stock solutions of rooibos standards were diluted with DI water containing 100 µg/mL mesityl oxide as EOF marker, except for **Quer** and **Asp**, for which separate calibration curves were constructed. Due to limited solubility, **Quer** was diluted with MeOH containing 100 µg/mL mesityl oxide, and **Asp** was diluted with DI water containing 20 mg/mL ascorbic acid, 0.1% formic acid and 100 µg/mL mesityl oxide. Stock solutions of honeybush standards were diluted with DI water containing 100 µg/mL mesityl oxide and 500 µg/mL ascorbic acid.

Calibration curves covered the expected concentration ranges in the samples (**Tables S1.** and **S2.**). Average corrected peak areas (area divided by migration time) of four analyses were used in the construction of calibration curves. **Hd** and **Eriot** were quantified at 283 nm, **Asp**, **Phlor-di-glc**, **Iri-di-glc** and **Iri-glc** at 330 nm, while the rest of the phenolics were quantified at 384 nm. Unfermented rooibos and *C. maculata* samples were diluted 1:1 and 1:3 for the quantification of **Asp** and **Mang**.

respectively. Selected unfermented (samples 1, 4, 6, 7, 8 and 10) and fermented (samples 2, 7 and 8) *C. subternata* samples were diluted as required for the quantification of **Scoly**.

3.2.6. Quantification of rooibos and honeybush phenolics by HPLC-DAD

Quantitative data for rooibos and honeybush phenolics were obtained for the same set of samples using validated RP-LC methods [16,19,22]. Values represent the average of duplicate measurements for both methods, and were compared by means of a paired sample t-test using the TTEST procedure of SAS software (Version 9.4; SAS Institute Inc, Cary, USA) for each compound.

3.3. Results and discussion

3.3.1. Method development

CZE and MEKC are the most common electrodriven modes used for the analysis of phenolics [25,27,28,33]. Initial experiments using both modes (using borate and SDS-containing BGEs) indicated the former mode to be more suitable for the target analytes (results not shown). Borate buffers at pH 8-10 are commonly used as BGEs in CZE [34-36] due to the ability of tetrahydroxyborate ($B[OH]_4^-$), triborate ($[B_3O_3(OH)_5^{2-}]$) and tetraborate ($[B_4O_5(OH)_4]^{3-}$) ions to form complexes with vicinal diol functional groups on the flavonoid backbone [35]. This increases both the effective charge and the hydrated radii of flavonoids, and enhances the selectivity of their separation. For CZE method optimisation, parameters that affect electrophoretic mobility were systematically optimised, as outlined below.

3.3.1.1. Optimisation of BGE pH and ionic strength

Optimisation of the BGE pH is arguably the most important step in CE method development. pK_a values of polyphenols range from ~4-12 for phenolic acids [37] and ~7-12 [38] for flavonoids. For optimisation of the BGE pH, therefore, a pH range between 8.50-9.50 was investigated using a 200 mM borate as BGE. Under these conditions, the net migration of analytes is towards the cathode, with their effective electrophoretic mobilities determined by their respective charge-to-size ratios. The effect of pH on the electrophoretic mobilities of the rooibos and honeybush phenolics is shown in **Figures 3.1.A** and **B**, respectively. Electrophoretic mobilities decreased with increasing pH for all analytes, but important differences in relative mobility are observed as a function of pH.

For rooibos phenolics, a pH range of 8.50-9.10 was evaluated. At the lower pH values, extensive co-migration was observed (for example **Orient**, **FA**, **Hyp**, **Chrys** and **Lut** at pH 8.50, **FA** and **Hyp** at pH 8.60, and **PPAG** and **Not** at pH 8.70). At higher pHs (9.00 and 9.10), **Asp** was not resolved from **Hyp**, whereas incomplete separation of **Asp** and **Orient** is observed at pH 8.85. Based on these results, a pH of 8.80 was selected as optimal for rooibos tea analysis.

A pH range of 8.50-9.50 was evaluated for the honeybush standards. Below pH 8.90, **Scoly** and **Eriot** were not resolved, whereas at pH 9.00 **Iri-glc** and **Isomang** as well as **Scoly** and **Eriot** were not baseline resolved (the electrophoretic mobility of **Iri-glc** especially is significantly altered within the studied pH range (**Figure 3.1.B**)). Co-migration of **Isomang** with ascorbic acid was observed at pH 9.10. All compounds were baseline separated at pH 9.50, although this pH resulted in long analysis times. The optimal pH for honeybush tea phenolics was therefore selected as 9.25.

An increase in the borate concentration influences the separation in two ways: (1) higher ionic strength results in compression of the double-layer at the capillary wall, and therefore a decrease in the zeta potential and magnitude of the EOF [39], and (2) at higher borate concentrations, the extent of complex formation with vicinal diol groups increases. Both of these phenomena are therefore expected to lead to longer analysis times, but also differences in separation selectivity. The effect of BGE ionic strength was evaluated at the optimal pHs for rooibos and honeybush phenolics within the range 50-200 mM. The results are summarised in **Figure S1**. in the Supporting Information (SI).

For the rooibos phenolics, **Lut** and **Quer** co-migrated at 50 mM, whereas between 100 and 200 mM the migration order of the critical peak pairs (**Not** and **PPAG**, **Isoorient** and **Isoquer** and **Orient** and **Asp**) shifted. Since co-migration of **Orient** and **Asp** was observed at 100 and 150 mM, a BGE concentration of 200 mM was selected as optimal. Honeybush phenolics were separated at all evaluated BGE concentrations. However, peak shapes of **Mang** and **Isomang** were poor at lower ionic strength, and resolution between **Scoly** and **Eriot** as well as **Isomang** and ascorbic acid was improved at 200 mM, which was therefore selected as optimal.

3.3.1.2. Optimisation of temperature

Analysis temperature and voltage were optimised using the optimal BGEs. Temperatures between 15 and 40°C were evaluated. At higher temperatures, the decrease in BGE viscosity results in an increased EOF, and therefore shorter analysis times (this factor outweighs a similar increase in

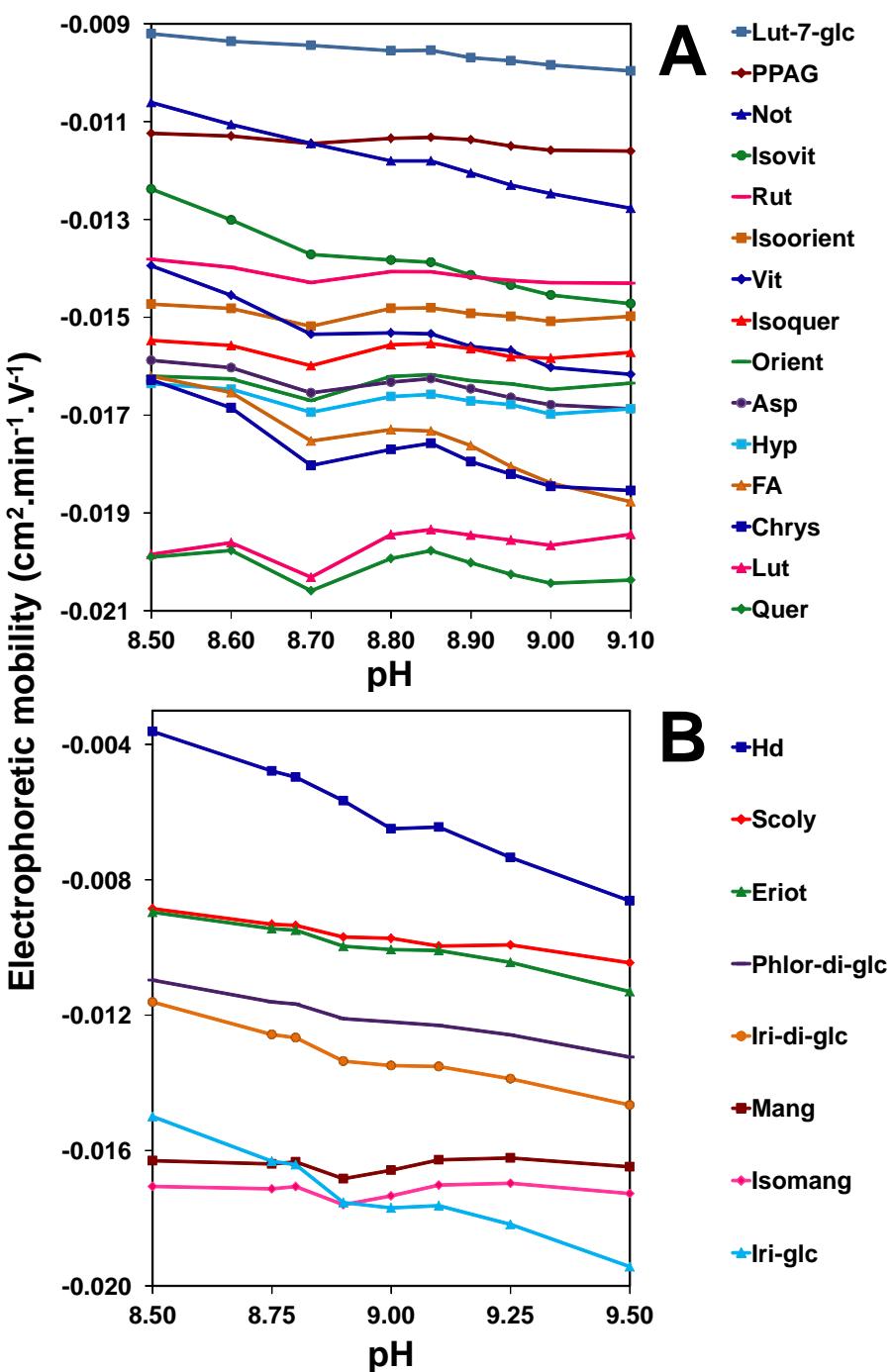


Figure 3.1. Effect of pH on the electrophoretic mobilities of rooibos (A) and honeybush (B) phenolics. Electrophoretic mobilities are the average of two measurements calculated using mesityl oxide as EOF marker. Experimental conditions: 200 mM borate, 30 kV, 30°C. (A): capillary 100 cm *l*/108.5 cm *L*, 1000 mbar.sec injection, (B) 80 cm *l*/88.5 cm *L* capillary, 250 mbar.sec injection. Compound names correspond to **Table 3.1..**

electrophoretic mobility). Higher temperatures (25–40°C) negatively affected the resolution of **Isoorient** and **Vit**, **Orient** and **Asp** and **Not** and **PPAG**. While all rooibos standards were separated at low temperature, the analysis time was excessive at 15°C; 20°C was therefore chosen as optimal. For honeybush standards, incomplete separation of ascorbic acid and **Iri-glc** was observed at low temperatures (15 and 20°C), whereas an increase in temperature resulted in increased efficiency. However, higher temperatures (35 and 40°C) negatively affected the peak shapes of **Phlor-di-glc** and **Iri-glc**. The optimal temperature was selected as 30°C.

3.3.1.3. Optimisation of capillary length and applied voltage

To investigate the feasibility of reducing analysis times, capillaries with dimensions 88.5/80 cm *L/l* and 80.5/72 cm *L/l* (both 50 µm i.d.) were evaluated. Incomplete separation of the critical pair **Orient** and **Asp** was observed on the shortest column, and high currents were measured. The 88.5 cm capillary provided complete resolution of all analytes and was therefore used for both methods. This implies that both rooibos and honeybush samples can facilely be analysed on a single instrument, since changing between methods involves a simple change in the BGE pH and temperature.

The occurrence of Joule heating was investigated by measuring the current of a BGE-filled capillary at applied voltages between 10 and 30 kV. A non-linear relationship between current and voltage was observed (SI, **Figure S2.**), indicating the presence of Joule heating at high voltages. On the other hand, a decrease in analysis time and increase in efficiency with increasing voltage were observed due to less diffusion associated with shorter migration times. Since higher voltages did not affect peak shapes or resolution negatively, and in the interest of reducing analysis times, the optimal applied voltage for both methods was selected as 30 kV (resulting in measured currents of 60 and 76 µA for rooibos and honeybush methods, respectively).

Electropherograms of the standard solutions for rooibos and honeybush phenolics using the optimised CZE methods for each are presented in **Figures 3.2.A** and **3.3.A**, respectively. In general peak shapes for the majority of compounds are very good; efficiencies range between 26 700 and 482 800 theoretical plates, determined using experimental peak widths at half height. The alternative selectivity of CZE resulted in a different migration order compared to HPLC, with especially critical peak pairs in the HPLC method for rooibos tea (**Hyp/Rut** and **Rut/Isovitrin**) being much better resolved. Indeed, resolution for all standard compounds was above 1.7 for both CZE methods (**Tables S1.** and **S2.**). Analysis times for the CZE methods are similar or slightly shorter compared to HPLC methods for the same analyses [10–23].

3.3.1.4. Analyte stability

Since tea phenolics are susceptible to oxidation, ascorbic acid was added to standard stock solutions (10 and 0.5 mg/mL for rooibos and honeybush phenolics, respectively). This was sufficient to avoid oxidation of most standards for a period of 20 months when stored at -20°C. Ascorbic acid is negatively charged under the CZE conditions used, but was separated from all target analytes and therefore did not interfere with the analyses.

For **Asp**, however, degradation was still observed in the presence of 20 mg/mL ascorbic acid. This is not unexpected, since oxidative degradation of **Asp** is one of the principal reactions occurring during rooibos fermentation: **Asp** oxidises rapidly to form the flavones **Isoorient** and **Orient** [1,2,13] as well as aspalathin dimers, dibenzofurans and unidentified polymers [40]. CZE analysis at high pH exacerbates the situation, since oxidation reactions are favoured at high pH. Surprisingly, degradation of **Asp** was observed in the course of consecutive injections (SI, **Figure S3.**). The electropherograms contain a broad ‘hump’, which increase in area with a decrease in the **Asp** peak area. This seems to indicate the presence of on-capillary degradation of **Asp**. For these reasons, calibration for **Asp** was performed independently, and several means of avoiding this phenomenon were explored (refer to SI, **Section S3.** for details); in the end preparation of **Asp** standard solutions containing 20 mg/mL ascorbic acid and 0.1% formic acid provided similar quantitative data compared to HPLC for the same samples (see below).

3.3.2. Analysis of rooibos and honeybush tea samples using the optimised CZE methods

Representative electropherograms obtained for the analysis of selected rooibos and honeybush samples using the optimised methods are presented in **Figures 3.2.** and **3.3.**, respectively. Several detection wavelengths were used for more selective detection: **PPAG** and the flavanones absorb maximally at 283 nm, the dihydrochalcones and benzophenones at 330 nm and the flavonols, flavones and xanthones at 384 nm. Compounds were identified by comparison of recorded UV-Vis spectra (**Figure S5.**) and calculated electrophoretic mobilities with those of the standards (as outlined in SI, **Section S4.**). Electrophoretic mobilities instead of migration times were used for compound identification, since the latter are highly dependent on small shifts in the EOF. Whereas RSDs for migration times varied between 1.3 and 4.8% between analyses, calculated mobilities were more stable (0.9-1.9%).

For the rooibos samples, good separation of most target analytes is observed in both fermented and unfermented samples (as confirmed using UV-vis spectra). **Isoorient** migrates close to an unidentified compound with a UV-vis spectrum characteristic of a flavonol, especially in fermented rooibos

samples, but is sufficiently resolved from it in unfermented samples to allow quantification. The unidentified compound could be quercetin-3-*O*-robinobioside, previously detected in rooibos tea [15,16]. **FA**, **Chrys**, **Lut** and **Quer** were not detected in any of the samples, whereas **Lut-7-glc** was only detected in one unfermented and one fermented rooibos sample. The content of the main phenolics, especially **Asp** and **Not**, differed drastically in the fermented samples, owing to their oxidation during fermentation [1]; levels of **Isoorient** and **Orient** would be less affected owing to their formation from **Asp** during fermentation. A somewhat surprising observation was the ‘hump’ observed at 283 nm at the end of the electropherograms (19-31.5 min), especially for fermented samples. This can presumably be ascribed to polymeric phenolic material, more of which is formed during fermentation. A similar ‘hump’ is less evident from HPLC chromatograms, since the polymeric material is spread across the retention window [15,16].

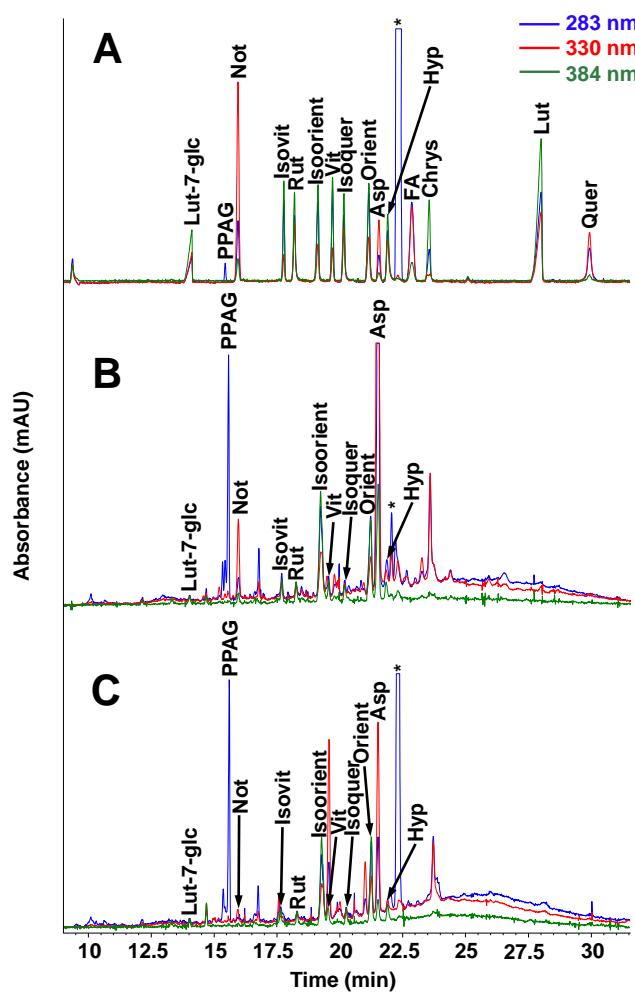


Figure 3.2. Representative electropherograms obtained for the separation of a standard rooibos phenolic mixture (**A**), unfermented (**B**) and fermented (**C**) rooibos samples using the optimised CZE method. Experimental conditions: BGE: 200 mM borate, pH 8.80; temperature: 20°C; applied voltage: 30 kV; injection: 500 mbar.sec; capillary dimensions: 50 µm i.d., 80 cm *l* × 88.5 cm *L*. Peak names correspond to **Table 3.1.** * denotes ascorbic acid.

Efficient separation of all standard honeybush phenolics was attained using the optimised method (**Figure 3.3.A**). The method also proved suitable for the separation of major phenolics in both *C. maculata* and *C. subternata*, indicating the potential of developing a ‘generic’ CE method for different honeybush species, something which is challenging by HPLC [23]. The differences in phenolic composition between the two honeybush species are clear from **Figure 3.3.**; **Scoly**, **Phlor-di-glc** and **Iri-di-glc** were not detected in the *C. maculata* samples. Furthermore, a clear decrease in the levels of especially **Mang**, **Isomang** and **Hd** was observed following fermentation [19,23], while **Iri-glc** was not detected in any of the fermented samples. Also for honeybush samples, a ‘hump’ likely associated with polymeric phenolic material is observed.

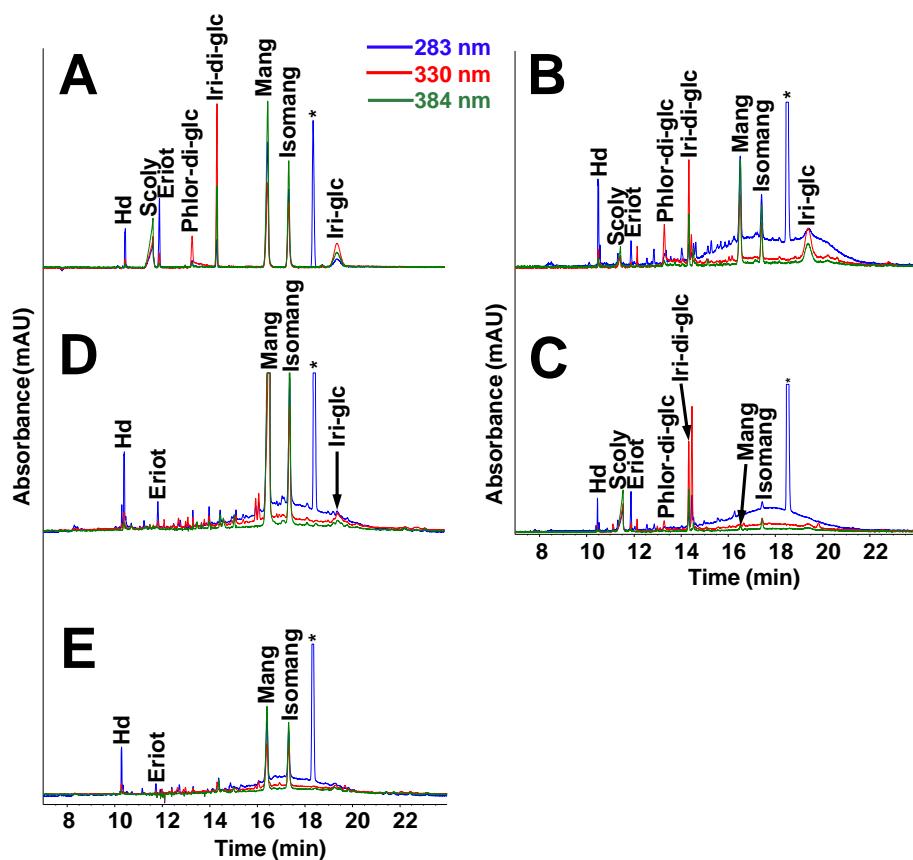


Figure 3.3. Representative electropherograms obtained for the analysis of a standard honeybush phenolic mixture (A), unfermented (B) and fermented (C) *C. subternata*, unfermented (D) and fermented (E) *C. maculata* samples using the optimised CZE method. Experimental conditions: BGE: 200 mM borate, pH 9.25; temperature: 30°C. Other conditions as in **Figure 3.2.**. Peak names correspond to **Tables 3.1.** and **S2.**. * denotes ascorbic acid.

3.3.3 Quantitative CZE analysis and comparison with HPLC data

To evaluate the performance of the optimised methods for the quantitative analysis of honeybush and rooibos phenolics, calibration was performed as outlined in **Section 2.5**. The data are summarised in **Tables S1** and **S2**. Note that **PPAG** and **Not** were not quantified due to insufficient amounts of reference standards. Linearity of calibration curves was good for all compounds, with R^2 -values ranging from 0.997-1.000. LODs and LOQs, determined from the standard deviation of repeated measurements at low levels according to [41], varied between 0.73-14.9 and 2.45-49.7 $\mu\text{g/mL}$, respectively. Higher LODs and LOQs were observed for compounds with relatively poor peak shapes, such as **Lut**, **Quer**, **Phlor-di-glc** and **Iri-glc**. These values are roughly an order of magnitude higher than those typically obtained by HPLC, and represent a well-known limitation of CE. Nevertheless, the major compounds could be quantified in the analysed samples (see below). **Lut-7-glc**, **Isoquer**, **Hyp**, **Rut** and **Vit** could not be detected and/or quantified in most of the samples, owing to their low levels.

To compare the quantitative performance of the CZE methods with HPLC, the same samples were analysed using the optimised CZE methods and validated HPLC methods described by Beelders *et al* [16] and de Beer *et al.* [19,22]. Quantitative data for rooibos tea samples ($n = 2$ for each method) are summarised in **Tables S3** and **S4**; visual comparisons of the data for selected rooibos and honeybush samples are shown in **Figure 4**. Quantitative data between CZE and HPLC methods were generally comparable for both sets of samples. For **Asp** overestimation of the concentration (15% on average) was observed for fermented samples compared to HPLC, whereas for unfermented rooibos samples, the discrepancy was notably less (3% on average). This could be due to possible co-migration in CZE. Levels of **Isoorient** and **Orient** were significantly lower in unfermented samples by CZE, possibly due to co-elution in HPLC.

For honeybush samples, CZE and HPLC data were more comparable (± 6 -11% on average for all compounds) (**Tables S5-S8.**), with few exceptions. The content of **Eriot** was consistently underestimated by CZE, and the same was observed for **Mang** in unfermented samples. **Iri-glc** and **Phlor-di-glc** could not be quantified in unfermented *C. maculata* and fermented *C. subternata*, respectively, owing to high LOQs, and **Mang** could not be quantified in fermented samples of *C. subternata*.

The overall agreement between CZE and HPLC data points to the utility of the former method as a cheap and more rapid alternative for the quantitative screening of phenolic composition of honeybush and rooibos teas, where the goal is more commonly to compare the contents of major compounds.

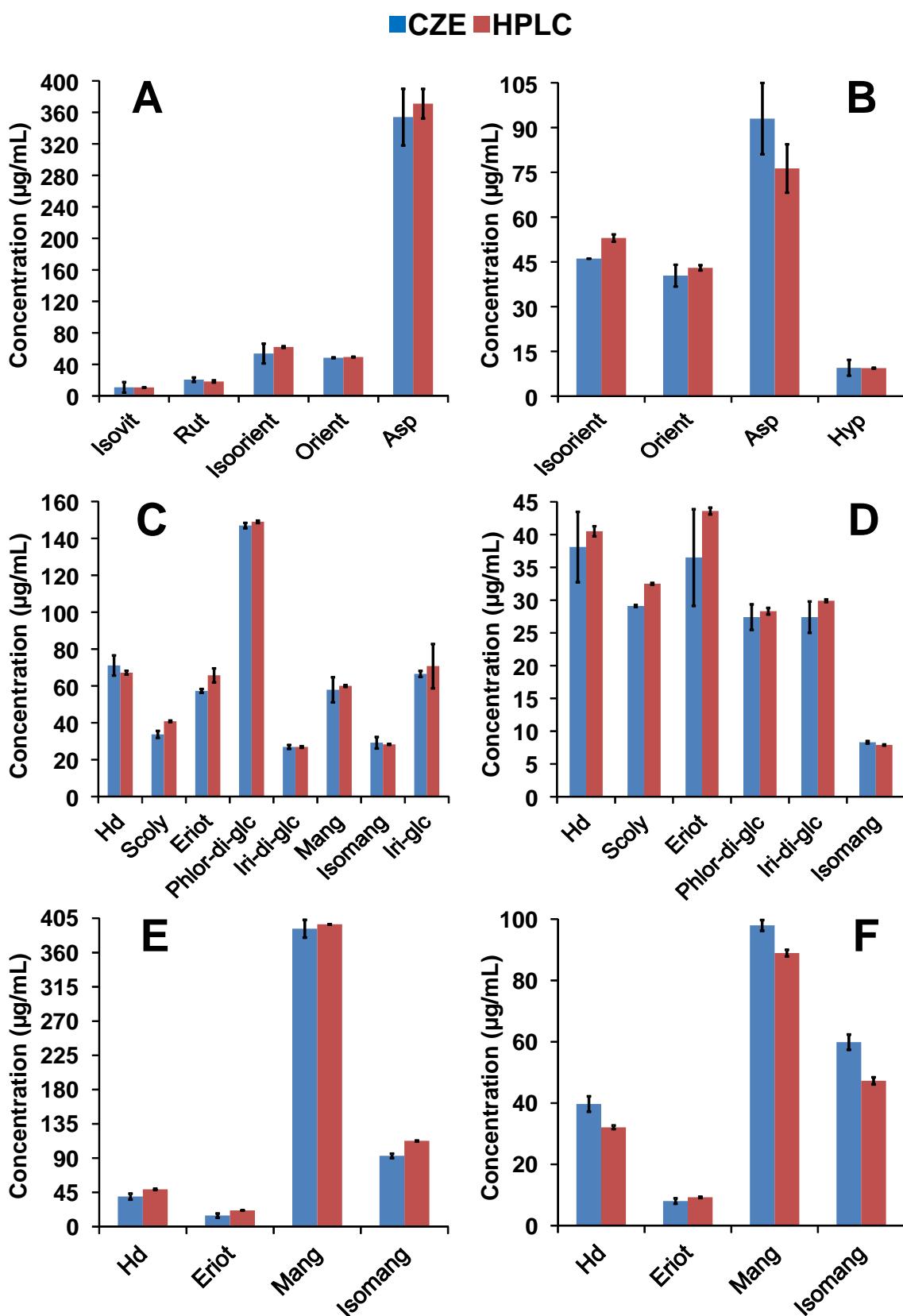


Figure 3.4. Comparison between quantitative data obtained by CZE and HPLC for selected unfermented (A) and fermented (B) rooibos samples (Tables S3. and S4.), unfermented (C) and fermented (D) *C. subternata* (Tables S5. and S6.) and unfermented (E) and fermented (F) *C. maculata* (Tables S7. and S8., respectively).

3.4. Concluding remarks

Two cost-effective quantitative CZE methods have successfully been developed for the analysis of rooibos and honeybush phenolics following optimisation of BGE composition and pH, ionic strength, applied voltage, temperature and capillary dimensions. Quantitative data obtained using the developed methods were generally comparable with HPLC data for the same set of samples, indicating the viability of the developed CZE methods for quantitative analysis. Clear differences in the phenolic profiles of fermented and unfermented rooibos tea samples were observed, in line with similar observations by HPLC. The same was observed for honeybush tea samples, where important additional variations in phenolic contents of *C. subternata* and *C. maculata* samples were also observed.

Compared to HPLC methods routinely used for rooibos and honeybush analyses, the reported CZE methods suffer from lower sensitivity and reproducibility, and are incompatible with MS detection due to the use of borate buffers dictated by separation considerations. On the other hand, the CZE methods offer the advantages of much lower solvent consumption, lower operating costs as well as slightly reduced analysis times. Taken together, these results indicate that CE may be used as a promising alternative to HPLC for the routine analysis of these samples, for example for quality control purposes. Future work should include the evaluation of CE for the analysis of other *Cyclopia* species.

3.5. References

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CHAPTER 3

SUPPORTING INFORMATION

S1. The effect of BGE ionic strength

The effect of ionic strength was investigated for BGEs of pH 8.80 and 9.25 for rooibos and honeybush phenolics, respectively, in the range 50–200 mM (in increments of 50 mM).

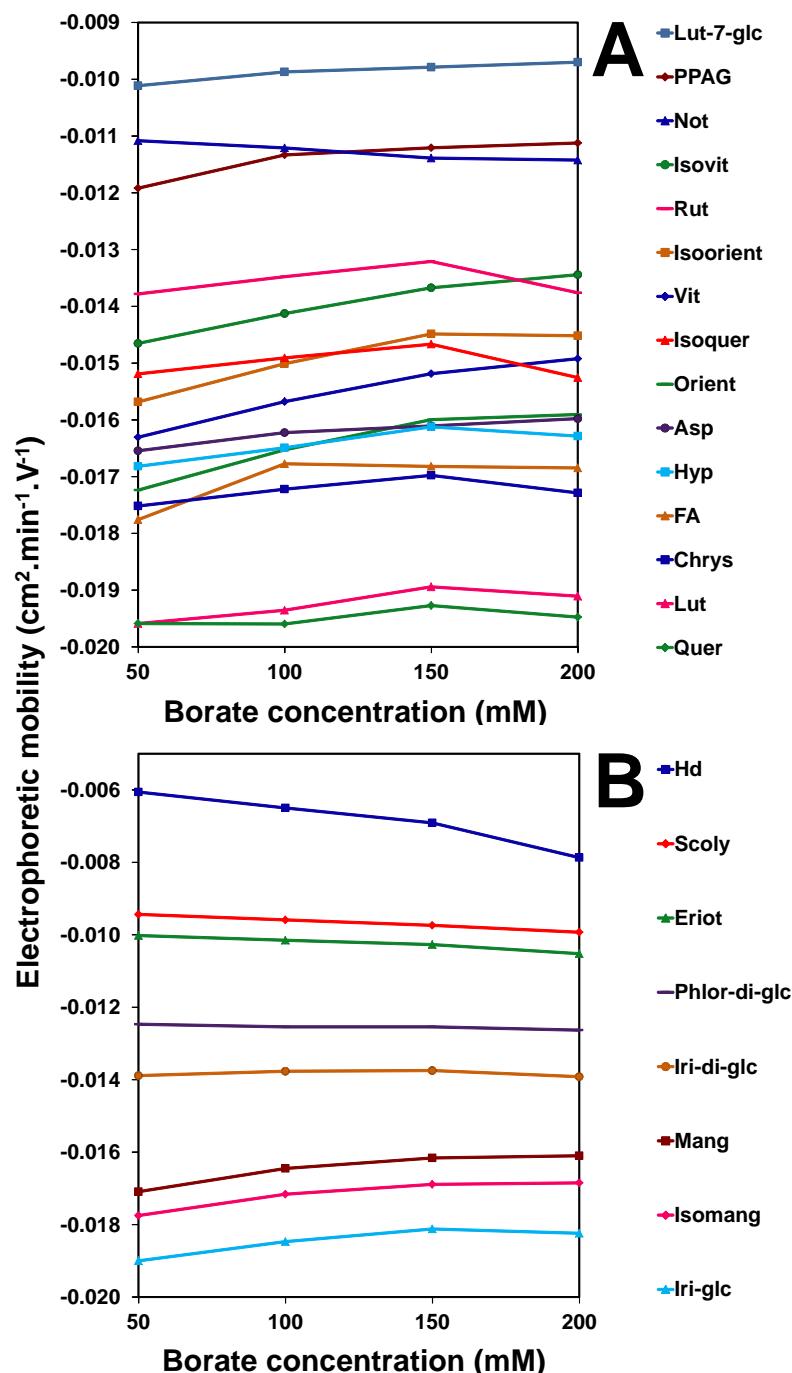


Figure S1. Effect of ionic strength on the electrophoretic mobilities of rooibos (A) and honeybush (B) phenolics. Electrophoretic mobilities are the average of two measurements calculated using mesityl oxide as EOF marker. Experimental conditions: 50–200 mM borate at pH = 8.80 (A) and 9.25 (B), 30 kV, 30°C. (A): capillary 100 cm (l) \times 108.5 cm (L), 1000 mbar.sec injection, (B) 80 cm l / 88.5 cm L capillary, 500 mbar.sec injection. Compound names correspond to Tables 3.1., S1. and S2.

S2. Evaluation of Joule heating

To evaluate the occurrence of Joule heating, the measured current was plotted as a function of the applied voltage (Ohm's law) for capillaries filled with the optimised BGEs (200 mM borate, pH 8.80 and 9.25 for rooibos and honeybush phenolics, respectively). A non-linear relationship indicates the occurrence of Joule heating. Experimental plots are presented for the rooibos and honeybush BGEs on a 50 µm (i.d.), 80 cm (effective length, l) and 88.5 cm (total length, L) capillary in **Figure S2.**. The effect of Joule heating is evident from the deviation from linearity observed for both these curves above 20 kV. However, higher voltages (25 and 30 kV) were found to not detrimentally affect analyte peak shapes and provided higher efficiencies for the studied phenolic compounds.

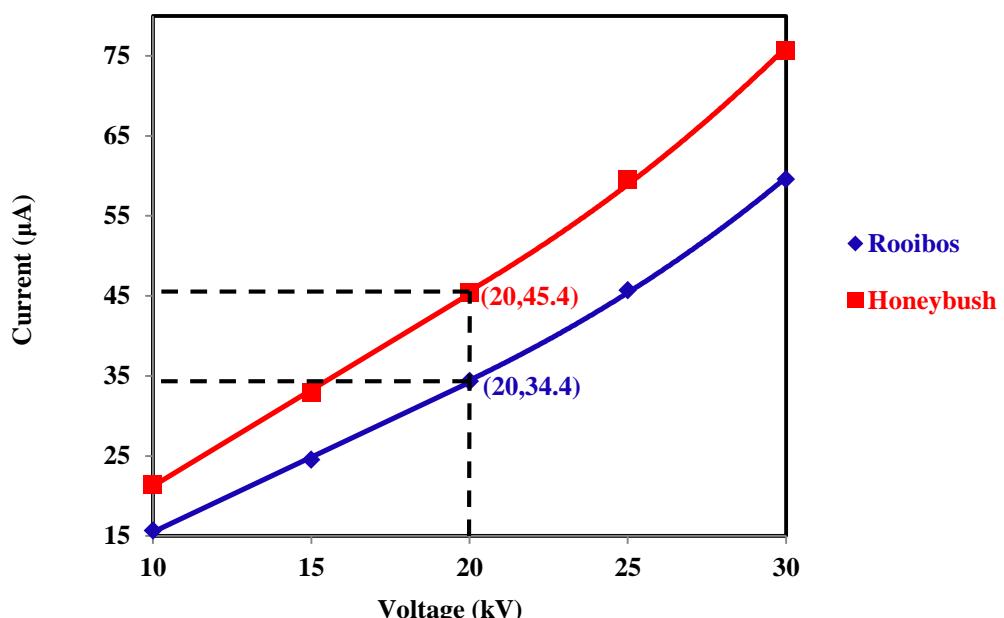


Figure S2. Plots of measured current as a function of applied voltage for the optimised rooibos and honeybush BGEs (200 mM borate, pH 8.80 and 9.25 for rooibos and honeybush phenolics, respectively). Data were obtained on a 50 µm (i.d.) capillary, 80 cm (l) × 88.5 cm (L) at 20 and 30°C for rooibos and honeybush phenolics, respectively.

S3. Degradation of aspalathin

A decrease in the peak areas of **Asp** and ascorbic acid was observed in four consecutive injections of a rooibos standard mixture (**Figure S3.**). An increase in the 'hump' (labelled * in **Figure S3.**), indicative of on-capillary degradation, coincided with the decrease in **Asp** peak area. The corrected area for **Asp** decreased drastically: by 52-94% from the first to the fourth injection.

Several approaches were evaluated to avoid **Asp** degradation: 1) higher concentrations of ascorbic acid (5-20 mg/mL) in the sample solution, 2) addition of 0.5 mM ethylenediaminetetraacetic acid (EDTA) to the **Asp** sample, 3) addition of 20 mg/mL ascorbic acid with 0.1% formic acid to the

sample solution, 4) addition of 20 mg/mL ascorbic acid to the sample and 100 µg/mL ascorbic acid to the BGE (adjusted to pH 8.80), and 5) addition of 20 mg/mL ascorbic acid and 0.1% formic acid to the sample solution and 100 µg/mL ascorbic acid to the BGE (adjusted to pH 8.80).

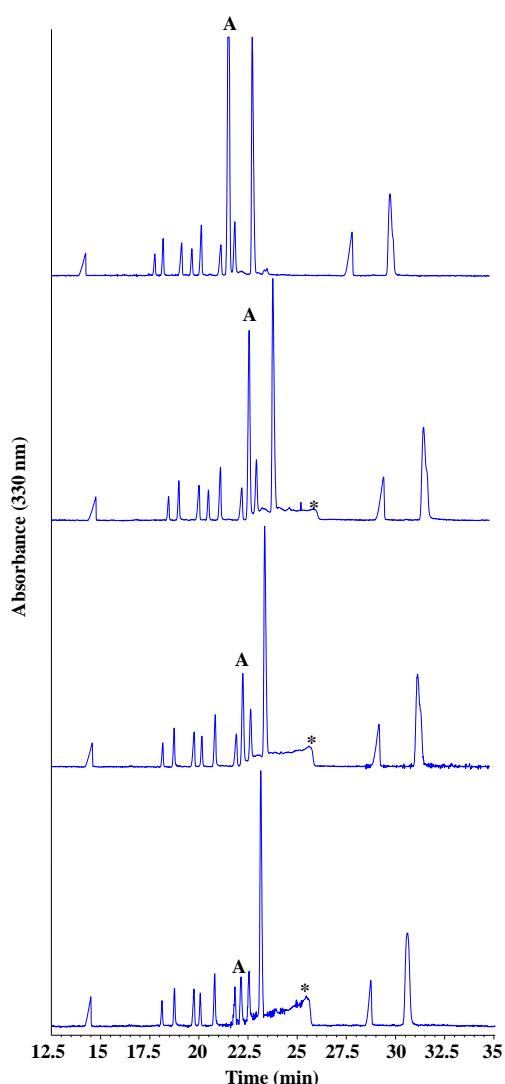


Figure S3. Overlayed electropherograms of four consecutive injections of a rooibos standard mixture (500 µg/mL **Asp**, labelled **A**, 200 µg/mL ferulic acid and 100 µg/mL for the rest of the compounds) preserved with 500 µg/mL ascorbic acid. The decrease in **Asp** peak areas corresponds with an increase in the area of a ‘hump’ designated by *, which likely indicates the presence of on-capillary degradation. Experimental conditions: 200 mM borate, pH 8.80, 30 kV, 20°C, 50 µm i.d. capillary, 80 cm (*l*) × 88.5 cm (*L*), 500 mbar.sec injection, electropherograms recorded at 330 nm.

Sample degradation was reduced by increasing the ascorbic acid concentration to 20 mg/mL, whereas acidification of the sample by the addition of 0.1% formic acid further increased the stability of **Asp**. In **Figure S4.** the corrected peak area for **Asp** is compared for three consecutive injections of three standard solutions containing 500 µg/mL ascorbic acid, 20 mg/mL ascorbic acid, and 20 mg/mL

ascorbic acid and 0.1% formic acid, respectively. It is clear that addition of 20 mg/mL ascorbic acid and 0.1% formic acid to the standard solution greatly improved the stability of **Asp**; these conditions were used for the calibration of **Asp**.

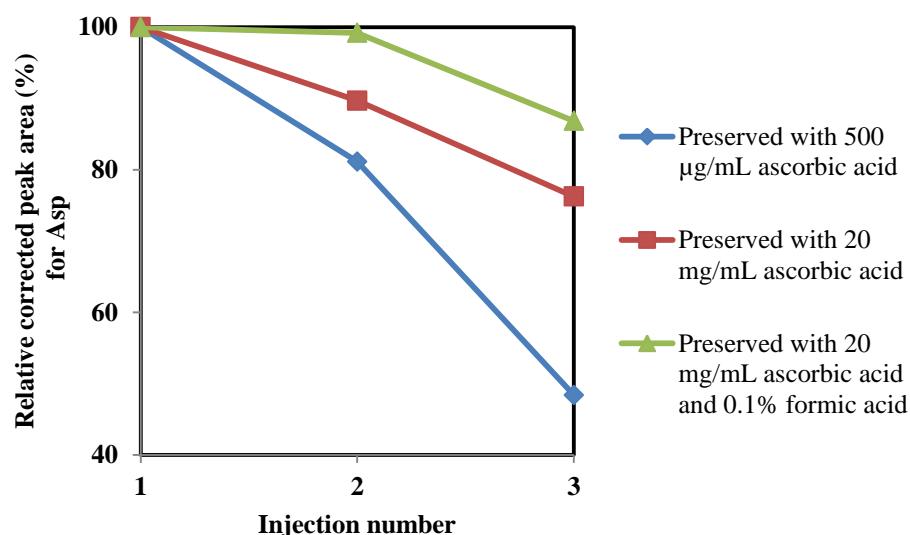


Figure S4. Comparison of degradation of a 500 µg/mL **Asp** standard solution under different preservation conditions. The relative corrected peak area (%) for **Asp** is presented as a function of consecutive injections for each sample solution.

S4. Identification of compounds in rooibos and honeybush extracts

Compounds were identified in the tea extracts by comparison of UV-Vis spectra and calculated electrophoretic mobilities with those of authentic standards. In the first instance, on-line UV-Vis spectra were compared with an in-house library created using authentic standards. Examples of UV spectra for each of the phenolic classes from the library are presented in **Figure S5..**

Secondly, electrophoretic mobilities were determined for the tea components using the following relationship:

$$\mu_a = \mu_e + \mu_{eof}$$

where, μ_a = the apparent mobility ($\text{cm}^2 \cdot \text{min}^{-1} \cdot \text{V}^{-1}$)

μ_e = the electrophoretic mobility ($\text{cm}^2 \cdot \text{min}^{-1} \cdot \text{V}^{-1}$)

μ_{eof} = the electroosmotic mobility, determined for the migration time of the EOF marker, mesityl oxide ($\text{cm}^2 \cdot \text{min}^{-1} \cdot \text{V}^{-1}$)

μ_a and μ_{eof} can be calculated with the follow equations, respectively:

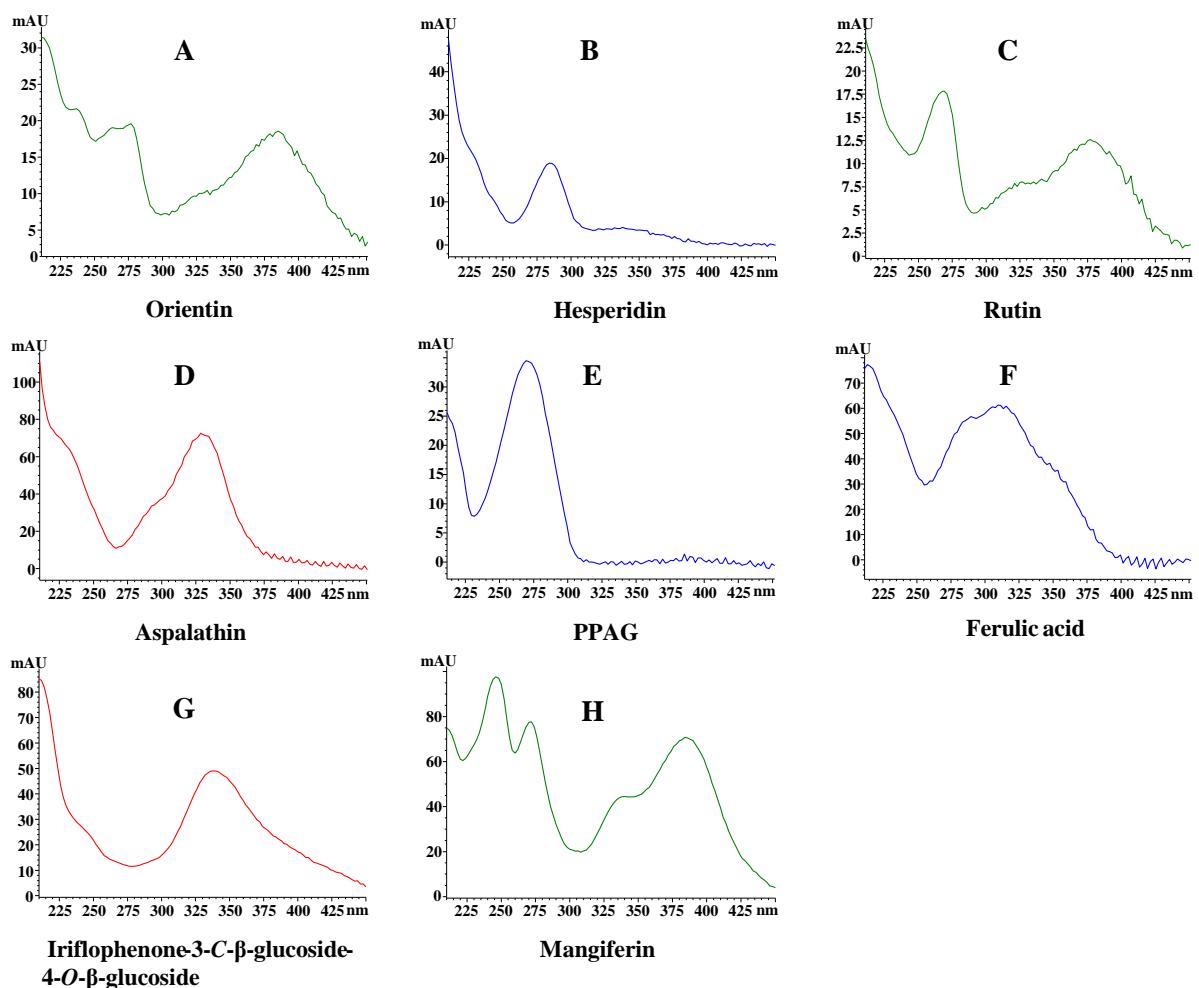


Figure S5. Examples of UV-vis spectra recorded for the CZE-DAD analysis of rooibos and honeybush phenolics. (A) flavones (**Orient**), (B) flavanones (**Hd**), (C) flavonols (**Rut**), (D) dihydrochalcones (**Asp**), (E) phenylpropanoid (**PPAG**), (F) hydroxycinnamic acid (**FA**), (G) benzophenones (**Iri-di-glc**) and (H) xanthones (**Mang**).

$$\mu_a = \frac{lL}{t_m V}$$

$$\mu_{eof} = \frac{lL}{t_{eof} V}$$

where, l (cm) = effective length of the capillary

L (cm) = total length of capillary

t_m (min) = migration time of an analyte

t_{eof} (min) = migration time of the EOF marker (mesityl oxide)

V (volts) = voltage applied during the analysis

The electrophoretic mobility (μ_e) of an analyte is then determined by the difference between μ_a and μ_{eof} .

$$\mu_e = \mu_a - \mu_{eof}$$

S5. Quantitative CZE analysis and comparison with HPLC data

Calibration was performed using the optimised CZE methods for rooibos and honeybush teas, respectively (Section 3.4.), as outlined in Section 3.5. of the manuscript. Calibration data for the two methods are summarised in Tables S1. and S2. below.

Table S1.: Calibration data for rooibos tea phenolics obtained using the optimised CZE method.

Compound	^a Wavelength (nm)	Peak name	^b R _s	Electrophoretic mobility (cm ² .min ⁻¹ .V ⁻¹) (× 10 ⁻³)	Calibration range (µg/mL)	r ²	^c LOD (µg/mL)	^c LOQ (µg/mL)
Luteolin-7-O-glucoside	384	Lut-7-glc	-	-8.26	5-100	0.999	5.13	17.1
Isovitexin	384	Isovitr	13.5	-11.8	5-100	0.999	3.08	10.3
Rutin	384	Rut	3.42	-12.1	5-100	0.998	4.90	16.3
Isoorientin	384	Isoorient	6.69	-12.7	5-100	0.999	2.81	9.36
Vitexin	384	Vit	4.21	-13.0	5-100	1.000	2.83	9.44
Isoquercitrin	384	Isoquer	3.43	-13.3	5-100	0.999	5.51	18.3
Orientin	384	Orient	6.55	-13.8	5-100	1.000	4.12	12.4
Aspalathin	330	Asp	2.40	-13.9	5-500	0.999	3.09	10.3
Hyperoside	384	Hyp	2.29	-14.2	5-100	1.000	2.76	8.27
Ferulic acid	330	FA	^d 2.10	-14.3	5-200	0.997	1.95	6.51
Luteolin	384	Lut	18.3	-16.4	10-100	0.997	7.98	26.6
Quercetin	330	Quer	6.78	-16.8	10-200	0.998	14.9	49.7

^aWavelength used for quantification.

^bResolution.

^cCalculated according to [41].

^dResolution calculated relative to ascorbic acid.

Table S2.: Calibration data for honeybush tea phenolics obtained using the optimised CZE method.

Compound	^a Wavelength (nm)	Peak name	^b R _s	Electrophoretic mobility (cm ² .min ⁻¹ .V ⁻¹) ($\times 10^{-3}$)	Calibration range (µg/mL)	r ²	^c LOD (µg/mL)	^c LOQ (µg/mL)
Hesperidin	283	Hd	-	-7.61	5-75	0.999	1.27	4.25
Scolymoside	384	Scoly	7.26	-9.49	10-80	0.997	1.79	5.96
Eriocitrin	283	Eriot	1.73	-10.3	5-100	0.997	0.97	3.24
Phloretin-3',5'-di-C-glucoside	330	Phlor-di-glc	14.2	-12.4	20-200	0.999	7.59	25.3
Iriflophenone-3-C-β-glucoside-4-O-β-glucoside	330	Iri-di-glc	10.6	-13.7	5-200	0.997	0.73	2.45
Mangiferin	384	Mang	17.2	-15.9	5-400	0.998	2.64	8.78
Isomangiferin	384	Isomang	5.89	-16.6	5-200	0.997	1.27	4.22
Iriflophenone-3-C-glucoside	330	Iri-glc	^d 3.37	-17.9	20-200	0.999	10.4	34.8

^a Wavelength used for quantification.^b Resolution.^c Calculated according to [41].^d Resolution calculated relative to ascorbic acid.

Table S3. Summary of quantitative data (in µg/mL) obtained by using the optimised CZE method and a routine HPLC method^a for the principal phenolics in unfermented rooibos tea samples. CZE values are listed above HPLC data (in brackets) for the same compound in the same sample (n = 2 replicates for each method).

Sample number	^b Compounds									
	Lut-7-glc	Isovitril	Rut	Isoorientin	Vit	Isoqueretin	Orientin	Asp	Hyp	
1	^c nd (nd)	10.8 ± 0.746 a ^d (10.7 ± 0.033) a	20.5 ± 0.307 a (18.2 ± 0.160) b	53.7 ± 1.40 b (62.0 ± 0.114) a	9.11 ± 0.194 (9.11 ± 0.194)	nd (4.06 ± 0.059)	48.3 ± 0.057 b (49.3 ± 0.055) a	354 ± 4.00 b (371 ± 2.08) a	nd (nd)	
2	nd (4.06 ± 0.008)	14.6 ± 0.379 b (18.4 ± 0.114) a	16.6 ± 0.334 a (16.7 ± 0.309) a	65.6 ± 1.92 b (89.7 ± 0.077) a	12.2 ± 0.185 b (14.5 ± 0.016) a	nq (6.42 ± 0.016)	49.8 ± 1.47 b (65.4 ± 0.307) a	498 ± 3.59 a (472 ± 3.99) b	nq (5.51 ± 0.066)	
3	nd (4.92 ± 0.062)	nq (9.98 ± 0.036)	nq (13.7 ± 0.153)	61.9 ± 2.57 a (64.2 ± 0.091) a	nq (8.48 ± 0.184)	nq (9.36 ± 0.096)	43.7 ± 0.005 b (49.3 ± 0.140) a	383 ± 11.1 b (466 ± 3.64) a	10.6 ± 0.062 b (11.0 ± 0.028) a	
4	nd (nd)	nq (8.71 ± 0.082)	nq (7.28 ± 0.061)	45.2 ± 0.416 b (50.6 ± 0.043) a	nq (7.76 ± 0.004)	nd (nd)	40.8 ± 0.206 b (43.0 ± 0.159) a	370 ± 3.89 a (358 ± 1.20) b	nd (nd)	
5	nq (5.94 ± 0.013)	nq (9.52 ± 0.118)	nq (15.6 ± 0.139)	55.3 ± 3.98 b (60.3 ± 0.115) a	nq (8.17 ± 0.102)	nq (9.29 ± 0.031)	46.7 ± 0.376 a (47.3 ± 0.029) a	449 ± 4.66 a (426 ± 2.87) b	10.5 ± 0.254 a (10.3 ± 0.128) a	
6	nd (nd)	nq (7.27 ± 0.038)	23.4 ± 0.887 a (24.2 ± 0.740) a	31.8 ± 2.87 a (41.3 ± 0.040) a	nq (6.14 ± 0.004)	nq (5.11 ± 0.005)	31.5 ± 0.578 a (33.5 ± 0.013) a	423 ± 11.2 a (392 ± 0.030) a	nd (nd)	
7	nd (nd)	nq (8.92 ± 0.077)	nq (16.3 ± 0.279)	44.4 ± 0.078 b (54.7 ± 0.073) a	nq (7.73 ± 0.066)	nq (7.20 ± 0.057)	30.9 ± 2.49 a (44.2 ± 0.011) a	491 ± 2.85 a (496 ± 7.41) a	nq (9.03 ± 0.010)	
8	nd (nd)	10.6 ± 1.22 a (11.0 ± 0.033) a	nq (10.8 ± 0.235)	59.1 ± 0.526 b (62.5 ± 0.218) a	nq (9.78 ± 0.115)	nq (6.00 ± 0.109)	48.1 ± 2.63 a (48.8 ± 0.050) a	517 ± 0.260 a (478 ± 1.27) b	nq (6.67 ± 0.038)	
9	nd (nd)	nq (7.93 ± 0.041)	nq (8.97 ± 0.091)	43.2 ± 0.022 b (47.6 ± 0.154) a	nq (6.77 ± 0.016)	nd (nd)	34.1 ± 0.554 b (38.9 ± 0.158) a	534 ± 4.47 a (507 ± 0.545) b	nd (nd)	
10	nd (nd)	10.4 ± 1.07 a (13.2 ± 0.004) a	nq (9.40 ± 0.237)	nq (69.6 ± 0.020)	10.1 ± 0.298 a (11.4 ± 0.047) a	nq (4.36 ± 0.044)	56.7 ± 0.501 a (54.7 ± 0.017) a	560 ± 2.21 a (473 ± 0.469) b	nq (4.82 ± 0.000)	
^d Mean	-	11.4 ± 2.16 a (13.3 ± 3.56) a	20.2 ± 3.07 a (19.7 ± 3.59) a	51.1 ± 10.6 b (59.2 ± 13.4) a	11.1 ± 1.48 a (13.0 ± 2.19) a	-	43.0 ± 8.59 b (47.4 ± 8.70) a	458 ± 72.9 a (440 ± 55.7) a	10.5 ± 0.070 a (10.6 ± 0.495) a	

^a Beelders, T., Sigge, G.O., Joubert, E., de Beer, D., de Villiers, A., *J. Chromatogr. A* 2012, 1219, 128-139.

^b Compound abbreviations are defined in **Tables 3.1.** and **S1..**

^c nd = not detected; nq = not quantified.

^d Different letters indicate significant (P < 0.05) differences between values obtained using CZE and HPLC.

Table S4. Summary of quantitative data (in µg/mL) obtained by using the optimised CZE method and a routine HPLC method^a for the principal phenolics in fermented rooibos tea samples. CZE values are listed above HPLC data (in brackets) for the same compound in the same sample (n = 2 replicates for each method).

Sample number	^b Compounds									
	Lut-7-glc	Isovitol	Rut	Isoorient	Vit	Isoquer	Orient	Asp	Hyp	
1	^c nd (nd)	^c nq (8.41 ± 0.107)	nq (9.11 ± 0.082)	nq (50.2 ± 0.040)	nq (8.33 ± 0.120)	nd (3.14 ± 0.073)	41.1 ± 0.893 b ^d (44.1 ± 0.219) a	79.3 ± 0.890 a (70.2 ± 0.377) b	nd (nd)	
2	nq (5.08 ± 0.008)	nq (7.51 ± 0.000)	nq (8.45 ± 0.017)	46.1 ± 0.002 b (53.0 ± 0.136) a	nq (7.04 ± 0.044)	nq (7.49 ± 0.089)	40.4 ± 0.407 b (43.0 ± 0.097) a	93.0 ± 1.33 a (76.3 ± 0.904) b	9.50 ± 0.295 a (9.40 ± 0.018) a	
3	nd (4.59 ± 0.066)	10.5 ± 0.460 b (13.6 ± 0.180) a	nq (8.74 ± 0.122)	nq (67.2 ± 0.263)	11.3 ± 0.378 a (11.8 ± 0.139) a	nq (4.29 ± 0.157)	49.6 ± 0.143 b (52.8 ± 0.158) a	93.5 ± 0.230 a (81.4 ± 0.033) b	nq (3.61 ± 0.166)	
4	nd (nd)	nq (7.00 ± 0.039)	nq (3.59 ± 0.067)	nq (41.1 ± 0.0735)	nq (7.24 ± 0.047)	nd (nd)	38.0 ± 0.277 a (38.0 ± 0.120) a	50.4 ± 0.036 a (38.0 ± 0.008) b	nd (nd)	
5	nq (6.60 ± 0.105)	nq (7.62 ± 0.098)	nq (9.23 ± 0.053)	nq (47.5 ± 0.016)	nq (7.43 ± 0.125)	nq (7.34 ± 0.154)	39.9 ± 0.141 a (40.1 ± 0.096) a	53.7 ± 1.18 a (45.7 ± 0.141) b	nq (7.98 ± 0.184)	
6	nd (nd)	nq (6.58 ± 0.105)	nq (17.3 ± 0.121)	36.0 ± 0.280 b (38.1 ± 0.089) a	nq (5.91 ± 0.346)	nq (5.36 ± 0.048)	39.9 ± 0.160 a (32.0 ± 0.331) b	85.1 ± 1.46 a (69.4 ± 0.163) b	nd (nd)	
7	nd (nd)	nq (7.72 ± 0.164)	nq (11.9 ± 0.000)	nq (49.0 ± 0.147)	nq (7.25 ± 0.082)	nq (5.69 ± 0.117)	29.8 ± 0.627 b (40.1 ± 0.093) a	70.6 ± 0.537 b (75.1 ± 0.048) a	nq (6.46 ± 0.050)	
8	nd (4.10 ± 0.036)	nq (8.50 ± 0.054)	nq (5.50 ± 0.033)	nq (53.3 ± 0.126)	nq (8.15 ± 0.051)	nd (4.53 ± 0.036)	41.6 ± 0.141 b (42.6 ± 0.061) a	62.5 ± 1.19 a (57.6 ± 0.269) b	nq (5.81 ± 0.036)	
9	nd (nd)	nq (6.26 ± 0.012)	nq (6.10 ± 0.063)	nq (40.3 ± 0.055)	nq (5.87 ± 0.149)	nd (nd)	32.1 ± 0.595 b (34.4 ± 0.071) a	119 ± 3.82 a (113 ± 1.41) a	nd (nd)	
10	nd (nd)	10.5 ± 1.19 a (9.76 ± 0.052) a	nq (5.13 ± 0.181)	nq (57.8 ± 0.096)	10.8 ± 0.859 a (9.11 ± 0.241) a	nd (3.30 ± 0.148)	41.6 ± 0.142 b (48.1 ± 0.200) a	57.7 ± 0.575 a (54.2 ± 0.419) b	nq (3.73 ± 0.121)	
^d Mean	-	10.5 ± 0.032 a (11.7 ± 2.71) a	-	41.1 ± 5.88 a (45.6 ± 8.57) a	11.1 ± 0.354 a (10.5 ± 1.90) a	-	39.4 ± 5.44 a (41.5 ± 6.12) a	76.5 ± 21.7 a (68.1 ± 21.1) b	-	

^a Beelders, T., Sigge, G.O., Joubert, E., de Beer, D., de Villiers, A., *J. Chromatogr. A* 2012, 1219, 128-139.

^b Compound abbreviations are defined in **Tables 3.1.** and **S1..**

^c nd = not detected; nq = not quantified.

^d Different letters indicate significant (P < 0.05) differences between values obtained using CZE and HPLC.

Table S5. Summary of quantitative data (in µg/mL) obtained using the optimised CZE method and a routine HPLC method^a for the principal phenolics in unfermented *Cyclopia subternata* samples. CZE values are listed above HPLC data (in brackets) for the same compound in the same sample (n = 2 replicates for each method).

Sample number	^b Compounds							
	Hd	Scoly	Eriot	Phlor-di-glc	Iri-di-glc	Mang	Isomang	Iri-glc
1	22.3 ± 0.032 b ^c (27.2 ± 0.129) a	97.0 ± 0.659 b (105 ± 0.159) a	30.7 ± 0.227 b (44.1 ± 0.486) a	145 ± 0.792 b (167 ± 0.036) a	20.6 ± 0.537 b (24.5 ± 0.515) a	62.8 ± 0.428 b (78.9 ± 0.019) a	35.2 ± 0.145 a (35.6 ± 0.128) a	120 ± 0.686 b (136 ± 0.590) a
2	20.6 ± 0.295 b (23.8 ± 0.072) a	26.7 ± 1.39 a (25.1 ± 0.470) a	25.1 ± 0.240 b (32.0 ± 0.178) a	43.6 ± 0.097 b (49.3 ± 0.141) a	9.18 ± 0.013 b (11.7 ± 0.166) a	89.1 ± 0.688 b (97.9 ± 0.226) a	30.0 ± 0.566 b (34.3 ± 0.168) a	30.6 ± 0.002 b (34.0 ± 0.048) a
3	75.2 ± 0.051 a (72.2 ± 0.025) b	20.3 ± 0.374 b (23.7 ± 0.014) a	14.5 ± 0.272 b (20.4 ± 0.275) a	108 ± 2.75 a (115 ± 0.072) a	56.7 ± 0.295 a (56.7 ± 0.825) a	62.1 ± 0.103 b (67.7 ± 0.356) a	29.3 ± 0.223 a (28.0 ± 0.062) b	114 ± 0.824 a (105 ± 0.013) b
4	28.0 ± 0.602 a (28.8 ± 0.148) a	134 ± 0.266 b (141 ± 0.278) a	43.0 ± 0.056 b (49.0 ± 0.273) a	132 ± 3.05 a (143 ± 0.046) a	49.6 ± 0.499 b (64.5 ± 0.108) a	43.9 ± 0.278 b (47.0 ± 0.107) a	23.4 ± 0.028 a (21.3 ± 0.083) b	98.1 ± 0.329 a (84.9 ± 0.017) b
5	19.9 ± 0.057 a (19.3 ± 0.137) b	66.5 ± 0.136 b (69.1 ± 0.038) a	22.4 ± 0.121 b (28.8 ± 0.151) a	123 ± 1.08 a (126 ± 0.274) a	9.79 ± 0.384 a (10.8 ± 0.250) a	88.8 ± 0.028 b (93.6 ± 0.161) a	30.6 ± 0.047 a (30.0 ± 0.232) a	39.8 ± 0.471 b (46.9 ± 0.257) a
6	28.2 ± 0.060 b (32.5 ± 0.051) a	94.4 ± 0.712 b (112 ± 0.387) a	34.0 ± 0.618 b (43.1 ± 0.351) a	73.3 ± 0.833 b (82.5 ± 0.023) a	32.3 ± 0.166 b (39.5 ± 0.079) a	34.6 ± 0.267 b (41.1 ± 0.014) a	20.1 ± 0.819 a (20.8 ± 0.105) a	^d nq (26.3 ± 0.007)
7	20.9 ± 1.34 a (21.1 ± 0.108) a	151 ± 2.23 b (159 ± 0.305) a	41.4 ± 0.747 b (48.7 ± 0.404) a	143 ± 0.126 b (150 ± 0.173) a	74.3 ± 1.11 a (78.9 ± 0.041) a	44.5 ± 0.429 a (48.1 ± 0.014) a	22.6 ± 0.488 a (22.1 ± 0.012) a	nq (33.9 ± 0.235)
8	24.2 ± 0.010 a (24.8 ± 0.252) a	147 ± 1.20 b (171 ± 0.466) a	47.6 ± 0.486 b (53.3 ± 0.224) a	66.9 ± 0.102 b (76.9 ± 0.465) a	41.5 ± 0.499 a (45.7 ± 1.43) a	74.9 ± 1.70 b (82.5 ± 0.390) a	35.4 ± 0.778 a (37.1 ± 0.246) a	63.8 ± 0.028 b (70.7 ± 0.291) a
9	71.1 ± 0.611 a (67.2 ± 0.112) b	33.7 ± 0.210 b (40.8 ± 0.047) a	57.3 ± 0.114 b (65.7 ± 0.424) a	148 ± 0.160 a (149 ± 0.073) a	26.9 ± 0.118 a (26.9 ± 0.050) a	57.9 ± 0.756 a (60.0 ± 0.060) a	29.2 ± 0.346 a (28.3 ± 0.032) a	66.5 ± 0.182 b (70.7 ± 1.34) a
10	20.4 ± 1.79 a (17.0 ± 0.025) b	85.8 ± 2.44 a (86.4 ± 0.014) a	40.9 ± 0.325 b (46.2 ± 0.098) a	169 ± 0.452 a (170 ± 0.146) a	45.5 ± 0.447 a (44.7 ± 0.079) a	52.4 ± 0.400 b (57.5 ± 0.147) a	29.1 ± 0.106 a (26.1 ± 0.007) b	96.4 ± 0.231 a (97.5 ± 0.604) a
^c Mean	33.1 ± 21.3 a (33.4 ± 19.7) a	85.6 ± 48.7 b (93.3 ± 53.7) a	36.2 ± 13.5 b (43.0 ± 13.9) a	112 ± 42.0 b (118 ± 40.3) a	35.2 ± 21.6 b (37.7 ± 22.0) a	60.9 ± 19.7 b (66.2 ± 20.8) a	28.5 ± 5.07 a (28.4 ± 5.95) a	78.6 ± 33.4 a (80.7 ± 32.7) a

^ade Beer,D., Schulze, A.E., Joubert, E., de Villiers, A., Malherbe, C.J., Stander, M.A., *Molecules* 2012, 17, 14602-14624.

^b Compound abbreviations are defined in **Tables 3.1.** and **S2..**

^c Different letters indicate significant (P < 0.05) differences between values obtained using CZE and HPLC.

^d nq = not quantified.

Table S6. Summary of quantitative data (in µg/mL) obtained using the optimised CZE method and a routine HPLC method^a for the principal phenolics in fermented *Cyclopia subternata* samples. CZE values are listed above HPLC data (in brackets) for the same compound in the same sample (n = 2 replicates for each method).

Sample number	^b Compounds						
	Hd	Scoly	Eriot	Phlor-di-glc	Iri-di-glc	Mang	Isomang
1	16.0 ± 0.377 b ^c (17.7 ± 0.086) a	63.5 ± 3.52 a (78.0 ± 0.045) a	23.2 ± 0.848 b (27.4 ± 0.433) a	31.4 ± 0.191 b (34.6 ± 0.024) a	33.4 ± 0.550 a (33.9 ± 1.11) a	^d nq (6.33 ± 0.050)	9.36 ± 0.403 a (10.8 ± 0.014) a
2	16.2 ± 0.173 b (18.9 ± 0.030) a	86.0 ± 1.15 b (102 ± 0.064) a	27.4 ± 0.106 b (34.5 ± 0.552) a	36.1 ± 0.271 b (38.7 ± 0.048) a	56.1 ± 0.281 b (64.7 ± 0.087) a	nq (5.09 ± 0.004)	6.90 ± 0.533 a (7.44 ± 0.044) a
3	38.7 ± 0.733 a (40.5 ± 0.008) a	16.6 ± 0.786 b (21.3 ± 0.332) a	9.74 ± 0.046 b (14.1 ± 0.076) a	nq (17.7 ± 0.003)	51.7 ± 0.033 b (61.4 ± 0.222) a	nq (5.79 ± 0.059)	7.41 ± 0.205 b (8.40 ± 0.048) a
4	13.4 ± 0.172 a (13.5 ± 0.011) a	19.5 ± 0.409 a (20.0 ± 0.095) a	21.5 ± 0.264 b (24.0 ± 0.094) a	nq (12.1 ± 0.020)	nq (12.7 ± 0.251)	nq (7.71 ± 0.012)	10.3 ± 0.849 a (10.1 ± 0.036) a
5	16.5 ± 0.647 a (17.9 ± 0.014) a	25.5 ± 0.420 b (34.9 ± 0.461) a	11.5 ± 0.034 b (18.8 ± 0.346) a	35.6 ± 1.64 a (36.4 ± 0.072) a	10.0 ± 0.128 b (11.9 ± 0.118) a	nq (6.49 ± 0.020)	7.52 ± 0.036 b (8.07 ± 0.031) a
6	22.5 ± 0.366 b (26.6 ± 0.027) a	64.3 ± 0.482 b (79.4 ± 0.078) a	20.3 ± 0.296 b (30.4 ± 0.124) a	nq (22.8 ± 0.0171)	37.0 ± 0.174 b (44.7 ± 0.038) a	nq (3.77 ± 0.035)	5.90 ± 0.105 b (6.31 ± 0.015) a
7	14.1 ± 0.756 a (15.6 ± 0.022) a	78.1 ± 0.796 b (97.3 ± 0.032) a	25.2 ± 0.338 b (33.4 ± 0.008) a	29.7 ± 0.480 a (31.2 ± 0.003) a	64.6 ± 0.631 b (72.6 ± 0.019) a	nq (4.58 ± 0.006)	6.46 ± 0.245 a (6.32 ± 0.020) a
8	18.1 ± 0.166 b (19.8 ± 0.011) a	87.8 ± 0.998 b (104 ± 0.081) a	26.5 ± 0.026 b (35.5 ± 0.102) a	nq (10.1 ± 0.010)	34.5 ± 0.438 b (39.8 ± 0.019) a	nq (7.01 ± 0.027)	8.94 ± 0.051 b (10.1 ± 0.042) a
9	38.1 ± 0.598 b (40.5 ± 0.086) a	29.1 ± 0.018 b (32.5 ± 0.014) a	36.5 ± 0.821 b (43.6 ± 0.058) a	27.4 ± 0.217 b (28.3 ± 0.055) a	27.4 ± 0.267 b (29.9 ± 0.022) a	nq (5.33 ± 0.035)	8.30 ± 0.020 a (7.89 ± 0.011) b
10	15.1 ± 0.012 b (16.6 ± 0.027) a	47.8 ± 0.103 b (58.1 ± 0.064) a	23.4 ± 0.065 b (32.1 ± 0.060) a	43.5 ± 0.045 b (52.1 ± 0.021) a	43.4 ± 0.418 b (49.1 ± 0.053) a	nq (4.47 ± 0.006)	7.56 ± 0.070 a (7.58 ± 0.031) a
^c Mean	20.9 ± 9.55 b (22.8 ± 9.94) a	51.8 ± 27.8 b (62.7 ± 33.5) a	22.5 ± 7.71 b (29.4 ± 8.62) a	34.0 ± 5.51 a (36.9 ± 7.92) a	39.8 ± 16.4 b (45.3 ± 19.1) a	-	7.87 ± 1.37 a (8.30 ± 1.56) a

^ade Beer, D., Schulze, A.E., Joubert, E., de Villiers, A., Malherbe, C.J., Stander, M.A., *Molecules* 2012, 17, 14602-14624.

^bCompound abbreviations are defined in **Tables 3.1.** and **S2..**

^cDifferent letters indicate significant (P < 0.05) differences between values obtained using CZE and HPLC.

^dnq = not quantified.

Table S7. Summary of quantitative data (in µg/mL) obtained using the optimised CZE method and a routine HPLC method^a for the principal phenolics in unfermented *Cyclopia maculata* samples. CZE values are listed above HPLC data (in brackets) for the same compound in the same sample ($n = 2$ replicates for each method).

Sample number	^b Compounds				
	Hd	Eriot	Mang	Isomang	Iri-glc
1	50.4 ± 0.319 b ^c (60.5 ± 0.021) a	14.9 ± 0.028 b (20.7 ± 0.275) a	346 ± 1.80 a (351 ± 0.167) a	89.4 ± 0.784 b (98.2 ± 0.015) a	^d nq (23.1 ± 0.170)
2	57.5 ± 0.410 a (56.6 ± 0.275) a	14.9 ± 0.017 b (22.1 ± 0.136) a	263 ± 2.08 b (339 ± 2.21) a	80.9 ± 2.09 b (96.8 ± 0.354) a	nq (27.7 ± 0.860)
3	39.5 ± 0.435 b (49.0 ± 0.087) a	14.4 ± 0.308 b (21.2 ± 0.016) a	391 ± 1.30 a (397 ± 0.021) a	92.9 ± 0.327 b (113 ± 0.049) a	nq (30.6 ± 0.022)
4	58.5 ± 1.86 a (63.9 ± 0.046) a	19.4 ± 0.238 b (25.2 ± 0.124) a	266 ± 2.00 a (282 ± 0.042) a	87.2 ± 1.49 a (85.4 ± 0.075) a	nq (20.7 ± 0.071)
5	48.1 ± 0.230 b (50.2 ± 0.103) a	17.0 ± 0.669 b (22.0 ± 0.140) a	307 ± 3.11 a (324 ± 0.084) a	92.6 ± 0.347 b (93.9 ± 0.138) a	nq (26.6 ± 0.040)
6	54.0 ± 1.18 b (63.3 ± 0.135) a	16.5 ± 1.82 a (20.3 ± 0.152) a	308 ± 4.01 b (375 ± 0.232) a	92.7 ± 0.341 b (106 ± 0.015) a	nq (27.8 ± 0.014)
7	54.1 ± 0.290 b (56.7 ± 0.139) a	19.3 ± 0.251 b (22.6 ± 0.088) a	251 ± 3.59 b (316 ± 0.402) a	98.7 ± 0.146 a (91.5 ± 0.079) a	nq (21.0 ± 0.018)
8	64.9 ± 0.195 a (65.0 ± 0.232) a	17.2 ± 0.079 b (20.6 ± 0.276) a	260 ± 2.33 b (335 ± 0.311) a	82.9 ± 1.38 b (96.2 ± 0.157) a	nq (26.1 ± 0.067)
9	68.3 ± 4.19 a (63.6 ± 0.010) a	20.3 ± 1.58 a (22.6 ± 0.225) a	326 ± 1.62 b (348 ± 0.025) a	112 ± 1.21 a (96.9 ± 0.015) b	nq (23.5 ± 0.050)
^c Mean	55.0 ± 8.69 a (58.8 ± 6.03) a	17.1 ± 2.15 b (21.9 ± 1.51) a	302 ± 47.0 b (341 ± 33.2) a	92.1 ± 9.22 a (97.5 ± 7.87) a	-

^a Schulze, A.E., de Beer, D., de Villiers, A., Manley, M., Joubert, E., *J. Agric. Food Chem.* 2014, 62, 10542-10551.

^b Compound abbreviations are defined in **Tables 3.1.** and **S2.**

^c Different letters indicate significant ($P < 0.05$) differences between values obtained using CZE and HPLC.

^d nq = not quantified.

Table S8. Summary of quantitative data (in µg/mL) obtained using the optimised CZE method and a routine HPLC method^a for the principal phenolics in fermented *Cyclopia maculata* samples. CZE values are listed above HPLC data (in brackets) for the same compound in the same sample (n = 2 replicates for each method).

Sample number	^b Compounds			
	Hd	Eriot	Mang	Isomang
1	31.7 ± 0.066 b ^c (34.1 ± 0.328) a	8.27 ± 0.155 a (9.25 ± 0.151) a	54.1 ± 0.292 a (56.2 ± 0.014) a	37.4 ± 1.31 a (36.7 ± 0.007) a
2	37.0 ± 0.805 a (33.4 ± 0.391) b	8.53 ± 0.018 a (10.7 ± 0.194) a	83.3 ± 3.60 a (85.9 ± 0.036) a	57.1 ± 0.160 a (48.6 ± 0.022) b
3	31.3 ± 2.72 a (35.2 ± 0.007) a	8.38 ± 0.139 b (9.95 ± 0.040) a	52.9 ± 0.448 b (62.4 ± 0.047) a	38.4 ± 1.31 a (41.1 ± 0.032) a
4	34.7 ± 0.499 b (41.7 ± 0.135) a	9.22 ± 0.101 b (12.5 ± 0.025) a	38.5 ± 0.748 b (45.5 ± 0.033) a	30.0 ± 0.783 a (32.8 ± 0.005) a
5	30.4 ± 0.490 b (38.8 ± 0.021) a	8.36 ± 0.351 b (13.2 ± 0.025) a	56.5 ± 1.35 b (75.7 ± 0.135) a	41.6 ± 0.359 b (47.4 ± 0.044) a
6	32.6 ± 1.59 a (32.4 ± 0.252) a	7.42 ± 0.153 b (9.01 ± 0.173) a	49.2 ± 2.82 a (51.2 ± 0.153) a	35.5 ± 1.33 a (34.9 ± 0.050) a
7	31.0 ± 0.096 a (31.4 ± 0.182) a	8.80 ± 0.298 a (10.7 ± 0.007) a	87.1 ± 1.05 a (92.1 ± 0.020) a	50.7 ± 2.07 a (47.8 ± 0.003) a
8	39.7 ± 0.281 a (32.1 ± 0.066) b	8.02 ± 0.095 b (9.22 ± 0.022) a	98.0 ± 0.194 a (88.9 ± 0.119) b	59.9 ± 0.278 a (47.3 ± 0.129) b
9	35.7 ± 0.073 a (25.3 ± 0.257) b	7.61 ± 0.159 b (8.30 ± 0.043) a	77.4 ± 0.489 a (60.3 ± 0.080) b	50.2 ± 0.000 a (35.1 ± 0.057) b
^c Mean	33.8 ± 3.19 a (33.8 ± 4.64) a	8.29 ± 0.556 b (10.3 ± 1.60) a	66.3 ± 20.4 a (68.7 ± 17.4) a	44.5 ± 10.3 a (41.3 ± 6.54) a

^a Schulze, A.E., de Beer, D., de Villiers, A., Manley, M., Joubert, E., *J. Agric. Food Chem.* 2014, 62, 10542-10551.

^b Compound abbreviations are defined in **Tables 3.1.** and **S2.**

^c Different letters indicate significant (P < 0.05) differences between values obtained using CZE and HPLC.

CHAPTER 4

GENERAL CONCLUSIONS AND RECOMMENDATIONS

4.1. General conclusions and future recommendations

The phenolic content of herbal teas is considered to be one of their most important characteristics, since phenolic compounds are responsible for many of the health promoting properties of these popular products. The phenolic composition of teas and herbal teas has therefore been studied extensively. Rooibos and honeybush, two globally popular South African herbal teas, have been found to contain high amounts of phenolics, including some unique compounds of this class.

Phenolics are most often analysed by reversed phase high performance liquid chromatography (RP-HPLC), which, although robust and sensitive, is relatively expensive and sometimes fails to resolve all compounds of interest in complex mixtures of phenolics such as encountered in rooibos and honeybush. Therefore, the main goal of this study was to explore the use capillary electrophoresis (CE) as a relatively inexpensive and fast alternative to HPLC for the analysis phenolics in these products.

Two cost-effective quantitative capillary zone electrophoresis (CZE) methods for the analysis of major phenolics in fermented and unfermented rooibos and honeybush tea were successfully developed for the first time. Systematic optimisation of major experimental variables that play a key role in separation in CZE, namely the background electrolyte (BGE) composition, pH, ionic strength, temperature, voltage, capillary dimensions and injection time was performed. The use of a borate BGE was found to be essential for the efficient separation of rooibos and honeybush phenolics. The optimal ionic strength was relatively high (200 mM), which was required to resolving several closely migrating compounds. The optimal pH was different for both methods (8.80 and 9.25 for rooibos and honeybush, respectively), reflecting the different phenolic constituents of these samples. Finally, relatively long capillaries of 80 cm effective length were used to provide optimal resolution.

The optimised CZE methods provided efficient separation of the fifteen principal rooibos and eight honeybush tea phenolics, respectively. Both methods offered slightly shorter analysis times than routine HPLC methods currently used for this application. Resolution and efficiency, evaluated using reference standards, were found to be good and generally higher than obtained by HPLC. Validation of the methods proved good linearity of calibration curves. Good reproducibility was achieved with %RSDs of electrophoretic mobilities and corrected areas ranging from 0.9-1.9% and 0.49-15.3%, respectively. The limits of detection (LODs) (0.73-14.9 µg/mL) and limits of quantification (LOQs) (2.45-49.7 µg/mL) were sufficient to allow quantification of the majority of compounds in real samples, with a few notable exceptions (luteolin-7-*O*-glucoside, rutin, isoquercitrin, luteolin and quercetin in rooibos and phloretin-3',5'-di-*C*-glucoside and iriflphenone-3-*C*-glucoside for honeybush). However, the sensitivity for both methods was lower than achieved by HPLC for all compounds. The optimised methods were subsequently used for the quantitative analysis of fermented and unfermented rooibos and honeybush samples.

With few exceptions, the quantitative results were statistically comparable to those obtained by reference HPLC methods. Aspalathin, the major phenolic constituent in rooibos tea, was over-estimated in fermented samples by CZE, possibly due to co-migration with an unidentified compound, although quantitative data for unfermented samples were similar to those obtained by HPLC. Luteolin-7-*O*-glucoside, rutin, vitexin, isoquercitrin and hyperoside were not quantified as a result of their low levels in some samples, while ferulic acid, chrysoeriol, luteolin and quercetin were not detected in any of the rooibos tea samples. Isoorientin co-migrated with a possible flavonol derivative, suspected to be quercetin-3-*O*-robinobioside, in most fermented samples. Apart from these discrepancies, quantitative data for the major phenolics in both fermented and unfermented samples were statistically comparable to those obtained by HPLC, indicating the suitability of the developed method for routine quantitative analysis of these samples.

Quantitative data for honeybush phenolics obtained using the developed CZE method were generally more comparable with those obtained using the HPLC reference method. The only exceptions were scolymoside and eriocitrin, which were under-estimated in most samples, possibly due to co-elution in HPLC. The same phenomenon was observed for mangiferin in unfermented *C. maculata* samples. Iriflophenone-3-*C*-glucoside was not detected in any of the fermented honeybush samples, as was the case for HPLC. Similar quantitative data for fermented and unfermented *C. subternata* and *C. maculata* samples were obtained compared to HPLC, again confirming the applicability of the CZE method as a generic separation method for honeybush tea.

Lower levels of most of the compounds in fermented rooibos and honeybush samples were observed, owing to oxidation occurring during fermentation. The exceptions were orientin and iriflophenone-3-*C*- β -glucoside-4-*O*- β -glucoside. Furthermore, significant variation in phenolic composition between the two *Cyclopia* species analysed (*C. subternata* and *C. maculata*) was also observed, in line with reported data for these samples. For example, scolymoside, phloretin-3',5'-di-*C*- β -glucoside and iriflophenone-3-*C*- β -glucoside-4-*O*- β -glucoside were not found in *C. maculata* samples.

In conclusion, the reported work demonstrates for the first time the potential of CE as alternative to HPLC for the analysis of herbal tea phenolics. The CZE methods developed offer cheaper routine operation, no solvent consumption and are slightly faster than the HPLC methods. Compared to HPLC methods, however, the CZE methods are less sensitive and reproducible, two inherent limitations of CE compared to HPLC. Another potential drawback of the CZE methods used here is that they are not compatible with ESI-MS detection due to the use of borate BGEs. This is a common constraint for CZE methods for the analysis of phenolics. Despite these shortcomings, both methods were found to be fit for routine use as possible cheap alternatives to HPLC for the analysis of major phenolics in rooibos and honeybush teas, for example in support of quality control purposes and to monitor production processes.

Possible future work in the field should focus on extending the applicability of the developed CZE methods to include the quantification of PPAG, nothofagin and another major rooibos constituent, quercetin-3-*O*-robinobioside. These analytes were not quantified in the current work due to lack of standards. Similarly, analysis and quantification of additional honeybush phenolics such as apigenin-6,8-di-*C*-hexoside (vicenin-2), 3-hydroxyphloretin-3',5'-di-*C*-glucoside and maclurin-3-*C*-glucoside should also be addressed in further work. Evaluation of CZE for analysis of phenolics in other commercially important *Cyclopia* species (*C. genistoides*, *C. intermedia*, *C. longifolia* and *C. sessiliflora*) should also be considered to confirm initial indications that the CZE method, unlike HPLC, might be equally applicable to all species.