

**HPLC METHOD DEVELOPMENT FOR
THE CHARACTERISATION OF THE
FLAVONOID AND PHENOLIC ACID
COMPOSITION OF ROOIBOS
(*ASPALATHUS LINEARIS*) INFUSIONS**

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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SUMMARY

Rooibos tea, produced from the endemic South African fynbos plant *Aspalathus linearis*, has various health-promoting benefits which can largely be attributed to its phenolic composition. In this study, the reversed-phase liquid chromatographic (RP-LC) separation of the principal phenolic constituents of aqueous rooibos infusions was optimised on conventional high-performance liquid chromatography (HPLC) instrumentation. The phenolic constituents comprised dihydrochalcones (aspalathin and nothofagin), flavones (orientin, isorientin, vitexin, isovitexin, luteolin, luteolin-7-O-glucoside and chrysoeriol), flavonols (quercetin, isoquercitrin, hyperoside and rutin), a hydroxycinnamic acid (ferulic acid) and a phenylpropanoid (phenylpyruvic acid glucoside, PPAG). A systematic approach towards method development was adopted: resolution was optimised by simultaneous optimisation of gradient conditions and temperature on a sub-2 μm phase to exploit the benefits of this phase for fast routine analyses. The developed HPLC method, utilising photodiode-array (PDA) detection, yielded complete separation of the 15 target analytes on the 1.8 μm C18 column, thermostatted to 37°C, within 37 min (total analysis time: 50 min). The method was successfully validated and demonstrated its suitability for the fast, quantitative analysis of aqueous infusions of unfermented and fermented rooibos. Mass spectrometric (MS) and tandem MS detection confirmed peak purity and permitted the tentative identification of 13 additional phenolic compounds, including a flavonol O-diglycoside (quercetin-3-O-robinobioside), a luteolin-6-C-pentoside-8-C-hexoside and a novel C-8-hexosyl derivative of aspalathin reported here for the first time.

The HPLC-PDA method was subsequently applied to a large number of fermented rooibos samples representative of different production seasons (2009, 2010 and 2011) and quality grades (grades A, B, C and D) to capture as much potential variation in the phenolic composition as possible. Production season had no clear effect on the levels of the individual phenolic compounds in 'cup-of-tea' rooibos infusions, whilst high quality tea (grades A and B) was associated with higher levels of phenolic compounds and soluble solids than low quality tea (grades C and D). Steam-pasteurisation of the plant material, required to obtain a product of high microbiological quality, induced significant reductions in the mean values of most of the phenolic compounds in rooibos infusions. The major phenolic constituents of steam-pasteurised, fermented rooibos were isorientin and orientin, whilst quercetin-3-O-robinobioside, PPAG and aspalathin were also present in high concentrations. Representative content values of the major phenolic compounds present in a typical 'cup-of-tea' rooibos infusion were thus obtained and the generated data are suitable for inclusion in food composition databases.

The application of comprehensive two-dimensional liquid chromatography (LCxLC) was investigated as an alternative approach for the detailed investigation of rooibos phenolics. The combination of hydrophilic interaction chromatography (HILIC) in the first dimension and RP-LC in the second dimension offered different separation selectivities and hence a high degree of orthogonality. HILICxRP-LC provided a significant improvement in resolution, as is evident from practical peak capacities in excess of 2000 and 800 for the off-line and on-line methods, respectively. Further optimisation, particularly of the first dimension separation, is however required to improve the LCxLC separation of complex rooibos phenolic fractions.

OPSOMMING

Gebruik van rooibostee, berei vanaf die eg Suid-Afrikaanse fynbosplant *Aspalathus linearis*, hou verskeie gesondheidsvoordele in wat grootliks toegeskryf kan word aan sy fenoliese samestelling. Die skeiding van die hoof fenoliese verbindings van 'n koppie rooibos is in hierdie studie deur middel van omgekeerde-fase vloeistof chromatografie (RP-LC) op konvensionele hoë-druk vloeistof chromatografie (HPLC) toerusting geoptimeer. Die fenoliese verbindings was verteenwoordigend van dihidrogalkone (aspalatien en notofagien), flavone (orientien, isoorientien, viteksien, isoviteksien, luteolien, luteolien-7-O-glukosied en krisoeriol), flavonole (kwersetien, isokwersetien, hiperosied en rutien), 'n hidrosiekaneulsuur (ferulasuur) en 'n fenielpropanoïed (fenielpirodruiwesuurglukosied, PPAG). Die ontwikkeling van die metode was sistematies benader: resolusie is op 'n geselekteerde 1.8 μm stationêre fase met welombekende kinetiese voordele geoptimeer deur die gradiëntkondisies en kolomtemperatuur gelyktydig te optimeer. Die ontwikkelde HPLC metode, gekoppel aan ultraviolet-fotodiode deteksie (PDA), het binne 37 min (totale analiese tyd: 50 min) volledige skeiding van die 15 standaard verbindings op die 1.8 μm C18 kolom teen 37°C bewerkstellig. Die metode is suksesvol gevalideer en het sy toepaslikheid vir vinnige, kwantitatiewe analiese van ongefermenteerde en gefermenteerde rooibos gedemonstreer. Piek suiwerheid is deur middel van massa spektrometrie (MS) en tandem MS bevestig, wat ook die identifikasie van 13 addisionele verbindings toegelaat het, insluitende 'n flavonol O-diglukosied (kwersetien-3-O-robinobiosied), 'n luteolien-6-C-pentosied-8-C-heksosied en 'n unieke C-8-heksosiel afgeleide van aspalatien wat vir die eerste keer hier gemeld is.

Die geoptimeerde HPLC-PDA metode is gevolglik toegepas vir die analiese van 'n groot aantal gefermenteerde rooibos monsters, verteenwoordigend van verskillende produksie seisoene (2009, 2010 en 2011) en kwaliteitsgrade (A, B, C en D). Hierdie lukraak-geselekteerde monsters het soveel as moontlik potensiële variasie in die fenoliese samestelling verseker. Produksie seisoen het geen definitiewe effek op die vlakke van die individuele fenoliese verbindings in 'n koppie rooibos gehad nie, terwyl hoë kwaliteit rooibos (grade A en B) geassosieër was met hoër vlakke van die individuele fenoliese verbindings en oplosbare vastestowwe in vergelyking met lae kwaliteit rooibos (grade C en D). Stoompasteurisasie van rooibos plantmateriaal, noodsaaklik om 'n produk van hoë mikrobiologiese gehalte te verseker, het gelei tot 'n betekenisvolle afname in meeste fenoliese verbindings in 'n koppie rooibos. Die hoof fenoliese verbindings van 'n koppie stoom-gepasteuriseerde, gefermenteerde rooibos was orientien en isoorientien, terwyl kwersetien-3-O-robinobiosied, PPAG en aspalatien ook in noemenswaardige hoeveelhede aanwesig was. Verteenwoordigende waardes van die hoof fenoliese verbindings aanwesig in 'n tipiese koppie rooibos is derhalwe verkry en die data is geskik vir insluiting in voedsel-samestelling databasisse.

Die analiese van rooibos fenole met omvattende twee-dimensionele vloeistof chromatografie (LCxLC) is bestudeer as 'n alternatiewe metode om verdere insig tot hierdie komplekse fenoliese fraksie te verkry. Die kombinasie van hidrofiliese interaksie chromatografie (HILIC) in die eerste dimensie en RP-LC in die tweede dimensie het 'n uiters gesogte lae graad van korrelasie verskaf. HILICxRP-LC het 'n besondere toename in resolusie teweeg gebring, gekenmerk deur praktiese piek kapasiteite hoër as 2000 en 800 vir die af-lyn en aan-lyn metodes, onderskeidelik. Verdere optimisering, veral van die eerste dimensie skeiding, is egter nodig om die LCxLC skeiding van rooibos fenole te verbeter.

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CHAPTER 1

General Introduction

The herbal tea, rooibos, is prepared from the unfermented green and fermented oxidised plant material of *Aspalathus linearis*, a leguminous shrub endemic to the Cape Floristic Region in South Africa (Dahlgren, 1968). Rooibos is recognised as one of the few economic plants that have made the transition from a local wild resource to a cultivated crop in the 20th century and the industry has seen tremendous growth, particularly in the international market segment. Total exports from South Africa were recorded at 6043 tons in 2010, relating to an estimated R120 million in foreign earnings, while current export until June 30th 2011 stands at 3293 tons (data from the Perishable Products Export Control Board, PPECB, supplied by South African Rooibos Council, SARC). The utilisation of rooibos has also been extended to intermediate value-added products such as extracts destined for the beverage, food, nutraceutical and cosmetic markets while its potential as phytopharmaceutical, shown in recent scientific studies, has yet to be exploited (Joubert & De Beer, 2011).

Rooibos tea is a unique beverage with a characteristic, sweet taste and is most notably characterised by the lack of caffeine and comparatively low tannin content (Blommaert & Steenkamp, 1978; Morton, 1983). Rooibos principally contains single ring phenolic acids and monomeric flavonoids from subclasses such as dihydrochalcones, flavanones, flavones and flavonols. The phenylpropanoid, phenylpyruvic acid glucoside, as well as coumarins, lignans and flavan-3-ols, are also typically present (Joubert *et al.*, 2008). The presence of other phenolic compounds such as di-C-glycosyl flavones in rooibos has also been established recently (Stalmach *et al.*, 2009; Breiter *et al.*, 2011; Iswaldi *et al.*, 2011). The flavonoid composition of rooibos is unique in that it contains the novel dihydrochalcone C-glucoside, aspalathin (Koeppen & Roux, 1965) and the cyclic dihydrochalcone, aspalalinin (Shimamura *et al.*, 2006). Rooibos also represents a source of C-glycosidic flavones (orientin, isoorientin, vitexin and isovitexin) not found in common food sources and thus the daily diet (Joubert, *et al.*, 2009a). Rooibos therefore contributes to a healthy diet by providing both unique and scarce bioactive flavonoids.

Phenolic compounds are regarded as phytochemicals, *i.e.* non-nutrient bioactive compounds that promote optimum human health and reduce the risk of chronic diseases (Gry *et al.*, 2007). In this sense, the various health-promoting benefits of rooibos (as reviewed by Joubert *et al.*, 2008 and Joubert & De Beer, 2011) may largely be attributed to its phenolic composition, as these compounds are known to exert antioxidant, antimutagenic, and phyto-oestrogenic activity, amongst others.

Qualitative and quantitative data on the phenolic constituents of rooibos are required to elucidate their role as potential bioactives in the diet and also to draw comparison with other teas and tea-like infusions. Data available in food composition databases are currently restricted to green, black and oolong teas prepared from *Camellia sinensis* plant material (Gry *et al.*, 2007; Nutrient Data Laboratory, 2007; Neveu *et al.*, 2010; Pérez-Jiménez *et al.*, 2010; De Menezes *et al.*, 2011; Moreno-Franco *et al.*, 2011; Obuchowicz *et al.*, 2011), which clearly illustrates the need for quality data regarding the phenolic content of rooibos infusion. Comprehensive characterisation of the flavonoid and phenolic acid composition of rooibos will furthermore positively impact on product authentication and quality control.

To date, reversed-phase liquid chromatography (RP-LC) coupled with ultraviolet-visible (UV-VIS) spectroscopy and/or mass spectrometry (MS) is the dominant analytical technique for the separation and characterisation of phenolic compounds (Stalikas, 2007). Various RP-LC methods have been employed in the quantitative analysis of rooibos, but these methods are hampered by limited resolution and/or excessive analysis times, restricting their suitability for routine application (Joubert, 1996; Bramati *et al.*, 2002, 2003; Schulz *et al.*, 2003; Kazuno *et al.*, 2005; Joubert *et al.*, 2009b; Stalmach *et al.*, 2009; Breiter *et al.*, 2011).

Recent developments in LC may, however, be used to improve conventional methods for the analysis of phenolics (De Villiers *et al.*, 2010). In this light, the current study will aim at optimising the RP-LC separation of the major phenolic constituents of rooibos on conventional HPLC instrumentation such as is typically available in laboratories charged with routine rooibos analyses. By subsequently validating and applying the optimised method to a large number of fermented rooibos infusions, representative content values on the major phenolic constituents present in a typical 'cup-of-tea' will be obtained. The generated data will accurately reflect natural variation and, due to the appropriate method of analysis, will be suitable for inclusion in food composition tables. This will be the first crucial step in calculating rooibos phenolic intake in populations which will allow the study of their association with health and disease.

In the last instance, the applicability of comprehensive two-dimensional LC (LCxLC) for the analysis of rooibos phenolics will be investigated as LCxLC represents a powerful technique for improved resolution of complex samples (Kalili & De Villiers, 2011). By subjecting rooibos samples to two complementary separations, further insight into its complex phenolic fraction could be obtained, pertaining specifically to minor phenolic constituents possibly impacting on bioactivity.

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CHAPTER 2

Literature Review

This literature review will give an overview of the basic principles regarding high-performance liquid chromatography (HPLC) coupled with ultraviolet-visible photodiode-array (UV-PDA) and mass spectrometric (MS) detection. Special attention will be devoted to HPLC method development as well as the application of HPLC in the separation of phenolic compounds, and their detection and characterisation by UV-PDA and MS detection. Furthermore, the classification of phenolic compounds and their importance from a health perspective will be discussed. An overview of rooibos tea covering various aspects related to the industry, processing, tea quality and chemical composition will be presented. Finally, current reversed-phase liquid chromatographic (RP-LC) methods employed in the quantitative analysis of rooibos phenolics will be discussed, with the emphasis on the limitations of these methods.

1. High-Performance Liquid Chromatography (HPLC)

In liquid chromatography, the separation of analyte molecules is based on their differential partitioning between two non-miscible phases, *i.e.* the stationary phase and the mobile phase. Separation is governed by various interactions between the analyte and the stationary and mobile phases. These include: dispersion interactions (contributing to hydrophobic interactions); dipole-dipole interactions; hydrogen bonding; ionic (coulomb) interactions; and charged transfer or π - π interactions (Snyder *et al.*, 2010). The stationary phase is either a solid, porous or surface-active material in small-particle form or, more commonly, a viscous liquid immobilised on these particles and is fixed in the system. The mobile phase is a liquid which carries the mixture to be separated. Liquid chromatographic techniques that utilise elevated pressure to force the mobile phase through the small-particle packed bed are collectively termed high-performance liquid chromatography (HPLC) (Meyer, 2010).

1.1 Separation modes in HPLC

Various HPLC separation modes have been developed for the analysis of a wide range of different analytes. Separation mechanisms vary from adsorption, partitioning, ionic interaction, size exclusion, etc. The most common LC modes include: normal-phase liquid chromatography (NP-LC); reversed-phase liquid chromatography (RP-LC); non-aqueous reversed-phase (NARP) chromatography; hydrophilic interaction chromatography (HILIC); size-exclusion chromatography (SEC); ion exchange chromatography (IEX); and ion-pair chromatography (IPC) (Snyder *et al.*, 2010). Only RP-LC and HILIC will be addressed in the following sections, since these modes were used in the current study.

1.1.1 Reversed-phase liquid chromatography (RP-LC)

RP-LC is the dominant HPLC mode, especially for the separation of mixtures of organic compounds (Snyder *et al.*, 2010). In RP-LC, the column, packed with derivatised silica particles, acts as a non-polar retaining stationary phase. Support-based stationary phases such as octadecyl (C18), octyl (C8), phenyl-hexyl (Ph-C6) and cyano (CN) phases are commonly employed (see also section 1.2.4). The binary mobile phase is of mid to high polarity and consists of an aqueous phase, which may be slightly acidified, and an organic component. Acetonitrile and methanol are the preferred organic solvents, whilst isopropanol and tetrahydrofuran may also be used (Snyder *et al.*, 2010). RP-LC provides separation according to hydrophobicity – analyte molecules partition between the non-polar stationary phase and the polar mobile phase, leading to increased retention of the less polar analytes (Meyer, 2010; Snyder *et al.*, 2010).

When discussing the mechanism of RP-LC retention, Horváth's solvophobic-interaction model (Horváth *et al.*, 1976, 1977; Melander & Horváth, 1980) is commonly cited: relatively hydrophobic solute molecules align with and adhere to the hydrophobic alkyl ligands attached to the silica surface. The interaction of the analyte molecules may also entail partitioning into the stationary phase and retention at the stationary-mobile phase interface, referred to as adsorption.

1.1.2 Hydrophilic interaction chromatography (HILIC)

HILIC may be regarded as an aqueous variant of NPC. In HILIC mode, the column is polar and the mobile phase is a mixture of water and an organic solvent (Snyder *et al.*, 2010). The type of stationary phase (e.g. cation-exchange, silica gel with low surface concentration of silanol groups, chemically bonded silica and zwitter-ionic phases) and the composition of the mobile phase play an important role in the HILIC retention mechanism and can be selectively adjusted to suit specific separation problems. HILIC columns are more polar than RP columns, and the more polar water serves as the stronger solvent in HILIC. Therefore, an increase in the percentage water typically leads to a decrease in analyte retention. It has been proposed that retention in HILIC entails a combination of adsorption and partitioning of the analyte into an adsorbed water layer that is formed on the surface of the stationary phase. The more polar analytes and/or ionised analytes tend to be more strongly retained, which implies that many samples that exhibit poor retention in RP-LC can be better separated by HILIC. HILIC is also characterised by other potentially advantageous features such as good peak shape for basic analytes and the compatibility with mass spectrometry coupled to LC, amongst others (Jandera & Hájek, 2009; Snyder *et al.*, 2010).

1.2 HPLC instrumentation

The essential components of an HPLC system are illustrated in Figure 1. The mobile phase is drawn from a reservoir via a pump, which controls the flow rate and generates sufficient pressure to drive the mobile phase through the column. An autosampler is used to inject the sample onto the column, which is typically placed within a column oven. The detector responds to changes in column effluent composition during the chromatographic run. A data system monitors the detector output and processes the data. The data system is nowadays often combined with a system controller that directs the functions of the various modules (Snyder *et al.*, 2010).

1.2.1 Solvent reservoir, solvent filtration and solvent degassing

For isocratic applications using a premixed mobile phase, only a single reservoir is required. When mobile phases are blended on-line for use in isocratic or gradient applications more than one reservoir is used. The mobile phase must be free of particulate matter and hence the mobile phase is typically filtered prior to filling the reservoir. The presence of air bubbles in the mobile phase may cause problems in the operation of the HPLC system, especially the pump, and therefore the mobile phase is degassed prior to use. Degassing may be done via ultrasonication and helium sparging, but the most popular technique is in-line degassing with the use of an in-line degasser (Meyer, 2010; Snyder *et al.*, 2010).

Besides selectivity considerations, the selection of the mobile phase is based on criteria such as UV transparency, refractive index, boiling point, purity, inertness with respect to sample analytes, toxicity and price. Mobile phase viscosity is also an important consideration as low-viscosity solvents lead to lower operating pressures. A low-viscosity solvent furthermore allows for faster chromatography as it increases the

diffusion coefficient of the analyte in the mobile phase and hence mass transfer takes place faster (Meyer, 2010).

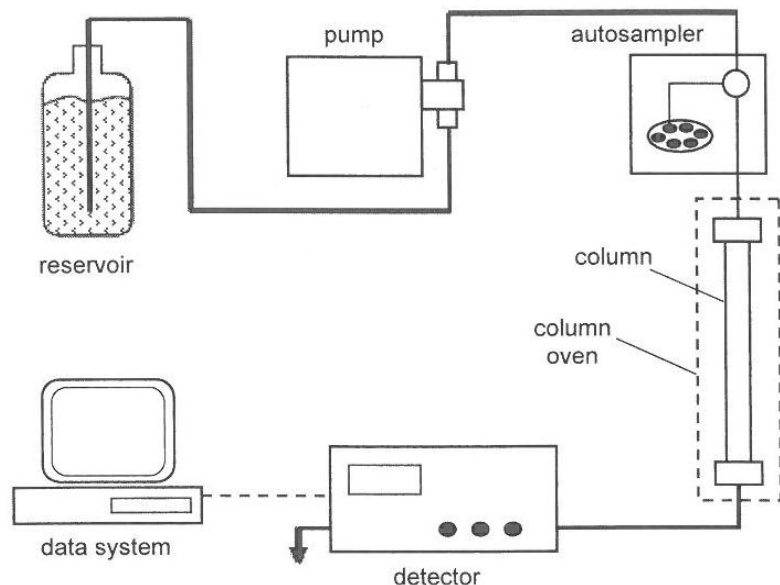


Figure 1 HPLC system diagram (Snyder *et al.*, 2010).

1.2.2 Pumping systems, gradient systems and on-line mixing

The HPLC pump unifies two different features: it must be capable of generating high pressures and must deliver flow accuracy and precision at the selected flow rate (Meyer, 2010). Nearly all HPLC pumping systems utilise some variation of the reciprocating-piston pump because it has a small internal volume and high output pressure. It also delivers constant flow rates and is compatible with gradient elution (Snyder *et al.*, 2010).

During the chromatographic separation process, the composition of the mobile phase may be kept constant (isocratic elution) or altered (gradient elution). The resolution of complex samples containing analytes with widely varying retention characteristics often cannot be accomplished at a constant mobile phase composition and therefore gradient elution is usually preferred. During gradient elution, the concentration of the strong solvent increases, leading to an increase in the elution strength of the mobile phase and a reduction in analyte retention. This also prevents peak broadening and reduces peak tailing effects. Mobile phase gradients can be classified according to the number of mobile phase components (binary, ternary etc.), the shape/profile of the gradient (linear, curved, stepped or segmented), and the chromatographic mode (Jandera, 2006).

Blending of the mobile phase for isocratic and gradient applications typically proceeds via on-line mixing (high-pressure mixing after or low-pressure mixing before the pump) (Snyder *et al.*, 2010).

1.2.3 Autosamplers and injection systems

The introduction of the sample into the column requires that a measured quantity of the sample be added to the flowing, pressurised mobile phase. For routine analysis, sample injection must furthermore be precise, accurate and automatic and hence an autosampler is typically employed. The six-port injection valve is the

core component of an autosampler and may be operated in filled-loop and partial-loop injection modes (Snyder *et al.*, 2010).

1.2.4 Column supports and stationary phases

The column may be considered as the 'heart' of the HPLC system as this is where the separation of analytes takes place. Most HPLC columns are made of stainless steel with column lengths of 50–250 mm and internal diameters (i.d.) of 2.0–4.6 mm (for analytical purposes) (Meyer, 2010).

The most commonly used support for the production of HPLC packings is silica, either in the form of particles or monoliths. Silica-based columns typically provide higher efficiency compared to other inorganic support materials such as zirconia, alumina, titania, and graphitised carbon (Snyder *et al.*, 2010). The utilisation of zirconia-based columns is, however, gaining popularity as it shows excellent thermal stability over a wide range of temperatures, making it ideally suited for high-temperature separations (Cacciola *et al.*, 2007a). Silica particles have high mechanical strength, allowing the formation of packed beds that are stable for long periods and high operating pressures. Spherical particles can be synthesised with a wide choice of pore sizes (e.g. 10, 30, 100 nm), particle sizes (e.g. 1.5, 2.7, 3.5, 5 μm) and in different particle configurations (totally porous, micro-pellicular, superficially porous/shell and perfusion particles). Totally porous silica particles are most common due to greater column capacity and typically have diameters in the 1.5–5 μm range. These particles are prepared by the aggregation of much smaller spheres (Meyer, 2010; Snyder *et al.*, 2010). Particle size is a primary factor in determining the efficiency of the column and will be discussed in section 1.3.1.1. Particle type and its particle-size distribution can also affect column efficiency, presumably by influencing the homogeneity of the packed bed (Snyder *et al.*, 2010); this will not be discussed.

The chemical nature of the unmodified silica surface strongly influences its properties. Hydrated silica is characterised by a surface layer of silanol groups ($-\text{SiOH}$) and three different types of silanol groups may be present: free (non-hydrogen-bonded) silanols, germinal silanols and associated (vicinal) silanols (Meyer, 2010; Snyder *et al.*, 2010). These silanol groups must be present for the reaction of silanes with silica to form a 'bonded' stationary phase.

Reversed phase chromatographic packings are typically made by covalently 'bonding' an organosilane with the silanols on the surface of the silica particle. The silane, denoted as $X_3\text{-Si-R}$, contains a functional group X (often $-\text{Cl}$, $-\text{OEt}$ and/or $-\text{CH}_3$) and a ligand R . Bonded-phase packings are typically made via a monofunctional reaction in which a single silane reagent (e.g. chlorodimethyl-octadecylsilane with $R = \text{C18}$) reacts with a single surface-silanol group, to form a monomeric C18 column (Snyder *et al.*, 2010).

The stationary phase determines retention and column selectivity. Retention typically increases with the carbon number of the silanol substituent and with higher percentage of bonded silanols. The percentage of bonded silanols also influences column performance; free silanols are relatively more acidic and have been associated with lower plate numbers and increased peak tailing of basic solutes (Snyder *et al.*, 2010).

1.2.5 Column ovens

Column temperature plays an important role in HPLC efficiency, retention and selectivity (sections 1.3.1.1 and 1.3.1.2) and hence temperature control is crucial. The use of a column oven (block-heater, air bath or Peltier heater design) is recommended to maintain constant column temperature (Snyder *et al.*, 2010). The

specific type of column oven should be specified, as this might impact on the effective column temperature, which is an important consideration during method transfer (Dolan, 2002; Spearman *et al.*, 2004)

It is also important to preheat the mobile phase entering the column to the temperature of the column (at least within 6°C when temperature > 40°C) to avoid the formation of axial and radial temperature gradients within the column, which leads to peak distortion. The extent of mobile phase preheating is of particular concern during high-temperature liquid chromatography (HTLC) and also during method transfer. If the column oven does not preheat the mobile phase, a simple preheater may be connected to the column inlet and mounted inside the column oven, preferably in close contact with the heated surface (Dolan, 2002; Teutenberg, 2009; Snyder *et al.*, 2010).

1.2.6 Detectors

The chromatographic detector may be defined as 'a transducer that converts a physical or chemical property of an eluted analyte into an electrical signal that can be related to analyte concentration' (Snyder *et al.*, 2010). The detector is positioned directly after the column. The characteristics of an ideal HPLC detector include: high sensitivity and predictable response; respond to all solutes, or else predictable specificity; be unaffected by changes in temperature and carrier composition; not contribute to extra-column peak broadening; be reliable and convenient to use; be robust and cheap; should provide a linear response; be non-destructive; and provide qualitative information on the detected peak (Meyer, 2010; Snyder *et al.*, 2010).

Four general types of detectors are used in HPLC. A *bulk property detector* can be considered as a universal detector as it measures a property that is common to all compounds. Bulk property detectors include the refractive index-, light scattering-, and corona discharge detectors. *Sample-specific detectors* measure a unique sample characteristic and the most commonly used sample-specific detector is the ultraviolet-visible (UV-VIS) detector. Other sample-specific detectors include fluorescence and electrochemical (amperometric) detectors, as well as radioactivity-, conductivity-, chemiluminescent- and chiral detectors. A *mobile phase modification detector*, *i.e.* a reaction detector, may also be used. *Hyphenated techniques*, referring to the coupling of an HPLC instrument to an independent analytical instrument, may also be used to provide detection. Liquid chromatography-mass spectrometry (LC-MS) is the most common hyphenated technique, whilst infrared (FTIR) and nuclear magnetic resonance (NMR) detectors are also commercially available with HPLC interfaces (Snyder *et al.*, 2010). HPLC-UV may also be considered as a hyphenated technique (Meyer, 2010).

Special attention will only be devoted to the instrumental aspects and principles of UV-VIS and MS detection, and their further application in the characterisation of phenolic compounds (section 2.4.2).

1.2.6.1 Ultraviolet-visible (UV-VIS) detection

UV-VIS detection is one of the most commonly used modes of detection in HPLC and is based on the absorption of light in the ultraviolet (UV) and/or visible (VIS) region (190–700 nm). UV detectors are ideal for use with gradient elution, are capable of high sensitivity and are relatively insensitive to changes in mobile-phase flow and temperature. The light source is typically a deuterium lamp, which provides acceptable light intensity from 190–400 nm. When measurements at visible wavelengths (400–700 nm) are required, a higher energy tungsten-halide lamp is often used. Light from the lamp passes through a UV-transmitting flow cell connected to the column and is transmitted onto a diode that measures the light intensity (I). Light from the lamp is also directed to a reference diode for measurement of the original light intensity (I_0). Sample

concentration in the flow cell is then related to the fraction of light transmitted through the cell according to Beer's law (Snyder *et al.*, 1997, 2010):

$$\text{Absorbance, } A = \log \frac{I_0}{I} = c \epsilon b \quad (1)$$

Where:

I_0 = intensity of the incident light

I = intensity of transmitted light

c = sample concentration (moles.L⁻¹)

ϵ = molar absorptivity or molar extinction coefficient (M⁻¹cm⁻¹)

b = cell-path length (cm)

This law states that absorbance is directly proportional to the concentration of the light absorbing species in the sample (c) and the molar absorptivity (ϵ) of the analyte at the specified wavelength. It is therefore imperative that, for this type of detection, the analyte possesses a chromophore which allows UV absorption. Proper selection of the mobile phase, based on acceptable UV-transmittance at the selected wavelength, is also crucial (Snyder *et al.*, 1997, 2010).

UV detectors come in three common configurations namely fixed-wavelength, variable-wavelength and photodiode-array (PDA) detectors. The fixed-wavelength detector relies on distinct wavelengths of light generated from the lamp, whereas the latter two detectors select one or more wavelengths generated from a broad-spectrum lamp. The PDA detector, also called diode-array detector (DAD), allows simultaneous monitoring of different wavelengths during a single chromatographic run. The entire UV spectrum of each analyte is obtained, which may aid in the selection of an optimum wavelength for the HPLC assay and may also provide information on peak-purity. These properties are at the basis of the popularity of the PDA detector (Snyder *et al.*, 1997, 2010).

1.2.6.2 Mass spectrometric (MS) detection

Mass spectrometers consist of three different features: the interface (ion source), where the eluate enters the MS and the ions are generated; the mass analyser, where ions of discrete mass-to-charge (m/z) ratios are separated and focused; and the detector, an electron multiplier which determines the ion beam intensity (Snyder *et al.*, 1997; Meyer, 2010).

LC-MS Interfaces

The primary functions of the interface are to evaporate the mobile phase and to generate analyte ions (McMaster, 2005; Snyder *et al.*, 2010). The mobile phase is converted from liquid to gas phase, leading to an expansion in volume. To reduce the pressure from atmospheric pressure (760 torr) to 10⁻⁵–10⁻⁶ torr, within the 5–10 cm flow path of the interface, most of the vaporised mobile phase and sample is pumped to waste and only a tiny fraction of the sample is drawn into the MS itself (Snyder *et al.*, 2010). It should be noted that typical LC flow rates overwhelm a high-vacuum mass spectrometer interface and therefore the eluent stream is often split prior to entering the interface (McMaster, 2005).

Modern LC-MS systems contain atmospheric pressure ionisation (API) interfaces in which the mobile phase is removed as the analyte molecules are ionised for introduction into the high-vacuum environment of the mass spectrometer analyser. The two most popular types of interfaces are the electrospray ionisation (ESI) and atmospheric-pressure chemical ionisation (APCI) interfaces. Other ionisation techniques include fast-atom bombardment (FAB), thermospray (TSP), laser desorption (LD) and so forth. In selecting an ion source, it must be ensured that the mass range and resolution of the instrument are compatible with the expected molecular weight of the analytes. The ionisation polarity of the interface can typically be switched between negative ionisation mode, which provides deprotonated ions, and positive ionisation mode, to give protonated molecular ions or solvent adducts (Snyder *et al.*, 1997; McMaster, 2005; Snyder *et al.*, 2010).

The ESI interface is recommended for use with highly polar and non-ionised materials and is suitable for thermolabile analytes and macromolecules. It is a very soft ionisation technique that results in very little fragmentation and is also less likely to cause undesirable analyte degradation. In the ESI interface, the HPLC eluent is passed through a heated capillary tube. A potential difference (typically 3–5 kV) is applied to the stainless-steel nebuliser spray-tip, adding a charge to analytes in the mobile phase. The mobile phase is sprayed out of the end of the tube and into the heated interface, where the solvent evaporates, leaving ions in the gaseous state. These ions are generated by ‘coulomb explosion’ (disintegration) of electrically charged droplets and may be of various charge states. In some models the charged capillary is surrounded by another capillary containing a nebulising inert gas to aid in final evaporation by reducing surface tension. The ESI interface yields a concentration-sensitive signal (Snyder *et al.*, 1997; McMaster, 2005; Niessen, 2006; Meyer, 2010; Snyder *et al.*, 2010).

In the APCI interface, the mobile phase is evaporated first, and then ions are generated by corona discharge. The APCI technique is typically used for compounds that do not ionise well with ESI, often more stable, lower molecular weight compounds and also nonpolar molecules. These compounds need some proton affinity and volatility. This technique is typically not suitable for thermolabile analytes, as harsher ionisation conditions are required, leading to increased risk of analyte degradation. The APCI interface yields a mass-sensitive signal (Meyer, 2010; Snyder *et al.*, 2010).

Mass Analysers

The core of a mass spectrometer is the mass analyser, which separates ions by their mass-to-charge (m/z) ratio and focuses the ions. The purpose of the analyser is to hold ions, select specific mass ions and move these into the electron multiplier for detection (McMaster, 2005).

Various mass analysers such as quadrupole, ion trap (ITP), linear ion trap, time-of-flight (TOF), magnetic and electrostatic sectors, and Fourier transform ion cyclotron resonance (ICR) analysers have been developed (Snyder *et al.*, 1997; McMaster, 2005). The two principal designs of mass analysers for single stage LC-MS applications include quadrupoles and ion traps. Time-of-flight analysers are also growing in popularity (Snyder *et al.*, 2010). Within the scope of this study, only (triple-)quadrupole and TOF mass analysers will be considered.

The quadrupole mass analyser consists of four symmetrically arranged, parallel quartz rods clamped in a pair of ceramic collars (Snyder *et al.*, 1997; McMaster, 2005). The exact hyperbolic spacing between diagonally opposed rods is critical for mass spectrometer operation (McMaster, 2005). Diagonally opposed rods are connected electrically to a radio frequency (RF) and direct current (DC) voltage generator (Snyder *et al.*, 1997). Ions extracted into the quadrupole region drift toward the detector as they are influenced by the

combined DC and oscillating RF fields. By selectively ramping the alternating electromagnetic fields, ions of successive m/z are permitted to pass to an electron multiplier for detection, providing a selective response for the desired ion. In this manner a mass spectrum, *i.e.* a plot of the relative abundance of the ions as a function of the m/z , is generated (Snyder *et al.*, 1997).

In a triple-quadrupole (MS/MS) instrument, the two terminal quadrupoles function as resolving elements, while the middle quadrupole becomes the source for collision induced dissociation (CID). Sample ions generated in the interface enter the first quadrupole, where ions of a given m/z are isolated. These parent/precursors ions are then sent to a second quadrupole, the collision cell, filled with an inert gas (nitrogen or argon), where fragmentation is induced. The generated product ions are then sent to a third quadrupole, which isolates specific ion fragments and passes them to the electron multiplier for measurement. The transition from the initial parent/precursor ion to daughter/product ion is characteristic for each analyte and therefore significantly increases the selectivity of the triple-quadrupole (MS/MS) over the quadrupole (MS) detector (Snyder *et al.*, 1997, 2010).

In the TOF mass analyser, ions generated at the interface are accelerated with a specific energy and directed through a drift tube to the detector. The velocity of an ion travelling through the drift tube is related to the amount of energy applied. Hence, if the same energy is applied, ions of lower mass will travel more quickly to the detector than ions of larger mass. The time it takes the ion to transit the drift tube is then correlated to the m/z of the ion. Mass resolution is related to the length of the drift tube, which is limited by spatial requirements. In order to overcome this limitation, an electrostatic mirror ('reflectron') is typically employed to increase the effective length of the drift tube, and thus improve resolution. In a quadrupole time-of-flight (Q-TOF) instrument, the quadrupole (first stage) is typically combined with the TOF (final stage) for MS/MS detection (Snyder *et al.*, 2010).

1.2.7 Data systems

Chromatographic data systems serve many functions in HPLC, of which the most notable functions include: experimental aids for method development; system control for all operational settings; data collection and processing; report generation; and regulatory functions (Snyder *et al.*, 2010).

1.3 Method development

During method development both the the quality of separation and the analysis time required for the separation are important considerations. The time required for the separation (run time \approx retention time for last analyte) should be as short as possible (Snyder *et al.*, 1997). The quality of separation is typically measured by the resolution, R_s , of two adjacent analyte zones or bands. Resolution may be defined as the difference in retention times between the peak centres divided by the average width at baseline (Snyder *et al.*, 1997, 2010):

$$R_s = \frac{[t_{R,1} - t_{R,2}]}{\frac{1}{2} [W_{B,1} + W_{B,2}]} \quad (2)$$

Where:

$t_{R,1}$ and $t_{R,2}$ = retention times of peaks 1 and 2, respectively.

$W_{B,1}$ and $W_{B,2}$ = widths at baseline for peaks 1 and 2, respectively.

Adequate resolution is a primary requirement in quantitative HPLC analysis. Baseline resolution ($R_s > 1.5$ for two peaks of similar size) favours maximum precision in reported results, and also greater method ruggedness. Baseline resolution occurs when the detector trace for the first band returns to the baseline before the second band elutes from the column (Snyder *et al.*, 1997, 2010).

However, during HPLC method development, the aim is usually to separate every peak of interest from the adjacent peaks with $R_s \geq 2$. This makes allowances for adjacent bands of dissimilar size, and the deterioration of the HPLC column. When more than two peaks are to be separated, the goal is usually $R_s \geq 2$ for the least resolved peak-pair, *i.e.* the critical peak-pair (Snyder *et al.*, 2010).

1.3.1 Optimising HPLC separations

The separation of any two bands in the chromatogram can be optimised by varying the experimental conditions. Therefore, for method development purposes, it is convenient to express resolution as a function of three parameters (k , α , N) which can be optimised by varying the experimental conditions:

$$R_s = \left(\frac{1}{4}\right) \left(\frac{\alpha - 1}{\alpha}\right) \sqrt{N} \left(\frac{k}{1 + k}\right) \quad (3)$$

The retention factor is represented by k and may be defined as the corrected retention time of the analyte (t_R), measured in units of column dead time (t_0):

$$k = \frac{t_R - t_0}{t_0} \quad (4)$$

Where:

$$t_0 = \frac{V_M}{F} \quad (5)$$

With:

V_m = column dead volume (mL), *i.e.* the volume of mobile phase in the column, approximated as $0.01 \times L$ (mm) for 4.6 mm i.d. columns (Snyder *et al.*, 2010).

F = volumetric flow rate (mL.min⁻¹)

α denotes the separation factor – a measure of separation selectivity or relative retention. The separation factor may be defined as (Snyder *et al.*, 2010):

$$\alpha = \frac{k_2}{k_1} \quad (6)$$

Where:

k_1 and k_2 = values of retention factor for two adjacent peaks 1 and 2, respectively.

N represents the column plate number or column efficiency, defined as:

$$N = 16 \left(\frac{t_R}{W_B}\right)^2 = 5.54 \left(\frac{t_R}{W_H}\right)^2 \quad (7)$$

Where:

t_R = retention time of the analyte

W_B and W_H = peak widths at baseline and half height, respectively.

Method development entails optimising resolution by optimising each of the terms in equation 3. In the following sections a systematic approach towards optimising the efficiency and selectivity will be discussed.

1.3.1.1 Optimising efficiency

The column plate number, N , is affected by various experimental parameters: flow rate, column length and particle size. Column conditions are usually selected at the beginning of method development, prior to optimising α .

During chromatographic separation, analyte molecules spread out to form a band during their migration through the column. When the band elutes from the column to form a corresponding peak in the chromatogram it is characterised by a certain width, which is related to the column efficiency according to equation 7. The peak width and retention time of a peak determine the value of N for the column. Various processes outside the column (extra-column) and within the column contribute to band broadening. There are two types of extra-column contributions: volumetric, derived from injection volume, detector volume and connection tubing volume; and time-related, stemming from the sampling rate and detector time constant. Ideally the extra-column contributions to band-broadening can be ignored. Small volume columns packed with small particles are, however, especially prone to extra-column band broadening, and hence these effects should be minimised to ensure optimum column performance (Snyder *et al.*, 1997; Fountain *et al.*, 2009; Snyder *et al.*, 2010). The contribution of physical processes that take place within the column to band broadening are described by the Van Deemter equation:

$$H = A + \frac{B}{u} + C u \quad (8)$$

Where:

H = theoretical plate height; relates the variance of a band to the distance travelled through the column (mm)

u = mobile phase linear velocity (mm.sec⁻¹)

A = eddy-dispersion term; flow independent

B = longitudinal diffusion term; time dependent

C = mobile-phase and stationary-phase mass transfer, also known as the resistance to mass transfer term; flow dependent

The theoretical plate height is related to the number of plates according to:

$$N = \frac{L}{H} \quad (9)$$

When operating a packed-particle column at optimal conditions, $H_{\min} \approx 2 d_p$. In this instance, equation 9 becomes:

$$N = \frac{L}{2 d_p} \quad (10)$$

Where:

L = column length (mm)

d_p = particle diameter (μm)

This implies that column efficiency may be increased by either increasing column length or decreasing particle size. Both approaches are, however, limited by pressure constraints. This is evident from Darcy's law (MacNair *et al.*, 1997), which relates the pressure drop across the column (ΔP) to the eluent viscosity (η), column length (L), linear velocity (u), column particle diameter (d_p) and column permeability (K_v):

$$\Delta P = \frac{\eta L K_v}{d_p^2} u = \frac{\eta N H K_v}{d_p^2} u \quad (11)$$

Effect of stationary phase particle size, d_p

Particle size is the primary factor in determining column efficiency, as measured by the plate number. By constructing Van Deemter curves, *i.e.* plots of plate height (H) as a function of linear velocity (u) for columns of different particle size (Figure 2), the following observations can be made: as the diameter of the particle size decrease, the plate height decreases, corresponding to an increase in column efficiency per mm of column length. This reduction in plate height further corresponds to an increase in the optimum linear velocity, u_{opt} . Hence, a decrease in particle size leads to an increase in separation efficiency and an increase in optimal linear velocity, allowing faster analyses. Plate height curves for the smaller particles also appear 'flat' at higher velocities and therefore increasing the velocity above the optimal value only leads to a relatively small decrease in efficiency, allowing even faster analyses. The reduction of the minimum plate height can partially be ascribed to a reduction in the eddy-dispersion term (A -term), as shorter, more uniform flow paths are obtained in the smaller particle-packed column. The increase in u_{opt} is due to a decrease in the C -term with decreasing particle size: small particles offer a short distance through which the analytes must diffuse in the mobile phase (Harris, 1999).

Columns with $d_p < 2 \mu\text{m}$ are becoming popular due to decreasing analysis times and increasing sample throughput (Snyder *et al.*, 2010). However, these sub-2 μm columns must be used with low dispersion, high-pressure instrumentation in order to achieve the full performance potential of these particles (Fountain *et al.*, 2009).

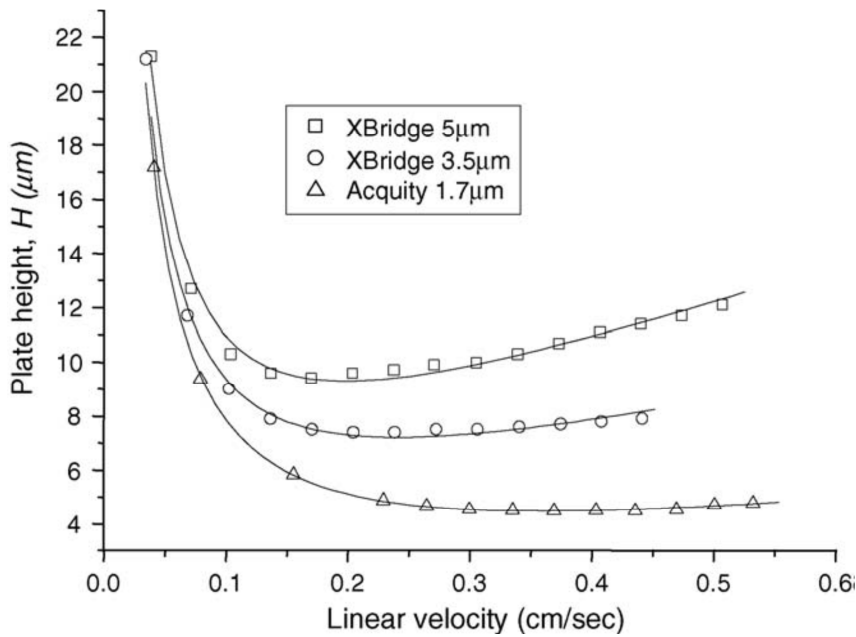


Figure 2 Experimental Van Deemter curves for 1.7, 3.5 and 5.0 μm columns (De Villiers *et al.*, 2006).

Column temperature

Temperature is an important parameter in liquid chromatography as it is known to influence efficiency, retention and selectivity. This subject has been reviewed extensively (e.g. Teutenberg, 2009); only a short summary of the major points regarding the effect of temperature on efficiency and selectivity will be presented.

It is generally assumed that the *A*-term of the Van Deemter equation (equation 8) does not depend on temperature, whilst the *B*- and *C*-terms are both temperature dependent. The *B*-term is directly proportional to the diffusion coefficient of the analyte (D_M) in the mobile phase, while the *C*-term is inversely proportional to D_M (Teutenberg, 2009). The diffusion coefficient of the analyte ($D_M, \text{cm}^2 \cdot \text{s}^{-1}$) may be calculated according to the Wilke-Chang equation (Wilke & Chang, 1955):

$$D_M = 7.4 \times 10^{-8} \frac{\sqrt{\psi_2 M_2}}{\eta V_1^{0.6}} T \quad (12)$$

Where:

T = absolute temperature (K)

M_2 = molecular weight of the solvent ($\text{g} \cdot \text{mol}^{-1}$)

V_1 = molecular volume of the solute ($\text{cm}^3 \cdot \text{mol}^{-1}$)

η = eluent viscosity (cP)

ψ_2 = association factor for the solvent (1 for nonpolar solvents, 1.9 for methanol and 2.6 for water)

From equation 12 it is clear that D_M is directly proportional to temperature, and inversely proportional to mobile phase viscosity. An increase in temperature will lead to an increase in the diffusion of the analytes in the mobile and stationary phases, and hence to a reduction in the *C*-term of the Van Deemter due to enhanced mass transfer kinetics. This effect is furthermore enhanced by reduced mobile phase viscosity at elevated temperature, which enhances the diffusion of the analytes (Teutenberg, 2009). Mobile phase

viscosity as a function of temperature may be calculated either according to Chen & Horvath (1993) or Guillaume *et al.* (2004); the latter calculation will be employed in this study.

The effect of temperature on column performance is clearly depicted in Figure 3 where theoretical Van Deemter curves obtained for a 5 μm column at different temperatures are shown. With an increase in temperature, the minimum plate height remains virtually unaltered, while the optimum linear velocity is shifted towards higher values. Therefore the primary advantage afforded by operating at elevated temperature is a reduction in analysis time. An increase in temperature can, however, improve the efficiency of typical chromatographic systems, but only at linear velocities higher than the optimal linear velocity.

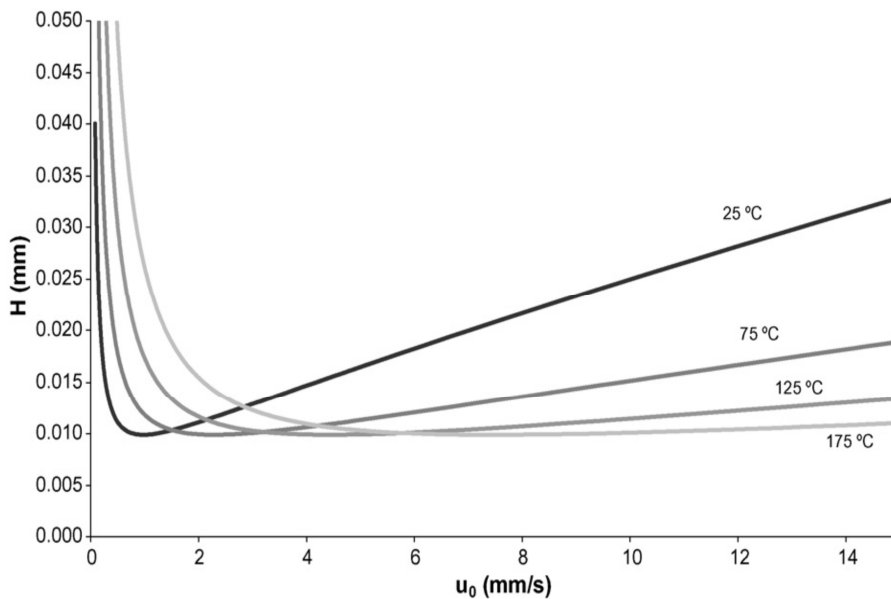


Figure 3 Theoretical Van Deemter curves on a 5 μm column, illustrating the effect of column temperature on the plate height, H , and the linear velocity, u (Lestremau *et al.*, 2007).

Kinetic evaluation of different column formats and temperatures

In evaluating different column formats and temperatures, traditional plate height representation methods such as Van Deemter curves provide limited information due to failure to take column permeability and pressure constraints into account. Therefore, an alternative geometry-independent plate height representation method has been proposed by Desmet *et al.* (2005). By using experimental plate height (H), linear velocity (u) and column permeability (K_v) data, and only requiring simple calculations (equations 13 and 14), a family of 'kinetic plots' can be constructed.

$$N = \left(\frac{\Delta P}{\eta} \right) \left[\frac{K_v}{u_0 H} \right]_{\text{exp}} \quad (13)$$

$$t_0 = \left(\frac{\Delta P}{\eta} \right) \left[\frac{K_v}{u_0^2} \right]_{\text{exp}} \quad (14)$$

These equations translate the experimental plate height data into the relevant, sought-after kinetic data, *i.e.* the minimal time needed to perform a separation requiring N plates for a given column (characterised by K_v) at a specified maximum pressure. Among the different kinetic plots, the $(N, t_0/N^2)$ are most often used for the graphical comparison of different experimental configurations. From this plot, illustrated in Figure 4, it can

easily be established for which range of efficiencies a particular experimental set-up will kinetically outperform the other. This can be visualised from the curve lying lowest on the x-axis. The $(N, t_0/N^2)$ plots have the additional advantage that the minimum of the Van Deemter curve (H_{\min}) can be directly visualised. In the kinetic plot, this point corresponds to the optimal plate number, N_{opt} , which can be considered as 'the plate number for which the support reaches its best possible kinetic performance/pressure cost ratio' (Desmet *et al.*, 2005). Furthermore, the vertical asymptote of the kinetic plots corresponds to the maximal number of plates (N_{max}) that can be achieved with the given support for a certain maximum operating pressure. In this plot, the diagonal lines correspond to t_0 times.

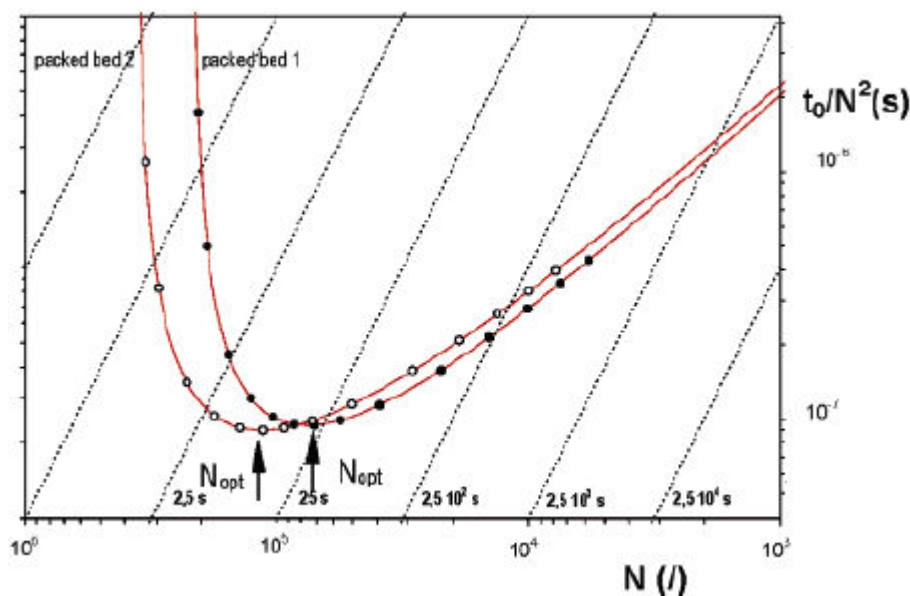


Figure 4 Plots of $(N, t_0/N^2)$ for two packed beds (adapted from Desmet *et al.*, 2005).

In this manner, these plots automatically yield the most relevant performance characteristics in terms of which experimental configuration will either yield faster separations for a required efficiency, or will achieve the maximum number of plates in a given analysis time. For a more comprehensive overview of kinetic plots, refer to Desmet *et al.* (2005).

Studies used to assess the kinetic performance of different column formats and temperatures typically use common organic molecules such as acetophenone as test analytes, since these compounds exhibit ideal thermodynamic behaviour (Oláh *et al.*, 2010). However, various authors have reported significant differences between the plate height behaviour of such test analytes and real-life compounds (De Villiers *et al.*, 2009; Oláh *et al.*, 2010; Fekete *et al.*, 2011). This indicates that the effect of analyte properties on the optimum kinetic configuration should also be taken into account.

1.3.1.2 Optimising selectivity

Selectivity is the most powerful factor to achieve improved separation. For non-ionised samples, selectivity may be optimised by changing the mobile phase composition, temperature or stationary phase. For samples that contain acids or bases (ionisable solutes), the mobile phase pH, buffer concentration and ion-pair reagent concentration may also be adjusted to improve selectivity (Snyder *et al.*, 2010). Special attention will only be given to the effect of solvent strength and column temperature on the separation selectivity.

Effect of solvent strength and column temperature

Under isocratic elution conditions, analyte retention as a function of solvent strength ($\varphi = 0.01\% \text{ B}$, where B is the stronger eluent in a binary mobile phase) can be described by the following well-known relationship (Zhu *et al.*, 1996a, 1996b):

$$\log k = \log k_W - S \varphi \tag{15}$$

Where:

k_W = the (extrapolated) value of k for $\varphi = 0$ (water as mobile phase)

S = constant for each analyte

This linear relationship of $\log k$ vs. % B (100φ) is depicted in Figure 5 for a 'regular' and 'irregular' sample. A sample comprising of analytes for which an increase in the % B leads to significant changes in relative retention is referred to as an 'irregular' sample. An 'irregular' sample is characterised by intersections of the individual curves (Snyder *et al.*, 2010). By optimally exploiting the relative changes in band position as a function of % B, the separation selectivity may be improved.

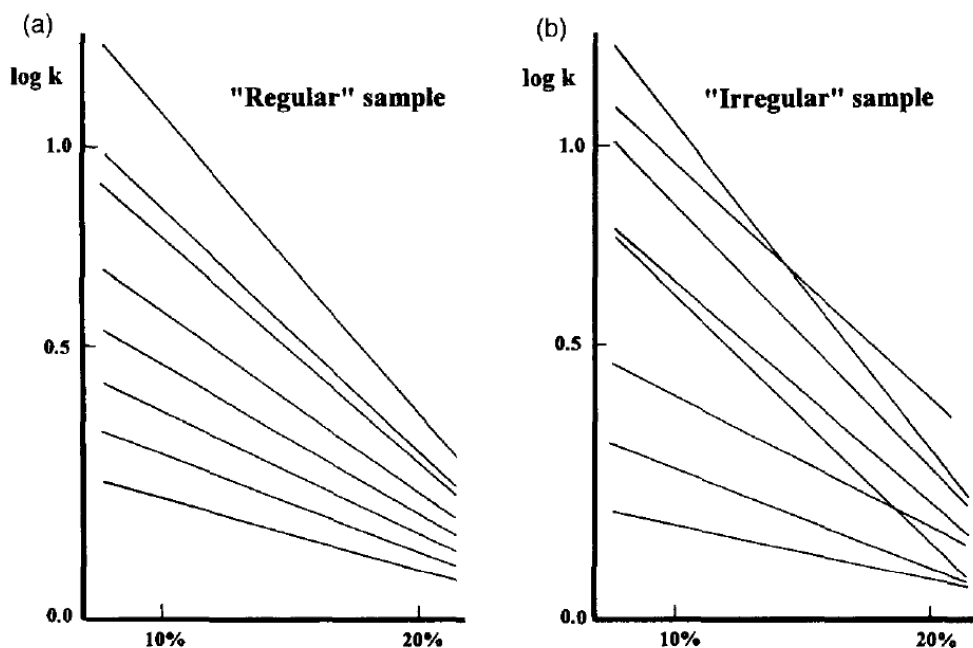


Figure 5 Illustration of (a) 'regular' and (b) 'irregular' sample behaviour for solvent strength selectivity (Zhu *et al.*, 1996b).

Temperature also has a significant effect on values of the retention factor, k . For most analytes, the influence of temperature on retention is given by the Van't Hoff equation (Zhu *et al.*, 1996a, 1996b), which can be expressed as:

$$\log k = A + \frac{B}{T} \tag{16}$$

For a given analyte and other conditions unchanged, A and B are constants and T is the absolute temperature (K). A is a function of the phase ratio and entropy of retention, ΔS , and B is proportional to the enthalpy of retention, ΔH . Values of ΔH are typically negative, so that retention decreases with increasing temperature (Hancock *et al.*, 1994).

According to the equation 16, plots of $\log k$ vs reciprocal temperature (Van't Hoff plots) should yield straight lines. Similar to the effect of solvent strength, even if all the analytes obey the Van't Hoff equation, a change in the separation selectivity may be obtained if the slopes of the Van't Hoff plots for the selected analytes differ (Hancock *et al.*, 1994; Teutenberg, 2009).

Deviations from the linear Van't Hoff equation are not uncommon, sometimes resulting in curved plots of $\log k$ vs reciprocal temperature or even increased retention with increasing temperature. These deviations may be ascribed to ionisation of the solute (Buckenmaier *et al.*, 2004; Heinisch *et al.*, 2006), changes in solute molecular conformation (Melander *et al.*, 1979), and changes in the stationary phase (Guillarme *et al.*, 2004) with a change in temperature.

Simultaneous optimisation of gradient conditions and temperature: the linear solvent strength (LSS) model

Selectivity effects as a function of changes in gradient conditions and temperature in RP-LC are often complementary and therefore simultaneous optimisation of these two variables may be useful in improving band spacing and resolution (Dolan, 2002; Jandera, 2006). Separation under gradient conditions as a function of temperature is described by the Linear Solvent Strength (LSS) model (Zhu *et al.*, 1996a, 1996b; Dolan *et al.*, 2000):

In gradient elution, retention time t_R for a well-retained band ($k_0 \gg 1$) is given by:

$$t_R = \frac{t_0}{b} \log (2.3 k_0 b) + t_0 + t_D \quad (17)$$

With:

$$b = \frac{V_M \Delta\phi S}{t_G F} = \frac{t_0 \Delta\phi S}{t_G} \quad (18)$$

Where:

t_0 = column dead time (min)

t_D = gradient dwell time (min)

t_G = gradient time (min)

$\Delta\phi$ = change in the volume fraction organic modifier during the gradient

V_m = column dead volume (mL)

F = volumetric flow rate (mL.min⁻¹)

k_0 is the value of k at the start of the gradient, given by:

$$\log k_0 = \log k_W + S \phi \quad (19)$$

By performing two gradient runs with different t_G 's and otherwise identical conditions, values for S and k_w can be determined from the measured values of t_R in combination with equations 17-19. This then allows prediction of t_R for different gradients (ϕ , F , t_G).

This approach can be extended to include the effect of both temperature and gradient profile on the retention of analytes. In this case, two gradient runs for different t_G times are performed at temperature T_1 . Two more gradient runs are performed at temperature T_2 using the same t_G values. t_R values at any temperature T_x ($T_1 < T_x < T_2$) can then be estimated for both t_G times using the following semi-empirical relationship (or derivatives thereof):

$$t_R = a' + b' T_x \quad (20)$$

Where a' and b' are constants for a given compound at every T . Finally, these values at t_R are used to calculate S and k_w at T_x using equations 17-19. In this manner the retention of any number of compounds may be calculated as a function of gradient conditions and temperature.

Computer simulation: Drylab

When dealing with more challenging, complex samples, or if the separation goals are particularly stringent, a large number of method-development runs may be required. A strictly experimental approach to method development is therefore not always feasible and hence computer simulation may be used as tool (Snyder *et al.*, 1997). Computer simulation software can predict separation as a function of one or more experimental conditions, by means of experimental data from a few preliminary separations.

In the mid 1980's Drylab software, developed by Snyder and Dolan, was introduced and subsequently expanded to the most comprehensive and widely used computer-simulation software presently available (Molnar, 2002). The experimental design of Drylab is based on initial gradient-elution experiments – four runs at two different gradient times and two different temperatures. This data are then used as input in the software program in order to simultaneously optimise % B and T for a given sample.

1.3.2 Completing the HPLC method

The final HPLC method should fulfill all the requirements that were defined at the beginning of method development in terms of resolution, speed and sensitivity. The method should also be robust for routine application and be suitable for all laboratories and personnel for which it is intended (Snyder *et al.*, 1997).

1.3.2.1 Quantitation

Most HPLC methods are used for routine quantitative analysis. A critical requirement for a quantitative method is the ability to measure a wide range of sample concentrations with a linear response for each analyte. With regards to this, the UV detector is most widely used for accurate and precise quantitation in HPLC (Snyder *et al.*, 1997).

Measurement of signals

Either peak height or peak area can be used for quantitation in HPLC, although the latter is more common. Various LC operating parameters affect the response measurement and these effects are different for peak height or peak area quantitation.

The area of a well resolved peak is defined as the integral of the signal response over time from the beginning until the end of the peak. This theoretical definition may appear relatively straightforward, but in practice the accurate and precise measurement of peak area relies on a number of factors. These factors typically include: establishing the correct baseline; accurately defining the beginning and end of the peak; and determining the number of data points that should be collected across the peak to accurately assess the actual peak area (sampling rate). Peak area is typically calculated using an integrator or computerised data system which is a very precise method of integration (Snyder *et al.*, 1997). However, the integration of non-ideal chromatograms where peaks are not fully resolved and/or the baseline is noisy or drifts remain problematic (Meyer, 2010; Snyder *et al.*, 2010).

Quantitation methods

Peak height or peak area measurements only provide a response in term of detector signal. This response must be related to the concentration or amount of the compound of interest and hence calibration must be performed. The four primary techniques for quantitation include the normalised peak area technique and three techniques using calibration: external standard, internal standard and standard addition (Snyder *et al.*, 1997, 2010). For the purpose of this study, however, only the external standard calibration method will be considered.

The external calibration method is the most general method for determining the concentration of an unknown sample and typically entails constructing a calibration plot using external standards. Standard solutions of known concentrations are prepared and a fixed volume of each standard solution is injected and analysed. The peak responses are subsequently plotted against concentration to yield a calibration plot. The concentration of the unknown sample may then be determined from the calibration plot. The calibration plot should be linear and have an intercept close to zero. The concentration range of the standards should accurately reflect the expected concentration range of the sample. If the sample concentration falls outside the range of the standards used, extrapolation of the calibration plot should be used with caution as non-linearity may occur at high concentrations (Snyder *et al.*, 1997).

1.3.2.2 Method validation

Once the HPLC method is finalised, it should be validated. Method validation establishes that the performance characteristics of the method meet the requirements of the intended analytical application. Several analytical performance characteristics may be investigated during method validation (Snyder *et al.*, 1997; Shabir *et al.*, 2007; Meyer, 2010; Snyder *et al.*, 2010) and these will be addressed briefly in the following paragraphs.

Accuracy

Accuracy is defined as the closeness of the measured/test/found value to the 'true value', *i.e.* an accepted reference value (Snyder *et al.*, 1997; Shabir *et al.*, 2007; Meyer, 2010; Snyder *et al.*, 2010). Accuracy can be determined by: comparison to a reference standard; recovery of the analyte spiked into blank matrix; or standard addition to the analyte (Snyder *et al.*, 1997; Shabir *et al.*, 2007).

Precision

Precision refers to the reproducibility of multiple measurements of a homogeneous sample and is typically expressed as the percentage relative standard deviation (% RSD) for a statistically significant number of samples. Precision criteria are usually $RSD \leq 2\%$. Different levels of precision are typically assessed as part of method validation and these include repeatability, intermediate precision, as well as reproducibility (Snyder *et al.*, 1997; Shabir *et al.*, 2007; Snyder *et al.*, 2010).

Repeatability (intra-day assay precision) is the precision of the method under the same operating conditions over a short time interval. One aspect of this is instrumental precision, measured by the sequential, repetitive injection of the same homogenous sample. A minimum of nine determinations is recommended and their repeatability should cover the specified range of the procedure, *i.e.* three concentrations at three repetitions each. A minimum of six determinations at 100% of the test concentration will also suffice. The peak area or peak height values are subsequently averaged and the % RSD calculated (Snyder *et al.*, 1997; Shabir *et al.*, 2007; Snyder *et al.*, 2010).

Intermediate precision (inter-day variation) refers to the agreement between the results from within-laboratory variations due to random events such as different days, analysts and equipment. In determining intermediate precision, an experimental design should be employed so that the effects of the individual variables can be monitored (Shabir *et al.*, 2007; Snyder *et al.*, 2010).

Reproducibility is determined by conducting collaborative studies among different laboratories; documentation of reproducibility should include the standard deviation, relative standard deviation and the confidence interval (Snyder *et al.*, 2010).

Linearity and range

Ideally a linear relationship between response and concentration is preferred because it is more precise, easier for calculations, and can be defined with fewer standards. The linearity of a method is a measure of how well a calibration plot of response vs. concentration approximates a straight line or how well the data fit to the linear equation:

$$y = m x + b \quad (21)$$

Where:

y = analyte response

x = analyte concentration

m = slope of a line fit to the data

b = intercept of a line fit to the data

Linearity can be assessed by performing single measurements at several analyte concentrations. The linear range is the interval between the upper and lower concentrations of the analyte that can be determined with acceptable precision, accuracy and linearity. Guidelines specify a minimum of five concentration levels for determining linearity and range. The data are processed using the linear least-squares regression, and the equation for the calibration curve line and the correlation coefficient (r^2 value) reported. Linearity is generally regarded in terms of the correlation coefficient (Snyder *et al.*, 1997, 2010).

Limit of detection (LOD) and limit of quantification (LOQ)

Two important characteristics of a method are the limit of detection (LOD) and the limit of quantification (LOQ). The LOD is defined as the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantified. The LOD may be estimated by visual evaluation, signal-to-noise-ratio, response standard deviation and slope, or based on % RSD. The LOD is typically based on a certain signal-to-noise ratio, of which a ratio of 3:1 is recommended for use in HPLC methods (Snyder *et al.*, 1997, 2010).

The LOQ is defined as the lowest concentration of an analyte in a sample that can be quantified with acceptable accuracy and precision. The LOQ may also be determined by three of the same procedures used in estimating the LOD, typically using a signal-to-noise-ratio of 10:1 (Snyder *et al.*, 1997, 2010).

Specificity

Specificity is the most important aspect of most analytical methods and can be defined as the ability to accurately quantify the compound of interest, also in the presence of other compounds (Snyder *et al.*, 1997; Meyer, 2010; Snyder *et al.*, 2010). Specificity takes the degree of interference from other sample constituents into account and therefore ensures that a peak's response is due to a single compound and that no peaks overlap. Specificity for a given compound is commonly measured by resolution, plate number (efficiency), and tailing factor. For identification purposes, specificity is demonstrated by separation from other compounds in the sample, *i.e.* resolution of two closely eluted compounds, and/or by comparison to known reference standards. With regards to the latter aspect, it is recommended that a peak-purity test based on PDA detection or MS be used to demonstrate specificity in chromatographic analyses (Snyder *et al.*, 2010)

Ruggedness

Method ruggedness is defined as 'the reproducibility of results when the method is performed under actual use conditions' (Snyder *et al.*, 1997) or 'the insensitivity of the method against external parameters' (Meyer, 2010). A rugged method is one that tolerates minor variations in experimental conditions, can be run easily by different analysts, and does not require an identical HPLC system for its use. Rugged methods are essentially trouble-free and transferable (Snyder *et al.*, 1997). The use of the term 'ruggedness' is, however, declining as ruggedness is now considered as part of intermediate precision and reproducibility (Snyder *et al.*, 2010).

Robustness

The robustness of an analytical procedure is defined as 'a measure of its capacity to obtain comparable and acceptable results when perturbed by small but deliberate variations in specified experimental conditions' (Snyder *et al.*, 2010). Robustness furthermore provides an indication of the suitability and reliability of the method during normal use. To measure and document robustness, Snyder *et al.* (2010) proposed that the following characteristics should be monitored: critical peak pair resolution; column plate number or peak width in gradient elution; retention time; tailing factor; peak area (and/or height) and concentration.

1.4 Two-dimensional liquid chromatography (2-D LC)

Despite recent advances aimed at increasing the resolving power of one-dimensional liquid chromatographic (1-D LC) methods, these attempts still fall short when very complex, multi-component samples need to be analysed (Snyder *et al.*, 2010). In this regard, two-dimensional liquid chromatographic (2-D LC) techniques provide a powerful approach to achieve improved separation of complex samples. In 2-D LC separations, two approaches may be employed: heart-cutting (LC-LC) and comprehensive (LCxLC) (Schoenmakers *et al.*, 2003). In both LC-LC and LCxLC, the two dimensions are coupled via an appropriate interface. The main difference between the two techniques is the amount of the primary column effluent that is transferred to the second dimension. In LC-LC only certain fractions of the first dimension effluent, containing the target analytes, are directed to the second dimension. Conversely, in LCxLC, the entire sample is subjected to both separation mechanisms. Several reviews (e.g. Stoll *et al.*, 2007; Dugo *et al.*, 2008a; Francois *et al.*, 2009) may be consulted for an overview of recent progress and applications of 2-D LC. Only some of the fundamental and instrumental aspects pertaining to LCxLC, the 2-D LC approach used in this study, will be highlighted.

1.4.1 Peak capacity, orthogonality and sampling frequency

1.4.1.1 Peak capacity

The resolving power of chromatographic methods is commonly measured in terms of peak capacity, n_c . Peak capacity may be defined as the maximum number of peaks that can be inserted into a given chromatographic space (from the first unretained peak to the last peak of interest) with resolution unity ($R_s = 1.0$) between all adjacent peaks. Already in 1967, Giddings reported the first equation for an isocratic peak capacity (equation 22; Giddings, 1967). This expression assumes that column plate number is independent of the retention factor.

$$n_c = 1 + \frac{\sqrt{N}}{4 R_s} \ln \left(\frac{k_1 + 1}{k_F + 1} \right) \quad (22)$$

Where:

N = efficiency

R_s = resolution; value depends on the goals of the separation, but is usually taken as unity.

k_1 and k_F = retention factors of the first and last eluting compounds, respectively.

The peak capacity for gradient separations is generally higher than for isocratic analysis, since the bandwidths are significantly narrower (Francois *et al.*, 2009). Gradient peak capacity is given by equation 23 in which W_{ave} is the average peak width at baseline and t_G is the gradient time (Neue & Mazzeo, 2001; Neue, 2005). This equation is only valid if the peak width pattern over the entire chromatogram is very similar (Figure 6).

$$n_c = 1 + \frac{t_G}{W_{ave}} \quad (23)$$

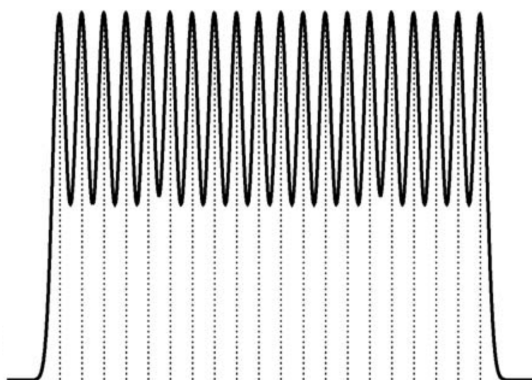


Figure 6 Resolution of a 20 component mixture at constant retention increments between the compounds and constant peak heights (Felinger, 1998).

The peak width is generally defined as four times the standard deviation (σ) of a peak, assumed to be Gaussian. However, this approximation does not take peak tailing/fronting effects into account and therefore it is potentially more correct to determine W_{ave} by actual measurement. The number of considered peaks together with their retention times strongly influences the determination of W_{ave} . For very complex samples it has been advised that average peak width should be estimated from the chromatogram of a relatively simple standard mixture, representative of the actual sample, and with an elution profile covering the entire separation space (Francois *et al.*, 2009).

Peak capacity delivers a measure of the maximum number of components that can theoretically be resolved during a single chromatographic analysis with defined run time (Francois *et al.*, 2009; Snyder *et al.*, 2010). However, due to the fact that sample components are not uniformly distributed throughout the chromatogram, the peak capacity should be significantly larger than the number of sample constituents to be separated. Davis and Giddings (1983) used the statistical method of overlap (SMO) theory to illustrate that resolution is severely compromised when the number of sample components exceeds 37% of the peak capacity. To resolve 98% of m randomly distributed sample components, the peak capacity should be $m \times 100$ (Giddings, 1995).

These calculations show that a single chromatographic dimension is inadequate to obtain complete separation of multi-component, complex samples. LCxLC offers a powerful approach to increase peak capacity which is reflected in the reduction of component overlap and is therefore ideally suited for complex samples. In LCxLC, the sample is subjected to two different separation mechanisms and the theoretical peak capacity ($n_{C,2D}$) is equal to product of the peak capacities in the first ($n_{C,1}$) and second ($n_{C,2}$) dimensions (Guiochon *et al.*, 1983; Giddings, 1987):

$$n_{C,2D} = n_{C,1} \times n_{C,2} \quad (24)$$

However, in practice the peak capacity is always lower than the theoretical maximum as there will always be a degree of similarity between the coupled dimensions, leading to retention correlations. In addition, extra peak broadening effects, including the sampling rate of the first dimension peaks, can also lead to a reduction in LCxLC peak capacity. Therefore, in the calculation of the practical LCxLC peak capacity ($n'_{C,2D}$), factors such as orthogonality and sampling frequency need to be considered.

1.4.1.2 Orthogonality

In LCxLC it is generally understood that the separation is orthogonal when the two separation mechanisms are independent of each other and show distinct retention profiles, *i.e.* provide different selectivities. The relative retention of each of the sample components in each dimension should therefore yield a plot that is optimally characterised by a random distribution of the compounds over the entire separation space (low correlation, high orthogonality). True orthogonality is technically difficult to achieve, as orthogonality not only depends on the separation mechanisms, but also on the analyte properties and the separation conditions. Successful orthogonal separations can be achieved by optimal selection of stationary and mobile phases with regards to the physicochemical properties of the sample constituents (size, charge, polarity, hydrophobicity, etc.) (Francois *et al.*, 2009).

In considering orthogonal LC modes, mobile phase compatibility/miscibility is an important technical consideration. If the mobile phase in the first dimension is a strong eluent in the second dimension, injection of analytes on this phase will lead to significant band broadening and hence the injection volume in the second dimension is usually limited to small volumes, which could lead to sensitivity problems.

Liu *et al.* (1995) proposed a geometric approach to factor analysis in which a correlation matrix was developed from the solute retention parameters in 2-D chromatography. The effective area of the 2-D space covered by the eluting peaks was used as a representative value of the orthogonality of the system. In this manner, a subtraction factor was applied to the theoretical peak capacity in order to determine the true practical value.

1.4.1.3 Sampling frequency

To maintain maximum first dimension resolution in a comprehensive configuration, a large number of fractions should be transferred along a peak eluting from the first dimension column. If sample or modulation periods are substantially larger than the peak widths emerging from the primary column, the first dimension resolving power will be severely compromised. It is therefore generally required that the peaks eluting from the first dimension column should be sampled at least three to four times across their width to avoid loss of first dimension resolution due to under-sampling (Murphy *et al.*, 1998; Seeley, 2002; Horie *et al.*, 2007).

Fast second dimension analyses are therefore a prerequisite in on-line comprehensive techniques, which unfortunately implies that little time is available for separation in the second dimension and hence the peak capacity of the second dimension is usually relatively low. The combination of high throughput and sufficient resolution is difficult to achieve and therefore the first dimension peaks are occasionally broadened by operating the primary column at a lower flow rate. This approach leads to a sufficient sampling number across the primary peak, but it also severely reduces the first dimension peak capacity by operating the column at a flow rate below the Van Deemter's optimum. Many practitioners have recognised the importance of sampling frequency on the peak capacity of the overall 2-D separation, but very few reports effectively deal with the issue (Francois *et al.*, 2009).

Murphy *et al.* (1998) and Seeley (2002) studied the broadening of individual first dimension peaks as a function of the first dimension sampling rate. Later Horie *et al.* (2007) applied the findings of Seeley to correct estimates of 2-D peak capacity. The work presented by these authors is based on the impact of under-sampling on a single pair of equal size peaks. Recently, Davis *et al.* (2008) established a more refined means of correcting 2-D peak capacity estimates by accounting for under-sampling in the first dimension. Their approach was based on averages over the entire LCxLC separation space rather than the behaviour of

a single peak. These authors incorporated an average first dimension peak broadening factor ($\langle\beta\rangle$) to correct for under-sampling in the first dimension. The peak broadening factor is defined as the ratio of the average effective first dimension peak width after sampling to its width prior to sampling, and is a function of the first dimension sampling time (t_s) and the first dimension peak standard deviation prior to sampling (σ_1):

$$\langle\beta\rangle = \sqrt{1 + 0.214 \left(\frac{t_s}{\sigma_1}\right)^2} \quad (25)$$

This equation is valid for 2-D separations of constituents having either randomly distributed or weakly correlated retention times over the range of $0.2 < (t_s / \sigma_1) < 16$. Determination of $\langle\beta\rangle$ may proceed via two approaches, namely the two-dimensional statistical overlap theory or the 'monte carlo simulation'. The total practical peak capacity is then calculated using the product rule (equation 26). For more detail, the reader is referred to Davis *et al.* (2008).

$$n'_{C,2D} = \left(\frac{n_{C,1}}{\langle\beta\rangle}\right) n_{C,2} \quad (26)$$

Based on these findings, the same group later derived a more accurate equation for the effective 2-D peak capacity taking under-sampling into account (Li *et al.*, 2009):

$$n'_{C,2D} = \left(\frac{n_{C,1} n_{C,2}}{\sqrt{1 + 3.35 \left(\frac{t_{C,2} n_{C,1}}{t_{G,1}}\right)^2}} \right) \quad (27)$$

Where:

$t_{C,2}$ = second dimension separation cycle time (gradient time + re-equilibration time)

$t_{G,1}$ = first dimension gradient time

Clearly, the multiplication rule represents severe over-estimation of 2-D peak capacity, and both orthogonality and under-sampling should be taken into account to obtain an accurate estimation of the practical peak capacity in comprehensive separations.

1.4.2 Method development and instrumentation in 2-D LC

Depending on the manner in which the primary column effluent is transferred onto the second dimension column, LCxLC separations can be further classified into stop-flow, off-line or on-line. In the stop-flow mode, fractions from the primary column are transferred to the second-dimension column and while a certain fraction is analysed, the flow in the first dimension is stopped. This process is repeated for the duration of the first dimension separation. The stop-flow mode will not be discussed further as this mode will not be employed in the current study. Off-line LCxLC is probably the most common approach since the execution is very simple: fractions of the first column effluent are collected (either manually or via a fraction collector) and subsequently re-injected on the secondary column. This approach places less demands on the second dimension analysis time and hence very high practical peak capacities are attainable. In the on-line set-up,

fractions of the first dimension are directly transferred to the second dimension for subsequent analysis by typically employing a switching valve. The principal advantages and disadvantages of on-line and off-line 2-D LC techniques are summarised in Table 1.

Table 1 Advantages and disadvantages of on-line and off-line 2-D LCxLC techniques (Kalili, 2009).

Approach	Advantages	Disadvantages
<i>Off-line</i>	<ul style="list-style-type: none"> • higher peak capacities • ease of operation • allows combination of non-compatible LC modes, with evaporation of solvents and redissolution prior to second dimension analysis • sample can be concentrated prior to second dimension analysis to enhance sensitivity 	<ul style="list-style-type: none"> • time consuming • difficult to automate • poor reproducibility • risk of sample loss, contamination and/or degradation/formation of artefacts
<i>On-line</i>	<ul style="list-style-type: none"> • automation • faster • better reproducibility 	<ul style="list-style-type: none"> • complicated and expensive instrumental configuration • specific interfaces are required

A typical on-line comprehensive 2-D LC system consists of two pumps and two columns, an injector, an interface and a detector. The record of the detector at the outlet of the second dimension column is transformed into a two-dimensional chromatogram, usually represented as a contour plot, *i.e.* a plot of the separation time in the second dimension vs separation time in the first dimension. A typical example of an on-line comprehensive LCxLC set-up is illustrated in Figure 7.

In this on-line set-up, a two-position/ten-port high-pressure switching valve is used as interface to hyphenate the two dimensions in a fully automated fashion. The switching valve is equipped with two sampling loops, which are alternately used for the collection and re-injection of the first dimension fractions onto the secondary column. In the absence of splitting, the loop sizes are determined by the mobile phase quantity per sampling period eluting from the first dimension. The time available for analysis (and re-equilibration when a gradient is applied) in the second dimension is equal to the sampling period in this set-up. The symmetrical arrangement (Figure 7a) is typically preferred over the asymmetrical arrangement (Figure 7b), where one of the loops is emptied in the forward-flush mode and the other loop in the back-flush mode, as identical peak shapes and retention times are obtained in the symmetrical arrangement (Van der Horst & Schoenmakers, 2003). The loop interface is the most widely used interface, but a packed loop interface, stop-flow interface, vacuum evaporation interface (VEI) and an interface with parallel second dimension columns may also be employed (Francois *et al.*, 2009).

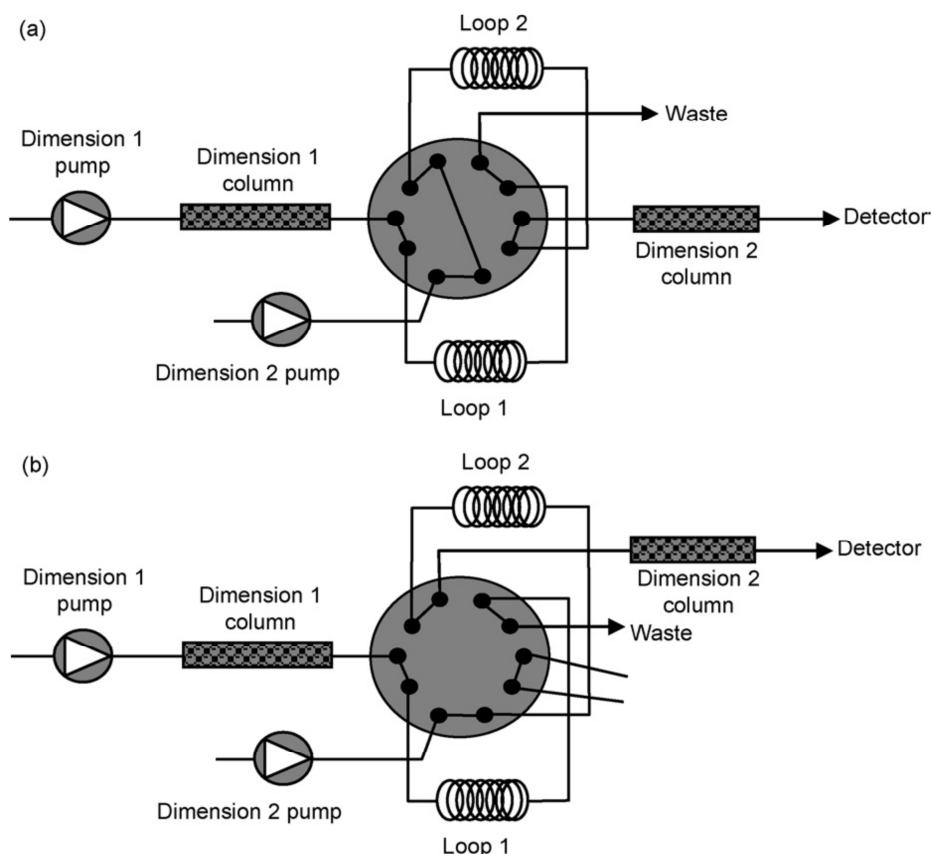


Figure 7 Common on-line comprehensive set-up with a two-position/ten-port switching valve in (a) symmetrical and (b) asymmetrical arrangements (Francois *et al.*, 2009).

The separation in the first dimension is typified by maximum resolution for the given application, since the analysis time is not limited. The first dimension may therefore consist of several columns coupled in series. To reduce band broadening and signal interferences caused by solvent incompatibilities, the first dimension is usually a micro- or narrow-bore column. In this regard, the use of a micro-bore HPLC column in the first dimension operated at a low flow rate and a conventional size column (4.6 mm i.d.) in the second dimension operated at a high flow rate is very convenient. The small column i.d. ensures minimal dilution and provides flow rates that are compatible with second dimension injection volumes. This avoids the requirement of a pre-concentration step at the head of the secondary column while problems associated with solvent incompatibility between different separation modes are minimised (Dugo *et al.*, 2008a; Francois *et al.*, 2009).

An alternative approach is to use a wider bore column and to split the effluent prior to the interface, but this leads to the loss of a large part of the sample components and hence decreased sensitivity. When a wider bore column is used in the first dimension, the flow rate is occasionally reduced to values below optimum (leading to a concomitant reduction in first dimension resolving power) in order to obtain a sufficient number of samplings per first dimension peak. The first dimension can be operated under isocratic or gradient conditions, depending on the application (Francois *et al.*, 2009).

It is essential that the fraction transferred to the second dimension column should be completely analysed prior to the subsequent transfer in order to avoid 'wrap around'. The 'wrap around' effect is characterised by analytes from the previous fraction eluting in the separation space of the next transfer (when the retention time of an analyte exceeds the sampling time) and results in chaotic band displacement (Grushka & Grinberg, 2006). Fast separation in the second dimension is therefore of utmost importance in

on-line LCxLC. Although the second dimension cycle time is restricted to the sampling period, various strategies may be employed to increase the analysis speed: the use of monolithic or superficially porous columns; short columns with small particles operated at ultra-high pressures; the use of multiple second dimension columns operated in parallel; and the application of elevated temperatures (Stoll *et al.*, 2007; Francois *et al.*, 2009).

To date, most of the LCxLC applications have included the use of a reversed-phase (RP) separation as the second dimension, operated in isocratic or gradient elution modes. Stoll *et al.* (2007) presented a qualitative comparison of the different elution modes in RP-LC for use as the second dimension in LCxLC. One of the greatest advantages of gradient elution is the potential to re-focus analyte bands eluting from the first dimension column, although the time required for column re-equilibration is wasted and cannot contribute to the overall useable peak capacity of the 2-D LC analysis (Stoll *et al.*, 2007).

1.4.3 LCxLC applications

Comprehensive LC techniques have been successfully applied in the separation of peptides and proteins, as well as polymers, but also to small molecules such as antioxidants, triacylglycerols and bioactive and pharmaceutical samples. Typical combinations of LC modes (refer to section 1.1 for abbreviations) providing orthogonal separations include IEXxRP-LC, SECxRP-LC, IEXxSEC for the separation of biological and organic compounds and NPxRP-LC for natural and pharmaceutical compounds, to name but a few (Dugo *et al.*, 2008a). Additionally, the same mode of separation can be used in both dimensions. Here, different selectivities and a certain degree of orthogonality can be achieved by using one type of column and two different sets of mobile phase solvents (e.g. different pH's), or by using two distinct stationary phases (Dugo *et al.*, 2008a).

A typical example in this regard is the RPxRP-LC separation of phenolic antioxidants. Cacciola *et al.* (2006; 2007a; 2007b) have developed a number of comprehensive LCxLC methods for the analysis of phenolic and/or flavone antioxidants in beer and wine samples. These authors optimally exploited the thermal stability of zirconia-based columns to obtain faster elution in the second dimension and shorter switching cycles between the first and second dimension by operating these columns at high temperature (120°C) (Cacciola *et al.*, 2007a). Kivilompolo & Hyötyläinen (2007) also described an on-line RPxRP-LC method for the quantitative analysis of antioxidant phenolic acids in Lamiaceae herbs. The optimised LCxLC method employed a combination of C18 and cyano columns and the same mobile phase was used with different gradient programs in each dimension. Dugo *et al.* (2008b) used a microbore phenyl-silica column in the first dimension and partially porous or monolithic columns in the second dimension for the comprehensive LCxLC analysis of polyphenolic antioxidants in red wine. Their results demonstrated the suitability of partially porous columns to obtain fast second dimension analysis and, although the resulting practical peak capacities were relatively low, they were still higher than those that could be achieved by 1-D LC. Other successful RPxRP systems in the field of phenolics and flavonoids have also been reported (Cesla *et al.*, 2009; Dugo *et al.*, 2009a, 2009b).

For the analysis of phenolic compounds, the combination of HILICxRP-LC provides a high degree of orthogonality due to different separation mechanisms in each dimension. Using an off-line approach, Kalili & De Villiers (2009; 2010) successfully applied comprehensive HILICxRP-LC method for the analysis of phenolic compounds in cocoa, apple and green tea extracts. Jandera and co-workers (Jandera & Hájek, 2009; Jandera *et al.*, 2010) demonstrated that dual retention mechanisms on a single stationary phase offer

the possibility of using complementary selectivity in the HILIC and RP modes for sequential 2-D separations of natural antioxidants on a single column. For a recent overview of 2-D LC analysis of phenolic compounds, the reader is referred to Kalili & De Villiers (2011).

In summary, the combination of LC modes with different selectivities may be exploited to resolve specific separation problems. LCxLC provides a significant increase in resolving power and a vast amount of additional information may be obtained from separation in the two dimensional space. These techniques offer a potentially powerful approach for the detailed investigation of complex phenolic fractions.

2. Phenolic compounds

2.1 Introduction

Phenolic compounds are secondary metabolites ubiquitous in the plant kingdom and constitute the largest class of non-nutrients typically found in vascular plants. Approximately 8000 naturally occurring compounds belong to the category of 'phenolics', all of which share a common structural feature: an aromatic ring bearing at least one hydroxyl substituent, *i.e.* a phenolic group. The class 'phenolic compounds' has more than a dozen subclasses, with hundreds of compounds within these subclasses. This large diversity arises from the extensive hydroxylation, methoxylation and glycosylation of the aglycone backbones and the acylation of the glycosides. Phenolic compounds are broadly classified into simple phenols, where one phenolic subunit is present, and polyphenols, where more than one phenolic subunit is present. The most recognised compounds are the phenolic acids (simple phenols), the flavonoids (monomeric polyphenols), and the flavonoid polymers (proanthocyanidins). More than 5000 different glycosylated flavonoids have been reported and phenolic acids are equally numerous (Anderson & Markham, 2006).

Flavonoids possess at least two phenolic subunits and belong to the broad category of monomeric polyphenols. These molecules are ubiquitous in plants and are formed from key precursors phenylalanine, obtained via the shikimate and arogenate pathways, and malonyl-CoA, derived from citrate produced by the tricarboxylic acid cycle (Anderson & Markham, 2006). The basic flavonoid structure is the flavan nucleus which consists of 15 carbon atoms arranged in three rings (C6-C3-C6; Figure 8).

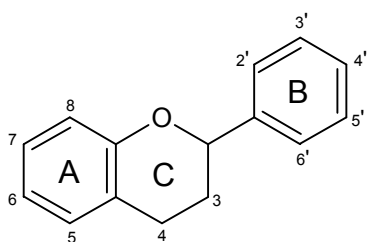


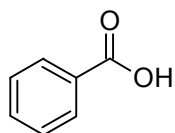
Figure 8 Basic flavonoid structure.

Flavonoids can be classified into several subclasses, based on cyclisation and degree of unsaturation and oxidation of the central heterocyclic ring. These comprise isoflavones, flavanols (catechins and tannins), anthocyanidins and, those of particular interest to this study, the dihydrochalcones, flavanones, flavones and flavonols (refer to section 3.5.2 Table 3 for the specific structures of these subclasses). Structural variation stems from degree and pattern of hydroxylation (positions 3, 5, 7, 3', 4' and/or 5'), whilst one or more of these hydroxyl groups may be methoxylated, acylated, prenylated or sulphated (Anderson & Markham, 2006).

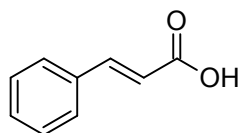
Flavonoids generally occur in plants as glycosylated derivatives, which increases their solubility in water and allows their storage in inactive forms. The most common saccharides are the pentose (L-arabinose and L-rhamnose) and the hexose (D-glucose and D-galactose) derivatives. O-glycosides, in which the saccharide is bound to the hydroxyl group(s) of the aglycone through the formation of an acid-labile glycosidic C-O bond, are most common. Glycosylation usually occurs at the hydroxyl groups located at C3 and C7. Flavonoid O-diglycosides are also frequently encountered, of which the most common disaccharides are rutinose [rhamnosyl-(α 1 \rightarrow 6)-glucose] and neohesperidose [rhamnosyl-(α 1 \rightarrow 2)-glucose]. Robinobiose [rhamnosyl-(α 1 \rightarrow 6)-galactoside] is also common. Glycosylation may also occur via direct linkage of the sugar to the basic flavonoid nucleus with the formation of an acid-resistant C-C bond to form C-glycosides. C-glycosylation sites are limited to the C6 or C8 positions. C-glycosides are further divided into mono-C-glycosyl-, di-C-glycosyl- and O,C-diglycosyl-flavonoids (Anderson & Markham, 2006).

The physiological roles of flavonoids in the ecology of plants are diverse. In considering their function in plants, it is important to distinguish between the functions of the red and blue flavonoids (anthocyanins and 3-deoxyanthocyanins) and those of the colorless (or yellow) remainder. Colorless flavonoids fulfill the following plant functions: stress protection (ultraviolet radiation; temperature stress; heavy metal tolerance; oxidative stress); reproduction and early plant development; signalling (defense against pathogenic microbes; legume nodulation); and protection from insect and mammalian herbivory (Anderson & Markham, 2006).

Phenolic acids are aromatic secondary plant metabolites possessing at least one carboxylic acid functionality. This group of organic acids contains two distinctive carbon frameworks: the hydroxybenzoic structure (C6-C1) and hydroxycinnamic structure (C6-C3) (Figure 9).



Hydroxybenzoic acid



Hydroxycinnamic acid

Figure 9 Basic hydroxybenzoic and hydroxycinnamic structures.

Variation stems from the number and positions of the hydroxyl groups on the aromatic ring, whilst methoxy groups (OCH₃) may also be present. Only a minor fraction of phenolic acids occur in free-acid form – the major fraction is linked through ester, ether, or acetal bonds to cellulose, proteins, lignin, flavonoids, glucose, terpenes and so forth. The type and concentration of phenolic acids differ between different stages of plant maturation, whilst growing conditions are also known to have an effect. Phenolic acids have been associated with diverse plant functions ranging from nutrient uptake to protein synthesis and enzyme activity, while other functions include photosynthesis, structural components and allelopathy (Macheix *et al.*, 1990).

2.2 Importance of phenolic compounds

Phenolic compounds are regarded as phytochemicals, *i.e.* non-nutrient bio-active compounds (Gry *et al.*, 2007). Phytochemicals are essential to fulfill the maximum, genetically-determined individual lifespan and are commonly referred to as 'lifespan essentials', a term coined by Holst & Williamson (2008).

It is believed that dietary flavonoids, present in relatively small quantities in plant products, play an important role in maintaining optimum human health, by exerting antioxidant, anti-inflammatory and anticarcinogenic activity. Min & Ebeler (2008) recently confirmed that flavonoids can act as antioxidants at low concentrations relevant to physiological levels achievable through the diet.

Many diseases with a strong dietary influence include oxidative damage as an initial event of disease progression, and therefore a significant focus is placed on antioxidant intervention (Holst & Williamson, 2008). Flavonoids and other phenolic compounds are especially important antioxidants due to their high redox potential which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers (Kahkonen *et al.*, 1999). It has furthermore been demonstrated that they possess metal chelating properties. By complexing iron (Fe) ions, flavonoids suppress the superoxide-driven Fenton reaction (Rice-Evans *et al.*, 1996). Copper (Cu) complexation is also an important activity of certain flavonoids, especially those with the catechol structure in the B-ring (Brown *et al.*, 1988). Flavonoids reduce the formation of reactive oxygen species (ROS; chemically reactive molecules that contain oxygen) and reactive nitrogen species (RNS; e.g., nitric oxide or peroxynitrite) due to their effect on enzymes that catalyze redox reactions and chelate metal ions (Fe and Cu) that are involved in ROS and RNS production (Kameoka *et al.*, 1999). The highly oxidising ROS are reduced by flavonoids, which are transformed to less aggressive aroxyl radicals (Pietta, 2000).

The traditional understanding has been that antioxidants promote health by removing free radicals and ROS that may otherwise exert harmful metabolic effects. However, as the complexity of cellular defense and cell signalling pathways, the role of oxidative stress, and the defense system that eliminates it are being uncovered, the role of antioxidants has become more complicated. Accumulating evidence suggests that ROS exert essential, normal metabolic functions and that the benefits of antioxidants are not necessarily from direct radical scavenging (Finley *et al.*, 2011).

It has been reported that flavonoids can selectively inhibit or increase the expression level or the biological activity of key proteins in various cell signalling cascades (Lee-Hilz *et al.*, 2008) whilst their role in herb-drug interactions are of concern as several dietary flavonoids affect key enzymes and transporters involved in drug metabolism (Cermak & Wolfram, 2006).

Most studies relate the potential health benefits of phenolic acids to their antioxidant activity. It has been established that the hydroxycinnamic acids have higher antioxidant activity compared to the corresponding hydroxybenzoic acids, due to the presence of a propenoic side chain instead of a carboxylic group (Natella *et al.*, 1999). Several studies have shown that hydroxycinnamic acid derivatives, such as ferulic acid, have effective radical scavenging activity (Chen & Ho, 1997; Pekkarinen *et al.*, 1999; Andreasen *et al.*, 2001; Vuorela *et al.*, 2004, 2005). The hydroxycinnamic acids furthermore show potential antioxidant activity in several oxidation model systems such as low-density lipoprotein (LDL) and liposome systems (Meyer *et al.*, 1998). Ferulic acid, in particular, has been shown to exhibit excellent antioxidant activity in inhibiting the formation of hexanal in liposomes (Vuorela *et al.*, 2005).

Increasing interest in dietary polyphenols and their effect on human health has led to the development of a number of databases on polyphenol contents in foods such as the USDA Database on Flavonoids (Nutrient Data Laboratory, 2007), Phenol-Explorer (Neveu *et al.*, 2010; Pérez-Jiménez *et al.*, 2010), EuroFIR-BASIS (Gry *et al.*, 2007) and the Brazilian Flavonoid Database (De Menezes *et al.*, 2011). Databases on phytochemicals have also been expanded to include data on the chemical structures and classification, spectra, metabolic pathways in plants, metabolism in humans and animals, biological properties and effects on health (Scalbert *et al.*, 2011). An Antioxidant Food Table, containing antioxidant

values for a wide array of samples including teas such as rooibos, was recently presented by Carlsen *et al.* (2010).

2.3 Characterisation of phenolic compounds

2.3.1 Colorimetric assays

Dietary polyphenols have often been determined using colorimetric assays such as Folin-Ciocalteu, to estimate the total polyphenol content of foods, and FRAP or oxygen radical absorbance capacity (ORAC), to estimate the total antioxidant capacity of foods (Tsao & Yang, 2003; Pérez-Jiménez *et al.*, 2010). These global assays do not, however, take into account the huge diversity of polyphenols in terms of their chemical structures, physico-chemical properties, bioavailability and biological properties. Because dietary polyphenols comprise this wide range of different properties, it is essential to consider them as individual chemical entities rather than as a whole undifferentiated group (Neveu *et al.*, 2010).

2.3.2 Analytical separation and detection techniques

Several separation techniques such as gas chromatography (GC), thin-layer chromatography (TLC), capillary electrophoresis (CE) and liquid chromatography (LC) have been applied in analysis of phenolic compounds. The analytical separation and detection methods for flavonoids and phenolic acids have been reviewed in full by De Rijke *et al.* (2006) and Stalikas (2007). Some of the major conclusions drawn in the review of De Rijke *et al.* (2006) will be discussed briefly below.

GC-based methods provide high resolution and low detection limits, but they are labour-intensive and time-consuming, as a derivatisation step is required to increase the volatility of the flavonoids and to improve their thermal stability. Various modes of TLC have been used, but the emphasis is mostly on rapid screening of plant or medicinal extracts for pharmacologically active constituents. Although CE allows higher separation efficiency than LC, it has found very limited application in flavonoid analysis. The CE modes primarily used are capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC). An excellent review of CE in the analysis of flavonoids has been presented by Suntornsuk (2002).

Hence, to date, the most widely used technique is based on RP-HPLC coupled to PDA detection and/or MS or tandem MS with atmospheric pressure ionisation techniques, *i.e.* ESI or APCI. PDA is an indispensable tool for the provisional identification of the main phenolic structures present in plants since they show characteristic UV-VIS spectra. Liquid chromatography coupled to mass spectrometry has, however, become the best alternative for their identification and structural characterisation (Rauha *et al.*, 2001; Abad-García *et al.*, 2009a). A review on the measurement of food flavonoids by HPLC has been presented by Merken & Beecher (2000).

2.4 RP-HPLC in the separation of phenolic compounds, and their detection and characterisation by UV-PDA and ESI-MSⁿ

For the investigation of structure-activity relationships and food quality control, it is important to have access to rapid and reliable methods for the analysis and identification of phenolic compounds in all of their many forms (Cuyckens & Claeys, 2004; Abad-García *et al.*, 2009a). Since flavonoids exist in complex natural matrices, these analytical methods should furthermore be selective and sensitive.

2.4.1 RP-HPLC separation

HPLC analysis of phenolic compounds is usually carried out in the RP mode employing octyl C8- or octadecyl C18-bonded silica columns (De Rijke *et al.*, 2006). RP columns are used because phenolic compounds are weak acids that can be separated as neutral, relatively hydrophobic compounds in a weak acid matrix (Harnly *et al.*, 2007). Almost exclusively, RP C18 phases with the following conventional dimensions are employed: column length: 100–250 mm; internal diameter: 3.9–4.6 mm; particle diameter: 3–10 μm (Stalikas, 2007). With regards to the selection of a suitable RP C18 column, a well end-capped column is preferred, as it has been demonstrated that residual silanol groups impair the separation of flavonoid glycosides (Cuyckens & Claeys, 2002). The choice of column also depends on the sample preparation technique because fairly crude plant extracts could damage the column. The main column is therefore usually protected by adding a small, in-line, guard column containing the same stationary phase (Stalikas, 2007).

Gradient elution is generally performed with a binary solvent system comprising of an organic modifier and a slightly acidified aqueous phase. Instead of linear elution gradients, complicated gradient profiles, comprising several steps and applying various slopes, are often used. In literature, details on how the gradient profiles were optimised are rarely supplied and hence one can deduce that trial-and-error often plays a large role in optimising gradient profiles (De Rijke *et al.*, 2006).

The two primary organic modifiers are methanol and acetonitrile. In some cases, acetonitrile leads to better resolution in a shorter analysis time than methanol. Generally, acetonitrile gives sharper peak shapes, although methanol is often preferred because of its less hazardous properties (Stalikas, 2007). The choice of organic modifier is also influenced by pressure and selectivity considerations.

In the HPLC analysis of phenolic compounds, separations are obtained by acidifying the mobile phase. A weakly acidic mobile phase suppresses ionisation, thereby increasing retention and decreasing peak broadening that is caused by formation of the deprotonated form, of especially phenolic acids (Cuyckens & Claeys, 2002; Harnly *et al.*, 2007; Stalikas, 2007). Most phenolic acids have $\text{p}K_{\text{a}}$ values of about 4, while the phenolic groups of flavonoids have $\text{p}K_{\text{a}}$ values above 9. The recommended pH range for the HPLC assay of phenolic compounds is therefore 2–4 (Stalikas, 2007).

The most common additives are formic acid, acetic acid, trifluoroacetic acid, ammonium-acetate and ammonium-formate. These compounds are volatile and thus compatible with mass spectrometric detection. Trifluoroacetic acid is, however, used to a lesser extent as it is known to suppress ionisation in the MS detector due to ion-pairing and surface tension phenomena (Cuyckens & Claeys, 2004; De Rijke *et al.*, 2006). The choice of acid may also affect resolution and retention times, for example, Cuyckens & Claeys (2002) demonstrated that the addition of formic and trifluoroacetic acid to the mobile phase solely affected chromatographic resolution, whereas the addition of acetic acid also resulted in a considerable decrease in the retention times of those flavonoid O-glycosides studied. This was attributed to acetic acid being a much weaker ion-pairing agent than formic and trifluoroacetic acid (Cuyckens & Claeys, 2002).

During reversed-phase gradient elution, the organic composition of the mobile phase is systematically increased, providing an increase in solvent strength, which leads to the elution of the more retained, non-polar compounds from the stationary phase. For RP C18 columns generally used, polyphenol subclasses generally elute in the following order: hydroxybenzoic acids, flavan-3-ols, hydroxycinnamic acids, coumarins, flavanones, dihydrochalcones, flavonols and flavones (Cuyckens & Claeys, 2004; Abad-García *et al.*, 2009a). Flavanones elute before their corresponding flavones due to the effect of unsaturation between

C2 and C3 (Anderson & Markham, 2006). Within the same polyphenol subclass, an increase in the number of hydroxyl groups and an increase in the degree of glycosylation leads to a reduction in retention time; acylation, prenylation, or methylation has the opposite effect (Cuyckens & Claeys 2004). The specific position of glycosylation (Harborne & Boardley, 1984) or methylation (Greenham *et al.* 1995) can also have a significant impact on the retention time. Flavone C-glycosides generally elute at lower retention times than the corresponding O-glycosides (Anderson & Markham, 2006). Furthermore, 8-C-glycosyl flavones elute at shorter retention times than the corresponding 6-C-glycosyl flavones (Anderson & Markham, 2006). For isomeric compounds, differing only with regards to the nature of the glycoside moiety, galactosides elute ahead of glucosides, glucosides ahead of arabinosides, and arabinosides ahead of rhamnosides. Furthermore, in all the studied cases, rutinosides elute before their corresponding neohesperidose analogues and hence the type of interglycosidic linkage also influences retention (Cuyckens & Claeys, 2004). Under the specific RP-LC conditions used by Abad-García *et al.* (2009a), the following elution order was established for glycosylated polyphenols in the same bond positions: di-O-glycoside, O-galactoside, O-glucoside, O-rutinoside, O-neohesperidoside, O-rhamnoside, aglycones.

HPLC analysis of phenolic compounds are mostly performed at ambient column temperature, but moderately higher temperatures ranging between 30–40°C have also been recommended, leading to a reduction in the analysis time and more reproducible elution times (De Rijke *et al.*, 2006; Harnly *et al.*, 2007; Stalikas, 2007).

If the main aim of the study is to determine the major phenolic compounds in a sample, analysis times of 0.5–1 hour usually suffice to separate the compounds of interest. If, on the other hand, a more extensive separation of the constituents is required, analysis times may extend up to 2 hours (De Rijke *et al.*, 2006).

2.4.2 Detection

2.4.2.1 Ultraviolet-photodiode-array (UV-PDA) detection

The multiple conjugate double and aromatic bonds make phenolic compounds strong chromophores, exhibiting absorption in the UV or UV-VIS region (Harnly *et al.*, 2007; Stalikas, 2007). A PDA detector allows recording of the UV-VIS spectra of each chromatographic peak. Each chromatographic peak may then be attributed to a polyphenol subclass, since each subclass exhibit characteristic UV-VIS spectra (De Rijke *et al.*, 2006; Stalikas, 2007; Abad-Garcia *et al.*, 2009a). UV-PDA detection furthermore supplies information on the purity of all peaks and offers the best means of quantifying phenolic compounds in routine applications (Harnly *et al.*, 2007; Stalikas, 2007).

Phenolic acids (Figure 9) with the hydroxybenzoic acid carbon framework have absorbance maxima in the 200–290 nm range, whereas the cinnamic acid derivatives exhibit a broad absorbance band from 270–360 nm due to additional conjugation (Stalikas, 2007). More recently, Abad-Garcia *et al.* (2009a) provided more narrow/defined absorption bands and reported that hydroxybenzoic acids, presenting a benzoyl group as the only chromophore, exhibit a band in the 255–280 nm range. The hydroxycinnamic acids were reported to exhibit a band in the 310–325 nm range (Abad-Garcia *et al.*, 2009a).

All flavonoid aglycones contain at least one aromatic ring and therefore efficiently absorb UV light, while each flavonoid subclass exhibits characteristic UV-VIS spectra (Markham, 1982). For an illustration of the basic structures of selected flavonoid subclasses, refer to section 3.5.2 (Table 3).

The UV spectra of flavones and flavonols exhibit two major absorption peaks in the region 240–400 nm, commonly referred to as band I (300–380 nm) and band II (240–280 nm). Band I is associated with the absorption due to the B-ring cinnamoyl system and band II with the absorption involving the A-ring benzoyl system. Flavones present an intense band I (339–350 nm) and band II (261–279 nm), whilst band I is shifted towards lower wavelengths for the polymethoxylated flavones due to the methoxylation of the B-ring. Flavonol-3-O-glucosides are characterised by a lower intensity of band I (347–370 nm) compared to band II (324–336 nm). Flavan-3-ols and dihydrochalcones are readily distinguished from other flavonoids by their UV spectra: flavan-3-ols typically exhibit an intense band II (269–279 nm) and do not present band I absorption due to the lack of conjugation between the A- and B-rings, whereas the dihydrochalcones presented a broader band II (286 nm) due to the benzoyl group, which appears as a shoulder. Flavanones have quite characteristic UV spectra, as the A- and B-rings are not conjugated: band I is not defined, but the benzoyl group shows an unresolved band at approximately 330 nm (Abad-García *et al.*, 2009a).

De Rijke *et al.* (2006) surmised that the characterisation of flavonoid aglycones also holds true for their conjugates and that simple substituents such as methyl, methoxy, and non-dissociated hydroxyl groups only induce minor changes in the position of the absorption maxima. More recently, however, Abad-García *et al.* (2009a) showed that the UV-spectra of flavonoids can actually aid in characterising the substitution patterns of the A- and B-rings. Additional hydroxyl groups in the A- or B-rings cause a bathochromic shift of bands II and I, respectively. Methylation of these hydroxyl groups lowers this effect, especially if they are in a resonant position (e.g. 4'). For flavone glycosides, O- and C-glycosylation in the A-ring had no or little effect on the UV-VIS spectra. Conversely, glycosylation in the B-ring shifted band I to lower wavelengths and this effect was more pronounced for a resonant (e.g. 4', see Figure 8) than for a non-resonant position (e.g. 3') (Abad-García *et al.*, 2009a). These authors ultimately concluded, however, that the detection of glycosylation or the differentiation of the type of glycosylation (O-, C- or O,C-) is impossible based only on UV-VIS spectra.

2.4.2.2. Mass spectrometric (MS) techniques

The coupling of LC to mass spectrometry represents a powerful tool for the analysis of natural products since the mass spectrometer provides high sensitivity. It furthermore provides information on the molecular weight as well as structural features of the sample analytes (Cuyckens & Claeys, 2002; Abad García *et al.*, 2009a). By resorting to MS/MS in combination with CID more detailed structural information can be obtained. With regard to structural characterisation of polyphenols, information can be obtained on: the aglycone moiety; the types of carbohydrates (mono-, di-, tri- or tetrasaccharides and hexoses, deoxyhexoses or pentoses) or other types of substituents present; the stereochemical assignment of terminal monosaccharide units; the sequence of the glycan part; the interglycosidic linkages; and the linkage point of the substituents to the aglycone (Cuyckens & Claeys, 2004).

Older ionisation techniques such as moving belt (MB), GC-MS and electron impact (EI) MS (Games & Martinez, 1989; Hedin & Phillips, 1992; Schmidt *et al.*, 1993; Franski *et al.*, 1999) were used in the earlier analysis of flavonoids. These methods presented several difficulties which were only overcome by the development of API interfaces (Rauha *et al.*, 2001). The efficiencies of various API interfaces, *i.e.* APCI, ESI and atmospheric pressure photoionisation (APPI), have been compared by Rauha *et al.* (2001).

Today, in the LC-MS analysis of flavonoids APCI and ESI are used almost exclusively and furthermore offer benefits with regards to ease of operation and high sensitivity (De Rijke *et al.*, 2006). Owing to the soft ionisation of APCI and ESI, flavonoid glycosides can be detected as protonated or deprotonated

molecules together with diagnostic fragment ions (Rauha *et al.*, 2001). According to most studies, for both APCI and ESI, the negative ionisation mode provides the highest sensitivity. Negative ionisation results in limited fragmentation, making it most suited to deduce the molecular mass of flavonoids, especially in cases where the concentrations are low (Cuyckens & Claeys, 2004). On the other hand, the first-order mass spectrum in the positive ionisation mode contains more structural information, which is useful to identify known compounds (Cuyckens & Claeys, 2004). The combined use of both ionisation modes yield complementary information which may aid in the identification of unknowns (De Rijke *et al.*, 2006).

Both APCI and ESI operated in positive or negative ionisation modes appear to be favoured by different authors, and in all these studies the elution conditions (pH and buffer components) were found to be critical. In the negative ionisation mode, a concentration of 0.1% formic acid (Cuyckens & Claeys, 2002) or ammonium acetate buffer adjusted to pH 4.0 (Rauha *et al.*, 2001) seems to be preferable. In the positive ionisation mode, the lowest detection limits are obtained with an overall concentration of 0.4–0.5% formic acid (Rauha *et al.*, 2001; Cuyckens & Claeys, 2002).

2.5 Structural characterisation of flavonoids obtained by ESI-MSⁿ

For structural elucidation, especially of flavonoids, special attention has been devoted to the use of mass spectrometric techniques for the characterisation of several important subclasses.

2.5.1 Flavonoid glycosides

Product ions of flavonoid glycosides are generally denoted according to the nomenclature proposed by Domon & Costello (1988) for glycoconjugates (Figure 10).

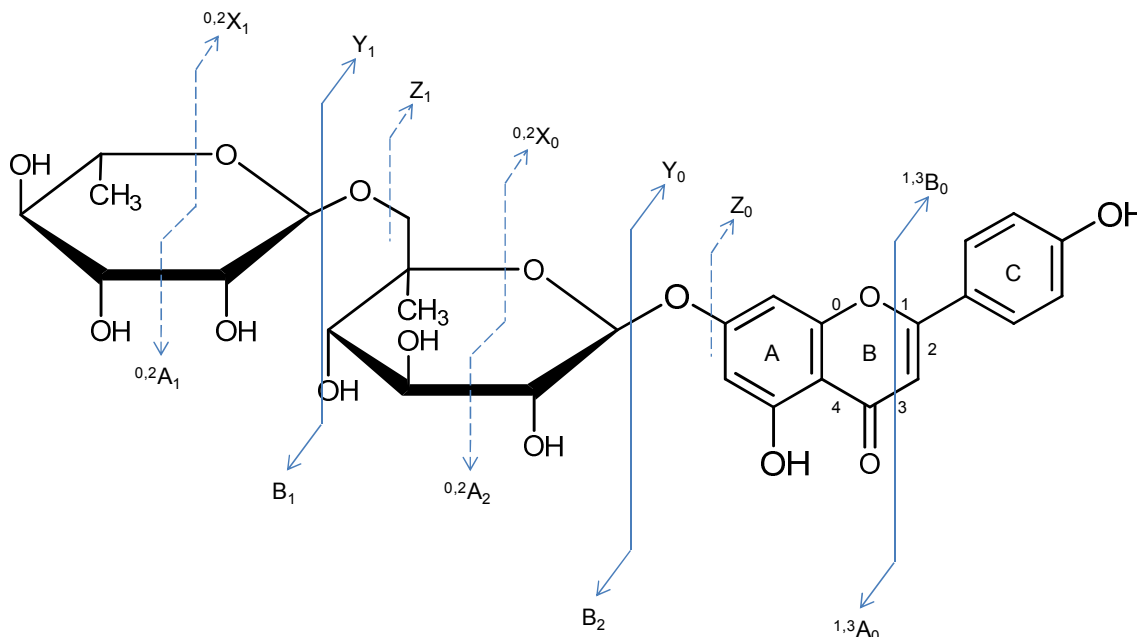


Figure 10 Fragmentation nomenclature used for MS analysis of flavonoid glycosides as illustrated for apigenin-7-O-rutinoside (Domon & Costello, 1988; Ma *et al.*, 1997; Cuyckens & Claeys, 2004; Abad-García *et al.*, 2009a). Numbering at C-ring correspond to specific bonds.

Ions containing the aglycone are labelled $^{k,l}X_j$, Y_j and Z_j . The subscript j denote the number of the interglycosydic bond cleaved, counting from the aglycone, while the superscripts k and l indicate cleavages

within the carbohydrate rings. The glycosidic bond linking the glycan part to the aglycone is numbered 0. When the charge is retained on the carbohydrate residue, fragments are designated $^{k,l}A_i$, B_i and C_i where i (≥ 1) represents the number of the glycosidic bond cleaved, counting from the non-reducing end. When more than one glycosylation position are involved, an additional superscript m , which denotes aglycone substitution, is used for ions $^{k,l}X_j^m$, Y_j^m .

2.5.1.1 Flavonoid O-glycosides

Flavonoids commonly occur as flavonoid O-glycosides; hydroxyl groups located at C3 and C7 are the typical glycosylation sites leading to the formation of an acid-labile glycosidic C-O bond.

O-monoglycosides

In the positive ion mode (ESI(+)-CID MS/MS), low collision energies in the range of 10 eV are enough to induce the main fragmentation in the O-monoglycosides (Abad-Garcia *et al.*, 2009a). Flavonoid O-monoglycosides are fragmented by cleavage of the glycosidic O-linkage with concomitant H-rearrangement, leading to the elimination of the monosaccharide residue, *i.e.* loss of 162 amu (hexose), 146 amu (deoxyhexose) or 132 amu (pentose), providing the corresponding Y_0^+ aglycone ion (Abad-Garcia *et al.*, 2009a; Cuyckens & Claeys, 2004). In flavonol-3-O-monoglycosides, the complementary monosaccharide ion, a terminal hexose or rhamnose unit, can also be detected as $[B_1]^+$ at m/z 163 and 147, respectively (Abad-Garcia *et al.*, 2009a).

Using negative ion ESI-MS/MS, in addition to the aglycone fragment $[Y_0]$, an abundant radical aglycone $[Y_0-H]^-$ may also be observed. The latter has been observed in the high-energy CID spectra of quercetin O-glycosides (Cuyckens & Claeys, 2004). This radical aglycone product ion is formed by a homolytic cleavage of the glycosidic bond between the aglycone and the glycan residue and has been studied in detail by Hvattum & Ekeberg (2003).

O-diglycosides (glycosylated flavonoids which have a disaccharide attached to a single hydroxyl group of the aglycone).

Flavonoid O-diglycosides are also frequently encountered, of which the most common disaccharides include rutinose [rhamnosyl-(α 1 \rightarrow 6)-glucose] and neohesperidose [rhamnosyl-(α 1 \rightarrow 2)-glucose]. These disaccharides only differ by the interglycosidic linkage between the glucose and rhamnose units. In the positive ionisation mode, Y_1^+ and Y_0^+ fragments are observed which correspond to losses of the terminal rhamnose unit and the rhamnosylglucose residue, respectively. Higher collision energies favour formation of the Y_0^+ aglycone fragment ion. In some cases, the complementary monosaccharide ions $[B_1]^+$ (m/z 147) and $[B_2]^+$ (m/z 309) can also be detected (Cuyckens & Claeys, 2004; De Rijke *et al.*, 2006; Abad-Garcia *et al.*, 2009a). A $[Y^*]^+$ ion, corresponding to the loss of the inner glucose residue ($[M+H-162]^+$), is also present in flavonol-3-O-rutinosides, flavanone-7-O-rutinosides and flavanone-7-O-neohesperidoses (Cuyckens & Claeys, 2004; Abad-Garcia *et al.*, 2009a). The $[Y^*]^+$ ion is formed by the migration of a hydrogen from the hydroxyl group at C5 of the aglycone to the terminal rhamnose and a rearrangement reaction. The rearrangement entails migration of the glucose acetalic oxygen to the terminal rhamnose anomeric carbon, losing the internal glycoside residue (Ma *et al.*, 2000). Formation of the $[Y^*]^+$ ion is favoured at low collision energies (Ma *et al.*, 2000) and this ion can reach very high relative abundances (Cuyckens *et al.*, 2001).

Differentiation of flavonoid O-diglycosides by LC/MS methods have been studied by various groups (Franski *et al.*, 1999; Ma *et al.*, 2000; Cuyckens *et al.*, 2001; Ma *et al.*, 2001; Ferreres *et al.*, 2004; Zhang & Brodbelt, 2005; Ablajan *et al.*, 2006; Clowers & Hill, 2006; Zhou *et al.*, 2006; Shi *et al.*, 2007; Kachlicki *et al.*, 2008). Recently, practical guidelines to differentiate between O-diglycosyl flavonoid isomers were presented by Abad-Garcia *et al.* (2009b). These guidelines are based on UV and MS² product ion spectra of [M+H]⁺ and allows determination of aglycone identity, the position of glycosylation, and the type of interglycosidic linkage.

Di-O-glycosides (glycosylated flavonoids which have two monosaccharides linked to two different hydroxyl groups of the aglycone)

Abad-Garcia *et al.* (2009b) reported that the ESI(+)-CID MS/MS spectra of flavonoid di-O-glycosides presented a very intense peak for the quasi-molecular ion at low collision energy (10 eV). Higher collision energy (20 eV) results in cleavage of one of the two glycosidic bonds, obtaining the ions [Y^{3'}₀]⁺ and/or [Y⁷₀]⁺ for flavones. The [Y^{3'}₀Y⁷₀]⁺ ion, formed by the cleavage of both sugar-aglycone linkages at C3' and C7, was also detected for the flavones.

The position of glycan substituents of different mass can easily be located in flavonol-3,7-di-O-glycosides because the protonated molecules more readily lose a glycan substituent at the C3 position compared to the C7 position (Sakushima *et al.*, 1988). Similarly, the aglycone product ion detected for protonated luteolin-5-O-glucoside is much more abundant than that detected for luteolin-7-O-glucoside (Grayer *et al.*, 2000). These observations indicate that the susceptibility of the sugar-aglycone bond to acid hydrolysis certainly depends on the position of the sugar, as has been demonstrated by Geiger & Schwinger (1980). Conversely, the nature of the sugar only has a negligible effect on the strength of the sugar-aglycone bond (Geiger & Schwinger, 1980).

2.5.1.2 Flavonoid C-glycosides

Glycosylation may also occur via direct linkage of the sugar to the basic flavonoid nucleus with the formation of an acid-resistant C-C bond to form C-glycosides. C-glycosides are further divided into mono-C-glycosyl-, di-C-glycosyl- and O,C-diglycosyl-flavonoids.

Mono-C-glycosides

Flavonoid C-glycosides need higher collision energies to fragment than O-glycosides, due to the absence of an acid-labile bond (Abad-Garcia *et al.*, 2009a). The main fragmentations concern cross-ring cleavages of the saccharide residue and the loss of water molecules (Figure 11; Cuyckens & Claeys, 2004; Abad-Garcia *et al.*, 2009a).

Abad-Garcia *et al.* (2009a) showed that the main product ions in the low collision energy (20 eV) ESI(+)-CID MS/MS spectra of the C-glycosides were as follows: [^{0,2}X]⁺, [^{0,1}X]⁺, [^{0,4}X-2H₂O]⁺ and [M+H-CH₂O-2H₂O]⁺. The latter fragment was formed by the loss of glucosidic methylol group as formaldehyde (Abad-Garcia *et al.*, 2009a). The product ions at [^{0,3}X]⁺, [^{0,2}X]⁺ and [^{0,1}X]⁺ correspond to losses of 90, 120 and 150 amu, respectively, for the hexose; and to losses of 60, 90 and 120 amu, respectively, for the pentose (Cuyckens & Claeys, 2004; Abad-Garcia *et al.*, 2009a).

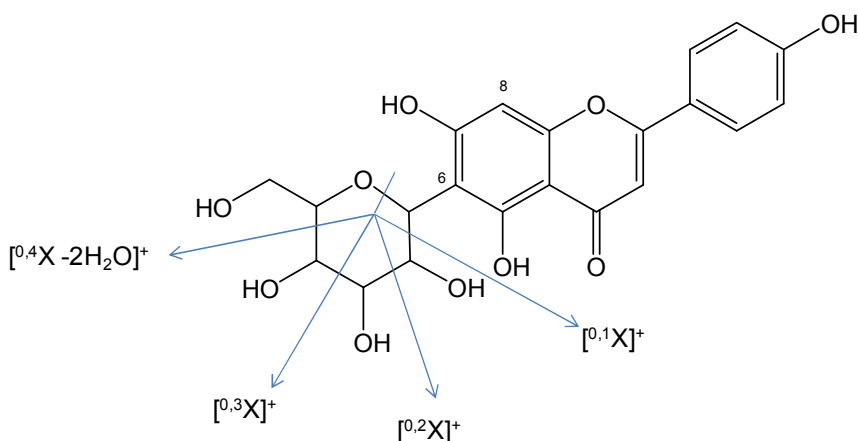


Figure 11 Characteristic product ions formed by cross-ring cleavages in a hexoside residue, illustrated for a protonated flavone C-6-mono-C-glucoside (Cuyckens & Claeys, 2004; Abad-Garcia *et al.*, 2009a).

As the C-glycosylation site is limited to the C6 and C8 positions of the flavonoid nucleus (Figure 11), the greatest challenge entails differentiation between the positional isomers. Abad-Garcia *et al.* (2008) proposed the use of the relative ratio of the product ions $[^{0.1}X]^+$ and $[^{0.2}X]^+$ (at high collision energies of 40 eV) to differentiate between flavone C-6- and C-8-mono-C-glucosides. For C-6-hexosides, $([^{0.1}X]^+ / [^{0.2}X]^+)$ yields a ratio of ~ 2 , whilst for C-8-hexosides, this value is ~ 1 . The fragmentation of the C6 isomers is also more extensive, attributable to the formation of an additional hydrogen bond which confers additional rigidity, and may aid in differentiation. $[^{0.3}X]^+$ is another diagnostic ion – this ion is present in low, but noticeable intensity for the C8 isomer, while its intensity is marginal for the C6 isomer (Abad-Garcia *et al.*, 2009a).

Di-C-glycosides

In di-C-glycosides, sugar residues of different mass can be located easily since the C6-sugar residue exhibits more extensive fragmentation than the C8-sugar residue (Cuyckens & Claeys, 2004). If the sugar residues are of different mass (for example a hexoside and pentoside), the di-C-glycoside is referred to as 'asymmetric', whilst sugar residues of similar mass yield 'symmetric' di-C-glycosides.

O,C-diglycosides

O,C-diglycosyl-flavonoids differ from O- and C-glycosylated flavonoids in that the O-glycoside moiety is either linked to a hydroxyl group of the aglycone or to a hydroxyl group of the C-bound glycosyl residue. Although the O,C-diglycosyl flavonoids present UV-vis spectra identical to their equivalent O- and C-glycosides, they can be distinguished based on ESI(+)-CID MS/MS spectra. Protonated O-diglycosides give rise to Y_1^+ and Y_0^+ ions, formed by rearrangement reactions at the interglycosydic bonds, whereas C-glycosides only yield $[M + H]^+$ ions together with cross-ring cleavages of the saccharidic residue and loss of water molecules. Conversely, at low collision energies protonated O,C-diglycosides only yield Y_1^+ ions, formed by fragmentation at the interglycosydic linkage (Cuyckens & Claeys, 2004). At higher collision energies, however, protonated O,C-diglycosides exhibit both types of fragmentations (Abad-Garcia *et al.*, 2009a).

2.5.2 Flavonoid aglycones

The flavonoid aglycone fragment ions are commonly designated according to the nomenclature proposed by Ma *et al.* (1997). For free aglycones, the $^{ij}A^+$ and $^{ij}B^+$ labels refer to the fragments containing intact A- and B-rings, respectively. The superscripts i and j indicate the C-ring bonds that have been broken. For conjugated aglycones, an additional subscript 0 to the right of the letter is used to avoid confusion with the A_i^+ and B_i^+ labels ($i \geq 1$) that have been used to designate carbohydrate fragments containing the a terminal (non-reducing) sugar unit (Figure 10).

2.5.2.1 Positive ionisation mode

In the structural characterisation of flavonoids, positive ion CID spectra are mostly used. The most useful fragmentations in terms of flavonoid aglycone identification are those that require cleavage of two C-C bonds of the C-ring, resulting in structurally informative $^{ij}A^+$ and $^{ij}B^+$ ions (Figure 10). These ions are the most diagnostic fragments for flavonoid identification since they provide information on the number and types of substituents on the A- and B-rings. Cleavage of the C-ring proceeds via a retro-Diels-Alder (RDA) reaction at bond positions 1/2, 0/2, 0/3, 0/4 or 2/4 of the C-ring (Cuyckens & Claeys, 2004; Abad-Garcia *et al.*, 2009a). RDA typically involves relocation of three pairs of electrons in the C-ring and the subsequent cleavage of two σ -bonds and the formation of two π -bonds (De Rijke *et al.*, 2006). The prevalent fragmentation pathways strongly depend on the substitution pattern and subclass of flavonoids studied (Cuyckens & Claeys, 2004; Abad-Garcia *et al.*, 2009a).

The $^{1,3}A^+$ ion, observed for all flavonoid subclasses, is generally the fragment most readily formed and often constitutes the most abundant fragment ion (Cuyckens & Claeys, 2004; De Rijke *et al.*, 2006). For flavones and flavonols cleavage of the 0,2 bonds (Figure 10) is a common C-ring cleavage pathway, leading to the formation of the $^{0,2}B^+$ ion. It has been proposed that the corresponding $^{0,2}A^+$ ion may be used to distinguish flavonols as this ion does not occur in the spectra of the other subclasses of flavonoids (De Rijke *et al.*, 2006).

In addition to the $^{ij}A^+$ and $^{ij}B^+$ ions discussed above, losses of 18 amu (H_2O), 28 amu (CO), 42 amu (C_2H_2O) and/or successive losses of these groups are commonly observed. The loss of 15 amu (CH_3) from the $[M+H]^+$ precursor ion, is prominent in many O-methylated isoflavones, flavones and flavonols, and the $[M+H-CH_3]^+$ radical product ion generally constitutes the base peak. Losses of 56 amu (C_4H_8) are indicative of the presence of a prenyl substituent (Cuyckens & Claeys, 2004; De Rijke *et al.*, 2006).

ESI(+)-CID MS/MS in the study of the fragmentation pathways of the different subclasses of flavonoid aglycones and phenolic acids

Abad-Garcia *et al.* (2009b) extensively studied the fragmentation pathways of different classes of flavonoid aglycones and phenolic acids by employing ESI(+)-CID MS/MS product ion spectra, obtained using the protonated aglycone $[Y_0]^+$ as precursor ion. A quick overview will only be given of the major flavonoid subclasses (flavones; flavonols; flavanones; dihydrochalcones) and phenolic acids considered in this study. Different collision energies in the range 5–40 eV were used.

For the hydroxylated flavones, apigenin and luteolin, the following ions, characteristic of the C-ring cleavage, were detected: $[^{0,4}B_0]^+$, $[^{0,4}B_0-H_2O]^+$, $[^{1,3}A_0]^+$ (base peak), $[^{0,2}B_0]^+$, $[^{1,3}B_0]^+$, $[^{1,3}A_0-C_2H_2O]^+$ and $[^{1,3}B_0-CO]^+$. In the case of luteolin two other ions, assigned as $[^{1,3}B_0-H_2O]^+$ and $[^{1,3}B_0-H_2O-CO]^+$, were also detected.

The hydroxylated flavonols, kaemferol, quercetin and myricetin, exhibited major fragment ions [$^{1,3}A_0$] $^+$ and [$^{0,2}B_0$] $^+$. Other fragment ions also detected include [$^{0,2}A_0$] $^+$, [$^{0,3}A_0$] $^+$, [$^{0,2}A_0-CO$] $^+$, [$^{1,3}A_0-H_2O$] $^+$, [$^{1,3}B_0-2H$] $^+$, [$^{1,4}A_0+2H$] $^+$, [$^{1,3}A_0-CO$] $^+$, [$^{1,3}A_0-C_2H_2O$] $^+$, [$^{0,2}A_0-C_2H_2O$] $^+$, [$^{0,2}A_0-2CO$] $^+$, [$^{1,3}A_0-H_2O-CO$] $^+$, [$^{1,3}B_0-2H-CO$] $^+$, [$^{0,2}B_0-CO$] $^+$ and fragment ions from losses of small molecules (CO, H₂O or ketene) from the aglycone.

For flavanone aglycones the ion [$^{1,3}A_0$] $^+$ was the base peak, while other fragment ions such as [$^{1,3}A_0-CO$] $^+$, [$^{1,3}A_0-C_2H_2O$] $^+$, [$^{1,3}B_0-2H$] $^{+}$ and [$^{0,4}B_0-H_2O-CO$] $^+$ were also detected. The relative abundances of these product ions were relatively high in comparison with the precursor ion ($[Y_0]^+$), since cleavage of the C-ring in the flavanones is easily induced (Abad-Garcia *et al.*, 2009b).

The dihydrochalcones differ from the other flavonoid subclasses in that they do not possess a heterocyclic C-ring, but an open and saturated 3-C chain, which fragments easily at low collision energies. Rupture of the bond between C1' from the A-ring and the carbon atom from the keto group and subsequent loss of a ketone, yields the product ions [$C_9H_9O_2$] $^+$ and [C_7H_7O] $^+$, respectively. Other ions characteristic to the dihydrochalcones include [$C_8H_9O_4$] $^+$ and [$C_6H_7O_3$] $^+$.

The ESI(+)-CID MS/MS product ion spectra of free hydroxycinnamic acids presented product ions caused by successive losses of H₂O and CO from the precursor ion. Losses of CH₃OH were also observed for acids containing a methoxy group, such as ferulic acid.

The fragmentation pathway of free hydroxybenzoic acids were characterised by losses of H₂O, CO and CO₂ molecules. In acids with methoxy groups, loss of CH₃OH or methyl radicals were also observed.

2.5.2.2 Negative ionisation mode

Although positive ionisation mode is more commonly employed, the negative ionisation mode is considered to be more sensitive. In comparison with positive ionisation mode, higher collision energies are required to yield adequate fragmentation (Cuyckens & Claeys, 2004).

The fragmentation patterns of various aglycones in the negative ionisation mode have been studied in detail by Fabre *et al.* (2001). RDA C-ring cleavage of the 1,3 bonds, forming $^{1,3}A^-$ and $^{1,3}B^-$ fragment ions, is the most important fragmentation pathway in the negative ionisation mode, similar to positive ionisation mode (De Rijke *et al.*, 2006). The $^{1,3}A^-$ fragment is often the major fragment ion (Cuyckens & Claeys, 2004), whereas a $^{0,3}B^-$ ion as the major peak is characteristic for isoflavones (Hughes *et al.*, 2001).

The degree of hydroxylation on the B-ring has an impact on fragmentation. For example, for flavonols containing more than two hydroxyl groups in the B-ring (e.g. quercetin), fragment ions corresponding to [$^{1,2}A-H$] $^-$ and [$^{1,2}B+H$] $^-$ may be observed. In the case of an unsubstituted B-ring, however, higher collision energies are required to obtain fragmentation, leading to many product ions (Cuyckens & Claeys, 2004).

In some cases, a direct cleavage of the bond between the B- and C-ring can be observed, resulting in an [M-B-ring] fragment (Fabre *et al.*, 2001). Neutral losses of 18 amu (H₂O), 28 amu (CO), 34 amu (CO₂), 42 amu (C₂H₂O) and/or successive losses of these groups may also be prominent (Cuyckens & Claeys, 2004; De Rijke *et al.*, 2006). Similar to positive ionisation mode, methylated compounds are characterised by the loss of 15 amu (CH₃), whilst, in contrast to positive ionisation mode, the characterisation of prenylated flavonoids is hampered as cleavage of the isoprenoid substituent is not observed (Cuyckens & Claeys, 2004).

3. Rooibos (*Aspalathus linearis*)

3.1 Introduction

3.1.1 Geographical distribution and botanical classification

The genus *Aspalathus* (Fabaceae; tribe Crotonarieae) comprises more than 270 species and is endemic to South Africa. *Aspalathus linearis* (Burm.f.) Dahlg. occurs over a wide geographical area in the western and south-eastern parts of the Western Cape Province (Figure 12) and in limited areas in the south-western part of the Northern Cape Province (Dahlgren, 1968; 1988).

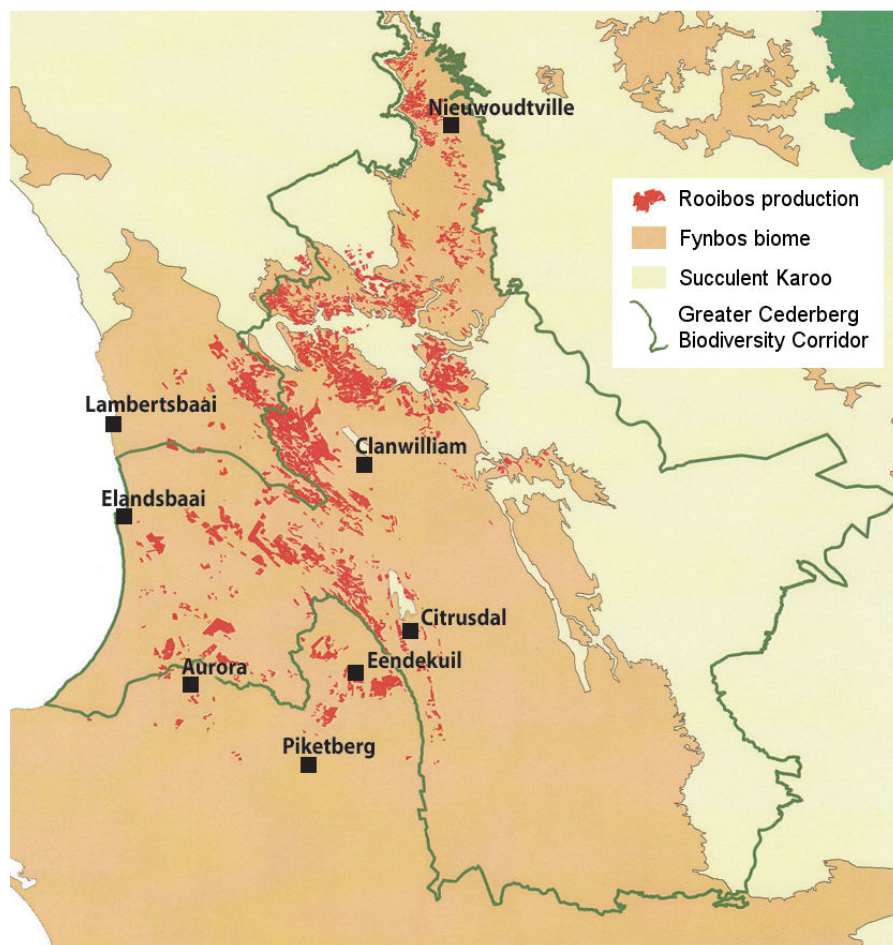


Figure 12 Distribution of *Aspalathus linearis* (map supplied by the South African Rooibos Council).

This specific occurrence of *Aspalathus linearis* in the Fynbos biome in the Cape Floristic Region, one of the 25 global biodiversity hotspots, has stimulated interest in acquiring geographical indication (GI) certification for rooibos. GI certification, reserved for products which acquire their characteristic qualities as a result of their geographical location, will protect rooibos from possible cultivation outside South Africa (Gerz & Bienabe, 2006; Joubert & De Beer, 2011).

Only one type of *Aspalathus linearis* namely the red or Rocklands type is currently used to produce tea and is of commercial importance. Initially different types of *Aspalathus linearis*, *i.e.* the grey, black and red-brown types, were also harvested in the wild for tea processing but marketing of the grey and black types were discontinued in 1966 due to poor quality (Anon., 1967). The red type is further divided into the wild-

growing Cederberg type and the selected and improved Nortier type (cultivated). The wild-growing Cederberg type is characterised by broader and coarser leaves (Morton, 1983). On a non-commercial scale, small amounts of a closely related species, *A. pendula* Dahlg., as well as several wild types of *A. linearis* are also used to make tea (Van Heerden *et al.*, 2003). Producers in the Cederberg (Wuppertal) and Southern Bokkeveld (Heiveld) harvest wild rooibos for commercial sale to niche organic and fair-trade markets (Nel *et al.*, 2007; Malgas *et al.* 2010).

3.1.2 Historical and modern uses

The first recorded consumption of rooibos by the native Hottentots dates back to 1772 (Morton, 1983), whilst modern use of rooibos as an alternative to Oriental tea (*Camellia sinensis*) only started in the early 1900s. Rooibos tea was initially prepared by boiling the leaves and stems in water, followed by consumption as a hot, strong brew with the addition of milk and sugar. Due to its more convenient form, the modern day consumer typically prefers the use of teabags instead of loose-leaf tea. The infusion is prepared by infusing one teabag (ca 2 g) per cup with freshly boiled water for 2–5 min to release the flavour and colour. The infusion may then be served hot, with or without milk and sugar according to taste (Joubert *et al.*, 2008a).

Rooibos tea is a unique beverage, with a characteristic flavour, and is most notably characterised by the lack of caffeine and a low tannin content (Morton, 1983). Various types of flavoured rooibos (such as vanilla and lemon) and tea mixtures (e.g. honeybush, buchu and fennel) are commercially available (Snyman, 2000) whilst several ready-to-drink, flavoured rooibos iced teas have also entered the market (Anon., 2010a). Another novel concept is that of Red Espresso™, a tea ‘espresso’ prepared from finely ground rooibos leaves (Anon., 2008). An African flavoured yoghurt range (Anon., 2010b) and ‘Centres’, which have a flavoured centre of rooibos tea, honeybush tea or buchu, with a carob coating (Anon., 2005a) are also two uniquely South African rooibos products.

3.1.3 Health-promoting benefits

Clinically, rooibos tea is often used for reducing nervous tension, for alleviating allergies and for calming digestive problems such as indigestion, heartburn and nausea. Anecdotal evidence furthermore suggests that rooibos improves the appetite and promotes sound sleep (Joubert *et al.*, 2008a). Topical applications of rooibos extract are believed to alleviate dermatological problems such as eczema and acne (Joubert *et al.*, 2008a) and it has also been established that rooibos has effects on dermatological diseases such as Behcet’s disease, Sweet disease and photosensitive dermatitis (Ferreira *et al.*, 1995).

The *in vitro*, *ex vivo* and *in vivo* biological properties of rooibos have been comprehensively reviewed by Joubert *et al.* (2008a). The beneficial physiological effects include antioxidant, anti-inflammatory, anticarcinogenic, hepatoprotective and phyto-oestrogenic properties. Other miscellaneous effects include: antispasmodic effects, immune system modulation, protection against cell transformation, proliferation and clastogenic effects, vasodilatory effect, antihemolytic effect, anti-ageing properties, and antimicrobial and antiviral effects (Joubert *et al.*, 2008a). Since then, the latest studies on the potential health-promoting properties of rooibos have been summarised by Joubert & De Beer (2011). These health-promoting benefits are attributed to rooibos’s phenolic composition.

3.2 Industry

The rooibos plant is recognised as one of the few economic plants that has made the transition from a local wild resource to a cultivated crop in the 20th century. Commercial sale of rooibos was initiated by Benjamin Ginsberg in 1904 when rooibos was marketed under the brand 'Eleven O'Clock' tea. The demand for rooibos increased during World War II as there was a shortage of Oriental tea but after the war the market collapsed due to the availability of cheap coffees and Oriental tea in more convenient forms. The Clanwilliam Tea Co-operative Company was subsequently established in 1948 to improve marketing conditions for rooibos tea producers. However, a further decrease in demand, overproduction and poor/inconsistent quality made rooibos production uneconomical by 1953/54. This led to the establishment of the Rooibos Tea Control Board (later known as the Rooibos Tea Board) with the objectives to regulate marketing, and to ensure quality grading and price stabilisation. This one-channel marketing system was abolished in October 1993, allowing new marketing companies to enter the industry and enabling access to a broader market (Joubert *et al.*, 2008a; Joubert & De Beer, 2011). With the abolishment of the Rooibos Tea Board, no organisation dealing with matters of mutual concern existed. This changed in 2005 when the South African Rooibos Council (SARC) was established to coordinate activities relating to generic marketing, research and development, and sustainable natural resource management (Joubert & De Beer, 2011).

The first exports of 524 tons of rooibos already occurred in 1955 and since then the international market demand for rooibos steadily grew from 750 tons in 1993 (Anon., 1994) to a record amount of 7176 tons (total exports) in 2007 (data from Perishable Products Export Control Board, PPECB, supplied by SARC). This increase coincided with the interest of consumers in natural antioxidants as rooibos gained prominence as an antioxidant-containing beverage with potential health-promoting properties (Von Gadow, 1996). However, a decline in the total amount of rooibos exported was observed during the past three years (Table 2), possibly due to the global economic crises and a strong Rand. Total tons of rooibos exported to the top ten countries for the 2003–2010 period are summarised in Table 2.

Table 2 Top ten export countries with the total tons of rooibos exported to each from 2003 to 2010 (data from PPECB, supplied by SARC).

Country	Year							
	2003	2004	2005	2006	2007	2008	2009	2010
Germany	4525.4	4063.1	3382.6	3354.0	3934.6	3495.1	3018.8	2512.0
Netherlands	455.0	520.0	721.0	798.0	781.0	753.8	955.8	1085.1
UK	169.7	150.4	307.3	425.6	527.2	782.6	787.3	860.3
USA	123.0	275.2	217.8	386.9	383.0	341.4	278.8	362.5
Japan	474.1	286.2	273.7	312.5	413.0	260.5	380.2	360.9
Poland	47.0	36.0	63.0	57.9	63.0	171.0	144.4	239.0
Russia	1.4	2.1	45.0	161.9	134.6	136.3	112.2	121.5
Australia	8.0	13.3	44.0	55.7	85.6	68.6	77.7	98.3
Sri Lanka	21.0	4.5	12.0	12.0	63.0	34.5	19.0	39.4
Spain	0.1	-	0.2	5.0	8.8	18.0	53.6	36.2
TOTAL ^a	5963.9	5490.1	5347.7	5894.1	7176.0	6885.9	6266.7	6042.9

^a Including other countries.

Germany represents the major export market, whilst exports to the United Kingdom (UK) and United States of America (USA) have, respectively, quadrupled and doubled over the past eight years. In 2010, these two

countries were the 3rd and 4th largest markets, respectively, for rooibos. Total exports of rooibos to Poland, Russia, Australia and Spain have also shown tremendous growth, although these countries still represent a relatively small market.

The export tons of conventional, organic and green rooibos during the 2003–2010 period is illustrated in Figure 13. In comparison with ‘conventional’ fermented rooibos, the export of organic and ‘green’ unfermented rooibos only represents a small market segment. Exports for organic rooibos were recorded at 828 tons in 2010 with Japan representing the major market (222 tons), followed by Germany, UK, Netherlands, and USA. Total exports of unfermented, ‘green’ rooibos was 253 tons in 2010, showing a significant increase from a mere 83 tons in 2003. The increased demand for unfermented rooibos possibly stems from the fact that it has a higher antioxidant capacity than its traditionally processed counterpart (as assessed by the DPPH radical scavenging method) (Von Gadow *et al.*, 1997). Seventy percent of the total tons of green rooibos exported in 2010 went to Germany, whilst 16% was destined for the Netherlands.

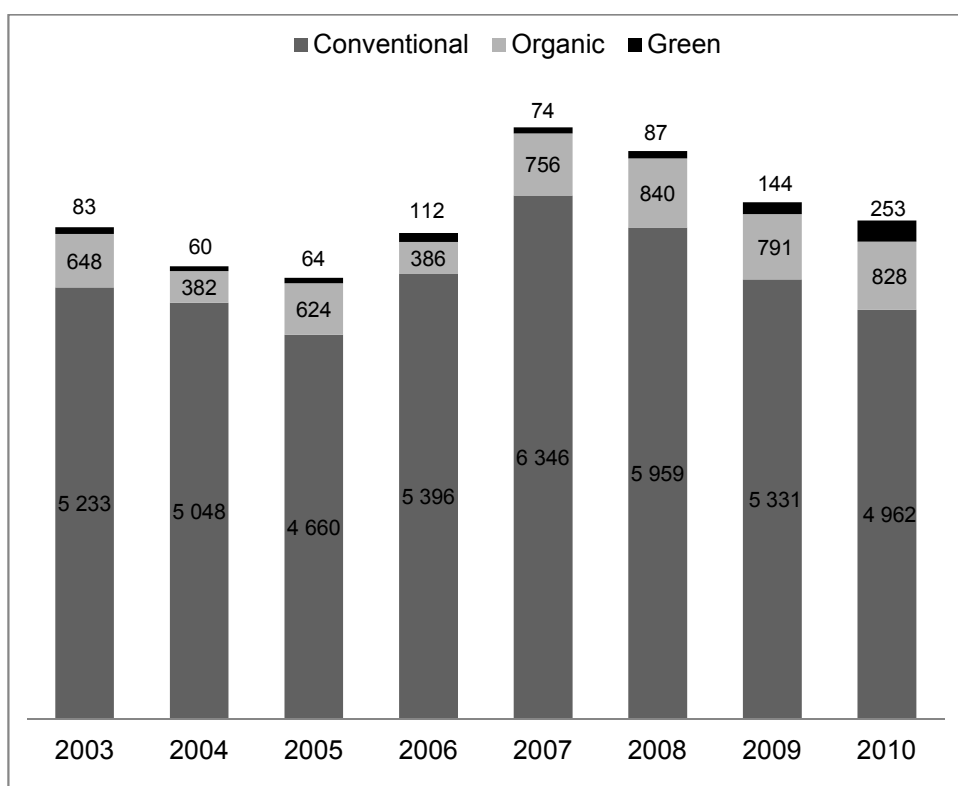


Figure 13 Tons of conventional, organic and green rooibos exported during the 2003-2010 period (data from PPECB, supplied by SARC).

3.3 Processing

3.3.1 Cultivation and harvest

Commercial cultivation of the Nortier type rooibos mainly occurs in the mountainous Cederberg region, but extends to areas as far as Darling and Nieuwoudtville. Seedlings (100 to 150 mm high) are used to propagate the plants and are planted between June and August at 8000–10000 plants per hectare. Approximately eight months after planting, the plants are topped to a height of about 30 cm to stimulate branching. The first harvest takes place after 18 months, but full production is only realised after three years

(Joubert & Schulz, 2006). It has been documented that the best harvest occurs in the fourth and fifth years after planting, and that some of the bushes start to die after the seventh harvest (Cheney & Scholtz, 1963).

Rooibos is harvested during the hot summer months and early autumn (January to April) by topping the whole bush to ca 45 cm. It has been established that the active growth of the shrub should not be more than 50 cm otherwise the plant material will produce a weak tea. Furthermore, no or few flowers should be present since they impart an unpleasant flavour to the rooibos tea, detrimental to quality (Joubert & Schulz, 2006). Based on observations, factors such as the presence of young growth, the age of the bush and the cultivation area could also affect the tea quality (Joubert, 1994), but none of these factors have been investigated to date.

After harvesting, the branches are bundled and transported to the processing yard. Most rooibos producers sell their produce to a central processing yard that processes ca 75% of all rooibos that is produced. This practice improves standardisation of tea quality since different quality grade production batches can be blended together to obtain a final product of acceptable quality (Koch, 2011).

3.3.2 Tea manufacture

Although rooibos tea is typically consumed in its traditional fermented form, the demand for unfermented, 'green' rooibos developed as it has a higher antioxidant capacity than its traditionally processed counterpart (Von Gadow *et al.*, 1997). Availability of good quality green rooibos (De Beer & Joubert, 2002) also stimulated interest in the product (E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, 2011, personal communication). During the processing of unfermented rooibos, the greatest challenge is to minimise oxidative changes so that the green leaf colour and aspalathin (the active principal) content are retained. This can be achieved by: drying the shredded plant material without delay under vacuum; drying the whole shoots to a critical moisture content before shredding; or by steaming the fresh shoots to inactivate enzymes before shredding (Joubert *et al.*, 2008a). Although these techniques are effective in yielding a high-end quality product, the cost implications are vast. Therefore, the industry currently produces unfermented rooibos by spreading the shredded plant material in a thin layer in the sun to facilitate quick drying. This technique still leads to a reduction in the aspalathin content, and, if not properly dried, slow browning of the tea will lead to poor quality green rooibos (Joubert *et al.*, 2008a; Joubert & De Beer, 2011).

Processing of traditional rooibos tea typically entails shredding the tea shoots into 3 to 4 mm lengths; bruising the plant material; the addition of water to the fermentation heap; and further bruising and mixing (Joubert & Schulz, 2006). Shredding of the shoots initiates enzymatic oxidation of the polyphenols, possibly via polyphenol oxidase and/or peroxidase activity, leading to rapid browning (Joubert, 1996). This oxidative process is commonly referred to as 'fermentation'. Bruising and the addition of water are required to accelerate this fermentation process by releasing and extracting some of the soluble polyphenols to the leaf surface. These polyphenols are then absorbed by the shredded stems, colouring it red-brown to yield a product of more uniform colour (Joubert & Schulz, 2006). Adequate aeration is required for oxidation and therefore the fermentation heap is turned over several times during the fermentation process (Joubert, 1994). The importance of this was demonstrated by Joubert (1998) who found that poor aeration resulted in a low quality product.

Fermentation occurs at ambient temperature, but the temperature of the fermentation heap naturally increases during fermentation. Joubert & De Villiers (1997) established that the temperature of the heap should preferably be between 38°C and 42°C, leading to the development of the sought-after characteristic

sweet rooibos tea aroma and taste. Lower fermentation temperatures result in under-fermented rooibos, characterised by a grassy note and astringent taste (Joubert & De Villiers, 1997). It was suggested that the change in taste with fermentation temperature could be attributed to the effect of temperature on the enzymatic and chemical oxidation of polyphenols and their subsequent loss of astringency with polymerisation. This is in accordance with findings by Haslam and co-workers (1988).

Fermentation takes place during the night with an average fermentation time of 12 to 14 hours. Fermentation time can, however, range anything from 8 to 24 hours, depending on the climatic and processing conditions, as well as the composition of the plant material (Joubert & Schulz, 2006). Fermentation time is also believed to be influenced by the presence of young growth, the age of the bush and the cultivation area (Joubert, 1994).

During fermentation, the leaves acquire the typical red-brown colour and the aroma of the moist tea changes from resinous, hay-like and grassy to sweet, apple-like or honey-caramel (Joubert, 1994). For more detail regarding rooibos aroma and its volatile constituents, refer to section 3.5.1. As soon as the characteristic sweet aroma has formed, the fermentation heap is spread open in a thin layer (15-20 mm) in the sun to dry. Drying of the tea should commence as soon as possible after completion of fermentation to prevent over-fermentation. Over-fermented rooibos is of poor quality, *i.e.* characterised by a dull, dark-brown leaf and extract colour and a very flat aroma (Joubert & De Villiers, 1997).

Fermentation of rooibos tea according to this traditional open-air method has various disadvantages including weather dependency, a lack of control over individual processing parameters affecting quality, leading to a product of inconsistent quality (Joubert & Müller, 1997). Du Plessis & Roos (1986) also stated that open-air processing is more likely to result in an unhygienic product. Therefore, controlled fermentation and drying of rooibos has been investigated as alternatives to ensure optimum and consistent quality (Joubert & De Villiers, 1997; Joubert & Müller, 1997; Joubert, 1998). High cost in terms of capital expenditure and energy requirements, however, does not make this approach feasible. The dried tea is ultimately sieved, graded and steam-pasteurised prior to packaging (Joubert and Schulz, 2006).

3.3.3 Steam-pasteurisation

The steam-pasteurisation treatment of rooibos was introduced in 1986 following an outbreak of *Salmonella* in 1984 which gave the rooibos industry a major setback. Exports were stopped, contaminated product was taken off the shelves and a major drop in sales occurred (Snyman, 2000). An investigation by Du Plessis & Roos (1986) led to the recovery of high numbers of coliform bacteria (*Escherichia coli* type I and *Salmonella* spp.) from processed tea. The recovery of *E. coli* type I from different batches of rooibos tea indicated a high incidence of faecal contamination, but it was not established how or during which processing stage(s) contamination had occurred. *Salmonella* serotypes were only recovered from the fermentation heaps after fermentation had occurred, which indicated that their numbers had significantly increased during the fermentation process. It was postulated that, due to the fact that different batches of tea are blended together to standardise the quality, a single batch containing high numbers of *E. coli* and *Salmonella* could have caused large-scale contamination and wide-spread occurrence of microbes in the packaged product. Steam-pasteurisation was therefore proposed as a decontamination process prior to packaging. It was established that pasteurising rooibos plant material at 99.5°C for 2 minutes was extremely effective in reducing coliform bacterial numbers, without imparting undesirable organoleptic qualities/properties to the tea (Du Plessis & Roos, 1986).

Koch (2011) investigated the effect of steam-pasteurisation of rooibos leaves on the composition and sensory characteristics of a tea infusion. With regards to the sensory characteristics, it was established that steam-pasteurisation did not impart a significant effect on the intensities of the taste attributes sweetness and bitterness, but that the astringency of the rooibos infusions was decreased slightly. The intensities of the aroma and flavour attributes, especially the 'green' and 'caramel' notes, significantly decreased as a result of steam-pasteurisation. The prominent 'green' flavour of the unpasteurised rooibos samples was typically replaced by a 'hay-like' flavour following steam-pasteurisation (Koch, 2011).

Currently the major South African rooibos processor steam-pasteurises rooibos at 96°C for 60 seconds. This treatment results in a slight increase in the moisture content of the dried rooibos, and therefore the leaves are subsequently dried to a moisture content of less than 10% (Koch, 2011). This is in accordance with the current South African regulations relating to quality standards for rooibos which stipulates that the moisture content of rooibos may not be more than 10% (Anon., 2002). Due to this low moisture content, rooibos tea is a well-preserved product and generally regarded as being microbiologically safe (Lund *et al.*, 2000). The microbiological quality of rooibos is monitored through laboratory testing to ensure that the regulatory standards are met.

3.3.4 Extract preparation

Further processing of rooibos includes the preparation of extracts and powders. In spite of early research conducted by Joubert in the 1980s (Joubert, 1984, 1988a, 1990a), showing potential for rooibos in extract form, aqueous extracts and extract powders only found commercial application in South Africa in 2000 (Anon., 2005b, 2005c). The types of rooibos extract and the raw material from which they are produced greatly depends on the final application (Joubert & De Beer, 2011).

Extracts produced from fermented rooibos, representing the bulk of the raw material for extract production, are primarily used in beverages and functional foods (Joubert & De Beer, 2011). Rooibos can be classified as a functional beverage product (Wilson, 2005), as it provides additional physiological benefits that may promote optimum health and prevent chronic disease (Hasler, 1996; see section 3.1.3). Food and beverage products containing rooibos extract include ready-to-drink iced teas and various yoghurt products (Anon., 2010a, 2010b), amongst others.

Extracts from unfermented rooibos are characterised by higher antioxidant levels (Von Gadow *et al.*, 1997) and are typically produced for the nutraceutical and cosmetic industries (Tiedtke & Marks, 2002; Otto *et al.*, 2003). Skin-care products, the first non-beverage application of rooibos extracts, were initiated by Anne Theron who brought an extensive cosmetic care range containing rooibos extract to the market (Joubert *et al.*, 2008a).

Aspalathin-enriched extracts can also be produced from unfermented rooibos plant material as this compound is present in significantly higher levels (Schulz *et al.*, 2003; Manley *et al.*, 2006). A patent was recently awarded to Grüner-Richter *et al.* (2008) and, according to their process, enrichment is achieved with organic solvents. The level of enrichment greatly depends on the level of purification and the extraction conditions. The effects of extraction time and temperature, water-to-leaf ratio and the specific extraction process (stirred batch-wise and fixed-bed with flow through) on the extraction of soluble solids and polyphenols from rooibos have been investigated. It was established that higher temperatures, higher water-to-leaf ratios and longer extraction times favour the extraction of soluble solids and polyphenols (Joubert, 1988b, 1990b, 1990c; Joubert & Hansmann, 1990). Furthermore, pretreatment of the plant material with

hydrolyzing enzymes expressed by food grade fungi was also shown to increase the yield of soluble solids and polyphenols from fermented rooibos during extraction (Pengilly *et al.*, 2008). The stem content and average particle size of rooibos plant material also influence the soluble solids content and levels of phenolic compounds of the extracts/infusions. Coarser fractions consisting mostly of stems (>10 mesh) provides lower levels of soluble solids and phenolic compounds when extracted (Joubert, 1984). Furthermore, it was shown that fermentation of rooibos plant material is accompanied by a decrease in soluble solids extracted. This was attributed to the polymerisation of polyphenolic compounds, which reduces their solubility (Joubert, 1984).

3.4 Tea quality aspects

The quality standards for fermented rooibos, as stipulated in the South African regulations, pertain only to moisture content, the percentage of white sticks allowed, pesticide residues and microbial contamination (Anon., 2002). The moisture content may not be more than 10% and the maximum percentage white sticks allowed is also 10%. The quality standards of rooibos destined for the export market is monitored by The Perishable Products Export Control Board (PPECB) (Snyman, 2000).

There is a dire need for strict quality criteria, which will positively impact on quality control, marketing and GI certification. New quality tools and criteria are currently under investigation (see section 3.4.2).

3.4.1 Sensory quality and the current grading system

With regards to the sensory quality attributes, the current regulations are extremely vague and merely state that all rooibos should have the 'clean, characteristic taste and aroma and clear, distinctive colour of rooibos' (Anon., 2002). No definitions or reference standards are, however, provided for the terms 'characteristic' and 'distinctive'. This signifies that producers have the freedom to set their own quality standards in terms of colour, flavour and mouthfeel of rooibos infusions (Joubert & De Beer, 2011).

This has significant implications for the industry as the current quality grading system is based exclusively on sensory evaluation. The most structured grading system is employed by Rooibos Ltd. Upon receipt, each batch of rooibos is mechanically sieved to obtain refined tea (<10 mesh; >40 mesh) which will then be graded, steam-pasteurised and packaged. Quality grading is performed by expert graders by evaluating the appearance of the dry and infused rooibos leaves; and by evaluating the overall colour and flavour of the rooibos infusions. A score out of 10 is allocated to each of these quality parameters and multiplied by a certain factor according to the weight assigned to the parameter. The weights assigned to the quality parameters differ according to the cut of the leaves which determines whether the tea will be used in teabags or as loose-leaf tea. The flavour, *i.e.* taste and aroma, of the rooibos infusion carries the most weight and is considered the most significant determinant of tea quality. High quality rooibos tea is expected to have a strong, full-bodied, sweet, 'characteristic', honey-like, 'rooibos' taste and aroma. After the preliminary grading has been completed, a panel of seven judges re-evaluates the tea and, if necessary, makes adjustments which may affect the final grade achieved by the batch. The quality grade assigned to each batch of fermented rooibos (AA, A, B, C, D, E or F) is subsequently based on the totals of the final scores. Rooibos is typically awarded a B or C grade, whilst rooibos of grades E and F is not distributed for consumption as tea (Koch, 2011).

Although the sensory evaluation of rooibos by expert tasters is inexpensive, quick and simple, such grading systems are hampered by subjectivity and the results cannot be scientifically validated (Feria-

Morales, 2002). Furthermore, as grading determines the market value of a specific batch of rooibos (Joubert, 1995), problems may arise due to conflicting interests between the producer and processor with regard to financial compensation/remuneration. In order to improve the sensory evaluation of rooibos tea, a flavour and mouthfeel wheel has been developed recently (Anon., 2011). This flavour and mouthfeel wheel incorporates both positive and negative sensory properties and comprises 17 descriptors. To aid interpretation of the descriptors, a preliminary sensory lexicon has also been developed for some of the descriptors (Koch, 2011).

With regards to unfermented rooibos tea, no general grading system is currently in place as the production volume is rather limited and the unfermented product only represents a small market segment. However, increased demand for unfermented rooibos due to its higher antioxidant levels (Von Gadow *et al.*, 1997), have stimulated interest in acquiring quality parameters for the reliable evaluation of green rooibos. Aspalathin, due to its instability under poor processing conditions, could be a good quality parameter for unfermented rooibos (Schulz *et al.*, 2003; Joubert & Schulz, 2006). The aspalathin content of green rooibos is not, however, routinely determined by processors and presently visual inspection of leaf colour serves as the only quality control parameter. The leaves should have a light green colour and impart a light yellow to orange tinge to the aqueous infusion prepared from it (Joubert & Schulz, 2006).

3.4.2 Other quality parameters

The rooibos industry is increasingly interested in establishing objective quality parameters in the evaluation of rooibos. Joubert (1995) investigated the use of tristimulus colour measurement of rooibos infusions as potential objective quality indicator. The potential of water-soluble solid content, total polyphenol content (TPC) and total antioxidant capacity (TAC) of rooibos infusions to serve as objective quality parameters are currently under investigation by the Agricultural Research Council (ARC) of South Africa (Joubert & De Beer, 2011). As of yet, no specifications exist for antioxidant activity and polyphenolic content, despite their associated importance in the health-promoting benefits of rooibos.

Extract manufacturers have begun to introduce product specifications such as minimum total polyphenol content and total antioxidant activity (TAA) values (Joubert & Schulz, 2006). Currently, two prominent South African extract producers use TPC and TAA as quality indicators (Joubert & De Beer, 2011). It has been established that a good correlation ($r^2 = 0.99$) exists between the TPC and the TAA of unfermented rooibos extracts, whilst the TAA of unfermented rooibos extracts furthermore correlate with their aspalathin content (Joubert *et al.*, 2008b).

Ninfali *et al.* (2009) proposed the combined use of two parameters to improve standardisation, namely the concentration of a specific 'marker compound' (MC) by HPLC and the antioxidant capacity, determined with the oxygen radical absorbance capacity (ORAC) method. A MC was defined as the 'active constituent' of an extract responsible for the intended pharmacological activity. In order to achieve elevated quality and batch-to-batch consistency, a fixed quantity of the specific MC should be guaranteed. Ninfali *et al.* (2009) proposed rutin as the MC for a freeze-dried extract of *Aspalathus linearis*, but they concluded that it is not representative of the antioxidant activity of the extract.

Currently, a major extract manufacturer produces rooibos extract standardised on total orientin and isoorientin content, while the use of aspalathin for standardisation of a fermented rooibos extract is currently under investigation (Joubert & De Beer, 2011). Aspalathin, unique to rooibos and one of the major phenolic constituents of cultivated rooibos, can be used as a very specific MC, also ensuring product authenticity. It

has, however, been demonstrated that certain wild rooibos types may contain little or even no aspalathin (Joubert & Schulz, 2006). Joubert & De Beer (2011) therefore stated that, if the presence of aspalathin becomes part of the quality parameters of rooibos, then some wild types may have to be excluded from the 'rooibos' label in the future. Moreover, large variation in the aspalathin content of unfermented rooibos (Joubert & Schulz, 2006) and uncontrolled processing conditions, which leads to oxidative degradation of aspalathin, further aggravate this variation (Joubert, 1996). This large variation would negatively impact on standardisation/quality control. Standardisation on total isoorientin and orientin content therefore represent a more viable option as these compounds, which are oxidation products of aspalathin, have been shown to be more stable under heat processing (Joubert *et al.*, 2009a) and varying pH conditions (De Beer *et al.*, 2011).

3.5. Chemical composition of rooibos

3.5.1 Caffeine content, non-phenolic metabolites, mineral composition and volatile compounds

Rooibos is valued as a caffeine-free herbal tea, but traces of the alkaloid sparteine in *Aspalathus* have been reported by Van Wyk & Verdoorn (1989). Non-phenolic constituents of rooibos include *p*-hydroxyphenylglycol and vanylglycol (Shimamura *et al.*, 2006) as well as the inositol, (+)-pinitol (Ferreira *et al.*, 1995).

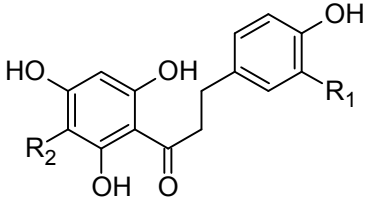
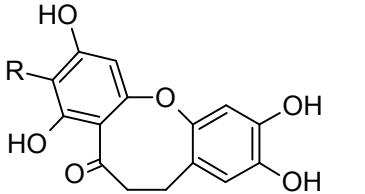
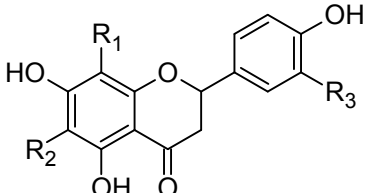
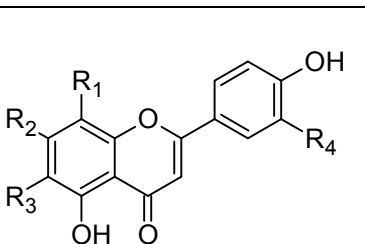
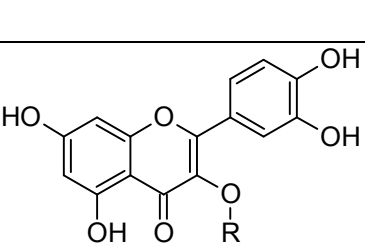
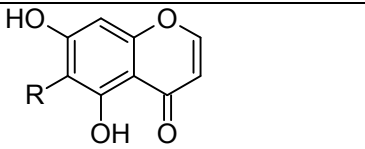
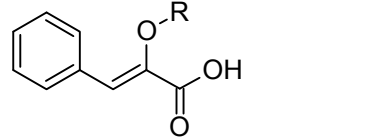
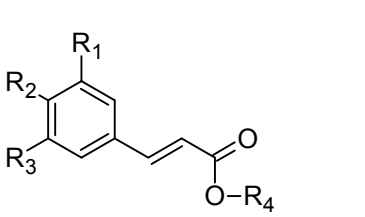
Little information on the mineral composition of rooibos is available. The mineral content of fermented rooibos plant material and infusions has been reviewed by Joubert *et al.* (2008a) who reported that potassium (K) and sodium (Na) are present at the highest concentrations, whilst magnesium (Mg), calcium (Ca) and phosphorous (P) are also present at relatively high levels. These results are in accordance with Malik *et al.* (2008), who conducted a study on the micro and macroelements in plant stimulants and their infusions. Malik *et al.* (2008) reported that aluminium (Al) is present at much lower levels in unfermented and fermented rooibos infusions in comparison with *Camellia sinensis* infusions. A considerable number of studies have reported the total concentration of Al in tea infusions due to its toxicity and association with disease pathologies, such as Alzheimer's disease, Parkinson's disease and dialysis encephalopathy (Exley & Korchazhkina, 2001). Touyz and Smit (1982) showed that a fermented rooibos infusion contains 1.29 µg fluoride.mL⁻¹.

The volatile fraction of fermented rooibos has been characterised by Habu *et al.* (1985) and Kawakami *et al.* (1993). Some of the major constituents of rooibos tea aroma are the degradation products of β-carotene, *i.e.* dihydroactinidiolide, 5,6-epoxy-β-ionone, damascenone, and β-ionone, amongst others (Habu *et al.*, 1985; Kawakami *et al.*, 1993). Damascenone (Buttery *et al.*, 1988) and β-ionone (Mihara *et al.*, 1987) have low threshold values and, together with their relatively high concentrations in rooibos tea aroma (Habu *et al.*, 1985; Kawakami *et al.*, 1993), it has been postulated that they could contribute significantly to the rooibos aroma (Joubert & De Villiers, 1997). The occurrence, sensory impact, formation, and fate of damascenone in foods and beverages, including rooibos, is currently the focus of a study by Sefton *et al.* (2011).

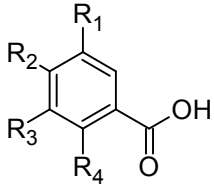
3.5.2 Phenolic composition

Joubert *et al.* (2008a) gave a comprehensive overview of the secondary phenolic metabolites which have been identified in fermented *Aspalathus linearis* plant material (Table 3).

Table 3 Secondary phenolic metabolites identified in fermented *Aspalathus linearis* plant material (adapted from Joubert *et al.*, 2008a).

General structure	Compound type, names and substituents
	Dihydrochalcones aspalathin ^{a,b,c,d,f,g,k} : R ₁ = OH, R ₂ = C-β-D-glucosyl nothofagin ^{e,f,g,k} : R ₁ = H, R ₂ = C-β-D-glucosyl
	Cyclic dihydrochalcone aspalalinin ^g : R = C-β-D-glucosyl
	Flavanones hemiphlorin ^g : R ₁ = C-β-D-glucosyl, R ₂ = R ₃ = H (<i>R</i>)/(<i>S</i>)-eriodictyol-8-glucoside ^{f,g,k} : R ₁ = C-β-D-glucosyl, R ₂ = H, R ₃ = OH (<i>R</i>)/(<i>S</i>)-eriodictyol-6-glucoside ^{f,g,k} : R ₁ = H, R ₂ = C-β-D-glucosyl, R ₃ = OH
	Flavones orientin ^{a,c,f,g,h,i,k} : R ₁ = C-β-D-glucosyl, R ₂ = R ₄ = OH, R ₃ = H isoorientin ^{c,f,h,i,k} : R ₁ = H, R ₂ = R ₄ = OH, R ₃ = C-β-D-glucosyl vitexin ^{a,c,f,g,k} : R ₁ = C-β-D-glucosyl, R ₂ = OH, R ₃ = R ₄ = H isovitexin ^{c,f,g,k} : R ₁ = R ₄ = H, R ₂ = OH, R ₃ = C-β-D-glucosyl luteolin ^{c,f,g,k} : R ₁ = R ₃ = H, R ₂ = R ₄ = OH luteolin-7- <i>O</i> -glucoside ^d : R ₁ = R ₃ = H, R ₂ = O-β-D-glucosyl, R ₄ = OH chrysoeriol ^{c,f,k} : R ₁ = R ₃ = H, R ₂ = OH, R ₄ = OCH ₃
	Flavonols quercetin ^{c,f,g,k} : R = H isoquercitrin ^{c,f,g,i,k} : R = O-β-D-glucosyl hyperoside ^{f,g,k} : R = O-β-D-galactosyl rutin ^{f,i,k} : R = O-β-D-rutinosyl quercetin-3- <i>O</i> -β-D-robinoside ^g : R = O-robinosyl
	Chromone 5,7-dihydroxy-6- <i>C</i> -β-D-glucosyl-chromone ^d
	Phenylpyruvic acid derivative 3-phenyl-2-glucopyranosyloxypropenoic acid ^{dj} : R = O-glucosyl (PPAG)
	Hydroxycinnamic acids and derivatives 3,4,5-trihydroxycinnamic acid ^c : R ₁ = R ₂ = R ₃ = OH; R ₄ = H <i>p</i> -coumaric acid ^{c,g,k} : R ₁ = R ₃ = H, R ₂ = OH; R ₄ = H caffeic acid ^{c,k} : R ₁ = R ₂ = OH, R ₃ = H; R ₄ = H ferulic acid ^{c,k} : R ₁ = OCH ₃ , R ₂ = OH, R ₃ = H; R ₄ = H sinapic acid ^c : R ₁ = R ₃ = OCH ₃ , R ₂ = OH; R ₄ = H chlorogenic acid ^k : R ₁ = R ₂ = OH, R ₃ = H; R ₄ = quinic acid

Phenolic carboxylic acids



p-hydroxybenzoic acid^{c,g,k}: R₁ = R₃ = H; R₂ = OH; R₄ = H

protocatechuic acid^{c,k}: R₁ = R₂ = OH, R₃ = R₄ = H

3,5-dihydroxybenzoic acid^k: R₁ = R₃ = H; R₂ = R₄ = OH

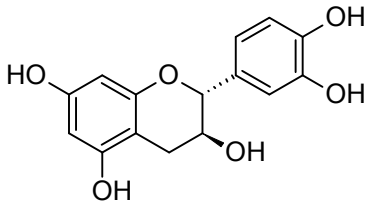
gentisic acid^k: R₁ = R₃ = OH; R₂ = R₄ = H

salicylic acid^k: R₁ = R₂ = R₃ = H; R₄ = OH

gallic acid^k: R₁ = R₂ = OH, R₃ = OH; R₄ = H

vanillic acid^{c,k}: R₁ = OCH₃; R₂ = OH; R₃ = R₄ = H

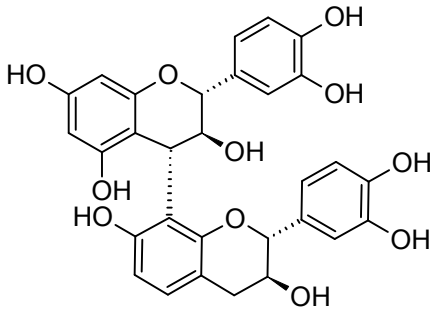
syringic acid^{d,k}: R₁ = R₃ = OCH₃; R₂ = OH; R₄ = H



Flavan-3-ols

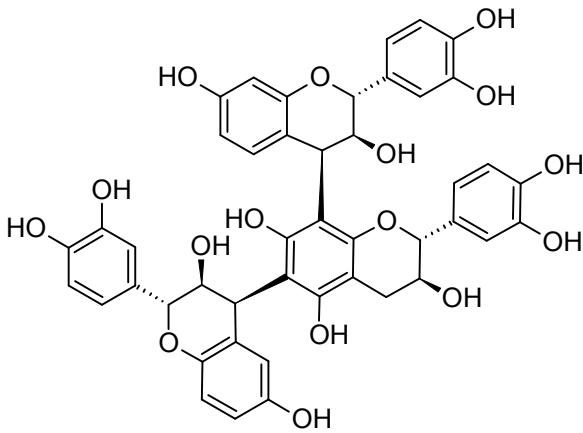
Monomeric flavan-3-ol

(+)-catechin^{d,k}



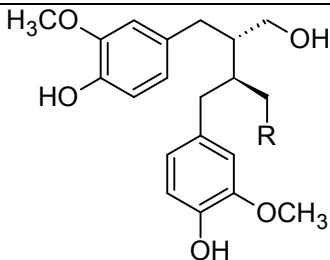
Oligomeric flavan-3-ol

procyanidin B₃^d



Oligomeric flavan-3-ol

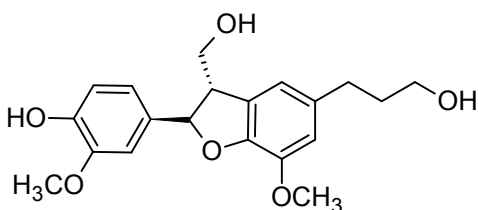
bis-fisetinidol-(4β,6:4β,8)-catechin^d



Lignans

secoisolariciresinol⁹: R = OH

secoisolariciresinol-O-glucoside⁹: R = O-glucosyl



vladinol F⁹

	<p>Lignan</p> <p>3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxy-1-(<i>E</i>)-propenyl)-2,6-dimethoxyphenoxy] propyl-β-D-glucopyranoside^g: R = O-β-D-glucosyl</p>
	<p>Coumarin esculetin^g</p>
	<p>Aldehyde syringin^g: R = O-glucosyl</p>

^a Koeppen & Roux, 1965a (identification by NMR).

^b Koeppen & Roux, 1966 (identification by NMR).

^c Rabe *et al.*, 1994 (identification by NMR).

^d Ferreira *et al.*, 1995 (identification by NMR).

^e Joubert, 1996 (identification by co-elution with pure standard).

^f Bramati *et al.*, 2002 (identification by LC-MS).

^g Shimamura *et al.*, 2006 (identification by NMR)

^h Koeppen & Roux, 1965b (identification by NMR)

ⁱ Koeppen *et al.*, 1962 (identification by NMR).

^j Marais *et al.*, 1996 (identification by NMR).

^k Krafczyk & Glomb, 2008.

Rooibos principally contains single ring phenolic acids and monomeric flavonoids from the following subclasses: dihydrochalcones, flavanones, flavones and flavonols. The monomeric flavonoid composition of rooibos is unique in the sense that it is the only known natural source of the dihydrochalcone C-glycoside, aspalathin (Koeppen & Roux, 1965a). β -Hydroxy-dihydrochalcones are extremely rare in nature and, apart from aspalathin, only pterosupin and nothofagin have been detected in plant material. Nothofagin, structurally similar to aspalathin except for the absence of a hydroxyl group on the B-ring, is also present in rooibos tea (Joubert, 1996). The cyclic dihydrochalcone, aspalalinin, has to date, also been isolated only from fermented rooibos (Shimamura *et al.*, 2006).

Rooibos contains diastereomeric mixtures of (*R*)- and (*S*)-eriodictyol-6-C- β -D-glucopyranoside, and (*R*)- and (*S*)-eriodictyol-8-C- β -D-glucopyranoside. Koeppen & Roux (1965a) postulated that these 6-C and 8-C flavanones are formed as intermediates during the oxidative conversion of aspalathin to its corresponding flavones, respectively isoorientin and orientin (refer to section 3.5.2.1). The presence of the 6-C-glycosides in fermented rooibos was unequivocally confirmed by Marais *et al.* (2000), whilst NMR data for the 8-C-glycosides were presented by Krafczyk & Glomb (2008). The presence of the novel compound, 5,7-dihydroxy-6-C- β -D-glucopyranosyl-chromone (Tabel 3), suggests further oxidative conversion of eriodictyol-6-C- β -D-glucopyranoside during fermentation (Ferreira *et al.*, 1995). Hemiphlorin, the flavanone analogue of isovitexin, has also been isolated from rooibos (Shimamura *et al.*, 2006).

Rooibos is also a source of C-glycosidic flavones not found in common food sources and thus the daily diet (Joubert, *et al.*, 2009b). C-glycosidic flavones present in rooibos include orientin, isoorientin, vitexin

and isovitexin. Their intake may be increased with the inclusion of unfamiliar foods such as buckwheat sprouts (Zielinska *et al.*, 2007), and medicinal plant extracts from bamboo (Zhang *et al.*, 2008) and lime leaves (Piccinelli *et al.*, 2008) to the diet. The flavone aglycone, luteolin, its 3-*O*-methyl analogue, chrysoeriol, and its *O*-glycoside, luteolin-7-*O*-glucoside are also present. The flavonol aglycone, quercetin, and its more abundant *O*-glycosides include isoquercitrin, hyperoside, rutin and quercetin-3-*O*-robinobioside (Joubert *et al.*, 2008a). Luteolin and quercetin with their known anti-spasmodic properties were among the first physiologically active compounds to be isolated from rooibos tea. The glycosides isoquercitrin and rutin are, however, equally important as far as antioxidant properties are concerned (Ferreira *et al.*, 1995).

Continued investigation of the secondary metabolites of *Aspalathus linearis* has revealed the presence of an enolic *O*- β -D-glucopyranoside of phenylpyruvic acid (Table 3). Phenylpyruvic acid (PPA) is an intermediate in the shikimic acid pathway for the biosynthesis of the essential aromatic amino acids, L-phenylalanine and L-tyrosine, in higher plants. Formation of the enolic glucoside presumably stabilises PPA, ensuring availability of the aglycone in specific metabolic processes. Since biosynthetic processes are often compartmentalised, it is postulated that phenylpyruvic acid glucoside (PPAG) represents the form that permits inter-compartmental transport (Ferreira *et al.*, 1995; Marais *et al.*, 1996).

Rooibos contains the hydroxybenzoic acids *p*-hydroxybenzoic-, protocatechuic-, vanillic-, and syringic acid, whilst the hydroxycinnamic acids include *p*-coumaric-, caffeic-, and ferulic acid (Table 3). The anti-microbial properties of hydroxybenzoic acids have been firmly established and hence these compounds may act as natural preservatives in rooibos. In rooibos, the hydroxycinnamic acids *p*-coumaric- caffeic- and ferulic acid co-exist with their 3,4,5-trihydroxy analogue, hence substantiating the central position of the activated hydroxycinnamic acids, *i.e.* as CoA esters, in the biosynthesis of various phenylpropanoid metabolites (Rabe *et al.*, 1994). The potential health benefits of phenolic acids, mostly attributed to its antioxidant activity, have been discussed in section 2.2.

The presence of (+)-catechin, the dimer, procyanidin B3, and the trimer, bis-fisetinidol-(4 β ,6:4 β :8)-catechin in rooibos has been established (Ferreira *et al.*, 1995; Kraczyk & Glomb, 2008). The leaf tannin content of fermented rooibos has been estimated at 3.2% (Reynecke *et al.*, 1949) and 4.4% (Blommaert & Steenkamp, 1978), which is very low in comparison to that of black tea (*Camelia sinensis*). It has, however, been shown that a dried water extract of fermented rooibos may contain up to 50% tannin-like substances (Joubert *et al.*, 2008a). Rooibos furthermore contains lignans (secoisolariciresinol, secoisolariciresinol-*O*-glucoside, vladinol, 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxy-1-(*E*)-propenyl)-2,6-dimethoxyphenoxy] propyl- β -D-glucopyranoside), the coumarin, esculetin, and the aldehyde, syringin (Shimamura *et al.*, 2006).

In addition to those compounds listed in Table 3, various other phenolic compounds have been identified in rooibos recently. Stalmach *et al.* (2009) tentatively identified diastereomeric isomers of naringenin-*C*-glucoside in fermented rooibos tea. Iswaldi *et al.* (2011) was the first to report the presence of the coumarin esculin, patuletin-7-glucoside, safflomin A, eriodictyol-5,3'-di-*O*-glucoside, quercetin-3-*O*-arabinoglucoside and scoparin in rooibos. Symmetric 6,8-di-*C*-glycosyl flavones (luteolin-6,8-di-*C*-hexoside and apigenin-6,8-di-*C*-hexoside) and asymmetric 6,8-di-*C*-glycosyl flavones (luteolin-6-*C*-glucoside-8-*C*-arabinoside, a non-specific luteolin-6-*C*-hexoside-8-*C*-pentoside, luteolin-6-*C*-arabinoside-8-*C*-glucoside and apigenin-6-*C*-arabinoside-8-*C*-glucoside) have also been identified in rooibos by LC-MS (Breiter *et al.*, 2011; Iswaldi *et al.*, 2011).

3.5.2.1 Factors affecting the phenolic composition of rooibos products

Various factors are known to influence the phenolic composition of plant material such as seasonal effects (Aherne & O'Brien, 2002; Yao *et al.*, 2005); climate (Aherne & O'Brien, 2002); day length and sunlight intensity (Harbowy & Balentine, 1997; Aherne & O'Brien, 2002; Yao *et al.*, 2005); development stage of the tea shoots (Nakagawa & Torri, 1964); cultivation methods (Ku *et al.*, 2010); plant distribution; and processing (Aherne & O'Brien, 2002). Although these factors have not been specifically investigated for rooibos, it is expected that they will also impact on the phenolic composition of rooibos. Research is, however, required.

More specifically, variation in the phenolic composition of rooibos owing to the genetic make-up of the seedling, the specific plantation and the geographical location (Joubert & Schulz, 2006) has been established. As an example of genetic variation, Joubert & De Beer (2011) demonstrated large variation in the aspalathin and orientin contents of unfermented rooibos leaves harvested at the same time from 21 individual plants of the same plantation. Harvest date was also shown to be an important factor influencing the phenolic composition of rooibos plants. Unpublished data reported by Joubert & De Beer (2011) stated that the aspalathin content of plant material harvested from the same plants ranged from 2.33 to 3.76 g.100 g⁻¹ on a dry matter basis over a one year period.

The above-mentioned studies only considered cultivated rooibos, but Van Heerden *et al.* (2003) have showed large qualitative and quantitative variation in the phenolic composition between populations and provenances of the wild tea types. Some populations even lacked aspalathin, which is the major flavonoid present in the red or Rocklands type of *Aspalathus linearis* used for commercial processing. Rutin was invariably the main compound of *Aspalathus pendula*, a close relative of *A. linearis* which is also used to make tea occasionally (Van Heerden *et al.*, 2003). Joubert & Schulz (2006) reported similar results in the comparison of cultivated and wild rooibos. Although the chemical compounds found in the leaves were highly variable across species, van Heerden *et al.* (2003) demonstrated that some tea types and various populations can be identified by unique and distinctive phenolic profiles or 'fingerprints'. In addition to phenolic variation, morphological (Van Heerden *et al.*, 2003; Malgas *et al.*, 2010), genetic (Malgas *et al.*, 2010), and ecological (Malgas *et al.*, 2010; Hawkins *et al.*, 2011) variation have also been demonstrated for the wild rooibos types.

Processing also has a significant influence on the phenolic composition of rooibos tea. In fermented rooibos, further variation in the phenolic composition may be induced during the uncontrolled fermentation process as it has been demonstrated that the dihydrochalcones are particularly susceptible to oxidative changes (Joubert, 1996). During fermentation aspalathin is photochemically and enzymatically converted to diastereomeric mixtures of (*R*)- and (*S*)-eriodictyol-6-*C*-glucoside, and (*R*)- and (*S*)-eriodictyol-8-*C*-glucoside. Degradation of aspalathin to eriodictyols may be explained by oxidation via the *o*-quinone which rearranges to form quinine methide. The latter compound is then reversibly converted to eriodictyols – (*R*)/(*S*)-eriodictyol-6-*C*-glucoside form preferentially, whilst (*R*)/(*S*)-eriodictyol-8-*C*-glucoside decompose readily. (*R*)/(*S*)-eriodictyol-6-*C*-glucoside are then further oxidised to their corresponding flavone, isoorientin, which is not the case for (*R*)/(*S*)-eriodictyol-8-*C*-glucoside and orientin. Orientin is formed irreversibly by the Wessely-Moser rearrangement from isoorientin (Krafczyk & Glomb, 2008). It has been postulated that the oxidation of nothofagin to isovitexin and vitexin also proceeds via similar intermediate flavanone compounds (naringenin-*C*-glucosides). Hillis & Inoue (1967), however, demonstrated that nothofagin failed to convert to its corresponding flavanones under conditions similar to those used by Koeppen & Roux (1965a).

Krafczyk *et al.* (2009) further demonstrated that during oxidation the dihydrochalcones aspalathin and nothofagin are also converted to higher molecular weight browning products. It was unequivocally established that aspalathin is the single most important compound within the reaction to form browning products and that the browning reactions are mainly non-enzymatic. Oxidation of nothofagin, differing only from aspalathin by lacking the *o*-dihydroxy function at the B-ring, proceeded much more slowly, and this comparably slow degradation was also observed for orientin and isoorientin. Krafczyk *et al.* (2009) therefore concluded that, in addition to the *o*-hydroquinone moiety of the B-ring, the configuration of the C-ring is of major importance to oxidation and hence to the browning of rooibos flavonoids. Oxidation of aspalathin also led to the formation of an aspalathin dimer, stemming from oxidative coupling of the A- to the B-ring. The mechanism of aspalathin oxidation is illustrated in Figure 14.

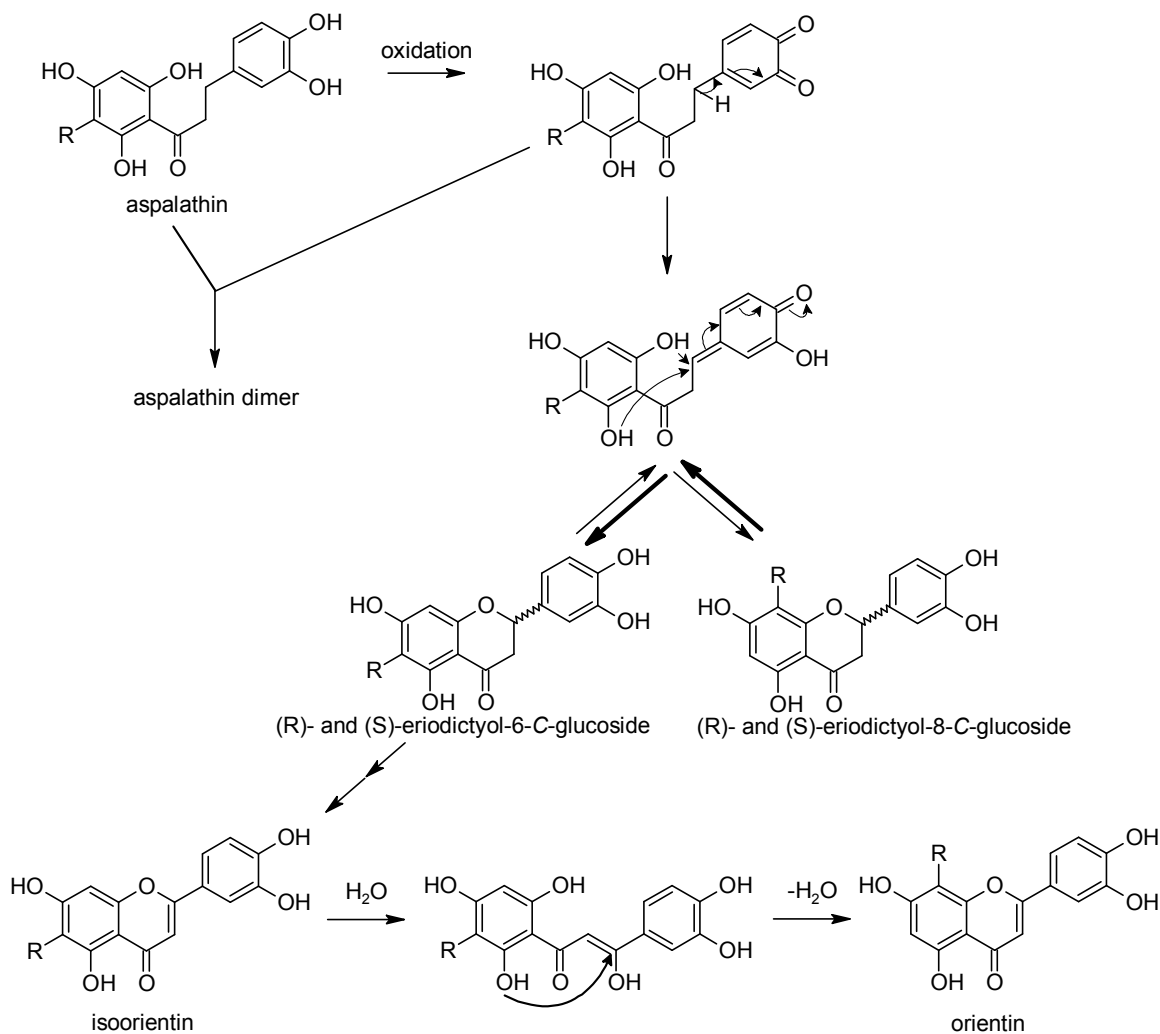


Figure 14 Mechanism of aspalathin oxidation (Joubert & De Beer, 2011).

Joubert *et al.* (2008c) investigated the effect of processing on the soluble solid and total polyphenol content of *Aspalathus linearis* material, by processing the harvested shoots ($n = 6$) into fermented and unfermented counterparts. The results indicated that fermentation induced a significant reduction in both the yield of dried aqueous extract and the total polyphenol content. Samples originated from the same plant material and hence the reduction in content values was solely attributed to the fermentation process.

Standley *et al.* (2001) reported that processing (fermentation, sun-drying, sieving and steam pasteurisation) decreased the total polyphenol content of the soluble solids of rooibos tea. Following the fermentation step, steam pasteurisation led to a significant increase in the percentage water-soluble solids of rooibos tea and also to a significant increase in the percentage total polyphenols. The latter observation was attributed to the removal of woody stems with a low water-soluble phenolic content from the samples prior to steam-pasteurisation. It should be noted, however, that the individual tea samples were collected randomly at the different processing stages and could therefore have originated from different plantations (ages, locations, etc). Factors other than the processing stage (such as natural variation) could therefore have been at play and hence the results are inconclusive.

Joubert *et al.* (2009a) investigated the effect of different heat treatments on the aspalathin, isoorientin and orientin contents of fermented rooibos iced tea. In the formulation containing only rooibos extract, without citric or ascorbic acid, the aspalathin content was significantly reduced by normal-temperature sterilisation (121°C for 15 min) and high-temperature sterilisation (135°C for 4 min), whilst pasteurisation (93°C for 30 min) only induced a small, albeit significant, reduction in the aspalathin content. Sterilisation also led to a significant reduction in the isoorientin and orientin contents. Heat-induced losses of isoorientin and orientin were remarkably lower than those observed for aspalathin, which was partially attributed to the oxidative conversion of aspalathin to these flavones. Conversely, it was found that pasteurisation actually led to small, but significant, increases in the isoorientin and orientin contents of the pasteurised iced tea. It was postulated that, upon heating, these flavones could have been released from an association with other compounds, such as proteins, in the rooibos matrix, leading to an observed increase in their content values. The contents of isoorientin and orientin could also have increased due to the oxidative degradation of aspalathin.

A study by Koch (2011), regarding the effect of steam-pasteurisation (96°C for 1 min) of rooibos leaves on the composition of aqueous rooibos infusions, only demonstrated significant reductions in the aspalathin content. Significant decreases in the soluble solids and total polyphenol contents of the infusions were also observed, which showed a strong correlation. It was therefore postulated that the decrease in soluble solids as a result of steam-pasteurisation may largely be attributed to a decrease in the level of soluble polyphenols.

3.5.2.2 Quantitative analysis of rooibos by RP-HPLC

Quantitative data on the phenolic constituents of rooibos are essential to understand their role as potential bioactives (Joubert *et al.*, 2009a). However, quantitative data on the phenolic constituents of rooibos are currently limited to either a few of the major compounds or to a small number of samples, which do not reflect the natural variation. A comprehensive discussion on the RP-HPLC methods which have been employed in the quantitative analysis of rooibos (Table 4) will ensue.

Joubert (1996) was the first to publish an HPLC method for the quantification of the C-glucoside dihydrochalcones aspalathin and nothofagin in rooibos tea as a function of processing parameters (Table 4). Separation was obtained under RP gradient elution conditions on a 5 µm C18 column (250 x 4.0 mm) thermostatted to 38°C; the chromatographic run time was 125 min. In addition to aspalathin and nothofagin, a standard mixture of different phenolic acids (*p*-hydroxybenzoic-, caffeic-, ferulic-, protocatechuic-, *p*-coumaric-, and vanillic acid) and other flavonoids (rutin, vitexin, isoquercitrin, orientin, and isoorientin) was also analysed. However, incomplete separation between vanillic and caffeic acid was obtained whilst orientin

and ferulic acid, and rutin and isoquercitrin coeluted. The reported method was subsequently applied to determine the effect of processing and drying method on the aspalathin and nothofagin content of rooibos tea.

The same method was applied in 2005 (Joubert *et al.*, 2005) to determine the phenolic composition of rooibos aqueous extracts and crude polymeric fractions. Results obtained for the aqueous extracts of unfermented ($n = 7$) and fermented rooibos ($n = 7$) are illustrated in Table 5.

Table 5 Phenolic composition of aqueous extracts of unfermented and fermented rooibos (results expressed as percentage of soluble solids) (Joubert *et al.*, 2005).

Compound	Unfermented rooibos	Fermented rooibos
isoorientin	0.81	0.85
orientin	0.59	0.76
aspalathin	12.29	0.61
vitexin	0.13	0.17
isoquercitrin + rutin	0.16	0.11
isovitexin	0.17	0.11
nothofagin	1.08	0.17
other ^a	trace	0.08

^a Other = quercetin, luteolin and chrysoeriol.

Bramati *et al.* (2002) developed an HPLC method for the quantitative determination of flavonoids in aqueous and methanolic infusions of fermented rooibos (commercially available rooibos tea-bags). Analyses were conducted at room temperature on a 5 μm C18 column (250 x 4.6 mm). The slightly acidified water-acetonitrile gradient was suitable for obtaining well-resolved and symmetrical peak separation for nearly all ten standard compounds (isoorientin, orientin, aspalathin, vitexin, rutin, isovitexin, isoquercitrin, luteolin, quercetin and chrysoeriol) within 30 min. Only isoorientin and orientin were incompletely separated in comparison to the other standards, although they could be accurately integrated. However, upon analysis of the rooibos sample, hyperoside (quercetin-3-O-galactoside) was found to co-elute with the standard compound, isoquercitrin (quercetin-3-O-glucoside). For quantitative purposes, the peak was quantified as isoquercitrin. The same method was subsequently applied in the analysis of aqueous extract of unfermented rooibos in 2003 (Bramati *et al.*, 2003). Content values for the flavonoids detected in unfermented ($n = 1$) and fermented ($n = 1$) rooibos aqueous extracts are summarised in Table 6 (page 65).

Schulz *et al.* (2003) developed an LC-UV-MS method for the reliable identification of the most relevant flavonoids occurring in rooibos. The study was aimed at addressing the lack of data on the variation of aspalathin and nothofagin content of rooibos, sampled from a large number of plantations/production areas. An HPLC method was subsequently established to provide a tool for the quantification of the two most important dihydrochalcones. Besides aspalathin and nothofagin other flavonoids (isoorientin, orientin, rutin and isoquercitrin) could also be reliably identified by LC-UV-MS measurements. A 3.5 μm C18 column (150 x 3.0 mm), thermostatted to 35°C, was utilised under gradient elution conditions. The method showed better sensitivity than earlier studies by Joubert (1996) in addition to a shorter analysis time (40 min). Quantitative data were only presented for aspalathin and nothofagin in both unfermented and fermented rooibos samples.

Table 6 Flavonoids detected in unfermented and fermented rooibos aqueous extract (results expressed as percentage of soluble solids) (Bramati *et al.*, 2002, 2003).

Compound	Unfermented rooibos	Fermented rooibos
isorientin	0.36	0.08
orientin	0.23	0.10
aspalathin	4.99	0.12
vitexin	0.05	0.03
rutin	0.17	0.13
isovitexin	0.07	0.03
isoquercitrin + hyperoside	0.03	0.04
luteolin	0.0022	0.0029
quercetin	0.0042	0.0107
chrysoeriol	0.0008	0.0022

Kazuno *et al.* (2005) developed a strategy for the determination and quantification of glycosyl flavonoids including dihydrochalcones using LC-tandem mass spectrometry in neutral loss scan mode. They demonstrated the suitability of their method in the analysis of unfermented rooibos tea, and quantified the identified glycosides by selected reaction monitoring (SRM). The method was suitable for the quantification of 11 glycosyl flavonoids within 50 min. This was the first report on quantitative data of a rutin 'isomer' in rooibos.

Joubert *et al.* (2009a) developed a HPLC method for the quantification of aspalathin and its corresponding flavones, orientin and isoorientin, in fermented rooibos (*Aspalathus linearis*) iced tea. The HPLC-PDA conditions (Table 4) were selected to obtain rapid separation of aspalathin, orientin and isoorientin. These three peaks were cleanly resolved, and, with the short analysis time (23 min), the method was suitable for the high through-put analysis of rooibos samples. This method has been applied in various studies pertaining to ready-to-drink rooibos iced teas since then (Joubert *et al.*, 2010; De Beer *et al.*, 2011).

More recently, two studies on the bioavailability and antioxidant potential of rooibos flavonoids following ingestion of rooibos teas were reported. Stalmach *et al.* (2009) presented quantitative data for diastereomeric isomers of eriodictyol-C-glucosides, aspalathin, nothofagin, isoorientin, orientin, vitexin, isovitexin, hyperoside, isoquercitrin, rutin, rutin isomer, luteolin and quercetin present in 500 mL of unfermented and fermented 'ready-to-drink' rooibos teas. Separation was achieved under gradient conditions on a 4 μm C12 RP column (250 x 4.6 mm) with a chromatographic run time of 75 minutes. Stalmach *et al.* (2009) were the first to report quantitative data for diastereomeric isomers of eriodictyol-C-glucosides, although the presence of these compounds in rooibos tea extract has been unambiguously established previously (Krafczyk & Glomb, 2008). Breiter *et al.* (2011) presented quantitative data for the major flavonoids (aspalathin, nothofagin, isoorientin, orientin, rutin, hyperoside, isoquercitrin, vitexin, isovitexin and luteolin-O-galactoside) in aqueous rooibos extracts, brewed with 500 mL of boiled water and 10 g of dried green rooibos tea leaves ($n = 12$). Separation was achieved on a 5 μm phenyl-hexyl column (250 x 4.6 mm) at a flow rate of 0.5 mL.min⁻¹. Analysis were conducted at room temperature and the analysis time was ca 80 min (Table 4).

Hence to conclude, various RP-HPLC methods have been used in the quantitative analysis of rooibos (Table 4). The methods typically employed 5 μm columns, operated at ambient and moderately

higher temperatures up to 40°C, with analysis times ranging up to 125 min. These methods are, however, hampered by limited resolution and/or excessive analysis times and are only suitable for the quantification of some of the major phenolic compounds.

Current practice at the Agricultural Research Council (ARC, Infruitec-Nietvoorbij, Post-harvest and Wine technology division, Stellenbosch, South Africa) entails the use of two methods for the quantification of the major phenolic constituents of aqueous rooibos infusions. The first method is adapted from the HPLC method published by Joubert (1996): the initial method was transferred to a 5 µm Gemini C18 column (150 x 4.6 mm) and the solvent program adjusted to improve resolution on an Agilent 1200 HPLC system leading to a run time of 90 min. The 2% formic acid aqueous solvent was also replaced with 2% acetic acid to simplify switching between methods. The adapted method is suitable for the quantification of orientin, isoorientin, nothofagin, vitexin, isovitexin, luteolin-7-O-glucoside, hyperoside, quercetin, luteolin and chrysoeriol; aspalathin and PPAG, and rutin and isoquercitrin coelute. The second method employed is the HPLC method developed by Joubert *et al.* (2009a) for the rapid separation and quantification of aspalathin and its corresponding flavones, isoorientin and orientin, in fermented rooibos iced tea. This method was also deemed suitable for the quantification of PPAG and nothofagin. Disadvantages of this current practice include the use of different organic modifiers and different stationary phases. In addition, the total analysis time is very long (113 min) which leads to increased solvent consumption and waste generation. Improved methods for the quantitative analysis of rooibos in routine application are therefore clearly required.

Table 4 Representative examples of HPLC methods for the analysis of flavonoids and phenolic acids in rooibos (*Aspalathus linearis*) extracts.

Main aim	Analytes	Stationary phase	Mobile phase	Analysis time	Column temperature	Reference
Quantification of the dihydrochalcones, aspalathin and nothofagin, as a function of processing parameters.	Protocatechuic acid; <i>p</i> -hydroxybenzoic acid; vanillic + caffeic acid; <i>p</i> -coumaric acid; aspalathin; orientin + ferulic acid; isoorientin; vitexin; nothofagin; rutin + isoquercitrin.	Merck LiChrospher 100 RP-18 (250 x 4.0 mm, 5 µm)	Gradient elution with A: methanol B: 2% formic acid in water (v.v ⁻¹) Flowrate: 0.4–1.2 mL.min ⁻¹	125 min	38°C	Joubert, 1996
Quantitative characterisation of flavonoid compounds in fermented and unfermented rooibos tea (<i>Aspalathus linearis</i>).	Isoorientin; orientin; aspalathin; vitexin; rutin; isovitexin; isoquercitrin + hyperoside; luteolin; quercetin; crysoeriol.	Waters Symmetry Shield C18 (250 x 4.6 mm, 5 µm)	Gradient elution with A: acetonitrile B: 0.1% acetic acid in water (v.v ⁻¹) Flowrate: 0.8 mL.min ⁻¹	30 min	Room temperature	Bramati <i>et al.</i> , 2002; Bramati <i>et al.</i> , 2003
Development of an LC-UV-MS method for the reliable identification of the most relevant flavonoids occurring in rooibos.	Isoorientin; orientin; aspalathin; rutin; isoquercitrin; nothofagin.	Agilent Zorbax SB-C18 (150 x 3.0 mm, 3.5 µm)	Gradient elution with A: acetonitrile B: 1% formic acid in water (v.v ⁻¹) Flowrate: 0.5 - 0.7 mL.min ⁻¹	40 min	35°C	Schulz <i>et al.</i> , 2003
Strategy for determination and quantification of glycosyl flavonoids using LC-MS.	Vitexin; isovitexin; nothofagin; isoorientin; orientin; luteolin-7-O-glucoside; aspalathin; hyperoside; isoquercitrin; rutin; rutin isomer.	Nomura Develosil ODS UG-5 (150 x 2.0 mm)	Gradient elution with A: 0.1% formic acid in 80% acetonitrile B: 0.1% formic acid in water (v.v ⁻¹) Flowrate: 0.2 mL.min ⁻¹	50 min	35°C	Kazuno <i>et al.</i> , 2005
Quantification of the dihydrochalcone aspalathin and its corresponding flavones orientin and isoorientin in fermented rooibos iced tea.	Aspalathin; orientin; isoorientin.	Agilent Zorbax Eclipse XDB-C18 (150 x 4.6 mm, 5 µm)	Gradient elution with A: acetonitrile B: 2% acetic acid in water (v.v ⁻¹) Flowrate: 0.8 mL.min ⁻¹	23 min	38°C	Joubert <i>et al.</i> , 2009a; Joubert <i>et al.</i> , 2010; De Beer <i>et al.</i> , 2011

Main aim	Analytes	Stationary phase	Mobile phase	Analysis time	Column temperature	Reference
Quantification of flavonoids in 500 mL of unfermented and fermented rooibos teas; and aspalathin and eriodictyol metabolites in urine.	Aspalathin; nothofagin; eriodictyol-C-glucosides (4); orientin; isoorientin; vitexin; isovitexin; hyperoside; isoquercitrin; rutin; rutin isomer; luteolin; quercetin in rooibos teas. Aspalathin and eriodictyol metabolites in urine.	Phenomenex Synergi (C12) RP-MAX 80Å (250 x 4.6 mm, 4 µm)	Gradient elution with A: acetonitrile B: 0.1% formic acid in water (v.v ⁻¹). Flowrate: 1.0 mL.min ⁻¹	Teas: 75 min Urine: 35 min	40°C	Stalmach <i>et al.</i> , 2009
Quantification of the major flavonoids in aqueous extract of dried green rooibos tea leaves; also unchanged flavonoids in plasma after ingestion of rooibos tea or active fraction isolated from rooibos tea.	Aspalathin; nothofagin; isoorientin; orientin; rutin; hyperoside; isoquercitrin; vitexin; isovitexin; luteolin-O-galactoside in aqueous extract of green rooibos. Aspalathin; isoorientin; and orientin in plasma.	Phenomenex Luna Phenyl-Hexyl (250 x 4.6 mm, 5 µm)	Gradient elution with A: acetonitrile B: 2% acetic acid in water (v.v ⁻¹) Flowrate: 0.5 mL.min ⁻¹	ca 80 min	Room temperature	Breiter <i>et al.</i> , 2011

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CHAPTER 3

*Kinetic optimisation of the reversed phase
liquid chromatographic separation of
rooibos tea (*Aspalathus linearis*) phenolics on
conventional high performance liquid chromatographic
instrumentation*

Abstract

Rooibos tea, produced from the endemic South African shrub *Aspalathus linearis*, has various health-promoting benefits which are attributed to its phenolic composition. Generating reliable, quantitative data on these phenolic constituents is the first step towards documenting the protective effects associated with rooibos tea consumption. Reversed phase liquid chromatographic (RP-LC) methods currently employed in the quantitative analysis of rooibos are, however, hampered by limited resolution and/or excessive analysis times. In order to overcome these limitations, a systematic approach towards optimising the RP-LC separation of the 15 principal rooibos tea phenolics on a 1.8 μm phase using conventional HPLC instrumentation was adopted. Kinetic plots were used to obtain the optimal configuration for the separation of the target analytes within reasonable analysis times. Simultaneous optimisation of temperature and gradient conditions provided complete separation of these rooibos phenolics on a 1.8 μm C18 phase within 37 minutes. The optimised HPLC-DAD method was validated and successfully applied in the quantitative analysis of aqueous infusions of unfermented and fermented rooibos. Major phenolic constituents of fermented rooibos were found to be a phenylpropanoid phenylpyruvic acid glucoside (PPAG), the dihydrochalcone C-glycoside aspalathin, the flavones isoorientin and orientin, and a flavonol O-diglycoside tentatively identified as quercetin-3-O-robinobioside. Content values for PPAG, ferulic acid and quercetin-3-O-robinobioside in rooibos are reported here for the first time. Mass spectrometric (MS) and tandem MS detection were used to tentatively identify 13 additional phenolic compounds in rooibos infusions, including a new luteolin-6-C-pentoside-8-C-hexoside and a novel C-8-hexosyl derivative of aspalathin reported here for the first time.

Keywords: Rooibos tea; phenolic compounds; high performance liquid chromatography; kinetic plots; mass spectrometry.

1. Introduction

Rooibos (*Aspalathus linearis*) is a leguminous shrub endemic to the Western Cape Province in South Africa. Herbal tea is prepared from both the unfermented green and fermented oxidised plant material, although consumption of the traditional fermented product is more common and has a long history of use. In less than 20 years the international market demand for rooibos steadily grew from 750 tons in 1993 to 5 633 tons in 2010. This increase has coincided with the interest of consumers in natural antioxidants as rooibos gained prominence as an antioxidant-containing beverage with potential health-promoting benefits. It has also led to value-added rooibos products such as extracts for the supplement/nutraceutical and functional food markets (Joubert & De Beer, 2011).

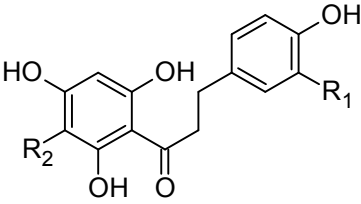
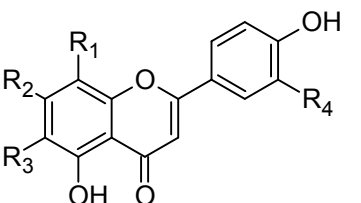
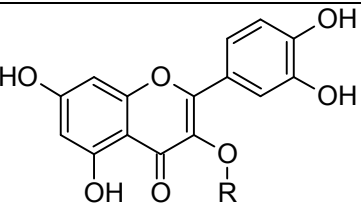
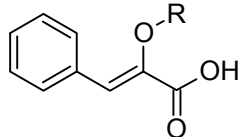
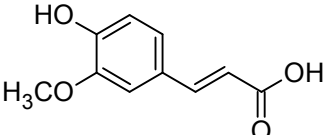
The secondary metabolites previously identified in fermented *Aspalathus linearis* plant material include single ring phenolic acids and monomeric flavonoids such as dihydrochalcones, flavanones, flavones, and flavonols (Joubert *et al.*, 2008). In addition, recently the presence of phenolic compounds such as di-C-glycosyl flavones has been established in rooibos by LC-MS (Stalmach *et al.*, 2009; Breiter *et al.*, 2011; Iswaldi *et al.*, 2011). Quantitative data on the phenolic constituents of rooibos are essential to understand their role as potential bioactives. However, insufficient data on the dietary exposure to rooibos flavonoids are currently available (Joubert *et al.*, 2009). Quantitative data on the rooibos phenolic compounds are either limited to a few of the major compounds such as aspalathin, nothofagin and their corresponding flavones, or to a limited number of samples, which do not reflect the natural variation.

Reversed-phase liquid chromatography (RP-LC) is commonly used for the analysis of rooibos infusions. However, RP-LC methods currently employed in the quantitative analysis of rooibos are hampered by limited resolution and/or excessive analysis times (Joubert, 1996; Bramati *et al.*, 2002, 2003; Schulz *et al.*, 2003; Kazuno *et al.*, 2005; Joubert *et al.*, 2009; Stalmach *et al.*, 2009; Breiter *et al.*, 2011). These methods typically employ 5.0 μm phases and are only suitable for the quantification of some of the major phenolic compounds in analysis times up to 125 min, restricting their suitability for routine application.

Recent developments in LC may be used to improve conventional methods for phenolic analysis (De Villiers *et al.*, 2010). Of these, particularly the use of columns packed with sub-2 μm particles operated at elevated temperatures provides an attractive alternative for the fast and efficient analysis of rooibos phenolics using conventional instrumentation. Iswaldi and co-workers (2011) recently presented a qualitative method on the analysis of rooibos phenolics utilising a column with 1.8 μm particles, although this method was not optimised for the separation of the most important compounds. Cabooter and co-workers (2011) recently reported a method for the separation of the principal rooibos phenolics on a 200 mm long column packed with 1.7 μm particles operated at 1000 bar. However, such ultra high pressure liquid chromatography (UHPLC) instrumentation is not commonly available in laboratories charged with routine analysis of rooibos.

In light of the above, the aim of the current paper was to develop a RP-LC method on a sub-2 μm particle phase suitable for the fast, routine quantification of the most important rooibos phenolics (Table 1) on conventional HPLC instrumentation. The kinetic benefits of this phase compared to conventional RP-LC phases were evaluated, followed by the systematic optimisation of gradient conditions to allow separation of the target analytes within a reasonable analysis time. The optimised method was applied to the analysis of aqueous infusions prepared from unfermented and fermented rooibos plant material, and high resolution mass spectrometry and tandem mass spectrometry were used to confirm peak purity and identify additional phenolic compounds present in each of these samples.

Table 1 Structures of the principal rooibos phenolic compounds.

General structure	No. ^a	Phenolic compound	Substituents
	4 12	Dihydrochalcones aspalathin nothofagin	R ₁ = OH, R ₂ = C-β-D-glucosyl R ₁ = H, R ₂ = C-β-D-glucosyl
	3 2 6 9 14 11 15	Flavones orientin isorientin vitexin isovitexin luteolin luteolin-7-O-glucoside chrysoeriol	R ₁ = C-β-D-glucosyl, R ₂ = R ₄ = OH, R ₃ = H R ₁ = H, R ₂ = R ₄ = OH, R ₃ = C-β-D-glucosyl R ₁ = C-β-D-glucosyl, R ₂ = OH, R ₃ = R ₄ = H R ₁ = R ₄ = H, R ₂ = OH, R ₃ = C-β-D-glucosyl R ₁ = R ₃ = H, R ₂ = R ₄ = OH R ₁ = R ₃ = H, R ₂ = O-β-D-glucosyl, R ₄ = OH R ₁ = R ₃ = H, R ₂ = OH, R ₄ = OCH ₃
	13 10 7 8	Flavonols quercetin isoquercitrin hyperoside rutin	R = H R = O-β-D-glucosyl R = O-β-D-galactosyl R = O-β-D-rutinosyl
	1	Phenylpropanoid phenylpyruvic acid-2-O-glucoside (PPAG)	R = O-glucosyl
	5	Hydroxycinnamic acid ferulic acid	

2. Experimental

2.1 Chemicals and columns

HPLC gradient-grade acetonitrile was purchased from Merck (Darmstadt, Germany), methanol from Riedel-de Haën (Sigma-Aldrich, St. Louis, MO, USA) and acetic acid from Fluka (Sigma-Aldrich, Johannesburg, South Africa). Deionised water, prepared using a Modulab (Continental Water Systems Corporation, San Antonio, TX, USA) water purification system, was further purified to HPLC-grade using a Milli-Q academic (Millipore, Milford, MA, USA) water purification system. Acetophenone and uracil were obtained from Fluka, while phenolic standards (Table 1) were from Extrasynthese (Genay Cedex, France), Roth (Karlsruhe, Germany), Sigma-Aldrich and Fluka. Enolic phenylpyruvic acid-2-O-glucoside (PPAG) was isolated and supplied by the Post Harvest & Wine Technology Division of the Agricultural Research Council of South Africa (ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa). Aspalathin and nothofagin were supplied by the PROMEC Unit of the Medical Research Council of South Africa (MRC, Cape Town, South Africa). Stock solutions of the phenolic standards were prepared in dimethyl sulfoxide (Fluka) at concentrations of approximately $1 \text{ mg}\cdot\text{mL}^{-1}$ and diluted with water according to experimental requirements. All diluted solutions contained $0.5 \text{ mg}\cdot\text{mL}^{-1}$ ascorbic acid (Sigma) and were filtered through $0.22 \mu\text{m}$ hydrophilic PVDF filters (Millipore) prior to use.

The following 4.6 mm i.d. columns were employed in this study: a 150 mm $5.0 \mu\text{m}$ d_p Phenomenex Gemini C18 column (Torrance, LA, USA); and 50 mm and 100 mm $1.8 \mu\text{m}$ d_p Agilent Zorbax SB-C18 columns (Agilent Technologies, Waldbronn, Germany).

2.2 Preparation of aqueous rooibos infusions

Randomly selected samples of unfermented ($n = 10$) and fermented ($n = 10$) rooibos plant material were obtained from South Africa. Duplicate infusions were prepared by infusing 2.5 g plant material for 5 min with 200 mL freshly boiled deionised water without agitation. The infusions were decanted through a tea strainer, filtered through Whatman No. 4 filter paper and cooled to room temperature. The soluble solid content of the infusions was determined in 20 mL aliquots using a gravimetric method. Aliquots (*ca* 1.5 mL) were stored at -20°C until analysis. For LC analyses, aliquots of the rooibos infusions were thawed at room temperature and filtered through $0.22 \mu\text{m}$ hydrophilic PVDF filters. Subsequently 1 mL of the filtrate was pipetted into a 1.5 mL amber autosampler vial and $100 \mu\text{L}$ 10% ascorbic acid in water ($v\cdot v^{-1}$) added. All samples were analysed within 24 hours of sample preparation.

2.3 Instrumentation

All analyses were performed using a mobile phase consisting of (A) 2% acetic acid in water ($v\cdot v^{-1}$) and (B) acetonitrile. HPLC analyses were conducted on an Agilent 1200 series instrument (maximum pressure 400 bar) equipped with an in-line degasser, quaternary pump, autosampler, column thermostat and diode-array detector (standard $13 \mu\text{L}$ flow cell, 10 mm path length) controlled by Chemstation software (all from Agilent Technologies). The dwell volume on this system was measured as 0.82 mL .

UHPLC analyses were conducted on an Acquity UPLC system (using a maximum pressure of 400 bar) equipped with a binary solvent manager, sample manager, column heating compartment and photodiode-array (PDA) detector (Waters, Milford, USA). The detector was equipped with a 500 nL flow cell (10 mm path length). The dwell volume of this system was 0.07 mL .

High Resolution LC-MS analyses were conducted on a UPLC-Synapt GT Q-TOF system (Waters) equipped with a PDA.

2.4 Chromatographic conditions

2.4.1 Construction of kinetic plots

For HPLC analyses on the Gemini column (25°C), the external volume of the system was minimised by using minimal lengths of 0.13 mm i.d. PEEK tubing. 10 μL injections were performed (100 μL injection loop). For UHPLC analyses on the 50 mm Zorbax column (25 and 50°C), injections were performed in the full loop mode (5 μL injection loop) using mobile phase A as weak needle wash solvent.

Phenolic compounds and acetophenone were dissolved in the mobile phase, together with uracil as unretained marker. Concentrations ranged between 2.5 and 12.5 $\text{mg}\cdot\text{L}^{-1}$. The organic content of the mobile phase was adjusted to yield constant retention factors for each of the analytes on the different stationary phases and at different temperatures. Plate height measurements were performed in isocratic mode by systematically increasing the flow rate from 0.1 $\text{mL}\cdot\text{min}^{-1}$ in increments of 0.1 $\text{mL}\cdot\text{min}^{-1}$ until the maximum column pressure was reached (in the case of the Zorbax column up to a maximum flow rate of 2 $\text{mL}\cdot\text{min}^{-1}$). UV detection was performed at 288 nm.

All experimental values represent the average of three measurements and were obtained after correction for the extra-column band broadening (σ_{extra}^2), t_0 -time (t_{extra}) and pressure drop (ΔP_{extra}). The extra-column peak variance for the Agilent 1200 series instrument ranged between 0.26-14 sec^2 (for flow rates 2.6-0.1 $\text{mL}\cdot\text{min}^{-1}$), resulting in 0.10-1.7% losses in efficiency on the Gemini column. For the Acquity UPLC system σ_{extra}^2 ranged between 0.027-7.0 sec^2 (flow rate 2.0-0.1 $\text{mL}\cdot\text{min}^{-1}$), leading to losses in efficiency of 0.0-1.4% on the 50 mm Zorbax column. For the construction of plate height curves, the number of theoretical plates (N) was calculated using the peak width at half height obtained from Agilent Chemstation software. The retention time of uracil (t_0) was used to calculate linear velocity (u_0) and retention factors (k). Kinetic plots were constructed using a freely downloadable spreadsheet template (Desmet *et al.*, 2005). Mobile phase viscosity (η , Pa.s) was calculated using water/acetonitrile as the mobile phases according to Guillaume *et al.* (2004), while diffusion coefficients (D_m , $\text{m}^2\cdot\text{s}^{-1}$) were calculated using the Wilke-Chang equation (Wilke & Chang, 1955). Column permeabilities (K_v , m^2) were determined using the general pressure drop equation (De Villiers *et al.*, 2009). For the maximum pressure (ΔP_{max}), values of 240 and 400 bar were used for the Gemini and Zorbax columns, respectively.

2.4.2 Evaluation of mobile phase composition and temperature

Optimisation experiments were conducted on the Agilent 1200 system with UV detection at 288 nm using the standards and uracil dissolved in the mobile phase. Experimental data were generated in isocratic mode on the 100 mm Zorbax column protected with an Acquity UPLC in-line filter (Waters) at a flow rate of 0.8 $\text{mL}\cdot\text{min}^{-1}$.

The effect of solvent strength was investigated by systematically increasing the volume fraction of the organic modifier (ϕ) from 0.075 to 0.400 in increments of 0.025. The column was thermostatted at 25°C throughout. For the construction of Van't Hoff plots, the column temperature was systematically increased from 25 to 60°C in increments of 5°C. The mobile phase composition was varied to obtain retention factors ranging between 5 and 50. Values represent the average of three measurements.

2.4.3 Optimised HPLC-DAD method for routine analysis

Routine analyses were performed on the Agilent 1200 system at 37°C using the 100 mm Zorbax column protected with an Acquity UPLC in-line filter and a 5.0 µm SB-C18 guard column (Agilent). An injection volume of 50 µL was used for all fermented rooibos infusions, whilst the injection volume for the unfermented rooibos infusions was adjusted to provide a response for aspalathin within the linear range. The flow rate was 1.0 mL.min⁻¹ and a multilinear gradient was performed as follows: 10% B (0-2 min), 10-14.8% B (2-19 min), 14.8-36.8% B (19-34 min), 36.8-100% B (34-37 min), 100% B isocratic (37-42 min), 100-10% B (42-45 min). The column was re-equilibrated for 5 min. UV spectra were recorded between 200-700 nm with selective wavelength monitoring at 288 and 350 nm.

Preliminary validation was performed in terms of linearity, intra- and interday analytical precision as well as analyte stability (Shabir *et al.*, 2007). The dihydrochalcones and PPAG were quantified at 288 nm, whilst the flavones, flavonols and ferulic acid were quantified at 350 nm. Quercetin-3-*O*-robinobioside was quantified using the calibration curve for rutin (quercetin-3-*O*-rutinoside) since no standard was available for this compound. Calibration mixtures were injected at nine different injection volumes corresponding to 0.025–1.2 µg on-column, covering the concentration ranges of the compounds in aqueous rooibos infusions. Linear regression was performed for each compound to determine the slope, *y*-intercept and correlation coefficients (*r*²).

2.4.4 Comparative HPLC-DAD methods for routine analysis

For comparative quantitative analyses, the method reported by Joubert *et al.* (2009) was used for the quantification of PPAG, isoorientin, orientin, aspalathin, rutin and nothofagin. An adapted method from Joubert (1996) was used for the quantification of vitexin, isovitexin, luteolin-7-*O*-glucoside, hyperoside, quercetin, luteolin and chrysoeriol. Adjustments to the method of Joubert (1996) led to the following chromatographic conditions: Analyses performed at 38°C using the 150 mm Gemini column protected with a Gemini C18 guard cartridge (4 x 3 mm). The flow rate was 0.4 mL.min⁻¹ and the binary mobile phase consisted of (A) 2% acetic acid in water (v.v⁻¹) and (B) methanol. A multilinear gradient was performed as follows: 20% B (0-3 min), 20-30% B (3-15 min), 30% B (15-18 min), 30-35% B (18-27 min), 35% B (27-29 min), 35-40% B (29-35 min), 40-60% B (35-54 min), 60-80% B (54-62 min), 80-60% B (62-68 min), 60-20% B (68-81 min). The column was re-equilibrated for 9 min at a flow rate of 1.2 mL.min⁻¹. All analyses were performed on the Agilent 1200 system.

2.4.5 LC-ESI-MS and LC-ESI-MS/MS analyses

UPLC-ESI-MS and MS/MS analyses were performed as outlined in 2.4.3, except that the in-line filter was replaced by the Acquity Column Stabilizer (Waters) and an analysis temperature of 38°C was used. Data were acquired in the high resolution (150-1500 amu) and MS/MS scanning modes and processed using MassLynx v.4.1 software (Waters). The instrument was operated in positive and negative ionisation modes and calibrated using a sodium formate solution. Leucine encaphalin was used for lockspray. The capillary voltage was 3.0 and -3.5 kV in positive and negative ionisation modes, respectively, and the sampling cone voltage was 20.0 V. The source temperature was 120°C, whilst desolvation temperature was set at 350°C. Desolvation gas flow (N₂) was 500 L.hr⁻¹ and cone gas flow (N₂) 50 L.hr⁻¹. For MS/MS experiments, a trap collision energy of 30 V was used. The rest of the parameters were optimised for best sensitivity. The eluent was split 3:2 prior to introduction into the ionisation chamber. Injection volumes of 5 and 10 µL were used for

the standard mixture and aqueous rooibos infusions, respectively. Injections were performed in the partial loop mode (10 μL injection loop) with 20% MeOH in water ($v.v^{-1}$) as weak needle wash solvent. PDA data were acquired over a wavelength range of 220–400 nm at a sampling rate of 10 points. sec^{-1} .

3. Results and Discussion

In order to develop a single method suitable for the fast, simultaneous quantification of the major rooibos phenolics on conventional instrumentation, a systematic approach towards optimising separation on a 1.8 μm RP-LC column was adopted. This entailed kinetic evaluation of this phase, followed by simultaneous optimisation of the gradient profile and temperature.

3.1 Kinetic evaluation of the effect of stationary phase particle size and temperature on the separation of rooibos phenolics

A 1.8 μm column was selected for this study due to the known benefits of such phases for speeding up routine RP-LC analyses (De Villiers *et al.*, 2006). Moreover, column lengths up to 100 mm of this phase may be used on conventional instrumentation to provide separations of the same efficiency as obtained on conventional (250 mm, 5.0 μm) phases. The mobile phase selected consisted of (A) 2% acetic acid in water ($v.v^{-1}$) and (B) acetonitrile. The slightly acidified aqueous phase is required to suppress ionisation (Harnly *et al.*, 2007), whilst the selection of acetonitrile as organic modifier, as opposed to methanol, was based primarily on lower viscosity (*i.e.* lower operating pressure) and, to a lesser extent, on selectivity considerations.

The 1.8 μm phase was kinetically evaluated at two different temperatures (25 and 50°C) and compared to a conventional 5.0 μm column, thermostatted at 25°C. The potential benefits provided by this strategy for improved separation of rooibos phenolics was determined by constructing van Deemter curves and kinetic plots (Desmet *et al.*, 2005) for these compounds. Plate height curves were measured on a 50 mm 1.8 μm column in order to obtain the maximum number of data points in the C-term regime. Note that although the final method optimisation was performed on a 100 mm column of the same phase, it has been shown practically (Cabooter *et al.*, 2009) that extrapolation of kinetic performance data for a particular phase to longer column lengths remains valid.

Studies used to assess the kinetic benefits provided by small particle-packed columns typically use common organic molecules such as acetophenone as test analytes, since these compounds exhibit ideal thermodynamic behaviour (Oláh *et al.*, 2010). Various authors have, however, reported significant differences in the plate height behaviour of such test analytes in comparison with real-life compounds (De Villiers *et al.*, 2009; Oláh *et al.*, 2010; Fekete *et al.*, 2011). While limited kinetic data on phenolic compounds have been reported (De Villiers *et al.*, 2010), it is to be expected that these relatively large, structurally diverse molecules will exhibit dissimilar plate height behaviour. Therefore, in the first instance, the kinetic behaviour of rooibos phenolics was investigated relative to acetophenone. Experimental plate height data obtained on a 1.8 μm column thermostatted at 25°C are illustrated in Figure 1. Relevant data for these van Deemter curves are summarised in Table 2.

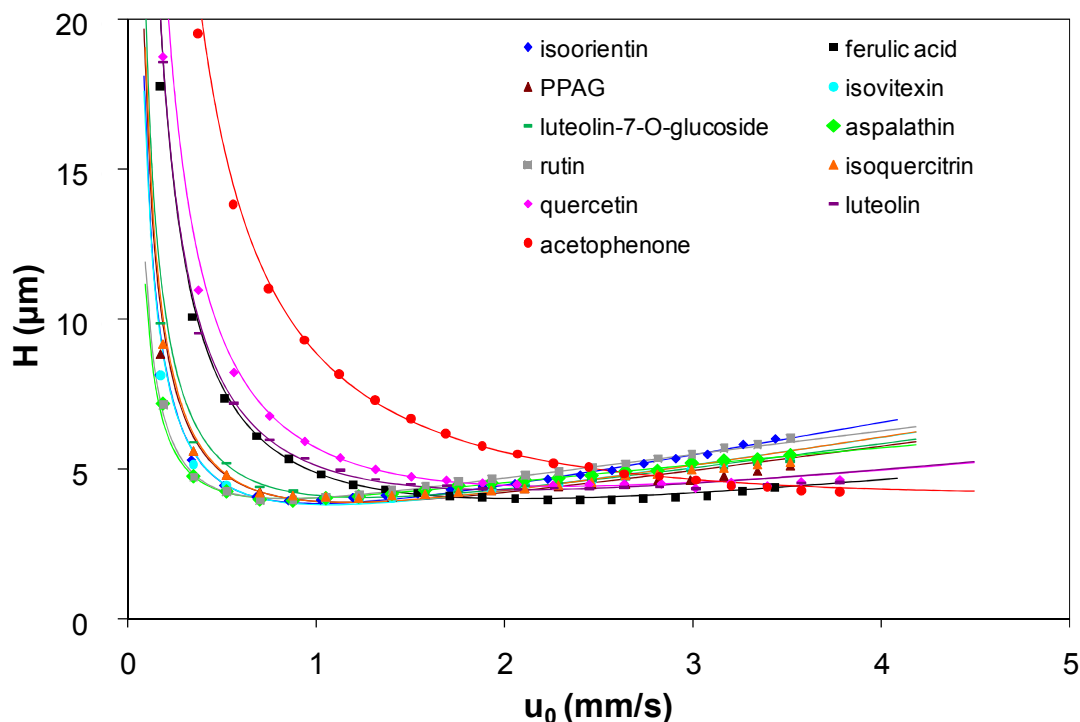


Figure 1 Van Deemter curves obtained for selected rooibos phenolic compounds and acetophenone on an Agilent Zorbax SB-C18 column (50 x 4.6 mm, 1.8 µm d_p), thermostatted at 25°C. For a summary of the plate height results, refer to Table 2.

Table 2 Summary of plate height data obtained for selected rooibos phenolic compounds and acetophenone on an Agilent Zorbax SB-C18 column (50 x 4.6 mm, 1.8 µm d_p) at 25°C.

Compound	D_M ($m^2 \cdot s^{-1}$)	k	H_{min} (µm)	u_{opt} ($mm \cdot s^{-1}$)
acetophenone	7.99×10^{-10} ^a	7.08	4.25 ^b	4.49 ^b
aspalathin	3.89×10^{-10} ^c	3.84	4.00	0.84
isoorientin	3.94×10^{-10} ^d	5.29	3.85	1.01
isovitexin	3.99×10^{-10} ^c	5.34	3.80	1.08
luteolin	5.32×10^{-10} ^a	6.21	4.30	2.05
luteolin-7-O-glucoside	3.93×10^{-10} ^c	6.78	4.05	1.25
quercetin	5.23×10^{-10} ^a	6.42	4.41	2.27
isoquercitrin	3.89×10^{-10} ^c	6.55	3.88	1.17
rutin	3.24×10^{-10} ^c	5.23	4.03	0.84
PPAG	4.53×10^{-10} ^c	3.36	3.91	1.16
ferulic acid	6.17×10^{-10} ^d	8.66	4.00	2.11

^a Calculated according to the Wilke and Chang equation (Wilke & Chang, 1955) using a mobile phase composition of 25/75 acetonitrile/2% acetic acid in water ($v \cdot v^{-1}$).

^b Extrapolated from experimental data acquired up to 3.79 $mm \cdot s^{-1}$.

^c Calculated as for ^a using a mobile phase composition of 15/85 acetonitrile/2% acetic acid in water ($v \cdot v^{-1}$).

^d Calculated as for ^a using a mobile phase composition of 12.5/87.5 acetonitrile/2% acetic acid in water ($v \cdot v^{-1}$).

These results were obtained using the same experimental configuration (instrument, column and temperature), and therefore variations in the shape of the van Deemter curves can primarily be attributed to the physicochemical properties of the analytes. Differences in analyte retention (k , Table 2) influence both the B - and the C -terms of the van Deemter equation (De Villiers *et al.*, 2009). For this reason, the mobile phase composition was adjusted experimentally to limit k between 3 and 10, and therefore the contribution of this factor can be considered as negligible.

Experimental data (Table 2) indicate that the minimum plate heights, and hence the maximum efficiencies attainable for a given column length, are approximately similar for all compounds. The slightly higher H_{\min} values obtained for acetophenone, quercetin and luteolin can presumably be attributed to the combined effect of slightly higher k values and the use of a less viscous mobile phase.

Significant differences were observed in the optimal linear velocity (u_{opt}) between all compounds. Isoorientin, isovitexin, luteolin-7-*O*-glucoside, isoquercitrin, and PPAG are characterised by optimal linear velocities ranging between 1.0 and 1.2 mm.s⁻¹, whilst u_{opt} values for luteolin, quercetin and ferulic acid are comparable at 2.0 to 2.2 mm.s⁻¹ (Table 2). Rutin and aspalathin have extremely low optimal linear velocities equal to 0.84 mm.s⁻¹, whereas this value is significantly higher for the test analyte, acetophenone ($u_{\text{opt}} = 4.49$ mm.s⁻¹, extrapolated). Practically, this implies that the optimal flow rate for aspalathin is approximately 0.5 mL.min⁻¹ (on a 4.6 mm i.d. column) and hence operating this column at conventional flow-rates of 1.0-1.5 mL.min⁻¹ will result in an efficiency loss of up to 20%. The relatively low optimal flow rates for the phenolics on the 4.6 mm i.d. 1.8 μm column (0.48-1.20 mL.min⁻¹) imply that analyses under optimal conditions may be performed on conventional instrumentation with maximum operating pressures of 400 bar. This is not the case for acetophenone, for which the optimal flow rate (2.38 mL.min⁻¹) would correspond to an operating pressure of ~570 bar for a 50 mm column length.

Variations in the shape of the experimental van Deemter curves can largely be ascribed to the different diffusion coefficients (D_M , Table 2) of the analytes under investigation. For compounds with lower D_M values, the B -term and C -term contributions to plate height are respectively smaller and larger, which together induce a significant reduction in the optimal linear velocity. For example, the optimal linear velocity for the flavonol aglycone, quercetin, was 2.27 mm.s⁻¹, while the values for its monoglycosylated (isoquercitrin) and diglycosylated (rutin) derivatives were significantly lower at 1.17 and 0.84 mm.s⁻¹, respectively. Considering the flavone phenolic subclass, a similar observation was made – the aglycone luteolin had an optimal linear velocity value of 2.05 mm.s⁻¹, while its mono-*O*-glycoside (luteolin-7-*O*-glucoside) and mono-*C*-glycoside (isovitexin), had lower optimal linear velocities of 1.25 and 1.08 mm.s⁻¹, respectively. It can therefore be concluded that, irrespective of the phenolic subclass and type of glycosidic bond ($C-C$ vs $C-O$), an increase in the degree of glycosylation (and hence molecular weight) leads to a decrease in the optimal linear velocity. In addition, an increase in the degree of glycosylation also results in a relatively steep increase in plate height in the C -term (high flow rate) regime. These results are in accordance with previous reports for large molecular weight analytes (Guiochon, 2006; De Villiers *et al.*, 2009; Oláh *et al.*, 2010).

Further discussion on the effect of stationary phase particle size and temperature will be limited to aspalathin as representative compound since this is the principal phenolic constituent of rooibos tea. Moreover, since aspalathin also exhibits the most conservative plate height behaviour, kinetic optimisation for this compound relatively accurately reflects the performance of this column for rooibos analysis.

Current RP-LC methods employed in the quantitative analysis of rooibos typically use 5.0 μm columns (Joubert, 1996; Bramati *et al.*, 2002, 2003; Joubert *et al.*, 2009; Breiter *et al.*, 2011) operated at ambient (Bramati *et al.*, 2002, 2003; Breiter *et al.*, 2011) and moderately higher temperatures up to 40°C (Joubert, 1996; Schulz *et al.*, 2003; Kazuno *et al.*, 2005; Joubert *et al.*, 2009; Stalmach *et al.*, 2009). Therefore a 5.0 μm Phenomenex column operated at 25°C was selected as reference in order to evaluate the kinetic benefits provided by the 1.8 μm column. Plate height curves obtained for aspalathin on each of the investigated phases at the studied temperatures are illustrated in Figure 2.

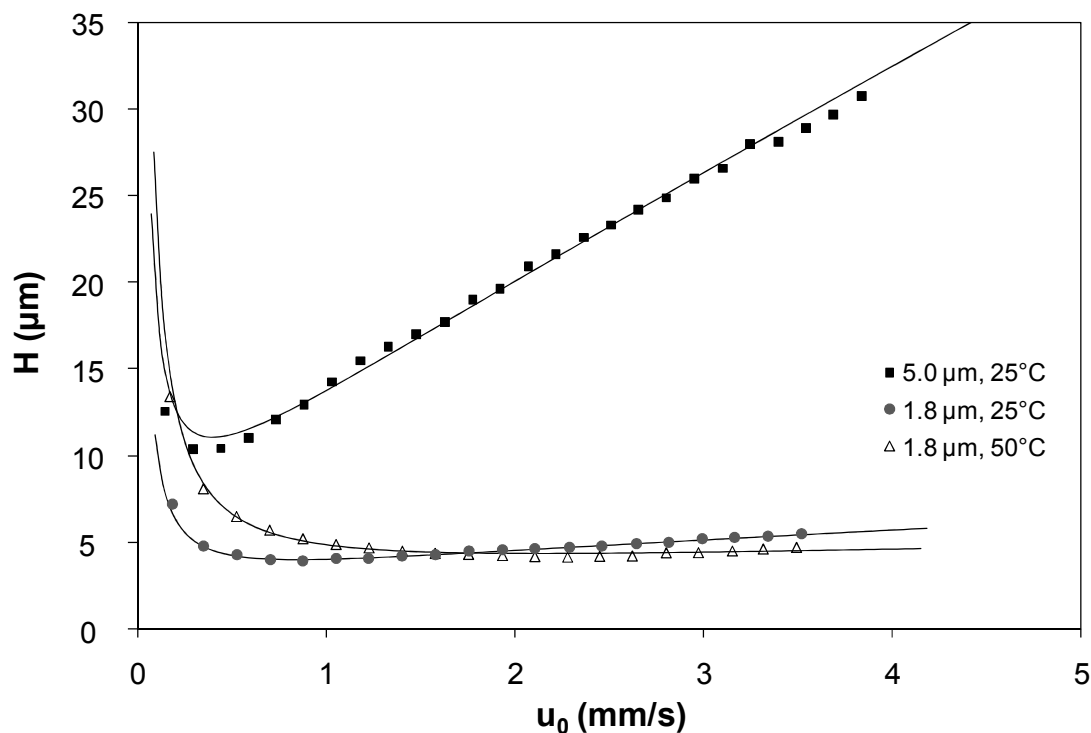


Figure 2 Van Deemter curves obtained for aspalathin on a 5.0 μm Phenomenex Gemini C18 column (150 x 4.6 mm) at 25°C, and a 1.8 μm Agilent Zorbax SB-C18 column (50 x 4.6 mm) at 25 and 50°C.

The minimum plate heights obtained for aspalathin on the 5.0 μm and 1.8 μm phases at 25°C are 11.02 and 4.00 μm , respectively, with corresponding u_{opt} values of 0.39 and 0.84 $\text{mm}\cdot\text{s}^{-1}$. As expected, a reduction in particle size resulted in an important reduction of the minimum plate height and an increase in the optimal linear velocity. Although not illustrated here, similar results were obtained for the other target phenolic compounds.

To evaluate the effect of temperature, a value of 50°C was chosen to avoid the potential on-column degradation of the thermally labile phenolic compounds. With an increase in temperature (compare the 1.8 μm phase at 25 and 50°C, Figure 2), the minimum plate height obtained for aspalathin remained virtually unaltered, while the optimum linear velocity was shifted towards a higher value (2.12 $\text{mm}\cdot\text{s}^{-1}$). This corresponds to an increase in the optimal flow rate from 0.48 to 1.22 $\text{mL}\cdot\text{min}^{-1}$ on this column. This increase in optimal linear velocity is surprisingly large, considering that the calculated diffusion coefficient of aspalathin increases from $3.9 \times 10^{-10} \text{ m}^2\cdot\text{s}^{-1}$ at 25°C (Table 2) to $6.0 \times 10^{-10} \text{ m}^2\cdot\text{s}^{-1}$ at 50°C. We currently have no explanation for this larger-than-expected increase in optimal linear velocity. However, it is clear that the primary advantage afforded by operating at elevated temperature is a reduction in analysis time.

Furthermore, the increase in temperature was accompanied by a decrease in the C -term (Guiochon, 2006), which may be exploited by applying higher flow rates to obtain fast separations without a significant loss in efficiency. This approach is, however, limited on conventional instrumentation with maximum pressure capabilities of 400 bar.

Kinetic plots were constructed according to Desmet *et al.* (2005) using experimental data for the rooibos phenolics on the studied columns. Kinetic plots automatically yield the most relevant performance characteristics in terms of which experimental configuration will either yield faster separations for a required efficiency, or will achieve the maximum number of plates in a given analysis time.

Since the goal of this work was to develop a fast routine method for rooibos phenolics on conventional HPLC instrumentation, kinetic plots were constructed using a maximum pressure of 400 bar. These plots were used to compare the kinetic performance of the different supports for aspalathin. By manipulation of the experimental plate height data using the retention factor (k), a plot of actual analysis time t_R versus N can be obtained (Desmet *et al.*, 2005) (Figure 3). These plots yield the most sought-after kinetic data, *i.e.* the minimum analysis time needed to perform a separation requiring N plates.

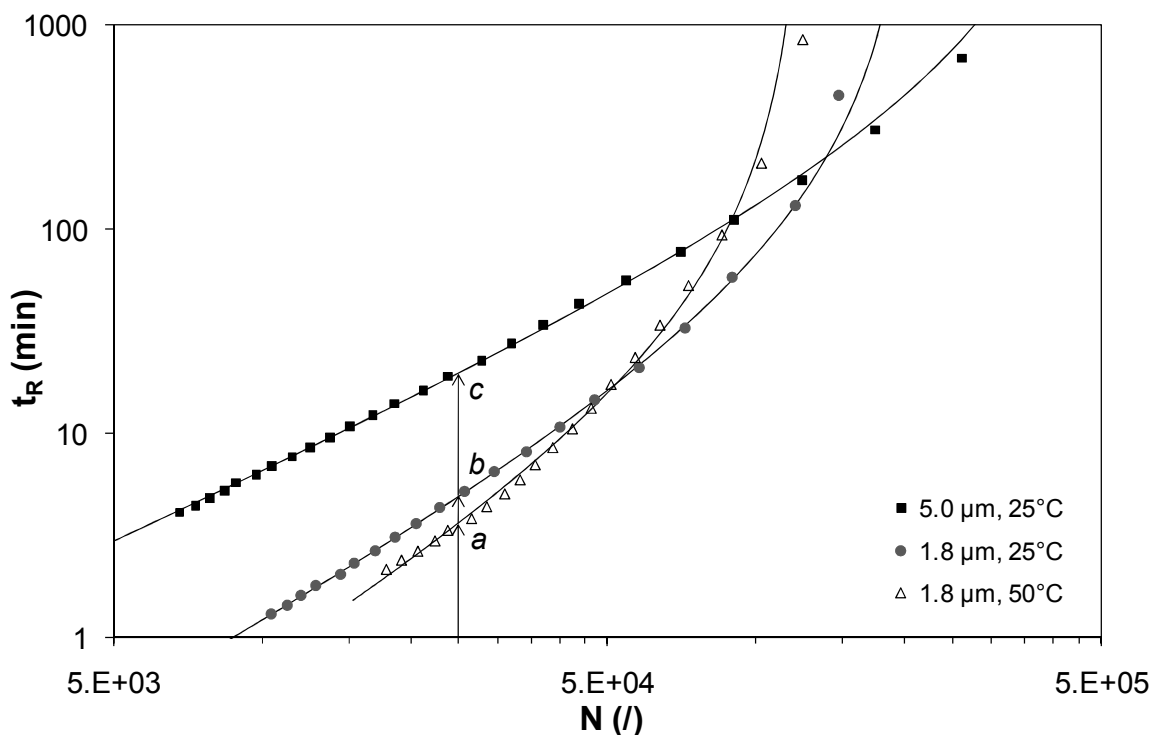


Figure 3 t_R vs N plots obtained for aspalathin on a 5.0 μm Phenomenex Gemini C18 column (150 x 4.6 mm) at 25°C, and a 1.8 μm Agilent Zorbax SB-C18 (50 x 4.6 mm) at 25 and 50°C.

From these plots, it is easy to establish the range of efficiencies (N) where a particular experimental setup will outperform the other. Note that for difficult separations requiring large N , the larger particle size will provide better results, albeit at the cost of unreasonably long analysis times. Figure 3 shows that for efficiencies less than $\sim 140\,000$ plates, the 1.8 μm phase will always permit faster analysis for a given efficiency, even at a maximum pressure of 400 bar. This can be attributed to a reduction in plate height and a decrease in mass-transfer resistance with reduced particle size, as discussed previously.

Considering the effect of temperature for aspalathin on the 1.8 μm phase, better performance is obtained in the range of lower plate counts. Utilizing this, elevated temperature can be used to either improve

N for a given analysis time, or to increase analysis speed for efficiencies up to ~65 000 plates (Figure 3).

Conventional routine HPLC methods for rooibos phenolics provide isocratic efficiencies of 15-25 000 plates. The latter value also roughly corresponds to the efficiency of a 100 mm 1.8 μm column (operated at optimal flow rate), the maximum length which may practically be used on conventional instrumentation at pressures up to 400 bar. Points a–c in Figure 3 correspond to the analysis times for aspalathin that will provide a required efficiency of 25 000 plates. The analysis times obtained on the 1.8 μm phase thermostatted to 50°C (point a) and 25°C (point b), and on the 5.0 μm phase at 25°C (point c), are approximately 4, 5 and 20 min, respectively. Therefore, for separations requiring 25 000 plates, a 75% decrease in analysis time can be obtained by switching to a smaller particle size, with a further 25% reduction obtained by increasing the temperature to 50°C. Clearly, for analyses providing relatively low efficiencies such as typically used in routine HPLC methods, the use of smaller particle-packed columns operated at elevated temperature will provide much better performance. Plots of t_0/N^2 vs N for aspalathin are presented in Supplementary Information (Figure S1) to allow comparison of the range of efficiencies where each configuration provides optimal results in terms of the conventional kinetic plot representation.

3.2 Simultaneous optimisation of mobile phase composition and temperature

Having established the suitability of the 1.8 μm phase for fast, efficient separation of rooibos phenolics on conventional instrumentation, the effect of solvent strength and temperature on the retention behaviour of rooibos phenolics were investigated by constructing plots of $\log k$ vs volume fraction of organic modifier (φ) and reciprocal temperature (Van't Hoff plots), for the 15 target analytes (Figure 4). Under isocratic elution conditions, analyte retention as a function of solvent strength ($\varphi = 0.01\%$ B) and temperature (T) can be described by the following well-known relationships (Zhu *et al.*, 1996):

$$\log k = \log k_w - S \varphi$$

$$\log k = A + B / T$$

where k_w is the (extrapolated) value of k for $\varphi = 0$ (water as mobile phase) and S is a constant for each analyte. A is a function of the phase ratio and entropy of retention, ΔS , and B is proportional to the enthalpy of retention, ΔH . Values of ΔH are typically negative, so that retention decreases with increasing temperature (Hancock *et al.*, 1994) as observed for the phenolic standards (Figure 4b).

The 15 rooibos phenolics represent an “irregular” sample, for which changes in solvent strength and temperature are associated with intersections of the individual curves and therefore retention order reversals (Zhu *et al.*, 1996). Note specifically the behaviour of the non-flavonoid compounds PPAG (1) and ferulic acid (5) and the flavones vitexin (6) isovitexin (9) and chrysoeriol (15), for which the decrease in retention as a function of increasing temperature especially was less pronounced compared to the other compounds. The behaviour of the acidic compounds (1 and 5) may be explained by the effect of temperature on $\text{p}K_a$ (increasing temperature leads to higher $\text{p}K_a$'s, and therefore lower ionisation) (Dolan, 2002), although the reason for the unique behaviour of the flavones is unclear.

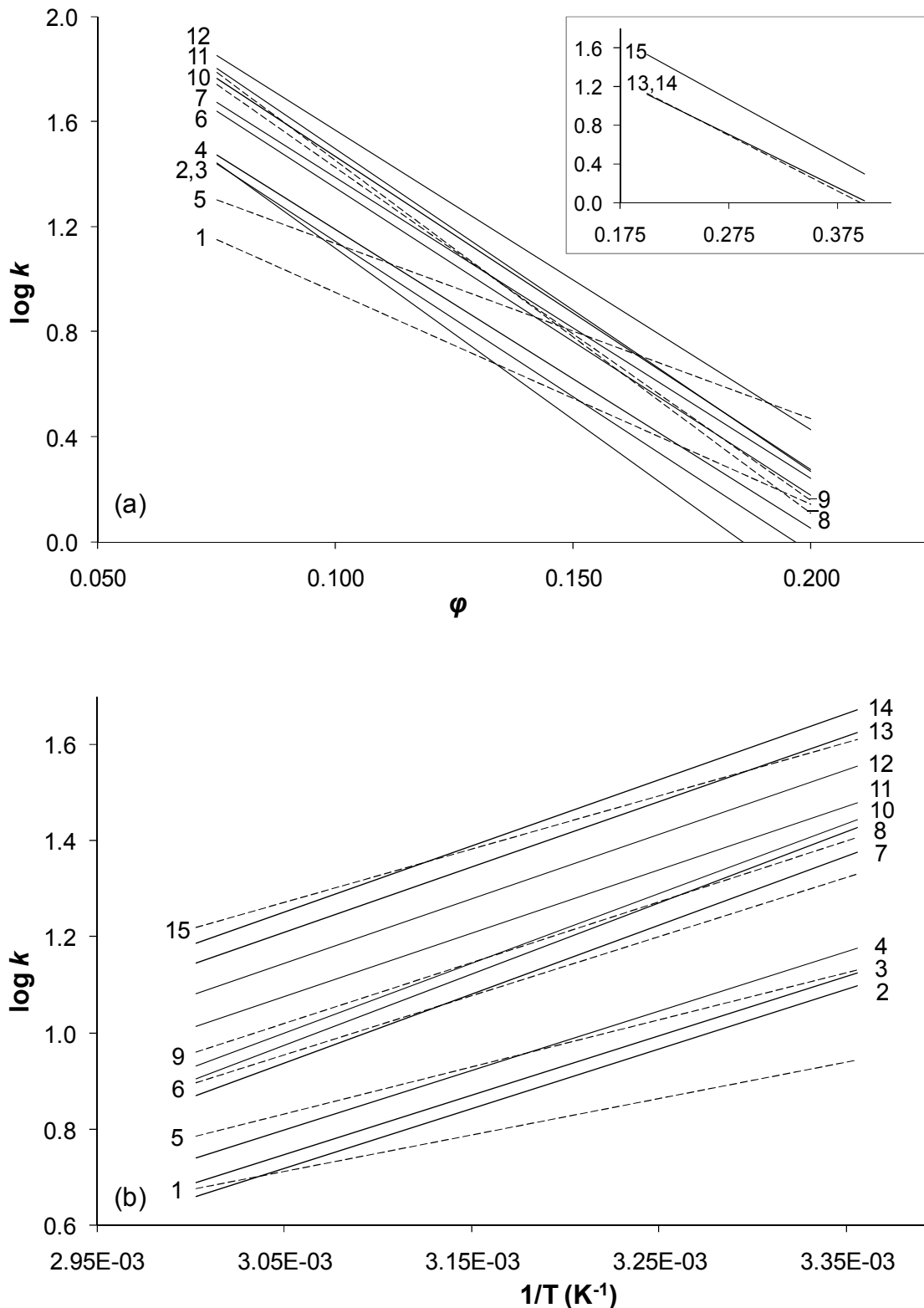


Figure 4 (a) Plots of $\log k$ vs. volume fraction of organic modifier (ϕ) and (b) plots of $\log k$ vs. reciprocal temperature (K^{-1}) for rooibos phenolics on the 1.8 μm Agilent Zorbax SB-C18 (100 x 4.6 mm) column. Data labels correspond to Table 1. The mobile phases consisted of 2% acetic acid (A) and acetonitrile (B). Note that for the more retained aglycones quercetin (13), luteolin (14) and chrysoeriol (15), the volume fraction of organic modifier was increased to 0.20–0.40 to obtain equivalent retention ($2.0 < \log k < 0.0$) in (a).

These data indicate that complete separation of all the compounds cannot be obtained under isocratic elution conditions. Selectivity effects as a function of changes in mobile phase composition and temperature in RP-LC are often complementary and therefore simultaneous optimisation of these two variables was used to improve resolution (Dolan, 2002; Jandera, 2006).

For this purpose, gradient runs were performed at two temperatures (25 and 60°C) and two gradient times (5-50% B in 20 and 70 minutes). These data were used as input for predictions based on computer simulations using DryLab software (Dolan *et al.*, 1990). DryLab is based on the Linear Solvent Strength (LSS) theory and uses two gradient runs where only gradient time (t_G) is varied to calculate values of S and k_w for each analyte. Although the prediction of retention times was sufficiently accurate, none of the simulated separations could provide baseline separation of the target phenolic compounds within acceptable pressures (results not shown). Important conclusions could however be drawn from the simulations to assist further experimental fine-tuning of the temperature and gradient.

Lower temperatures (23°C) provided the best separation of the critical compounds 6-10, with the apigenin-glycosides (vitexin and isovitexin) eluting before the quercetin-glycosides (hyperoside, rutin and isoquercitrin) in the elution order vitexin < isovitexin < hyperoside < rutin < isoquercitrin. However, at this temperature, pressure was excessive and perfect co-elution of quercetin and luteolin occurred, which could not be improved by manipulation of the gradient profile. As the temperature was systematically increased (gradient unchanged) the apigenin-glycosides were shifted towards higher retention times in comparison with the quercetin-glycosides, leading to the positioning of hyperoside and rutin between vitexin and isovitexin, while isoquercitrin eluted after isovitexin. This is in accordance with the isocratic retention data presented for these compounds as a function of temperature (Figure 4b). The elution order at 37°C was: vitexin < hyperoside < rutin < isovitexin < isoquercitrin. By further increasing the temperature, the apigenin-glycosides were more retained than the quercetin-glycosides, leading to perfect co-elution of vitexin + hyperoside and isovitexin + isoquercitrin. Hence the optimal column temperature was established at an intermediate temperature of 37°C, where baseline separation of the two aglycones quercetin and luteolin could also be achieved.

An increase in gradient steepness (% B) was found to have a similar effect on the separation selectivity of compounds 6-10 as observed for an increase in temperature. Trial-and-error adjustments yielded an optimal slope of 0.28% B.min⁻¹ following an isocratic hold period of 2 min at 10% B in the beginning to provide separation of compounds 1-12 within 19 min. The gradient steepness was subsequently increased to elute compounds 13-15 in an acceptable time, followed by a rinsing step 100% B for 3 min (essential for the analysis of rooibos infusions). The optimised gradient provided minimum resolution of 1.25 and 0.92 for critical pairs 7/8 and 8/9, respectively. A UV chromatogram (288 nm) for the optimised RP-LC separation of the phenolic standards is indicated in Figure 5.

Preliminary validation of the optimised HPLC-DAD method was subsequently performed to demonstrate suitability for accurate quantitative analysis. Linearity of the calibration curves for standards over the studied concentration ranges was excellent, with correlation coefficients larger than 0.99997 and y-intercept values close to zero (± 1.9 mAU). The stability of all phenolic compounds over a 27 hour period ($n = 8$) was very good, with percentage relative standard deviation (% RSD) < 2%. Intraday ($n = 6$) and interday ($n = 3$) values were highly reproducible for all phenolic standards (% RSD < 5%).

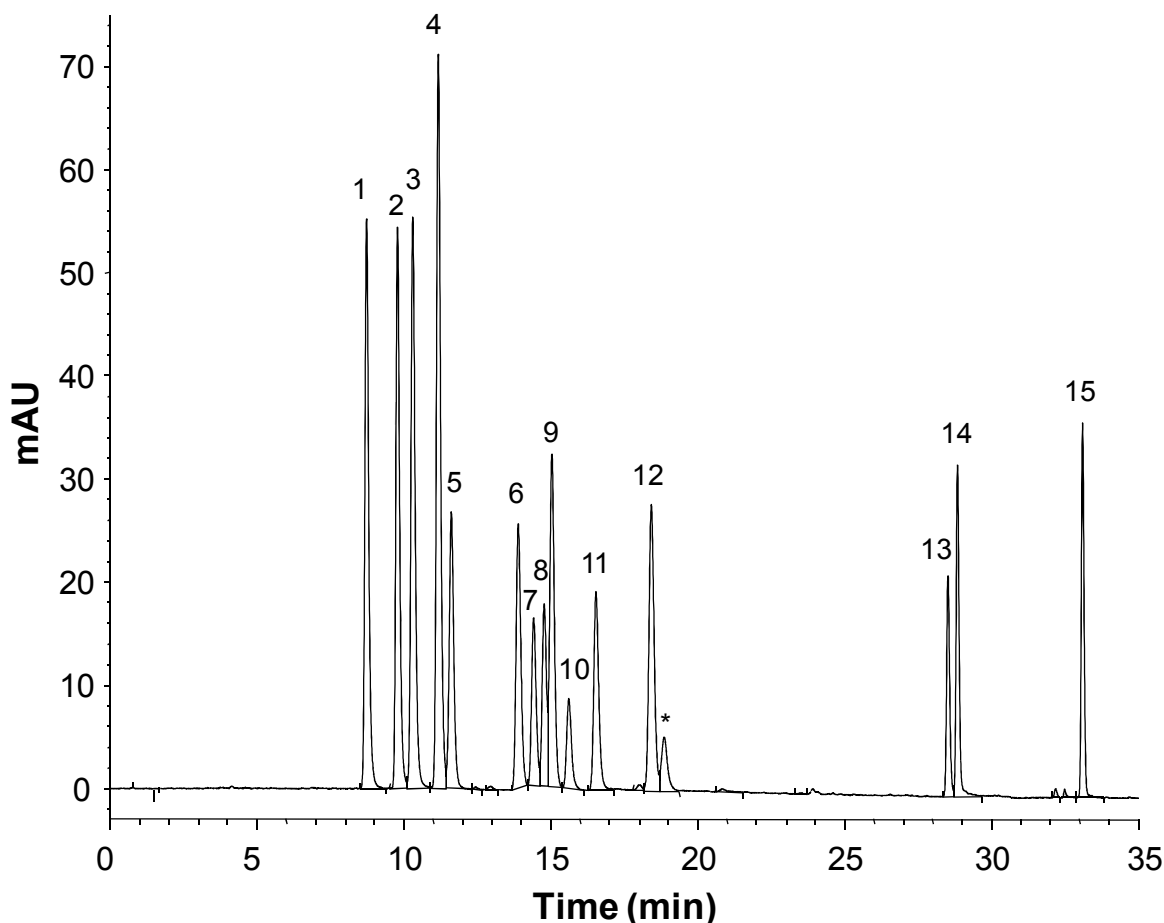


Figure 5 UV chromatogram (288 nm) for the optimised RP-LC-UV separation of the phenolic standards on the 1.8 μm Agilent Zorbax SB-C18 (100 x 4.6 mm) column at 37°C. Peak labels correspond to Table 1. * denotes a contaminant. For further experimental details, refer to section 2.4.3.

3.3 Quantitative analysis of phenolic compounds in aqueous rooibos infusions

Although numerous HPLC methods suitable for the quantification of selected rooibos phenolics have been reported, the developed method is, to our knowledge, the first method suitable for the simultaneous quantification of these 15 phenolic compounds on conventional instrumentation. In our group, for example, two diverse methods (section 2.4.5) were previously employed for the quantification of most of the compounds (excluding isoquercitrin and ferulic acid, which could not be quantified due to co-elution). The first method was adapted from the HPLC method published by Joubert (1996), initially developed for the quantification of the dihydrochalcones aspalathin and nothofagin in rooibos tea. This method (Joubert, 1996) was transferred to a 5 μm Gemini column (150 x 4.6 mm) and the solvent program adjusted to improve resolution on an Agilent 1200 HPLC system, leading to a run time of 90 min. The 2% formic acid aqueous solvent was also replaced with 2% acetic acid to simplify switching between methods. The adapted method was subsequently used for the quantification of vitexin, isovitexin, luteolin-7-O-glucoside, hyperoside, quercetin, luteolin and chrysoeriol. The second method employed in our group was a HPLC method of 23 min developed by Joubert *et al.* (2009) for the rapid separation and quantification of aspalathin and its corresponding flavones, isoorientin and orientin, in fermented rooibos iced tea. This method was also deemed suitable for the quantification of PPAG, nothofagin and rutin.

The optimised HPLC-DAD method described in this paper was subsequently applied to the analysis of aqueous infusions of unfermented ($n = 10$) and fermented ($n = 10$) rooibos plant material. Quantitative data on the fermented samples were compared to those obtained for the identical samples using HPLC-DAD methods previously employed in our group. The mean content values of the major phenolic compounds, expressed as g per 100 g soluble solids, together with the standard deviations are summarised in Table 3.

Table 3 Mean content values (g.100 g SS⁻¹ ± standard deviation) of the major phenolic compounds present in aqueous infusions of unfermented and fermented rooibos plant material.

Phenolic compound	Unfermented	Unfermented	Fermented	Fermented
	rooibos, P ₁ ($n = 5$) ^a	rooibos, P ₂ ($n = 5$) ^a	rooibos ($n = 10$) ^a	rooibos, ($n = 10$) ^{a,b}
PPAG	0.25 ± 0.051	0.44 ± 0.056	0.53 ± 0.10	0.60 ± 0.15
isoorientin	0.72 ± 0.12	1.3 ± 0.16	1.3 ± 0.084	1.2 ± 0.16
orientin	0.48 ± 0.080	0.81 ± 0.080	0.92 ± 0.053	0.92 ± 0.13
aspalathin	8.4 ± 1.4	12.4 ± 1.0	0.64 ± 0.17	0.68 ± 0.19
ferulic acid	nd ^d	nd ^d	0.088 ± 0.040	nq ^e
quercetin-3- <i>O</i> -robinobioside ^c	0.43 ± 0.068	0.91 ± 0.12	0.94 ± 0.16	nd ^d
vitexin	0.093 ± 0.015	0.18 ± 0.017	0.19 ± 0.016	0.18 ± 0.013
hyperoside	0.046 ± 0.0079	0.18 ± 0.046	0.26 ± 0.076	0.26 ± 0.073
rutin	0.30 ± 0.047	0.42 ± 0.018	0.23 ± 0.14	1.2 ± 0.30
isovitexin	0.13 ± 0.021	0.23 ± 0.029	0.20 ± 0.017	0.21 ± 0.014
isoquercitrin	0.090 ± 0.015	0.24 ± 0.038	0.18 ± 0.11	nq ^e
nothofagin	0.69 ± 0.12	1.7 ± 0.16	0.10 ± 0.026	0.071 ± 0.013

^a Mean content values (g.100 g SS⁻¹). P₁ and P₂ refer to producers 1 and 2, respectively.

^b Data for the same fermented samples, obtained using previous comparative HPLC methods.

^c Quantified as rutin equivalents.

^d nd – not detected.

^e nq – not quantified due to coelution.

The C-glycosyl dihydrochalcones aspalathin and nothofagin are, respectively, the most and second most abundant phenolic compounds present in unfermented rooibos infusions (Table 3). Despite its considerable decrease during fermentation, aspalathin remains one of the major phenolic constituents of fermented rooibos, whilst nothofagin is degraded to extremely low concentrations. These results are in agreement with previous reports (Joubert, 1996) and indicate that C-glycosyl dihydrochalcones are not stable during the fermentation process. Conversely, the proportions of the other C-glycosyl flavonoids in unfermented rooibos were similar to those in fermented rooibos. It is important to emphasise that the fermented and unfermented rooibos did not originate from the same plant material and therefore differences in the content values could be attributed to factors other than the fermentation process, such as natural variation. Furthermore, even amongst the unfermented samples substantial variation was observed in the content values. The soluble solids of unfermented rooibos from producer 2 (P₂) contained almost twice the amount of phenolic compounds compared to the unfermented rooibos supplied by producer 1 (P₁). This was attributed to a more effective vacuum-drying process employed by producer 2, although natural variation could once again also be responsible for this observation.

Other major phenolic constituents of rooibos infusions include PPAG, isoorientin, orientin and a compound tentatively identified as quercetin-3-O-robinobioside (refer to LC-ESI-MS results in section 3.4). The presence of PPAG, an enolic β -D-glucopyranoside of phenylpyruvic acid, in *Aspalathus linearis* plant material was first reported by Marais and co-workers in 1996 and identified using nuclear magnetic resonance (NMR) (Marais *et al.*, 1996). Luteolin-7-O-glucoside and the aglycones quercetin, luteolin and chrysoeriol were only present in trace amounts in both unfermented and fermented samples and could not be accurately quantified. Ferulic acid (not detected in unfermented rooibos) was present at low concentrations (0.088 g.100 g SS⁻¹) in fermented rooibos. Slight over-estimation of ferulic acid in some of the fermented samples was due to partial co-elution with an unknown compound (refer to LC-ESI-MS results in section 3.4), present in low quantities.

The content values obtained using the optimised method are in good agreement with those obtained previously and, on average, only differs with $ca \pm 0.039$ g compound.100 g SS⁻¹. The exception is rutin, for which the calculated content value is less than 20% of that obtained using the previous methods. This is due to perfect co-elution of rutin and the other quercetin-O-diglycoside, quercetin-3-O-robinobioside, in the previous method. These compounds exhibit identical UV and MS/MS spectra (Table 4), making it impossible to distinguish between them. This hypothesis is further supported by the combined content value of quercetin-3-O-robinobioside and rutin (1.2 g.100 g SS⁻¹), which is equal to the content value of rutin (1.2 g.100 g SS⁻¹) obtained using the previous method (Table 3). The high content values reported for rutin in other studies could also be overestimated for the same reason (Bramati *et al.*, 2002, 2003). Stalmach *et al.* (2009) and Kazuno *et al.* (2005) have, however, discriminated between rutin and a rutin isomer and have reported individual content values for both compounds. It is assumed that the rutin isomer quantified in these reports (Kazuno *et al.*, 2005; Stalmach *et al.*, 2009) is in fact quercetin-3-O-robinobioside, which was identified in the current study by LC-MS and LC-MS/MS (see further). Comparison of previously reported content values to those obtained in the current study is difficult due to different sample preparation techniques. However, the higher relative concentration of the isomer compared to rutin (Kazuno *et al.*, 2005; Stalmach *et al.*, 2009), is in agreement with our results for quercetin-3-O-robinobioside. Similar difficulties in the separation and quantification of the isomeric pair isoquercitrin and hyperoside have also been reported (Bramati *et al.*, 2002, 2003).

The developed HPLC-DAD method therefore demonstrated its suitability for the quantitative analysis of the principal phenolic constituents of rooibos. It is characterised by better resolution, higher sensitivity and shorter analysis times than previously reported methods and is therefore tailored for routine application. The method furthermore allowed us to report content values for PPAG, ferulic acid and quercetin-3-O-robinobioside in rooibos tea for the first time.

3.4 LC-ESI-MS and tandem MS analyses of aqueous rooibos infusions

LC-ESI-MS was used to confirm peak purity of the 15 standard phenolic compounds in aqueous rooibos infusions. In both unfermented and fermented rooibos samples, peak purity of the standards was established by comparison of MS spectra with reference compounds. The TOF-MS base peak chromatogram of a fermented rooibos infusion is depicted in Figure 6. Note that LC-ESI-MS analyses were conducted on the Acquity UPLC system and in order to obtain equivalent separation (Dolan, 2002) compared to the 1200 instrument the temperature was increased by 1°C and the solvent preheated using an Acquity Column Stabilizer.

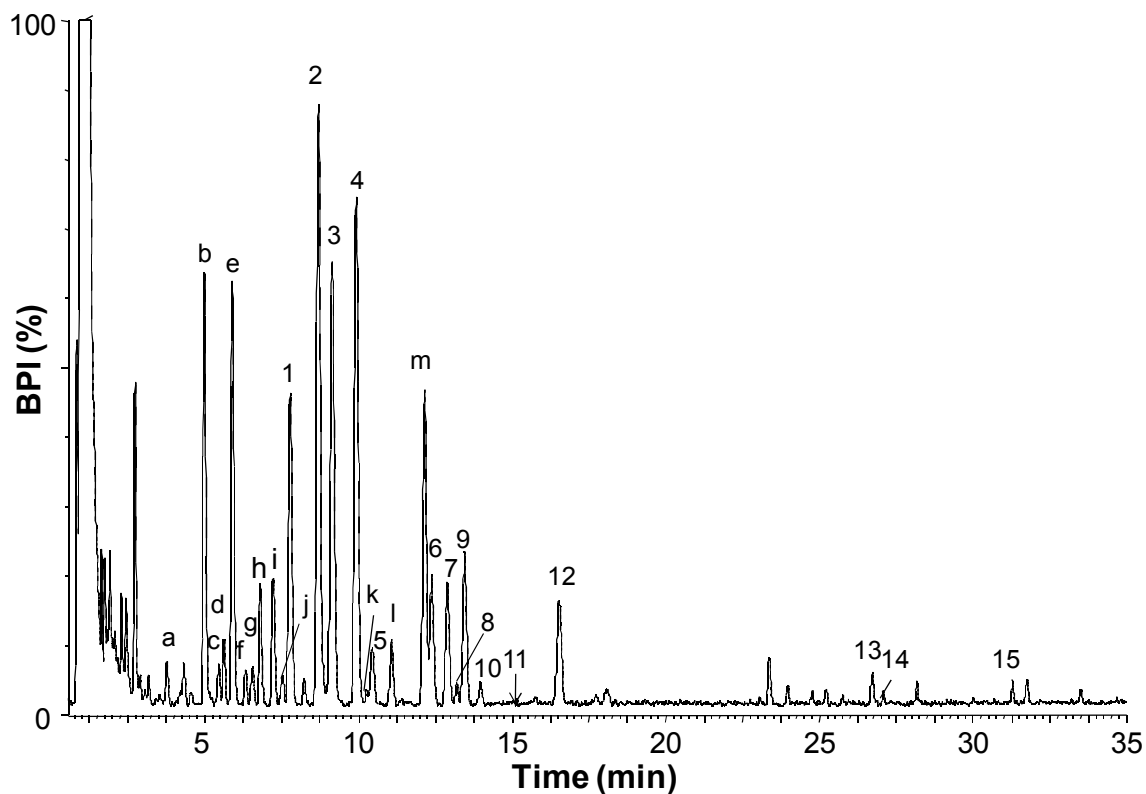


Figure 6 Base peak chromatogram (acquired in negative ionisation mode) of a fermented rooibos infusion. Peak labels correspond to Table 4.

Identification of standard phenolic compounds (1-15) was based on comparison of t_R , UV-VIS, MS and MS/MS data with those of authentic standards. The non-standard phenolic compounds (a-m) were tentatively identified by interpretation of UV and mass spectra in comparison with relevant literature reports. UV-VIS spectrometry delimited the class of phenolic compounds (Abad-García *et al.*, 2009), while accurate mass measurement and fragmentation patterns obtained by MS/MS were used to confirm the proposed structures. A summary of the phenolic compounds identified in aqueous infusions of fermented and unfermented rooibos (*Aspalathus linearis*) by LC-ESI-MS and LC-ESI-MS/MS is presented in Table 4.

Table 4 Phenolic compounds identified in aqueous infusions of fermented and unfermented rooibos (*Aspalathus linearis*) by LC-ESI-MS and LC-ESI-MS/MS.

<i>t_R</i> , min ^a	Peak	Negative			Positive			Phenolic compound	
		Accurate mass		Accurate mass	Proposed molecular formula		Error (ppm)		
		exp	[M-H] ⁻	exp	[M+H] ⁺	calc			
3.77	a	609.0861	611.1605	611.1612	C ₂₇ H ₃₁ O ₁₆	-1.1	425, 395*, 365, 353, 341*	230, 276, 340	luteolin 6,8-di-C-hexoside
4.99	b	449.0543	451.1236	451.124	C ₂₁ H ₂₃ O ₁₁	-0.9	231, 219, 195*, 165	234, 287	(S)-eriodictyol-6-C-β-D-glucopyranoside
5.45	c	579.0714	581.149	581.1506	C ₂₆ H ₂₉ O ₁₅	-2.8	425*, 407*, 395*, 365, 353, 341	231, 273, 340	carlinoside, isocarlinoside, neocarlinoside or isocarlinoside isomer
5.64	d	593.0682	595.165	595.1663	C ₂₇ H ₃₁ O ₁₅	-2.2	457, 427, 409, 379*, 355, 337, 325*	231, 273, 331	apigenin 6,8-di-C-hexoside (vicenin-2)
5.89	e	449.045	451.1239	451.124	C ₂₁ H ₂₃ O ₁₁	-0.2	231, 219, 195*, 165	233, 288	(R)-eriodictyol-6-C-β-D-glucopyranoside
6.33	f	579.0608	581.1497	581.1506	C ₂₆ H ₂₉ O ₁₅	-1.5	425, 407, 395*, 365, 353, 341	231, 273, 340	carlinoside, isocarlinoside, neocarlinoside or isocarlinoside isomer
6.55	g	579.0819	581.1509	581.1506	C ₂₆ H ₂₉ O ₁₅	0.5	425*, 407*, 395*, 365, 353, 341	233, 273, 340	carlinoside, isocarlinoside, neocarlinoside or isocarlinoside isomer
6.81	h	449.0357	451.1238	451.124	C ₂₁ H ₂₃ O ₁₁	-0.4	231, 219, 195*, 165	233, 289	(S)-eriodictyol-8-C-β-D-glucopyranoside
7.23	i	449.045	451.1242	451.124	C ₂₁ H ₂₃ O ₁₁	0.4	231, 219, 195*, 165	232, 289	(R)-eriodictyol-8-C-β-D-glucopyranoside
7.55	j	579.0714	581.1497	581.1506	C ₂₆ H ₂₉ O ₁₅	-1.5	425, 407, 395*, 365, 353, 341	235, 276, 331	carlinoside, isocarlinoside, neocarlinoside or isocarlinoside isomer
7.77	1	325.0378	327.1075	327.108	C ₁₅ H ₁₉ O ₈	-1.5	119*, 91	237, 280	phenylpyruvic acid glucoside (PPAG)
8.7	2	447.0376	449.1088	449.1084	C ₂₁ H ₂₁ O ₁₁	0.9	329, 299*	233, 270, 349	luteolin-6-C-glucoside (isoorientin)
9.15	3	447.0284	449.1083	449.1084	C ₂₁ H ₂₁ O ₁₁	-0.2	329*, 299*	236, 268, 348	luteolin-8-C-glucoside (orientin)
9.92	4	451.0661	453.1401	453.1397	C ₂₁ H ₂₅ O ₁₁	0.9	247, 235, 205, 193, 165, 137, 123*	235, 288	aspalathin
10.28	k	613.105	615.1929	615.1925	C ₂₇ H ₃₅ O ₁₆	0.7	435, 411, 369, 247, 235, 205, 193, 165*, 137, 123	235, 288	8-C-hexoside derivative of aspalathin
10.45	5	193.0039	195.064	195.0657	C ₁₀ H ₁₁ O ₄	-8.7		239, 323	ferulic acid
11.08	l	449.045	451.1234	451.124	C ₂₁ H ₂₃ O ₁₁	-1.3	379, 367, 325, 313, 301*, 285, 271, 163	240, 287	aspalathin ^b

t_R , min ^a	Peak	Negative			Positive						Phenolic compound
		Accurate mass		exp [M+H] ⁺	Accurate mass		Proposed molecular formula	Error (ppm)	MS/MS fragment ions	λ_{max} , nm	
		exp	[M-H] ⁻		calc						
12.16	m	609.0969		611.1608	611.1612	C ₂₇ H ₃₁ O ₁₆	-0.7	303*	256, 355	quercetin-3-O-robinobioside	
12.37	6	431.0317		433.1132	433.1135	C ₂₁ H ₂₁ O ₁₀	-0.7	313*, 283*	240, 269, 340	apigenin-8-C-glucoside (vitexin)	
12.89	7	463.0437		465.1024	465.1033	C ₂₁ H ₂₁ O ₁₂	-1.9	303*	256, 355	quercetin-3-O-galactoside (hyperoside)	
13.21	8	609.9438		611.1613	611.1612	C ₂₇ H ₃₁ O ₁₆	0.2	303*	256, 355	quercetin-3-O-rutinoside (rutin)	
13.47	9	431.0317		433.1135	433.1135	C ₂₁ H ₂₁ O ₁₀	0	313, 283*	241, 271, 340	apigenin-6-C-glucoside (isovitexin)	
13.96	10	463.0155		465.1036	465.1033	C ₂₁ H ₂₁ O ₁₂	0.6	303*	256, 355	quercetin-3-O-glucoside (isoquercitrin)	
14.81	11	447.0468		449.1078	449.1084	C ₂₁ H ₂₁ O ₁₁	-1.3	287*	255, 348	luteolin-7-O-glucoside	
16.52	12	435.0605		437.1459	437.1448	C ₂₁ H ₂₅ O ₁₀	2.5	107*	238, 287	nothofagin	
26.73	13	300.9807		303.0509	303.0505	C ₁₅ H ₁₁ O ₇	1.3		255, 371	quercetin ^b	
27.06	14	284.989		287.0549	287.0556	C ₁₅ H ₁₁ O ₆	-0.7		253, 349	luteolin ^b	
31.28	15	299.0051		301.0719	301.0712	C ₁₆ H ₁₃ O ₆	2.3		250, 348	chrysoeriol ^b	

^a t_R – retention time of the phenolic constituent in a fermented rooibos infusion.

^b only detected in fermented samples.

* base peak(s) in the MS/MS spectrum.

The nomenclature used here to describe the fragmentation is in accordance with (Kazuno *et al.*, 2005; Abad-García *et al.*, 2008, 2009), represented in Figure 7 for a dihydrochalcone-C-6-hexoside. ${}^{c,d}X^{e+}$ indicates the aglycone ion, where fragmentation on the carbohydrate ring is specified by the superscripts c and d. The position of glycosylation on the flavonoid A-ring (*i.e.* C-6 or C-8) is specified by the superscript e, and is similar for both positions (indicated only for the C-6 configuration in Figure 7). For flavonoid-pentosides, additional characteristic losses of 60 m/z are common. Note that for high collision energies such as used here, additional loss of one or more water molecules were observed. For the specific case of dihydrochalcones, A^+ and B^+ denote charged fragments containing the A- and B-rings, respectively.

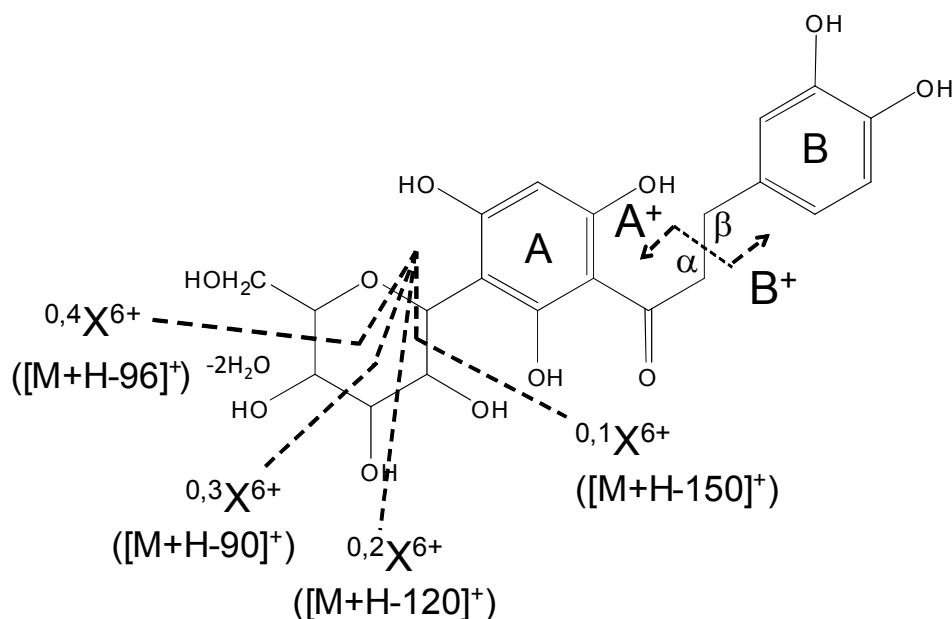


Figure 7 General MS fragmentation pattern of flavonoid C-glycosides illustrated for the dihydrochalcone-C-6-glucoside aspalathin.

Flavone di-C-glycosides. Peaks c, f, g and j with m/z 581 ($[M+H]^+$) were detected at retention times of 5.45, 6.33, 6.55 and 7.55 min, respectively, and had the same proposed molecular formula ($C_{26}H_{29}O_{15}$). These compounds presented a major fragment ion at m/z 395 corresponding to $[M+H-150-2H_2O]^+$. Compounds c and g additionally presented major fragment ions (intensities > 90%) at m/z 425 and m/z 407, corresponding to losses of $[M+H-120-2H_2O]^+$ and $[M+H-120-3H_2O]^+$, respectively. These fragments were also present in the MS/MS spectra of f and j, albeit at lower intensities (*ca* 30%). These compounds exhibited identical UV spectra with maximum absorbance at *ca* 230, 270 and 340 nm.

Based on similar results reported by Iswaldi and co-workers (2011), three of these peaks were tentatively assigned as carlinoside (Li *et al.*, 2006), neocarlinoside (Xie *et al.*, 2003) and isocarlinoside (Gallo *et al.*, 2006). Carlinoside is a 6,8-di-C-glycoside of luteolin, with glucopyranosyl and arabinopyranosyl groups attached at the C-6 and C-8 positions, respectively. Neocarlinoside differs from carlinoside with regards to the arabinose configuration, whilst isocarlinoside differs from carlinoside in that the hexoside and pentoside moieties are attached at the C-8 and C-6 positions, respectively. The presence of luteolin-6-C-hexoside-8-C-pentoside (*i.e.* either carlinoside or neocarlinoside) in rooibos was recently proposed by Breiter *et al.* (2011). Iswaldi and co-workers (2011) tentatively identified a fourth peak with the same molecular ion as 2''-O- β -arabinopyranosyl-orientin (Li *et al.*, 2006). However, the four compounds c, f, g and j displayed similar

MS/MS spectra which are consistent with asymmetric 6,8-di-C-glucosides of luteolin. We therefore propose that the fourth peak is more likely an isomer of the luteolin-6-C-pentoside-8-C-hexoside (isocarlinoside), differing only with regard to the conformation of one of the sugar moieties.

Due to the absence of standards, it was impossible to distinguish between these isomers and allocate absolute peak identity. However, Abad-García *et al.* (2008) showed that the relative ratio of the product ions $[M+H-120]^+$ and $[M+H-150]^+$ can be used to differentiate between flavone C-6- and C-8-mono-C-glucosides. For C-6-hexosides, $([M+H-150]^+ / [M+H-120]^+)$ yields a ratio of ~ 2 , whilst for C-8-hexosides, this value is ~ 1 . Extrapolation of this principle to flavone di-C-glucosides suggests that the hexoside is in the C-6 position for compounds f and j (ratio ~ 2 ; luteolin-6-C-hexoside-8-C-pentoside, carlinoside and neocarlinoside). Accordingly, it is proposed that the hexoside is in the C-8 position for compounds c and g (ratio ~ 1 ; luteolin-6-C-pentoside-8-C-hexoside, isocarlinoside and an isomer).

Peak a ($t_R = 3.77$ min) exhibited pseudomolecular ions at m/z 609 ($[M-H]^-$) and m/z 611 ($[M+H]^+$) and maximum UV absorbance at $\lambda = 230, 276$ nm. Fragment ions of the protonated molecule were detected at m/z 395 ($[M+H-2 \times 90-2H_2O]^+$) and 341 ($[M+H-150-120]^+$). This fragmentation pattern, corresponding to losses of 90, 120 and 150 amu from the C-glycoside, was almost identical to those of c, f, g and j and compound a was therefore tentatively identified as another luteolin 6,8-di-C-glycoside. Due to the mass difference of 30 amu, it is proposed that peak a is a symmetric luteolin-di-C-hexoside. The presence of luteolin-6,8-di-C-hexoside in rooibos has been proposed by Breiter *et al.* (2011).

Peak d ($t_R = 5.64$ min) with m/z 593 ($[M-H]^-$) and 595 ($[M+H]^+$) and proposed molecular formula $C_{27}H_{31}O_{15}$ ($[M+H]^+$) was tentatively identified as the anti-inflammatory compound apigenin 6,8-di-C-glucoside, also known as vicenin-2. This compound showed a UV spectrum of a flavone derivative (λ_{max} at 273 and 331 nm), which is in line with λ_{max} values reported in (Xie *et al.*, 2003; Iswaldi *et al.*, 2011). Breiter *et al.* (2011) and Iswaldi *et al.* (2011) proposed the presence of this compound in rooibos, based on its occurrence in species such as *Viola vedoensis* MAKINO (Xie *et al.*, 2003), *Citrus aurantifolia* (Piccinelli *et al.*, 2008) and *Lychnophora ericoides* Mart. (De Moraes *et al.*, 2007). The ESI-MS/MS spectrum of the protonated vicenin-2 molecule (m/z 595, $[M+H]^+$) showed the most abundant fragments at m/z 379 and 325, with further fragments detected at m/z 457, 427, 409 and 355 in accordance with (De Moraes *et al.*, 2007).

Flavanones. Peaks b, e, h and i, with m/z 451 ($[M+H]^+$) were detected at retention times of 4.99, 5.89, 6.81 and 7.23 min, respectively, and had the same proposed molecular formula ($C_{21}H_{23}O_{11}$). The ESI-MS/MS spectra of the protonated compounds yielded major product ions at m/z 195 and minor ions at m/z 231, 219 and 165. These compounds exhibited identical UV response with maximum absorbance at ca 233 and 288 nm. In accordance with literature, peaks b and e, and h and i were assigned as (S)- and (R)-eriodictyol-6-C- β -D-glucopyranoside, and (S)- and (R)-eriodictyol-8-C- β -D-glucopyranoside, respectively (Bramati *et al.*, 2002; Shimamura *et al.*, 2006; Philbin & Swartz, 2007; Krafczyk & Glomb, 2008).

Koeppen & Roux (1965) postulated that these flavanone 6-C and 8-C compounds are formed as intermediates during the oxidative conversion of aspalathin to the flavones, isoorientin and orientin. The presence of the 6-C-glycosides in fermented rooibos was unequivocally confirmed by Marais *et al.* (2000), whilst NMR data for the 8-C-glycosides were presented by Krafczyk & Glomb in 2008 (Krafczyk & Glomb, 2008). Eriodictyol-6-C- β -D-glucopyranoside forms more readily than eriodictyol-8-C- β -D-glucopyranoside, which results in a ratio of $\sim 2:1$ for these compounds (Krafczyk & Glomb, 2008), as can be visualised by the relative ratios of b and e compared to h and i in Figure 6.

Dihydrochalcones. Compound k, eluting at 10.28 min, revealed molecular ions at m/z 613 ($[M-H]^-$) and m/z 615 ($[M+H]^+$). The experimental accurate mass (615.1929, $[M+H]^+$) was in good accordance with the accurate mass calculated for the molecular formula $C_{27}H_{35}O_{16}$. Furthermore, UV and MS/MS spectra for compound k showed similar characteristics to those of aspalathin (m/z 453, $[M+H]^+$) (Figure 8).

Due to the mass difference of 162 amu, compound k was proposed to be a glycosylated derivative of aspalathin. To confirm this hypothesis, analysis of the MS/MS spectra of these molecules was performed using the calculated molecular formulas for each fragment. The mass spectrum of aspalathin shows a base peak ion at m/z 123 ($C_7H_7O_2$), resulting from the cleavage of the bond between α - and β -carbons (B^+ , Figure 7). Furthermore, related B^+ fragments at m/z 165 ($C_9H_9O_3$) and 137 ($C_8H_9O_2$), resulting from cleavage of the bonds on either side of the carbonyl group, were also observed for aspalathin. The same three ions were also present in the MS/MS spectrum of compound k, indicating that the B-ring is the same as for aspalathin, and therefore that the additional hexosyl residue is attached to the A-ring.

The fragment detected at m/z 235 ($[M+H-218]^+$, $C_{12}H_{11}O_5$) for aspalathin results from ($^{0,4}X^+$) fragmentation of the C-glucose and loss of two water molecules. Ions at m/z 193 ($[M+H-260]^+$, $C_{10}H_9O_4$) result from ($^{0,2}X^{6+}$) fragmentation of the C-glucose, followed by the loss of one water molecule. Similarly, m/z 205 ($[M+H-248]^+$, $C_{11}H_9O_4$) results from ($^{0,3}X^{6+}$) fragmentation of the C-glucose, followed by the loss of two water molecules. The ion detected at m/z 247 ($[M+H-206]^+$, $C_{13}H_{11}O_5$) results from loss of the B-ring, followed by $^{0,4}X^{6+}$ fragmentation of the A⁺ C-glucoside and loss of two water molecules. Significantly, fragments with m/z 247, 235, 205 and 193 are all common to aspalathin and compound k, indicating that the latter also contains a C-6-hexoside functionality.

Finally, in the higher MW range, the MS/MS spectrum of compound k shows several ions consistent with a flavonoid C-6,C-8-dihexoside [37]: m/z 525 ($[M+H-90]^+$), m/z 495 ($[M+H-120]^+$), m/z 477 ($[M+H-120-H_2O]^+$), m/z 465 ($[M+H-150]^+$), m/z 447 ($[M+H-150-H_2O]^+$), m/z 435 ($[M+H-2\times 90]^+$), m/z 423 ($[M+H-2\times 96]^+$), m/z 411 ($[M+H-90-96-H_2O]^+$), m/z 399 ($[M+H-96-120]^+$), m/z 381 ($[M+H-96-120-H_2O]^+$), m/z 369 ($[M+H-96-150]^+$), m/z 345 ($[M+H-120-150]^+$) and m/z 327 ($[M+H-120-150-H_2O]^+$). The fragmentation pattern for this compound clearly corresponds with the simultaneous fragmentation of 2 C-hexosyl groups, as opposed to a C-6-dihexosyl (where the inter-sugar bond would preferentially be cleaved), or a O-hexoside (which would not result in cleavage within the second carbohydrate ring but rather loss of 162 amu).

These data lead us to propose that compound k is most likely the C-8-hexosyl derivative of aspalathin. This is to our knowledge the first time that this compound has been reported in literature. As a derivative of aspalathin, a compound unique to rooibos, it would be interesting to isolate this compound and study its chemical properties.

Peak I ($t_R = 11.08$ min) with an accurate mass of m/z 451.1234 ($[M+H]^+$, experimental) and the proposed molecular formula $C_{21}H_{23}O_{11}$ ($[M+H]^+$) was tentatively assigned to aspalalinin. The presence of this cyclic dihydrochalcone possessing an intramolecular ether linkage has been established in rooibos by NMR (Shimamura *et al.*, 2006). The ESI-MS/MS spectrum of the protonated aspalalinin molecule (m/z 451, $[M+H]^+$) showed the most abundant fragment at m/z at 301, which corresponds to ($[M+H-150]^+$). Other fragments were found at m/z 379, 367, 325, 313, 285, 271 and 163. This compound exhibited UV λ_{max} at 240 and 287 nm and was only detected in fermented rooibos.

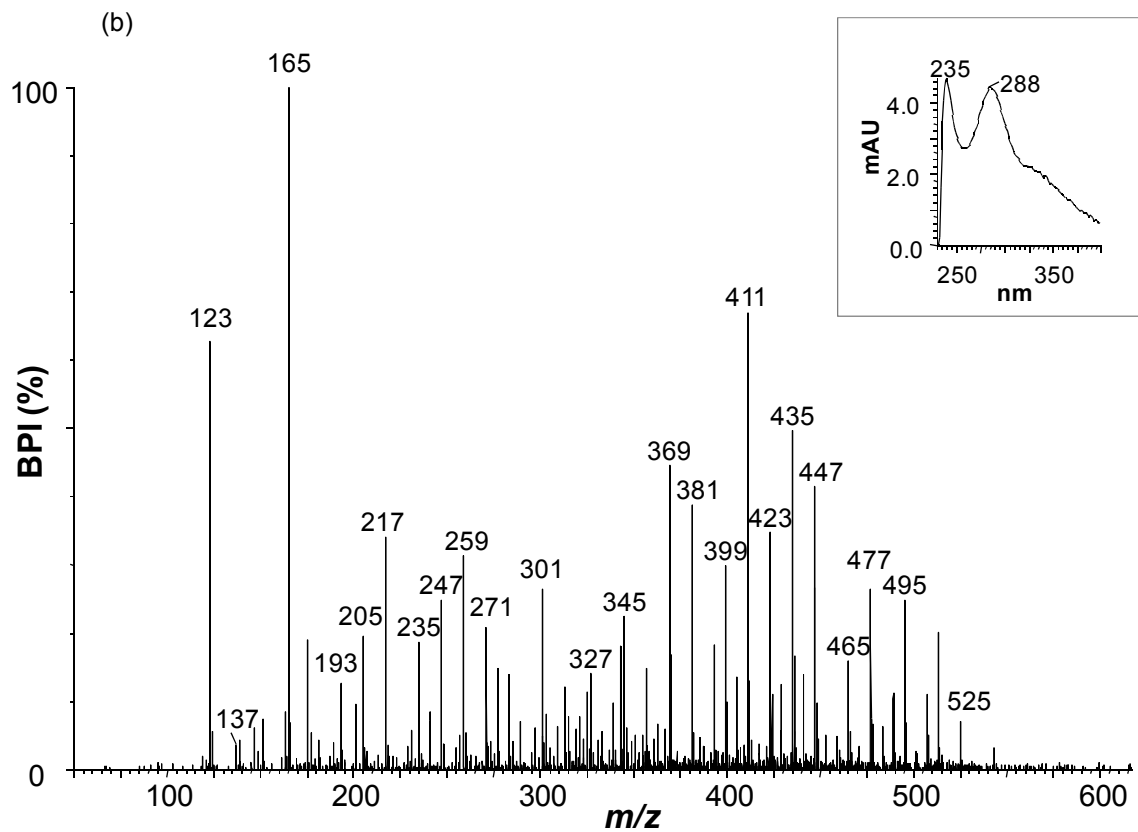
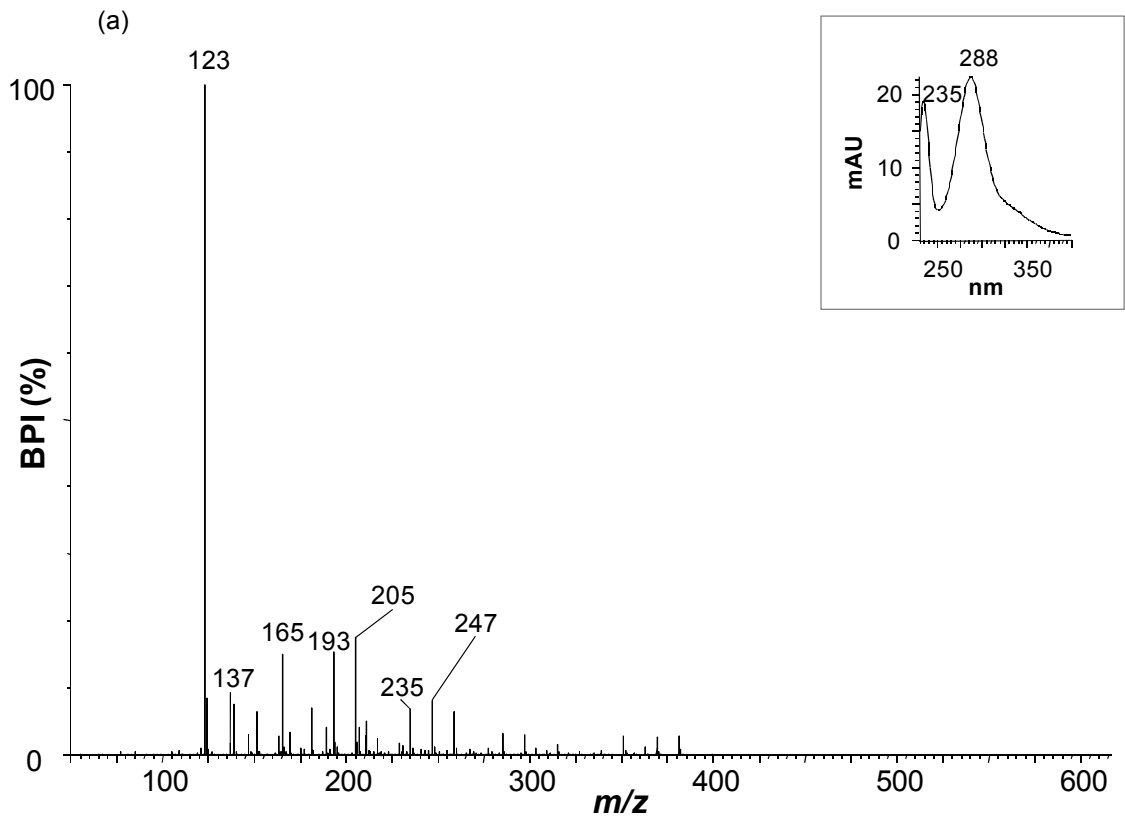


Figure 8 MS/MS and UV spectra (insert) of (a) aspalathin and (b) unknown compound **k**.

Flavonol O-glycosides. In contrast to flavonoid C-glycosides, the flavonol O-glycosides (compounds 7 and 10, Table 4), and flavonol-3-O-dihexoside (compound 8, Table 4) displayed much simpler MS/MS spectra, with the prevalent fragmentation involving cleavage at the glycosidic O-linkage. Rutin, quercetin-3-O-glucoside and quercetin-3-O-galactoside all showed only one fragment at m/z 303, corresponding to the aglycone ion. A similar fragment was observed for compound m ($t_R = 12.16$ min). ESI-MS showed a molecular ion similar to rutin at m/z 611 ($[M+H]^+$), while maximum UV absorbance at 256 and 355 nm was similar to that found for the other flavonol-O-glycosides (Table 4). Peak m was therefore tentatively identified as quercetin-3-O-robinobioside. The presence of this compound in rooibos has been unequivocally established by NMR (Shimamura *et al.*, 2006). This compound was also tentatively identified in rooibos by Iswaldi *et al.* (2011), although the relative retention time and MS/MS spectra did not match our data, which are in agreement with other reports on this compound (Ozga *et al.*, 2007; Lin *et al.*, 2008; Breiter *et al.*, 2011).

Phenylpropanoid. PPAG (peak 1, $t_R = 7.77$ min) produced a protonated molecular ion at m/z 327 ($[M+H]^+$). The ESI-MS/MS spectrum of the protonated compound showed a base peak at m/z 119. This ion is likely formed as a result of cleavage of both the glucoside and carboxylic acid groups at C2. A second ion at m/z 91 was presumably formed by the subsequent loss of formaldehyde resulting from cleavage of the C2-C3 bond.

4. Conclusions

An HPLC-DAD method has been successfully developed for the quantitative determination of the 15 principal phenolic constituents of rooibos on conventional HPLC instrumentation. A systematic approach towards method development was used to optimise resolution on a selected sub-2 μm column to exploit the benefits of this phase for fast analysis. Under the optimised gradient conditions and temperature, complete separation of the 15 principal phenolics could be obtained on a 1.8 μm C18 phase within 37 min. This method was successfully applied to the quantitative analysis of aqueous infusions of fermented and unfermented rooibos. Quantitative data for ferulic acid, PPAG and quercetin-3-O-robinobioside are reported here for the first time.

Coupling of the optimised method to MS and tandem MS confirmed that no co-elution for the 15 target analytes occurred in fermented and unfermented rooibos infusions, and enabled the tentative identification of 13 additional phenolic constituents. These included 4 flavanones, a cyclic dihydrochalcone, and a flavonol-O-diglycoside previously reported in rooibos. The presence of the symmetric 6,8-di-C-glycosyl flavones (luteolin 6,8-di-C-hexoside and apigenin 6,8-di-C-hexoside) and the asymmetric 6,8-di-C-glycosyl flavones (carlinoside, neocarlinoside, isocarlinoside) have been tentatively re-confirmed in this study. Based on MS/MS data, a fourth asymmetric di-C-glycosyl flavone is proposed to be an isomer of isocarlinoside, differing only with regard to the sugar configuration. The presence of this compound in rooibos is reported here for the first time. Moreover, another phenolic compound (MW 614) is tentatively identified in rooibos for the first time, displaying structural characteristics in accordance with a C-8-hexosyl derivative of the dihydrochalcone aspalathin.

Establishment of this HPLC method will enable the comprehensive analysis of a cup of rooibos tea and the subsequent calculation of the contribution of rooibos polyphenols to dietary intake. Further application to a large number of samples would allow inclusion of data in food composition tables to provide

typical fingerprints for product authenticity and quality control purposes. Future applications of the method will include authentication of extracts used in nutraceutical products on the basis of their flavonoid fingerprints; selection of plant material for propagation of genotypes producing high levels of specific phenolic markers; quantification of the effect of seasonal and geographical influences on composition; and correlating pharmacological activities with specific constituents.

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Supplementary Information

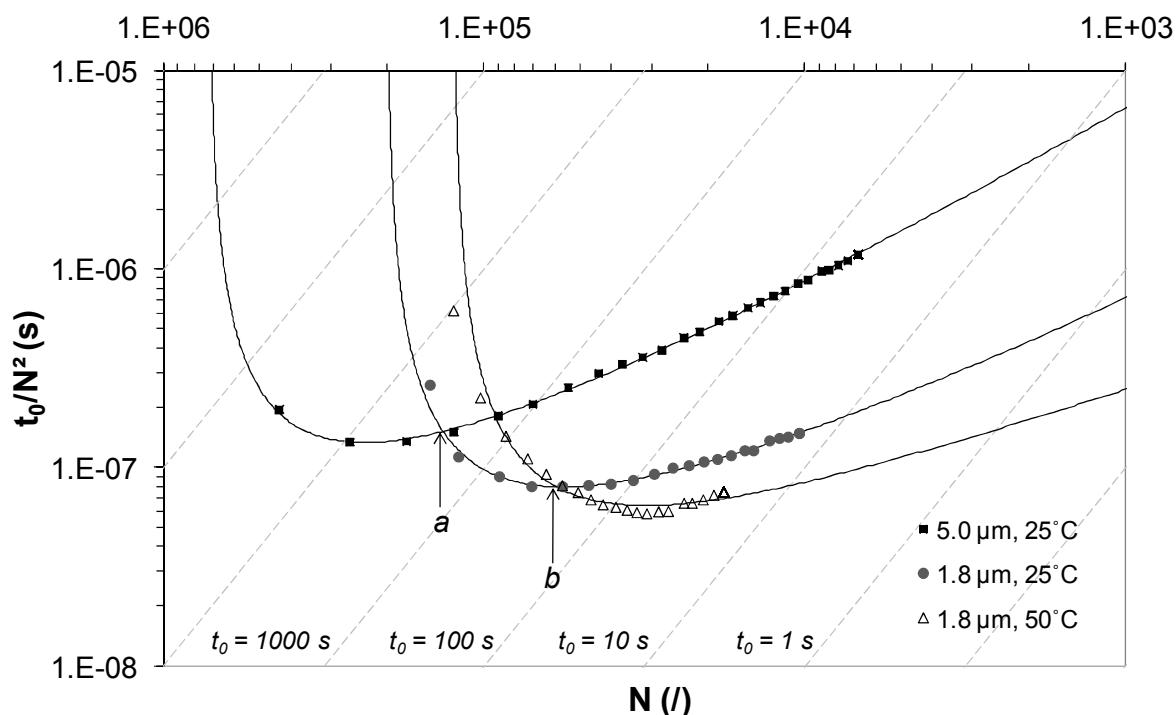


Figure S1 t_0/N^2 vs N plots obtained for aspalathin on a 5.0 μm Phenomenex Gemini C18 column (150 x 4.6 mm) at 25°C, and a 1.8 μm Agilent Zorbax SB-C18 (50 x 4.6 mm) at 25 and 50°C.

From these plots, it is easy to establish the range of efficiencies (N) where a particular experimental setup will outperform the other – this can be visualised by the curve lying lowest on the x-axis. The diagonal lines in this figure correspond to t_0 times, as indicated at the bottom of each line. Figure S1 illustrates that by decreasing the particle size, both the optimal plate count (N_{opt} , corresponding to the curves' respective minima) and the maximum efficiency (N_{max} , determined from the vertical asymptotes) are reduced. N_{opt} is the plate number for which the support achieves its best possible kinetic performance/pressure cost ratio, whereas N_{max} refers to the maximum number of plates attainable for a given support.

It is clear that for efficiencies less than ~140 000 plates (indicated by point *a* in Figure S1), the 1.8 μm phase will always permit faster analysis for a given efficiency, even at a maximum pressure of 400 bar. Considering the effect of temperature for aspalathin on the 1.8 μm phase (Figure S1), it is evident that the curve obtained at higher temperature is shifted towards the right, implying better performance in the range of lower plate counts. Utilizing this, elevated temperature can be used to either improve N for a given analysis time, or to increase analysis speed for efficiencies up to ~65 000 plates (point *b* in Figure S1).

CHAPTER 4

*Quantitative characterisation
of the monomeric phenolic composition
of aqueous infusions of fermented rooibos
(Aspalathus linearis) by HPLC-PDA*

Abstract

Use of the herbal tea, rooibos, made from the indigenous South African fynbos plant, *Aspalathus linearis*, has gained tremendous popularity on the international markets during the past twenty years. This is largely due to the associated health-promoting benefits of rooibos, which are attributed to its phenolic composition, in particular the flavonoids. Insufficient data are, however, available on the dietary exposure to rooibos flavonoids. Furthermore, most of the studies on its health-promoting properties do not provide a chemical fingerprint of the extracts or infusions used. Investigations on the bioactivity of rooibos extracts and infusions in many instances fail to give repeatable results because minor differences in composition, which impact on bioactivity, are not taken into account. Estimating flavonoid and phenolic acid intake is the first step toward documenting the protective effects of these two phenolic groups in rooibos against the risk of lifestyle diseases. Therefore the aim of this study was to generate representative content values for the principal monomeric phenolic compounds present in a 'cup-of-tea' rooibos infusion. An optimised high-performance-liquid chromatographic photodiode-array detection (HPLC-PDA) method was applied to a large number of fermented rooibos samples ($n = 114$), representative of different production seasons and quality grades. Samples were obtained from different geographical areas and different producers to capture as much potential variation in the phenolic composition as possible. Rooibos tea is typically steam-pasteurised to ensure a product of high microbiological quality and therefore the effect of steam-pasteurisation on the phenolic composition of rooibos infusions was investigated. Steam-pasteurisation treatment led to a significant reduction in the mean values ($\text{mg}\cdot\text{L}^{-1}$) of most phenolic compounds in the infusions, partly due to a reduction in the soluble solids content. The major phenolic constituents of steam-pasteurised, fermented rooibos were found to be the flavone C-glycosides isoorientin and orientin. The flavonol O-diglycoside, quercetin-3-O-robinobioside, the phenylpropanoid, phenylpyruvic acid glucoside, and the novel dihydrochalcone C-glycoside, aspalathin, were also present in large quantities. Substantial variation was observed in the individual content values of the phenolic compounds, particularly in those of quercetin-3-O-robinobioside and aspalathin. The effect of production season (2009, 2010 and 2011) on the levels of individual phenolic compounds was inconclusive. Conversely, the quality grade allocated to the rooibos samples by expert graders showed a slight correlation with the mean values of the individual phenolic compounds and the soluble solids content. High quality tea (grades A and B) was associated with higher levels of phenolic compounds and soluble solids than lower quality tea (grades C and D).

1. Introduction

Rooibos is gaining prominence as an antioxidant-containing beverage with potential health-promoting benefits (Joubert & De Beer, 2011). This is reflected in the increased demand for rooibos, specifically in the international market segment, with exports showing steady growth from the 750 tons exported in 1993 to the 6043 tons exported in 2010 (data from the Perishable Products Export Control Board, PPECB, supplied by South African Rooibos Council, SARC). The various health-promoting benefits of rooibos can largely be attributed to its phenolic composition, principally comprising single ring phenolic acids and monomeric flavonoids from the following subclasses: dihydrochalcones, flavanones, flavones and flavonols. The phenolic composition of rooibos is unique in the sense that it is, to date, the only known natural source of the dihydrochalcone C-glycoside, aspalathin, which is also one of its major phenolic constituents (Joubert *et al.*, 2008). Rooibos is also a source of C-glycosidic flavones not found in common food sources and thus the daily diet (Joubert *et al.*, 2009a). Both marketers and scientists place great emphasis on the unique flavonoid composition of rooibos when differentiating it from other herbal teas, *Camellia sinensis* teas and plant extracts, yet very little quantitative and representative data are available.

Dietary flavonoids, present in relatively small quantities in plant products, are believed to play an important role in maintaining optimum human health, largely through their antioxidant, anti-inflammatory and anticarcinogenic properties. It was confirmed that flavonoids can act as antioxidants at low concentrations relevant to physiological levels achievable through the diet (Min & Ebeler, 2008). It has also been reported that flavonoids can selectively inhibit or increase the expression level or the biological activity of key proteins in various cell signalling cascades (Lee-Hilz *et al.*, 2008). Furthermore, their role in herb-drug interactions are of concern as several dietary flavonoids affect key enzymes and transporters involved in drug metabolism (Cermak & Wolfram, 2006).

However, in order to better elucidate the role of dietary polyphenols in human health, comprehensive data on the nature and quantities of the polyphenols found in the main foods consumed in our diet are required. This information is needed for researchers in food science and nutrition, food manufacturers, regulatory authorities, dieticians and consumers. Neveu *et al.* (2010) stated that representative content values for polyphenols in foods will enable: the comparison of polyphenol content between foods; the calculation of polyphenol intake in populations and the study of its association with health and disease; and the evaluation of the relative contribution of a particular food product to the intake of a specific polyphenol as compared to other food sources. Although it has been established that phenolic compounds are important dietary constituents, the essentiality of phenolic compounds is still being debated and none have Dietary Recommended Intakes (DRIs). Sufficient data should be collected to set up a DRI or to allow public health recommendations for one or more of the subclasses of flavonoids (Harnly *et al.*, 2007; Williamson & Holst, 2008). Taking the above into consideration, it is clear that food composition databases are required for these compounds.

To date, several databases on polyphenol contents in foods have been developed, such as the USDA Database on Flavonoids, Phenol-Explorer, EuroFIR-BASIS and the Brazilian flavonoid database (Gry *et al.*, 2007; Neveu *et al.*, 2010; Pérez-Jiménez *et al.*, 2010; De Menezes *et al.*, 2011). With specific regard to tea and tea-like infusions, data are currently restricted to green, black and oolong tea prepared from *Camellia sinensis* plant material (Neveu *et al.*, 2010; Pérez-Jiménez *et al.*, 2010; De Menezes *et al.*, 2011). The USDA database mainly provides content values for the flavonoid aglycones from subclasses such as flavan-3-ols, flavonols and flavones, whilst Phenol-Explorer contains information on specific glycosides and

esters. This is an important distinction as the nature of the glycosidic entity impacts on the biological activity and bioavailability of the polyphenols (Arts *et al.*, 2004). Data are mostly expressed as mg.100 g⁻¹ or mg.100 mL⁻¹ edible portion, with the mean, minimum, maximum and standard deviation values indicated. A flavanol database for green and black teas was also recently presented by Obuchowicz *et al.* (2011). This is the largest tea database worldwide, containing 295 black- and 358 green-origin teas. Two new ISO standard methods were used to determine the characterisable constituents of black and green teas and also aided in developing chemical parameters for both black and green teas. ISO standards 14502-1 and 14502-2 specify methodologies for the determination of total polyphenolic content by a colorimetric assay (Folin-Ciocalteu) and determination of total catechin content by a HPLC method, respectively. The first database on dietary alkylresorcinols and lignans content values, adapted to the Spanish diet, has furthermore been presented as the Aalignia Database by Moreno-Franco and co-workers (2011)

Besides databases on the occurrence and concentrations of phytochemicals in foods, databases on phytochemicals have been further expanded to include data on the chemical structures and classification, spectra, metabolic pathways in plants, metabolism in humans and animals, biological properties and effects on health (Scalbert *et al.*, 2011). An Antioxidant Food Table containing data on the total antioxidant content of more than 3100 foods, beverages, spices, herbs and supplements was recently presented by Carlsen *et al.* (2010). A modified version of the ferric-reducing antioxidant power (FRAP) assay was used to determine the total antioxidant content of samples procured worldwide. This database contains antioxidant values for a wide variety of teas, including rooibos. By combining the polyphenol content, or the concentration of a specific 'marker compound', with the antioxidant activity as quality parameters in the evaluation of herbal extracts, botanical standardisation of rooibos may be improved (Ninfali *et al.*, 2009).

In a recent study in humans, Marnewick *et al.* (2011) demonstrated that daily consumption of six cups of fermented, traditional rooibos infusions over a six-week period significantly improved the lipid profile as well as redox status, both relevant to heart disease, in adults at risk of developing cardiovascular disease. This has led to a recommended consumption of six cups of rooibos daily. However, no information on the composition of individual phenolics in the tea used in this study was made available, and as a result the daily intake of these compounds cannot be calculated. The spectrophotometric methods used in the latter study to determine total polyphenol/flavonol/flavanol content do not take into account the huge diversity of the chemical structures of rooibos phenolic compounds nor their contrasting biological properties. The data generated by these spectrophotometric methods are not suitable for inclusion in food composition databases (Pérez-Jiménez *et al.*, 2010).

Conversely, high performance liquid chromatography (HPLC) permits the study of dietary polyphenols as individual chemical entities rather than as a whole undifferentiated group. Various HPLC methods utilising photodiode-array (PDA) detection for the quantitative analysis of rooibos have been reported (Joubert, 1996; Bramati *et al.*, 2002, 2003; Schulz *et al.*, 2003; Kazuno *et al.*, 2005; Joubert *et al.*, 2009b, Stalmach *et al.*, 2009, Breiter *et al.*, 2011), but these methods are merely suitable for the quantification of some of the major phenolic constituents. Recently, however, an optimised HPLC-PDA method, suitable for the fast quantification of the 15 principal rooibos phenolics was developed (Chapter 3). This method yielded separation of monomeric polyphenols from five phenolic subclasses (dihydrochalcone, flavone, flavonol, phenylpropanoid and hydroxycinnamic acid) within 37 min, making it suitable for routine analyses. Quantitative data available in literature are furthermore only representative of a limited sample size and do not accurately reflect natural variation. Large variation in content values for the dihydrochalcones, for

instance, has been observed and this was attributed to genetic variation in the cultivated plants, which stems from the use of seedlings for propagation (Joubert & Schulz, 2006). In fermented rooibos, further variation in the phenolic composition is induced by the uncontrolled fermentation process, whilst the effects of production season, geographical distribution and steam-pasteurisation on the phenolic composition of rooibos have yet to be determined.

In light of the above, the aim of this study was to apply the optimised HPLC-PDA method to a large number of fermented rooibos infusions, in order to obtain representative quantitative data on the major phenolic constituents present in a 'cup-of-tea' rooibos infusion. Fermented rooibos samples were representative of different production seasons and quality grades. The effect of steam-pasteurisation on the phenolic content of the infusions was investigated, since rooibos plant material is typically steam-pasteurised prior to packaging to ensure microbiological quality (Joubert & Schulz, 2006).

2. Experimental

2.1 Materials

HPLC gradient-grade acetonitrile was purchased from Merck (Darmstadt, Germany) and acetic acid from Fluka (Sigma-Aldrich, Johannesburg, South Africa). Deionised water, prepared using an Elix (Millipore, Milford, MA, USA) water purification system, was further purified to HPLC-grade using a Milli-Q academic (Millipore) water purification system. Isovitexin, hyperoside, orientin, luteolin and chrysoeriol standards were obtained from Extrasynthese (Genay, France) and vitexin, isoorientin and luteolin-7-O-glucoside from Roth (Karlsruhe, Germany). Ferulic acid, rutin and quercetin were obtained from Sigma-Aldrich (St. Louis, MO, USA) and isoquercitrin from Fluka. Enolic phenylpyruvic acid-2-O-glucoside (PPAG) was isolated and supplied by the Post Harvest & Wine Technology Division of the Agricultural Research Council of South Africa (ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa). Aspalathin and nothofagin were supplied by the PROMEC Unit of the Medical Research Council of South Africa (MRC, Cape Town, South Africa). Stock solutions of the phenolic standards were prepared in DMSO (Fluka) at concentrations of approximately 1 mg.mL⁻¹ and diluted with water according to experimental requirements. All diluted solutions were filtered through 0.22 µm PVDF filters (Millipore) prior to use.

2.2 Rooibos plant material

Fermented rooibos samples of different production batches ($n = 114$), randomly selected and representing unrefined plant material, were kindly supplied by Rooibos Limited (Clanwilliam, South Africa). The plant material was sampled after processing, but prior to sieving and steam-pasteurisation. The different batches of rooibos were produced at different geographical locations and harvested over three production seasons ($n_{2009} = 39$, $n_{2010} = 40$, $n_{2011} = 35$). Each sample was allocated a quality grade (A, B, C or D) according to the in-house grading system of Rooibos Limited, giving 30 grade A and grade B samples each, 29 grade C samples and 25 grade D samples.

2.3 Preparation of refined rooibos plant material

In accordance with industry practice, unrefined fermented rooibos plant material (400 g) was sieved through 10 and 40 mesh sieves for 1.5 min at 190 rpm using a SMC Mini-sifter (JM Quality Services, Cape Town, South Africa). The fraction >40 mesh and <10 mesh represents refined rooibos.

2.4 Steam-pasteurisation of rooibos plant material

In order to determine the effect of steam-pasteurisation on the phenolic composition of rooibos, a portion of each sample comprising the refined fraction was subjected to steam-pasteurisation. The plant material (2 x 40 g) was spread out in a thin layer on stainless steel, 40-mesh trays, which were placed in a steam cabinet at $\pm 96^{\circ}\text{C}$ for 2 min. The steam pressure at the cabinet inlet, generated with a THE 400 NM Electropac electrode boiler (John Thompson Boilers, Cape Town), was maintained at 2.76 N.m^{-2} . In order to reduce the moisture content of the steam-pasteurised rooibos samples below 10%, drying in a dehydrator (Decon Humidifier, Continental Fan Works CC, Cape Town, South Africa) at 40°C for 20 min ensued.

2.5 Preparation of 'cup-of-tea' rooibos infusions

Duplicate infusions were prepared according to a one-cup-serving strength by infusing 2.5 g refined plant material for 5 min with 200 mL freshly boiled deionised water, without agitation. The infusions were decanted through a tea strainer, filtered through Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, UK) and cooled to room temperature. Aliquots (*ca* 1.5 mL) of the rooibos infusions were stored at -20°C until analysis.

For HPLC-DAD analyses, aliquots of the rooibos infusions were thawed at room temperature and filtered through $0.22 \mu\text{m}$ PVDF filters (Millipore). Subsequently 1 mL of the filtrate was pipetted into 1.5 mL amber autosampler vials and 0.100 mL 10% ascorbic acid (Sigma) in water ($v.v^{-1}$) added. All samples were analysed within 24 hours of sample preparation.

2.6 Determination of soluble solids (SS) content

The soluble solids content of the aqueous rooibos infusions were determined on 20 mL aliquots using a gravimetric method (Joubert, 1988).

2.7 Instrumentation and chromatographic conditions

HPLC-PDA analyses were performed on an Agilent 1200 system (Agilent, Santa Clara, CA, USA) equipped with an in-line degasser, quaternary pump, autosampler, column thermostat and diode-array detector (standard $13 \mu\text{L}$ flow cell, 10 mm path length), controlled by Chemstation software (Agilent Technologies, Waldbronn, Germany). Chromatographic conditions were used as reported in Chapter 3.

2.8 Method validation

Three standard calibration mixtures, each containing a different combination of phenolic standards at concentrations ranging between $0.012\text{-}0.026 \mu\text{g}.\mu\text{L}^{-1}$, and a randomly selected fermented rooibos infusion were used for method validation. Ascorbic acid was added to the standard calibration mixtures and the rooibos infusion to prevent oxidative degradation and was present at final concentrations of 0.5 and $0.9 \mu\text{g}.\mu\text{L}^{-1}$, respectively.

2.8.1 Linearity and range

Nine-point calibration curves were set up for all standards to test the linearity of the UV-DAD response. UV spectra were recorded between 200-700 nm with selective wavelength monitoring at 288 and 350 nm. The dihydrochalcones and PPAG were quantified at 288 nm, whilst the flavones, flavonols and ferulic acid were quantified at 350 nm. The calibration mixtures were injected at different injection volumes (2, 5, 10, 15, 20,

25, 30, 40, 50 μL) leading to levels of 0.025–1.2 μg on-column. Concentration ranges were selected to cover the different quantities of the compounds present in aqueous rooibos infusions. Linear regression, using the least squares method (Microsoft Excel 2003, Microsoft Corporation, Redmond, WA, USA), was performed on the calibration curve data for each compound to determine the slope, y -intercept and correlation coefficients (r^2).

2.8.2 Stability

Stability of the phenolic compounds, both as part of standard calibration mixtures and a fermented rooibos infusion, were tested by repeated injection ($n = 8$) over 27 h. Standard calibration mixtures were injected at 20 μL and the rooibos infusion at 50 μL . The percentage change (% change) in the peak areas over the 27 h period, as well as percentage relative standard deviation (% RSD) over eight time-points during the period, were used to evaluate the stability of the compounds. The effect of ascorbic acid on the stability of these compounds was also investigated by omitting ascorbic acid from the standard calibration mixtures and fermented rooibos infusion.

2.8.3 Intra- and inter-day precision

Intra- and inter-day precision were determined by injecting the standard calibration mixtures and the fermented rooibos infusion six times each on three consecutive days. Standard calibration mixtures were injected at 20 μL and the rooibos infusion at 50 μL . % RSD was determined for replicate injections on each day (intra-day precision) and for mean values per day (inter-day precision) by considering the respective peak areas.

2.9 Application

2.9.1 Quantification of monomeric phenolic compounds by HPLC-PDA

Aqueous rooibos infusions were injected in duplicate using an injection volume of 50 μL . Phenolic compounds were identified by comparing their retention times and UV–VIS spectra with those of authentic standards. Calibration curves, as described in section 2.8.1, were constructed weekly. Quercetin-3-*O*-robinobioside was quantified using the calibration curve for rutin (quercetin-3-*O*-rutinoside) since no standard was available for this compound.

2.9.2 Statistical analysis

Quantitative data were subjected to analysis of variance (ANOVA) using SAS[®] version 9.2 (SAS Institute, Cary, NC, USA). The Shapiro-Wilk test was performed to test for normality (Shapiro & Wilk, 1965). Student's *t*-test was used to calculate the least significant difference (LSD) at the 5% level to compare mean values.

3. Results and Discussion

3.1 Method validation

Method validation was performed to ensure that the HPLC-DAD method can produce reliable and reproducible results (Shabir *et al.*, 2007). Method validation parameters included linearity and range, stability, as well as intra- and inter-day analytical precision.

3.1.1 Linearity and range

Linearity was assessed by performing single measurements at several analyte concentrations (μg on-column). Nine concentration levels were considered which conform to guidelines specifying a minimum of five levels (Snyder *et al.*, 1997, 2010). Linearity of the calibration curves for authentic standards was excellent (Table 1), with correlation coefficients larger than 0.9999. The y -intercept values were also relatively low.

Table 1 Characteristics of calibration curves obtained for phenolic standards.

Standard	Wavelength (nm)	Linearity range (μg on-column)	Regression equation ^a	Correlation coefficient, r^2
aspalathin	288	0.026-0.650	$y = 2205.64x - 1.34$	0.9999
nothofagin	288	0.025-0.619	$y = 1849.25x + 0.58$	0.9999
orientin	350	0.051-1.275	$y = 2983.38x + 0.16$	0.9999
isoorientin	350	0.050-1.256	$y = 2205.12x - 0.08$	0.9999
vitexin	350	0.025-0.630	$y = 2467.00x - 1.80$	0.9999
isovitexin	350	0.025-0.631	$y = 2425.36x - 1.46$	0.9999
isoquercitrin	350	0.025-0.623	$y = 1907.43x - 1.89$	0.9999
hyperoside	350	0.025-0.619	$y = 2148.55x - 1.00$	0.9999
rutin	350	0.025-0.628	$y = 1533.91x + 0.32$	0.9999
PPAG ^b	288	0.025-0.625	$y = 2796.95x - 1.80$	0.9999
ferulic acid	350	0.029-0.719	$y = 1828.60x - 0.59$	0.9999

^a y = peak area (mAU) and x = amount of standard compound injected (μg).

^b Phenylpyruvic acid-2-O-glucoside.

3.1.2 Stability

Data presented in Table 2 indicate that the stability of the phenolic compounds in the standard calibration mixtures that did not contain ascorbic acid were very poor. The percentage change (% change) in the respective peak areas over the 27 hour period ranged between -7.01% and -24.45%, with the greatest loss observed for aspalathin. The percentage relative standard deviation (% RSD) ranged between 2.70% (isoorientin) and 9.49% (aspalathin), which signifies instability of the phenolic compounds when ascorbic acid is omitted from the standard calibration mixtures.

Conversely, the stability of the phenolic compounds in the aqueous rooibos infusion that did not contain ascorbic acid was remarkably better. An additional phenolic compound in the fermented rooibos infusion, tentatively identified as quercetin-3-O-robinobioside (Chapter 3), was also stable over the considered time period. The % change and % RSD values calculated for most compounds were slightly higher than or equal to 2. The exceptions were isoquercitrin and aspalathin for which % change in respective areas were calculated at 7.1% and -18.9%, respectively. The apparent 'increase' in isoquercitrin may be due to its small peak area and hence integration inaccuracy. The large decrease in the peak area of aspalathin indicated that this compound was particularly prone to oxidation, as previously demonstrated by Joubert (1996). The reason for the improved stability of the phenolic compounds as constituents of the fermented rooibos infusion, as opposed to the standard calibration mixtures, may be attributed to matrix effects, *i.e.* the

constituents of the rooibos infusion that were not quantified may have protected those compounds quantified from degradation.

Table 2 Stability of phenolic compounds (without and with ascorbic acid), as constituents of standard calibration mixtures and an aqueous infusion of fermented rooibos, over a 27 hour period.

Compound	Initial concentration (µg on-column)	Without ascorbic acid		With ascorbic acid	
		% change ^a	% RSD ^b	% change ^a	% RSD ^b
Calibration mixtures					
aspalathin	0.260	-24.45	9.49	0.52	0.19
nothofagin	0.248	-9.24	3.34	0.40	0.14
orientin	0.510	-8.06	2.90	1.23	0.38
isorientin	0.504	-7.01	2.70	1.36	0.71
vitexin	0.252	-19.42	7.20	1.20	0.42
isovitexin	0.252	-19.56	7.24	0.42	0.21
isoquercitrin	0.249	-7.54	2.93	3.86	1.44
hyperoside	0.248	-7.47	2.90	3.55	1.29
rutin	0.251	-8.24	2.96	1.47	0.49
PPAG ^c	0.250	-15.07	5.66	3.47	1.15
ferulic acid	0.288	-7.68	3.15	0.06	0.18
Fermented rooibos infusion					
aspalathin	0.202	-18.90	7.52	0.85	0.49
nothofagin	0.074	-1.88	2.45	2.73	1.34
orientin	0.553	-0.02	0.52	-1.76	1.15
isorientin	0.827	-0.74	0.49	-1.71	0.84
vitexin	0.120	-0.14	1.17	-3.43	1.80
isovitexin	0.148	-0.29	0.97	-2.52	1.25
isoquercitrin	0.018	7.10	4.24	1.23	1.44
hyperoside	0.087	0.67	0.74	-1.40	0.91
rutin	0.056	2.34	1.34	-0.69	1.87
ferulic acid	0.130	0.17	0.85	-2.40	1.26
PPAG	0.314	-0.34	0.47	-2.16	1.02
quercetin-3-O-robinobioside ^d	0.404	-0.52	0.62	-1.45	0.93

^a Percentage change in peak area.

^b Percentage relative standard deviation over eight time-points during the 27 hour period.

^c Phenylpyruvic acid-2-O-glucoside.

^d Constituent of fermented rooibos tentatively identified as quercetin-3-O-robinobioside and quantified as rutin equivalents (refer to Chapter 3).

The stability of the phenolic compounds in the standard calibration mixtures and fermented rooibos infusion containing ascorbic acid was very good (Table 2). The % change in the respective peak areas of each compound over the 27 hour period was less than 4%. The % RSD over the eight time-points were all less than 2%, which indicates that no significant decrease in any of the compounds occurred over this 27 hour period. Note specifically the improved stability of aspalathin with the addition of ascorbic acid.

To conclude, the results indicated that the addition of ascorbic acid to the standard calibration mixtures, and to a lesser extent to the rooibos infusion, greatly improved the stability of the phenolic compounds over a 27 hour period. Practically, this signified that a large number of samples could be prepared simultaneously for injection with the autosampler, which greatly simplified sample preparation during subsequent routine analysis.

3.1.3 Intra- and inter-day precision

Intra-day analytical precision refers to the precision of the method under the same operating conditions over a short time interval and was assessed by repetitive injection of the same homogenous sample ($n = 6$). Inter-day precision was determined by repeating the aforementioned procedure on three consecutive days and subsequently pooling the data to calculate the % RSD ($n = 3$). Although precision criteria are usually % RSD < 2 % (Snyder *et al.*, 1997, 2010), values lower than 5% were deemed acceptable within the context of this study. Intra- and inter-day precision values were reproducible for all phenolic compounds (% RSD < 4.4%, Table 3) from which it was concluded that the analytical precision of the HPLC-PDA method is good.

3.2. Application

The optimised HPLC-PDA method (Chapter 3) suitable for the quantification of the 15 principal rooibos phenolic compounds, was applied to aqueous infusions of fermented rooibos ($n = 114$). Infusions were prepared at a one-cup-serving strength according to the recommendations of the South African Rooibos Council.

3.2.1 Phenolic composition of aqueous rooibos infusions and the effect of steam-pasteurisation

To ensure a product of high microbiological quality, rooibos plant material is typically steam-pasteurised prior to packaging (Joubert & Schulz, 2006). This follows from an outbreak of *Salmonella* in 1984, which gave the rooibos industry a major setback (Snyman, 2000). Therefore, composition data for the database should reflect those of pasteurised rooibos as consumed. The effect of steam-pasteurisation on the phenolic composition is, however, not known. For this reason infusions were also prepared from unpasteurised rooibos so that the effect of this decontamination process on the phenolic composition could be determined. The mean, standard deviation, minimum and maximum values for the phenolic compound and soluble solids content of aqueous rooibos infusions (unpasteurised vs pasteurised) are indicated in Table 4.

Table 3 Intra- and interday analytical precision (% RSD) of phenolic compounds as constituents of standard calibration mixtures and an aqueous infusion of fermented rooibos.

Compound	Intra-day			Inter-day
	day 1 (<i>n</i> = 6)	day 2 (<i>n</i> = 6)	day 3 (<i>n</i> = 6)	pooled (<i>n</i> = 3)
Calibration mixtures^a				
aspalathin	3.61	0.99	0.04	4.29
nothofagin	0.15	2.13	0.16	3.10
orientin	0.08	2.15	0.05	3.23
isoorientin	0.76	0.14	0.07	1.89
vitexin	3.30	0.84	0.05	4.28
isovitexin	3.33	0.89	0.03	4.33
isoquercitrin	0.75	0.48	0.22	1.15
hyperoside	0.79	0.43	0.18	1.03
rutin	0.18	2.19	0.23	3.44
PPAG ^b	2.86	0.63	0.22	3.59
ferulic acid	0.73	0.62	0.31	1.07
Fermented rooibos infusion^a				
aspalathin	0.56	0.44	0.32	0.64
nothofagin	0.98	0.96	0.39	2.43
orientin	0.23	0.19	0.28	1.31
isoorientin	0.05	0.14	0.14	1.35
vitexin	0.87	0.97	0.83	0.91
isovitexin	0.57	0.65	0.59	1.09
isoquercitrin	2.49	3.51	1.80	2.10
hyperoside	0.62	0.68	1.01	1.43
rutin	0.82	1.67	1.61	1.26
ferulic acid	0.54	0.51	0.65	0.64
PPAG	0.35	0.38	0.20	0.33
quercetin-3- <i>O</i> -robinobioside ^c	0.17	0.23	0.40	0.60

^a For concentrations of the compounds refer to Table 2.

^b Phenylpyruvic acid-2-*O*-glucoside.

^c Constituent of fermented rooibos tentatively identified as quercetin-3-*O*-robinobioside and quantified as rutin equivalents (refer to Chapter 3).

Table 4 Mean values ($\text{mg.L}^{-1} \pm$ standard deviation) as well as minimum and maximum values (indicated in brackets) for the phenolic compound and soluble solids content of aqueous infusions of fermented rooibos, representative of different treatments (unpasteurised vs pasteurised).

Parameter	Unpasteurised ($n = 114$)		Pasteurised ($n = 114$)	
Phenolic compound				
aspalathin	$6.31a^a \pm 3.61$	(nd ^b - 15.70)	$5.84b \pm 3.26$	(nd - 15.66)
nothofagin	$0.99a \pm 0.54$	(nd - 2.31)	$0.95b \pm 0.51$	(nd - 2.76)
orientin	$11.22a \pm 1.85$	(6.29 - 14.46)	$10.84b \pm 1.58$	(10.33 - 14.31)
isoorientin	$16.12a \pm 2.92$	(7.63 - 23.04)	$15.03b \pm 2.50$	(7.40 - 20.47)
vitexin	$2.41a \pm 0.40$	(1.28 - 3.17)	$2.33b \pm 0.37$	(1.30 - 3.32)
isovitexin	$2.58a \pm 0.44$	(1.38 - 3.55)	$2.40b \pm 0.39$	(1.35 - 3.29)
luteolin	trace ^c		trace	
luteolin-7-O-glucoside	trace		trace	
chrysoeriol	trace		trace	
quercetin	trace		trace	
isoquercitrin	$1.13a \pm 1.09$	(nd - 6.03)	$1.08b \pm 1.00$	(nd - 5.79)
hyperoside	$2.32a \pm 1.23$	(nd - 6.10)	$2.22b \pm 1.16$	(nd - 6.79)
rutin	$1.66a \pm 1.29$	(nd - 6.66)	$1.65a \pm 1.22$	(nd - 5.71)
PPAG ^d	$7.18a \pm 2.09$	(2.62 - 15.60)	$6.91b \pm 2.03$	(2.72 - 14.81)
ferulic acid	$1.48a \pm 0.61$	(nd - 3.02)	$1.51a \pm 0.84$	(nd - 3.07)
quercetin-3-O-robinobioside ^e	$8.87a \pm 3.92$	(0.72 - 18.85)	$8.34b \pm 3.66$	(0.89 - 18.41)
Soluble solids	$1261a \pm 166$	(872 - 1622)	$1172b \pm 143$	(843 - 1666)

^a Means in the same row with the same letter are not significantly different ($P \leq 0.05$).

^b Not detected.

^c Compound detected but not quantifiable due to peak area below minimum of standard curve and/or due to elution on polymer 'hump' at the end of the chromatographic run (luteolin, chrysoeriol, quercetin).

^d Phenylpyruvic acid-2-O-glucoside.

^e Constituent of fermented rooibos tentatively identified as quercetin-3-O-robinobioside and quantified as rutin equivalents (Chapter 3).

Steam-pasteurisation led to a significant decrease in the mean values (mg.L^{-1}) of most phenolic compounds, excluding rutin and ferulic acid which remained unaffected. This decrease can partially be ascribed to the significant reduction in the soluble solids content with steam-pasteurisation (Table 4). The lower soluble solids content of the steamed samples indicates that the content of the water-soluble compounds in the plant material was reduced by steam-pasteurisation. This observation is in agreement with results presented by Koch (2011) who demonstrated significant reduction in the levels of total polyphenols, soluble solids and aspalathin in rooibos infusions following steam-pasteurisation. The reduction in the levels of the other monomeric phenolic compounds was, however, found to be insignificant (Koch, 2011). The latter study used shorter steam-pasteurisation (1 min) and drying periods (10 min) than those employed in the current study (respectively 2 min and 20 min) and it can therefore be deduced that, by subjecting rooibos plant material to prolonged periods of heat-treatments, the reduction in the levels of phenolic compounds becomes more significant. Heat-induced changes in polyphenolic compounds and a reduction of the levels of polyphenolic compounds have also been previously demonstrated for *Camellia sinesis* (Wang *et al.*, 2000) and *Cyclopia subternata* tea leaves (Joubert *et al.*, 2010).

By taking the soluble solids (SS) contents of the rooibos infusions into account (*i.e.* by expressing the compositional data as g.100 g SS⁻¹) significant differences were still found between the mean values of the monomeric phenolic compounds in the unpasteurised and pasteurised rooibos infusions (Table 5).

Table 5 Mean values (g.100 g SS⁻¹ ± standard deviation) as well as minimum and maximum values (indicated in brackets) for the phenolic compounds in aqueous infusions of fermented rooibos, representative of different treatments (unpasteurised vs pasteurised).

Phenolic compound	Unpasteurised (n = 114)		Pasteurised (n = 114)	
aspalathin	0.484a ^a ± 0.250	(nd ^b – 1.127)	0.486a ± 0.240	(nd – 1.205)
nothofagin	0.077b ± 0.040	(nd – 0.165)	0.080a ± 0.040	(nd – 0.185)
orientin	0.889b ± 0.085	(0.627 – 1.076)	0.925a ± 0.089	(0.629 – 1.127)
isorientin	1.276a ± 0.147	(0.760 – 1.620)	1.285a ± 0.173	(0.776 – 1.756)
vitexin	0.191b ± 0.021	0.128 – 0.246)	0.199a ± 0.023	(0.139 – 0.299)
isovitexin	0.205a ± 0.023	(0.138 – 0.253)	0.206a ± 0.026	(0.126 – 0.271)
isoquercitrin	0.085a ± 0.076	(nd – 0.410)	0.087a ± 0.074	(nd – 0.416)
hyperoside	0.179a ± 0.082	(nd – 0.415)	0.184a ± 0.080	(nd – 0.416)
rutin	0.126b ± 0.092	(nd – 0.495)	0.134a ± 0.092	(nd – 0.438)
PPAG ^c	0.573b ± 0.163	(0.223 – 1.084)	0.596a ± 0.183	(0.251 – 1.262)
ferulic acid	0.119b ± 0.053	(nd – 0.291)	0.126a ± 0.056	(nd – 0.289)
quercetin-3-O-robinobioside ^d	0.685a ± 0.261	(0.072 – 1.162)	0.699a ± 0.261	(0.094 – 1.322)

^a Means in the same row with the same letter are not significantly different ($P \leq 0.05$).

^b Not detected.

^c Phenylpyruvic acid-2-O-glucoside.

^d Constituent of fermented rooibos tentatively identified as quercetin-3-O-robinobioside and quantified as rutin equivalents (refer to Chapter 3).

In this case, the pasteurised rooibos infusions contained higher levels of all phenolic compounds. This difference was, however, only significant ($P \leq 0.05$) for nothofagin, orientin, vitexin, hyperoside, PPAG and ferulic acid. This indicated that the monomeric polyphenols extracted from the pasteurised plant material represent a larger percentage of the total soluble solids of these infusions. From the perspective of the consumer, the results practically signify that a typical 'cup-of-tea' prepared from pasteurised rooibos will contain a lower amount of phenolic compounds.

Based on mean values presented in Table 4, the major phenolic constituents of steam-pasteurised, fermented rooibos infusion are the flavone C-glycosides, isorientin (15.03 mg.L⁻¹) and orientin (10.84 mg.L⁻¹). The flavonol O-diglycoside, quercetin-3-O-robinobioside, the phenylpropanoid, PPAG, and the novel dihydrochalcone C-glycoside, aspalathin, were also present in relatively large quantities of 8.34, 6.91 and 5.84 mg.L⁻¹, respectively. Quercetin-3-O-robinobioside was quantified as rutin-equivalents, since no standard was available for this compound. This was deemed appropriate as quercetin-3-O-robinobioside and rutin are both O-diglycosides of quercetin, therefore expected to exhibit similar detector response. The presence of quercetin-3-O-robinobioside in rooibos has been established by Shimamura *et al.* (2006) via identification by NMR and was tentatively re-confirmed using LC-MS in this study (Chapter 3). The presence of PPAG, an enolic β -D-glucopyranoside of phenylpyruvic acid in *Aspalathus linearis* plant material, was first reported and identified using NMR by Marais *et al.* (1996). The hydroxycinnamic acid, ferulic acid, was quantified at a

minor concentration of 1.51 mg.L^{-1} . Slight over-estimation of ferulic acid occurred due to partial co-elution with an unknown compound in some of the samples (Chapter 3). Luteolin-7-O-glucoside and the aglycones, quercetin, luteolin and chrysoeriol, were only present in trace amounts and could therefore not be quantified accurately. This is in accordance with Toyoda *et al.* (1997) who reported small quantities of these aglycones in rooibos tea.

In this study, substantial variation was observed in the individual content values of the phenolic compounds (Table 4). For instance, the minimum and maximum values for aspalathin and quercetin-3-O-robinobioside differed by factors of more than 15 and 20, respectively. This range was smaller for the other phenolic compounds (Table 4). It has been demonstrated that water temperature, water-to-leaf-ratio and extraction time during sample preparation can influence the extraction of polyphenols from rooibos (Joubert *et al.*, 2008). However, due to the fact that sample preparation was standardised, differences in phenolic composition can solely be attributed to sample variation.

Various factors are known to influence the phenolic composition of plant material. Phenolic compounds fulfill important secondary functions in plants (Gry *et al.*, 2007) and are usually generated in response to environmental stress conditions such as predation, attack by microorganisms or UV light levels (Harnly *et al.*, 2007). Other sources of variability include: seasonal effects (Yao *et al.*, 2005; Aherne & O'Brien, 2002); climate (Aherne & O'Brien, 2002); day length and sunlight intensity (Harbowy & Balentine, 1997; Aherne & O'Brien, 2002; Yao *et al.*, 2005); development stage of the tea shoots (Nakagawa & Torri, 1964); cultivation methods (Ku *et al.*, 2010); plant distribution and processing (Aherne & O'Brien, 2002). Although these factors and their effect on phenolic composition were not specifically investigated for rooibos, it is expected that these factors will have a similar effect on the phenolic composition of rooibos.

More specifically, variation in the phenolic composition of rooibos has been established owing to the genetic make-up of the seedling, the specific plantation and the geographical location (Joubert & Schulz, 2006). As an example of genetic variation, Joubert & De Beer (2011) demonstrated large variation in the aspalathin and orientin contents of rooibos leaves of 21 individual plants harvested at the same time from the same plantation. Harvest date was also shown to be an important factor influencing the phenolic composition of rooibos plants (Joubert & De Beer, 2011). In addition, in fermented rooibos, further variation in the phenolic composition may be induced during the uncontrolled fermentation process as it has been shown that the dihydrochalcones are particularly susceptible to oxidative changes (Joubert, 1996). This could also explain the large variation observed in the aspalathin content (Table 4).

As the aim of this study was to characterise the phenolic composition of a typical 'cup-of-tea' rooibos infusion, this large degree of variation merely emphasises the importance of using a large sample size. The samples of the present study were obtained from different geographical locations and were harvested over three production seasons to capture as much variation as possible. Different production seasons would allow for age of the bush and influence of climate on the plant to be taken into consideration. The reported content values (Table 4) are therefore representative and accurately reflect the amounts of phenolic compounds present in a typical 'cup-of-tea' rooibos infusion.

The large degree of variation observed in the levels of the individual phenolic compounds also has significant implications with regards to the standardisation of rooibos extracts for quality control/assurance purposes. Interest in methods to improve the botanical standardisation of commercial freeze-dried herbal extracts has been expressed recently. Ninfali *et al.* (2009) proposed the combined use of two parameters to improve standardisation, namely the concentration of a specific 'marker compound' (MC) by HPLC and the

antioxidant capacity, determined with the oxygen radical absorbance capacity (ORAC) method. A MC was defined as the 'active constituent' of an extract responsible for the intended pharmacological activity and in order to achieve elevated quality and batch-to-batch consistency, a fixed quantity of the specific MC should be guaranteed. However, the large degree of variation observed in the levels of individual phenolic compounds in rooibos infusions, indicate that this might be problematic. A marker compound for rooibos, which adequately represents the quality of the extract, should therefore be carefully selected. Ninfali *et al.* (2009) proposed rutin as the MC for a freeze-dried extract of *Aspalathus linearis*, but these authors ultimately concluded that it is not representative of the antioxidant activity of the extract.

Currently, a major extract producer standardises rooibos extract on combined orientin and isoorientin contents, while standardisation of a fermented rooibos extract based on aspalathin content is currently underway. Two prominent South African extract producers use total polyphenol content (TPC) and total antioxidant capacity (TAC) as quality indicators (Joubert & De Beer, 2011).

4.2.2 Effect of production season

In order to investigate the effect of production season on the phenolic composition of aqueous rooibos infusions, the samples were divided according to production season ($n_{2009} = 39$, $n_{2010} = 40$, $n_{2011} = 35$). Mean values ($\text{mg.L}^{-1} \pm$ standard deviation) for the phenolic compound and soluble solids content of steam-pasteurised, fermented rooibos from different production seasons are summarised in Table 6.

Table 6 Mean values ($\text{mg.L}^{-1} \pm$ standard deviation) for the phenolic compound and soluble solids content of aqueous infusions of fermented rooibos, representative of different production seasons ($n_{2009} = 39$, $n_{2010} = 40$, $n_{2011} = 35$).

Parameter	2009	2010	2011
Phenolic compound			
aspalathin	5.85a ^a \pm 2.25	5.48a \pm 3.59	6.17a \pm 3.89
nothofagin	1.10a \pm 0.37	1.02a \pm 0.63	0.69b \pm 0.40
orientin	10.96a \pm 1.24	11.02a \pm 1.93	10.49a \pm 1.49
isoorientin	14.85b \pm 1.73	14.33b \pm 2.75	16.05a \pm 2.67
vitexin	2.27a \pm 0.31	2.42a \pm 0.46	2.31a \pm 0.32
isovitexin	2.29b \pm 0.28	2.43ab \pm 0.45	2.50a \pm 0.39
isoquercitrin	1.23a \pm 0.98	0.96a \pm 0.85	0.90a \pm 0.86
rutin	2.01a \pm 1.28	1.53ab \pm 1.20	1.39b \pm 1.11
hyperoside	2.25a \pm 1.00	2.24a \pm 1.18	2.15a \pm 1.34
PPAG ^b	6.48b \pm 1.18	5.65c \pm 1.31	8.84a \pm 2.07
ferulic acid	1.35a \pm 0.62	1.55a \pm 0.58	1.48a \pm 0.72
quercetin-3-O-robinobioside ^c	8.97a \pm 3.16	8.42a \pm 3.82	7.54a \pm 3.94
Soluble solids	1183a \pm 127	1210a \pm 129	1115b \pm 159

^a Means in the same row with the same letter are not significantly different ($P \leq 0.05$).

^b Phenylpyruvic acid-2-O-glucoside.

^c Constituent of fermented rooibos tentatively identified as quercetin-3-O-robinobioside and quantified as rutin equivalents (Chapter 3).

Considering mean values for the phenolic compounds of samples harvested over different production seasons (Table 6), no distinct trends were observed. The aspalathin, orientin, vitexin, isoquercitrin, hyperoside, ferulic acid and quercetin-3-O-robinobioside contents were not significantly affected by the different production seasons. Mean values for nothofagin and rutin were the highest for the 2009 production season, and did not differ significantly from the 2010 production season, although they did differ significantly from the 2011 season. Isoorientin and isovitexin were present at the highest levels in 2011, and at the lowest levels in 2009 and 2010, respectively, although mean values for these phenolic compounds over the 2009 and 2010 production season did not differ significantly. PPAG was the only phenolic compound which exhibited significant differences over all three production seasons and was quantified at levels of 6.48, 5.65 and 8.84 mg.L⁻¹ for the 2009, 2010 and 2011 production seasons, respectively. Collectively, these results signify that data obtained from a single production season are, to a certain extent, representative. This was, in part, facilitated by the random selection of samples employed in this study.

Selective differences in the mean values of the phenolic compounds over the different production seasons can be attributed to a higher/lower degree of sensitivity of the particular phenolic compound towards changes in environmental conditions (climate, drought, stress etc.). It is well known that changes in environmental conditions can selectively inhibit/stimulate the biosynthesis of certain phenolic compounds in plant tissue (Yao *et al.*, 2005). Yao *et al.* (2005) demonstrated large seasonal variations in the principal flavanols of *Camellia sinensis* tea which were attributed to day length, sunlight and/or temperature, which varied markedly across seasons. It was also suggested that the flavonoid profile of green tea leaves should be correlated with conditions such as rainfall and humidity. Although comparable studies have not been conducted on *Aspalathus linearis* plant material, it may be assumed that these factors could also be responsible for the observed seasonal variation (Table 6).

It should, however, be stressed that the rooibos samples were randomly selected for each production season and did not originate from the same plantations or even the same geographical locations which could have introduced additional variation. It is therefore recommended that, in subsequent studies, the samples should be harvested from a defined geographical location so that the effect of different production seasons on the phenolic composition of rooibos may be elucidated.

The combined content values for the phenolic compounds quantified in rooibos samples from the 2009, 2010 and 2011 production seasons averaged 59.61, 57.35 and 60.51 mg.L⁻¹, respectively. Highest levels of the quantified phenolic compounds were therefore found in the rooibos samples of 2011. The average total values for the phenolic compounds were inversely correlated to the mean soluble solids content, for which the lowest value was obtained for the 2011 samples (Table 6). These results are contradictory to those reported by Koch (2011), who illustrated a positive correlation between the levels of phenolic compounds and the levels of soluble solids. This discrepancy could once again be explained by considering the composition of the soluble solids which comprise polyphenolic compounds and tannins in addition to the monomeric phenolic compounds quantified in this study. As the exact composition of the soluble solids is undefined, the percentage monomeric phenolic compounds extracted in samples from different production seasons could be relatively less/more, leading to the observed discrepancy.

The distributions of the major phenolic constituents (aspalathin, isoorientin, orientin, PPAG and quercetin-3-O-robinobioside; mg.L^{-1}) of steam-pasteurised, fermented rooibos infusions as a function of production season are illustrated in Figure 1.

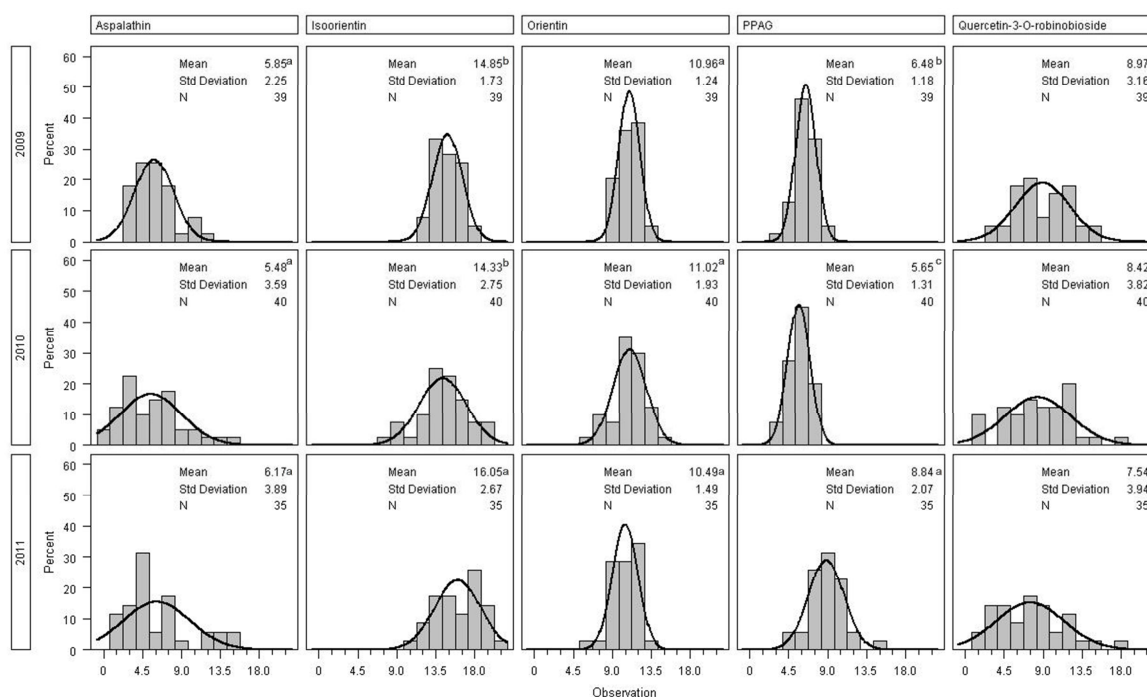


Figure 1 Distributions of the major phenolic constituents (aspalathin, isoorientin, orientin, PPAG and quercetin-3-O-robinobioside; mg.L^{-1}) of steam-pasteurised, fermented rooibos infusions as a function of production season, 2009, 2010 and 2011.

By examining the distributions in Figure 1 a number of observations can be made. In the first instance, the Gaussian distributions for orientin and PPAG, and to a lesser extent for isoorientin, were narrow, with standard deviations less than three. The range of values for orientin was the lowest, with standard deviations less than 2. Conversely, the distributions for aspalathin and quercetin-3-O-robinobioside were broader, which was also exemplified by the minimum and maximum values for these compounds as indicated in Table 6. This is indicative of larger natural variation in the concentration of these two major phenolic compounds in fermented rooibos plant material.

Secondly, the distributions for each of the phenolic compounds over the different production seasons did not notably differ, although the lowest standard deviations for all compounds were found in 2009 (Figure 1). The distributions for aspalathin and quercetin-3-O-robinobioside were broad over all three production seasons. A substantially narrower distribution for the former compound was, however, found in 2009. Also note that the distribution for PPAG was strikingly broader in 2011 and shifted towards higher mean values.

3.2.3 Correlating phenolic composition and soluble solids content of aqueous rooibos infusions with the allocated quality grade

Quality grading was performed by expert graders associated with Rooibos Limited (Clanwilliam, South Africa) that provided the samples. Quality grading was performed by evaluating the appearance of the dry and infused rooibos leaves, and by evaluating the overall colour and flavour of the rooibos infusions. A score

out of 10 was allocated to each of these four parameters and multiplied by a certain factor according to the weight assigned to each quality parameter. The flavour of the rooibos infusion carried the most weight. The quality grade assigned to each sample (A, B, C or D) was subsequently based on the totals of the final scores (Koch, 2011). In order to correlate the phenolic composition and soluble solids content of aqueous rooibos infusions with the allocated quality grade, the samples were divided according to their quality grade ($n_A = 30$, $n_B = 30$, $n_C = 29$, $n_D = 25$). Mean values ($\text{mg}\cdot\text{L}^{-1} \pm$ standard deviation) for the phenolic compound and soluble solids content of steam-pasteurised, fermented rooibos representative of different quality grades are summarised in Table 7.

Table 7 Mean values ($\text{mg}\cdot\text{L}^{-1} \pm$ standard deviation) for the phenolic compound and soluble solids content of aqueous infusions of steam-pasteurised, fermented rooibos representative of different quality grades ($n_A = 30$, $n_B = 30$, $n_C = 29$, $n_D = 25$).

Parameter	Grade A	Grade B	Grade C	Grade D
Phenolic compound				
aspalathin	7.70a ^a \pm 3.33	5.84b \pm 2.83	5.83b \pm 3.30	3.53c \pm 2.24
nothofagin	1.04a \pm 0.38	1.02a \pm 0.52	1.04a \pm 0.57	0.64b \pm 0.48
orientin	11.37a \pm 1.38	11.15a \pm 1.29	10.72ab \pm 1.60	9.95b \pm 1.80
isoorientin	15.92a \pm 2.35	15.55a \pm 1.93	14.95a \pm 2.73	13.44b \pm 2.37
vitexin	2.45a \pm 0.36	2.42a \pm 0.26	2.22b \pm 0.39	2.21b \pm 0.42
isovitexin	2.53a \pm 0.35	2.49a \pm 0.32	2.35ab \pm 0.43	2.22b \pm 0.40
isoquercitrin	1.82a \pm 1.11	0.98b \pm 0.65	0.69b \pm 0.55	0.59b \pm 0.62
rutin	2.47a \pm 1.50	1.57b \pm 0.88	1.38b \pm 0.95	1.08b \pm 1.01
hyperoside	3.22a \pm 1.41	2.06b \pm 0.67	1.66b \pm 0.78	1.83b \pm 0.97
PPAG ^b	7.29a \pm 2.09	7.39a \pm 1.98	6.86a \pm 2.27	5.94b \pm 1.40
ferulic acid	1.22a \pm 0.56	1.56a \pm 0.55	1.56a \pm 0.70	1.48a \pm 0.72
quercetin-3-O-robinobioside ^c	11.07a \pm 3.58	8.17b \pm 2.83	6.89b \pm 3.00	6.95b \pm 3.70
Soluble solids	1247a \pm 147	1173b \pm 132	1118b \pm 126	1142b \pm 136

^a Means in the same row with the same letter are not significantly different ($P \leq 0.05$).

^b Phenylpyruvic acid-2-O-glucoside.

^c Constituent of fermented rooibos tentatively identified as quercetin-3-O-robinobioside and quantified as rutin equivalents (Chapter 3).

Considering mean values for the phenolic compounds of different quality grade samples (Table 7), it can be observed that the higher quality grade samples tend to associate with higher levels of the phenolic compounds. The grade A samples had the highest mean values for most phenolic compounds, excluding PPAG and ferulic acid. For the latter phenolic constituent, the grade A samples had the lowest mean content value, although it did not statistically differ from the other quality grade samples. Furthermore, the grade A samples contained the highest levels of the dihydrochalcone C-glycoside, aspalathin, and the flavonol O-glycosides, isoquercitrin, rutin, hyperoside and quercetin-3-O-robinobioside. Mean content values for these phenolic compounds in the grade A samples differed significantly from all the other quality grade samples. It is therefore possible that, based on the current quality grading system, these phenolic constituents are the major contributors to the positive quality attributes of the rooibos infusions. This is in accordance with results

presented by Koch (2011), who reported strong correlations of rutin with the appearance of the rooibos leaves; and of rutin and isoquercitrin with the flavour of the rooibos infusions.

Conversely, the grade D samples had the lowest mean content values for most phenolic compounds, excluding hyperoside, quercetin-3-O-robinobioside and ferulic acid. Mean values for these latter compounds were lowest in the grade C samples. It should be stressed, however, that rooibos plant material graded as a D quality grade, rarely reaches the tea-drinking consumer and is mostly utilised in the preparation of extracts for the supplement/nutraceutical and functional food markets.

Considering mean values for the soluble solids content over the different quality grade samples (Table 7), it is clear that the high quality samples (grades A and B) are related to higher concentrations of soluble solids. The low quality samples (grades C and D) had lower levels of soluble solids, which may be attributed to a larger percentage of stems in the rooibos samples (Joubert, 1984) and/or to lower solubility of the phenolic compounds resulting from over-fermentation (Joubert, 1994). The soluble solids contents of the grade A samples differed significantly from the contents of the grade B, C and D samples, although these samples did not differ significantly from one another. To conclude, it was established that the chemical data (mean values for the phenolic compounds and the soluble solids) show a tentative correlation with the quality grade allocated to the rooibos samples by expert graders. High quality tea (especially grade A, and to a lesser extent grade B as well) was associated with higher levels of phenolic compounds and soluble solids.

The distributions of the major phenolic constituents (aspalathin, isoorientin, orientin, PPAG and quercetin-3-O-robinobioside; mg.L^{-1}) of steam-pasteurised, fermented rooibos infusions as a function of quality grades are illustrated in Figure 2.

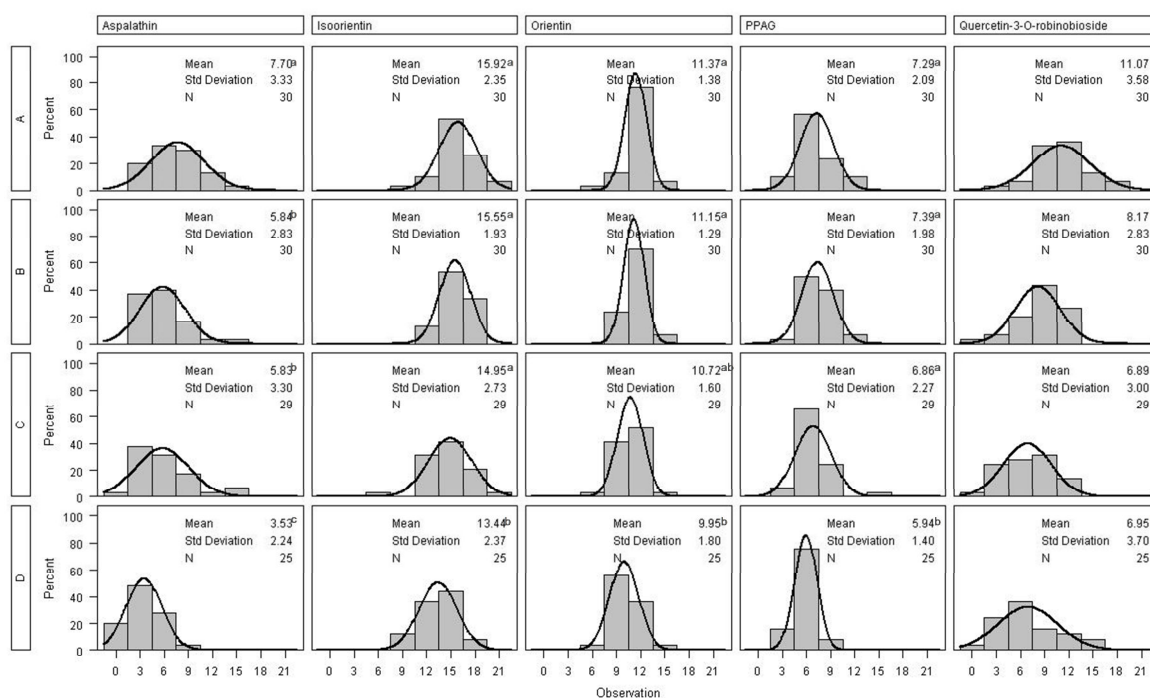


Figure 2 The distributions of the major phenolic constituents (aspalathin, isoorientin, orientin, PPAG and quercetin-3-O-robinobioside; mg.L^{-1}) of aqueous infusions of steam-pasteurised, fermented rooibos as a function of quality grade, A, B, C and D.

For the distributions according to quality grade (Figure 2) the same conclusions can be drawn as from the distributions according to production season (Figure 1). Orientin and PPAG, and to a lesser extent isoorientin, exhibited the most narrow distributions over all the different quality grades, whilst the distributions for aspalathin and quercetin-3-*O*-robinobioside were substantially broader. A narrower distribution for aspalathin was, however, observed in the grade D samples. Furthermore, the mean values of aspalathin and quercetin-3-*O*-robinobioside in the grade A samples were 35–50% higher than those of the grade D samples, while the mean values for the other major compounds were only slightly higher (12–19%) in the grade A samples when compared to the other quality grades.

4. Conclusions

An optimised HPLC-PDA method, suitable for the quantification of the 15 principal rooibos phenolic compounds, was rigorously validated and successfully applied in the analysis of a large number of fermented rooibos samples ($n = 114$), representative of different production seasons and quality grades. Rooibos samples, obtained from different geographical locations and different producers, captured the maximum potential variation in the phenolic composition. Owing to the large number of representative samples analysed, the generated data accurately reflect natural variation and is therefore suitable for inclusion in food composition tables. The subsequent calculation of the contribution of rooibos polyphenols to dietary intake is now possible. In addition, the representative content values will provide the foundation for product authenticity and quality control purposes based on chemical ‘fingerprints’.

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CHAPTER 5

*Comprehensive two-dimensional liquid
chromatographic analysis of rooibos
(Aspalathus linearis) phenolics*

Abstract

The analysis of rooibos tea (*Aspalathus linearis*) phenolics by off-line and on-line comprehensive two-dimensional liquid chromatographic (2-D LC) methods was investigated. Phenolic compounds were separated according to polarity by hydrophilic interaction liquid chromatography (HILIC) in the first dimension, whilst reversed-phase liquid chromatography (RP-LC) on a C18 column provided separation according to hydrophobicity in the second dimension. Ultraviolet photodiode-array (UV-PDA) and electrospray ionisation mass spectrometry (ESI-MS) were used for identification purposes. Comprehensive HILICxRP-LC demonstrated its applicability for the analysis of a diverse range of phenolic compounds in unfermented and fermented rooibos samples. The combination of these orthogonal separations provided a significant improvement in resolution, as is evident from practical peak capacities in excess of 2000 and 800 for the off-line and on-line methods, respectively. Further optimisation, especially of the first dimension separation, is however required to improve the applicability of LCxLC for the in-depth analysis of rooibos tea phenolics.

1. Introduction

Considerable interest in natural phenolic compounds stems from the mounting evidence suggesting that these compounds promote optimal human health and reduce the risk of chronic diseases (Gry *et al.*, 2007). In this sense, phenolics are associated with various health-promoting benefits of rooibos tea, a beverage prepared from the endemic Cape fynbos plant, *Aspalathus linearis*. The beneficial physiological effects of rooibos (as reviewed by Joubert *et al.*, 2008; Joubert & De Beer, 2011) typically include antioxidant, anti-inflammatory, anticarcinogenic, hepatoprotective and phyto-oestrogenic properties. The phenolic constituents of rooibos comprise monomeric flavonoids such as phenolic acids, flavan-3-ols, lignans and coumarins, amongst others (Joubert *et al.*, 2008). The monomeric flavonoid composition of rooibos is unique in that it contains the novel dihydrochalcone C-glycoside, aspalathin (Koeppen & Roux, 1965), as well as the cyclic dihydrochalcone, aspalalinin (Shimamura *et al.*, 2006). Rooibos tea furthermore represents an important dietary source of uncommon C-glycosyl flavones, such as orientin and isoorientin (Joubert *et al.*, 2009a), which are also two of the major phenolic constituents of fermented rooibos infusions (Koch, 2011) and extracts (Joubert *et al.*, 2009b). In order to exploit the full potential of rooibos as phytopharmaceutical and to realise authentication of rooibos extracts, comprehensive data regarding the phenolic composition of rooibos are required.

Reversed-phase high performance liquid chromatographic (RP-HPLC) coupled with ultraviolet-visible (UV-VIS) spectroscopy and mass spectrometry (MS) is the dominant analytical technique for the separation and characterisation of phenolic compounds (De Rijke *et al.*, 2006). In the quantitative analysis of rooibos phenolics, various RP-HPLC methods have been reported (Joubert, 1996; Bramati *et al.*, 2002, 2003; Schulz *et al.*, 2003; Kazuno *et al.*, 2005; Joubert *et al.*, 2009b; Stalmach *et al.*, 2009; Breiter *et al.*, 2011) but these are generally limited in terms of the number of compounds investigated. This limitation is largely due to shortcomings associated with analytical methods for phenolic determination as the large chemical diversity of rooibos phenolics present severe analytical challenges. The complexity of rooibos phenolics, from both a quantitative and qualitative perspective, is further enhanced by natural variation (Van Heerden *et al.*, 2003; Joubert & Schulz, 2006) and the uncontrolled fermentation process (Krafczyk & Glomb, 2008; Krafczyk *et al.*, 2009). As a result, the large number of diverse phenolic compounds in rooibos extracts typically exceed the separation capacity of a single HPLC method.

Recently, the advantages of new developments in HPLC such as the use of sub-2 μm phases and ultra high pressures have also been explored for rooibos analysis (Cabooter *et al.*, 2011; Iswaldi *et al.*, 2011). However, despite the benefits of these methods for fast separation of the principal phenolics in rooibos, complete separation of all constituents in a single analysis remains elusive. Detailed investigation of the minor phenolic constituents of rooibos which might possibly impact on bioactivity is therefore still required.

In this regard two-dimensional liquid chromatography (2-D LC) represents a powerful alternative method for the improved separation of complex samples such as rooibos due to the increased peak capacity afforded by complementary separations. In comprehensive 2-D LC (LCxLC), the entire sample is subjected to two different separation mechanisms and the theoretical peak capacity ($n_{C,2D}$) is equal to the product of the peak capacities in the first ($n_{C,1}$) and second ($n_{C,2}$) dimensions (Guiochon *et al.*, 1983; Giddings, 1987):

$$n_{C,2D} = n_{C,1} \times n_{C,2} \quad (1)$$

In order to fully utilise the increased peak capacity offered by LCxLC separations, a number of criteria must be satisfied: the two separation mechanisms have to be orthogonal (Giddings, 1995) and the sampling of the first dimension effluent must be fast enough to avoid resolution loss due to 'under-sampling' effects (Murphy *et al.*, 1998).

LCxLC may be performed in three different ways, *i.e.* on-line, off-line and stop-flow, depending on the manner in which the effluent is transferred to the second-dimension column. In the on-line configuration, fractions of the first dimension are directly transferred to the second-dimension column, typically by employing a switching valve. Off-line LCxLC entails collection of fractions from the first dimension (either manually or via a fraction collector) and subsequent re-injection onto the second-dimension column. In the stop-flow mode, fractions from the primary column are transferred to the second-dimension column and while this fraction is analysed, the flow in the first dimension is stopped (Kalili & De Villiers, 2011).

In recent years, LCxLC has also been successfully applied to the analysis of phenolic compounds (Kalili & De Villiers, 2011). The most common combination of LC modes involves RPxRP-LC (Cacciola *et al.* 2006, 2007a, 2007b; Kivilompolo & Hyötyläinen, 2007; Cesla *et al.*, 2009; Dugo *et al.*, 2008, 2009a, 2009b) although these methods suffer from limited orthogonality. In contrast, the combination of hydrophilic interaction chromatography (HILIC) and RP-LC provides a high degree of orthogonality for the separation of phenolic compounds due to different separation mechanisms in each dimension. Using an off-line approach, Kalili & De Villiers (2009, 2010) have successfully applied comprehensive HILICxRP-LC for the analysis of diverse phenolic compounds in cocoa, apple and green tea extracts.

In light of the above, the aim of the current study was to investigate the applicability of HILICxRP-LC for the analysis of rooibos phenolics. By subjecting the rooibos sample to two complementary separations, additional information regarding minor phenolic constituents possibly impacting on bioactivity may be obtained. This approach will also be used to facilitate comparison of rooibos samples as a function of processing parameters by analysing both unfermented and fermented rooibos samples.

2. Experimental

2.1 Materials

HPLC gradient-grade acetonitrile was purchased from Merck (Darmstadt, Germany), methanol from Riedel-de Haën (Sigma-Aldrich, St. Louis, MO, USA) and acetic acid from Fluka (Sigma-Aldrich). Deionised water, prepared using a Modulab (Continental Water Systems Corporation, San Antonio, TX, USA) water purification system, was further purified to HPLC-grade using a Milli-Q academic (Millipore, Milford, MA, USA) water purification system. Phenolic standards (Table 1, Chapter 3) were obtained from Extrasynthese (Genay, France), Roth (Karlsruhe, Germany), Sigma-Aldrich and Fluka. Enolic phenylpyruvic acid-2-O-glucoside (PPAG) was isolated and supplied by the Post Harvest & Wine Technology Division of the Agricultural Research Council of South Africa (ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa). Aspalathin and nothofagin were supplied by the PROMEC Unit of the Medical Research Council of South Africa (MRC, Cape Town, South Africa). Stock solutions of the phenolic standards were prepared in DMSO (Fluka) at concentrations of approximately $1 \text{ mg}\cdot\text{mL}^{-1}$ and diluted with water according to experimental requirements. All diluted solutions contained $0.5 \text{ mg}\cdot\text{mL}^{-1}$ ascorbic acid (Sigma) and were filtered through $0.22 \mu\text{m}$ PVDF filters (Millipore) prior to use.

2.2 Sample preparation

A freeze-dried sample of unfermented and fermented rooibos aqueous infusion (*ca* 150 mg each) was transferred to 50 mL volumetric flasks and dissolved in 50 mL deionised water. Two SPE cartridges (Discovery DSC-18, 60 mL, 10 grams, SUPELCO, Sigma-Aldrich) were conditioned with 60 mL methanol, followed by 60 mL deionised water. Thereafter ~ 25 mL of the fermented sample and ~ 50 mL of the unfermented sample was loaded onto separate cartridges. Deionised water (60 mL) was passed through the SPE column to elute polar compounds such as polysaccharides and these fractions were discarded. The flavonoids were eluted with 60 mL acetonitrile. The eluents were transferred to 250 mL round-bottomed flasks and the acetonitrile evaporated under vacuum (180 millibar, 40°C). The samples were then transferred to amber vials, frozen overnight and freeze-dried for *ca* 24 hours. The freeze-dried samples (*ca* 14 mg) were subsequently reconstituted in $1000 \mu\text{L}$ acetonitrile and methanol (70:30, v.v⁻¹), to which $100 \mu\text{L}$ 2% acetic acid in water (v.v⁻¹) was added. The reconstituted samples were filtered through hydrophilic $0.22\text{-}\mu\text{m}$ PVDF filters and kept at 4°C until analysis.

2.3 Instrumentation and chromatographic methods

2.3.1 Off-line HILICxRP-LC analyses

Off-line HILICxRP-LC analyses were performed on a Waters Acquity UPLC system equipped with a binary solvent manager, sample manager, column heating compartment, photodiode-array (PDA) detector (500 nL flow cell, 10 mm path length) and controlled by Waters Empower software (Waters, Milford, USA). The mixing chamber on the UPLC was replaced with a low dead-volume union, while the pump was connected to the injection valve using 0.1 mm i.d. tubing (Waters) to reduce the system delay volume.

In the first dimension, HILIC separations were performed on a Nomura Chemical Develosil Diol-100 column ($5 \mu\text{m}$, $250 \text{ mm} \times 1 \text{ mm}$ i.d., Aichi, Japan) using method(s) adapted from Kalili & De Villiers (2009, 2010). The binary mobile phase consisted of (A) acetonitrile and acetic acid (98:2, v.v⁻¹) and (B) methanol,

water and acetic acid (93.05:4.95:2.00, v.v⁻¹). The following off-line gradient was applied at a flow rate of 0.050 mL.min⁻¹: 0-10% B (0-45 min); 10-100% B (45-65 min); 100% B isocratic (65-75 min); 100-0% B (75-77 min). The column was re-equilibrated for 23 min. Separations were performed at ambient temperature and 10 µL was injected in the 'partial loop with needle overfill' mode using solvent A as the week needle wash solvent.

One-minute fractions (corresponding to 50 µL each) of the HILIC effluent were automatically collected between 0 and 70 min using a programmable BIO-RAD Model 2110 fraction collector (Corston, UK). The collected fractions were transferred to 1.5 mL amber autosampler vials containing 250 µL inserts and kept under N₂ until analysed by the off-line RP-LC gradient as described below. All fractions were analysed within 48 hours of collection.

In the second dimension, RP-LC separations were performed on an Agilent Zorbax SB-C18 column (1.8 µm, 50 x 4.6 mm i.d., Waldbronn, Germany) protected by an Acquity UPLC BEH C18 pre-column (1.7 µm, 5 x 2.1 mm, Van Guard, Waters) and thermostatted to 48°C. The binary mobile phase consisted of (A) 1% acetic acid in water (v.v⁻¹) and (B) acetonitrile. The following off-line gradient was applied at a flow rate of 1.0 mL.min⁻¹: 10-14.8% B (0-8.5 min); 14.8-50% B (8.5-20.5 min); 50-100% B (20.5-21.5 min); 100% B isocratic (21.5-24 min); 100-10% B (24-25 min). The column was re-equilibrated for 4 min. The fractions (2 µL) were injected in the 'partial loop with needle overfill' mode using solvent A as the week needle wash solvent.

UV spectra were recorded between 200-500 nm with selective wavelength monitoring at 288 and 350 nm at an acquisition rate of 10 points.s⁻¹. Raw UV data for the RP-LC analyses were exported to STATISTICA 8 (Statsoft Inc., US) to create 2-D contour plots.

2.3.2 On-line HILICxRP-LC analyses

In the first dimension, HILIC analyses were conducted on a Hewlett Packard 1050 system equipped with a quaternary pump, PDA detector and autosampler, which were controlled by Chemstation software (Agilent). First dimension separations were performed using the same column and mobile phases as for the off-line system. The following on-line gradient was applied at a flow rate of 0.025 mL.min⁻¹: 0-10% B (0-90 min); 10-100% B (90-130 min); 100% B isocratic (130-150 min); 100-0% B (150-155 min). The column was re-equilibrated for 25 min. Separations were performed at ambient temperature and 10 µL was injected.

The Hewlett Packard 1050 system was interfaced through a two-position/ten-port switching valve (Cheminert C72NX-4690D, VICI, Valco Instruments Co., Schenkon, Switzerland) to the Waters Acquity UPLC-PDA system. The switching valve was equipped with two 5 µL loops and the first dimension flow split was 1:25. Two minute fractions of the first dimension effluent, corresponding to 2 µL, were alternately collected and analysed in the second dimension.

In the second dimension, RP-LC separations were performed as specified for the off-line system, with the flow rate changed to 1.2 mL.min⁻¹ according to the following gradient: 12-50% B (0-1.1 min); 50-100% B (1.1-1.2 min); 100-12% B (1.2-1.3 min). The column was re-equilibrated for 0.7 min. Detection and construction of 2-D contour plots were performed as for the off-line system.

2.3.3 LC-ESI-MS analyses

The individual HILIC and RP-LC methods, as described above for the off-line analysis, were combined with ESI-MS detection. ESI-MS analyses were conducted on the Waters Acquity UPLC system interfaced through

an electrospray ionisation (ESI) ion source to a Waters Ultima API quadrupole time-of-flight (Q-TOF) mass spectrometer. The mass spectrometer was operated in both positive and negative ionisation modes. Masses were scanned from 150–1500 amu and data were collected and processed using MassLynx v.4.0 software (Waters). The instrument was calibrated using a sodium formate solution. The capillary voltage was 3.5 kV in the positive ionisation mode and -3.7 kV in the negative ionisation mode. The following parameters were used in both ionisation modes: cone voltage: 35 V; source temperature: 100°C; desolvation temperature: 350°C; desolvation gas flow (N₂): 350 L.hr⁻¹; and cone gas flow (N₂): 50 L.hr⁻¹. These instrumental parameters were the same for HILIC and RP-LC-ESI-MS analyses, except that the desolvation temperature was lowered to 300°C for the former.

For HILIC- and RP-LC-ESI-MS analyses, the samples were injected at 2 µL in the 'partial loop' mode using solvent A as the weak needle wash solvent, while the eluent was split 3:2 prior to introduction into the ionisation chamber for RP-LC-ESI-MS only.

3. Results and discussion

3.1 1-D analyses

3.1.1 HILIC analyses

The HILIC methods reported by Kalili & De Villiers (2009, 2010) were adapted by increasing the acetic acid content of both A and B solvents to 2% v.v⁻¹, and by selectively adjusting the gradient to improve the separation of the rooibos tea phenolics (see experimental section 2.3.1 for details). The HILIC analysis of an unfermented rooibos extract is illustrated in Figure 1. Compounds were identified by co-injection of standard compounds and based on MS and MS/MS spectra compared to literature reports (section 3.1.3; Table 1).

HILIC provides separation according to polarity, with the more polar compounds being more retained on the polar DIOL-100 phase. The flavone aglycones chrysoeriol (11) and luteolin (9) eluted at 4.38 and 7.07 min, respectively. The former compound contains a methoxy group at C5' on the C-ring, leading to decreased polarity and hence to a decrease in retention in HILIC mode compared to luteolin. Both these flavone aglycones eluted before their glycosylated derivatives, which showed an elution order of apigenin-8-C-glucoside (7) + apigenin-6-C-glucoside (8) < luteolin-7-O-glucoside (10) < luteolin-8-C-glucoside (5) + luteolin-6-C-glucoside (6). The 6-C and 8-C glucosides of apigenin exhibited perfect coelution in HILIC, as the position of glycosylation does not impact on the polarity of the molecule. This was also observed for the 6-C and 8-C glucosides of luteolin, (5) and (6). However, the apigenin-glucosides eluted before their 5'-hydroxy (luteolin) analogues due to their lower polarity. The type of glycosidic linkage also influenced retention in HILIC as the flavone mono-O-glucoside, luteolin-7-O-glucoside (10), eluted before the mono-C-glucosides luteolin-8-C-glucoside (5) + luteolin-6-C-glucoside (6). This may be ascribed to the loss of a hydroxyl functionality, and hence decreased polarity, as the monosaccharide is bound to the C7 hydroxyl group of the flavone aglycone to form a C-O glycosidic linkage in the mono-O-glucoside.

For the flavonol subclass, retention in HILIC was also found to increase with an increase in the degree of glycosylation, with mono-O-glycosides eluting before their corresponding O-diglycosides. The general elution order was established as quercetin (12) < quercetin-3-O-glucoside (13) + quercetin-3-O-galactoside (14) < quercetin-3-O-rutinoside (15) + quercetin-3-O-robinobioside (X). Perfect coelution of (13) and (14) illustrated that the nature of the monoglycoside does not impact on the retention of the analyte in

HILIC. This was also true for (15) and (X) in which the diglycoside group only differs with regard to the nature of the internal glycosidic moiety. The C-glycosyl dihydrochalcone aspalathin (1) also eluted before compound (Y), postulated to be a C-8-hexosyl derivative of aspalathin based on LC-ESI-MS/MS results (refer to Chapter 3).

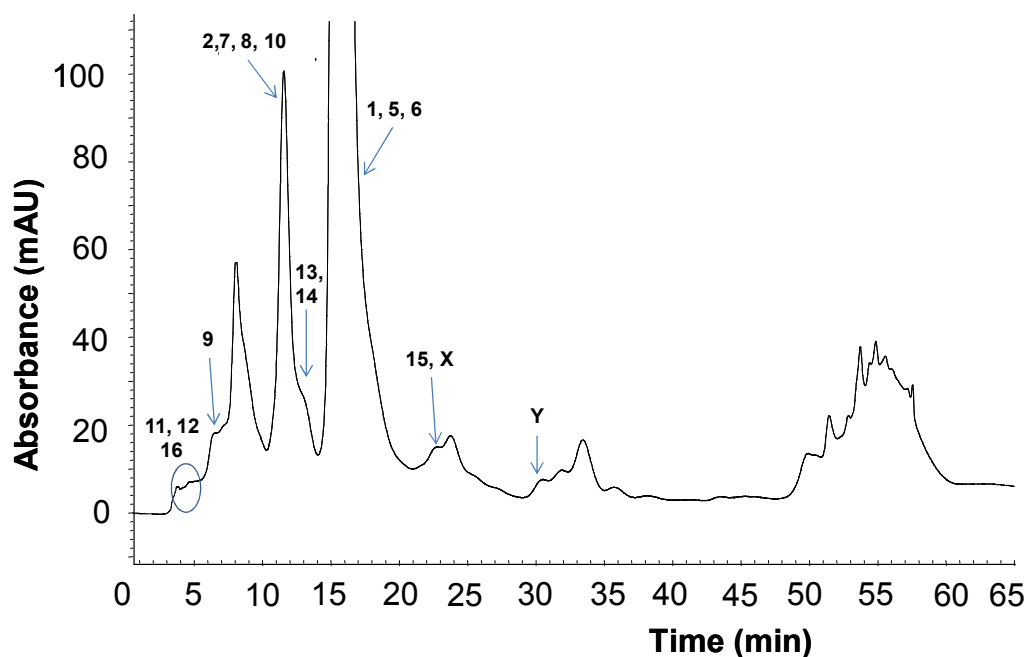


Figure 1 UV chromatogram (288 nm) for HILIC analysis of an unfermented rooibos extract. Peak numbers correspond to Table 1.

HILIC separation using this off-line gradient afforded a peak capacity (n_C) of 27, calculated according to (Neue & Mazzeo, 2001; Neue, 2005):

$$n_C = 1 + \frac{t_G}{W_{ave}} \quad (2)$$

Where t_G represents the gradient time (*i.e.* time until highest % B is reached, 65 min) and W_{ave} the average width at baseline, estimated by actual measurement of the major peaks ($n = 5$) from the chromatogram of the unfermented rooibos sample (2.48 min). Clearly, HILIC does not provide high resolution separation of rooibos phenolics, although the group-type separation could prove valuable in combination with RP-LC in a LCxLC system.

3.1.2 RP-LC analyses

Conditions for the RP-LC analyses of rooibos tea phenolics were adapted from the optimised HPLC-PDA method reported in Chapter 3. The optimised method was adapted to a column length of 50 mm and temperature increased to 48°C (see experimental section 2.3.1 for the gradient details). The RP-LC separation of an unfermented rooibos extract under these conditions is illustrated in Figure 2.

Within the same phenolic subclass, an increase in the number of hydroxyl groups led to a reduction in RP-LC retention time as observed for luteolin-6-C-glucoside and luteolin-8-C-glucoside eluting before apigenin-6-C-glucoside and apigenin-8-C-glucoside. Furthermore, within the same phenolic subclass, an

increase in the degree of glycosylation also led to a reduction in RP-LC retention time. This was exemplified by quercetin-3-*O*-robinobioside (X) eluting before quercetin-3-*O*-galactoside (15) and quercetin-3-*O*-rutinoside (14) eluting before quercetin-3-*O*-glucoside (13). Flavone *C*-glycosides (5) and (6) eluted at lower retention times than the corresponding *O*-glycoside (10) (Table 1). These observations are in accordance with RP-LC results reported by Cuyckens & Claeys (2004) as well as Anderson & Markham (2006).

Contrary to HILIC, RP-LC provided clear separation based on both the glycosylation position and nature of the glycoside entity. Although some reports on RP-LC state that C8 isomers generally elute before their C6 homologues (Anderson & Markham, 2006; Abad-Garcia *et al.*, 2008), this elution order was only observed for apigenin-8-*C*-glucoside (7) and apigenin-6-*C*-glucoside (8). Luteolin-6-*C*-glucoside eluted before luteolin-8-*C*-glucoside and, in the fermented rooibos sample, the (*R*)/(*S*)-eriodictyol-6-*C*-glucosides (4a and b) also eluted before their C8 isomers (3a and b). For isomeric compounds differing only with regards to the nature of the glycoside moiety, galactosides eluted before glucosides (e.g. quercetin-3-*O*-galactoside (14) < quercetin-3-*O*-glucoside (13)).

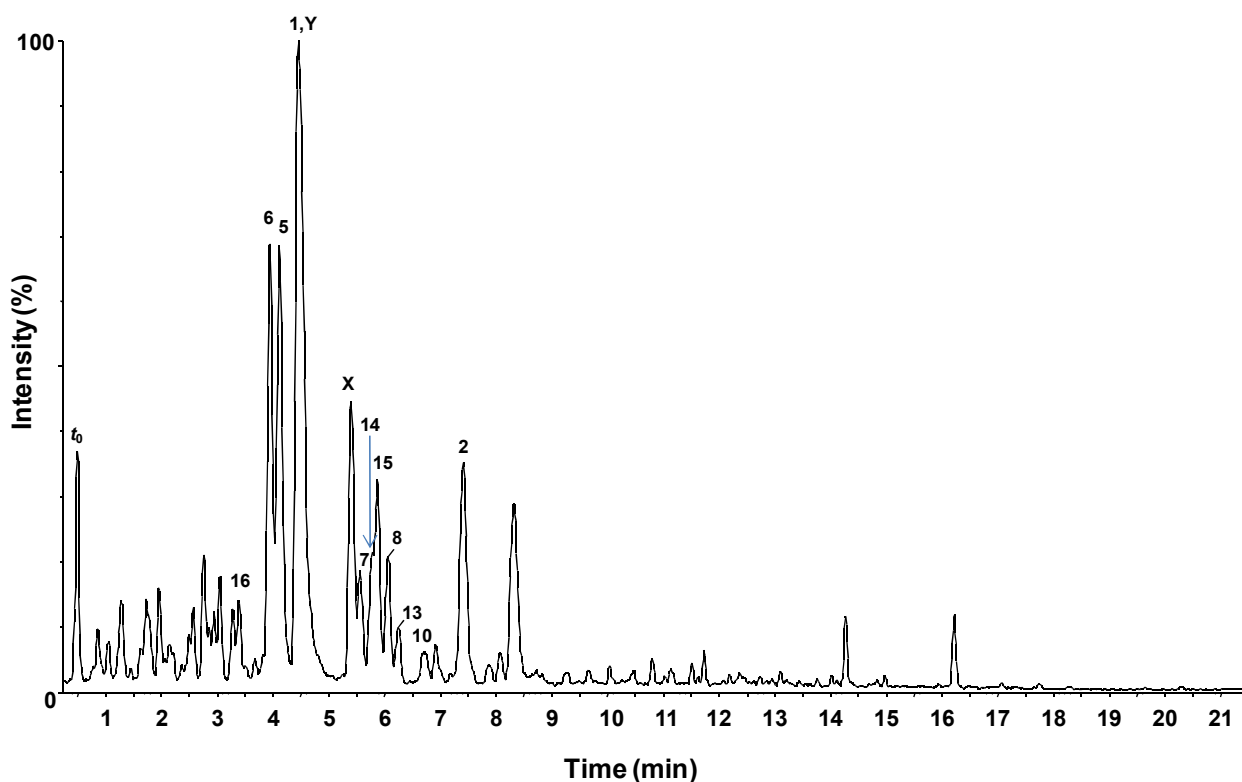


Figure 2 RP-LC-MS analysis of an unfermented rooibos extract using the off-line RP-LC gradient. Peak numbers correspond to Table 1.

The peak capacity afforded by the off-line RP-LC gradient was 96, calculated according to equation 2 with t_G and W_{ave} equal to 21.5 min and 0.228 min, respectively. W_{ave} was estimated from the chromatogram of a standard phenolic mixture ($n = 12$) representative of the actual rooibos samples.

3.1.3 LC-ESI-MS identification of rooibos phenolics

ESI-MS, conducted in positive and negative ionisation modes, permitted the identification of phenolic compounds in unfermented and fermented rooibos extracts under HILIC and RP-LC conditions as described in section 2.3.1. Identification was based on MS and MS/MS spectra, and by comparison of retention times and UV data with those of authentic standards when available (Table 1).

Table 1 Results for the LC-ESI-MS analysis of unfermented and fermented rooibos extracts.

Phenolic compound	No.	[M+H] ⁺	[M-H] ⁻	HILIC		RP-LC	
				t _R ^a , min	range-scaled retention factor	t _R ^a , min	range-scaled retention factor
Dihydrochalcones							
aspalathin ^b	1	453	451	14.80	0.405	4.46	0.283
nothofagin ^b	2	437	435	11.25	0.269	7.43	0.495
Flavanones							
(S)-eriodictyol-8-C-glucoside ^{c,d}	3a	451	449	11.10	0.263	3.14	0.188
(R)-eriodictyol-8-C-glucoside ^{c,d}	3b	451	449	11.10	0.263	3.32	0.201
(S)-eriodictyol-6-C-glucoside ^{c,d}	4a	451	449	11.10	0.263	2.36	0.133
(R)-eriodictyol-6-C-glucoside ^{c,d}	4b	451	449	11.10	0.263	2.74	0.160
Flavones							
luteolin-8-C-glucoside (orientin) ^b	5	449	447	15.35	0.426	4.12	0.258
luteolin-6-C-glucoside (isorientin) ^b	6	449	447	15.35	0.426	3.95	0.246
apigenin-8-C-glucoside (vitexin) ^b	7	433	431	11.17	0.266	5.57	0.362
apigenin-6-C-glucoside (isovitexin) ^b	8	433	431	11.17	0.266	6.07	0.398
luteolin ^b	9	287	285	7.07	0.109	12.37	0.848
luteolin-7-O-glucoside ^{b,d}	10	449	447	12.34	0.310	6.72	0.444
chrysoeriol ^b	11	301	299	4.38	0.006	14.50	1
Flavonols							
quercetin ^b	12	303	301	4.23	0	12.19	0.835
quercetin-3-O-glucoside (isoquercitrin) ^b	13	465	463	13.94	0.372	6.26	0.411
quercetin-3-O-galactoside (hyperoside) ^b	14	465	463	13.94	0.372	5.78	0.377
quercetin-3-O-rutinoside (rutin) ^{b,e}	15	611	609	22.12	0.685	5.89	0.385
Phenyl propanoid							
phenylpyruvic acid-2-O-glucoside ^b	16	327	325	4.42	0.007	3.50	0.214
Additional compounds							
quercetin-3-O-robinobioside ^c	X	611	609	22.12	0.685	5.42	0.351
C-8-hexosyl derivative of aspalathin ^{c,e}	Y	615	613	30.34	1	4.52	0.287

^a Retention times (min) represent the average retention of the analyte in the standard mixture, as well as unfermented and fermented rooibos extracts (where applicable) in positive and negative ionisation modes.

^b Identified using t_R, MS and MS/MS data compared to authentic standards.

^c Tentative identification based on UV, MS/MS spectra and relevant literature reports (refer to Chapter 3).

^d Only detected in the fermented rooibos extract.

^e Only detected in the unfermented rooibos extract.

3.1.4 Orthogonality

For successful LCxLC methods, the separations used in each dimension should be based on different separation mechanisms, *i.e.* be totally uncorrelated or orthogonal. Orthogonality may be estimated using the correlation coefficients (r^2) obtained from the range-scaled retention factors in each dimension (Liu *et al.*, 1995). The range-scaled retention factors for the rooibos phenolics under HILIC and RP-LC conditions (Table 1) were calculated based on LC-ESI-MS retention times according to:

$$\text{range-scaled retention factor} = \frac{t_R - t_0}{t_L - t_0} \quad (3)$$

Where t_R and t₀ are the retention times (min) of the considered compound and the first unretained compound, respectively, and t_L is the retention time of the last compound of interest (min).

The range-scaled retention factors for the rooibos phenolics under HILIC and RP-LC conditions were subsequently plotted against one another in the two dimensional separation space (Figure 3). The low correlation coefficient ($r^2 = 0.0074$) illustrates that the combination of these two separation modes provide a high degree of orthogonality and that their combination in a comprehensive two-dimensional set-up therefore holds promise for the improved separation of rooibos phenolics.

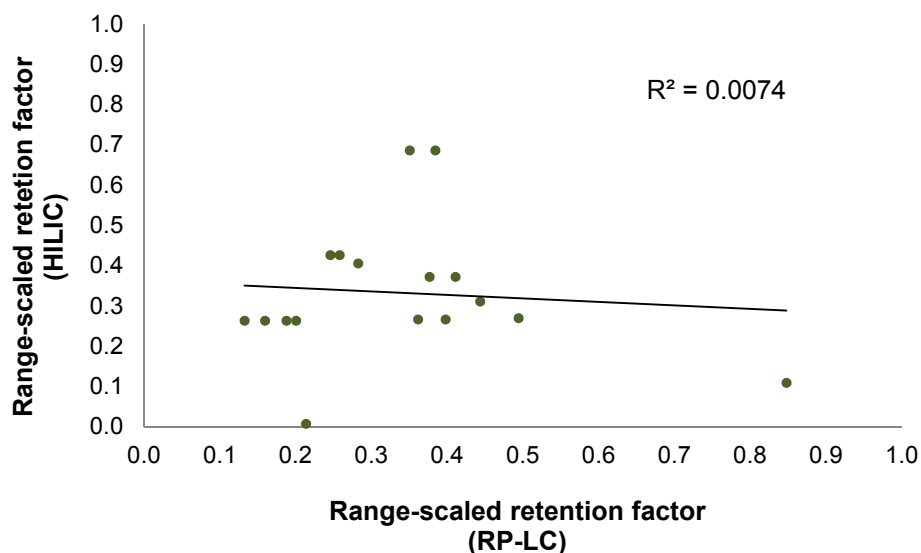


Figure 3 Plot of the range-scaled retention factors of each analyte under HILIC and RP-LC conditions.

3.2 HILICxRP-LC analyses

Following the successful separation of rooibos phenolics by HILIC and RP-LC and confirmation of the high degree of orthogonality provided by these separation modes, they were combined in both off-line and on-line comprehensive configurations. The rooibos extracts were freeze-dried and reconstituted in acetonitrile and methanol (70:30, v.v⁻¹) to allow higher loadability on the HILIC column and ultimately better sensitivity of the LCxLC separation. Maximally 10 μ L of the unfermented and fermented rooibos extracts could be injected on the HILIC column without significant peak distortion. Furthermore, the addition of acetic acid to the extracts was required to increase the solubility of the phenolic compounds in the organic solvents and to avoid on-column precipitation.

3.2.1 Off-line HILICxRP-LC analysis of rooibos phenolics

For off-line analyses, one-minute fractions of the HILIC effluent ($n(f) = 70$), corresponding to 50 μ L each, were automatically collected and subsequently analysed by RP-LC. As the HILIC mobile phase is a strong solvent in RP-LC, maximally 2 μ L of the fractions were injected onto the secondary column to avoid band-broadening. The contour plot obtained for the off-line HILICxRP-LC analysis of an unfermented rooibos extract is illustrated in Figure 4. Note that, contrary to common practice, contour plots are represented with the second dimension separation on the x-axis since this facilitates visual interpretation with reference to RP-LC-MS analysis (see Figure 2).

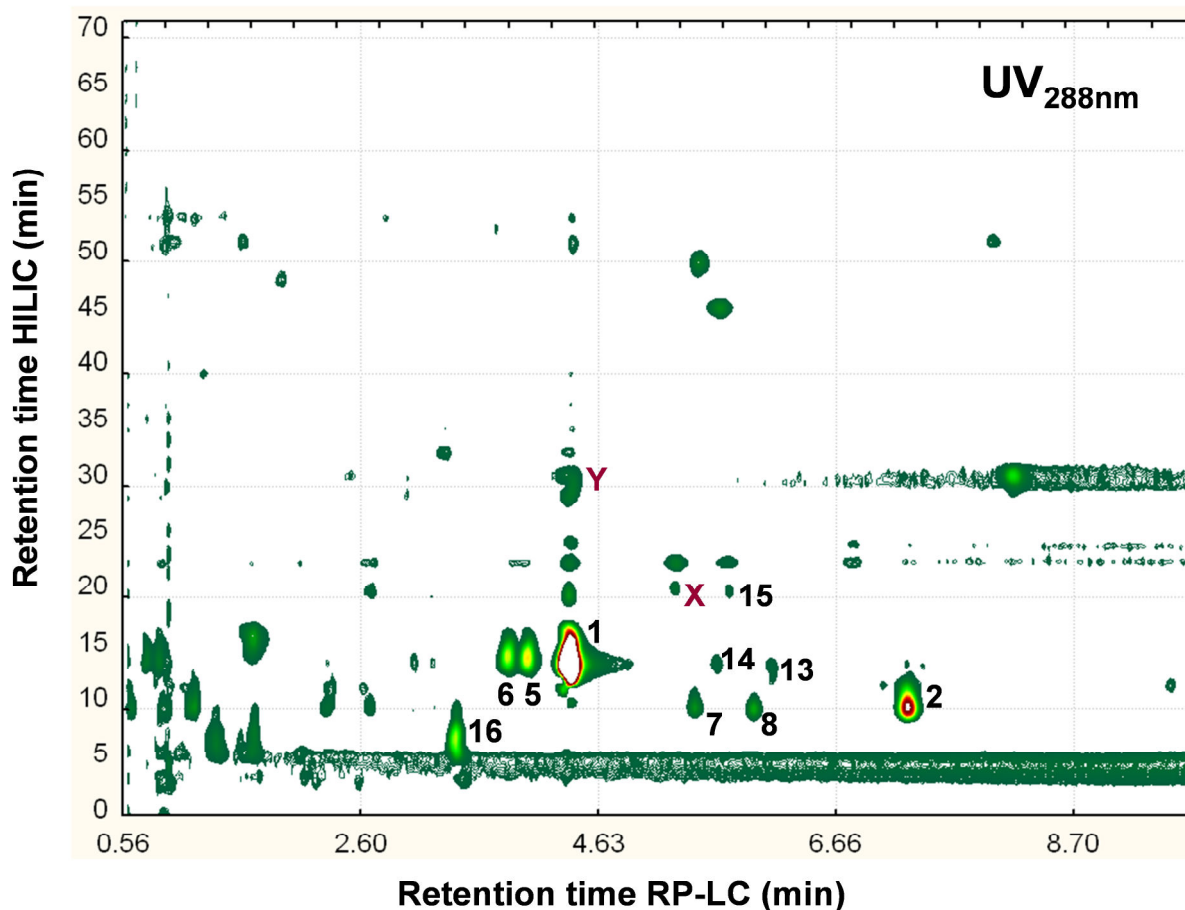


Figure 4 Contour plot obtained for the off-line HILICxRP-LC analysis of an unfermented rooibos extract. Peak numbers correspond to Table 1.

The improved resolution offered by the HILICxRP-LC system is clearly evident in Figure 4 – the combination of these two modes enabled the separation of compounds coeluting in either one of the one-dimensional separations. For example, aspalathin (1) and compound Y (the C-8-hexosyl derivative of aspalathin) coelute in RP-LC, but are clearly separated in the HILIC dimension. On the other hand, compound X (quercetin-3-O-robinobioside) and quercetin-3-O-rutinoside (15) exhibit perfect coelution in HILIC, but are separated by RP-LC. These examples are of particular interest as these two sets of compounds exhibit identical UV-VIS spectra, making it impossible to distinguish between them by 1-D HPLC with PDA detection.

Off-line HILICxRP-LC afforded a theoretical peak capacity ($n_{C,2D}$) of 2599, as calculated from the product of the peak capacities in the first ($n_{C,1}$) and second ($n_{C,2}$) dimensions (Guiochon *et al.*, 1983; Giddings, 1987). However, the practical peak capacity is always lower than the theoretical maximum as factors such as orthogonality and under-sampling need to be taken into account. Liu *et al.* (1995) proposed a geometric approach to factor analysis in which a correlation matrix was developed from the solute retention parameters in 2-D chromatography. In this method, the effective area of the 2-D space covered by the eluting peaks is used as a representative value of the orthogonality of the system. This may be calculated using the correlation coefficient of the range-scaled retention factors in each dimension (Figure 3). A subtraction factor is then applied to the theoretical peak capacity in order to determine the true practical value (equations 4-7). By taking orthogonality into account, the practical peak capacity of the off-line

separation was calculated according to Liu *et al.* (1995) as 2503. Clearly the HILIC and RP-LC separations used here are highly orthogonal, which results in practical peak capacities close to the theoretical values.

$$n'_{C,2D} = n_{C,1} n_{C,2} - 0.5 [n_{C,2}^2 \tan(\gamma) + n_{C,1}^2 \tan(\alpha)] \quad (4)$$

Where:

$$\gamma = \frac{\pi}{2} - \alpha - \beta \quad (5)$$

With:

$$\alpha = \alpha' \left(1 - \frac{2\beta}{\pi}\right) = \left[\tan^{-1} \left(\frac{n_{C,2}}{n_{C,1}}\right)\right] \left(1 - \frac{2\beta}{\pi}\right) \quad (6)$$

$$\beta = \cos^{-1} \sqrt{r^2} \quad (7)$$

r^2 = the correlation coefficient of the range-scaled retention factors in each dimension (refer to Figure 3).

Furthermore, in order to maintain maximum first-dimension resolution in a comprehensive configuration, it is generally required that the peaks eluting from the first dimension column should be sampled at least three to four times across their width to avoid loss of first dimension resolution due to under-sampling (Murphy *et al.*, 1998; Seeley, 2002; Horie *et al.*, 2007). Li *et al.* (2009) derived an accurate equation for the effective 2-D peak capacity taking under-sampling into account:

$$n'_{C,2D} = \left(\frac{n_{C,1} n_{C,2}}{\sqrt{1 + 3.35 \left(\frac{t_{G,2} n_{C,1}}{t_{G,1}}\right)^2}} \right) \quad (8)$$

Where $t_{G,2}$ and $t_{G,1}$ represent the second dimension separation cycle time (*i.e.* gradient time + re-equilibration time) and first dimension gradient time, respectively.

In off-line LCxLC, the first dimension sampling time is independent of the second dimension cycle time, and therefore under-sampling is generally not a problem, provided that a sufficiently short sampling time is selected. Very high sampling rates are theoretically possible, the limiting factor being the maximum feasible total analysis time. The sampling time of 1 min employed in this study was sufficient to provide a minimum of two fractions per first dimension peak, facilitated by the relatively broad peaks eluting from the HILIC column. By taking under-sampling into account, the practical peak capacity as calculated using equation 8 was 2069.

The effective peak capacities of the HILICxRP-LC separation, taking both orthogonality and under-sampling into account, clearly illustrate the considerable benefit of off-line comprehensive 2-D LC analysis, when compared to the peak capacities obtained in each one-dimensional separation. Despite the long total analysis time (~35 h; equation 9), the practical peak capacity afforded by off-line HILICxRP-LC is much higher than the maximum peak capacity attainable using a single dimensional analysis.

$$t_{\text{total,off-line}} = t_{G,1} + n(f) \times t_{C,2} \quad (9)$$

With $t_{G,1}$ the gradient time in the first dimension (min), $t_{C,2}$ the cycle time in the second dimension (min), and $n(f)$ the number of fractions analysed.

The contour plot obtained for the off-line HILICxRP-LC analysis of a fermented rooibos extract is illustrated in Figure 5. Figures 4 and 5 allow visual comparison of the two samples, in which large qualitative differences were found to exist. The fermented extract contained diastereomeric mixtures of (*S*)- and (*R*)-eriodictyol-6-*C*-glucoside (4a and 4b, respectively) and (*S*)- and (*R*)-eriodictyol-8-*C*-glucoside (3a and 3b, respectively), which are formed as intermediates during the oxidative conversion of aspalathin to its corresponding flavones, isoorientin and orientin (Koeppen & Roux, 1965; Marais *et al.* 2000; Krafczyk & Glomb, 2008). The fermented extract also contained luteolin-7-*O*-glucoside (10), which is absent in the unfermented extract. On the other hand, compounds Y and (15) were detected only in the unfermented rooibos extract. It should however be noted that the plant material used in this study did not originate from the same plant and hence the observed differences may not only be attributable to the fermentation process, but also to natural variation in rooibos plant material (refer to Chapter 3).

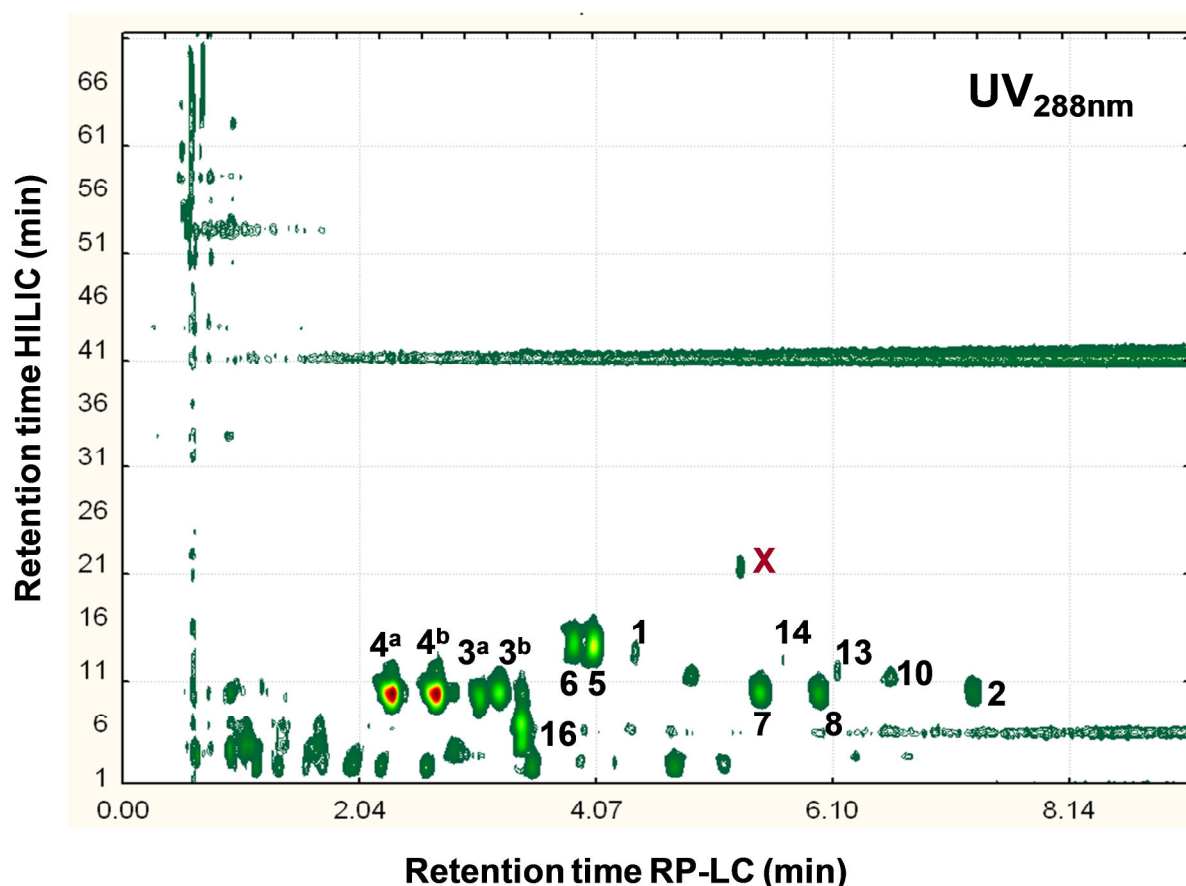


Figure 5 Contour plot obtained for the off-line HILICxRP-LC analysis of a fermented rooibos extract. Peak numbers correspond to Table 1.

3.2.2 On-line HILICxRP-LC analysis of rooibos phenolics

Following the successful off-line coupling of the two separation methods, an on-line approach was also investigated. On-line LCxLC provides several potential benefits compared to off-line methods such as automation, better reproducibility and operation reliability, and minimal risk of analyte degradation (Kalili, 2009). Before on-line coupling of the two methods, each one-dimensional separation was optimised individually to maximise the overall peak capacity.

For on-line LCxLC analyses, the analysis speed of the second dimension is of utmost importance – the second dimension separation has to be fast to enable sufficient sampling of the first dimension peaks, whilst still affording acceptable resolution. The time available for analysis (and re-equilibration when a gradient is applied) in the second dimension is therefore equal to the sampling period (Francois *et al.*, 2009). In this study a cycle time of 2 min was employed and hence the optimised gradient of the off-line RP-LC separation had to be adapted to an analysis time of 2 min. The 50 mm 1.8 μm Zorbax column operated at 48°C was ideally suited for these fast analyses as the short column length and small particle size permitted the use of higher flow rates ($F = 1.2 \text{ mL}\cdot\text{min}^{-1}$) without a loss in efficiency.

Different volumes of the samples dissolved in HILIC solvents were injected onto the RP column to determine the maximum allowable injection volume without compromising chromatographic performance. The maximum injection was found to be 2 μL , which, for a 2 min cycle time, implied a flow rate of 1 $\mu\text{L}\cdot\text{min}^{-1}$ in the first dimension. This would translate into analyses performed at flow rates far below the optimal values (corresponding to $\sim 50 \text{ }\mu\text{L}\cdot\text{min}^{-1}$ on the 1 mm i.d. column), resulting in important loss of efficiency and first dimension resolution, in addition to excessive analysis times. Therefore, a flow rate of 25 $\mu\text{L}\cdot\text{min}^{-1}$ was selected as suitable compromise in terms of practical analysis times and efficiency. To ensure an injection volume of 2 μL on the secondary column, a 1:25 split was employed prior to the two-position/ten-port switching valve (Figure 6) used as interface to hyphenate the two dimensions.

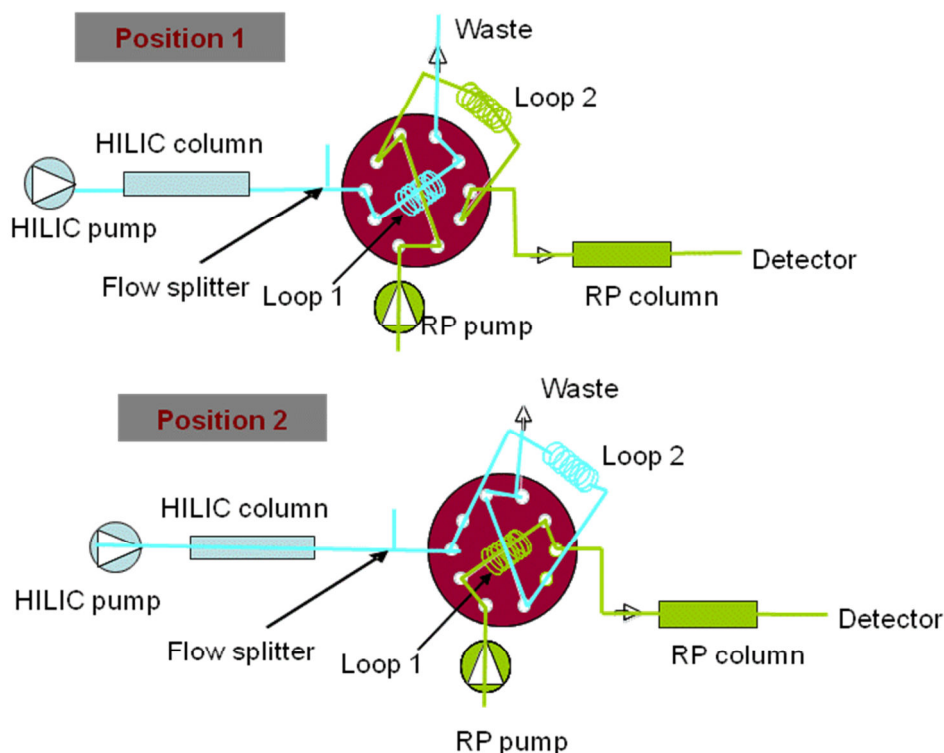


Figure 6 Schematic illustration of the instrumental configuration used for the on-line HILICxRP-LC analysis of rooibos phenolics.

The switching valve was equipped with two 5 μ L sampling loops, which were alternately used for the collection and re-injection of the first dimension fractions onto the secondary column. Although the splitting of the first dimension effluent results in decreased sensitivity, the reduced on-column dilution of the 1 mm i.d. column and the use of fast second dimension analyses ensured sufficient sensitivity for the on-line system (see Figure 7).

The contour plot obtained for the on-line HILICxRP-LC analysis of an unfermented rooibos extract is illustrated in Figure 7. This plot is characterised by much lower resolution compared to off-line HILICxRP-LC, yet still afforded a theoretical peak capacity of 1075. This difference may largely be attributed to lower second dimension peak capacity (40 on-line vs 96 off-line; Table 2) due to the 2 min analysis time. This was exacerbated by the fact that the RP-LC analysis had to be conducted under gradient conditions, a prerequisite for the analysis of rooibos samples in order to avoid 'wrap around' for highly retained species. The 'wrap around' effect is characterised by analytes from the previous fraction eluting in the separation space of the next transfer (when the retention time of an analyte exceeds the sampling time) and results in chaotic band displacement (Grushka & Grinberg, 2006). In fact, the effective analysis time in the second dimension was only 1.2 min, with 0.8 min being required for column re-equilibration. Another factor contributing to the low second dimension peak capacity is that the most important rooibos phenolics elute in a small window of ca 0.2 min (Figure 7).

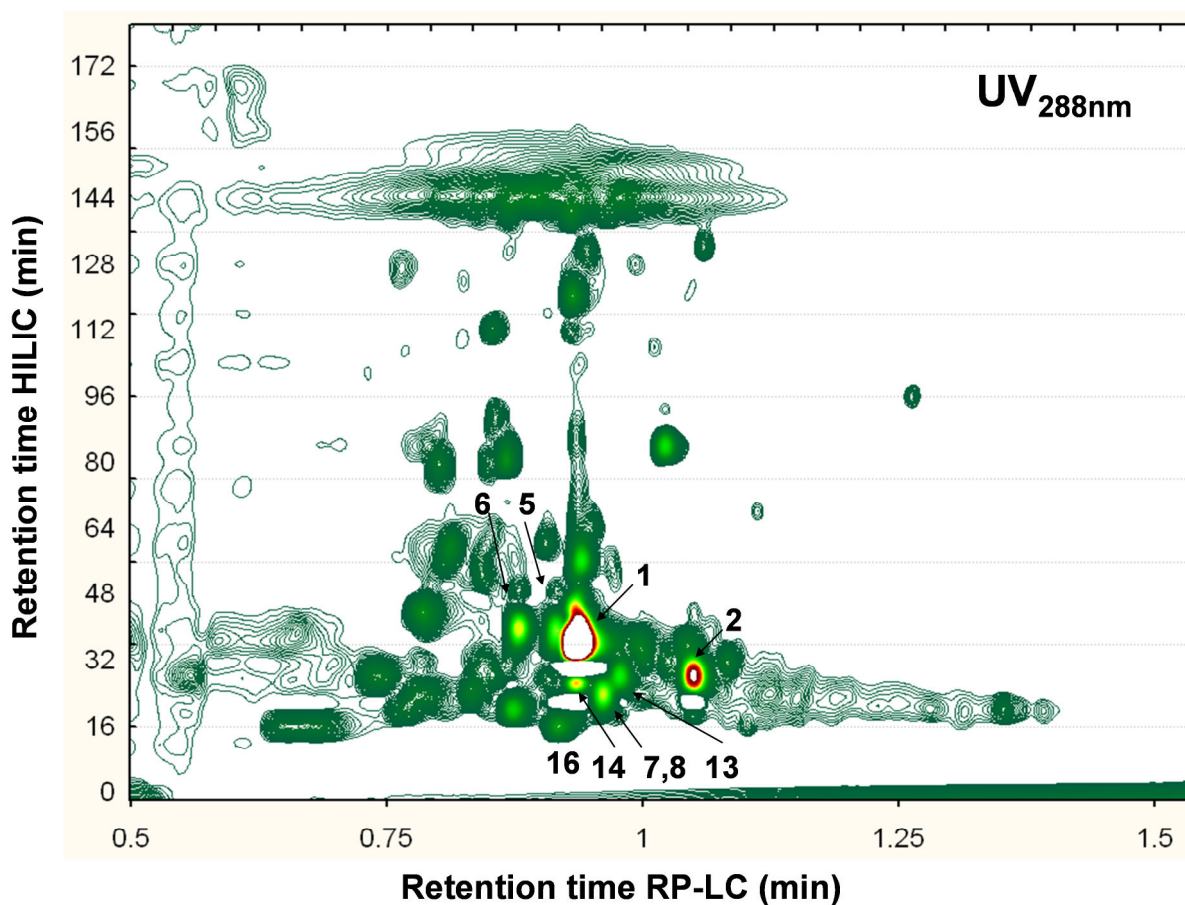


Figure 7 Contour Plot obtained of the on-line HILICxRP-LC analysis of an unfermented rooibos extract. Peak numbers correspond to Table 1.

However, a significant advantage afforded by the on-line approach is a reduction in the total analysis time. The total analysis time of the on-line approach, calculated according to equation 10, is only 2.2 hours (*i.e.* ~16 times faster than the off-line approach).

$$t_{\text{total,on-line}} = t_{G,1} + t_{C,2} \quad (10)$$

With $t_{G,1}$ the gradient time in the first dimension (min) and $t_{C,2}$ the cycle time in the second dimension (min).

By taking orthogonality (Liu *et al.*, 1995) and under-sampling (Li *et al.*, 2009) into account, the practical peak capacities for the on-line system were calculated as 905 and 856, respectively. In the on-line comprehensive set-up, under-sampling is more important than under off-line conditions. However, due to the fact that the first dimension HILIC peaks were relatively broad in the on-line set-up, a sampling time of 2 min did not result in significant reduction of the practical peak capacity.

3.2.3 Evaluation of the off-line and on-line HILIC x RP-LC systems

In order to compare the off-line and on-line comprehensive 2-D LC modes, one may consider the peak capacity production rate, defined as the practical peak capacity afforded by the 2-D LC separation, divided by the total analysis time (Horváth *et al.*, 2009). A summary of the parameters used to calculate the practical peak capacities and the peak capacity production rates of the off-line and on-line HILICxRP-LC systems is presented in Table 2. The peak capacity production rates were *ca* 1.09 and *ca* 6.67 for the off-line and on-line modes, respectively. Although the rate is much higher for the on-line system, the absolute value of the practical peak capacity is more than twice as high for the off-line mode

Table 2 Summary of the parameters used to calculate the practical peak capacities and the peak capacity production rates of the off-line and on-line HILICxRP-LC systems.

Parameter	Off-line	On-line
^a HILIC peak capacity, $n_{C,1}$	27	27
^a RP peak capacity, $n_{C,2}$	96	40
^b Correlation coefficient, r^2	0.0074	0.0990
^c Theoretical peak capacity, $n_{C,2D}$	2599	1075
^d Practical peak capacity, $n'_{C,2D}$	2503	905
^e Practical peak capacity, $n'_{C,2D}$	2069	856
Average practical peak capacity	2286	880.5
^f Total analysis time (t_{total} , min)	2095	132
^g Peak capacity production rate	1.09	6.67

^a Calculated according to Neue & Mazzeo (2001) and Neue (2005); equation 2.

^b Calculated according to Liu *et al.* (1995); equation 3.

^c Calculated according to Guiochon *et al.* (1983) and Giddings (1987); equation 1.

^d Calculated according to Liu *et al.* (1995); equations 4-7 to account for orthogonality.

^e Calculated according to Li *et al.* (2009); equation 8 to account for under-sampling.

^f Total analysis times for the off-line and on-line systems calculated according to equations 9 and 10, respectively (De Villiers & Kallii, 2011).

^g Peak capacity production rate = average $n'_{C,2D} / t_{\text{total}}$ (Horváth *et al.*, 2009).

4. Conclusions

In the current study, HILICxRP-LC methods have been successfully developed for the analysis of a diverse range of unique phenolic compounds in rooibos samples. HILIC provided separation according to polarity, with flavones generally eluting before flavonols, and flavonoid monoglycosides eluting before corresponding diglycosides of the same parent aglycone. In HILIC, *O*-glycosides also eluted before *C*-glycosides of the same parent aglycone, while both the position of glycosylation and the nature of the glycosidic moiety did not influence retention. In contrast, RP-LC on a C18 column provided separation according to hydrophobicity and clear separation based on both the glycosylation position and nature of the glycosidic entity. On-line HILICxRP-LC offers the advantage of automation and less risk of sample alteration, while the off-line combination offers increased resolution, albeit at the cost of longer analysis times. This approach shows promise for the in-depth investigation of rooibos phenolics as a vast amount of additional information may be gained on minor constituents coeluting in 1-D HPLC methods. Further optimisation, particularly of the 1st dimension separation, is however required.

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CHAPTER 6

General Discussion and Conclusions

In this study, an optimised HPLC-PDA method was successfully developed for the quantitative determination of the 15 principal phenolic constituents of rooibos tea. The target phenolic compounds were representative of five different phenolic subclasses including dihydrochalcones (aspalathin and nothofagin), flavones (orientin, isorientin, vitexin, isovitexin, luteolin, luteolin-7-*O*-glucoside and chrysoeriol), flavonols (quercetin, isoquercitrin, hyperoside and rutin), a hydroxycinnamic acid (ferulic acid) and a phenylpropanoid (phenylpyruvic acid glucoside, PPAG). The goal was to develop a quantitative method for use on conventional HPLC instrumentation, as these instruments are typically used in laboratories where rooibos is routinely analysed. A sub-2 μm reversed phase C18 column was selected for method development due to the well-known benefits of these phases for fast separation. The connection tubing volume of the HPLC system was minimised to reduce extra-column band broadening in order to exploit the full performance potential of these particles (Fountain *et al.*, 2009). Kinetic evaluation of this phase compared to a conventional 5 μm column demonstrated higher optimal flow rates and higher efficiency per unit column length for the 1.8 μm column. Furthermore, for rooibos phenolic compounds, the relatively low optimal flow rates (compared to common test analytes) meant that optimal use of the 1.8 μm column was possible on instrumentation with maximum pressure capabilities of 400 bar. Higher operating temperatures further improved the kinetic performance of this column.

A systematic approach towards optimising selectivity on a high-efficiency column was adopted. Selectivity effects as a function of mobile phase composition and temperature were found to be complementary and therefore simultaneous optimisation of these two variables was used to improve band spacing and resolution of the 15 phenolic standards on the 1.8 μm phase. The rooibos phenolic mixture represented an irregular sample, characterised by retention reversals with changes in mobile phase composition and/or temperature, which presented several challenges during method development. Most notably, complete separation of vitexin, isovitexin, isoquercitrin, hyperoside and rutin proved to be problematic and is marred by minimum resolution of 1.25 and 0.92 for the critical pairs hyperoside + rutin and rutin + isovitexin, respectively. The separation of these compounds was also extremely temperature sensitive, which impacted negatively during method transfer between instruments, as the extent of solvent preheating and absolute column oven temperatures differed for different systems. Optimal separation of the target analytes was obtained on the 1.8 μm column thermostatted to 37°C within 37 min (total analysis time: 50 min).

Although this method showed some limitations in terms of robustness, it is the first reported method suitable for the quantification of all 15 target analytes within a single chromatographic run on conventional HPLC instrumentation. Furthermore, the total analysis time of 50 min is relatively short, making it ideally suited for high through-put quantitative analyses.

For the analysis of unfermented and fermented rooibos samples, clear separation of another flavonol *O*-diglycoside, tentatively identified as quercetin-3-*O*-robinobioside, could also be achieved. It is suspected that under the RP-LC conditions previously employed by the ARC for the quantitative analysis of rooibos this compound exhibited perfect co-elution with rutin (quercetin-3-*O*-rutinoside). Due to identical UV-VIS spectra it would have been impossible to distinguish between them since peak purity testing using a PDA detector would have indicated a pure peak. From a quantitative perspective, over-estimation of rutin would therefore have occurred and this hypothesis was confirmed experimentally. The combined content values of quercetin-3-*O*-robinobioside and rutin, as obtained using the newly developed method, were in good accordance with the content value for rutin determined with the previous methods employed by the ARC. In this sense, the

high content values reported for rutin in other studies (Bramati *et al.*, 2002, 2003) are also brought into question. Quantitative data for a 'rutin isomer' have, however, been presented by other authors (Kazuno *et al.*, 2005; Stalmach *et al.*, 2009) and it is supposed that this compound is quercetin-3-*O*-robinobioside. This is further substantiated by the relative ratios reported for quercetin-3-*O*-robinobioside/'rutin isomer' and rutin – the former is present at much higher concentrations, whilst the latter compound was even found to be absent in some of the samples analysed in this study. Note that throughout this study quercetin-3-*O*-robinobioside was quantified as rutin-equivalents since no standard was available for this compound. This was deemed valid as these compounds are both *O*-hexose-rhamnosides of quercetin and is therefore expected to exhibit near-identical UV detector response. These flavonol *O*-diglycosides merely differ with regard to the internal glycosidic entity – in quercetin-3-*O*-robinobioside the internal glycosidic entity is a galactoside, while in rutin (quercetin-3-*O*-rutinoside) this is a glucoside. Considering the flavonol *O*-monoglycosides hyperoside (quercetin-3-*O*-galactoside) and isoquercitrin (quercetin-3-*O*-glucoside), the galactoside entity is also present at much higher concentrations (roughly double) than its glucoside equivalent. This highlights an interesting aspect for further research, pertaining to the differential biosynthesis of the galactoside/glucoside flavonoids in *Aspalathus linearis* plant material.

The aforementioned points exemplified the importance of using a representative sample during method development. This is especially true for complex botanical samples such as rooibos, due to large qualitative and quantitative differences stemming from the fermentation state and natural variation. The uncontrolled fermentation process induces additional variation between fermented rooibos teas, as rooibos tea is typically consumed in its fermented 'oxidised' form due to development of the sought-after organoleptic properties associated with fermentation. Therefore, in the first instance, a small subset of both unfermented and fermented rooibos samples was analysed to ensure variation and complexity that could be expected in routine analysis.

Coupling of the optimised method to ESI-MS confirmed that no co-elution for the 15 target analytes occurred in the unfermented and fermented rooibos infusions. MS and tandem MS detection furthermore enabled the tentative identification of 13 additional phenolic constituents in rooibos. These included four flavanones ((*R*)- and (*S*)-eriodictyol-6-*C*- β -*D*-glucopyranoside and (*R*)- and (*S*)-eriodictyol-8-*C*- β -*D*-glucopyranoside) and a cyclic dihydrochalcone (aspalalinin), only detected in fermented rooibos, as well as the flavonol *O*-diglycoside, quercetin-3-*O*-robinobioside, present in both unfermented and fermented samples. These compounds have previously been identified in rooibos by NMR (Shimamura *et al.*, 2006). The presence of the symmetric 6,8-di-*C*-glycosyl flavones (luteolin-6,8-di-*C*-hexoside and apigenin-6,8-di-*C*-hexoside) and the asymmetric 6,8-di-*C*-glycosyl flavones (carlinoside, neocarlinoside, isocarlinoside) in rooibos (Breiter *et al.*, 2011; Iswaldi *et al.*, 2011) was also tentatively re-confirmed in this study. Based on MS/MS data, a fourth asymmetric di-*C*-glycosyl flavone, proposed to be an isomer of isocarlinoside, was also identified in the present study.

Moreover, ESI-MS and tandem MS permitted the identification of another phenolic compound (MW 614) in rooibos, displaying structural characteristics in accordance with a *C*-8-hexosyl derivative of aspalathin. This is the first report on the presence of a 6,8-di-*C*-glycosyl dihydrochalcone and, seeing as aspalathin is a bioactive compound unique to rooibos, it could have an important contribution to biological activity for instance. This compound eluted within close proximity of aspalathin and exhibited partial coelution with ferulic acid. This was, however, not perceived as a problem as the content of this compound in the fermented infusions was very low, leading only to slight over-estimation of ferulic acid, while the unfermented

samples analysed did not contain detectable limits of ferulic acid. In plants, most phenolic acids are linked through ester, ether, or acetal bonds to cellulose, proteins, lignin, flavonoids, glucose, terpenes and so forth (Macheix *et al.*, 1990). Our results would therefore suggest that ferulic acid is released from bound form during fermentation.

The optimised method therefore demonstrated its suitability for the quantitative analysis of real-life samples and was subsequently validated to ensure accuracy and precision of the generated data. The method was successfully validated in terms of linearity and range ($r^2 > 0.9999$), as well as intra- and inter-day analytical precision (% RSD < 4.4%). The stability of the analytes of interest was also monitored by considering the respective peak areas at eight time-points over a 27 hour period. The addition of ascorbic acid greatly improved the stability of the analytes over the studied time period (% RSD < 2%) and therefore facilitated the use of an autosampler for subsequent routine analyses.

The optimised method was subsequently applied to the analysis of a large number of fermented rooibos samples ($n = 114$), representative of different production seasons (2009, 2010 and 2011) and quality grades (A, B, C and D). These samples were obtained from different geographical locations and different producers to capture as much potential variation in the phenolic composition as possible. Aqueous infusions of fermented rooibos were prepared in duplicate according to 'cup-of-tea' strength, as the second research aim was to obtain representative content values of the major phenolic constituents present in a 'cup-of-tea' rooibos infusion. The average values for the flavonoid and phenolic acid content of a 'cup-of-tea' rooibos infusion will for the first time enable the calculation of their contribution to dietary intake. Estimating flavonoid and phenolic acid intake is the first step toward documenting the protective effects of these two phenolic groups in rooibos against the risk of lifestyle diseases (Neveu *et al.*, 2010). Data created for a large number of infusions of different batches of rooibos will serve as basis for the compilation of a food composition table, giving the average flavonoid and phenolic acid content of a 'cup-of-tea' rooibos infusion. A short-term output of the study will be making these data available on the website of the South African Rooibos Council for use by industry and dieticians.

In the first instance, the effect of steam-pasteurisation on the phenolic composition of rooibos infusions was investigated as rooibos plant material is steam-pasteurised prior to packaging to ensure a product of high microbiological quality (Joubert *et al.*, 2008). The results indicated that steam-pasteurisation led to a significant reduction in the mean concentration values (mg.L^{-1}) of most phenolic compounds in the infusions partly attributed to a reduction in the soluble solids content. However, by expressing the data on a soluble solids (SS) basis (g.100 g SS^{-1}), significant differences were still found between the mean values of the phenolic compounds in the unpasteurised and pasteurised rooibos infusions. The pasteurised rooibos infusions contained higher levels of all phenolic compounds on a SS basis, which demonstrate that the monomeric polyphenols extracted from the pasteurised plant material represent a larger percentage of the total soluble solids of these infusions. From the perspective of the consumer, the results practically signify that a typical 'cup-of-tea' prepared from pasteurised rooibos will contain a lower amount of phenolic compounds. Steam-pasteurisation is, however, a prerequisite and therefore composition data for the database should reflect those of pasteurised rooibos as this is the form reaching the consumer.

The major phenolic constituents of steam-pasteurised, fermented rooibos were found to be isoorientin (15.03 mg.L^{-1}) and orientin (10.84 mg.L^{-1}), while quercetin-3-O-robinobioside (8.34 mg.L^{-1}), PPAG (6.91 mg.L^{-1}) and aspalathin (5.84 mg.L^{-1}) were also present in substantial quantities. Ferulic acid was quantified at a low mean concentration of 1.51 mg.L^{-1} , which represents a slight over-estimation due to

partial co-elution of ferulic acid with a C-8-hexosyl derivative of aspalathin in some of the samples (as discussed above). This is the first report on representative, quantitative data for quercetin-3-O-robinobioside, PPAG and ferulic acid in 'cup-of-tea' rooibos infusions. Using a preliminary HPLC method developed during this study prior to final optimisation, Koch (2011) reported content values for PPAG in rooibos, but the sample size was significantly smaller ($n = 69$) and all samples were from the same production year (2009). Furthermore, the samples of Koch (2011) were prepared at a slightly stronger cup-strength as they were subjected to sensory evaluation.

Luteolin-7-O-glucoside was only present in trace quantities in steam-pasteurised fermented rooibos infusions and could therefore not be accurately quantified. Furthermore, the highly retained flavonoid aglycones quercetin, luteolin and chrysoeriol were also present in trace amounts and eluted on a polymer 'hump' at the end of the chromatogram and could not be accurately quantified. It is therefore recommended that, in the subsequent analysis of aqueous infusions, the gradient be 'ramped' following elution of the last known standard compound of interest (nothofagin, $t_R \sim 16.5$ min). By speeding up the gradient after the crucial linear segment comprising 10-14.8% B (2-19 min) to ensure that all compounds are eluted from the column, an analysis time of *ca* 35 min (including re-equilibration) may be obtained for the compounds of interest. This will increase sample through-put and reduce operating costs and waste generation, the latter being an important environmental consideration as the acetonitrile used in the binary mobile phase is toxic. However, if different extraction procedures are employed or if selective fractions containing high concentrations of the less polar aglycones are to be analysed, the complete gradient as reported in this study should be used.

Production season (2009, 2010 and 2011) was found to have no clear effect on the levels of individual phenolic compounds in aqueous rooibos infusions. This may be attributed to additional variation resulting from the use of samples originating from different geographical regions. It is therefore recommended that, in subsequent studies, samples should be harvested from a defined geographical region so that the effect of production season may be better elucidated. Ideally samples from the same plantations should be analysed over several production seasons. This was however not within the scope of the current study.

The quality grade allocated to the rooibos samples by expert graders showed a slight correlation with the mean values of the individual phenolic compounds and the soluble solids content. High quality tea (grades A and B) was associated with higher levels of phenolic compounds and soluble solids than low quality tea (grades C and D). This demonstrates that, although the current grading system employed in the industry is based exclusively on sensory evaluation, it does, to a certain extent, reflect the chemical compositional data. Further studies are required to determine the role of the phenolic compounds in specific quality criteria.

Owing to the large number of representative samples analysed here, the generated data accurately encompass natural variation and are therefore suitable for inclusion in food composition tables. Representative content values for rooibos, specifically, will enable the comparison of polyphenol content with other teas and tea-like infusions as well as the evaluation of the relative contribution of rooibos to the intake of a specific polyphenol as compared to other herbal beverages and/or food sources.

The representative content values will furthermore provide typical chemical 'fingerprints' for product authenticity and quality control purposes. 'Fingerprinting' addresses the complex issues of comparison between products of similar nature but different origins or different sources. In most cases, the differences

searched for are in terms of the presence and/or relative concentrations of a few components of complex mixtures, components that are most often unknown early on in the project. In this sense, it would be too time-consuming and costly to identify and quantify all the components of the samples considered and therefore chromatograms of samples are often compared. This method relies heavily on data analysis and on the use of chemometric methods, particularly multi-variate data analysis (Van Mispelaar *et al.*, 2005), and therefore represents a potentially useful tool in the authentication of rooibos extracts used in nutraceutical products on the basis of their flavonoid 'fingerprints'. The 1-D method developed for rooibos in this study would form the basis of such fingerprinting comparisons.

By ensuring a minimum amount of a specific 'marker compound', as determined for example by this optimised method, in combination with antioxidant activity, botanical standardisation of rooibos extract may also be improved as proposed by Ninfali *et al.* (2009). This will positively impact on the strict quality control measures required in obtaining Geographical Indication (GI) certification for rooibos. Future applications of this method may furthermore include selection of plant material for propagation of genotypes producing high levels of specific phenolic markers; quantification of the effect of geographical influences on composition; and correlating pharmacological activities with specific constituents.

The third research aim of this study entailed investigating the applicability of comprehensive two-dimensional liquid chromatography (LCxLC) for the analysis of rooibos phenolics. LCxLC offered improved resolution of the complex rooibos samples due to the increased peak capacity provided by complementary separations. The utilisation of HILIC in the first dimension and RP-LC in the second dimension presented a highly orthogonal combination by providing separation according to polarity and hydrophobicity, respectively. Their combination in off-line and on-line modes afforded practical peak capacities in excess of 2000 and 800, respectively, which are significantly higher than the maximum peak capacity obtainable in a 1-D separation. On-line HILICxRP-LC offered the advantage of automation and less risk of sample alteration, while the off-line approach was characterised by increased resolution, albeit at the cost of increased analysis times. In this regard, the peak capacity production rate for the on-line system was found to be much higher than that of the off-line mode, although the absolute value of the practical peak capacity will always be higher in the latter.

This approach showed promise for the in-depth investigation of rooibos phenolics as a vast amount of additional information may be gained on minor constituents coeluting in 1-D HPLC methods. Contour plots greatly facilitated the visual comparison of rooibos phenolic composition as a function of production parameters and therefore also has potential for 2-D 'fingerprinting'. This will enable the identification and characterisation of minor phenolic compounds possibly impacting on bioactivity, such as the phenolic acids for example. Further optimisation, particularly of the 1st dimension separation, is however required. Alternative HILIC phases should be evaluated to increase the resolving power of the first dimension separation. A further reduction in the first dimension column internal diameter (for example using capillary LC) would also avoid the need for splitting in on-line LCxLC. Stop-flow LCxLC can be explored as automated alternative to the off-line configuration to reduce the risk of analyte degradation. Future work could also include on-line LCxLC-ESI-MS analyses, which would facilitate identification.

The analysis of rooibos phenolics by HILICxRP-LC presented several practical challenges, pertaining specifically to sample preparation and mobile phase compatibility. Extensive sample preparation was required to ensure maximum concentration of phenolic compounds in the rooibos extracts to be analysed. This was partially facilitated by using freeze-dried samples of aqueous rooibos infusions and

performing solid phase extraction. As water is the strong eluent in HILIC, these samples had to be reconstituted in acetonitrile and methanol (70:30, v.v⁻¹). This was problematic as the freeze-dried samples were obtained from an aqueous matrix, leading to reduced solubility in less-polar organic solvents. In this regard, a small amount of acetic acid was added to the extracts to improve solubility and to avoid on-column precipitation. This was more of an issue in the fermented rooibos extract, due to the concomitant formation of polymeric compounds with the oxidation of aspalathin during fermentation (Krafczyk *et al.*, 2009). Alternative sample preparation techniques should therefore be investigated.

To conclude, in this study an HPLC-PDA method has been successfully developed for the quantitative analysis of the 15 principal phenolic constituents of rooibos. By applying this method to a large number of aqueous infusions of fermented rooibos, representative content values for the major flavonoids and ferulic acid present in a typical 'cup-of-tea' was obtained. Due to appropriate method of analysis, the generated data is suitable for inclusion in food composition databases, to which data were previously restricted to *Camellia sinensis* tea. The relative contribution of rooibos phenolics to dietary polyphenols may now be calculated which will facilitate a study on their association with health and disease. Further insight into the minor phenolic constituents is however required and in this regard HPLC in combination with MS and tandem MS as well as LCxLC separation have been shown to be particularly powerful analytical approaches for complex rooibos samples.

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