Rooibos fermentation-Characterising phenolic changes using chemometric analysis and kinetic modelling

by

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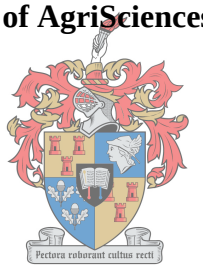

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DECLARATION

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SUMMARY

Rooibos is a popular herbal tea produced from *Aspalathus linearis*, a South African endemic fynbos plant species. Health-promoting properties associated with rooibos are closely linked to its phenolic composition. 'Fermentation' (oxidation) is essential to the development of characteristic sensory attributes, including colour, of traditional fermented rooibos, however oxidation reactions are detrimental to the quality of green rooibos. In this study, advanced chemometric methods were used to uncover the phenolic changes occurring in rooibos during fermentation in an unbiased manner. Chromatographic fingerprints of green, semi-fermented and fermented rooibos extracts were constructed using a recently improved high-performance liquid chromatography with diode-array detetction (HPLC-DAD) method. Pre-processing techniques were applied to eliminate unwanted variation that may be related to the nature of the data or the analytical instrument used, *i.e.* signal enhancement (baseline elimination and signal denoising), peak alignment, peak detection and integration, normalisation, centering and transformation. The 'size effect', an artefact related to the overall differences in concentration between samples, was eliminated by application of a new normalisation method called robust pair-wise log ratios (rPLR). rPLR also allowed for easy identification of phenolic compounds showing significant ($p<0.01$) changes between treatments. Results showed that all 56 peaks detected were significantly ($p<0.01$) changing between green, semi-fermented and fermented rooibos. A 30% change in peak area threshold was used to ensure that the selected markers showed reasonable degradation, which reduced their number to 34. Given that this is still a relatively large number of compounds, the number of phenolics examined in targeted analysis of rooibos fermentation is small in comparison to the number of compounds significantly changing. To gain insight into the reaction kinetics of rooibos phenolics during elevated temperatures and humid conditions, rooibos plant material was subjected to a 6 h simulated fermentation under highly controlled conditions, at four different temperatures between 37 and 50°C. Preceding the determination of the temperature dependence, two preliminary experiments were performed. Firstly, an extraction optimisation experiment showed that heating is not necessary for extraction and that a 5 min sonication period was sufficient. It was also necessary to investigate the role of endogenous rooibos enzymes since no direct evidence of their involvement in fermentation was available. For the second experiment, a dry heat treatment (170°C for 30 min) prior to simulated fermentation was postulated to partially inactivate the enzymes based on aspalathin and nothofagin content changes and differences in reaction rate constants between the control and heat-treated plant material. The fractional conversion reaction kinetics model, based on first-order kinetics, best described the degradation of aspalathin and nothofagin, as well as the formation of four eriodictyol-glucoside isomers, during simulated fermentation. The kinetic parameters estimated from the model (*i.e.* reaction rate constant and equilibrium concentration) suggested that the reactions proceeded faster, but, achieved a lower extent of degradation/formation at higher temperatures. Based on these results, it was postulated that enzymes were partially inactivated at higher fermentation temperatures or that there was a limiting factor during the simulated fermentation, *e.g.* oxygen depletion.

OPSOMMING

Rooibos is ‘n gewilde kruie-tee wat van *Aspalathus linearis*, ‘n endemiese Suid-Afrikaanse fynbos plantspesie, geproduseer word. Die gesondheidsvoordele van rooibos is geassosieer met sy fenoliese samestelling. “Fermentasie” (oksidasie) van die plantmateriaal is van kardinale belang vir die ontwikkeling van tradisionele rooibos se karakteristieke sensoriese eienskappe, inlsuitend kleur, maar oksidasiereaksies is nadelig vir die kwaliteit van groen rooibos. Chemometriese metodes is in hierdie studie gebruik om die fenoliese veranderinge wat tydens fermentasie plaasvind op ‘n onsydige manier te onthul. Chromatografiese vingerafdrukke van groen, semi-fermenteerde en gefermenteerde rooibos ekstrakte is saamgestel deur van ‘n onlangs verbeterde hoë druk vloeistof chromatografiese metode met ultraviolet-diode deteksie (HPLC-DAD) gebruik te maak. Data prosesseringstegnieke is toegepas on ongewensde variasie verwant aan die inherente eienskappe van die data of die analitiese instrument wat gebruik is uit te skakel. Dit het seinversterking (verwydering vab agtergrond en seingeraas), piek belyning, piek deteksie en integrasie, normalisering, sentrering en transformasie behels. Die “grootte effek”, wat ‘n gevolg is van oorhoofse verskille in konsentrasie tussen monsters, is ge-elimineer deur gebruik te maak van ‘n nuwe normaliseringsmetode bekend as robuuste paargewyse logaritmiese verhoudings (rPLR). Hierdie tegniek het ook die identifisering van fenoliese verbindings wat beduidend ($p<0.01$) tussen behandelings verskil het vergemaklik. Die resultate het getoon dat al 56 pieke wat detect is beduidend ($p<0.01$) verander het tussen groen, semi-fermenteerde en gefermenteerde rooibos. ‘n Verandering van ten minste 30% is geselekteer om te verseker dat die merkers ‘n redelike degradasie ondergaan het, wat die aantal na 34 verminder het. Gegewe dat dit steeds ‘n groot aantal is, is die aantal fenole wat in doelgerigte analise van rooibos fermentasie ondersoek is redelik min. Rooibos plantmateriaal is onderworpe aan ‘n 6 uur gesimuleerde fermentasie onder streng beheerde toestande, by vir temperature tussen 37 en 50°C, om insig te verkry oor die reaksie kinetika van rooibos fenole inderworpe aan hoë temperatuur en vogtigheid. Eerstens het ‘n ekstraksie optimeringseksperiment getoon dat ‘n 5 min sonikeringstydperk voldoende was. Dit was ook nodig om die rol van endogene rooibos ensieme te ondersoek, aangesien direkte bewys van hul betrokkenheid nie beskikbaar was nie. Vir die tweede eksperiment is ‘n droë hitte behandeling (170°C vir 30 min) voor gesimuleerde fermentasie gepostuleer om die ensieme gedeeltelik te inaktiveer, gebaseer op die verandering in aspalatien en nothofagin inhoud en die verskille in reaksietempo konstantes tussen die kontrole en hitte-behandelde plantmateriaal. Die gedeeltelike omskakeling reaksie kinetika model, wat baseer is op eerste orde reaksie kinetika, het die degradasie van aspalatien en nothofagin, sowel as die vorming van vier eriodictyol-glukosied isomere, tydens gesimuleerde fermentasie die beste beskryf. Die kinetiese parameters wat deur die model beraam is (maw reaksie tempo konstante en ekwilibrium konsentrasie) het getoon dat die reaksies vinniger was, maar ‘n verlaagde degradasie-/vormingsomvang bereik het by hoër temperature. Hierdie waarnemings het gelei to die postulasie dat ensieme gedeeltelik ge-inaktiveer word by hoër temperature of dat ‘n beperkende faktor ;n rol speel tydens gesimuleer fermentasie, bv. opgebruik van suurstof.

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NOTES

This thesis is presented in the format prescribed by the Department of Food Science at Stellenbosch University. The structure is in the form of one or more research chapters (papers prepared for publication) and is prefaced by an introduction chapter with the study objectives, followed by a literature review chapter and culminating in a chapter for elaborating a general discussion, recommendations and conclusions. Language, style and referencing format used are in accordance with the requirements of the International Journal of Food Science and Technology. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore been unavoidable.

TABLE OF CONTENTS

CHAPTER 1

General introduction

Rooibos, made from the endemic South African fynbos species *Aspalathus linearis*, is one of South Africa's most commercially successful herbal teas alongside honeybush (Joubert *et al.*, 2008). In 1904 rooibos had its first commercial appearance (Joubert *et al.*, 2008) and since then the tea has seen tremendous growth on both a local and global scale. In 2015, the total rooibos exports reached a high of 7000 tons (data supplied by the South African Rooibos Council) with Germany, Japan, UK, USA and the Netherlands listed as some of the major exporting countries (Joubert and De Beer, 2011). The tea was exclusively sold in its fermented form until 2001, when the first commercial green rooibos was produced (Joubert *et al.*, 2008). Green rooibos is differentiated from fermented rooibos based on its green tea leaf colour and higher polyphenolic content (Joubert and De Beer, 2014). These characteristic attributes of green rooibos are difficult to retain as the onset of oxidation during manufacturing is rapid under the ambient production conditions. To date, research has been focused on changes of major phenolic compounds, such as aspalathin and nothofagin, during fermentation, often neglecting the effect of compounds occurring in lower concentrations. The use of advanced chemometric methods could help uncover the phenolic changes occurring in rooibos tea during fermentation in an unbiased manner. Furthermore, gaining insight into the reaction kinetics of rooibos phenolic compounds under conditions conducive to fermentation may be the first step in optimising parameters for minimising the loss of phenolics during green rooibos manufacturing.

Rooibos fermentation briefly entails cutting of the plant shoots, bruising, wetting and allowing the plant material to ferment for 12-14 h at uncontrolled temperatures (Joubert and De Beer, 2011). Based on the common occurrence of polyphenol oxidising enzymes in nature (Yoruk and Marshall, 2003) and their role in black tea fermentation (Subramanian *et al.*, 1999), it has been postulated that the onset of oxidation in rooibos is initiated by enzymatic reactions. With this in mind, optimal conditions for enzymatic reactions are created when the plant material is bruised and hydrated during processing. Enzymes and polyphenolic compounds localised in the chloroplast and the plant vacuole, respectively, are released and allowed to come into contact when the plant material is bruised (Subramanian *et al.*, 1999). The presence of water is essential as it serves as a reacting medium and also facilitates the transportation of substrates and reactants during enzymatic reactions (Acker, 1969). Based on findings in literature of black tea, it is evident that 'fermentation' can be divided into two distinct steps, which can be explained by the mechanism of the polyphenol oxidising enzymes (Subramanian *et al.*, 1999; Stodt *et al.*, 2014). First, monophenols and diphenols are converted into highly reactive *o*-quinones, and then chemical reactions proceed to produce complex brown polymers (Melberg *et al.*, 2009). Endogenous enzymes in rooibos have only briefly been mentioned by Joubert (1996), and although postulations have been made, there has been no further research aimed at investigating their role in rooibos fermentation. On the other hand, the chemical oxidation has been studied in much greater detail. Colour formation (Krafczyk *et al.*, 2009; Heinrich *et al.*, 2012), formation of eriodictyol-glucopyranoside isomers via aspalathin oxidation (Marais *et al.*, 2000), conversion of aspalathin to flavone analogs (isoorientin and orieintin) via eriodictyol-glucopyranoside isomers (Krafczyk and Glomb, 2008) and the quantitative analysis of phenolic changes (Joubert, 1996; Bramati *et al.*, 2003; Beelders *et al.*, 2015; Walters *et al.*, 2017) are some of the many aspects of chemical oxidation during rooibos fermentation covered in the literature.

Processing of green rooibos was instigated to meet a demand by the international market for a product with a higher antioxidant activity than the traditional fermented tea (Joubert *et al.*, 2008). Fermentation is an essential step for the development of the characteristic colour, flavour and aroma of fermented rooibos, but, these reactions are detrimental to the quality of green rooibos (Joubert and De Beer, 2014). Minimising the oxidative changes is no trivial task, as once the plant material has been cut the onset of oxidation is rapid. A high-end-quality product can be produced either by rapid drying, under suitable sun drying conditions, or mechanically (for example drying under vacuum). Sun drying is typically used in industry because it is more cost effective, since it does not require any additional equipment to that used for processing of fermented rooibos. However, the ambient conditions at which the tea is manufactured are conducive to oxidation and if the tea is not properly dried this will lead to slow browning (Joubert and De Beer, 2014).

The aforementioned studies, focused on rooibos fermentation, have all been governed by targeted approaches where selected compounds are monitored during fermentation. For this reason, much of the research has been focused on the oxidative conversion of major phenolic compounds, such as aspalathin. Implementing an untargeted analysis approach is essential to obtaining a comprehensive understanding of the effect of fermentation on the phenolic content of rooibos. The high performance liquid chromatography (HPLC) method recently developed by Walters *et al.* (2017) fulfilled the first step toward performing untargeted analysis of rooibos extracts. The good separation of rooibos phenolic compounds attained with this method makes it ideal for constructing rooibos fingerprints. Additionally, the detection and quantification of intermediate aspalathin oxidation products ((*S*)/(*R*)-eriodictyol-6-*C*-β-D-glucopyranoside and (*S*)/(*R*)-eriodictyol-8-*C*-β-D-glucopyranoside) allows for a better comparison between plant material of different oxidation states.

Fingerprints are described as "a characteristic profile or pattern which chemically represents the sample composition and in which, usually, as much information as possible is reflected" (Goodarzi *et al.*, 2013). Among other analytical methods, chromatographic fingerprint analysis has been recommended by the World Health Organisation (WHO), the American Food and Drug Administration (FDA), the European Medicine Evaluation Agency (EMEA) and the Chinese State Food and Drug Administration (SFDA) as a reliable method for the identification and quality regulation of herbal products (Tistaert *et al.*, 2011; Goodarzi *et al.*, 2013). Examples of fingerprint analysis applied in studies focused on herbal products include, similarity analysis and quality control of green tea (Alaerts *et al.*, 2012), classification and quality control of *Artemisia* species (Alaerts *et al.*, 2014), development of a prediction model for the antioxidant activity of *Tunera diffusa* (Lucio-Gutiérrez *et al.*, 2012), qualitative and quantitative analysis of Chinese herbal medicines (Fan *et al.*, 2006; Xie *et al.*, 2006; Liang *et al.*, 2009) and prediction of potential antioxidant compounds in *Mallotus* species (Tistaert *et al.*, 2012).

HPLC is the preferred method for analysis of non-volatile components in herbal products (Tistaert *et al.*, 2011; Goodarzi *et al.*, 2013) and with the more robust hyphenated techniques (such as HPLC coupled to diode array or mass detection) additional information could be gained. These hyphenated techniques generate massive amounts of data and, therefore, require specialised chemometric analysis methods. Chemometric analysis of fingerprints can be divided into two distinct steps. First, pre-processing should be performed

depending on the nature of the data, before the second step, which is multivariate data analysis. Unwanted variation may arise as a result of the type of samples, data collection methods or analytical instrument used. These artefacts may deter data analysis efforts by misrepresentation of the true differences between samples within the data, and pre-processing techniques should be applied to correct them (Amigo *et al.*, 2010; Tistaert *et al.*, 2011). Typical pre-processing techniques applied to chromatographic data include signal enhancement (background elimination and signal denoising), peak alignment, normalisation, centering and scaling or transformation. Each technique is used to correct a particular artefact. In brief, signal enhancement is used to eliminate noise and background components (Reis *et al.*, 2009), peak alignment corrects retention time shifts (Jellema, 2009), normalisation is used to remove variation between samples that may arise from differences in concentration (Bylesjo *et al.*, 2009) and lastly, centering, scaling and transformation techniques adjust for variation between individual compounds of a sample (Van den Berg *et al.*, 2006).

Fingerprints are classified as correlated, high-dimensional data because measurements are often performed according to a predefined experimental design and, for this reason, they require advanced analysis methods to highlight all aspects of the experimental design (Smilde *et al.*, 2012). ANOVA-simultaneous component analysis (ASCA), ANOVA-target projection (ANOVA-TP) and regularised-multivariate-ANOVA (rMANOVA) are some of the methods that combine the ideas of univariate analysis (*i.e.* analysis of variance, ANOVA) and the advantages of multidimensional techniques designed for handling high-dimensional, highly correlated data (Marini *et al.*, 2017). Fingerprints and chemometric techniques have been used as tools for discrimination analysis (Fan *et al.*, 2006; Alaerts *et al.*, 2012; Lehallier *et al.*, 2012), classification (Chen *et al.*, 2008; Sârbu *et al.*, 2012; Thiangthum *et al.*, 2012; Alaerts *et al.*, 2014), quality assessment (Chen *et al.*, 2008; Schulze *et al.*, 2016) and biomarker identification (Kebede *et al.*, 2015a,b,c; Marini *et al.*, 2015; Wibowo *et al.*, 2015a,b) studies.

Food quality changes are related to chemical, biochemical and physical changes taking place in a food matrix and kinetic modelling gives insight into these specific reactions by allowing quantification of parameters such as reaction rates and activation energies (Van Boekel, 2008). Reactions will typically follow one of three reaction order models depending on the reaction mechanism. The zero-, first- and second-order models are used to determine the rate of the reaction based on the substrate concentration and the time. Another, less frequently used model is the fractional conversion model which allows for non-zero equilibrium concentrations and is based on first-order kinetics (Levenspiel, 1999). The reaction rate constant can be used to estimate how fast or slow a reaction will proceed under different conditions, for example under varying temperature, oxygen concentration or pH. The reaction kinetics are generally affected by the temperature at which the reaction proceeds, with the Arrhenius model of temperature dependency used to estimate the activation energy. The activation energy is described as the energy barrier that molecules need to cross in order to react; the higher the activation energy the more sensitive the reaction is to changes in temperature (Van Boekel, 2008). Reaction rate constants and activation energies were used to establish the relative thermal sensitivities of polyphenolics in cloudy apple juice (De Paepe *et al.*, 2014), benzophenones and xanthones in *Cyclopia genistoides* (Beelders *et al.*, 2015) and selected phenolics in model solutions (Chaaban *et al.*, 2017).

The aims of this study were to investigate phenolic changes occurring during rooibos fermentation using a ‘hypothesis-free’ approach in contrast to previous studies, and to determine the reaction kinetics of rooibos phenolic compounds during typical manufacturing conditions as this has not been done before.

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CHAPTER 2

Literature review

This literature review will provide a general introduction to rooibos, the industry, marketing, processing and background to its chemical composition. Emphasis will be placed on the phenolic composition of the tea and its associated health promoting properties. A discussion of high performance liquid chromatography (HPLC) analysis of rooibos will include a brief introduction to the principles of HPLC and a detailed description of how HPLC analysis of rooibos has progressed over the years. Chromatographic fingerprint analysis and the importance of fingerprints in investigation of food quality changes will be highlighted. A broad spectrum of methods used for data analysis of fingerprints will be discussed, paying special attention to the pre-processing techniques used and multivariate data analysis. This section will also demonstrate the relevance of chemometrics to fingerprint analysis. The literature discussion will conclude with an introduction to kinetic modelling, its use in studies focussing on changes in food quality and the possible outcomes of a 'fingerprint-kinetics' approach.

2.1. Rooibos (*Aspalathus linearis*)

2.1.1. Introduction

2.1.1.1. Geographical distribution and botanical classification

Production of rooibos is mainly concentrated in the Clanwilliam area of the Cederberg mountain region in the Western Cape (Figure 2.1). The distribution of the plant is very limited as it occurs naturally only in the Western Cape and select parts of the south western areas of the Northern Cape, *i.e.* Nieuwoudtville (Morton, 1983).

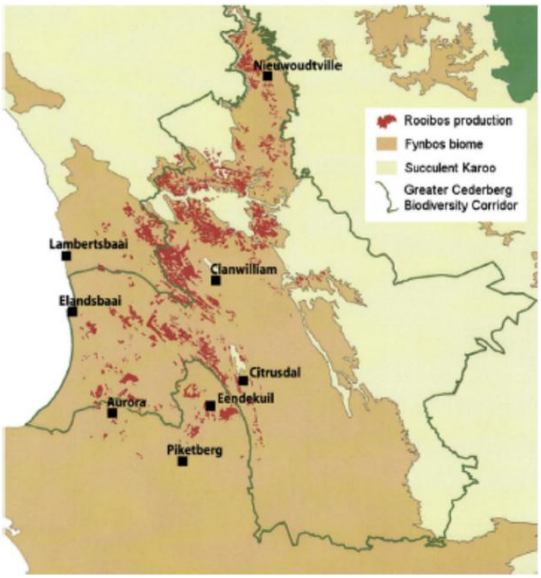


Figure 2.1 Graphical illustration of the distribution of rooibos (*Aspalathis linearis*) in the Western and Northern Cape regions (Map supplied by South African Rooibos Council).

The recently granted Geographical Indication (GI) status of rooibos offers ownership of the name to South Africa and ensures that the term will only be applicable to the rooibos and rooibos products cultivated in South

Africa (Wynberg, 2017). Geographical indication (GI) is a form of intellectual property right given to products that are produced in a certain way and that attribute their quality and reputation to their place of origin (Quinones-Ruiz *et al.*, 2016).

Rooibos belongs to the genus *Aspalathus* (Fabaceae; tribe Crotalarieae) and is described by Dahlgren (1968) as having branches that are 60 cm in length and are red/brown in colour, the leaves are needle-like and 2-6 cm in length and has yellow flowers that occur in short clusters (Figure 2.2).

Figure 2.2 Typical rooibos plantation (Photo- E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch).

The 'red-type' which is used for tea production can be divided into two types, namely the Nortier (cultivated) and Cederberg (wild growing). The Cederberg type is described as having broader and coarser leaves in comparison to the Nortier type (Morton, 1983).

Wild harvesting practices were diminished up until recently (Malgas *et al.*, 2010). Small-scale farmers in Wupperthal and on the Suid Bokkeveld plateau are marketing tea from wild forms of *Aspalathus*; these species vary significantly in growth form and reproductive strategy (Malgas *et al.*, 2010). The four main growth forms identified by local land users include an 'erect form', a 'prostate form', a 'shrub form' and a 'tree form' (Malgas *et al.*, 2010).

2.1.1.2. Historical and modern uses

Anecdotal evidence collected since the late 1960s indicated that rooibos was used to alleviate infantile colic, allergies, asthma and dermatological problems (Morton, 1983; Joubert *et al.*, 2008). Although listed in Smith *et al.* (1966) as a medicinal plant, no indication of use was provided (Van Wyk and Gorelik, 2017). The tea was prepared in the hot summer months to facilitate 'fermentation' (this was incorrectly labelled fermentation but is in actual fact an oxidation step). The wild bushes were harvested, the shoots were chopped into small pieces using an axe and the plant material was allowed to 'ferment' followed by sun-drying. These basic steps, although some mechanised, are still used today in the manufacture of rooibos.

The beverage was prepared by steeping loose leaves in boiling water and allowing it to brew at a low heat. More tea was added according to desirability of strength (Morton, 1983). For modern day use both loose tea and tea bags are available, tea bags usually contain 2 g of rooibos. Rooibos tea is prepared by steeping one bag in a cup of freshly boiled water for 2-5 min, and it can be served with milk and sugar. In summer the tea is also enjoyed as a cold beverage usually served with honey, lemon and sugar. Variations of the traditional

tea are common and typically include blends of herbs and other teas as well as flavoured rooibos (Joubert *et al.*, 2008).

Annetjie Theron was the first person to identify rooibos for its ability to alleviate infantile colic (Joubert *et al.*, 2008). Her baby was cured from its colic symptoms after administering regular doses of rooibos. She has since developed her own range of skincare products containing rooibos extract (Joubert *et al.*, 2008). Extracts are also popular for use as additives in food and beverages such as yoghurt, bread, ready-to-drink iced-tea, jam and 'instant rooibos cappuccino' (Joubert and De Beer, 2011). Some recent applications in beverages include exposure of wine to rooibos and honeybush wood to produce sulphite- and preservative-free wines available from Audacia wine farm (Anon., 2016). In a pilot study by Cullere *et al.* (2013), the antioxidant potential of unfermented and fermented rooibos in meat products was investigated. Results were promising, which prompted a second study by Hoffman *et al.* (2014) where fermented rooibos extract was added as a natural antioxidant to dröewors. Due to the interference of external factors the results regarding the antioxidant capabilities of the extract in the dröewors were inconclusive and require further research (Hoffman *et al.*, 2014). Besides its use in cosmetics and as a food additive, antioxidant rich extracts are commonly used in the nutraceutical industry, however more research is required to gain insight into the therapeutic dose needed for bioactivity (De Beer *et al.*, 2017).

Since the early 2000s, unfermented rooibos, also known as green rooibos, has been marketed and sold for its increased phenolic content (Joubert and De Beer, 2014). The taste, aroma and infusion colour is unlike that of fermented rooibos (Koch *et al.*, 2012). Based on sensory analysis results, a 'plant-like' taste/aroma is strongly associated with green rooibos in comparison to fermented rooibos tea, which was associated with a 'rooibos-woody' taste/aroma (Viljoen *et al.*, 2017). However, the relatively high phenolic content makes its use in the nutraceutical and cosmetics industries popular (De Beer *et al.*, 2017).

Since its initial 'medicinal' use, extensive research has been conducted on the potential health-promoting properties of rooibos tea. Table 2.1 summarises research conducted on fermented and unfermented rooibos aimed at uncovering or providing evidence for its potential health promoting properties.

Table 2.1 A selection of studies conducted on health promoting properties associated with rooibos (Adapted from Joubert and de Beer, 2011).

Bioactivity	Extract/Infusion	Reported by
Hypoglycaemic activity	Fermented[a,c] and unfermented[a,b,c,d]	Joubert *et al.* (2010)[a]; Muller *et al.* (2012)[b]; Mazibuko *et al.* (2013)[c]; Kamakura *et al.* (2015)[d]
Anti-inflammatory activity	Fermented[a,b] and unfermented[c,d,e]	Hendricks and Pool (2010)[a]; Mueller *et al.* (2010)[b]; Schloms *et al.* (2012)[c]; Smith and Swart (2016)[d]; Magcwebeba *et al.* (2016a)[e]
Anti-oxidant activity	Fermented[a,b,c] and unfermented[a]	Villaño *et al.* (2010)[a], Marnewick *et al.* (2011)[b]; Wanjiku (2009)[c]
Cell regeneration	Fermented	Uličná *et al.* (2008)
Chemoprotection	Fermented[a] and unfermented[a,b]	Marnewick *et al.* (2009)[a] ; Magcwebeba *et al.* (2016b)[b]
Anti-carcinogenic and photoprotective	Fermented and unfermented	Petrova (2009)
Cytoprotective	Fermented	Yoo *et al.* (2009)
Anti-obesity	Fermented	Mueller and Jungbauer (2009); Beltrán-Debón *et al.* (2011); Sanderson *et al.* (2014)
Cardiovascular protection	Fermented	Persson (2012); Marnewick *et al.* (2011); Dludla *et al.* (2014)
Estrogenic activity	Fermented and unfermented	Monsees and Opuwari (2017)

Aspalathin, the major flavonoid in rooibos, has shown anti-inflammatory (Magcwebeba *et al.*, 2016a), anti-mutagenic (Snijman *et al.*, 2007) and hypoglycaemic activity (Kawano *et al.*, 2009; Joubert *et al.*, 2010a) as well as phytoestrogenicity (Shimamura *et al.*, 2006). However, the phenolic composition of the plant material, extracts, infusions and value-added products may vary depending on many factors (see section 2.1.4.3).

Isoorientin and orientin, major flavones present in rooibos, have also been studied in detail because of their link to health-promoting properties of rooibos. Isoorientin has been associated with anti-mutagenicity (Sezik *et al.*, 2005; Snijman *et al.*, 2007), anti-hyperlipidaemic activity (Sezik *et al.*, 2005), anti-inflammatory activity (Küpeli *et al.*, 2004; Zucolotto *et al.*, 2009), anti-nociceptive activity (Küpeli *et al.*, 2004), gastroprotective activity (Küpeli *et al.*, 2004) and hepatoprotective activity (Deliorman Orhan *et al.*, 2003). Orientin has been associated with anti-mutagenicity (Snijman *et al.*, 2007) and anti-adipogenesis activity (Kim *et al.*, 2010). Lam *et al.* (2016) reviewed the medicinal properties of orientin, including anti-inflammatory, cardioprotective and anti-viral properties, among many others.

2.1.2.Marketing and export

Rooibos was first marketed and packaged under the brand name Eleven O'clock by Benjamin Ginsberg in 1904 (Joubert *et al.*, 2008). Since then it has become somewhat of a staple in South African homes. A growing

demand initially came about as a result of a shortage of oriental tea during the Second World War (Joubert *et al.*, 2008). The market has seen growth on both local and global scales. Total international exports for rooibos between the years 2004 and 2015 can be seen in Figure 2.3. Major export countries include Germany, Japan, UK, USA, and Netherlands. Table 2.2 shows the distribution of product exported to the major international market contributors.

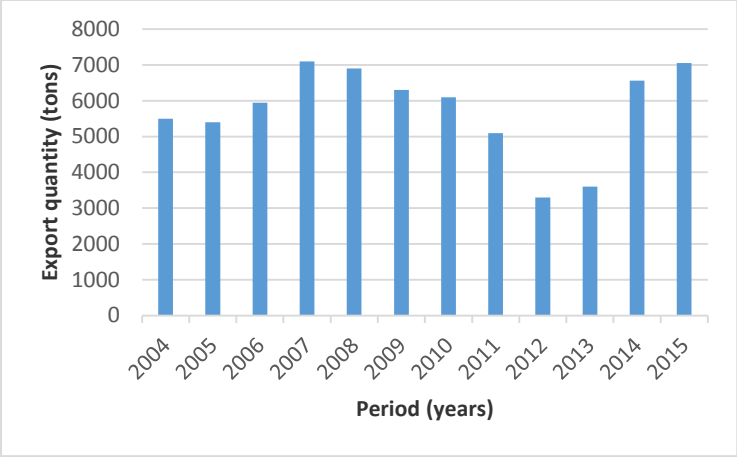


Figure 2.3 Total international exports of rooibos teas for the period of 2004-2015 (Data supplied by the South African Rooibos Council).

Table 2.2 Commercial rooibos exports as distributed to international countries between 2004 and 2015 (Data supplied by the South African Rooibos Council).

	Quantity exported (tons) (percentage of total exports)										
Country	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**	**2010**	**2011**	**2012**	**2013**	**2014-2015**
Germany	3817 (69.4)	3126 (57.90)	3126 (52.54)	3720 (52.40)	3203 (46.42)	2740 (43.50)	2209 (36.21)	1850 (36.27)	1884 (57.10)	1058 (29.40)	1563 (33.76)
Netherlands	496.41 (9.03)	674.46 (12.49)	773.20 (13.00)	742.99 (10.46)	699.13 (10.13)	861.42 (13.67)	932.60 (15.29)	796.30 (15.61)	570.09 (17.28)	164.17 (4.56)	914.12 (19.74)
UK	129.45 (2.35)	209.67 (3.88)	363.53 (6.11)	440.13 (6.20)	665.30 (9.64)	662.75 (10.52)	736.34 (12.07)	815.73 (16.00)	566.68 (17.17)	275.98 (7.67)	602.12 (13.00)
USA	237.44 (4.32)	116.72 (2.16)	299.69 (5.04)	225.59 (3.18)	219.64 (3.18)	186.54 (2.96)	241.84 (3.96)	199.75 (3.92)	198.88 (6.03)	67.29 (1.87)	148.24 (3.20)
Japan	183.56 (3.34)	160.48 (2.97)	205.45 (3.45)	215.99 (3.04)	98.04 (1.42)	166.83 (2.65)	127.83 (2.10)	162.24 (3.18)	176.5 (5.35)	207.41 (5.67)	527.48 (11.39)

2.1.3. Cultivation, harvesting and processing

2.1.3.1. Cultivation and harvesting

The first attempts at cultivation were conducted in the 1930s by Dr. P. le Fras Nortier and local farmers O. Bergh and H. Riordan. Their efforts laid the foundation for the modern day cultivation of rooibos (Cheney and Scholtz, 1963; Joubert and De Beer, 2011).

Seed propagation is responsible for major genetic variation within rooibos manufactured from the same species (Joubert and De Beer, 2011). Planting of seeds occurs between June and August and 8-12 months after planting the plants are cut to approximately 30 cm high to promote branching. The first harvest takes place a year and a half later, but full production is only realised 3 years later. The plants are harvested in the hot summer months where they are topped off to approximately 45 cm. Bunches of harvested plants are tied together in bundles (Figure 2.4) and transported to the processing yard and after a wilting period fermentation is initiated.

Figure 2.4 Harvesting of a rooibos bush (Photo- E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch).

It has been noted that the active shrub growth should not be more that 50 cm, as this results in weaker tea production. Additionally, it is critical that the flowering part of the plant be avoided when harvesting as this is known to impart an unpleasant flavour to the infusions (Joubert and Schulz, 2006). Due to overseas product demand, cultivation areas and production have expanded. The cultivation area now covers approximately 60 000 ha (Wynberg, 2017), with cultivation areas mainly concentrated in the Cederberg mountain area and also extending to areas such as Darling and Niewoudtville.

Processing takes place outside and involves the following steps: plant shoots are cut into small pieces (≤5 mm), the shredded plant material is placed in 'fermentation' heaps approximately 15–30 cm deep; heaps are bruised by rolling a heavy object over them and hydrated (10 L per 35 kg of plant material) to initiate fermentation; fermentation continues for 12-14 h at temperatures between 38-42°C (Joubert and De Villiers, 1997). The fermentation time can vary depending on the presence of young growth, the age of the bush and the cultivation area (Joubert, 1994). Aeration of fermentation heaps, by flipping, is important as some of the plant material may not oxidise properly and a low quality product may be produced (Joubert *et al.*, 1998). The fermentation step is complete once the plant material has obtained a good colour and a sweet aroma; thereafter the tea is spread out and allowed to sun dry to <10% moisture content. The sun drying should be carried out as soon as the fermentation is complete to halt any further chemical changes. Figure 2.5 illustrates the change in the colour of the plant material during fermentation as a result of oxidation.

Prior to packaging, the dry sieved tea is steam pasteurised (93°C for 60 s), a processing step introduced in the 1980's to ensure a microbiologically safe product after *Salmonella* was detected on rooibos (Joubert and De Beer, 2011). Although pasteurisation efficiently reduces the microbial load, the presence of *Salmonella* has been detected even after pasteurisation (Du Plessis and Roos, 1986). Gouws *et al.* (2014) recommended

the application of other, less abrasive and more efficient methods such as radiation, specific bacteriophages and ozone treatment to control *Salmonella* levels in rooibos.

Figure 2.5 Fermentation heaps in their a) unfermented and b) fermented state (Photo- E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch).

Joubert *et al.* (1998) investigated controlled processing as an alternative to traditional open-air processing. The aim of the study was to improve quality control methods during rooibos manufacturing. Good quality tea was obtained by controlling fermentation temperature, relative humidity and velocity of the inlet air. Fermentation was conducted at the following optimum conditions, 34-40°C, >0.95 relative humidity and an air velocity of 0.2-0.7 m/s (Joubert *et al*., 1998).

Green or unfermented rooibos was first produced in 2001 as a result of consumer interest in a tea with higher antioxidant activity, as demonstrated by Von Gadow *et al*. (1997). To maintain the green leaf colour and high phenolic content, oxidative changes during green rooibos manufacturing must be kept to a minimum (Joubert *et al.*, 2008). This is achieved by rapid drying, under suitable sun drying conditions or mechanically, to arrest the onset of oxidation (E. Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, 2017, personal communication). Sun-drying cut shoots can be slow and if the plant material does not reach a critical moisture content in time, slow browning will occur resulting in a green product of poor quality (Joubert *et al.*, 2008).

2.1.3.2. Commercial extract production and value-added products

The idea of a rooibos extract with commercial application in the cosmetic, supplement, food and beverage industries was conceived from a study by Joubert (1984). Although the initial studies for powdered extracts were done in the 1980s, the concept only found commercial application in 2000 (Joubert *et al.*, 2008). Optimisation experiments found that higher temperatures, higher water-to-plant material ratios and longer extraction times resulted in products with increased total soluble solids, total flavonoid and higher polyphenolic content (Joubert, 1988a, 1990a,b; Joubert and Hansmann, 1990). The fermented powdered extract had poor solubility in water and to address this agglomeration was investigated. This improved the wettability, sinkability and dispersability, resulting in a typical instant powder after agglomeration (Joubert, 1988b).

The extract is mainly used as an intermediate value-added product. The first application of value-addition was done by A. Theron when she used rooibos extract as an ingredient in her skin care product range (Joubert and De Beer, 2011). One of the first food applications of the rooibos extract was as a flavoured

ingredient of yoghurt (Joubert *et al*., 2008). The product was only introduced to the market many years later after consumers started taking interest in health-promoting foods and ingredients. Additionally, the extract is often used in value-added products in the form of a mixture containing other plant and fruit extracts, for example in the US a soft drink developed in 2009 contain a of mixture of rooibos and pomegranate extracts (Joubert and De Beer, 2011). In South Africa, extracts are popular for use in ready-to-drink iced teas (Joubert and Schulz, 2006).

Based on trends seen in literature, there has been an increased interest in the development and quality control of an aspalathin-enriched extract (from green rooibos) for use in the nutraceutical industry (Muller *et al.*, 2012; Mazibuko *et al.*, 2013; De Beer *et al*., 2017).

2.1.4. Chemical composition

2.1.4.1. Caffeine content, non-phenolic metabolites, mineral constituents and volatile compounds

Rooibos is often prized as a caffeine-free tea. Trace amounts of the alkaloid sparteine were found in rooibos (Van Wyk and Verdoorn, 1989). Non-phenolic compounds identified to date include, *p*-hydroxyphenylglycol and vanylglycol (Shimamura *et al.*, 2006), as well as inositol and (+)-pinitol (Ferreira *et al.*, 1995).

The mineral content of rooibos, as reported on three different occasions as well as the fluoride content reported by Touyz and Smit (1982) is summarised in Table 2.3. Ca, K, Mg and Na occur in the highest concentrations in both the leaves and infusion. Rooibos had considerably lower concentrations of most minerals when compared to other traditional black and green teas (Olivier *et al.*, 2012).

Table 2.3 Mineral and fluoride content (mean±SD) of fermented rooibos plant material ($\mu g.g^{-1}$) and infusions ($\mu g.mL^{-1}$).

Mineral	Leaves[a]	Leaves[b]	Leaves[c]	5 min infusions[b]
Al	98±4	99±13	82±14	0.05±0.03
B	-	30±6	-	0.26±0.07
Ba	7±0.23	-	-	-
Ca	2130±30	2017±276	1792±302	6.35±1.69
Co	-	<0.05	5±1	Trace
Cr	-	0.61±0.22	-	Trace
Cu	-	2.26±1.01	-	0.07±0.04
Fe	-	90±12	117.6±24	<0.02
K	-	4083±944	2762±806	58.63±17.72
Mg	2360±35	1531±156	1704±323	10.17±1.72
Mn	70±1	83±19	57±9	0.40±0.11
Na	-	2467±184	2797±293	43.33±6.95
P	-	679±127	680±169	7.57±1.97
S	-	-	874±45	-
Zn	12±0.3	11±3	20±6.1	0.08±0.02
Fluoride[d]	-	-	-	1.29±0.27

[a] Mokgalaka *et al.* (2004),[b] Joubert *et al.* (2008),[c] Olivier *et al.* (2012),[d] Touyz and Smit (1982).

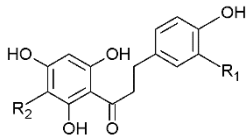
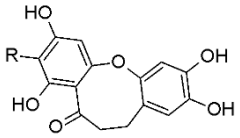
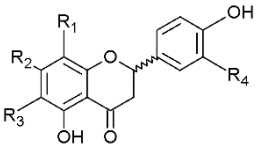
The characteristic flavour and aroma of fermented rooibos is influenced by the presence of numerous volatile constituents (Joubert and Schulz, 2006). The volatile fraction of rooibos is diverse and 99 different compounds have been identified to date including, guaiacol, a 6-methyl-3,5-heptadien-2-one isomer, damascenone, geranylacetone, β-phenylethyl alcohol and 6-methyl-5-hepten-2-one as its major constituents (Habu *et al.*, 1985). The presence of these compounds (except for damascenone) was later confirmed by Kawakami *et al.* (1993).

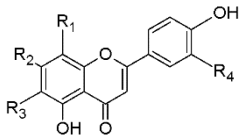
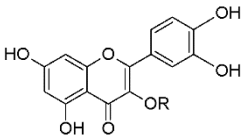
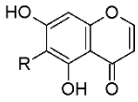
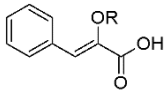
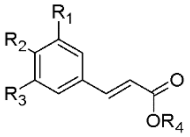
2.1.4.2. Phenolic composition

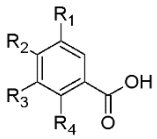
Phenolic compounds, also known as polyphenols, constitute a class of chemically diverse compounds generally synthesised by plants as secondary metabolites (Kalili and De Villiers, 2011). They can be loosely classified into flavonoid and non-flavonoid groups (Kalili and De Villiers, 2011). Their distribution in nature is wide with frequent occurrences in many fruits, vegetables and herbs. Common occurrences of these sought after compounds has sparked extensive research into the role they play in food quality and the human diet (Stalikas, 2007).

Studies on rooibos have therefore been focused on identifying the phenolic composition of extracts and infusions and obtaining evidence of their health promoting properties (Joubert *et al.*, 2008; Joubert and De Beer, 2011). The phenolic fraction of rooibos covers a broad spectrum of different sub-groups, with some novel or rare compounds and others commonly occurring in nature. Most of the major phenolics present belong to the sub-groups dihydrochalcones, flavanones, flavones, flavonols, cinnamic acids and benzoic acids. A detailed illustration of the compound structures and their names can be found in Table 2.4.

Table 2.4 Compound structures and names of major phenolics and phenolic precursors found in rooibos (adapted from Joubert *et al.*, 2008).

General structure	Compound type, name and substituents
	**Dihydrochalcones** Aspalathin[a,b,c,d,f,g,k]: R_1 = OH, R_2 =β-D-glucopyranosyl Nothofagin[e,f,g,k]: R_1 = H, R_2 = β-D-glucopyranosyl Phloretin-3',5'-di-*C*-β-D-glucopyranoside[l]: R_1 = β-D-glucopyranosyl, R_2 = β-D-glucopyranosyl
	**Cyclic dihydrochalcone** aspalalinin[g]: R = *C*-β-D-glucopyranosyl
	**Flavanones** Hemiphlorin[g]: R_1 = β-D-glucopyranosyl, R_2 = OH, R_3 = R_4 = H *(R)/(S)*-eriodictyol-8-*C*-glucopyranoside[f,g,k]: R_1 = β-D-glucopyranosyl, R_2 = OH, R_3 = H, R_4 = OH *(R)/*(S)-eriodictyol-6-*C*-glucopyranoside[f,g,k]: R_1 = H, R_2 = OH, R_3 = β-D-glucopyranosyl, R_4 = OH

	Hesperidin[l]: R_1 = H, R_2 = *O*-rutinosyl, R_3 = H, R_4 = OCH_3
	**Flavones** Orientin[a,c,f,g,h,i,k] : R_1 = β-D-glucopyranosyl, R_2 = R_4 = OH, R_3 = H Isoorientin[c,f,h,i,k] : R_1 = H, R_2 = R_4 = OH, R_3 = β-D-glucopyranosyl Vitexin[a,c,f,g,k] : R_1 = β-D-glucopyranosyl, R_2 = OH, R_3 = R_4 = H Isovitexin[c,f,g,k] : R_1 = R_4 = H, R_2 = OH, R_3 = β-D-glucopyranosyl Luteolin[c,f,g,k] : R_1 = R_3 = H, R_2 = R_4 = OH Luteolin-7-*O*-glucopyranoside[d] : R_1 = R_3 = H, R_2 =β-D-glucopyranosyloxyl, R_4 = OH Chrysoeriol[c,f,k] : R_1 = R_3 = H, R_2 = OH, R_4 = OCH_3 Vicenin-2[l,m]: R_1 = β-D-glucopyranosyl, R_2 = H, R_3 = = β-D-glucopyranosyl, R_4 = H Scolymoside[l]:R^1 = H, R^2 = *O*-rutinosyl, R^3 = H, R^4 = OH
	**Flavonols** Quercetin[c,f,g,k] : R = H Isoquercitrin[c,f,g,i,k] : R = β-D-glucopyranosyloxy Hyperoside[f,g,k] : R = β-D-galactopyranosyloxy Rutin[f,i,k] : R = *α*-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyloxy Quercetin-3-*O*-β-D-robinobioside[g] : R = *α*-L-rhamnopyranosyl-(1→6)-β-D-galactopyranosyloxy
	**Chromone** 5,7-dihydroxy-6-*C*-β-D-glucosyl-chromone[d]
	**Phenylpropenoid** *Z*-2-(β-D-Glucopyranosyloxy)-3-phenylpropenoic acid (PPAG)[d,j] : R = 2-β-D-glucopyranosyloxy
	**Hydroxycinnamic acids and derivatives** 3,4,5-trihydroxycinnamic acid[c]: R_1 = R_2 = R_3 = OH; R_4 = H *p*-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] : R_1 =R_3 = H; R_2 = OH; R_4 = H

protocatechuic acid[c,k] : R_1 = R_2 = OH, R_3 = R_4 = H

3,5-dihydroxybenzoic acid[k] : R_1 = R_3 = H; R_2 = R_4 = OH

gentisic acid[k] : R_1 = R_3 = OH; R_2 = R_4 = H

salicylic acid[k] : R_1 = R_2 = R_3 = H; R_4 = OH

gallic acid[k] : R_1 = R_2 = OH, R_3 = OH; R_4 = H

vanillic acid[c,k] : R_1 = OCH_3,; R_2 = OH; R_3 = R_4 = H

syringic acid[d,k] : R_1 = R_3 = OCH_3; R_2 = OH; R_4 = H

[a] Koeppen and Roux (1965b) (identification by NMR); [b] Koeppen and 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 and Roux, (1965a) (identification by NMR); [i] Koeppen *et al.* (1962) (identification by NMR); [j] Marais *et al.* (1996) (identification by NMR); [k] Krafczyk *et al.* (2009) (identification by NMR); [l] Walters *et al.* (2017a) (identification by LC-MS); [m] Beelders *et al.* (2012a) (identification by LC-MS).

Other phenolic compounds present in considerable quantities are the flavones carlinoside (Beelders *et al.*, 2012), neocarlinoside (Beelders *et al.*, 2012), isocarlinoside (Beelders *et al.*, 2012), lignans vladinol F (Shimamura *et al.*, 2006), secoisolariciresinol (Shimamura *et al.*, 2006), secoisolariciresinol glucoside (Shimamura *et al.*, 2006), and scolymoside (Walters *et al.*, 2017b), as well as coumarins esculetin and esculin (Shimamura *et al.*, 2006) and the aldehyde syringin (Shimamura *et al.*, 2006). Ferreira *et al.* (1995) identified the presence (in low concentrations) of the procyanindin dimer, procyanindin B3, the trimers, bis-fisetinidol-(4β,6:4β,8)-catechin, isetinidol-(4β,6:4β,8)-catechin, and a pentamer.

A dried water extract of fermented rooibos was shown to contain up to as much as 50% tannin-like substances (Joubert *et al.*, 2008). This is however relatively low when compared to *Camellia sinensis* (*ca.* 45-55 $mg.L^{-1}$) (Lima *et al.*, 2012). Limited information is available about the structural information of rooibos tannins.

2.1.4.3. Factors affecting phenolic composition of rooibos products

Plant production and harvesting

De Beer *et al.* (2017) demonstrated the variation between plants from the same species by analysing the phenolic content of 47 production batches of green rooibos. Plant material was obtained from different plants on the same plantation. Although the plant material came from a single species of rooibos, the aspalathin content varied between 2.50 and 4.49% (De Beer *et al.*, 2017).

Different components of rooibos plants (*i.e.* leaves and stems) also have varying phenolic contents (Joubert *et al.*, 2013). PPAG contents in unfermented rooibos samples were reported as inconsistent (Joubert *et al.*, 2013). The leaves in certain samples, but not the stems, contained PPAG, and the opposite was true for other samples. Additionally, De Beer *et al.* (2017) reported that the aspalathin content in leaves were much higher than that found in stem samples.

Harvest date has also been identified as another factor that may influence the phenolic composition of rooibos. The aspalathin content differed in plant material harvested from the same plants for a period of a year

(De Beer *et al.*, 2017). The experiment used plant material harvested from random plants on three plantations at specific intervals, ranging from early summer to late spring, for a period of a year. Results indicated that aspalathin and nothofagin contents were highest in summer, where after the levels decreased to the lowest in mid-spring.

Processing of fermented rooibos

'Fermentation' (which is in actual fact an oxidation step) is essential in the production of traditional rooibos. One of the main changes that occurs as a result of oxidation is the conversion of aspalathin to coloured compounds that impart the characteristic red/brown colour to the plant material (Krafczyk and Glomb, 2008). It was later reported by Krafczyk et al. (2009) that nothofagin, under similar oxidation conditions, undergoes degradation which is much slower, but comparable to that of aspalathin. The conversion of aspalathin to its oxidation products is summarised in Figure 2.6.

Isoorientin and orientin are products of aspalathin oxidation (Walters *et al.*, 2017a). Aspalathin is converted to isoorientin via *(R)/*(S)-eriodictyol-6-*C*-β-D-glucopyranoside (Krafczyk and Glomb, 2008). Orientin does not form from *(R)/*(S)-eriodictyol-8-*C*-β-D-glucopyranoside as isoorientin does from *(R)/*(S)-eriodictyol-6-*C*-β-D-glucopyranoside (Krafczyk and Glomb, 2008). Instead, orientin is formed as a result of isoorientin undergoing opening of its vinyl ester structure, bond rotation and closing of the ring structure (Heinrich *et al.*, 2012). Further oxidation of aspalathin leads to the formation of high molecular weight brown compounds via polymerisation reactions (Heinrich *et al.*, 2012).

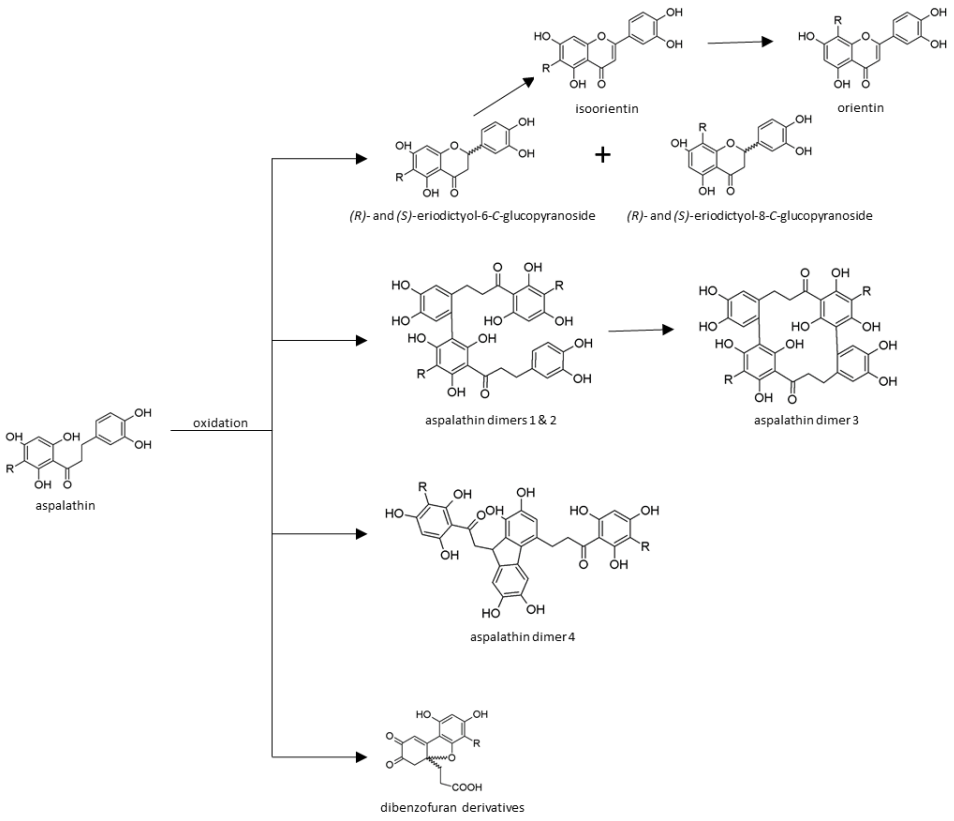


Figure 2.6 Schematic diagram of aspalathin and its oxidation products formed during fermentation. R = β-D-glucopyranosyl (Joubert and De Beer, 2014).

Walters *et al.* (2017a) reported quantitative data of 15 major rooibos phenolics and PPAG in green, semi-fermented and fermented rooibos samples. It was concluded that the largest decreases with fermentation were observed for the dihydrochalcones (aspalathin and nothofagin), with moderate to small decreases for flavonols (quercetin-3-O-robinobioside, rutin, hyperoside and isoquercitrin) (Walters *et al.*, 2017a). The concentrations of intermediate products of aspalathin oxidation, eriodictyol-glucopyranoside isomers, increased as a result of fermentation. Trends observed for the flavones isoorientin and orientin were less predictable and distinct in comparison to those of the flavanones, dihydrochalcones and flavonols. This was to be expected, as isoorientin and orientin participate in both degradation and formation reactions during fermentation (Walters *et al.*, 2017a). Vitexin and isovitexin, the flavone products of nothofagin oxidation, had small but significant decreases following fermentation, which can be explained by lower rates of formation via oxidation of nothofagin than their degradation rates.

Steam pasteurisation

Steam pasteurisation was introduced during the 1980s for food safety reasons (Joubert and De Beer, 2014). After its introduction, it was reported by consumers that there was a softening of the flavour and prominent 'medicinal aroma'. This was followed by an increase in acceptability for some consumers, while others preferred the flavour of the unpasteurised tea (Koch *et al.*, 2013). These sensorial changes prompted the development of the sensory wheel.

By development of the rooibos sensory wheel, researchers were provided with a tool for comprehensive profiling of the sensory attributes of rooibos products (Koch *et al.*, 2012). Subsequently, the effect of steam pasteurisation on the sensory attributes of fermented rooibos and the link between rooibos sensory attributes and its phenolic composition were investigated (Koch *et al.*, 2013). Steam pasteurisation was found to significantly reduce the aspalathin content of fermented rooibos, however, the levels of the other major flavonoids were not significantly affected (Koch *et al.*, 2013). Contradictory findings by Standley *et al.* (2001) stated that the radical scavenging ability of pasteurised rooibos was slightly higher than that of unpasteurised rooibos. However, it was speculated that the variation in antioxidant activity reported by Standley *et al.* (2001) was related to the natural variation of phenolic content in the plant material since samples came from different plants

Stanimirova *et al*. (2013) and Marini *et al.* (2015) observed a significantly lower phenolic content in pasteurised rooibos when compared to unpasteurised rooibos (results reported in these two papers are based on the same data). The phenolic compounds affected by pasteurisation included aspalathin, isoorientin, orientin, quercetin-3-*O*-β-D-robinobioside, vitexin, hyperoside, rutin, isovitexin and isoquercitrin (Stanimirova *et al.*, 2013; Marini *et al.*, 2015). These findings differ from the results reported by Koch *et al.* (2013) because of the differences in sampling methods. The latter compared quantitative results for averages of 69 random plants, whereas the experiment for the aforementioned studies was designed in such a manner that only the variation due to post-production processing (*i.e.* steam pasteurisation) was reflected.

Processing of ready-to-drink iced teas

Rooibos is typically incorporated into ready-to-drink iced teas in the form of powdered aqueous extracts. The main concern with these ready-to-drink (RTD) products is that their rooibos content is not being regulated. Joubert *et al.* (2009, 2010b) investigated the effects of processing (*i.e*. extract production and iced tea manufacturing) on the retention of the rooibos active ingredients (*i.e*. aspalathin, isoorientin and orientin). Aspalathin is highly susceptible to oxidative changes. The rate of these changes are affected by heat processing which is used in the manufacturing of the extract and during the iced tea manufacturing. Therefore, low levels of aspalathin in ice tea samples indicated the use of over oxidised rooibos for extract production or poor stability during the manufacturing of the extract or iced tea.

The oxidation products of aspalathin were absent in certain brands of iced tea and this suggested that no extract was used during the manufacturing of the RTD product (Joubert *et al.*, 2009). Furthermore, Joubert *et al.* (2009) concluded that the intensity of the heat treatment affected the stability of the active ingredients. Pasteurisation had minimal effects, while standard temperature sterilisation (121°C for 15 min) and high temperature sterilisation (135°C for 4 min) resulted in significant losses.

The low aspalathin levels reported for RTD rooibos iced teas prompted a study aimed at incorporating aspalathin-enriched extracts and acidic additives to enhance aspalathin stability in RTD rooibos iced teas (De Beer *et al.*, 2011). Green, fermented and aspalathin-enriched extracts (ascorbic acid solubilisate) were compared for aspalathin stability in the presence of citric acid and ascorbic acid. De Beer *et al.* (2011) reported increased aspalathin stability for green and fermented extracts in the presence of ascorbic and citric acid. The

aspalathin-enriched extract had improved stability without the addition of citric acid. Lower pH favoured aspalathin stability and this was later confirmed by De Beer *et al.* (2015), where pure aspalathin had better stability at pH 3 (91% remaining after 29 h) compared to pH 7 (45% remaining after 29 h). It was postulated that differences observed between formulations by De Beer *et al.* (2011) was a result of differences in the matrices.

2.1.5. The role of endogenous enzymes in tea fermentation

Fermentation of black tea, *Camellia sinensis*, is initiated by endogenous enzymes, polyphenol oxidase and peroxidase (Subramanian *et al.*, 1999). It is postulated that, under similar conditions, rooibos undergoes the same biochemical reactions (Gouws *et al.*, 2014).

Polyphenol oxidase (PPO), also known as tyrosinase, is widely found in nature. They are said to have an almost universal distribution in animals, plants, fungi and bacteria (Mayer, 2006). It is commonly known for their role in post-harvest browning of fresh produce (Melberg *et al.*, 2009). The active site of PPOs is a dinuclear copper centre responsible for the catalysis of *ortho*-diphenols to their corresponding *ortho*-quinones (Yoruk and Marshall, 2003; Mayer, 2006). The activity of the enzymes catalyses non-enzymatic oxidative condensation to yield melanins (coloured polymers). A schematic of the general reaction that occurs between diphenols and PPO in the presence of oxygen is illustrated in Figure 2.7.

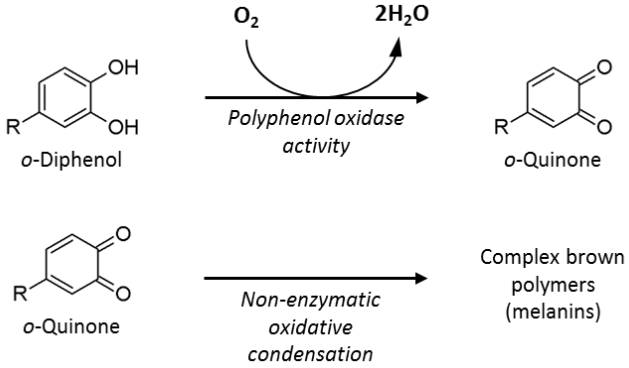


Figure 2.7 Schematic representation of enzymatic and subsequent non-enzymatic condensation as a result of PPO in the presence of diphenols (Melberg *et al.*, 2009).

Peroxidase (POD), a heme-containing enzyme, is most recognisable for its high abundance in horseradish, which has proved useful as a biotechnological tool (Melberg *et al.*, 2009). Apart from plant species, their presence has also been noted in fungi and bacteria. POD catalyses a reaction between hydrogen peroxide and hydrogen donors such as phenols. The result is an oxidised radical which participates in polymerisation reactions to yield coloured products. The reaction between POD and a 4-chloronaphthol is used as an example to illustrate the effect of POD on phenols in the presence of peroxide (Figure 2.8).

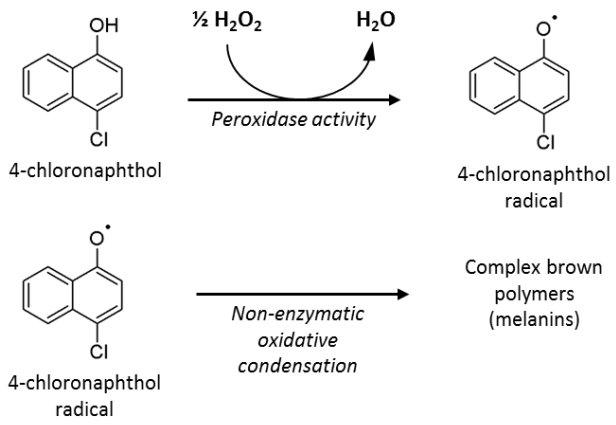


Figure 2.8 Example of enzymatic and subsequent non-enzymatic condensation as a result of POD in the presence of phenols and hydrogen peroxide (Melberg *et al.*, 2009).

In *Camellia sinensis*, PPOs and PODs are responsible for catalysing the oxidation of catechins which results in the formation of orange-red theaflavins and reddish-brown thearubigins (Subramanian *et al.*, 1999; Baruah and Mahanta, 2003; Stodt *et al.*, 2014). These pigments are responsible for sensory characteristics unique to black tea (Subramanian *et al.*, 1999).

Postulation that rooibos tea undergoes similar biochemical reactions to black tea is attributed to the high abundance of PPOs and PODs in nature and the similarities in processing conditions that they share. The enzymes and polyphenolic compounds are spatially separated in the tea leaves; enzymes are localised in the chloroplast, while polyphenols are localised in the plant vacuole. Mechanical maceration of the tea leaves disrupts the internal cell structure, which allows the enzymes to come into contact with the phenolic substrates. The biochemical reactions produce highly reactive *o*-quinones and radicals that subsequently lead to chemical, non-enzymatic oxidation reactions.

As discussed in section 2.1.4.3, Krafczyk *et al.* (2009) showed the oxidative conversion of aspalathin (the major dihydrochalcones of aspalathin) from a chemical point of view. The role of biochemical reactions in the initiation of rooibos fermentation is only briefly mentioned by Joubert, (1996).

2.1.6. Quantitative analysis of rooibos phenolic compounds by high performance liquid chromatography (HPLC)

Chromatography entails the distribution of molecules between two immiscible phases, one stationary and one mobile. High-Performance Liquid Chromatography (HPLC) is the most popular method used for non-volatile phenolic separation (Kalili and De Villiers, 2011). The mobile phase is liquid and hence the name liquid chromatography, as opposed to a gaseous mobile phase used in gas chromatography or supercritical fluid used in supercritical fluid chromatography. The general components of an HPLC instrument are depicted in Figure 2.9.

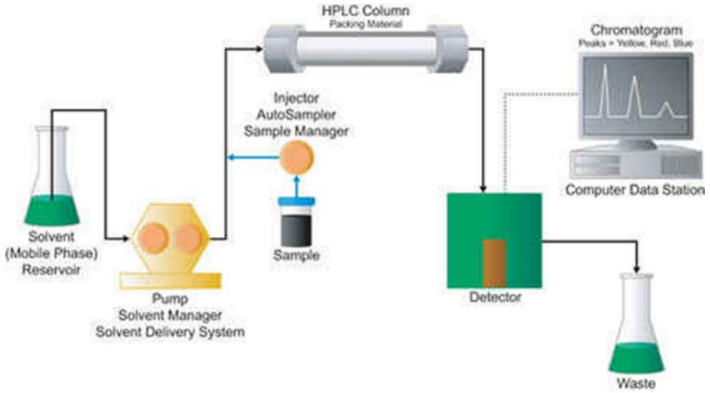


Figure 2.9 HPLC diagram depicting different compartments of the instrument (Anon., 2012).

HPLC is favoured for its high sensitivity and separation efficiency that is achieved through the use of tightly packed columns (Siouffi, 2000). Solvents and columns can be easily changed according to the type of analytes to be separated and this offers users a wide range of selectivity for a broad spectrum of analytes (Siouffi, 2000).

With respect to separation modes, reversed-phase liquid chromatography (RP-LC) is the dominant HPLC mode, especially for the separation of mixtures of organic compounds (Snyder *et al.*, 2010). It is used for approximately 75% of all HPLC methods and its popularity is attributed to its reproducibility and broad applicability (Anon., 2012). Reversed-phase, as opposed to normal-phase chromatography, involves a non-polar stationary phase (*e.g.* C_{18}) and a polar mobile phase (*e.g.* water and an organic solvent like acetonitrile) (Snyder *et al.*, 2010). Analytes are separated based on their hydrophobicity, less polar analytes have increased interaction with the non-polar stationary phase and therefore elute later than more polar compounds (Snyder *et al.*, 2010). Other separation modes include normal-phase liquid chromatography, non-aqueous reversed-phase chromatography, hydrophilic interaction chromatography, size-exclusion chromatography, ion-exchange chromatography and ion-pair chromatography (Snyder *et al.*, 2010).

Method development for an analytical instrument is a trial and error approach and the goal is to create ideal conditions for separating a group of analytes of a sample. The process involves sample pre-treatment (extraction method), selection of separation conditions (stationary phase, mobile phase, column temperature and flow rate), detection of analytes, quantitation and method validation (Snyder *et al.*, 2010). The evolution of RP-HPLC methods developed for analysis of rooibos phenolic compounds is summarised in Table 2.5.

Table 2.5 RP-HPLC methods used for quantitative analysis of rooibos (*Aspalathus linearis*) phenolics (adapted from Beelders, 2011).

Objectives	Extract preparation/analyte matrix	Analytes	Stationary phase and column temperature	Mobile phase and flow rate	Analysis time	Reported by
Quantitative analysis of aspalathin and nothofagin as affected by processing	Hot water extraction followed by liquid-liquid extraction with ethyl acetate	Protocatechuic acid; *p*-hydroxybenzoic acid; vanillic + caffeic acid; *p*-coumaric acid; aspalathin; orientin + ferulic acid; isoorientin; vitexin; nothofagin; rutin + isoquercitrin (n=7)	Merck LiChrospher 100 RP-$_{18}$ (250x4 mm i.d., 5 µm) Column temp.: 38°C	Gradient elution with A: methanol B: 2% formic acid in water ($v.v^{-1}$) Flowrate: 0.4–1.2 $mL.min^{-1}$	125 min	Joubert (1996)
Quantitative characterisation of rooibos flavonoids	Hot water and cold methanol infusions	Isoorientin; orientin; aspalathin; vitexin; rutin; isovitexin; isoquercitrin; luteolin; quercetin; chrysoeriol (n=10)	Waters Symmetry Shield C_{18} (250x4.6 mm i.d., 5 µm) Column temp.: room temp.	Gradient elution with A: acetonitrile B: 0.1% acetic acid in water ($v.v^{-1}$) Flowrate: 0.8 $mL.min^{-1}$	30 min	Bramati *et al.* (2002) Bramati *et al.* (2003)
Quantification of select flavonoids for use as quality parameters for green rooibos quality	Hot water extraction	Aspalathin; nothofagin; isoorientin; orientin; rutin; isoquercitrin (n=6)	Agilent Zorbax SB-C_{18} (150x3.0 mm i.d., 3.5 µm) Column temp.: 35°C	Gradient elution with A: 1% formic acid in water ($v.v^{-1}$) B:acetonitrile Flowrate: 0.5-0.7 $mL.min^{-1}$	40 min	Schulz *et al.* (2003)
Identification and quantification of glycosyl flavonoids in rooibos using LC-MS	Hot water extraction	Vitexin; orientin; isovitexin; isoorientin; nothofagin; aspalathin; luteolin-7-*O*-glucoside; isoquercitrin; hyperoside; rutin; daidzin (n=12)	Nomura Develosil ODS UG-5 (150x2.0 mm i.d.) Column temp.: 35°C	Gradient elution with A: 0.1% formic acid in 80% acetonitrile ($v.v^{-1}$) B: 0.1% formic acid in water($v.v^{-1}$) Flow rate: 0.2 $mL.min^{-1}$	50 min	Kazuno *et al.* (2005)
Determination of the effect of heat on major phenolic compounds in rooibos iced-teas	Commercial rooibos iced teas/fruit teas	Aspalathin; nothofagin; isoorientin; orientin (n=4)	Agilent Zorbax Eclipse XDB-C_{18} (150x4.6 mm i.d., 5 µm) Column temp.: 38°C	Gradient elution with A: 2% acetic acid in water ($v.v^{-1}$) B: acetonitrile Flow rate: 0.8 mL.min-^{1}	23 min	Joubert *et al.* (2009) Joubert *et al.* (2010b) De Beer *et al.* (2011)
Quantification of flavonoids in unfermented and fermented rooibos; and aspalathin and erioditol metabolites in urine	'Ready-to-drink' rooibos tea; urine	Aspalathin; nothofagin; eriodictyol-*C*-glucosides (4); orientin; isoorientin; vitexin; isovitexin; hyperoside; isoquercitrin; rutin; rutin isomer; luteolin; quercetin (n=16) Aspalathin and eriodictyol metabolites in urine	Phenomenex Synergi (C_{12}) RP-MAX 80Å (250x4.6 mm i.d., 4 µm) Column temp.: 40°C	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	Stalmach *et al.* (2009)
Determination of bioavailability and metabolism of rooibos flavonoids	Hot water extraction; blood and urine samples from volunteers who consumed rooibos drinks (rooibos tea and isolated active fraction)	Aspalathin; nothofagin; isoorientin; orientin; rutin; hyperoside; isoquercitrin; vitexin; isovitexin; luteolin-*O*-galactoside (n=10)	Phenomenex Luna Phenyl–Hexyl (250x4.6 mm i.d., 5 µm) Column temp.: room temp.	Gradient elution with A: 2% acetic acid in water ($v.v^{-1}$) B: acetonitrile Flow rate: 0.5 $mL.min^{-1}$	*ca*. 80 min	Breiter *et al.* (2011)

		Aspalathin; isoorientin; and orientin in urine (n=3)				
Development of HPLC method for analysis of rooibos phenolics with improved resolution and analysis time	Hot water extraction	Aspalathin; nothofagin; orientin; isoorientin; vitexin; isovitexin; luteolin; luteolin-7-*O*-glucoside; chrysoeriol; quercetin; isoquercitrin; hyperoside; rutin; phenylpyruvic acid-2-*O*-glucoside (n=14)	Agilent Zorbax SB-C_{18} (100x4.6 mm i.d., 1.8 µm) Column temp.: 37°C	Gradient elution with A: 2% acetic acid in water ($v.v^{-1}$) B: acetonitrile Flow rate: 1.0 $mL.min^{-1}$	45 min	Beelders *et al.* (2012) Joubert *et al.* (2012) Coetzee *et al.* (2014) Joubert *et al.* (2016)
Development for rapid separation of four major phenolics in preparation for isolation of aspalathin and nothofagin using HPCCC	Hot water extraction	Aspalathin; nothofagin; isoorientin; orientin (n=4)	Agilent Poroshell SB-C_{18} column (50×4.6 mm i.d., 2.7 µm) Column temp.: 30°C	Gradient elution with A: 0.1% formic acid in water ($v.v^{-1}$) B: acetonitrile Flow rate: 1.0 $mL.min^{-1}$	16 min	De Beer *et al.* (2015) De Beer *et al.* (2017)
Development of method targeted at the separation and quantification of flavanone intermediate oxidation products of aspalathin	Hot water and 40% acetonitrile extraction	Eriodictyol-glucopyranoside stereo-isomers (4); PPAG; Isoorientin; orientin; vitexin; isovitexin; luteolin-7-*O*-glucopyranoside; aspalathin, nothofagin, quercetin-3-*O*-robinobioside; hyperoside; rutin; isoquercitrin (n=16)	Agilent Poroshell 120 C_{18} (150x4.6 mm i.d., 2.7 µm) Column temp.: 44.5°C	Gradient elution with A:2% acetic acid in water ($v.v^{-1}$) B: acetonitrile Flow rate: 1 $mL.min^{-1}$	46 min	Walters *et al.* (2017a)

The first instance of rooibos phenolic separation for quantification of the dihydrochalcones, aspalathin and nothofagin, was reported by Joubert (1996). The compounds were separated using a Merck LiChrospher 100 C_{18} column (250x4.0 mm i.d., 5 µm) and gradient elution with methanol and 2% aqueous formic acid as solvent. The column was thermostatted to 38°C and an analysis time of 125 min was employed. In addition to the separation of the dihydrochalcones, other flavonoids were also separated (Table 2.5). The method was subsequently used in an investigative study on the antioxidant and pro-oxidant activities of aqueous extracts and crude polyphenolic fractions of rooibos (Joubert *et al.*, 2005). A comparison was made between unfermented and fermented rooibos aqueous extracts (n=7 each), confirming substantially less aspalathin and nothofagin in fermented rooibos extract (Table 2.6).

Table 2.6 Quantitative data on phenolic composition of aqueous extracts of unfermented and fermented rooibos expressed as mass percentage of solids (Joubert *et al.*, 2005).

Compound	Unfermented	Fermented
Aspalathin	12.29	0.61
Isoorientin	0.81	0.85
Orientin	0.59	0.76
Nothofagin	1.08	0.17
Vitexin	0.13	0.17
Isovitexin	0.17	0.11
Isoquercitrin+rutin	0.16	0.11
Other	trace	0.08

Bramati *et al.* (2002) developed a HPLC method for quantification of rooibos flavonoids using a C_{18} Symmetry column (250x4.6 mm i.d., 5 µm) and gradient elution using 0.1% aqueous acetic acid and acetonitrile. Separation of 10 flavonoids was achieved (Table 2.5). The slightly acidified water/acetonitrile mobile phase created favourable conditions for well separated, symmetrical peaks for all 10 standards in 30 min. There was only a slight issue with the separation of isoorientin and orientin, but the peaks could still be integrated. Extracts were prepared by hot water infusion and cold methanol infusion. The main compounds identified in the extracts were aspalathin, rutin and orientin, followed by isoorientin and isoquercitrin. Hyperoside coeluted with isoquercitrin at 16.4 min and therefore the fractions were collected and analysed by micellar electrokinetic capillary electrophoresis, which could effectively separate the two compounds. There were no major differences between the phenolic content of the cold methanol and hot water extractions, which is relevant for *in vivo* studies where aqueous extracts are used to administer rooibos phenolics (Bramati *et al.*, 2002). This method was applied in a subsequent study by Bramati *et al.* (2003) where the phenolic content of unfermented rooibos was quantified and compared to the results of fermented rooibos phenolics reported by Bramati *et al.* (2002) (Table 2.7). The peak at 16.4 min was quantified as isoquercitrin, because the absorption was essentially based on the aglycon moiety.

Table 2.7 Quantitative data of flavonoids detected in unfermented and fermented rooibos aqueous extracts ($mg.g^{-1}$) (Bramati *et al.*, 2003).

Compound	Unfermented	Fermented
Isoorientin	3.57	0.833
Orientin	2.336	1.003
Aspalathin	49.92	1.234
Vitexin	0.504	0.330
Rutin	1.69	1.269
Isovitexin	0.659	0.265
Isoquercitrin+hyperoside	0.326	0.429
Luteolin	0.022	0.029
Quercetin	0.042	0.107
Chrysoeriol	0.0008	0.022

Aspalathin and nothofagin are commonly regarded as markers for determining the presence or extent of fermentation in rooibos. Thus, Schulz *et al.* (2003) developed a method for detecting the most relevant flavonoids with improved sensitivity and shorter analysis time than the earlier method reported by Joubert (1996). A Zorbax column (150x3.0 mm i.d., 3.5 µm) with gradient elution using 1% aqueous formic acid and acetonitrile achieved separation of aspalathin, nothofagin, isoorientin, orientin, rutin and isoquercitrin in 40 min. In comparison to Joubert (1996), the analysis time was halved and sensitivity of the method had improved. The method was also used in the development of a near infrared spectroscopy (NIRS) prediction model for aspalathin, nothofagin and total dihydrochalcones in rooibos (Manley *et al.*, 2006).

LC-MS with neutral loss scan strategy was employed for the determination and quantification of glycosylated flavonoids in rooibos (Kazuno *et al.*, 2005). The efficacy of the HPLC method was tested on unfermented rooibos extracts. Separation of 11 glycosyl flavonoids was achieved within 50 min using a Develosil ODS UG5 column (150x2.0 mm i.d., 5 µm). Quantitative data on a rutin 'isomer' found in rooibos tea was reported here for the first time.

Rapid quantification of aspalathin and its corresponding flavones, isoorientin and orientin, by HPLC analysis was reported by Joubert *et al.* (2009). Separation was achieved using a Zorbax Eclipse XDB-C_{18} column (150x4.6 mm i.d., 5 µm) and gradient elution with acetonitrile and 2% aqueous acetic acid. The column was thermostatted at 38°C and the analysis time was reduced to 23 min. The method was applied to determine the aspalathin, orientin and isoorientin content of a large number of commercial ready-to-drink rooibos iced teas (Joubert *et al.*, 2009). Application of this method was also seen in two other studies on rooibos (Joubert *et al.*, 2010b; De Beer *et al.*, 2011), one of food ingredient extract of fermented rooibos (Joubert and De Beer, 2012) and the other of green rooibos plant material (Joubert *et al.*, 2013).

Stalmach *et al.* (2009) developed a method for quantitative analysis of rooibos phenolic compounds in unfermented and fermented 'ready-to-drink' teas and the urine plasma samples of subjects who had consumed the tea. Compounds that were separated includes aspalathin, nothofagin, isoorientin, orientin, vitexin, isovitexin, hyperoside, isoquercitrin, rutin, rutin isomer, luteolin, quercetin and diasteriomeric mixtures of eriodictyol-*C*-glucosides. Separation was achieved under gradient conditions on a 4 6m C_{12} RP column

(250x4.6 mm) with a chromatographic run time of 75 minutes. A similar study by Breiter *et al.* (2011) detected the presence of seven metabolites of both aspalathin and nothofagin by HPLC-MS/MS analysis, thus confirming the metabolism of aspalathin and nothofagin in the human body.

Beelders *et al.* (2012) used a kinetic optimisation approach to develop a method with good quality resolution of important peaks and a reasonable analysis time. Temperature and gradient elution conditions were simultaneously optimised on a Zorbax SB-C_{18} (100x4.6 mm i.d., 1.8 µm) column. Separation of 15 key phenolics was achieved within 37 min. Quantitative data for PPAG, ferulic acid and quercetin-3-*O*-robinobioside were reported for the first time in this study. Content values for rutin were 20% less than those reported in previous studies. Higher levels of rutin reported in previous studies was due to the perfect co-elution of rutin and quercetin-3-*O*-robinobioside (Beelders *et al.*, 2012). This method was used in many subsequent studies, including analysis of rooibos infusions (Joubert *et al.*, 2012, 2016) and extracts used in bioactivity studies (Muller *et al.*, 2012, 2016; Sanderson *et al.*, 2014; Kamakura *et al.*, 2015; Van Der Merwe *et al.*, 2015). Additionally, it was concluded that this method would serve well in fingerprint analysis studies of rooibos. Thus, it was utilised by Orzel *et al.* (2014) to model the antioxidant capacity of rooibos and by Stanimirova *et al.* (2013) to determine the effect of production season, quality grade and steam pasteurisation on fermented rooibos.

De Beer *et al.* (2015) developed a method for rapid detection of aspalathin, nothofagin, isoorientin and orientin in green rooibos extracts, using a Poroshell SB-C_{18} column (50×4.6 mm i.d., 2.7 µm), thermostatted at 30°C and gradient elution using 0.1% aqueous formic acid and acetonitrile. The 16 min analysis time allowed for rapid separation and quantification of the four phenolic compounds during the development of an isolation protocol using high-performance countercurrent chromatography (HPCCC). This method was also applied in a subsequent study by De Beer *et al.* (2017) for analysis of aspalathin, nothofagin, orientin and isoorientin in green plant material and freeze-dried extracts.

Recently, Walters *et al.* (2017a) developed a method specifically targeting the separation of the flavone intermediate oxidation products of aspalathin (*i.e.* eriodictyol-glucopyranoside stereoisomers). The method developed by Beelders *et al.* (2012) was used as a starting point. A core shell column was singled out as being ideal for the desired separation, suitable for the separation and quantification of 15 rooibos phenolic compounds and PPAG (Table 2.8). Eriodictyol-glucopyranoside stereoisomers were quantified in rooibos extract for the first time in this study. In this regard, the method reported by Walters *et al.* (2017a) is suitable for construction of chromatographic fingerprints of rooibos as it enables further study of the oxidation of aspalathin.

Table 2.8 Phenolic composition (g.100 g^{-1} plant material) of green, semi-fermented and fermented rooibos plant material (n =10 each) extracted with 40% aqueous acetonitrile (Walters *et al.*, 2017a).

Compounds	Green	Semi-fermented	Fermented
(*S*)-eriodictyol-6-*C*-β-D-glucopyranoside	nq[1]	0.023	0.047
(*R*)-eriodictyol-6-*C*-β-D-glucopyranoside	nq[1]	0.021	0.049
(*S*)-eriodictyol-8-*C*-β-D-glucopyranoside	nq[1]	0.006	0.014
(*R*)-eriodictyol-8-*C*-β-D-glucopyranoside	nq[1]	0.007	0.014
PPAG	0.100	0.081	0.083
Aspalathin	1.20	0.610	0.170
Nothofagin	0.098	0.043	0.015
Isoorientin	0.157	0.124	0.124
Orientin	0.149	0.120	0.118
Quercetin-3-*O*-robinobioside	0.084	0.067	0.063
Vitexin	0.021	0.015	0.018
Hyperoside	0.016	0.011	0.012
Rutin	0.032	0.024	0.018
Isovitexin	0.034	0.025	0.023
Isoquercitrin	0.014	0.009	0.011
Luteolin-7-*O*-β-D-glucopyranoside	0.007	0.008	0.011

[1]Compounds not quantified due to low signal-to-noise ratios

2.2. Fingerprints

2.2.1.Introduction to fingerprints

Similar to humans, herbal products are also distinguishable by fingerprints, but in this case the fingerprint is "a characteristic profile or pattern which chemically represents the sample composition and in which, usually, as much information as possible is reflected" (Goodarzi *et al.*, 2013). Fingerprint analysis is important, because previously herbal products were analysed using targeted, single response approaches focussing on the effects of one or two major compounds. In doing so the effects of compounds present in lower concentrations were being neglected (Hendriks *et al.*, 2005). Herbal products are known to be chemically complex and compounds that occur even in the lowest concentrations could adversely affect their health promoting capabilities (Tistaert *et al.*, 2011). As the popularity of herbal products increased, it became mandatory to find analytical tools powerful enough for their analysis (Alaerts *et al.*, 2010).

Fingerprints provide an analytical platform that accommodates the multi-response, hypothesis-free analysis that is necessary for the evaluation and quality control of herbal products. Over the past decade the World Health Organisation (WHO), the American Food and Drug Administration (FDA), the European Medicine Evaluation Agency (EMEA) and the Chinese State Food and Drug Administration (SFDA) have all

promoted the use of fingerprinting as a method for the assessment and quality regulation of herbal products (Tistaert *et al.*, 2011; Xie et al., 2006).

Fingerprints can be obtained by spectroscopic, chromatographic and electrophoretic methods. Spectroscopic methods include: Infrared, Raman, nuclear magnetic resonance and mass spectrometry. Chromatographic methods include but are not limited to: thin-layer chromatography, HPLC, ultra high pressure liquid chromatography (UHPLC) and GC. HPLC is the preferred method for analysis of non-volatile components in herbal material (Tistaert *et al.*, 2011; Goodarzi *et al.*, 2013).

2.2.2. Chromatographic fingerprints

A chromatographic fingerprint should be a chemical representation of a sample and its reliability is dependent on the degree of separation and the concentration distribution of chemical components (Gong *et al.*, 2003). Chromatography is the ideal analytical technique for fingerprinting (Gong *et al.*, 2003). Its powerful separation capabilities allows the analyst the opportunity to view a complex system in the form of simpler sub-systems (Fan *et al.*, 2006). Samples with similar fingerprints suggest that the samples are similar at a chemical level and should most likely exhibit the same pharmacological properties (Lucio-Gutiérrez *et al.*, 2012).

Generally, a fingerprint used for quality control testing is constructed of a single chromatogram developed by HPLC-UV. However, single detection methods yield inadequate representations of samples as they neglect valuable information regarding chemical complexities of herbal products (Liang *et al.*, 2004). Currently more robust hyphenated techniques are being utilized, such as HPLC-diode array detector (DAD), as these techniques can provide additional spectral information and are more commonly used for development of herbal fingerprints (Liang *et al.*, 2004).

2.2.3. Role and application of fingerprints in the investigation of changes in food quality

The effects of processing, preservation or storage on food quality are often investigated with the main aim of identifying potential marker compounds. When attempting to identify marker compounds, it is necessary to have a comprehensive overview of all relevant chemical changes taking place. This is unachievable when implementing a single response study. In this regard, fingerprint analysis is the more recommendable choice as opposed to targeted, single response approaches- because it is not biased toward preselected compounds. Often fingerprints are used as a screening step to identify compounds that are influenced by food quality changes. The potential of these compounds as markers is tested by means of kinetic modelling.

Recent studies on food quality changes have implemented the use of hyphenated chromatographic techniques and chemometric methods to successfully identify marker compounds for accelerated shelf-life testing (ASLT) (Kebede *et al.*, 2015a,b). Fingerprinting was used as a prerequisite for identifying different types of volatile compounds (sulphur containing compounds, fatty acid derivatives, furanic compounds and Strecker aldehydes) that showed significant changes in concentration during storage of broccoli and carrot puree (Kebede *et al.*, 2015a,b). In a subsequent study, Kebede *et al.* (2015c) compared the reaction kinetics of key volatiles (identified by Kebede *et al.* (2015a)) in carrot puree samples that had been sterilised using

different thermal preservation techniques. Results showed that the concentration of volatiles decreased to almost the same level when using either sterilisation method.

Wibowo *et al.* (2015a) investigated the quality changes of pasteurised mango juice after the addition of potential precursors (ascorbic acid, citric acid and sugars). A combination of targeted and untargeted data was used to identify potential markers for quality changes of different formulations of mango juice. It was evident that the various precursors had different effects on the degradation kinetics of compounds in mango juice and therefore individual markers were selected for each formulation. Kinetic modelling was subsequently performed on the shelf-life markers and preselected targeted parameters of pasteurised mango juice (Wibowo *et al.* 2015b).

The phenolic changes occurring during rooibos pasteurisation was characterised by Marini *et al.* (2015). A targeted analysis approach was used to identify eight compounds that were significantly changing during pasteurisation, which could potentially be used as phenolic markers for rooibos pasteurisation.

2.3. Chemometric analysis of fingerprints

Chromatographic data can only be used to extract useful information once they have been properly pre-processed and analysed (Amigo *et al.*, 2010). Variation between chromatograms of the same sample is common. This may be the result of variation stemming from instrumental artefacts or from variation that exists within the samples (Amigo *et al.*, 2010). This can be corrected by application of relevant pre-processing techniques. The importance of pre-processing to fingerprint analysis is stressed in section 2.3.1 and a broad overview of the different types of techniques used and the artefacts corrected for are also provided.

Data analysis methods can be divided into univariate and multivariate analysis. Univariate analysis entails the examination of one changing element at a time, whereas in multivariate analyses two or more predefined experiments are performed and several influential factors are examined simultaneously. The range of chemometric methods that can be used for analysing the large amounts of data generated by fingerprints is not limited (Tistaert *et al.*, 2011). In the sections to follow the use of multivariate analysis techniques in fingerprint analysis will be discussed.

2.3.1. Pre-processing of chromatographic data

For constant reproduction of ideal chromatographic data, experimental conditions should remain constant, however, this is not always the case and ideal chromatographic data are often not obtained (Gong *et al.*, 2005; Amigo *et al.*, 2010). Artefacts may arise in the form of peak retention time shifts, baseline offset and poor signal-to-noise ratio (Amigo *et al.*, 2010). The disregard of these undesirable variations may lead to data analysis producing inaccurate results. Artefacts in raw data therefore must be corrected for by application of pre-processing techniques. Pre-processing is a crucial prerequisite for data analysis of fingerprints (Tistaert *et al.*, 2011). Figure 2.10 is a basic decision tree illustrating the type of pre-processing technique that should be applied when a particular artefact is observed.

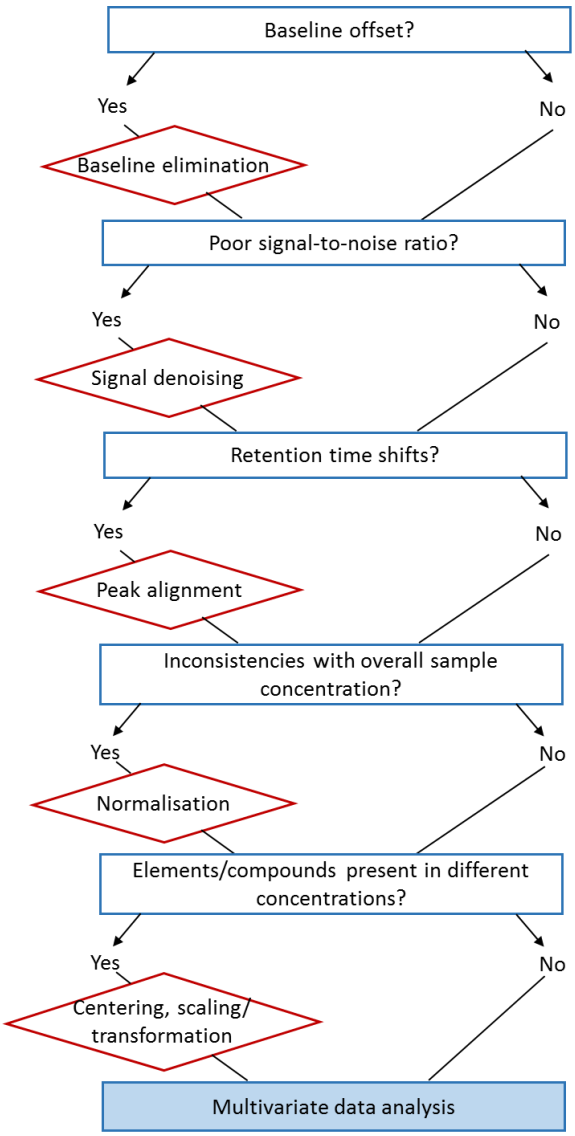


Figure 2.10 Decision tree of common chromatographic artefacts and the corresponding pre-processing techniques applied to correct them.

2.3.1.1. Baseline elimination

A varying baseline can greatly influence measures of similarity used to compare chromatograms such as the correlation coefficient or the Euclidean distance (Daszykowski and Walczak, 2006). General methods used to correct for baseline offset include fitting a polynomial curve and subtracting this from the overall signal or separating the baseline from the signal in a low rank factor model (Skov, 2008; Amigo *et al.*, 2010). Of these approaches, the polynomial fitting approach asymmetric least squares proposed by Eilers (2004), is one of the most cited baseline elimination tools (Daszykowski and Walczak, 2006; Skov, 2008). It works by fitting an initial polynomial of a specified order to all data points in the chromatogram. Iterations of asymmetric weighting (positive deviations are penalised to a higher degree than negative deviations) is applied until a baseline is approximated within a certain, predefined limit (Skov, 2008; Engel *et al.*, 2013). The estimated baseline can then be subtracted from the overall signal (Skov, 2008).

2.3.1.2. Denoising

Noise is described as fluctuations observed in the original signal that are not representative of the behaviour of the primary variable(s) (Reis *et al.*, 2009). Any measured data are most likely to contain noise and require the application of signal enhancement techniques such as filtering and smoothing to increase the signal-to-noise ratio and reduce the noise (Skov, 2008). Denoising techniques cannot be used to improve precision or accuracy. Their capabilities are extended mainly to identify hidden peaks in a noisy background (Skov, 2008). Common techniques used for removing noise involve the smoothing of a signal by use of the Savitzky-Golay algorithm or by means of wavelets (Engel *et al.*, 2013). The Savitzky-Golay algorithm applies the least squares fit of a polynomial to small data windows in a chromatogram to replace the noisy data elements with fitted polynomial elements (Skov, 2008). The wavelet approach is closely related to Fourier transformation, where noise is removed by filtering the high frequency parts of the transformed signal (Reis *et al.*, 2009; Engel *et al.*, 2013).

2.3.1.3. Peak alignment

Variations in retention time are the result of instrumental artefacts including, but not limited to, stationary phase decomposition, changes in temperature or changes in mobile phase composition (Jellema, 2009). Alignment is defined by Amigo *et al.* (2010) as "a mathematical operation where similar chemical features are repositioned so that they appear at the same elution time, in different runs". The term alignment covers a broad spectrum of techniques used to correct retention time differences, including both techniques that only allow the movement of peaks by a certain degree along the time axis, as well as techniques that are flexible enough to allow for the swapping of peaks (more prevalent in NMR data). However, the term warping, as opposed to alignment, strictly refers to the stretching or compressing of chromatograms (Bloemberg *et al.*, 2013). It is important to verify the identity of aligned peaks (Liang *et al.*, 2004). Detectors (*e.g.* DAD) provide structural information of the analytes and this can be used to avoid the misalignment of non-corresponding peaks (Tistaert *et al.*, 2011).

Target selection

Target selection is crucial for any alignment process. It entails the selection of a target or reference chromatogram to which the rest of the chromatograms will be aligned (Bloemberg *et al.*, 2013). Choosing a good representative target out of many chromatograms is no trivial task. Some chemometricians propose the use of a random chromatogram from a series, while others strongly believe that because this step is so critical there should be some sort of reasoning behind the selection. Different methods have been discussed in detail by Daszykowski and Walczak (2007). Two main methods have been proposed. The first suggests using a chromatogram from the given data set. This could entail the use of the first chromatogram of the data set or even one from the middle point. A more popular choice is choosing the chromatogram that is most similar on average to the other chromatograms- this can differ based on the type of similarity measurement used. Daszykowski and Walczak (2007) concluded that using the chromatogram with the highest mean correlation

coefficient yields optimal warping results, while Amigo *et al.*(2010) suggests using the product of the absolute value of the pair wise correlation coefficient. The second method known as target creation entails the creation of a chromatogram that contains the chemical information of several other chromatograms (Daszykowski and Walczak, 2007). Generally, a mean chromatogram is used. However, this method is not a popular choice because the averaging of a series of chromatograms is likely to introduce unique peaks from atypical chromatograms which can result in alignment difficulties (Bloemberg *et al.*, 2013).

Dynamic time warping

Dynamic time warping (DTW) originated from the field of speech recognition. The algorithm was first proposed by Sakoe and Shiba (1971, 1978) and was later adapted for alignment of chromatographic shifts. It involves the stretching and compression of the time axis to align a query chromatogram to a target by means of dynamic programming. The non-linear warping aims to align chromatograms so that the cumulative distance between the chromatograms is minimised (Pravdova *et al.*, 2002). Individual data points of the query chromatogram are moved so that similar events of the target chromatogram are matched (Jellema, 2009). The method used to determine the optimal warping path is described schematically in Figure 2.11. A grid configuration is used with the target or reference signal on the y-axis and the query signal on the x-axis. If the 2 signals were perfectly aligned the warping path would be represented by a straight diagonal line, but the warping path will differ when there are points on the y- and x-axis that do not match up. The 11th point on the query chromatogram (x-axis) does not match the events on the y-axis and the warping path indicates the movement of this point to match that of y-axis (Figure 2.11).

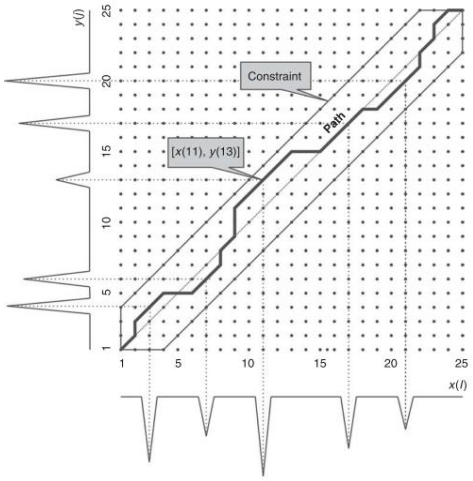


Figure 2.11 Illustration of warping function of DTW. The grid depicts data points of query chromatogram (x-axis) and target chromatogram (y-axis) and the optimised warping path with pre-selected constraints (Jellema, 2009).

With so much flexibility in terms of movement of the data points, some kind of constraint is necessary to obtain sensible warping results. The first restriction is that the beginning and end points are fixed and the second restriction provides a limit on the extent to which the query signal can be moved to obtain the optimal warping path and this is dependent on the maximum expected shift within a chromatogram (Pravdova *et al.*,

2002). DTW has two different operating modes, namely: symmetric and asymmetric modes. The symmetric algorithm sees both the target and query chromatogram as equally important and therefore, if the positions of the chromatograms on the axes are changed, the same warping path will be obtained in both instances (Pravdova *et al.*, 2002). With the asymmetric algorithm one of the chromatograms are labelled as the target and if the roles of the 2 signals are interchanged it will result in a different warping path and minimum distance (Pravdova *et al.*, 2002).

Correlation optimised warping

Correlation optimised warping (COW) was originally introduced by Nielsen *et al.* (1998) with the specific purpose to correct time axis shifts in chromatographic data. The technique has been praised for its simplicity (Amigo *et al.*, 2010) and also criticised for its lengthy computation time (Tistaert *et al.*, 2011), but of all the alignment techniques COW is the most popular and also the most studied. Instead of the global alignment of signals where an entire chromatogram is shifted either left or right to match a target chromatogram, COW uses a local approach (Tomasi *et al.*, 2004). Both the target and query chromatogram are divided into segments of equal length and each segment is aligned to the target by a stretching or compressing motion using linear interpolation (Tomasi *et al.*, 2004). By locally aligning the segments within a chromatogram, COW is capable of handling more complex, non-linear shifts. The computation time of the algorithm is dependent on two parameters that must be set by the user. The first parameter is the number of data points within a segment, or the size of the segments, and the second is the slack, which determines the number of data points the segment can be adjusted by in either direction. The warping path that maximises the correlation between the two chromatograms, also known as the optimal warping path, is found by means of dynamic programming (Nielsen *et al.*, 1998). This means that all possible combinations of transformations are examined to determine the optimal warping path. The parameters can be changed and the algorithm will find the best correlation for those parameters. Parameters must be optimised for each individual chromatogram and this adds to the computation time (Tistaert *et al.*, 2011). Important to note is that peaks at the beginning and end of chromatograms are more difficult to align as these points are fixed, unlike peaks found toward the centre where the warping flexibility increases (Van Nederkassel *et al.*, 2006). When compared to DTW, it was concluded that COW was more suitable for alignment of chromatograms, as DTW proved to be too unconstrained (Tomasi *et al.*, 2004). Where the COW algorithm works on segments of data (keeping consecutive points together), DTW works on individual data points (Tomasi *et al.*, 2004). The restriction on movement of the data points avoids extreme stretching or compressing of the chromatogram that can result in peak deformations (Jellema, 2009). Figure 2.12 illustrates how COW aligns sections of the query chromatogram to match the target chromatogram.

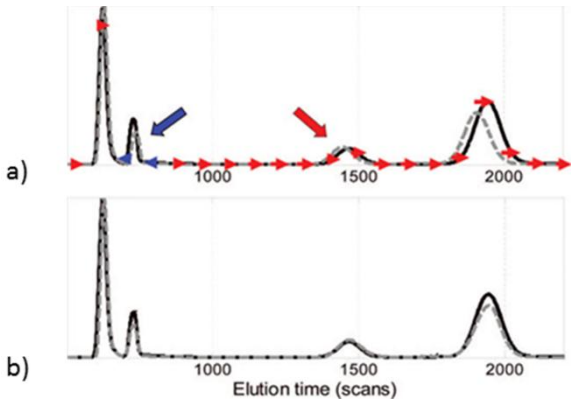


Figure 2.12 a) Illustration of how COW works to align a query chromatogram (dotted line) to a target chromatogram (solid line). The number of arrows depict the number of movable segments and the direction of the arrow shows which direction the segment is moved to align with the target chromatogram. b) The result after alignment using COW (Amigo *et al.*, 2010).

Parametric time warping

Parametric time warping (PTW) was first introduced by Eilers (2004) as a method for aligning chromatograms without the undesirable features experienced when using dynamic programming methods such as dynamic time warping and correlation optimised warping. A second degree polynomial is calculated so that the optimal polynomial coefficients minimise the sum of squares between the target and query chromatogram (Eilers, 2004; Van Nederkassel *et al.*, 2006). The method is fast and has small demands on computing memory. It also avoids artefacts such as peak flattening or jumping of peaks that may occur in DTW (Eilers, 2004). The gradual changes experienced in the chromatographic data are modelled as a true function and used to correct simple shifts that may occur as a result of column aging (Jellema, 2009). The technique can be described as parametric as opposed to the non-parametric methods, DTW and COW. Which means that PTW does not cope well with sudden disturbances in signals such as background noise. The mechanism of warping by PTW is two-fold. Firstly, a set of calibration chromatograms are aligned to a target to determine the individual polynomial equations (warping function) and the second step uses the warping functions to estimate a global warping function that can be used on the data set of interest (Jellema, 2009). Implementing the use of a global warping function reduces the flexibility of the function, making the use of PTW ideal in cases where only systematic or non-complex shifts are seen (Skov, 2008). Certain prerequisites need to be met for the warping function to work effectively (Jellema, 2009). Baseline removal is essential as a difference in baselines between query samples and the target can contribute to the sum of squares (Van Nederkassel *et al.*, 2006; Jellema, 2009). Additionally, the warping function is sensitive to sharp peaks; it is therefore suggested that they are smoothed by means of the Whittaker smoother (Van Nederkassel *et al.*, 2006; Jellema, 2009). The chromatograms should have approximately the same average values and normalisation should be performed after the baseline has been removed (Van Nederkassel *et al.*, 2006; Jellema, 2009).

Semi-parametric time warping

To counter the problem of inflexibility experienced with PTW, Eilers (2004) developed semi-parametric time warping (STW). Similar to PTW, STW finds the best possible match of the query chromatogram to a target by minimising the sum of squares between the two (Jellema, 2009). The warping function makes use of B-splines instead of polynomial coefficients as seen in PTW. The B-splines are constructed by joining polynomial pieces together at certain values on the time axis (Bloemberg *et al.*, 2013). By increasing the number of B-splines (40 B-splines are generally used by default) the flexibility of the warping function increases and this makes it possible for correction of more complex shifts that occur within a short time interval. However, the computation time increases as a result of this. Increasing the B-splines may result in more variation than can be explained by the data and in order to correct for this a penalty term is added to the warping function. Important to note is that the same prerequisites (normalisation, denoising and centering) applied to PTW are required when using STW (Van Nederkassel *et al.*, 2006). In a comparison study, STW proved to have the shortest computation time and its alignment was comparable to that of COW (Van Nederkassel *et al.*, 2006). However, COW is said to be easier to explain and visualise and also has better overall alignment in terms of peak preservation (Skov, 2008).

Automated alignment

A great deal of research has been invested in the development of an automated alignment technique (Skov *et al.*, 2007; Daszykowski *et al.*, 2010; Tomasi *et al.*, 2011). An approach based on the principles of COW was proposed by Skov *et al.* (2007). It makes use of a so-called simplicity value as the goodness of fit criterion. To reduce the computational time, the required parameters (slack and segment length), including the selection of the reference sample, are automated (Skov *et al.*, 2007). A second approach proposed by Daszykowski *et al.* (2010) shares more similarities with STW. The aim was to obtain the perfect balance between restraint and flexibility by use of B-splines functions. The number of B-spline functions are automatically optimised to achieve the best fit by increasing the correlation coefficient between the reference and query chromatogram (Daszykowski *et al.*, 2010).

2.3.1.4. Normalisation

Normalisation, also referred to as row scaling, is described as a pre-processing technique used to remove undesirable variability between samples that result from concentration differences or other systematic effects (Bylesjö *et al.*, 2009). It is relevant for use where peak intensity is proportional to the concentration of a compound and it is not possible to use the same amount of matter for each sample (Li Vigni *et al.*, 2013). This phenomenon is often experienced with biological data or data generated from hyphenated chromatographic techniques (*e.g.* HPLC-DAD, LC-MS and UPLC-MS) and it has been labelled the 'size-effect' (Filzmoser and Walczak, 2014). Leaving the signals unprocessed may lead to bias towards the variation in overall sample concentration (Hendriks *et al.*, 2005).

There is no one single normalisation strategy that is suitable under all conditions. The selection of a normalisation strategy is dependent on the data characteristics and how well the data fit the underlying assumptions that are related to the chosen methods (Bylesjö *et al.*, 2009). A commonly used technique is the total area normalisation, which is simply applied by taking the inverse of the total area of a spectrum (row vector) and using it as a scaling factor for each row vector (Bylesjö *et al.*, 2009). For quantitative measures, signal intensities may also be normalised by means of an internal standard. If one internal standard is used globally, it is assumed that all the peaks behave in the same way and can be corrected for by the same internal standard (Amigo *et al.*, 2010). In the case where peaks behave differently from one another, more than one internal standard must be used to normalise local regions (Amigo *et al.*, 2010). Other types of normalisation also include setting the maximum peak height at the same value for all samples and dividing each signal value for one sample by the sum of all signal values for that sample, or alternatively the median instead of the sum of all the signal values can be used (Hendriks *et al.*, 2005). Probabilistic quotient normalisation (PQN) and pairwise log-ratios (PLR) are highly recommended for normalisation of chromatographic data (Filzmoser and Walczak, 2014). For PQN a dilution factor is estimated based on the median of the ratios of the elements of $\mathbf{x}_i$ (*i*th signal of a data set) and the corresponding elements of $\mathbf{x}_{ref}$ (a preselected standard signal) (Dieterle *et al.*, 2006). PQN, however, does have limitations as it assumes that the majority of the observations do not show significant differences between two studied groups (Filzmoser and Walczak, 2014). PLR makes use of a variation matrix, of which the elements are defined as $\log(x_{ij}/x_{ik})$, where j,k=1,…n (Filzmoser and Walczak, 2014; Walach *et al.*, 2017). Total sum normalisation (TSN), PQN, PLR and other compositional data (CODA) methods have been compared by Filzmoser and Walczak (2014) for their applicability to eliminate the size effect in chromatographic data.

2.3.1.5. Centering, scaling and transformation

Centering is applied to adjust for differences in compound concentrations and encompasses the conversion of concentrations to be centred on zero instead of the mean (Skov, 2008). It is typically applied in combination with a scaling or transformation method (Van den Berg *et al.*, 2006).

Unimportant elements (*i.e.* compounds) may mask the variability of the more important ones even after normalisation and this may be detrimental for data analysis techniques such as principal component analysis (PCA), which is used to describe the largest variation in the data. In such instances, scaling can be used to equalise the potential contribution each element will have in the eventual model (Engel *et al.*, 2013). Auto-scaling is a commonly used method whereby all the measured values for a signal are divided by the standard deviation of these values. However, this method may increase the apparent importance of compounds that have low standard deviations because they only contain noise. Other well-known scaling methods include pareto scaling, range scaling, level scaling and variable stability scaling (VAST) (Van den Berg *et al.*, 2006; Engel *et al.*, 2013).

Transformations differ from scaling because they modify individual elements of the data matrix rather than entire variables (Engel *et al.*, 2013). Examples of transformations include power and log transformation, which are easily applied by taking the log or square root of each data point in the matrix (Engel *et al.*, 2013).

Transformations achieve correction of heteroscedastic noise, conversion of multiplicative relations into additive relations and making skewed distributions more symmetric (Van den Berg *et al.*, 2006). Additionally, the transformations also have a 'pseudo-scaling' effect as they reduce large values in the data set relatively more than they do smaller values, however, the use of this pseudo-scaling effect is rarely sufficient to adjust for the magnitude of differences seen in concentration (Van den Berg *et al.*, 2006). One drawback of the log transformation is that it cannot deal with the value zero or any values from relatively low concentration compounds. On the other hand, the power transformation has no trouble with zero values or values close to zero. However, it does not have the ability to make multiplicative effects additive (Van den Berg *et al.*, 2006).

2.3.2.Multivariate data analysis

The advancement of chromatographic instruments has assisted in the migration of data analysis from a single response approach to a multivariate response approach (Grauwet *et al.*, 2014). Consequently, the amount of data being generated has increased significantly, and in response chemometricians have developed methods to properly analyse these enormous data sets. Multivariate data analysis (MVDA) can follow one of two paths, *i.e.* methods may either be unsupervised or supervised. Unsupervised data analysis techniques make use of only the fingerprint matrix (**X**). Data visualisation and exploration are performed without any *a priori* information on the samples. On the other hand, supervised analysis techniques use a **Y** matrix in conjunction with the **X** matrix. The **Y** matrix is also known as the response matrix and it contains additional information about the samples, for example quality grades (Marini *et al.*, 2015), processing state (Marini *et al.*, 2015), formulations (Wibowo *et al.*, 2015a) and storage times in the case of shelf-life testing (Kebede *et al.*, 2015a,b,c; Wibowo *et al.*, 2015c).

2.3.2.1. Unsupervised multivariate data analysis

Exploratory analysis

Exploratory analysis techniques are applied to visualise the data for identifying groups within the set and also for identifying outliers or strange occurrences (Goodarzi *et al.*, 2013). Techniques used include principal component analysis (PCA), robust PCA (rPCA), independent component analysis (ICA), projection pursuit (PP) and cluster analysis (CA) (Alaerts *et al.*, 2010). PCA is a variable reduction technique that defines new latent variables from the original data; these variables are known as principal components (PCs) (Wold *et al.*, 2009). The variation described by the PCs decrease successively, for example PC 1 will describe the highest variation, followed by PC 2, (Wold *et al.*, 2009). Interpretation is done visually by looking at the resulting scores and loading plots (Tistaert *et al.*, 2011). The x and y axes of the score plot are represented by the two highest PCs and the manner in which the scores are plotted will give an indication of the (dis)similarity between samples (Goodarzi *et al.*, 2013). The loadings plot is composed in a similar manner, but instead the individual loadings give insight into how samples differ in terms of sample variables (Goodarzi *et al.*, 2013). *A priori* information is used to reveal groups based on for example processing state or quality grades (Marini *et al.*, 2015).

Cluster analysis can be divided into two types, hierarchical and non-hierarchical. Hierarchical clustering results in tree-like structures known as dendograms, based on a measurement of (dis)similarity or an applied linkage method (Alaerts *et al.*, 2010). Larger groups are divided into smaller groups by means of divisive methods or, *vice versa* by agglomerative methods (Thiangthum *et al.*, 2012). Non-hierarchical methods produce a table of clusters that are not necessarily in any form of hierarchical order. Clusters are established by assigning observations based on their closeness to an assigned number of seed points or based on how close the observation is to a predefined distance from a seed number (Tistaert *et al.*, 2011).

Similarity analysis

Numerous parameters have been used to distinguish fingerprints based on their (dis)similarities (Goodarzi *et al.*, 2013). The most common measurement of (dis)similarity used is the correlation coefficient (r) (Alaerts *et al.*, 2010; Tistaert *et al.*, 2011). The correlation coefficient between two signals is defined as the scalar product of the normed mean centred signals (Alaerts *et al.*, 2010). Similarity ranges from 0 to 1, with 0 being an indication of no similarity and 1 indicating high similarity or identical fingerprints (Tistaert *et al.*, 2011). A reference signal from the sample set or a standardised extract can be used for comparison (Schulze *et al.*, 2016). The problem with using standardised extracts is that it is not always available and in this instance the mean or median signal of the data set is used (Tistaert *et al.*, 2011).

The congruence coefficient is another popular measurement of similarity and it differs from the correlation coefficient in the way that the two are calculated. The correlation coefficient is calculated by taking the mean signal into consideration, whereas the congruence coefficient computation does not subtract the mean prior to its calculation (Alaerts *et al.*, 2010). Distance calculations such as Euclidean and Mahalanobis distances form part of the parameters used as similarity measurements. The choice between distance calculations and correlation calculations are dependent on the objective of the study (Alaerts *et al.*, 2012).

2.3.2.2. Supervised multivariate data analysis

It is advisable to perform exploratory data analysis prior to any form of supervised methods. This is important as the models produced from these methods are often used to make predictions and it is crucial that outliers are removed prior to this step.

Pattern recognition or multivariate classification

Information about the **X** matrix is presented in the **Y** matrix and this is used to classify observations (in a non-continuous manner) into categories, for example, high concentration/medium concentration/low concentration (Alaerts *et al.*, 2010; Grauwet *et al.*, 2014). The model produced from this analysis must be validated on an independent test set to determine the confidence in its predictability. Popular methods used include linear and quadratic discriminant analysis, k-nearest neighbours, classification and regression trees, partial least squares discriminant analysis (PLS-DA) and Soft Independent Modelling of Class Analogy (SIMCA) (Alaerts *et al.*, 2010; Tistaert *et al.*, 2011). PLS-DA uses variance-covariance relationships between observations to separate them into classes. The variance-covariance between classes is maximised while variance-covariance within

classes are minimised to yield observations that fall within distinct categories (Chen *et al.*, 2008). The resulting latent variables will describe the maximum variance of observations based on the division of classes. The modelling system formed from SIMCA, in theory, designs a box for each category and the centre of the box is the mean value for that class (Chen *et al.*, 2008). PCs are calculated for each class and observations are placed into classes based on their distances from the boxes. Observations may fall into none or even more than one class (Chen *et al.*, 2008; Tistaert *et al.*, 2011). This is not often viable with other discrimination techniques because of issues arising with collinearity (Tistaert *et al.*, 2011).

Multivariate calibration

Multivariate calibration techniques are used to model a continuous property, for example, antioxidant activity (Tistaert *et al.*, 2011). Similar to multivariate classification, a model is designed based on a data set and its predictability is validated before the model can be used to predict values of activity for other samples. Popular methods used include stepwise multiple linear regression, principal component regression, partial least squares (PLS) and orthogonal projections to latent structure (OPLS) (Alaerts *et al.*, 2010). PLS creates latent variables that explain the variance of the model by maximising the covariance between the data matrix and the response variables. To determine which parameters are significant to the model a 'leave one out' cross validation procedure can be used, where a single parameter is left out and the root mean square error (RMSE) for each model generated is compared. The model with the lowest RMSE is the optimum model. Orthogonal variation in data matrix **X** can cause complications in PLS analysis and in this case OPLS can be used. The variation in data matrix **X** that cannot be correlated to the **Y** matrix (orthogonal variations) is removed to improve the predictive power of the model (Thiangthum *et al.*, 2012).

2.3.2.3. Analysis of high-dimensional data sets

Complex systems are often observed by measuring many variables according to a predefined experimental design (Jansen *et al.*, 2006; Smilde *et al.*, 2012). Experimental factors involved are typically a combination of dose, time and treatment, which results in high dimensional data sets. Using typical multivariate methods such as PCA or hierarchical cluster analysis do not take in account the experimental design, which means that important variation is lost in the model (Jansen *et al.*, 2006). More advanced methods have to be used to highlight all aspects of the experimental design (Smilde *et al.*, 2012). In this regard, there are several methods that combine the ideas of univariate analysis (*i.e.* analysis of variance, ANOVA) and the advantages of dimensionality techniques designed for handling high dimensional, highly correlated data (Stanimirova *et al.*, 2013; Marini *et al.*, 2015). A general framework for application of these methods is proposed by Smilde *et al.* (2012). First, a linear model is selected based on its suitability to the data and the biological question at hand. Second, the factor effects are estimated and these should be collected in matrices. Lastly, a dimension reduction (*e.g.* PCA or PLS) is used to summarise the variation. Current methods used include, but are not limited to, ANOVA-simultaneous component analysis (ASCA) (Smilde *et al.*, 2005), ANOVA-target projection (ANOVA-TP) (Marini *et al.*, 2015) and regularised-multivariate ANOVA (rMANOVA) (Engel *et*

al., 2015). The aforementioned methods were compared in a study by Marini *et al.* (2017) and it was concluded that ASCA and ANOVA-TP result in similar interpretations, but, ANOVA-TP had better estimation of within class variation. Additionally, loading patterns were more difficult to interpret with respect to rMANOVA. Overall, all the methods were concluded to be well suited to deal with high dimensional, highly correlated data sets (Marini *et al.*, 2017).

2.3.2.4. The effect of pre-processing techniques on multivariate data analysis

Pre-processing techniques in many cases have favourable effects on MVDA. In particular for chromatographic fingerprinting, alignment techniques have been shown to improve results for multivariate analysis methods. Gong *et al.* (2004) corrected the retention time shifts of 79 chromatograms belonging to four different herbal medicines and PCA was performed on data before and after alignment as shown in Figure 2.13.

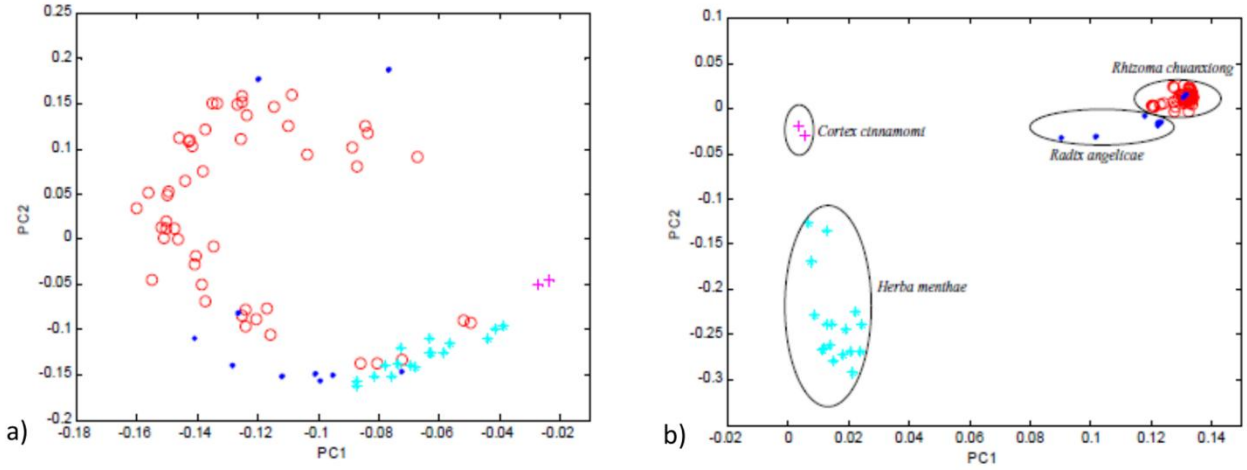


Figure 2.13 PCA score plots of four different plant species (indicated by coloured points). a) Before and (b after peak alignment (Gong *et al.*, 2004).

After alignment there is an improvement in grouping that corresponds to the four kinds of herbal medicines (Figure 2.13b). This was less evident in the PCA score plot before alignment (Figure 2.13a), where the observations were more scattered and not grouped (Gong *et al.*, 2004).

The importance of target selection was highlighted by Daszykowski and Walczak (2007), who simulated data sets from a set of 40 chromatograms and introduced linear and non-linear shifts to the chromatograms to mimic artefacts commonly occurring with instrumentation. The data were then aligned using COW and PCA was used to visualise the data. A snippet of the data and results produced can be seen in Figure 2.14.

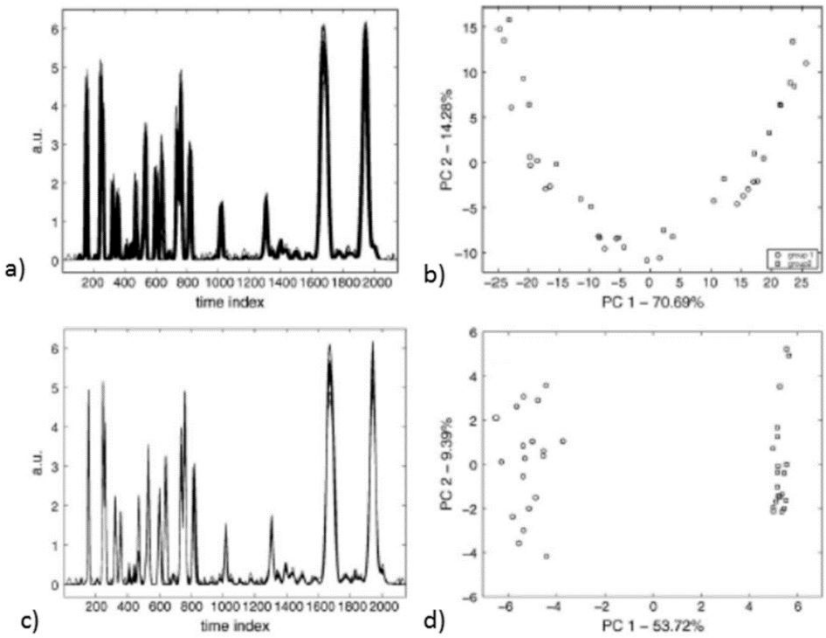


Figure 2.14 a) Chromatographic data and b) PCA plot before alignment. c) Chromatographic and d) PCA plot after alignment (Daszykowski and Walczak, 2007).

The retention time shifts evident in Figure 2.14a influences the grouping in the PCA plot (Figure 2.14b). After alignment the peaks match well (Figure 2.14c) and this consequentially results in two distinct groups in the PCA plot (Figure 2.14d) (Daszykowski and Walczak, 2007).

2.3.2.5. Application of multivariate data analysis techniques to fingerprints

Chemometrics offer a variety of multivariate data analysis techniques for analysing the enormous data sets generated by state of the art analytical instrumentation. Details of different studies showing the application of chemometrics (MVDA and in some cases pre-processing techniques) to fingerprints generated from hyphenated chromatographic techniques are listed in Table 2.9.

Table 2.9 Application of chemometic methods to various types of chromatographic data.

Purpose of study	Method of analysis	Pre-processing technique/s	Chemometric methods	Plant species/food type/product	References
Discrimination	HPLC-UV	Peak alignment	SA	*Camellia sinensis*	Alaerts *et al.* (2012)
Discrimination	HPLC-MS	-	SA	Danshen dropping pill	Fan *et al.* (2006)
Discrimination and classification	HPLC-DAD	-	PCA and SIMCA	*Artemisia* species	Alaerts *et al.* (2014)
Quality assessment, classification and discrimination	HPLC-DAD	-	HCA, PCA, PLS-DA and SIMCA	*Ganoderma lucidum*	Chen *et al.* (2008)
Biomarker identification by kinetic modelling	GC-MS	-	PCA and PLS	Carrot puree and broccoli puree	Kebede *et al.* (2015a,b,c)
Discrimination by origin and biomarker identification	GC-MS	Normalisation	PCA and LDA	Vegetable oils, aged meat	Lehallier *et al.* (2012)
Biomarker identification	HPLC-DAD	Baseline elimination and peak alignment	ANOVA-TP	*Aspalathus linearis*	Marini *et al.* (2015)
Modelling of antioxidant activity	HPLC-DAD	Baseline elimination and peak alignment	PCA, PLS and UVE-PLS	*Aspalathus linearis*	Orzel *et al.* (2014)
Classification	HPLC-DAD	-	CA and PCA	Kiwi and pomelo subspecies	Sârbu *et al.* (2012)
Quality assessment	HPLC-DAD-MS	Background elimination, normalisation and peak alignment	PCA	*Cyclopia maculata*	Schulze *et al.* (2014)
Discrimination and Classification	HPLC-DAD	Normalisation, centering and scaling	PCA, HCA, PLS and OPLS	*Mallotus* and *Phyllanthus*	Thiangthum *et al.* (2012)
Biomarker identification and kinetic modelling	GC-MS	Denoising and peak alignment	PCA and PLS	Mango juice	Wibowo *et al.* (2015a,b)
Assessing applicability of fingerprints as a screening tool	HPLC-DAD	Peak alignment, denoising, baseline elimination and normalisation	SA	*Cyclopia subternata*	Schulze *et al.* (2016)

Simultaneous analysis of factors (production season, quality grade and stem pasteurisation) affecting the phenolic composition of fermented rooibos	HPLC-DAD	Peak alignment, baseline elimination and signal denoising	ASCA	*Aspalathus linearis*	Stanimirova *et al.* (2013)

ANOVA-TP, analysis of variance-target projection; ASCA, analysis of variance-simultaneous component analysis, CA, cluster analysis; GC-MS, gas chromatography-mass spectrometry; HCA, hierarchal cluster analysis; HPLC-DAD, high performance liquid chromatography-diode array detector; HPLC-DAD-MS, high performance liquid chromatography-diode array detector-mass spectrometry; HPLC-UV, high performance liquid chromatography-ultraviolet; LDA, linear discriminant analysis; OPLS, orthogonal partial least squares; PCA, principle component analysis; PLS, partial least squares; PLS-DA, partial least squares-discriminant analysis; SA, similarity analysis; SIMCA, soft-independent modelling of class analogy; UVE-PLS, uninformative variable elimination-partial least squares.

2.4. Kinetic modelling of food quality changes during thermal processing

2.4.1. Food quality as affected by food processing

Quality in a broad sense can be described as satisfying the expectation of the consumer (Van Boekel, 2008). To maintain a good quality standard, food quality attributes such as texture, taste, appearance and nutrient content need to match consumer expectations. These attributes are, however, susceptible to changes due to the application of food processing techniques.

Food processing covers a broad range of treatments used for either one or multiple purposes, including sensorial enhancement, inhibition of microbiological growth or decreasing microbiological load and inactivation of enzymes (Van Boekel, 2008). In many cases, processing may lead to unintentional, undesired consequences such as loss of nutrients (leaching of vitamins by blanching) (Selman, 1994) or formation of toxic compounds (acrylamide production from Maillard reaction) (Van Boekel, 2001).

Food quality changes are related to chemical, biochemical and physical changes taking place in the food, and kinetic modelling gives insight into these specific reactions by quantifying parameters such as reaction rates and activation energies. With the help of kinetic modelling food scientists can achieve three main goals: a better understanding of reactions responsible for quality changes; predictability of expected quality changes, and processing parameters can be better controlled to ensure beneficial effects are promoted and undesirable effects are counteracted (Van Boekel, 2008). For the purpose of this discussion thermal processing, the experimental conditions required to perform kinetic modelling and the equations and methods used to quantify the relative kinetic parameters will be focused on.

2.4.2. Thermal processing

Popular thermal processing applications include cooking, roasting, baking and frying, all of which increase the palatability of food. Moreover, blanching, pasteurisation and sterilisation are used for increasing the shelf-life of food and minimising food-borne illnesses. Food quality degradation is a major concern during these steps and kinetic information concerning the changes is essential for proper design of thermal processing (Lund, 1988).

It is advisable that preliminary tests are conducted to select test conditions so that an adequate extent of degradation is observed over the maximum span of time for a selected temperature (Ling *et al.*, 2015). There are two methods widely used for kinetic modelling, namely direct and indirect heating. During direct heating, pre-packaged samples are immersed directly into the heating media (*e.g.* water bath or oil bath). This is not suitable for kinetic studies because heat transfer is non-uniform as the centre of the product takes longest to heat. Indirect heating methods are a more viable choice as they make use of small volumes or thin products to achieve isothermal conditions**.** When selecting the temperature-time combinations it must be taken into consideration that the reaction must be carried far enough so that it is possible to distinguish between the orders of reactions. Lund (1977) concluded that it is necessary to have at least 2-3 log changes in concentration or physical property for meaningful determination of the reaction order**.**

2.4.3.Fundamentals of kinetic modelling

The main objective of thermal processing kinetic studies is to quantify a quality attribute as a function of heating time. For an ideal food system reaction kinetics are obtained from a general rate law in eqn. (2.1):

$$\frac{dC}{dt} = \pm kC^n \qquad (2.1)$$

Where k is the rate constant (min^{-1}), t is the reaction time (min) and n is the reaction order. In general, C represents the concentration of the analyte, but it could also represent a quantitative value for a quality attribute, enzyme activity or population of microorganisms. A particular temperature is used to determine the reaction order (n), which is used to determine a temperature-dependent reaction rate constant. Kinetics of food quality changes generally follow pseudo zero-, first- or second- order reactions (Ling *et al.*, 2015).

The integrated, linearised forms of zero-, first-, and second-order kinetic models are given in eqn. 2.2-2.4:

Zero-order: $C = C_0 - kt$ (2.2)

First-order: $\ln C = lnC_0 - kt$ (2.3)

Second-order: $\frac{1}{C} = \frac{1}{C_0} + kt$ (2.4)

where C_0 is the concentration of the reactant at t=0. Different models are tested by changing relevant differential equations to their respective linearised forms and fitting them to the experimental data. Goodness-of-fit criteria (R^2, χ^2, run test and serial correlation) are used to decide which model predicts best the change in the concentration of the reactant.

Instead of using an absolute value to indicate quality changes, the concept of fractional conversion is commonly used (Levenspiel, 1999). The fractional conversion is defined as the fraction of reactant that has been converted to products at a given time. It should be applied when reactions have an appreciable amount of reactant remaining after a long enough reaction time (non-zero equilibrium concentration). For the fractional-model is defined as:

$$C = C_\infty + (C_0 - C_\infty)\, e(-kt) \qquad (2.5)$$

where C_∞ is the equilibrium concentration of the reactant after prolonged heating.

The temperature sensitivity of degradation rate constants is investigated using the Arrhenius equation (eqn. (2.6)).

$$k = Ae^{\frac{-Ea}{RT}} \qquad (2.6)$$

where A is the pre-exponential factor or the frequency factor, E_a is the activation energy (kJ mol $^{-1}$), defined as the minimum amount of energy required to start a reaction, T is the absolute temperature (K) and R is the universal gas constant (8.3145 J mol^{-1} K^{-1}). A represents the reaction rate at infinite temperature. For practical applications the equation is reformulated without A in the following manner:

$$k = k_{ref} e^{\frac{-Ea}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)} \qquad (2.7)$$

where k_{ref} is the reaction rate at a reference temperature (T_{ref}). The reference temperature should preferentially be chosen in the middle of the studied temperature range which should include at least five temperatures (eqn. (2.8)):

$$T_{\text{refi}} = \frac{1}{n} \sum_{i=1}^{n} T_i \qquad (2.8)$$

The Q_{10} value or temperature coefficient is another kinetic concept used to describe the rate of quality changes with temperature (eqn. (2.9)).

$$Q_{10} = \frac{k_{T+10°C}}{k_T} \qquad (2.9)$$

The Q_{10}-value is typically used for data where only a few temperatures are available. It is commonly used to predict shelf-life using accelerated shelf-life testing. Since it is strongly dependent on temperature, it is applied in small temperature ranges of 10-20 °C. For a more in depth review on general kinetic modelling refer to Van Boekel and Tijskens (2001) and Van Boekel (2008).

2.4.4.Food matrix effects and kinetic modelling

Peleg *et al.* (2012) provides a critical review of the Arrhenius equation. The author challenges the universal acceptance of the equation for modelling the temperature effect in various more complex systems, as the model was originally developed for gas molecules. In particular, its applicability to enzymatic reactions, microbial growth and reactions occurring in complex food systems is questioned (Peleg *et al.*, 2012). In that regard, Capuano *et al.* (2017) explains that the complexity of food matrices warrants the application of models that can explain and predict the effect different food matrices have on chemical reactivity. The proposed solution is the application of mechanistic and empirical approaches (Capuano *et al.*, 2017). Mechanistic models aim at quantifying the effects of food matrices based on detailed understanding of chemical and physical phenomena occurring in the food. However, their applicability is limited to very simple food systems. Empirical modelling on the other hand, is based on reliable analytical techniques and data mining methods (Figure 2.15). This form of empirical modelling has been referred to in literature as a 'fingerprinting-kinetics' approach (Kebede *et al.*, 2015c). 'Fingerprinting-kinetics' makes use of chemical fingerprints to accurately predict the effect of the food matrix on reactivity and for identification of key characteristics of interest prior to kinetic modelling (Capuano *et al*., 2017).

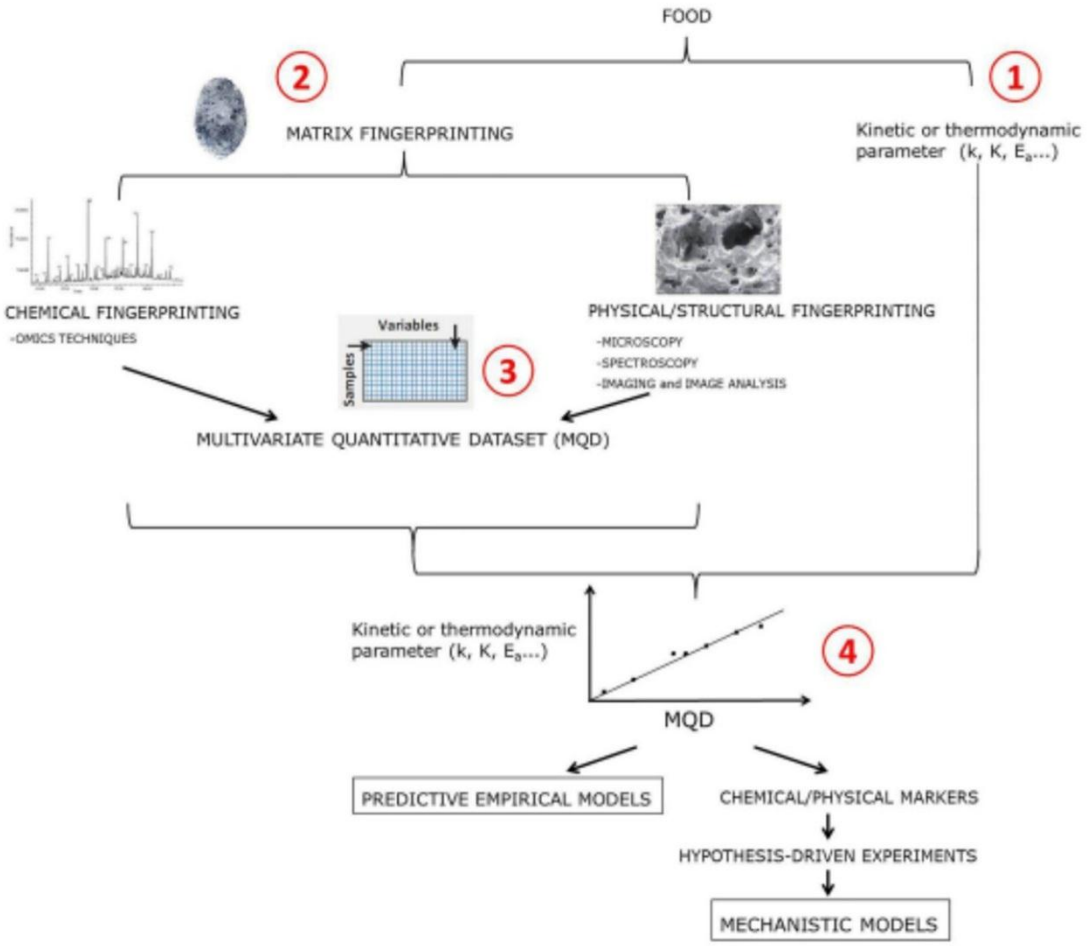


Figure 2.15 Empirical modelling of the food matrix based on fingerprinting (Capuano *et al.*, 2017).

2.4.5. Application of kinetic modelling to food quality changes

The application of fundamental kinetic modelling in a food quality study is presented by Zimeri and Tong (1999). Degradation kinetics of (-)-epigallocatechin gallate (EGCG), a polyphenol commonly occurring in black tea and many fruits, as a function of pH and dissolved oxygen concentration were determined. Degradation was modelled as a fractional conversion model, rate constants under different conditions were determined and the temperature dependency was investigated using the Arrhenius equation. From this a mathematical model relating the rate constant to temperature, pH and dissolved oxygen was developed. The results were shown to successfully predict losses of EGCG during processing and/or storage. Similarly, Wibowo *et al.* (2015c) used kinetic modelling to investigate colour instability in pasteurised orange juice. A simple kinetic model was implemented where the degradation of reactants and the formation of browning products were tracked over time and the reaction order was determined by means of best fit. The Arrhenius equation was used to determine the temperature dependency of the reactions. Total colour difference was highly correlated to degradation of sugars and ascorbic acid. Furthermore, PLS regression was used to rank the importance of the quality parameters for colour degradation. From this, acid-catalysed degradation of sugars and ascorbic acid degradation were identified as major contributors to the browning development in pasteurised orange juice.

The ‘fingerprinting-kinetics’ approach has been applied in studies by Kebede *et al.* (2015a,b,c) as a means to determine shelf-life of canned broccoli and carrot puree by accelerated shelf-life testing. Kinetic modelling was performed on compounds identified from data mining of chemical fingerprints constructed by GC-MS. ‘Fingerprinting-kinetics’ successfully identified marker compounds for food quality degradation of these products during storage, which could potentially be used to predict their longevity. Vervoort *et al.* (2012, 2013) used a similar approach to compare differences between processing techniques in pasteurisation of orange juice and high-pressure processing of carrots, respectively. Based on the results of these studies it was concluded that unexpected and unintended effects of novel processing methods could be easily detected when using fingerprinting as opposed to a targeted method.

2.5. Summary

Due to the complex phenolic composition of rooibos an untargeted analysis method such as fingerprintanalysis is essential to gain a broader understanding of the changes occurring during fermentation. The recently improved HPLC-DAD method for rooibos phenolic analysis provides a good phenolic representation of rooibos, which is ideal for producing chromatographic fingerprints. The application of relevant pre-processing and MVDA techniques may potentially identify marker compounds for rooibos fermentation. Pairing fingerprint analysis with kinetic modelling, in a ‘fingerprinting-kinetics’ approach, will provide greater insight into the chemical reactions that lead to the phenolic changes in fermented rooibos and the process can potentially be optimised.

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CHAPTER 3

Chromatographic fingerprint analysis of phenolic changes occurring during rooibos fermentation: removal of the 'size effect' and identification of potential marker compounds using chemometric methods

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3.1. Abstract

Rooibos (*Aspalathus linearis*) is well known for its health-promoting properties which are closely associated to its phenolic composition. However, rooibos phenolic compounds are highly susceptible to degradation during fermentation and the phenolic changes occurring during this process are still poorly understood. Previous work has been governed by targeted approaches whereby pre-selected analytes are closely monitored during processing. This approach is limited as changes in other important compounds are being neglected. In this study, an untargeted approach employing fingerprint analysis is used to uncover the changes occurring to phenolic compounds during fermentation. Chromatographic fingerprints of green, semi-fermented and fermented rooibos samples (n=10 each) were obtained by HPLC analysis with diode array detection (mean chromatogram; 250-450 nm). Chromatographic signals were pre-processed using the following techniques: background elimination, signal denoising, peak alignment, peak detection, normalisation, centering and transformation. With respect to normalisation, two methods were investigated for elimination of the 'size-effect' namely, probabilistic quotient normalisation (PQN) followed by log transformation and pair-wise log ratios (PLR). The suitability of the normalisation methods was determined based on analysis of variance using ANOVA-target projection (ANOVA-TP). Results indicated that PQN followed by log transformation was not suitable for normalisation because the underlying assumption of PQN, that the majority of the sample features should not differ, was not met. The PLR approach was better suited, however, the results are not expressed in terms of the original features (peaks). Thus, a new approach called robust PLR (rPLR) was proposed as a solution and this method simplified the identification of marker compounds. Based on rPLR results, it was concluded that green, semi-fermented and fermented samples differed significantly (p=0.01) in terms of phenolic composition and all the detected peaks (n=56) contributed to the differences between the chromatographic profiles.

3.2. Introduction

Rooibos, made from the endemic South African fynbos plant *Aspalathus linearis*, has gained tremendous popularity on a local and global scale. The commercial potential of rooibos phenolics made the tea the subject of many studies focused on optimising phenolic extraction for use in nutraceutical and cosmetic markets (Coetzee *et al.*, 2014; De Beer *et al.*, 2017; Miller *et al.* 2017). Fermentation (in actual fact an oxidation step) is important for the colour, taste and aroma characteristic to rooibos tea (Joubert and Schulz, 2006). However, rooibos phenolic compounds are highly susceptible to degradation during 'fermentation' (Walters *et al.*, 2017).

Previous studies focussed on investigating the phenolic changes occurring during fermentation have been guided by targeted approaches, where one or more pre-selected analytes are closely monitored (Joubert, 1996; Bramati *et al.*, 2003; Walters *et al.*, 2017). These methods often neglect the potential contribution of unidentified or lower quantity compounds (Hendriks *et al.*, 2005). In order to gain a broader understanding of the phenolic changes occurring during fermentation an untargeted analysis approach should be implemented. Chromatographic fingerprint analysis is one way of doing this (Grauwet *et al.*, 2014; Capuano *et al.*, 2017). Recent optimisation of a HPLC-DAD (High performance liquid chromatography-diode array detector) method specific for rooibos phenolic separation (Walters *et al.*, 2017), addressed the first requirement toward chromatographic fingerprint analysis of rooibos.

The aim of the current study was to uncover the phenolic changes occurring during fermentation by means of untargeted analysis. Chromatographic fingerprints of green (unfermented), semi-fermented and fermented rooibos were pre-processed using the following techniques: background elimination, signal denoising, peak alignment, peak detection, normalisation, centering and transformation. The 'size-effect' (caused by differences in the overall concentrations of the samples) is common to fingerprints of biological samples or fingerprints obtained from hyphenated chromatographic techniques like HPLC-DAD (Filzmoser and Walczak, 2014). Thus, two normalisation methods namely, probabilistic quotient normalisation (PQN) and pair-wise log ratios (PLR) were investigated as methods to remove the 'size-effect'. Marini *et al.* (2017) compared the efficiency of different multivariate analysis of variance methods using 16 targeted rooibos compounds. Based on the recommendations of this pilot study, ANOVA-target projection (ANOVA-TP) was used to determine the suitability of the scaling/transformation methods.

3.3. Experimental

3.3.1. Chemicals

HPLC grade water was prepared by purifying water using Millipore Elix and Milli-Q Advantage A^+ (Millipore, Bedford, USA) water purification systems in series. HPLC gradient grade acetonitrile for HPLC-DAD analysis was purchased from Merck (Darmstadt, Germany) and glacial acetic acid from Sigma-Aldrich (St Louis, MO, USA). Authentic reference standards (purity ≥ 95%) were purchased from Extrasynthese (Genay, France; isoorientin, orientin, eriodictyol-7-*O*-glucopyranoside, isovitexin, and hyperoside), Karl Roth (Karlsruhe, Germany; vitexin, luteolin-7-*O*-glucopyranoside), Sigma-Aldrich (isoquercitrin), Transmit

(Gießen, Germany; rutin) and the PROMEC unit of the Medical Research Council of South Africa (MRC, Cape Town, South Africa; aspalathin and nothofagin). *Z*-2-(β-D-Glucopyranosyloxy)-3-phenylpropenoic acid (PPAG) was sourced from the compound library of the Plant Bioactives Group of the Agricultural Research Council (Infruitec-Nietvoorbij) of South Africa. The phenolic standard stock solutions were prepared in dimethyl sulphoxide (DMSO) at concentrations of approximately 1 $mg.mL^{-1}$ and diluted with water as required. All the diluted standard mixtures contained *ca.* 1% ascorbic acid ($m.v^{-1}$) (Sigma-Aldrich) and were filtered through 0.22 µm hydrophilic PVDF filters (Millipore) before use.

3.3.2. Plant material and extract preparation

Plant material and extract preparation was conducted as described by Walters *et al.* (2017). Briefly, shoots (leaves and stems) from 10 bushes on a commercial plantation were harvested and kept separate. Green rooibos was prepared by drying *ca*. one-third of the 'intact' shoots of a sample without delay at 40°C for 12 h in a cross-flow drying tunnel. The remaining shoots were shredded into small pieces and a sub-sample was dried 1 h after shredding, representing semi-fermented rooibos. For preparation of fermented rooibos, 300 g shredded plant material was moistened with 130 mL water, incubated at 38°C for 12 h and then dried as described above. The dried plant material was coarsely milled using a Retsch mill (1 mm sieve; Retsch GmbH, Haan, Germany) followed by fine milling with a Retsch MM301 ball mill. The fermented (n=10), semi-fermented (n=10), and green (n=10) plant material were extracted in duplicate (Figure 3.1). Extraction proceeded as follows: 3 mL 40% acetonitrile-water was added to *ca*. 80 mg of plant material in a 4 mL reaction vial, followed by heating in a digital heating block (Stuart, Bibby Scientific Limited, Stone, UK) at 100°C for 20 min. After removal from the heating blocks, samples were sonicated for 5 min and then cooled to room temperature. The extracts were filtered using a 0.45 µm hydrophilic PVDF syringe filters (Millipore), diluted and ascorbic acid solution (final concentration 0.1-0.2%) added.

3.3.3. High-performance liquid chromatography (HPLC) analysis

HPLC analysis of the extracts was performed in duplicate (Figure 3.1) as described by Walters *et al.* (2017). An Agilent HPLC 1200 series instrument (maximum pressure limit 400 bar) equipped with an in-line degasser, quaternary pump, autosampler, column thermostat with an in-line 3 µL sample preheater and a DAD was used for chromatographic analysis. The instrument was controlled using Chemstation software (Agilent Technologies, Waldbronn, Germany). Separation was achieved on a Poroshell 120 SB-C_{18} (150 × 4.6 mm i.d. 2.7 µm) column, protected by a ZORBAX SB-C_{18} analytical guard column (12.5 × 4.6 mm i.d. 5 µm) (Agilent) and a Waters Acquity in-line filter. The mobile phase was composed of acetonitrile (B) and 2% aqueous acetic acid (A) ($v.v^{-1}$). The flow rate was 1 $mL.min^{-1}$ using the following multilinear gradient: 10-14.8% B (0-28.5 min), 14.8-19.2% B (28.5-33 min), 19.2-100% B (33-33.5 min), 100% B isocratic (33.5-38 min), 100-10% B (38-39 min), 10% B isocratic (39-46 min). The column oven temperature was set to 44.5 °C. UV spectra were recorded between 200 and 700 nm.

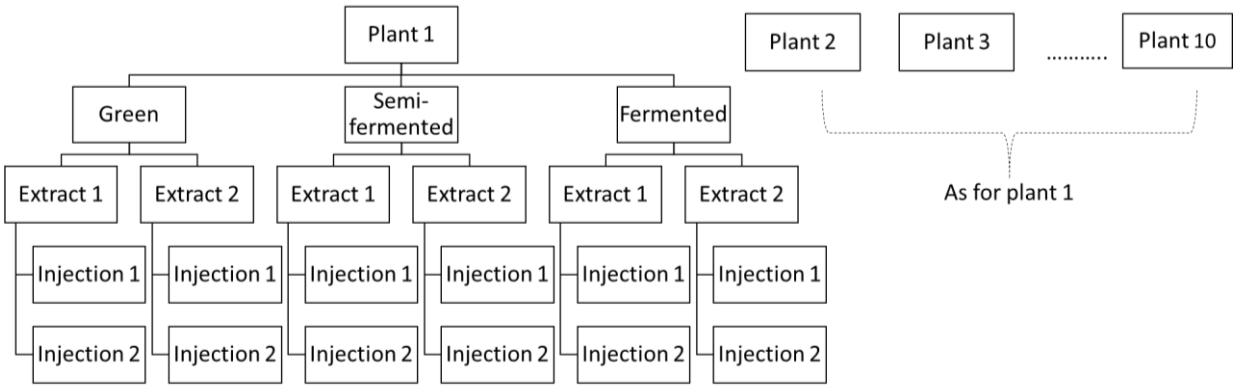


Figure 3.1 Schematic diagram of experimental design.

3.3.4. Data handling, pre-processing and analysis of variance

The data handling and pre-processing applied to chromatographic fingerprints are illustrated in Figure 3.2. Absorbance matrices for green, semi-fermented and fermented rooibos were exported from Chemstation software (Agilent) as *.csv files for the wavelength range 250-450 nm (0.2 nm increments) and 0-35 min using a ChemStation macro, export3D. The *.csv files were transformed to comma delimited format in Microsoft Excel (2013) and imported into MATLAB R2016a (The MathWorks, Natick, MA, USA). The adapted data (120 samples × 21000 time points × 101 wavelengths) were averaged across all wavelengths to give a matrix with dimensions 120 samples × 21000 time points. The elution time during which ascorbic acid eluted (0-2 min) was removed from all chromatograms, resulting in a data matrix of 120 samples × 19771 time points. Since the majority of the peaks were well separated, a peak table (120 samples × 56 peak areas) was constructed instead of analysing the entire chromatograms.

Analysis of variance using ANOVA-TP requires the decomposition of the centered data matrix ($\boldsymbol{X}_c$) into separate components associated with the studied effects and their interactions according to the principles of ANOVA. Eqn. (3.1) explains how this was done for the studied data set. There are two factors, namely batches and treatments (these refer to the harvested plants and the fermentation treatment, respectively). To determine the influence of the 'treatment' factor and to identify individual peaks contributing to the effect of fermentation, it is necessary to remove the data mean and the effect of 'batches'. The peak table after centering was decomposed into the following components:

$$\boldsymbol{X}_c = \boldsymbol{X} - \boldsymbol{X}_m = \boldsymbol{X}_{Batches} + \boldsymbol{X}_{Treatment} + \boldsymbol{X}_{BatchesTreatment} + \boldsymbol{X}_{error} \qquad (3.1)$$

where $\boldsymbol{X}$ is the original data matrix, $\boldsymbol{X}_m$ is the matrix of means, $\boldsymbol{X}_{Batches}$ is the variance present as a result of the biological diversity of rooibos plant material, $\boldsymbol{X}_{Treatment}$ is the effect of the studied treatments (green, semi-fermented and fermented), $\boldsymbol{X}_{BatchesTreatment}$ is the interaction effect and $\boldsymbol{X}_{error}$ is the error component.

ANOVA-TP can be applied to two classes only, therefore, for illustrative purposes the results for the comparison between green and fermented rooibos samples will be discussed in the sections to follow.

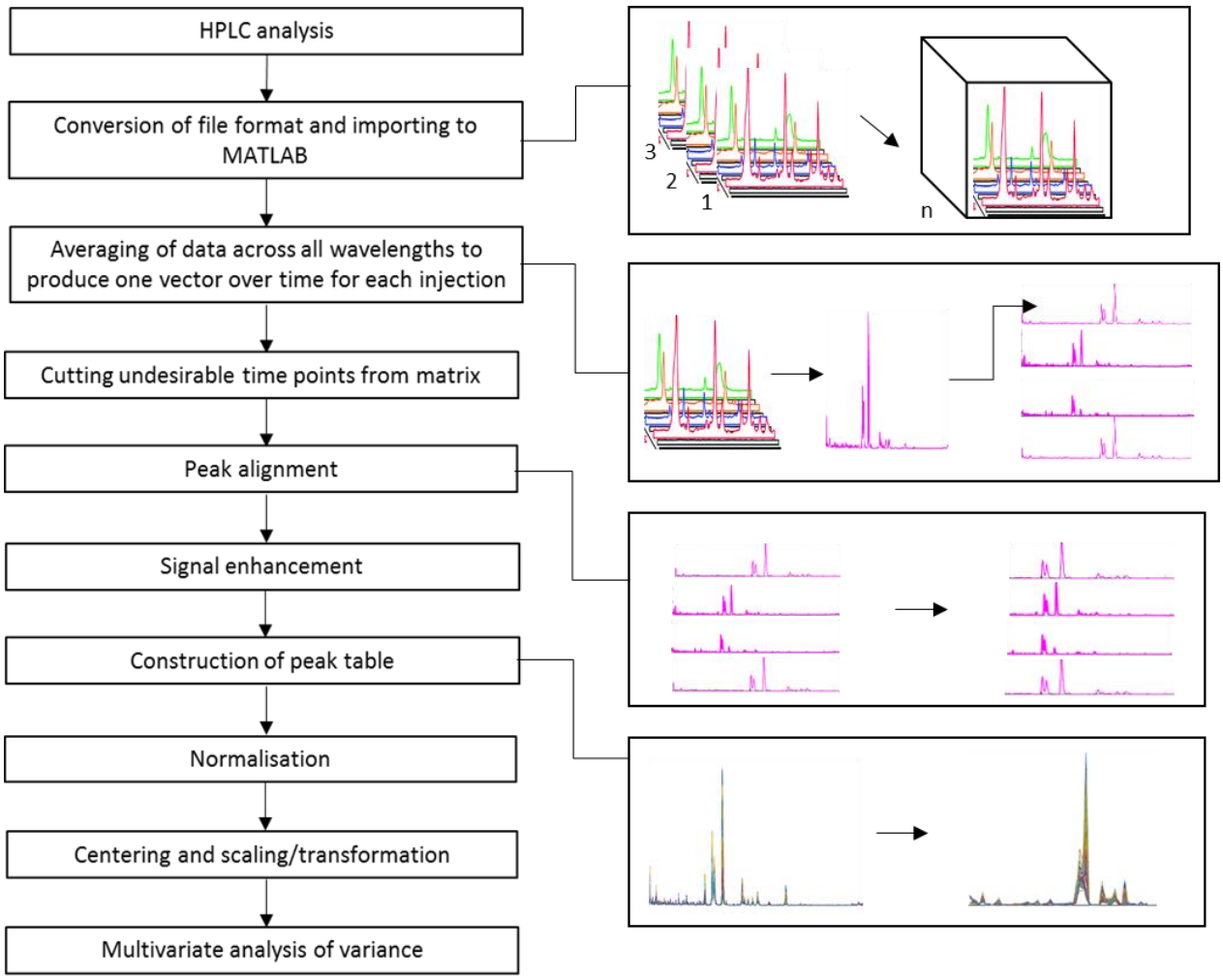


Figure 3.2 Data-handling and pre-processing steps as applied to chromatographic fingerprints of rooibos.

3.4. Theory

3.4.1. Pre-processing

Typical pre-processing techniques used on chromatographic fingerprints include signal enhancement (background elimination and signal denoising), peak alignment, normalisation, centering and scaling or transformation.

3.4.1.1. Peak alignment

Correlation optimised warping (COW) was performed to correct retention time shifts (Nielsen *et al.*, 1998). COW is a popular choice among chemometricians as it is efficient, easy to use and freely available (Amigo *et al.*, 2010). It aligns a query chromatogram to a target chromatogram by means of piece-wise linear stretching and compression of the time axis. A target chromatogram is chosen based on having the highest mean correlation within a data set (Daszykowski and Walczak, 2007). Parameters to be set by the user include the number of segments the chromatogram will be divided into and the slack (maximum number of data points

along the time axis that the segments are allowed to move either left or right). These parameters have to be optimised for each signal individually as the differences between individual chromatograms and the query chromatograms vary.

3.4.1.2. Signal enhancement

As for any other instrumental signal, chromatograms are made up of three major components, *i.e.* chromatogram = analytical signal + noise + background (Daszykowski and Walczak, 2006; Amigo *et al.*, 2010). These components differ based on their frequency. Noise is the highest frequency component, background the lowest frequency component and the signal of an intermediate frequency. Signal enhancement is achieved by eliminating the noise and background components (Daszykowski and Walczak, 2006).

Background elimination

The background is not related to the analyte signal and typically shows some sort of systematic behaviour (Amigo *et al.*, 2010). It is dependent on the chromatographic conditions and is often referred to as the baseline (Skov, 2008; Amigo *et al.*, 2010). Background estimation was performed by the asymmetric penalised least squares (APLS) method (eqn. (3.2)) proposed by Eilers (2003). APLS is the most cited background elimination method.

The objective function of the method, *Q*, is defined as:

$$Q = \sum_i v_i (y_i - \hat{y}_i)^2 + \lambda \sum_i (\Delta^2 \hat{y}_i)^2 \qquad (3.2)$$

where y is the experimental signal (*e.g.* chromatogram), $\hat{y}$ is the smooth trend or baseline approximation, v_i is the prior weights, λ is the positive parameter weighting the second term in eqn. (3.2) and Δ denotes the derivatives of $\hat{y}$. The second term explains the roughness of $\hat{y}$ by squaring and summing the differences, while the first term measures the lack of fit of the data by the sum of squares of differences (Eilers, 2003). The larger λ is, the stronger the influence of the second term on the goal, *Q*, and the smoother $\hat{y}$ will be (the penalty is that the fit of the data may get worse) (Eilers, 2003; Daszykowski and Walczak, 2006).

The weights are chosen in an asymmetric way:

$$v_i = p \quad \text{if} \quad y_i > \hat{y}_i \qquad (3.3)$$

$$v_i = 1 - p \quad \text{if} \quad y_i < \hat{y}_i \qquad (3.4)$$

where $0 < p < 1$. Thus, the positive and negative deviations from the trend $\hat{y}$ can be differently weighted. For example, if p=0.01, all data points with a positive deviation from the approximation $\hat{y}$ will exert minimal influence on the baseline approximation. Determining $\hat{y}$ with unknown weight can be a problem and this can only be done iteratively (Li Vigni *et al.*, 2013; Daszykowski and Walczak, 2006). Using v_i=1 for all the data points, the first approximation of the signal can be calculated and then, for all the points above this first approximation, take p=0.01 and find the second approximation of the signal and so on. Basically, the points below the estimated baseline are assumed to be more significant in fitting the baseline and get higher weights

in the next iteration of fit (Li Vigni *et al*., 2013). The background is eliminated once the baseline is approximated in a satisfactory manner and subtracted from the studied signal.

Signal denoising

Reducing the contribution of noise without adversely affecting the underlying signal is referred to as denoising (Reis *et al.*, 2009). Chromatograms are non-stationary signals, so they should be denoised in the wavelet domain. A signal is transformed from its original domain to another domain so that operations such as denoising can be carried out more easily (Walczak and Massart, 1997). Projection of a signal onto wavelet basis functions is called wavelet transform (Walczak and Massart, 1997). Wavelets are a family of basis functions that have good localisation in the time and frequency domains (Pasti *et al.*, 1999; Reis *et al.*, 2009). The shapes of these basis functions vary according to the region they occupy, namely having good time localisation in the high-frequency regions and good frequency localisation in the low-frequency region (Reis *et al.*, 2009). Signal denoising was performed in the wavelet domain using discrete wavelet transform (DWT) with filter no. 4 from the Daubechies family of filters, 12 levels of decomposition and universal thresholding.

Wavelet transform of signal, f (*e.g.* chromatogram), can be presented as a linear transformation involving an orthonormal matrix $\boldsymbol{W}$:

$$\boldsymbol{w} = \boldsymbol{W}f \qquad (3.5)$$

where $\boldsymbol{w}$ is a vector containing wavelet transform coefficients and $\boldsymbol{W}$ is the matrix of wavelet filter coefficients. The wavelet coefficients calculated by DWT are related to the 'basis wavelet'. This particular basis wavelet is specified by a set of numbers called wavelet filter coefficients. For example, the Daubechies family of wavelets includes 10 members ranging from highly localised to highly smooth (Walczak and Massart, 1997) where its first member is characterised by two coefficients and the next one is characterised by four coefficients. Generally, the wavelet number n is characterised by 2n coefficients.

Once the filters are defined, the recursive decomposition algorithm (also known as the tree algorithm) proposed by Mallat (1989) is applied (Figure 3.3). Each row, presented in Figure 3.3, represents a level of decomposition (Pasti *et al.*, 1999). The original signal N (Figure 3.3) is passed through a low-pass filter and a high pass filter from a specified wavelet to yield two sets of coefficients. The high-pass filtered data are the wavelet transform detail coefficients ($d^1 = \{d_0^1, d_1^1, \ldots, d_{N/2-1}^1\}$) at the first level of resolution. The low-pass filtered data are the approximation coefficients ($a^1 = \{a_0^1, a_1^1, \ldots, a_{N/2-1}^1\}$) at the first level of resolution. This process of filtering (using the same wavelet filters) is repeated for each approximation coefficient until the limit of the unit interval is reached (Walczak and Massart, 1997).

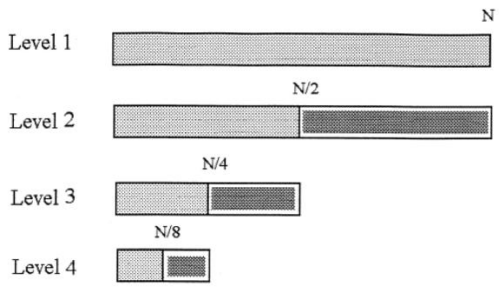


Figure 3.3 Tree algorithm used in dynamic wavelet transform (DWT) as proposed by Mallat (1989). N at level one indicates the length of original signal, lighter and darker grey regions represent approximation coefficients and detail coefficients at different levels of decomposition (Pasti *et al.*, 1999).

A universal threshold is used to estimate the noise level (Pasti *et al.*, 1999). It is defined as:

$$th = \sigma\sqrt{2\log N} \qquad (3.6)$$

where N is the signal length and σ is the standard deviation of the noise. The latter is estimated from the median of the detail coefficients at the first resolution level (d^1) of signal decomposition:

$$\sigma = |median(d^1)/0.674| \qquad (3.7)$$

3.4.1.3. Normalisation

Probabilistic quotient normalisation (PQN)

In PQN, all elements (*i.e.* peak areas) of the signal $\boldsymbol{x}$ (*i.e.* chromatogram) are divided by the median of the element wise ratios between a given signal $\boldsymbol{x}$ and a selected target $\boldsymbol{x}_{ref}$ (*e.g.* the mean chromatogram) (Dieterle *et al.*, 2006):

$$\mathbf{x}_{\mathrm{PQN}} = \frac{\boldsymbol{x}}{median\left(\frac{\boldsymbol{x}}{\boldsymbol{x}_{ref}}\right)} \qquad (3.8)$$

The choice of reference spectrum is not critical, however, using the median spectrum is considered as the most robust candidate (Dieterle *et al.*, 2006). When PQN is performed on entire chromatograms, it should be stressed that the points on the baseline should be eliminated to avoid division by 0 and to eliminate the influence of the baseline points on the calculation of the scaling constant. If the peaks in the studied chromatogram are well separated a peak table can be used as an alternative to working with entire chromatograms. The assumption that the majority of the sample elements cannot vary between classes has to be met in order to apply PQN (Bylesjö *et al.*, 2009).

Pair-wise log ratios (PLR)

Peak ratios are scale invariant, but because variance(x_j/x_k) ≠ variance(x_k/x_j), log transformation is required, since variance(log(x_j/x_k)) = variance(log(x_k/x_j)). The use of PLR has an additional advantage of eliminating the need to perform further scaling aimed at stabilising the data variance (Filzmoser and Walczak, 2014).

For an individual chromatogram, there are k= ½ (n-1) n pair-wise log-ratios (an unfolded upper part of matrix $\boldsymbol{M}$ $(n \times n)$). For m chromatograms, data can be organised as matrix $\boldsymbol{P}$, its dimensionality being equal to $m \times k$. Matrix $\boldsymbol{P}$, instead of the original data matrix $\boldsymbol{X}$, can be further used for data analysis, using such methods as, *e.g*. ASCA (Smilde *et al.*, 2005) or ANOVA-TP (Marini *et al.*, 2015). When working with matrix $\boldsymbol{P}$ it is, however, difficult to estimate the significance of the n individual features (original variables). For the two class discrimination problems, another approach, *i.e.* robust PLR proposed by Walach *et al.* (2017), can be applied, with its main idea described in section 3.4.3.

3.4.1.4. Centering and transformation

Centering is performed by subtracting the mean data matrix from the original data matrix. This is done so that the data fluctuate around zero and not the mean. It is usually applied in combination with scaling or transformation (Van den Berg *et al.*, 2006).

Transformations are non-linear conversions of the data (Van den Berg et al., 2006). Log transformation is performed by taking the log of each element in the data matrix (Van den Berg *et al.*, 2006). However, a particular drawback of log transformation is that it is unable to deal with zero values (Hendriks *et al.*, 2005).

3.4.2.Multivariate analysis of variance

Correlated, high dimensional data require advanced analysis methods to highlight all aspects of the experimental design (Smilde *et al.*, 2012). In this regard, there are several methods that combine the ideas of univariate analysis (*i.e*. ANOVA) and the advantages of multidimensional techniques designed for handling high dimensional, highly correlated data (Stanimirova *et al.*, 2013; Marini *et al.*, 2015). One such method is ANOVA-target projection and the principles of the method will be elaborated on further in the section to follow.

3.4.2.1. ANOVA-target projection (ANOVA-TP)

In ANOVA-TP, analysis of variance is based on the principles of partial least squares (PLS) and target projection. For a single dependent variable $\boldsymbol{y}$, the PLS model with f_{opt} components may be expressed as:

$$\boldsymbol{X} = \boldsymbol{T}\boldsymbol{P}' + \boldsymbol{E} \qquad (3.9)$$

$$\boldsymbol{y} = \boldsymbol{T}\boldsymbol{q} \quad + \boldsymbol{f} \qquad (3.10)$$

where $\boldsymbol{X}$ $(m \times n)$ denotes the centered independent data matrix of interest, $\boldsymbol{T}$ $(m \times f_{opt})$ and $\boldsymbol{P}$ $(n \times f_{opt})$ denote the $\boldsymbol{X}$-scores and loadings, respectively, and $\boldsymbol{E}$ and $\mathbf{f}$ denote the residuals matrix and vector, respectively. $\boldsymbol{y}$ $(n$

$\times 1$) denotes the vector of dependent variables, $\boldsymbol{q}$ ($f_{op\,x\,1t}$) denote the vector of $\boldsymbol{y}$ loadings, and k denotes number of responses.

The PLS model can also be expressed in the following form:

$$\boldsymbol{y} = \boldsymbol{X}\boldsymbol{b}_{PLS} + \boldsymbol{R} \qquad (3.11)$$

where $\boldsymbol{b}_{PLS}$ is the vector ($n \times 1$) of the regression coefficients. The number of latent variables, f_{opt}, is determined based on cross validation.

In the target projection method, the PLS model is replaced by the model containing a single latent variable (the target projected component). It is constructed by projecting the data matrix onto the regression vector $\boldsymbol{b}_{PLS}$ of the optimal PLS model. The PLS model in eqn. (3.9) can be replaced by the target projection model:

$$\boldsymbol{X} = \boldsymbol{t}_{TP}\boldsymbol{p}'_{TP} + \boldsymbol{E}_{TP} = \boldsymbol{X}_{TP} + \boldsymbol{E}_{TP} \qquad (3.12)$$

where $\boldsymbol{t}_{TP}$ denotes the target projected scores which are defined as:

$$\boldsymbol{t}_{TP} = \boldsymbol{X}\boldsymbol{b}_{PLS}/\|\boldsymbol{b}_{PLS}\| = \hat{\boldsymbol{y}}/\|\boldsymbol{b}_{PLS}\| = \boldsymbol{X}\boldsymbol{w}_{TP} \qquad (3.13)$$

and the target projected loadings, $\boldsymbol{p}_{TP}$, are calculated as:

$$\boldsymbol{p}'_{TP} = \boldsymbol{t}'_{TP}\boldsymbol{X}/(\boldsymbol{t}'_{TP}\boldsymbol{t}_{TP}) = \boldsymbol{b}'_{PLS}/\|\boldsymbol{b}_{PLS}\| * (\boldsymbol{X}'\boldsymbol{X})/\|\boldsymbol{t}_{TP}\|^2 \qquad (3.14)$$

Target projected scores are proportional to the vector of the predicted response, *i.e.* to $\hat{\boldsymbol{y}}$. The target projected loadings are the product of the normalised regression coefficients of the optimal PLS model and the covariance matrix of the data $\boldsymbol{X}$, scaled by the inverse of the variance of the target projected scores. In the case where there is more than one response variable the same concepts are applicable and as many target projected components as the number of response variables can be extracted (Marini *et al.*, 2015). The effect of the treatment is calculated as the sum of squares of the element of its target projection reconstructed matrix $\boldsymbol{X}_{TP}$ and its significance is determined based on a permutation test. The target projection loadings are used to determine the significance of considered features using a bootstrap procedure.

3.4.3. Marker identification based on robust pair-wise log ratio (rPLR) approach

Identifying significant elements when working with unfolded log ratios is possible, however it becomes problematic when trying to link the significant log ratios back to the compounds in the sample. In the proposed approach, instead of working with the unfolded log ratios, it is possible to work with a single symmetric variation matrix or matrix $\boldsymbol{V}$ ($n \times n$) (Walach *et al.*, 2017), the elements of which are defined as:

$$V_{ij} = \frac{m_1\sqrt{W1_{ij}} + m_2\sqrt{W2_{ij}}}{2(m_1+m_2)\sqrt{T_{ij}}} \qquad for\ j = 1, \ldots, n \qquad (3.15)$$

where m_1, and m_2 denote the numbers of samples in class 1 and 2, respectively; $W1_{ij}$, $W2_{ij}$ and T_{ij} represent the within-class variances and the total variance, respectively, for the log ratio of variables *i* and *j* and they are defined as:

$$W1_{ij} = var\left(log\left(\frac{x(class\ 1,i)}{x(class\ 1,j)}\right)\right)$$ variance within class 1 for the log ratio of variables *i* and *j*

$$W2_{ij} = var\left(log\left(\frac{x(class\ 2,i)}{x(class\ 2,j)}\right)\right)$$ variance within class 2 for the log ratio of variables *i* and *j*

$$T_{ij} = var\left(log\left(\frac{x(:,i)}{x(:,j)}\right)\right)$$ total variance for the log ratio of variables *i* and *j*

where ***x(:,i)*** denotes a column vector containing the *i*-th parameter for all *m* samples, ***x(:,j)*** denotes a column vector containing the *j*-th parameter for all samples, and 'var' denotes the variance for the predefined set of samples. *Class 1* and *class 2* are the vectors containing indices of the objects belonging to *class 1* and *class 2*, respectively. Diagonal elements of matrix ***V*** are set to zeros.
For the balanced data set (m_1=m_2), eqn. (3.15) reduces to:

$$V_{ij} = \frac{\sqrt{W1_{ij}}+\sqrt{W2_{ij}}}{2\sqrt{T_{ij}}} \qquad for\ j\ =\ 1,2,\dots,n \qquad (3.16)$$

The proposed statistics are defined, as follows:

$$v_{.j} = \sum_{i=1}^{n} V_{ij} \quad for\ j = 1,\dots,n \qquad (3.17)$$

The resulting variation statistic, or ***v*** statistic is used to explain whether or not the variable in question is significant in explaining the difference between two studied classes.

The aforementioned method can easily be made robust (referred to as rPLR), by replacing the square root of variance (*i.e.*, standard deviation), in eqn. (3.15) and (3.16), by its robust equivalent. For instance, the median absolute deviation (MAD) can be used as a highly robust estimator of standard deviation. It is defined as:

$$\text{MAD}(\mathbf{x})=1.48\ \text{median}|\mathbf{x}\text{-median}(\mathbf{x})| \qquad (3.18)$$

A randomisation test is used to estimate the null distribution of the ***v*** statistic since it is unknown. In the case of designed data, a randomisation test should be performed on the reduced model residuals (Anderson and Ter Braak, 2003). This means that a randomisation test should be applied to the centered data matrix after the removal of the batch effect. A threshold is calculated based on the null distribution and a significance level (α=0.01). Values of the $\mathbf{v}_{.j}$ statistics are compared to the null distribution obtained for the randomised data. If the ***v*** statistic of an element is smaller than the threshold then that element significantly contributes to the variance observed between classes.

3.5. Results and discussion

Results presented below follow a series of events in an attempt to remove the 'size effect', starting with PQN followed by log transformation, because of its efficiency as a signal scaling method (Filzmoser and Walczak, 2014). Incompatibility of PQN with the presented data prompted the investigation of PLR as an alternative approach. Based on ANOVA-TP results, PLR was better suited, however, there were difficulties with identification of marker compounds. A new approach, robust PLR, was used as a solution. Final results are therefore presented based on the robust pair-wise log ratio approach.

3.5.1.Pre-processing

3.5.1.1. Peak alignment

If retention time shifts are not corrected, the variations modelled by the chemometric methods will not correspond to the chemical sources but rather to the retention time shifts (Daszykowski and Walczak, 2007). The presence of retention time shifts are most likely the result of column aging, slight temperature differences, variability in mobile phase composition, matrix effects, irreproducible sample injections, interaction between analytes, pressure fluctuations, fluctuations in flow rates or small gas bubbles in the chromatographic system (Jellema, 2009; Alaerts et al., 2010; Daszykowski et al., 2010).

Ascorbic acid was added to aliquots of extracts to preserve the phenolics during the time between sample preparation and analysis, it does not form part of the rooibos phenolic content and was therefore removed from all chromatograms (Figure 3.4). Chromatogram number 69 (Plant 8, extraction 1, injection 1 from semi-fermented rooibos) was chosen as the target chromatogram based on its highest mean correlation coefficient with all other chromatograms. Segment sizes between 50 to 100 data points and a slack range between 4 and 8 data points proved to work well for alignment. Segment size and slack were selected based on the highest correlation with the target chromatogram. Alignment with COW successfully removed all unwanted variation as a result of retention time shifts. Chromatograms (n=120) before and after alignment with COW are illustrated in Figure 3.4.

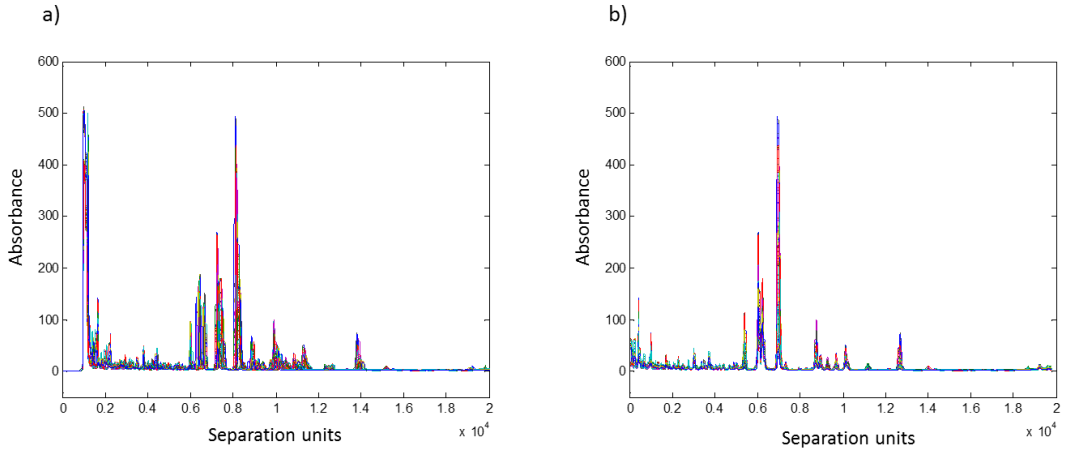


Figure 3.4 Initial set of chromatographic signals (n=120) a) before and b) after alignment and removal of ascorbic acid from the time axis.

3.5.1.2. Signal enhancement

Background elimination

Background elimination is important because the offset could potentially hinder peak detection, identification and integration (Skov, 2008). A varying baseline can also greatly influence measures of similarity used to compare data (*e.g.* correlation coefficient or Euclidean distance) (Daszykowski and Walczak, 2006). It is typically the result of interference contributed by solvents or impurities (Bloemberg *et al.*, 2013).

Figure 3.5 represents the chromatograms after performing background elimination using asymmetric penalised least squares (APLS). The offset was concentrated on the left hand side of the chromatograms (Figure 3.4). Application of APLS removed unwanted baseline variation (Figure 3.5).

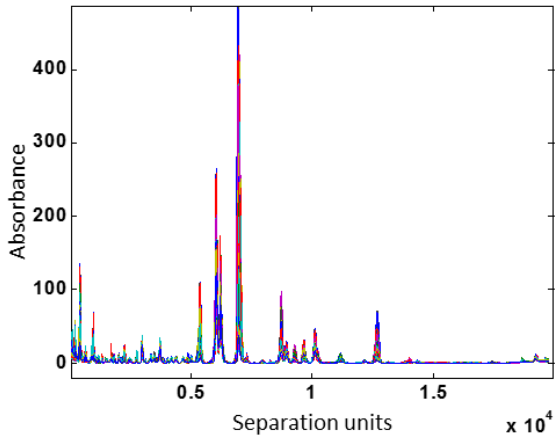


Figure 3.5 Set of rooibos chromatographic signals (n=120) after background elimination using the asymmetric penalised least squares (APLS) method proposed by Eilers (2003).

Signal denoising

All measured data are likely to contain noise and the quality of chemometric and data mining tasks is dependent on the quality of denoising (Reis *et al.*, 2009). As an example, Figure 3.6 illustrates the effect of DWT on a segment of a rooibos chromatogram obtained from the sample set. The signal after denoising (shown in green) is smoothed (Figure 3.6).

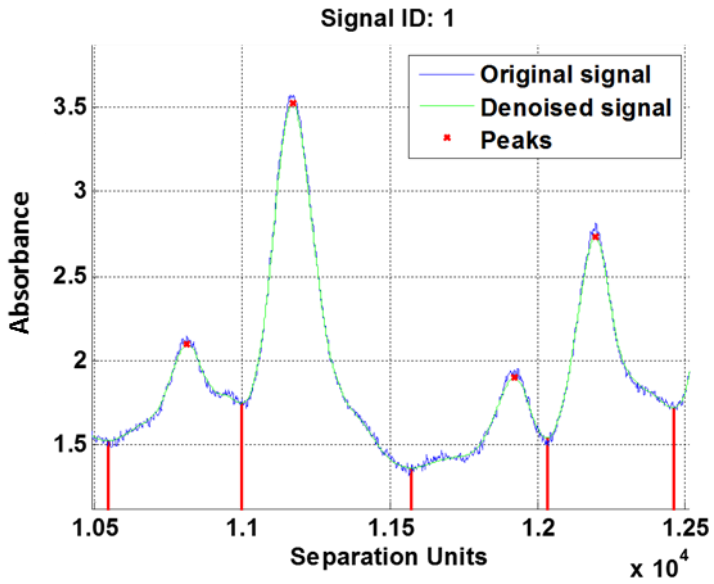


Figure 3.6 Segment of a rooibos chromatogram illustrating the effects of signal denoising using dynamic wavelet transform (DWT).

3.5.1.3. Peak detection and identifiable compounds

Peak detection was performed for the mean chromatogram (Figure 3.7) and then peak areas for individual chromatograms were calculated. After peak detection, the resulting data matrix had dimensions of 120 (samples) × 56 (peak areas). Based on identification by Walters *et al.* (2017), 16 compounds were identified from the mean chromatogram (Table 3.1). These compounds are also quantifiable with the use of authentic reference standards.

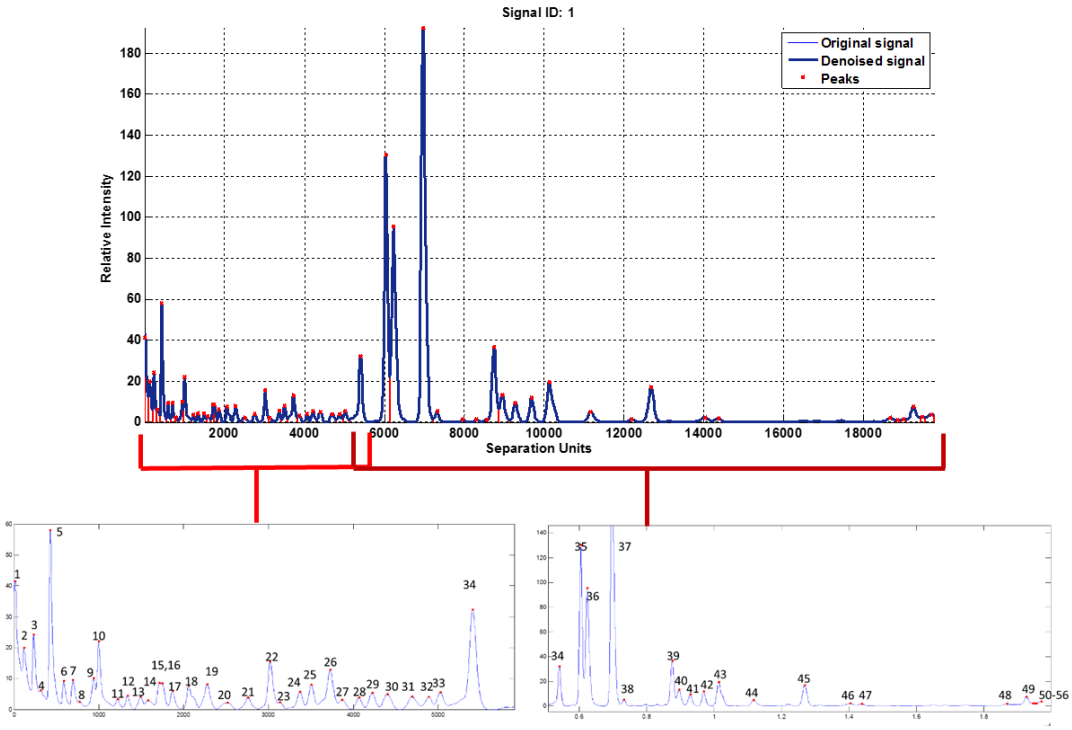
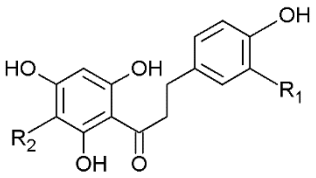
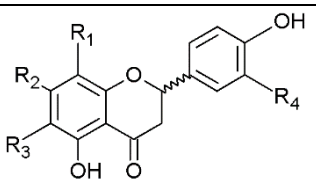
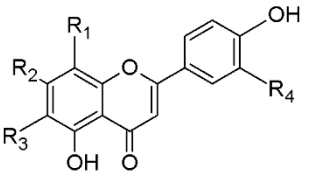
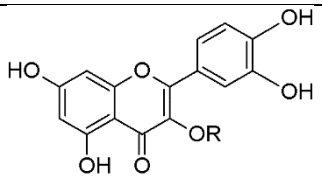
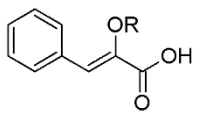


Figure 3.7 Mean rooibos chromatogram (n=120) depicting 56 detected peaks used to construct a peak table.

Table 3.1 Corresponding compound types, names and structures of selected peaks from Figure 3.7.

Compound type	Peak nr.	Compound name and substituents	General structure
Dihydrochalcones	37	Aspalathin: R_1 = OH, R_2 = β-D-glucopyranosyl	
	45	Nothofagin: R_1 = H, R_2 = β-D-glucopyranosyl	
Flavanones	[a]22 [b]26	(*R*)[b]/(*S*)[a]-eriodictyol-6-*C*-glucopyranoside: R_1 = β-D-glucopyranosyl, R_2 = OH, R_3 = H, R_4 = OH	
	[c]30 [d]31	(*R*)[d]/(*S*)[c]-eriodictyol-8-*C*-glucopyranoside: R_1 = H, R_2 = OH, R_3 = β-D-glucopyranosyl, R_4 = OH	
Flavones	35	Isoorientin: R_1 = H, R_2 = R_4 = OH, R_3 = β-D-glucopyranosyl	
	36	Orientin: R_1 = β-D-glucopyranosyl, R_2 = R_4 = OH, R_3 = H	
	40	Vitexin: R_1 = β-D-glucopyranosyl, R_2 = OH, R_3 = R_4 = H	
	43	Isovitexin: R_1 = R_4 = H, R_2 = OH, R_3 = β-D-glucopyranosyl	
	44	Luteolin-7-*O*-glucopyranoside: R_1 = R_3 = H, R_2 = β-D-glucopyranosyloxy, R_4 = OH	
Flavonols	43	Isoquercitrin: R = β-D-glucopyranosyloxy	
	41	Hyperoside: R = β-D-galactopyranosyloxy	
	39	Quercetin-3-*O*-β-D-robinoside: R = α-L-rhanopyranosyl-(1→6)-β-D-galactopyranosyloxy	
	42	Rutin: R = α-L-rhanopyranosyl-(1→6)-β-D-glucopyranosyloxy	
Phenylpropenoid	34	*Z*-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid (PPAG): R = 2-β-D-glucopyranosyloxy	

3.5.1.4. Normalisation

To illustrate the effects of normalisation, the studied peaks before and after PQN followed by log transformation are seen in Figure 3.8. Green peaks represent green rooibos samples, red peaks represent semi-fermented samples and blue peaks represent fermented samples (n=40 each) (Figure3.8). The variation as a result of the treatments is preserved after normalisation while the variation as a result of differing concentrations (*i.e.* the 'size effect') is corrected.

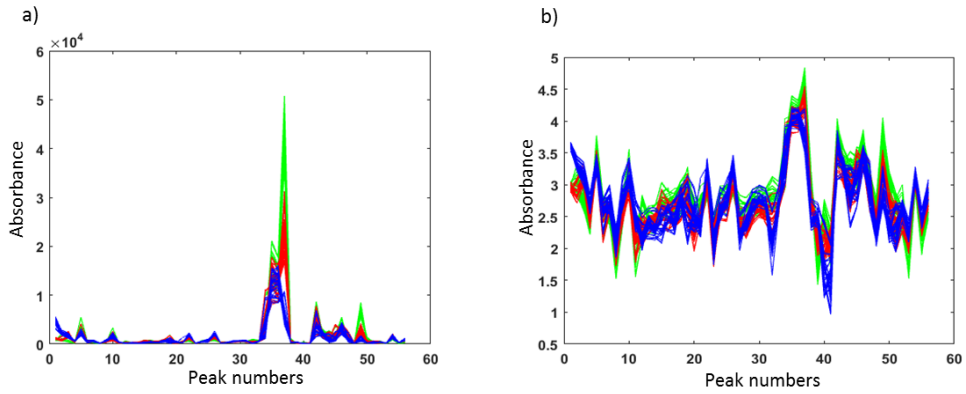


Figure 3.8 The effect of normalisation on peak table of 56 rooibos compounds (n=120), a) raw data and b) data after probabilistic quotient normalisation (PQN) and log transformation.

3.5.1.5. Centering and transformation

PQN was followed by log transformation to correct heteroscedastic variance and ensure that all compounds have comparable peak areas. To illustrate the effect of log transformation, the centered data matrix ($\boldsymbol{X}_c$) before and after transformation is shown in a colour map (Figure 3.9). Colour maps are an effective way to visualise data (Moreland, 2009). Studied peaks are represented by the x-axis and samples are represented by the y-axis. Green to yellow tones indicate higher peak areas and blue tones indicate lower peak areas.

The centered data matrix is solely dominated by the peak areas of aspalathin (peak nr. 37) (Figure 3.9a). This is because the aspalathin content in rooibos is much higher in comparison to the other phenolic compounds, especially in green rooibos (Bramati *et al.*, 2003). Additionally, an increase in sample number was followed by a decrease in aspalathin peak area (Figure 3.9a). This was to be expected since aspalathin oxidises during fermentation and the data matrix was constructed in the order: green, semi-fermented and fermented. Once the data had been log transformed (Figure 3.9b), the variance of all peaks was properly balanced and their influence on the differences between the studied classes was investigated.

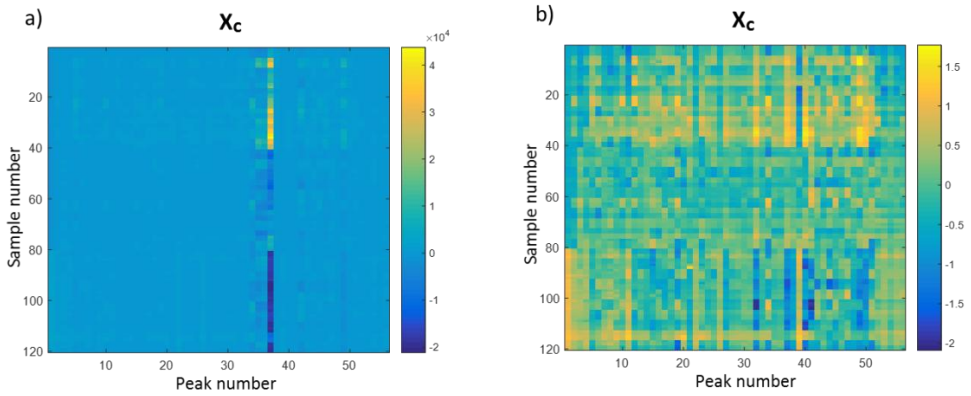


Figure 3.9 Centered data matrix, $\boldsymbol{X}_c$, composed of rooibos peak areas with the dimensions 120 (samples) × 56 (peak areas) (a) before and b) after log transformation.

3.5.2. Multivariate analysis of variance based on pre-processed data

3.5.2.1. Probabilistic quotient normalisation (PQN) and log transformation

ANOVA-TP results are summarised in Figure 3.10. The effect matrix is illustrated in Figure 3.10a, with the mean profile for green rooibos samples denoted by pink peaks and the mean profile of fermented samples are denoted by red peaks. The null distribution of the treatment effect is shown in Figure 3.10b (red point indicates observed sum of squares). Significant/insignificant compounds are identifiable from the loadings and bootstrap confidence interval (Figure 3.10c). The red line represents the loadings and the blue lines represent the confidence interval (Figure 3.10c). If the bootstrap confidence interval contains loading values of zero, there is a 99% probability that the loading of the compound could also be zero, thus the compound is deemed insignificant.

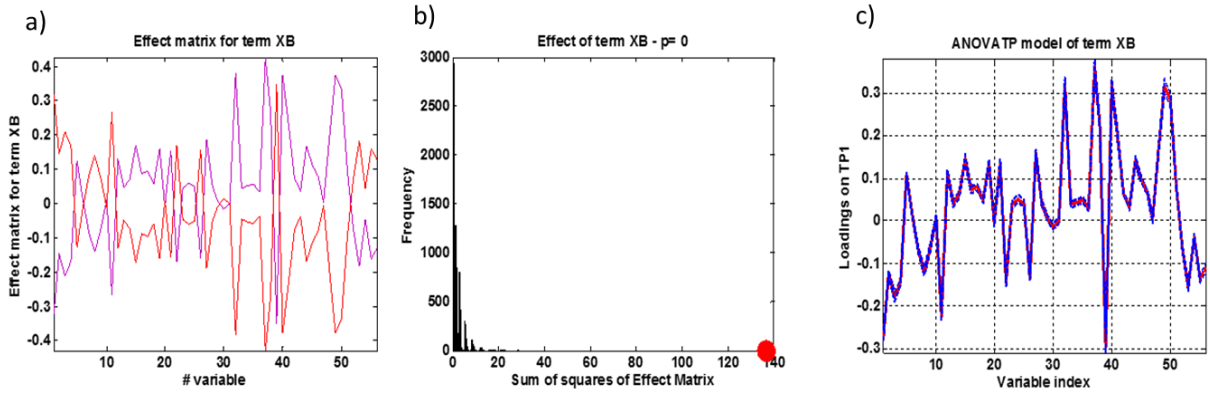


Figure 3.10 Summary of ANOVA-target projection (ANOVA-TP) results as applied to green and fermented rooibos peak tables after pre-processing using probabilistic quotient normalisation (PQN) followed by log transformation. a) Effect matrix of 'treatment', b) null distribution of the effect 'treatment' and c) loadings and bootstrap confidence intervals.

Based on ANOVA-TP results, four peaks (peak nr. 20, 29, 31 and 47) out of 56 studied peaks were identified as insignificant. This indicates that the above mentioned compounds are not significantly affected by the fermentation process. This conclusion is inconsistent with the main assumption of the PQN method, which states that it can only be applied to data for which the majority of the studied features do not differ. Therefore, PQN is not suitable as a normalisation method in this case. The assumption that the majority of the compounds are not affected by the treatment is highly unrealistic and it can lead to incorrect estimation of the scaling constants. PQN typically works well for metabolomics or proteomics data, where the majority of the features do not vary due to the studied effects.

Apart from the underlying assumption of PQN not being met, the ANOVA-TP results based on PQN are inconsistent with quantitative data reported for the intermediate product of aspalathin oxidation. ((*R*)-eriodictyol-8-*C*-β-D-glucopyranoside, peak nr. 31). Walters *et al.* (2017) reported (*R*)-eriodictyol-8-*C*-β-D-glucopyranoside content in green rooibos to be unquantifiable due to low signal-to-noise ratios. This is in relation to 0.400 g.100 g^{-1} plant material reported for fermented rooibos. For reference, the (*R*)-eriodictyol-8-*C*-β-D-glucopyranoside content in semi-fermented rooibos (0.152 g.100g^{-1} plant material) was significantly different to that of fermented rooibos (Walters *et al.*, 2017).

3.5.2.2. Pair-wise log ratios (PLR)

Additional data scaling is not necessary when using the PLR approach, as the relative number of features do not depend on the 'size effect'. Moreover, the log transformation is recommended to deal with data heteroscedasticity, so that further data transformation aimed at variance stabilisation is not required. A summary of the ANOVA-TP results is presented in Figure 3.11. The figures are analogous to those illustrated in the PQN section. However, results of ANOVA-TP performed on matrix ***P*** are expressed in terms of log ratios and not the peaks (Figure 3.11), making it difficult to identify marker compounds.

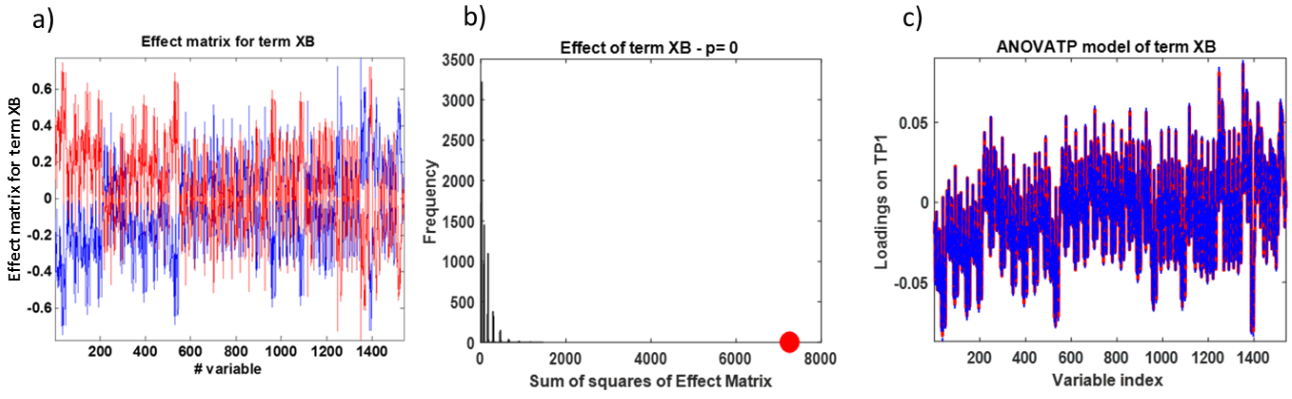


Figure 3.11 Summary of ANOVA-target projection (TP) results as applied to pair-wise log ratios of green and fermented rooibos samples. a) Effect matrix of 'treatment', b) null distributions of the effect 'treatment' and c) loadings and bootstrap confidence intervals.

3.5.3. Marker identification based on robust pair-wise log ratio (rPLR) approach

The statistics introduced (eqn. 3.17) allows identification of the significant features, which differentiate the studied classes of samples. After the data was log transformed, the log ratios were replaced with differences of the log transformed data. Thus, the log ratio $log(x_i/x_j)$ can be expressed as $log(x_i)$-$log(x_j)$. This means that the rPLR approach can be applied to complicated designs with the factor of interest at two levels.

To study the effect of 'treatment', it is necessary to remove the effect of 'batches' from the centered data. The resulting data are used to calculate a variation matrix (*i.e.* matrix ***V***) and the variation statistics (*i.e.* ***v*** statistics). While computing ***V***, it is not necessary to compute the entire matrices ***W1***, ***W2*** and ***T***. Elements of matrix ***V*** can be calculated directly. Only for illustrative purposes, the matrices representing the within class variances (***W1*** and ***W2***) and the matrix representing the total variance (***T***) are presented as colour maps in Figure 3.12. Yellow to red tones indicate higher variances, while blue tones indicate lower variances. Matrices are symmetrical, as they represent the variance calculated for the same pairs of samples.

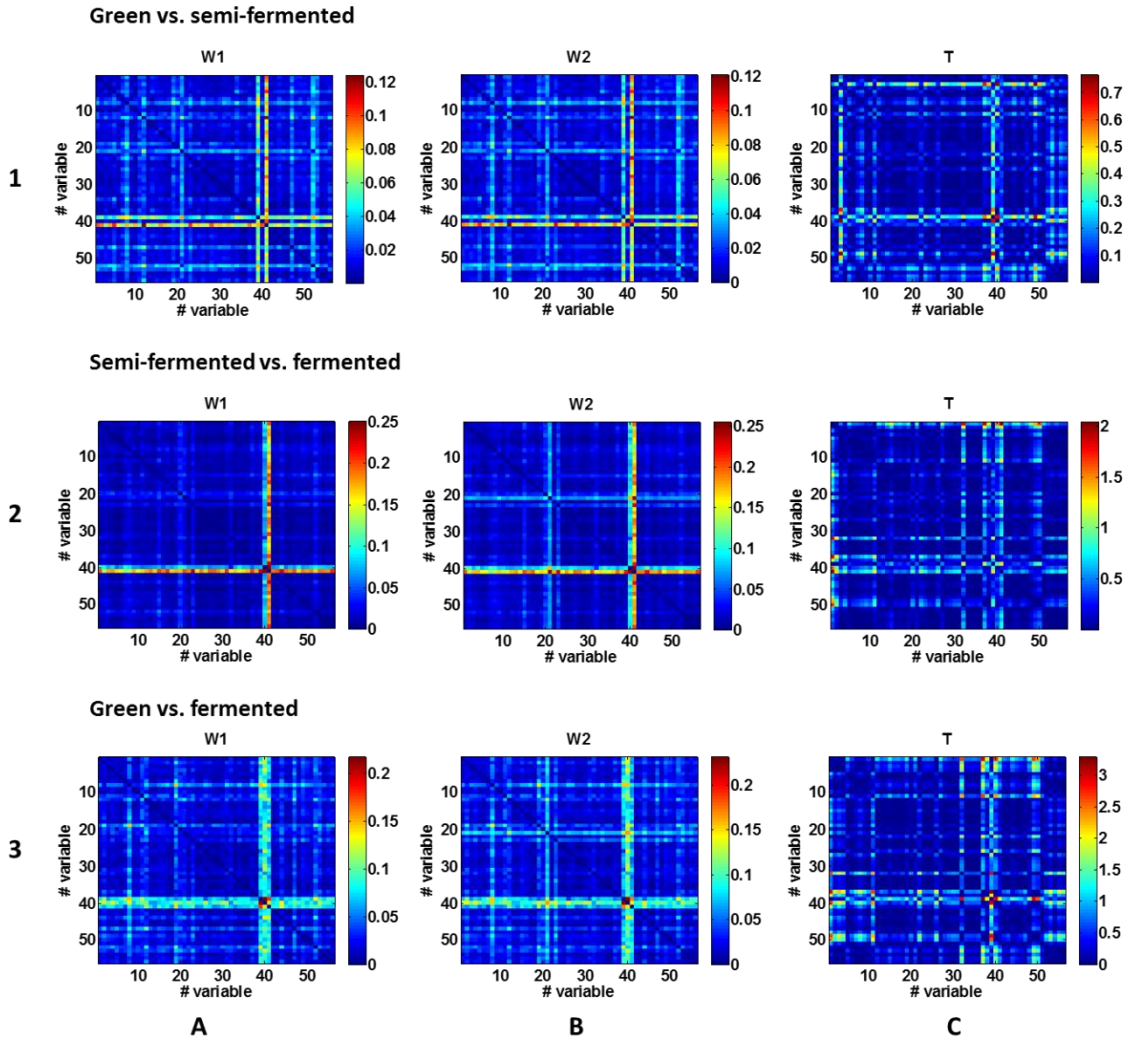


Figure 3.12 A) Matrix ***W1***, B) matrix ***W2*** and C) matrix ***T*** representing the within class 1, the within class 2 and the total variance, respectively. 1) Green vs. semi-fermented, 2) semi-fermented vs. fermented and 3) green vs. fermented.

The rPLR results are summarised in Figure 3.13. The ***V*** matrix, the corresponding **v** statistics and the null distributions of ***v*** statistics for all comparisons between treatments can be seen here. The ***V*** matrices were calculated as described by eqn. (3.16) and illustrated by the colour map in Figure 3.13A. As described above, yellow to red tones indicate higher values and blue tones indicate lower values. Similar to ***W1***, ***W2*** and ***T***, matrices illustrated in Figure 3.13 are symmetrical. The ***v*** statistic is calculated by taking the sum of all the values from the matrix ***V*** for individual compounds, this can be done by following either a horizontal or vertical direction (Figure 3.13B). The ***v*** statistic values range anywhere between zero and one (Figure 3.13B). In order to estimate the statistical significance of an individual peak, the observed value of its statistic is compared with the null distribution of ***v*** statistics which is derived based on the randomisation test. The test is performed 10 000 times for the reduced model residuals. To this effect, matrix ***V*** was recalculated 10 000 times for the matrix $\boldsymbol{X}_c = \boldsymbol{X}_{Treatment} + \boldsymbol{X}_{Error}$, using the permuted objects of the two studied classes of samples. The resulting null distributions is presented in Figure 3.13C. A compound is deemed as significant if it lies to the left hand side of the critical value which is 1% of the null distribution (Figure 3.13C). The null hypothesis applicable to all individual compounds is as follows: the compound has no significant influence on the observed differences between treatments.

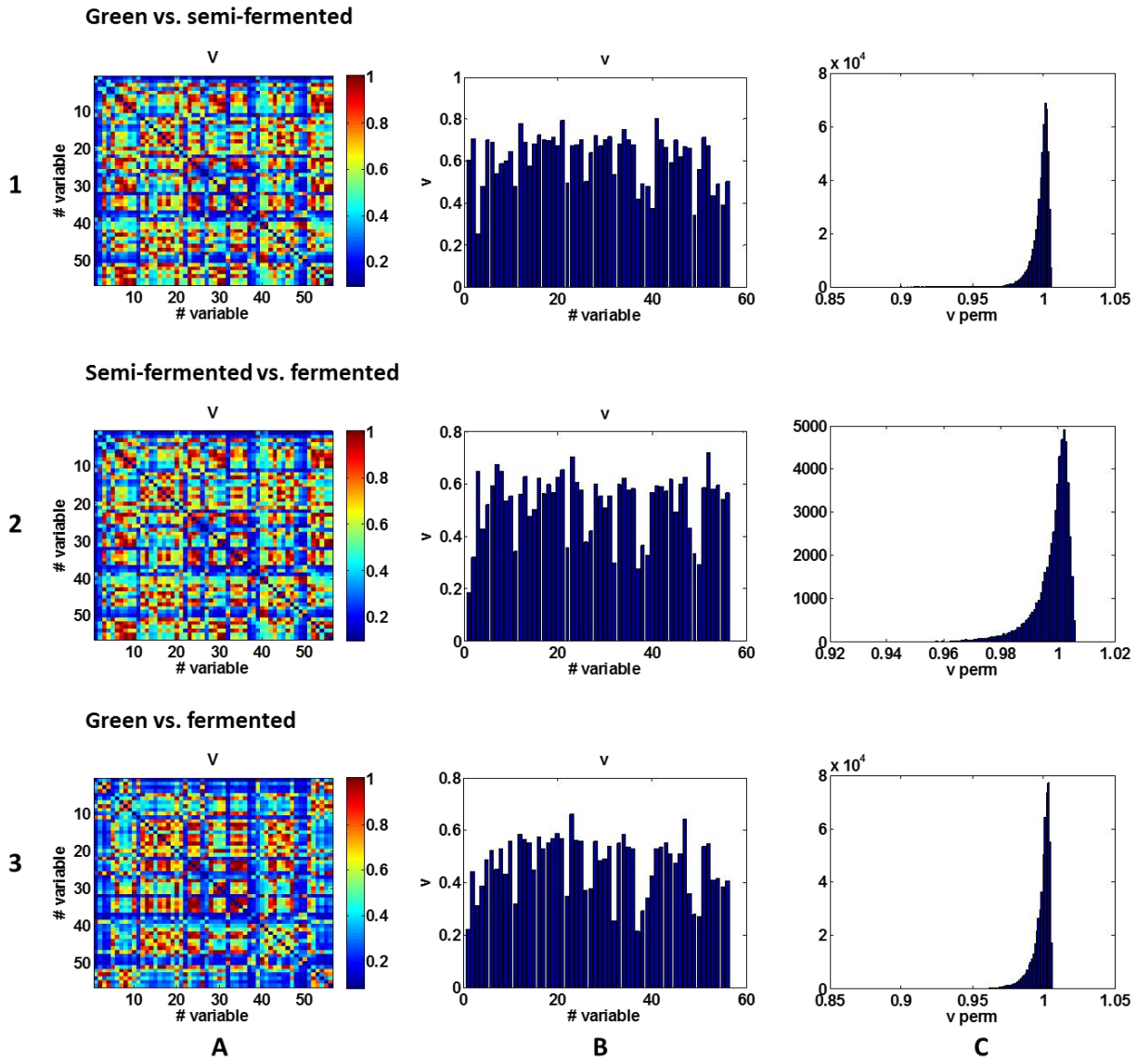


Figure 3.13 Robust pair-wise log ratio (rPLR) results for comparison of three pairs of two classes. A) Matrix ***V***, B) statistics ***v*** and C) distributions of the null hypothesis for 1) green vs. semi-fermented, 2) semi-fermented vs. fermented and 3) green vs. fermented.

The ***v*** statistics for the comparison between green and semi-fermented range between 0.2 and 0.8 (Figure 3.13B-1). All observed values of the ***v*** statistics are much smaller that the minimal value of the null distribution, indicating that p=1/10000 (Figure 3.13C-1). Therefore, the null hypothesis is rejected at a significance level of p=0.01 and all (56) peaks contribute significantly to the differences observed between green and semi-fermented rooibos. A similar trend is observed for the comparison between semi-fermented vs. fermented samples and green vs. fermented samples. It is important to note that no conclusions can be made with regard to the weight of the ***v*** stastics. For example, a compound cannot be reported as having a greater influence than another compound on the differences observed between treatments.

The percentage change in peak area was used to measure the degree of formation or degradation of compounds as a result of fermentation (Table 3.2). Quantitative data for the 16 identified peaks are reported

by Walters *et al.*, 2017 and in theory the percentage difference in peak areas (Table 3.2) should be similar to the percentage difference in content (g. $100g^{-1}$).

The percentage change in peak area observed for PPAG was relatively low (22.39%; Table 3.2), but, comparable to the percentage change in content (17%) reported by Walters *et al.*, (2017). However, contradictory to results reported here, PPAG in fermented rooibos (0.083 g. 100 g^{-1} plant material) was reported as showing no significant difference when compared to green (0.1 g. 100 g^{-1} plant material) and semi-fermented rooibos (0.081 g. 100 g^{-1} plant material) (Walters *et al*., 2017). It is likely that PPAG in fermented rooibos was marginally classified as having no significant differences to the other treatments in the study by Walters *et al*. (2017). The contradiction in results could be the effect of the differences between the analysis of variance methods used. Similar trends are observed for isoorientin, orientin, quercetin-3-*O*-robinobioside, vitexin, hyperoside, rutin, isovitexin, isoquercitrin and luteolin-7-*O*-glucopyranoside.

The aspalathin content was significantly different between all three treatments and the percentage change in content (86%) (Walters *et al*., 2017) was similar to the percentage change in peak area (85%) (Table 3.2). Degradation of aspalathin to this degree was expected as it is known to undergo rapid oxidation during fermentation (Walters *et al*., 2017). The percentage change in content from green to fermented rooibos reported for nothofagin (85%) is considerably different from percentage change reported here (40%). These differences in percentage change can be ascribed to the ambiguity that comes with integration of chromatograms.

Percentage change in peak areas for eriodictyol-6-*C*-glucopyranosides (~50%) are comparable to results reported by Walters *et al*. (2017). However, this was not the case for eriodictyol-8-*C*-glucopyranosides, which can be related to integration and peak detection methods (see above). 8-*C*-glucopyranosides occur in fermented rooibos at lower concentrations than 6-*C*-glucopyranosides. Lower contents of 8-*C*-glucopyranosides is attributed to the relatively slow formation via Wessely-Moser rearrangement in relation to the formation of 6-*C*-glucopyranosides via oxidative cyclisation of aspalthin.

A total of 22 compounds including (*S*)-eriodictyol-8-*C*-glucopyranoside, isoorientin, orientin, rutin, isovitexin and isoquecitrin, show less than 30% change in peak area (Table 3.2). In this regard, these compounds can be excluded from consideration for potential marker compounds of rooibos fermentation.

Table 3.2 Percentage change in peak area for 56 rooibos peaks calculated based on mean green and fermented peak tables. + and – symbols indicate an increase or decrease in peak area as a result fermentation, respectively.

Peak nr.	Change in peak area (%)	Peak nr.	Change in peak area (%)	Peak nr.	Change in peak area (%)	Peak nr.	Change in peak area (%)
1	+77.1	**16**	-34.2	**31**[d#]	-1.3	**46**	-32
2	+45.8	**17**	-36.3	**32**	-80.7	**47**[#]	+1.5
3	+62	**18**[#]	-23.7	**33**[#]	-18.9	**48**	-60.4
4	+52.5	**19**	-44.9	**34**[e#]	-22.4	**49**	-83
5	-45	**20**[#]	+5.5	**35**[f#]	-25.9	**50**	-79.4
6[#]	-9.1	**21**	-49.1	**36**[g#]	-16.5	**51**	-34.6
7	+30.8	**22**[a]	+53.9	**37**[h]	-85.5	**52**[#]	+20.6
8	+42.6	**23**[#]	-19.2	**38**	-70.3	**53**	+54.1
9[#]	+29.2	**24**[#]	-23.9	**39**[i]	+77.6	**54**[#]	+18
10[#]	-10.3	**25**[#]	-20	**40**[j]	-78.4	**55**	+50.7
11	+67.3	**26**[b]	+51.4	**41**[k]	-60.1	**56**	+42.9
12	-50.9	**27**	-57.7	**42**[l#]	-28		
13[#]	-19	**28**[#]	-20.1	**43**[m#]	-18.6		
14[#]	-28.9	**29**[#]	-4.3	**44**[n]	-48.8		
15	-54.4	**30**[c#]	+4.4	**45**[o]	-39.8		

[a](*S*)-Eriodictyol-6-*C*-β-D-glucopyranoside; [b](*R*)-eriodictyol-6-*C*-β-D-glucopyranoside; [c](*S*)-eriodictyol-8-*C*-β-D-glucopyranoside; [d](*R*)-eriodictyol-8-*C*-β-D-glucopyranoside; [e]*Z*-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid (PPAG); [f]isoorientin; [g]orientin; [h]aspalathin; [i]quercetin-3-*O*-β-D-robinobioside; [j]vitexin; [k]hyperoside; [l]rutin; [m]isovitexin+isoquercitrin; [n]luteolin-7-*O*-glucopyranoside; [o]nothofagin.
[#]Change in content = less than 30%.

3.6. Conclusion

Pre-processing methods need to be tailored to suit the type of data being analysed. The rPLR approach removed the 'size-effect' efficiently. When performing any type of chemometric analysis it is vital to always consider the body of knowledge on the topic before making conclusions. That being said, realistic and credible conclusions can be drawn when all limitations of the chemometric methods are considered. By application of untargeted analysis, 56 rooibos compounds were identified as potential markers of rooibos fermentation. This was narrowed down to 34 compounds based on a 30% peak area change threshold. Marker compounds identified for rooibos fermentation can be used to optimise the process.

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CHAPTER 4

Modelling of reaction kinetics of rooibos phenolic compounds during simulated fermentation

4.1. Abstract

Polyphenolic compounds of *Aspalathus linearis* (rooibos) are susceptible to degradation during processing, this is evident from the differences in polyphenolic content between green and fermented rooibos. Changes as a result of oxidation ('fermentation'), such as phenolic composition and colour changes, are detrimental to the quality of green rooibos. Minimising these changes is difficult as oxidation occurs rapidly and is easily initiated by elevated moisture levels or elevated temperatures, conditions which are often encountered during transportation or storage of green tea. Understanding the reaction kinetics of rooibos phenolics under simulated high temperature and high moisture conditions is essential as they might play a key role in prevention of phenolic losses in green rooibos. In this study, a kinetic modelling approach was used to establish reaction kinetic parameters of selected rooibos compounds during simulated fermentation (mini-scale, controlled process). A preliminary experiment entailed heating green rooibos at 170°C for 30 min in an attempt to inactivate endogenous enzymes that might play a role in rooibos fermentation. Degradation of aspalathin and nothofagin during fermentation of heat-treated samples was considerably less than for control samples - this reinforces the postulation that rooibos endogenous enzymes participate in the oxidation of polyphenolic compounds during fermentation. The degradation/formation of selected rooibos phenolic compounds during simulated fermentation of untreated plant material at four different temperatures between 37 and 50°C was best described by the fractional conversion model based on first-order kinetics, which allows for non-zero equilibrium concentrations. Reaction rates for the degradation/formation of compounds during fermentation increased as temperature increased, following the Arrhenius law. A lower extent of phenolic degradation (higher equilibrium concentration) was observed at higher temperatures, which is contrary to general findings in literature relating to phenolic degradation. This poses the possibility that enzymes in the tea were partially inactivated at the higher temperatures, or that there was a limiting factor during the simulated fermentation.

4.2. Introduction

Rooibos is produced from an endemic South African fynbos species called *Aspalathus linearis*. The tea is well known for its rich polyphenolic content and the health-promoting properties linked to it. Common health-promoting properties associated with rooibos polyphenolics include anti-inflammatory (Magcwebeba *et al.*, 2016; Smith and Swart, 2016), anti-obesity (Beltrán-Debón *et al.*, 2011; Sanderson *et al.*, 2014), anti-diabetic (Muller *et al.*, 2016) and anti-carcinogenic (Petrova, 2009) activity, among many others.

During processing rooibos polyphenolics are susceptible to degradation (Joubert and De Beer, 2014). An oxidation step (commonly known as 'fermentation') is essential to the development of the characteristic colour and flavour of rooibos tea. However, fermentation has been shown to cause significant losses to the phenolic content of rooibos (Walters *et al.*, 2017). The fermentation process employed by the rooibos industry briefly entails bruising and wetting of cut plant material and allowing the plant material to 'ferment' in heaps at uncontrolled temperatures for 12-14 h (Joubert *et al.*, 2008). It is postulated that rooibos fermentation, similar to black tea (*Camellia sinensis*) fermentation, is initiated by enzymatic reactions (Joubert and De Beer, 2011). Initiation of enzymatic oxidation is induced by bruising of the plant material, which causes cell disruption, allowing enzymes (localised in the plant chloroplast) to come into contact with phenolic compounds (localised in the plant vacuole) (Subramanian *et al.*, 1999). The enzymatic reaction produces highly reactive compounds that are responsible for the onset of chemical oxidation during the fermentation process (Yoruk and Marshall, 2003).

Increased consumer interest in natural antioxidants has led to many studies focused on rooibos phenolic compounds and their degradation during processing. The oxidative conversion of aspalathin to intermediate products, (*R*)/(*S*)-eriodictyol-6-*C*-β-D-glucopyranoside and (*R*)/(*S*)-eriodictyol-8-*C*-β-D-glucopyranoside, was first reported by Koeppen and Roux (1965). Joubert (1996) followed by reporting quantitative data of aspalathin and nothofagin, in green, semi-fermented and fermented rooibos. The mechanism of formation of isoorientin and orientin via the aspalathin oxidation intermediate product, eriodictyol-6-*C*-β-D-glucopyranoside, was illustrated by Krafczyk and Glomb (2008). Colour formation during rooibos fermentation was investigated by Heinrich *et al.* (2012), and it was found that coloured dibenzofurans, that add to the red/brown colour of fermented rooibos, are formed as products of aspalathin oxidation.

The focus to date has been on the identification and quantification of phenolic compounds in green and fermented rooibos infusions and extracts (Bramati *et al.*, 2002, 2003; Stalmach *et al.*, 2009; Beelders *et al.*, 2012; Walters *et al.*, 2017). In keeping with the focal point, the untargeted analysis of changes in phenolic content due to fermentation concluded that 56 compounds in rooibos change significantly between unfermented, semi-fermented and fermented plant material (Chapter 3). Of the 56 reported compounds, 16 are identifiable and quantifiable by means of authentic reference standards using the HPLC-DAD method reported by Walters *et al*. (2017).

The aim of the following study was to gain insight about the reaction kinetics of selected rooibos phenolic compounds during simulated fermentation so that their losses may be predicted. Understanding more about the reactions kinetics of rooibos phenolics may help prevent phenolic losses during green rooibos

processing, transportation and storage. To date, there has been no research conducted on the reaction kinetics of rooibos phenolics in a model system or within the plant matrix. Thus, kinetic parameters (*i.e* reaction order and reaction rate constant) governing the degradation of selected rooibos compounds in the plant material matrix were established using a kinetic modelling approach. Parameters estimated from the Arrhenius equation (*i.e.* activation energy and the pre-exponential factor) was used to describe the temperature dependence of the reactions. Q_{10} values were also calculated as an alternative measurement of the temperature dependence of the reaction rates. In addition to the reaction kinetics modelling, two preliminary experiments were performed. First, the conditions for extraction of rooibos phenolics was optimised and, lastly, the role of endogenous enzymes in rooibos fermentation was investigated in brief.

4.3. Experimental

4.3.1. Chemicals

General laboratory chemicals (ascorbic acid and DMSO) were reagent grade, provided by Sigma-Aldrich (St. Louis, MO, USA). HPLC grade water was prepared by purifying deionised water using Millipore Elix and Milli-Q Advantage A^+ (Millipore, Bedford, USA) water purification systems in series. HPLC gradient grade acetonitrile was purchased from Merck (Darmstadt, Germany) and glacial acetic acid and formic acid from Sigma-Aldrich (St Louis, MO, USA). Authentic reference standards (purity ≥ 95%) were purchased from Extrasynthese (Genay, France; isoorientin, orientin, eriodictyol-7-*O*-β-D-glucopyranoside, isovitexin and hyperoside), Karl Roth (Karlsruhe, Germany; vitexin and luteolin-7-*O*-β-D-glucopyranoside), Sigma-Aldrich (isoquercitrin), Transmit (Gieβen, Germany; rutin) and the PROMEC unit of the Medical Research Council of South Africa (MRC, Cape Town, South Africa; aspalathin and nothofagin). *Z*-2-(β-D-Glucopyranosyloxy)-3-phenylpropenoic acid (PPAG) was sourced from the compound library of the Plant Bioactives Group of the Agricultural Research Council (Infruitec-Nietvoorbij) of South Africa. Stock solutions of the phenolic standards were prepared in DMSO at concentrations of approximately 1 $mg.mL^{-1}$ and diluted with water as required. All the diluted standard mixtures contained *ca.* 1% ascorbic acid ($m.v^{-1}$) (Sigma-Aldrich) and were filtered through 0.22 µm hydrophilic PVDF filters (Millipore) before use.

4.3.2. Plant material

Green rooibos was kindly supplied by Rooibos Limited (Clanwilliam, South Africa). The shoots (leaves and stems) were coarsely milled using a Retsch mill (1 mm sieve; Retsch GmbH, Haan, Germany). Plant material was then finely milled using a Retsch MM301 ball mill to improve homogeneity for mini-scale extraction and simulated fermentation. The finely milled plant material was passed through Endecott test sieves and the fraction between 210 and 500 µm was used. Different batches of green plant material were used in the enzyme inactivation trial and the temperature dependence experiment.

4.3.3. Optimisation of extraction conditions

A standardised 40% acetonitrile-water extraction solvent (optimised by Walters *et al*., 2017) was used during optimisation of the extraction conditions. Treatments 1-5 were used to compare the effect of different periods of sonication. The control, as reported by Walters *et al*. (2017), entailed a heating step (100°C for 20 min) and a sonication step (5 min). Treatments were applied in triplicate using the same batch of green rooibos plant material (Table 4.1).

Table 4.1 Six treatments applied to 40% acetonitrile rooibos extracts.

Treatment	**1**	**2**	**3**	**4**	**5**	**control**
Heat	-	-	-	-	-	100°C for 20 min
Sonication	5 min	10 min	15 min	20 min	30 min	5 min

Approximately 80 mg of plant material and 3 mL of 40% aqueous acetonitrile were mixed together in 24 mL glass vials with screw caps. Samples that required heat treatment were heated in a digital heating block (Stuart, Bibby Scientific Limited, Stone, UK), thermostatted to 100°C. Sonication was performed using a sonication bath (Branson Ultrasonic Corporation, Danbury, CT, USA). After sonication, the extracts were diluted with water, ascorbic acid solution (final concentration 0.1-0.2%) was added and the extracts were filtered using 0.45 μm hydrophilic PVDF syringe filters (Millipore).

4.3.4. Simulated fermentation

Fermentation of rooibos was simulated on mini-scale and under highly controlled conditions. Approximately 40 mg of plant material was hydrated with 200 μL of HPLC-grade water in 24 mL clear glass vials and closed with screw caps. Samples were vortexed to ensure that the bottom of the vials were covered in a thin layer of the wetted plant material. A Stuart digital heating block was used to heat the vials at a specified fermentation temperature. Separate vials were removed from the heating block at specified time points up to a maximum time of 6 h (10 time-points in total, including the control, *i.e.* 0 min). Glycerol (*ca.* 1-2 drops) was added to the cavities of the heating blocks to facilitate heat transfer between the aluminium blocks and the vials.

4.3.5. Enzyme inactivation trial

A dry heat treatment was applied to green rooibos plant material in an attempt to inactivate endogenous rooibos enzymes. The heat treatment entailed covering the bottom of a nickel moisture dish with a thin layer of plant material and heating it in a convection-type laboratory oven at 170°C for 30 min. Samples of control and heat-treated rooibos were then subjected to the simulated fermentation process at 37°C for 6 h. This simulated fermentation was performed in triplicate for both heat-treated and control samples. After removal from the heating blocks, the phenolic fraction of the plant material was extracted using the optimised 40% acetonitrile extraction procedure (Treatment 1: 5 min sonication).

4.3.6. Determination of temperature dependence of rooibos phenolic compounds during simulated fermentation

To quantitatively describe changes occurring to selected rooibos phenolics, a kinetic modelling approach was used for monitoring degradation during oxidation as a function of controlled time and temperature conditions. The effect of temperature on the phenolic compounds in rooibos tea was investigated at four different temperatures (*i.e.* 37, 40, 43 and 50°C). Samples were prepared in triplicate per fermentation temperature. After fermentation, the phenolic fraction of the samples was extracted using the optimised 40% acetonitrile extraction procedure (Treatment 1: 5 min sonication).

4.3.7. Quantitative high performance liquid chromatography-diode array detector (HPLC-DAD) analysis

HPLC analysis of extracts from the extraction optimisation and temperature dependence experiments was performed as described by Walters *et al.* (2017). Briefly, an Agilent HPLC 1200 series instrument (maximum pressure limit 400 bar) equipped with an in-line degasser, quaternary pump, autosampler, column thermostat with an in-line 3 µL sample preheater and a DAD was used for chromatographic analysis. The instrument was controlled using Chemstation software (Agilent Technologies, Waldbronn, Germany). Separation at 44.5°C was achieved on a Poroshell 120 SB-C_{18} (150×4.6 mm i.d., 2.7 µm particle size) column, protected by a ZORBAX SB-C_{18} analytical guard column (12.5×4.6 mm i.d., 5 µm particle size; Agilent) and a Waters Acquity in-line filter. Acetonitrile (B) and 2% aqueous acetic acid (A) were used as mobile phases at a flow rate of 1 mL.min^{-1} using a multilinear gradient as follows: 10-14.8% B (0-28.5 min), 14.8-19.2% B (28.5-33 min), 19.2-100% B (33-33.5 min), 100% B isocratic (33.5-38 min), 100-10% B (38-39 min) and 10% B isocratic (39-46 min). UV-Vis spectra were recorded between 200 and 700 nm with selected wavelength monitoring at 288 nm (aspalathin, nothofagin, PPAG, (*S*)/(*R*)-eriodictyol-6-*C*-β-D-glucopyranoside and (*S*)/(*R*)-eriodictyol-8-*C*-β-D-glucopyranoside) and 350 nm (isoorientin, orientin, quercetin-3-*O*-robinobioside, vitexin, hyperoside, rutin, isovitexin, isoquercitrin and luteolin-7-*O*-β-D-glucopyranoside). The eriodictyol derivatives were quantified as eriodictyol-7-*O*-β-D-glucoside equivalents as no authentic reference standards were available. For the same reason, quercetin-3-*O*-robinobioside was quantified as rutin equivalents. Response factors for nothofagin (1.346) and PPAG (1.016) relative to aspalathin were obtained by preparing calibration curves (8 points) of each and dividing the slopes for nothofagin and PPAG with that of aspalathin. To cover the range of expected concentration in the samples, the calibration mixtures were injected at eight different injection volumes between 0.009 and 2.6 µg 'on-column'.

HPLC analysis of extracts from the enzyme inactivation trial was performed as described by De Beer *et al.* (2015). An Agilent 1200 HPLC system (see above) was used to determine the aspalathin, nothofagin, orientin and isoorientin content of extracts. Separation at 30°C was achieved on a Poroshell SB-C_{18} column (50×4.6 mm i.d., 2.7 µm particle size) protected by a Waters Acquity in-line filter and a Waters Acquity UPLC VanGuard pre-column (stationary phase: BEH C_{18} 1.7 µm). Gradient elution was performed using 0.1% aqueous formic acid (A) and acetonitrile (B) at 1 mL.min^{-1} as follows: 12.4-16.6% B (0-10 min); 16.6-80% B (10-10.5 min); 80-12.4% B (11.5-12 min); 12.4% B (12-16 min). UV-Vis spectra were recorded for all

samples from 220 to 450 nm. Aspalathin and nothofagin were quantified using peak areas at 288 nm, while peak areas at 350 nm were used to quantify orientin and isoorientin. To cover the range of expected concentration in the samples, the calibration mixtures were injected at seven different injection volumes between 0.01 and 1.7 µg 'on-column'.

4.3.8. Kinetic modelling and statistical analysis

Kinetic modelling was only performed on the quantitative data of compounds that showed a minimum of *ca.* 10% change in content after 6 h for at least one temperature. The degradation/formation of the selected phenolic compounds during fermentation was modelled with empirical kinetic models, namely the zero-order, first-order, second-order and first-order fractional conversion model (Levenspiel, 1999):

Zero-order: $$C = C_0 - kt \quad (4.1)$$

First-order: $$\ln C = lnC_0 - kt \quad (4.2)$$

Second-order: $$\frac{1}{C} = \frac{1}{C_0} + kt \quad (4.3)$$

Fractional conversion model: $$C = C_\infty + (C_0 - C_\infty)\, e(-kt) \quad (4.4)$$

where C is the concentration of a compound at a given time t (min), C_0 is the initial concentration before fermentation, C_∞ is the equilibrium concentration or the value of the stable fraction and k is the reaction rate constant. Model evaluation and selection were performed by examining R^2 values and by visual inspection of the parity plot (estimated concentration values versus measured concentration values).

Furthermore, the effect of temperature on the reaction rate constants of each compound was evaluated by calculating the activation energy according to the Arrhenius equation (Van Boekel, 2008):

$$k = Ae^{\frac{-Ea}{RT}} \quad (4.5)$$

The linearised form is:

$$lnk = -\left(\frac{E_a}{R}\right).\frac{1}{T} + lnA \quad (4.6)$$

where A is the so-called pre-exponential factor (min^{-1}), E_a is the activation energy ($kJ.mol^{-1}$), R is the universal gas constant (8.314 J mol^{-1} K^{-1}) and T is the absolute temperature (K). For a comparison, the activation parameters were computed by estimation of the slope and y-intercept by linear regression of *ln k* vs *1/T* according to eqn. (4.6).

The temperature coefficient (Q_{10}) was determined as an additional measurement of the temperature dependence of the rate constants:

$$Q_{10} = \frac{k(T+10°C)}{k(T)} \quad (4.7)$$

where *k* is the reaction rate constants at temperature *T* and *T+10°C*.

The experimental design for the extraction optimisation experiment was a completely randomised one factor design with three random replicates for each of the extraction conditions. Quantitative data were subjected to analysis of variance (ANOVA) using the GLM procedure of SAS® statistical software (Version 9.4, SAS Institute, Cary, NC, USA). The Shapiro-Wilk test was performed to test for normality and identify outliers. Fisher's least significant difference (LSD) was calculated at a significance level of 5% to compare treatment means. Values of $p<0.05$ were considered to be significant.

For temperature dependence experiments the content of compounds was observed at ten time intervals for each experimental replicate. These observations over time were combined in a split-plot analysis of variance with fermentation temperature as main plot factor and time as sub-plot factor. Estimation of kinetic parameters were performed by application of linear (zero-order model, linearised form of Arrhenius equation) and non-linear modelling (first-order, second-order and fractional conversion model) with time as independent variable for each experimental replicate, using the NLIN procedure of SAS®. Regression parameters obtained were used as input in a one-way ANOVA to compare regression parameters for fermentation temperatures.

4.4. Results and discussion

4.4.1. Optimisation of extraction conditions

Heat and sonication treatments are commonly used in extraction processes (Smith, 2003; Chemat and Khan, 2011), but whether both treatments are necessary for the extraction of rooibos phenolics has not yet been determined. The method reported by Walters *et al*. (2017) was used as a control and other treatments used various sonication times without a heat treatment step (Table 4.2). In the present investigation it was important to avoid changes as a result of heat exposure other than treatment of the plant material.

ANOVA was performed on the quantitative data of 9 major phenolic compounds and PPAG. No significant ($p<0.05$) differences between any of the treatments were observed for all compounds across all treatments (Table 4.2). Thus, it can be concluded that the heat treatment is not essential for the extraction of the phenolic fraction from rooibos plant material and only minimal sonication is required, which is likely due to the very small particle size of the plant material after sieving.

Table 4.2 Quantitative data (mean±SD, g. 100 g^{-1} plant material) of 9 major rooibos phenolics and *Z*-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid (PPAG) as measured for various extraction conditions.

Compounds	Sonication					Heat+5 min sonication
	5 min	10 min	15 min	20 min	30 min	
PPAG	0.083±0.006$_a$	0.084±0.006$_a$	0.081±0.004$_a$	0.085±0.005$_a$	0.085±0.008$_a$	0.087±0.003$_a$
Aspalathin	4.5±0.4$_a$	4.6±0.4$_a$	4.3±0.2$_a$	4.5±0.1$_a$	4.5±0.2$_a$	4.4±0.1$_a$
Nothofagin	0.248±0.025$_a$	0.261±0.028$_a$	0.245±0.014$_a$	0.26±0.008$_a$	0.259±0.012$_a$	0.255±0.008$_a$
Isoorientin	0.34±0.04$_a$	0.35±0.04$_a$	0.33±0.02$_a$	0.34±0.01$_a$	0.34±0.01$_a$	0.35±0.02$_a$
Orientin	0.248±0.021$_a$	0.252±0.023$_a$	0.238±0.012$_a$	0.249±0.008$_a$	0.248±0.007$_a$	0.26±0.009$_a$
Quercetin-3-*O*-robinobioside	0.35±0.04$_a$	0.36±0.04$_a$	0.33±0.02$_a$	0.35±0.01$_a$	0.35±0.02$_a$	0.36±0.01$_a$
Vitexin	0.047±0.004$_a$	0.048±0.003$_a$	0.045±0.003$_a$	0.048±0.002$_a$	0.047±0.002$_a$	0.049±0.000$_a$
Hyperoside	0.101±0.012$_a$	0.102±0.008$_a$	0.096±0.006$_a$	0.101±0.007$_a$	0.102±0.007$_a$	0.103±0.002$_a$
Rutin	0.162±0.016$_a$	0.166±0.015$_a$	0.156±0.007$_a$	0.162±0.006$_a$	0.162±0.007$_a$	0.169±0.002$_a$
Isovitexin	0.069±0.007$_a$	0.07±0.006$_a$	0.066±0.003$_a$	0.067±0.004$_a$	0.065±0.007$_a$	0.073±0.005$_a$
Isoquercitrin	0.168±0.020$_a$	0.174±0.017$_a$	0.16±0.014$_a$	0.172±0.011$_a$	0.174±0.006$_a$	0.171±0.008$_a$

Values in the same row with different letters are significantly different from one another ($p<0.05$).

4.4.2.Enzyme inactivation trial

Upon visual inspection, a noticeable colour change was observed in the plant material (green to red/brown) and the extracted fractions (yellow to red/brown) as fermentation progressed. These observations were expected as colour change is an essential part of the fermentation process (Joubert and De Beer, 2014). A less pronounced, but noticeable, colour change from green to red/brown was also observed for the dry heat-treated tea samples.

The reaction order was determined based on the highest coefficient of determination (R^2) between measured values and estimated values of the zero-order, first-order, second-order and fractional conversion models. The R^2 values observed for the fractional conversion model ($R^2 \geq 0.99$) were better than the R^2 values observed for the other models (Table 4.3), indicating that the degradation of aspalathin and nothofagin, in both control and heat-treated samples, was best described by this model. Degradation kinetics of polyphenolics typically follow first-order kinetics. This has been illustrated in literature for procyanidins and dihydrochalcones in cloudy apple juice during heat treatment (De Paepe *et al.*, 2014), benzophenones and xanthones in *Cyclopia genistoides* during 'fermentation' (Beelders *et al.*, 2015) and pure naringin (predominantly found in grapefruit) during photodegradation (Cordenonsi *et al.*, 2016). The use of the fractional conversion model to describe the degradation kinetics of polyphenolics is rare. Nonetheless, the

fractional conversion model was previously shown to be well suited for modelling the thermal degradation (80°C to 100°C) of epigallocatechin gallate (a flavan-3-ol commonly found in black tea) in a model system (Zimeri and Tong, 1999). In other instances, thermal degradation of total phenolic and total flavonoid content in Irish York cabbage (Jaiswal *et al.*, 2012), thermal degradation of ascorbic acid and dehydroascorbic acid in Cupuaçu nectar (Vieira *et al.*, 2000) and colour change in peach puree (Avila and Silva, 1999) were modelled using the fractional conversion model.

Table 4.3 The coefficients of determination (R^2) between average measured and estimated contents for empirical models namely, zero-order, first-order, second-order and fractional conversion models, of aspalathin and nothofagin. Results are based on the degradation of aspalathin and nothofagin in heat-treated and untreated (control) samples.

	Zero-order	First-order	Second-order	Fractional conversion
Aspalathin				
Control	0.7443	0.8667	0.9222	0.9923
Heat-treated	0.8812	0.9056	0.9255	0.9796
Nothofagin				
Control	0.7209	0.8675	0.9328	0.9943
Heat-treated	0.9058	0.9408	0.9669	0.9864

The measured and estimated quantitative data for aspalathin and nothofagin in heat-treated samples and control samples are presented in Figure 4.1. Estimated initial and equilibrium concentrations and reaction rates are given in Table 4.4.

The reaction rate of aspalathin degradation in the control samples (0.012 min^{-1}) was significantly higher than that of the heat-treated samples (0.007 min^{-1}) and a similar observation was made for the reaction rate of nothofagin degradation (Figure 4.1; Table 4.4). A significantly higher equilibrium concentration was obtained for aspalathin in heat-treated samples (2.52 g.100 g^{-1} plant material) in comparison to control samples (1.47 g.100 g^{-1} plant material) and again a similar observation was made for nothofagin (Figure 4.1; Table 4.4). These results indicate a more rapid and greater extent of degradation of the dihydrochalcones in the control samples compared to the heat-treated samples. As stated previously, it is postulated that the onset of rooibos fermentation is initiated by enzymatic oxidation (Joubert, 1996). In this regard, two deductions can be made from the decrease in the extent of degradation and reaction rate of aspalathin and nothofagin in heat-treated samples. Firstly, the results support the postulation that endogenous enzymes in rooibos participate in the oxidation of polyphenolic compounds during rooibos fermentation and secondly, these enzymes are inactivated or partially inactivated as a result of the applied dry heat-treatment. Based on the common occurrence of polyphenol oxidase (PPO) and peroxidase (POD) in nature and the role they play in black tea fermentation, it is most likely that these enzymes are also responsible for the enzymatic oxidation in rooibos.

Inactivation or partial inactivation of endogenous enzymes in rooibos was achieved by application of a relatively intensive dry heat treatment (170°C for 30 min). Exposure time and temperature required for inactivation of PPO and POD are known to vary among different plant sources (Yoruk and Marshall, 2003).

Thermal stability tests on PPO and POD in peaches indicated that PPO was inactivated at temperatures above 80°C, while POD was inactivated at temperatures above 60°C (Lopes *et al.*, 2014). PPO and POD in green coconut water showed 90% reduction in activity after microwave treatment at 93°C for 16.5 s and 91.5°C for 44s, respectively (Matsui *et al.*, 2007). Complete inactivation of PPO activity in blueberries was achieved after a 20 min treatment at 85°C (Siddiq and Dolan, 2017). At 78 and 75°C, PPO and POD in table grapes lost >90% of their relative activity after 5 min, respectively (Fortea *et al.*, 2009). In all these cases, the enzymes were exposed to heat at a high moisture content. Since water is essential for the activity of enzymes (Rezaei *et al.*, 2007), it seems reasonable that an intense temperature-time combination would be necessary for inactivation during a dry heat treatment. However, further investigation is required on this matter.

The initial aspalathin content (at time 0 min) in heat-treated samples (3.70 g.100 g^{-1} plant material) was significantly lower than the initial aspalathin content in control samples (5.48 g.100 g^{-1} plant material) (Figure 4.1a; Table 4.4). No significant differences were observed for the initial nothofagin content in heat-treated (0.43 g.100 g^{-1} plant material) and control samples (0.46 g.100 g^{-1} plant material) (Figure 4.1b; Table 4.4). Nothofagin has a lower oxidation potential than aspalathin (due to one less catechol group) (De Beer *et al.*, 2015), therefore, it is sensible that during the dry heat treatment, where mainly chemical oxidation is taking place, that nothofagin would be more thermally stable. However, apart from the effect of the dry heat treatment, there is a strong resemblance between the degradation plots of nothofagin and aspalathin (Figure 4.1 and 4.2). The high moisture conditions during simulated fermentation are conducive to enzymatic reactions (Rezaei *et al.*, 2007). During polyphenolic oxidation via PPO, monophenols (such as nothofagin) are oxidised to diphenols (such as aspalathin) prior to formation of *o*-quinones (McEvily *et al.*, 1992). This means that nothofagin is equally susceptible to oxidation as aspalathin during enzymatic oxidation and hence, the same degradation trends for the aspalathin and nothofagin were observed during simulated fermentation.

The orientin and isoorientin content of the plant material did not change over the fermentation period in either the control or heat-treated samples (Figure A1), which can be explained by the formation (via aspalathin oxidation) and degradation of the compounds occurring simultaneously during oxidation (Walters *et al.*, 2017).

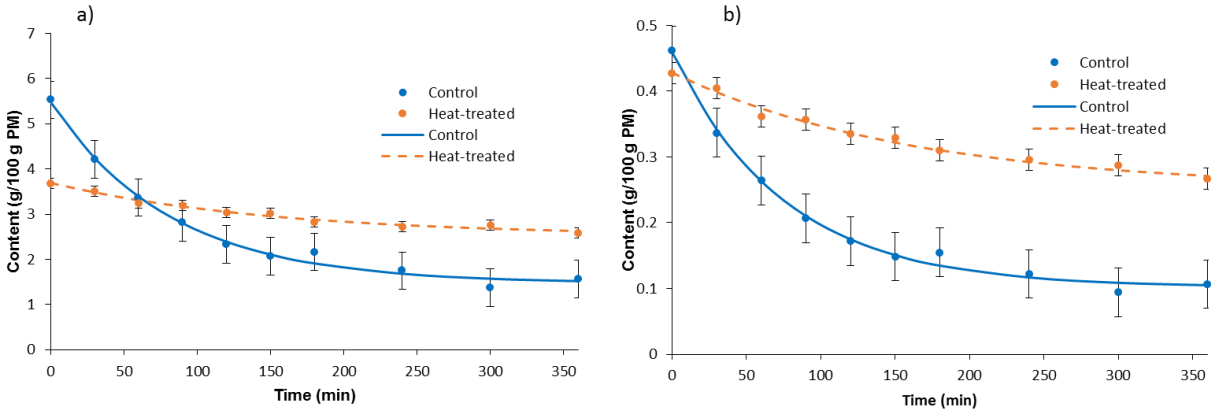

Figure 4.1 Content (mean±SD) of a) aspalathin and b) nothofagin, in heat-treated (orange) and untreated samples (blue), during simulated fermentation at 37°C. Estimated values according to the fractional

conversion model (eqn. 4.4) are indicated by smooth and dashed lines. Measured values (mean±SD) are indicated by dots.

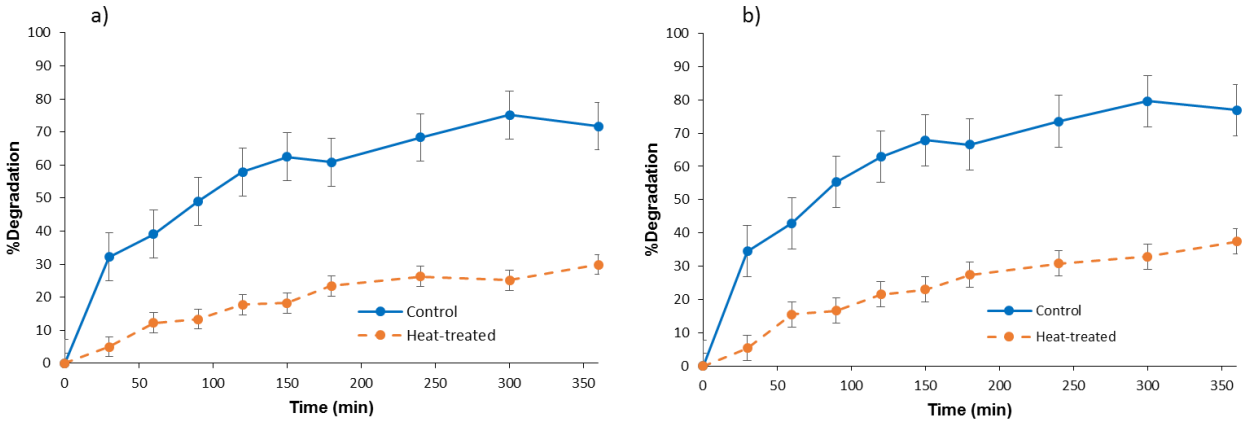

Figure 4.2 Percentage degradation (mean±SD) of a) aspalathin and b) nothofagin, in heat-treated (orange) and untreated samples (blue), during simulated fermentation at 37°C.

Table 4.4 Estimated fractional conversion kinetic parameters (equilibrium concentration, C_∞; initial concentration C_0 and reaction rate constant, k) for the degradation of aspalathin and nothofagin at 37°C in heat-treated and untreated (control) samples.

	Control	Heat-treated
Aspalathin		
C_0, g.100 g^{-1} PM	5.48±0.07$_a$	3.70±0.25$_b$
C_∞, g.100 g^{-1} PM	1.47±0.06$_b$	2.52±0.11$_a$
k, min^{-1}	0.012±0.001$_a$	0.007±0.002$_b$
Nothofagin		
C_0, g.100 g^{-1} PM	0.46±0.02$_a$	0.43±0.01$_a$
C_∞, g.100 g^{-1} PM	0.102±0.005$_b$	0.251±0.028$_a$
k, min^{-1}	0.013±0.002$_a$	0.006±0.002$_b$

Values in the same row with different letters are significantly different from one another ($p<0.05$).
PM, plant material.

4.4.3. Determination of temperature dependence of rooibos phenolic compounds during simulated fermentation

The measured mean content values of rooibos compounds in green samples (samples at time 0 min) and the percentage degradation after fermentation are shown in Table 4.5. Based on the % degradation, aspalathin, nothofagin, hyperoside, rutin and isoquercitrin showed at least 10% change for one temperature after 6 h and were selected for modelling of the oxidation kinetics of rooibos phenolics (Table 4.5). The formation of the aspalathin intermediate oxidation products, (*S*)/(*R*)-eriodictyol-6-*C*-β-D-glucopyranoside and (*S*)/(*R*)-eriodictyol-8-*C*-β-D-glucopyranoside, were modelled as well.

The degradation of the dihydrochalcones was more pronounced in comparison to the flavonols (Table 4.5) and this is in line with findings reported by Walters *et al.* (2017). For example, % degradation of

aspalathin during fermentation at 37°C is 67.6% in comparison to the 11.9% degradation of hyperoside (Table 4.5). The lower extent of degradation observed for the flavonols infer that they have a higher thermal stability than the dihydrochalcones; this can likely be attributed to the higher C-ring oxidation state of the flavonols.

Table 4.5 Composition of green rooibos including 15 rooibos compounds and *Z*-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid (PPAG) (g.100 g^{-1}; mean±SD). The percentage change in content after 6 h of fermentation are provided in brackets. Degradation or formation of compounds are indicated by negative and positive symbols, respectively.

Compounds	Content			
	37ºC	40ºC	43ºC	50ºC
PPAG	0.238±0.015 (-1.7)	0.250±0.009 (+2.8)	0.241±0.015 (-3.1)	0.245±0.010 (+1.3)
Aspalathin[#]	6.2±0.2 (-67.6)	6.4±0.2 (-63.1)	6.6±0.3 (-55.1)	6.6±0.2 (-43.1)
Nothofagin[#]	0.472±0.012 (-70.6)	0.495±0.012 (-67.3)	0.512±0.025 (-60.5)	0.512±0.002 (-43.0)
Isoorientin	0.64±0.02 (-6.7)	0.65±0.04 (-6.0)	0.53±0.04 (+0.3)	0.52±0.01 (-5.3)
Orientin	0.523±0.016 (-3.6)	0.531±0.014 (-4.9)	0.407±0.012 (+0.4)	0.418±0.009 (-2.8)
Quercetin-3-*O*-robinobioside	0.516±0.014 (-8.1)	0.544±0.018 (-8.0)	0.447±0.008 (-3.0)	0.450±0.007 (-6.8)
Vitexin	0.106±0.008 (+0.9)	0.111±0.002 (-0.5)	0.057±0.005 (-0.1)	0.069±0.002 (-3.9)
Hyperoside[#]	0.161±0.013 (-11.9)	0.175±0.004 (-13.1)	0.140±0.007 (-9.1)	0.152±0.005 (-9.5)
Rutin[#]	0.171±0.015 (-14.2)	0.187±0.004 (-11.2)	0.126±0.015 (-18.2)	0.137±0.005 (-12.2)
Isovitexin	0.122±0.004 (-3.7)	0.132±0.004 (-5.7)	0.117±0.003 (+0.5)	0.114±0.003 (-3.4)
Isoquercitrin[#]	0.155±0.001 (-11.1)	0.161±0.003 (-9.4)	0.107±0.010 (-8.8)	0.130±0.003 (-9.7)
Luteolin	nq[1]	nq[1]	nq[1]	nq[1]

[1]Compounds not quantified in green or fermented rooibos due to low signal-to-noise ratios.
[#]Compounds selected for modelling of reaction kinetics based on *ca*. 10% change in content for at least one temperature.

The zero-order, first-order, second-order and first-order fractional conversion models were fitted to quantitative data of nine selected compounds. The data were again best described by the first-order fractional conversion model based on higher R^2 values pertaining to measured and estimated content values for all compounds and temperatures (Table 4.6). Refer to Table A1 for the complete set of R^2 values calculated for all replications at 37°C. The R^2 values for the fractional conversion model based on the reactions of 9 major rooibos phenolics during fermentation at temperatures between 37 and 50°C are given in Table 4.6. Excellent R^2 values were obtained for degradation of the dihydrochalcones (aspalathin and nothofagin) and formation of the flavanones ((*S*)- and (R)-eriodictyol-6-*C*-β-D-glucopyranoside and (*S*)- and (R)-eriodictyol-8-*C*-β-D-glucopyranoside) across all investigated fermentation temperatures (R^2>0.98) (Table 4.6). Although the fractional conversion model best described the degradation of the flavonols hyperoside, rutin and isoquercitrin, R^2 values were still poor (Table 4.6). The change in content of the flavonols was much lower in comparison to the other compounds studied here. Thus, the poor R^2 values reported for these compounds at certain

temperatures (in particular hyperoside at 43°C, R^2=0.4434) could be the result of only minimal changes occurring during fermentation (Table 4.6). Due to the models being poorly fitted, kinetic parameters for flavonols will not be discussed further.

Figures 4.3 and 4.4 represent the values estimated by the fractional conversion model over the 6 h simulated fermentation at the different temperatures. Estimated kinetic parameters (*i.e.* initial (C_0) and equilibrium (C_∞) concentrations, reaction rates (k)) are given in Table 4.6.

Degradation patterns of aspalathin and nothofagin during simulated fermentation at 37, 40, 43 and 50°C were similar (Figure 4.3). At higher temperature, a lower extent of degradation (*i.e* higher equilibrium concentration) was observed for both aspalathin and nothofagin (Figure 4.3). The extent of formation was higher (*i.e.* higher equilibrium concentration) for the eriodictyol-6-*C*-β-D-glucopyranosides than that of the eriodictyol-8-*C*-β-D-glucopyranosides (Figure 4.4). A similar trend was observed by Walters *et al.* (2017). This is the result of faster formation of eriodictyol-6-*C*-β-D-glucopyranosides via oxidative cyclisation of aspalathin, in contrast to the slower formation of the eriodictyol-8-*C*-β-D-glucopyranosides via subsequent Wessely-Moser rearrangement (Marais *et al.*, 2000).

For the degradation of dihydrochalcones, an increase in temperature was accompanied by an increase in the reaction rate constant (Table 4.6). For example, the reaction rate constant of aspalathin at 50°C (0.033 min^{-1}) was significantly higher than the reaction rate constant reported for the lower temperatures (≤0.025 min^{-1}) (Table 4.6). The extent of degradation was significantly lower at the higher temperatures, this was indicated by higher equilibrium concentrations obtained at higher temperatures (Figure 4.3; Table 4.6). For example, the equilibrium concentrations for aspalathin at 43 and 50°C (2.81 and 3.71 g. 100 g^{-1} plant material, respectively) were significantly higher than the equilibrium concentrations at 37 and 40°C (≥2.09 g.100 g^{-1} plant material) (Table 4.6). The lower extent of degradation could be perceived as a higher apparent thermal stability of the dihydrochalcones at the higher temperatures.

The increase in reaction rate with an increase in temperature is typical behaviour of polyphenolics. For example, De Paepe *et al.* (2014) reported an increase in the rate constants for degradation reactions of dihydrochalcones, phloretin and hydroxyphloretin, with an increase in thermal treatment of cloudy apple juice samples. Beelders *et al.* (2015) reported on thermal degradation of benzophenones and xanthones in *Cyclopia genistoides*, and it was found that an increase in thermal treatment temperature resulted in an increase in reaction rate. Similar trends were also reported by Chaaban *et al.* (2017) for the thermal degradation of rutin, naringin, eriodictyol, mesoquitol, luteolin and luteolin-7-*O*-β-D-glucoside in model systems. However, it is peculiar that at higher temperatures the equilibrium concentrations of aspalathin and nothofagin were higher (lower extent of degradation after 6 h). This phenomenon can be explained by two postulated scenarios. The first possibility is that enzymes were partially inactivated under the simulated fermentation conditions at temperatures >43°C. The enzymatic activity of PPOs and PODs typically increase as the optimum temperature is approached, thereafter the activity decreases steadily as the inactivation temperature is approached. PPO in blueberries have exhibited activity over a wide range of temperatures (25-60°C), with an optimum activity observed at 35°C (Siddiq and Dolan, 2017). PPO activity in strawberries was higher after treatments between 40-70°C and only slight inactivation was observed between 80-100°C (Shiferaw *et al.*, 2010). Anthon and

Barrett (2002) elaborated on the subject further by explaining that enzymes retain their activity over a broad range of temperatures due to the presence of both heat stable and heat labile isoenzymes. In a case study on carrots and potatoes, thermal treatment between 76 and 85°C resulted in inactivation of heat labile POD isoenzymes and, consequently, rapid decrease (*ca.* 50%) in POD activity (Anthon and Barrett, 2002). In this context, it is reasonable to postulate that the unexpected apparent higher thermal stability of the rooibos dihydrochalcones at higher temperatures could be ascribed to the inactivation of heat labile polyphenolic oxidising isoenzymes. The second, more practical, possibility is that there was a limiting factor during the simulated fermentation experiment. Although the open air fermentation of rooibos tea was simulated by using miniscule amounts of plant material (40 mg) and water (200 µL) in a relatively large enclosed environment (24 mL glass vial with an air-tight seal), it appears that the availability of oxygen may have been a limiting factor. If this is true, at higher temperatures the limited amount of oxygen was depleted faster because of the subsequent increase in reaction rate. The cause of higher equilibrium concentrations being linked to higher fermentation temperatures is yet to be established and will require further experimentation.

The general trend of the reaction rate constants for the formation of eriodictyol-isomers was an increase with temperature. The extent of formation of the eriodictyol-glucopyranoside isomers was lower at higher temperatures (Figure4.4; Table 4.6). This corresponds well with the apparent increase in thermal stability of aspalathin as temperature increased.

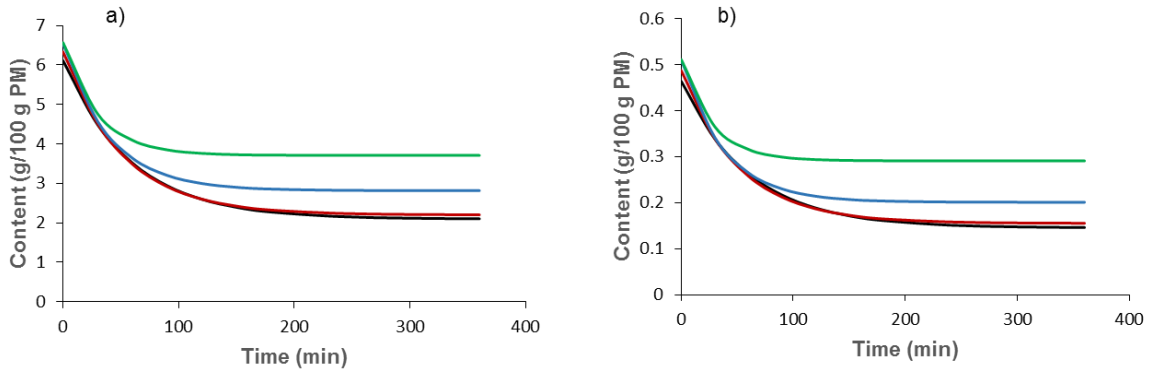

Figure 4.3 Estimated concentration (g.100 g^{-1} plant material) of a) aspalathin and b) nothofagin during simulated fermentation at 37°C (black), 40°C (red), 43°C (blue) and 50°C (green). Estimated concentrations were determined according to kinetic parameters reported in Table 4.4 using a fractional conversion model.

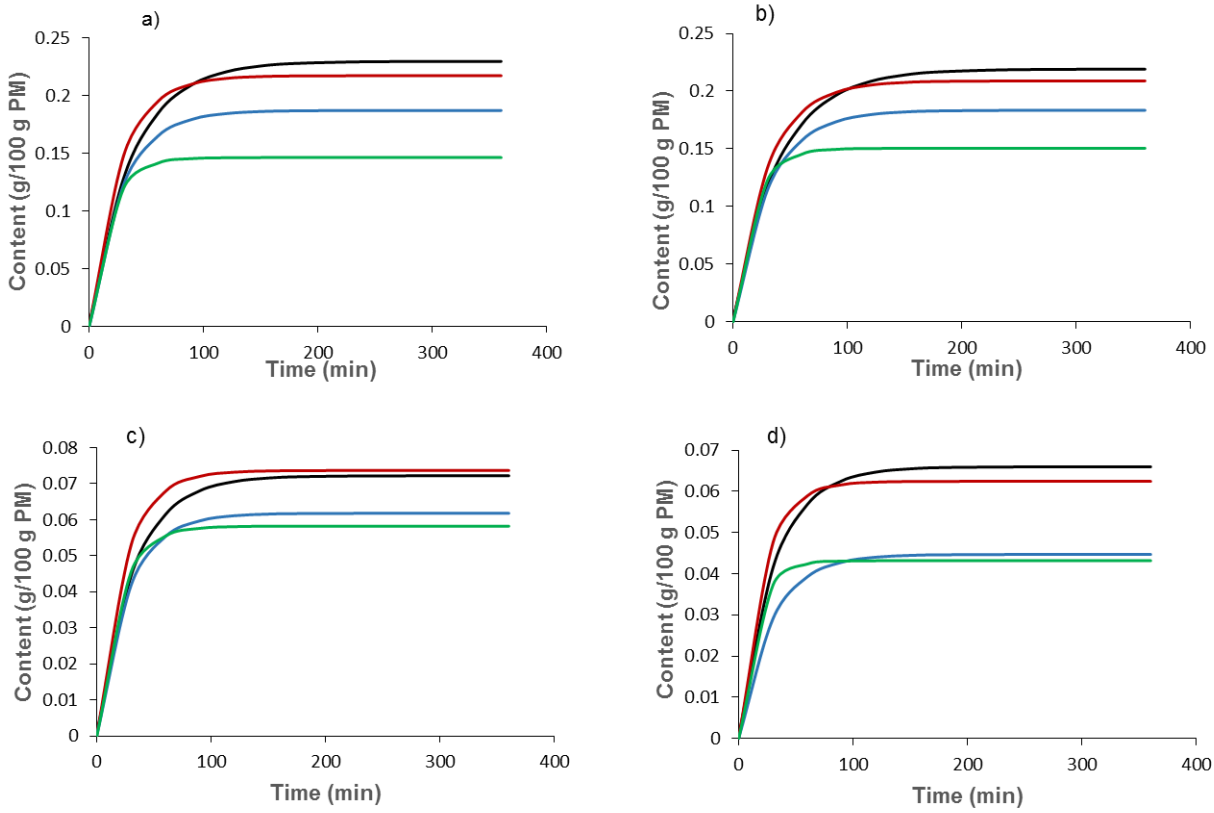

Figure 4.4 Estimated concentration (g.100 g^{-1} plant material) of intermediate products of aspalathin oxidation a) (*S*)-eriodictyol-6-*C*-β-D-glucopyranoside, b) (*R*)-eriodictyol-6-*C*-β-D-glucopyranoside, c) (*S*)-eriodictyol-8-*C*-β-D-glucopyranoside and d) (*R*)-eriodictyol-8-*C*-β-D-glucopyranoside during simulated fermentation at 37°C (black), 40°C (red), 43°C (blue) and 50°C (green). Estimated concentrations were determined according to kinetic parameters reported in Table 4.4 using a fractional conversion model.

Table 4.6 Estimated fractional conversion kinetic parameters (equilibrium concentration, C_∞; initial concentration, C_0 and reaction rate constant, k) for the degradation of selected rooibos phenolic compounds at various fermentation temperatures.

	37°C	40°C	43°C	50°C
Aspalathin				
C_0, g.100 g^{-1} PM	6.1±0.1$_b$	6.3±0.2$_{ab}$	6.5±0.3$_a$	6.6±0.1$_a$
$C\infty$, g.100 g^{-1} PM	2.09±0.06$_c$	2.20±0.13$_c$	2.81±0.18$_b$	3.71±0.21$_a$
k, min^{-1}	0.017±0.002$_c$	0.019±0.002$_c$	0.025±0.001$_b$	0.033±0.003$_a$
R^2	0.9900	0.9818	0.9885	0.9792
Nothofagin				
C_0, g.100 g^{-1} PM	0.464±0.009$_b$	0.487±0.009$_{ab}$	0.510±0.026$_a$	0.511±0.001$_a$
$C\infty$, g.100 g^{-1} PM	0.145±0.008$_c$	0.155±0.007$_c$	0.201±0.006$_b$	0.291±0.009$_a$
k, min^{-1}	0.017±0.001$_d$	0.020±0.001$_c$	0.026±0.002$_b$	0.036±0.001$_a$
R^2	0.9886	0.9812	0.9928	0.9842
(*S*)-eriodictyol-6-*C*-β-D-glucopyranoside				
C_0, g.100 g^{-1} PM	0.0017±0.0022$_a$	0.0007±0.0005$_a$	-0.0004±0.0017$_a$	0±0.0002$_a$
$C\infty$, g.100 g^{-1} PM	0.230±0.001$_a$	0.217±0.004$_a$	0.187±0.013$_b$	0.146±0.006$_c$
k, min^{-1}	0.027±0.001$_c$	0.038±0.002$_b$	0.036±0.005$_b$	0.056±0.005$_a$
R^2	0.9956	0.9911	0.9879	0.9803
(*R*)-eriodictyol-6-*C*-β-D-glucopyranoside				
C_0, g.100 g^{-1} PM	0.0027±0.0020$_a$	0.0010±0.0008$_{ab}$	0.0001±0.0013$_b$	-0.0002±0.0003$_b$
$C\infty$, g.100 g^{-1} PM	0.219±0.002$_a$	0.209±0.004$_a$	0.183±0.010$_b$	0.151±0.003$_c$
k, min^{-1}	0.025±0.002$_c$	0.034±0.002$_b$	0.032±0.005$_b$	0.056±0.005$_a$
R^2	0.9925	0.9887	0.9857	0.9858
(*S*)-eriodictyol-8-*C*-β-D-glucopyranoside				
C_0, g.100 g^{-1} PM	0.00094±0.00012$_a$	0.00046±0.00014$_b$	0.00028±0.00043$_{bc}$	0±0.00004$_c$
$C\infty$, g.100 g^{-1} PM	0.0722±0.0004$_a$	0.0737±0.0015$_a$	0.0618±0.0018$_b$	0.0582±0.0038$_b$
k, min^{-1}	0.032±0.003$_c$	0.042±0.005$_{bc}$	0.038±0.007$_{ab}$	0.051±0.004$_a$
R^2	0.9870	0.9854	0.9814	0.9941
(*R*)-eriodictyol-8-*C*-β-D-glucopyranoside				
C_0, g.100 g^{-1} PM	0.00070±0.00016$_a$	0.00021±0.00013$_b$	0.00011±0.00003$_{bc}$	0±0.00004$_c$
$C\infty$, g.100 g^{-1} PM	0.0660±0.0005$_a$	0.0624±0.0013$_b$	0.0447±0.0027$_c$	0.0432±0.0013$_c$
k, min^{-1}	0.033±0.003$_c$	0.048±0.004$_b$	0.035±0.008$_c$	0.067±0.004$_a$
R^2	0.9886	0.9887	0.9892	0.9942
Hyperoside				
C_0, g.100 g^{-1} PM	0.161±0.015$_{ab}$	0.175±0.004$_a$	0.139±0.008$_c$	0.153±0.005$_{bc}$
$C\infty$, g.100 g^{-1} PM	0.137±0.019$_a$	0.145±0.006$_a$	0.121±0.011$_a$	0.139±0.009$_a$
k, min^{-1}	0.008±0.005$_a$	0.012±0.009$_a$	0.014±0.005$_a$	0.034±0.032$_a$
R^2	0.6726	0.8113	0.4434	0.7314
Isoquercitrin				
C_0, g.100 g^{-1} PM	0.156±0.003$_a$	0.161±0.003$_a$	0.108±0.010$_c$	0.132±0.001$_b$
$C\infty$, g.100 g^{-1} PM	0.141±0.0034$_a$	0.136±0.006$_a$	0.093±0.010$_b$	0.103±0.030$_b$
k, min^{-1}	0.0184±0.0230$_a$	0.0100±0.0097$_a$	0.0133±0.0023$_a$	0.0172±0.0221$_a$
R^2	0.6269	0.7586	0.5784	0.7619
Rutin				
C_0, g.100 g^{-1} PM	0.174±0.017$_a$	0.189±0.003$_a$	0.126±0.016$_b$	0.138±0.005$_b$
$C\infty$, g.100 g^{-1} PM	0.141±0.014$_a$	0.140±0.007$_a$	0.095±0.020$_b$	0.121±0.007$_a$
k, min^{-1}	0.005±0.004$_a$	0.004±0.002$_a$	0.012±0.009$_a$	0.012±0.004$_a$
R^2	0.5200	0.7379	0.5550	0.7224

Values in the same row with different letters are significantly different from one another ($p<0.05$).
PM, plant material.

The linearity (determined by the R^2 values) of the Arrhenius plots (*ln k* vs. 1/T) in Figure 4.5 served as indication of the model's validity. R^2 values reported in Figure 4.5 were calculated based on the average *ln k* values obtained for each of the four fermentation temperatures. The Arrhenius parameters were estimated based on linear regression (eqn. 4.6) of the *k* values for each replicate experiment (Table 4.7). The R^2 values reported in Table 4.7 differ from those reported in Figure 4.5 because they were determined using the *ln k* values of all the replications performed per fermentation temperature. Activation energies for the degradation of aspalathin and nothofagin are 43.3 and 51 kJ.mol^{-1}, respectively. These values are comparable to activation energies obtained for degradation of ascorbic acid in cape gooseberry (45.4 kJ.mol^{-1}; Olivares-Tenorio *et al.* 2017), quercetin arabinoside in apple juice (57 kJ.mol^{-1}; Van Der Sluis *et al.* 2005), α-terpinolene in carrot puree (49 kJ.mol^{-1}; Kebede *et al.* 2015) and luteolin in a model solution (51.4 kJ.mol^{-1}; Chaaban *et al.*, 2017). In other instances, the activation energies observed for the degradation of epigallocatechin gallate in a model solution (78 kJ.mol^{-1}; Zimeri and Tong, 1999), procyanidins in cloudy apple juice (>93.3 kJ.mol^{-1}), naringin (100.6 kJ.mol^{-1}), rutin (107.3 kJ.mol^{-1}) and luteolin-7-*O*-β-D-glucoside (120 kJ.mol^{-1}) in model solutions (Chaaban *et al.*, 2017) were relatively higher than activation energies reported in this study. Van Boekel (2008) describes activation energy as the energy barrier that molecules need to cross in order to react. The moderate activation energies paired with the relatively high pre-exponential factors (aspalathin=3.33×10^5 min^{-1}, nothofagin=6.46×10^6 min^{-1}) are an indication that the degradation of the dihydrochalcones is dependent on temperature, meaning that the reactions will run very slowly at low temperatures but relatively fast at high temperatures (Van Boekel, 2008). The activation energy for the formation of (*S*)-eriodictyol-8-*C*-β-D-glucopyranoside (27.05 kJ.mol^{-1}) is approximately half the magnitude of activation energy required for the formation of the rest of the eriodictyol-isomers (≥40 kJ.mol^{-1}) (Table 4.7). The pre-exponential factor for (*S*)-eriodictyol-8-*C*-β-D-glucopyranoside (1.23×10^3 min^{-1}) is also lower than those of the rest of the eriodictyol-isomers (<2×10^3 min^{-1}). This observation is yet to be explained. The fact that the degradation of the selected compounds obeys the Arrhenius law does not contradict the possibility that enzymes participate in rooibos fermentation. Fortea *et al.* (2009) and Anthon and Barrett (2002) showed that polyphenol oxidising enzymes in table grapes and carrots and potatoes, respectively, obey the Arrhenius equation up until the inactivation temperature is reached.

The Q_{10} value is an alternate way of expressing temperature dependence of a reaction; it can be defined as the ratio of reaction rate constants at temperatures differing by 10°C. Based on the Q_{10} values for aspalathin and nothofagin, there was a 1.72 and 1.85 fold increase in degradation rate with an increase in temperature from 40 to 50°C, respectively (Table 4.8). The formation of the eriodictyol-isomers showed 1.21-1.66 fold increase with an increase in temperature from 40-50°C (Table 4.8). Q_{10} values can be used to estimate the expected increase in reaction rate for temperature increases of 10°C increments.

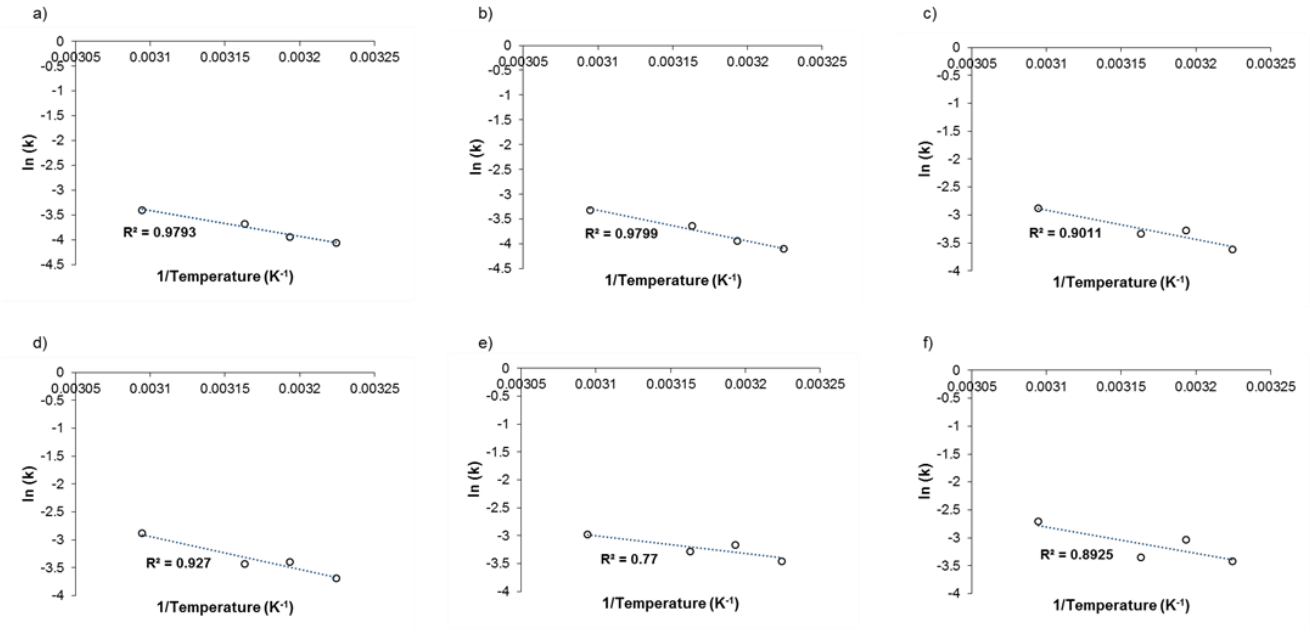

Figure 4.5 Arrhenius plots (linearised form of Arrhenius equation, eqn. (4.6)) using average reaction rate constants of selected rooibos phenolic compounds fermented at four different temperatures (*i.e.* 37, 40, 43 and 50°C). a) Aspalathin, b) nothofagin, c) (*S*)-eriodictyol-6-*C*-β-D-glucopyranoside, d) (*R*)-eriodictyol-6-*C*-β-D-glucopyranoside, e) (*S*)-eriodictyol-8-*C*-β-D-glucopyranoside and f) (*R*)-eriodictyol-8-*C*-β-D-glucopyranoside.

Table 4.7 Estimated values for the Arrhenius parameters (activation energy, E_a; pre-exponential factor, A; and coefficient of determination R^2) for the degradation of the dihydrochalcones, aspalathin and nothofagin, and the formation of the eriodictyol-isomers. Regression were performed for all replicate reaction rate constant values at each temperature. The values for the 95% confidence intervals are provided in brackets.

Parameter	Aspalathin	Nothofagin	(*S*)-eriodictyol-6-*C*-*β*-D-glucopyranoside	(*R*)-eriodictyol-6-*C*-*β*-D-glucopyranoside	(*S*)-eriodictyol-8-*C*-*β*-D-glucopyranoside	(*R*)-eriodictyol-8-*C*-*β*-D-glucopyranoside
Linear regression (eqn. 4.6)						
ln*A*	12.72 (8.44-17.00)	15.68 (12.43-18.94)	13.34 (8.05-18.63)	15.31 (11.09-19.54)	7.11 (1.51-12.72)	12.28 (6.06-18.50)
E*$_a$*/R	5205 (3855-6556)	6131 (5104-7158)	5241 (3573-6909)	5883 (4551-7215)	3255 (1488-5023)	4848 (2886-6811)
R^2	0.8941	0.953	0.8488	0.9173	0.6586	0.7763
with computed values for:						
***E*$_a$, kJ.mol^{-1}**	43.26	50.95	43.55	48.89	27.05	40.29
***A*, min^{-1}**	3.33×10^5	6.46×10^6	6.22×10^5	4.48×10^6	1.23×10^3	2.15×10^5

Table 4.8 Temperature coefficient values (Q_{10}) determined for dihydrochalcones, aspalathin and nothofagin and intermediate aspalathin oxidation products, eriodictyol-6-*C*-β-D-glucopyranosides and eriodictyol-8-*C*-β-D-glucopyranosides.

Compound	Q_{10} value
Aspalathin	1.72
Nothofagin	1.85
(*S*)-eriodictyol-6-*C*-β-D-glucopyranoside	1.49
(*R*)-eriodictyol-6-*C*-β-D-glucopyranoside	1.66
(*S*)-eriodictyol-8-*C*-β-D-glucopyranoside	1.21
(*R*)-eriodictyol-8-*C*-β-D-glucopyranoside	1.39

4.5. Conclusion

Results from this work support the postulation that endogenous enzymes in rooibos participate in the oxidation of polyphenolic compounds during fermentation. Rooibos is prone to oxidation when exposed to elevated temperatures and in the presence of moisture, and these conditions should be avoided to prevent browning and the loss of aspalathin during the manufacturing, storage and transportation of green rooibos. As temperature increases, the rate of oxidation will increase, although the eventual extent of degradation may decrease. The role of endogenous enzymes in rooibos was postulated based on the reaction kinetics of major rooibos phenolics and thus, further research is required to elucidate the properties of these enzymes. The kinetic parameters determined from the Arrhenius equation along with the Q_{10} values can be used as rough guidelines to estimate the expected degradation/formation of major rooibos phenolic compounds during processing of rooibos products.

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Addendum

Table A1 The coefficient of determination (R^2) between average and estimated contents of 9 rooibos phenolic compounds using empirical modelling with zero-order, first-order, second-order and fractional conversion models.

Compounds	Zero-order	First-order	Second-order	Fractional conversion
Aspalathin	0.6580	0.8257	0.9446	0.9740
	0.6579	0.7538	0.8123	0.9924
	0.6335	0.7627	0.8577	0.9833
Nothofagin	0.6803	0.8627	0.9633	0.9657
	0.6517	0.7585	0.8176	0.9942
	0.6503	0.7772	0.8656	0.9809
(*S*)-eriodictyol-6-*C*-β-D-glucopyranoside	0.4608	0.4469	0.4327	0.9904
	0.4690	0.4527	0.4365	0.9920
	0.5054	0.4846	0.4640	0.9833
(*S*)-eriodictyol-6-*C*-β-D-glucopyranoside	0.4800	0.4658	0.4514	0.9900
	0.4984	0.4798	0.4614	0.9890
	0.5407	0.5194	0.4983	0.9815
(*S*)-eriodictyol-6-*C*-β-D-glucopyranoside	0.4469	0.4417	0.4365	0.9870
	0.4746	0.4670	0.4595	0.9793
	0.4908	0.4830	0.4753	0.9769
(*S*)-eriodictyol-6-*C*-β-D-glucopyranoside	0.4003	0.3965	0.3926	0.9870
	0.4264	0.4211	0.4159	0.9827
	0.5062	0.4997	0.4932	0.9855
Hyperoside	0.6564	0.6582	0.6582	0.6726
	0.3973	0.3818	0.3660	0.4800
	0.6675	0.6760	0.6834	0.9120
Isoquercitrin	0.3263	0.3170	0.3072	0.5272
	0.2270	0.2198	0.2123	0.2917
	0.1988	0.1859	0.1733	0.3409
Rutin	0.7484	0.7501	0.7515	0.7719
	0.5927	0.5828	0.5714	0.6095
	0.5570	0.5501	0.5427	0.6942

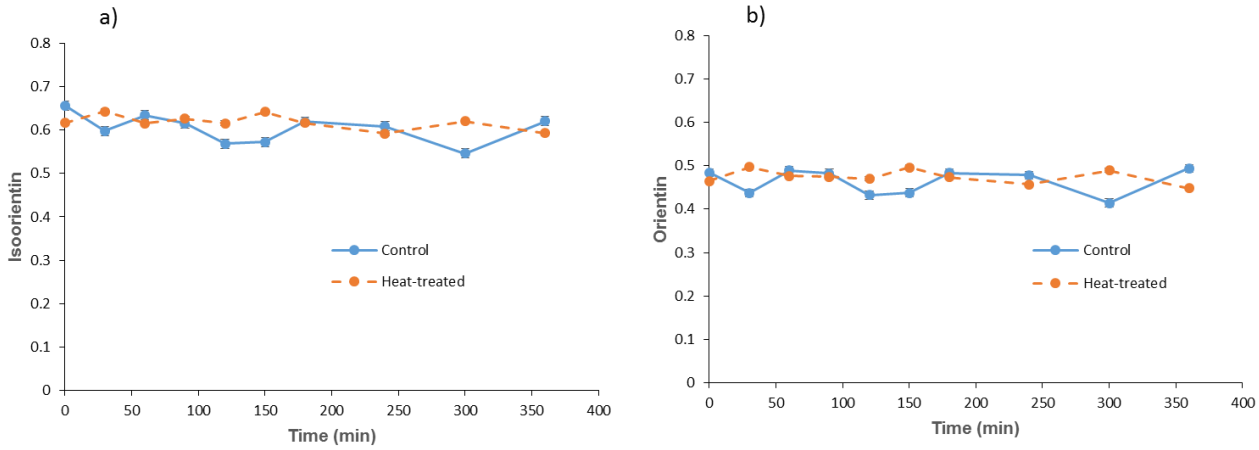

Figure A1 Content (mean±SD) of a) isoorientin and b) orientin, in heat-treated (orange) and untreated samples (blue), during simulated fermentation at 37°C.

CHAPTER 5

General discussion and conclusions

Rooibos 'fermentation' is an oxidation step essential to the development of the characteristic colour, flavour and taste of fermented rooibos (Joubert and De Beer, 2014). Batches of plant material are cut, bruised, hydrated, placed into heaps and allowed to ferment in the open air at uncontrolled temperatures, usually for 12-14 h, before being spread out and allowed to sun dry (Joubert and De Beer, 2011). Although characteristic fermented rooibos attributes such as the honey-like aroma, sweet taste and red-brown leaf colour are developed during fermentation, the reactions responsible for these changes are detrimental to the quality of green rooibos (Joubert and De Beer, 2014). Characteristic features of green rooibos such as the green leaf colour and the relatively high phenolic content are not only vulnerable during fermentation conditions, but, also under storage and transportation conditions where slight increases in temperature and/or humidity may occur.

Studies on the phenolic changes occurring during fermentation have been geared toward targeted analysis of select, quantifiable rooibos phenolic compounds (Joubert, 1996; Bramati *et al.*, 2002, 2003; Krafczyk *et al.*, 2009; Heinrich *et al.*, 2012; Walters *et al.*, 2017a). Thus, the aim of the current study was to investigate the phenolic changes occurring during oxidation by means of an untargeted approach, and to establish reaction kinetic parameters of selected phenolic compounds under similar conditions.

The first objective was to uncover phenolic changes between green, semi-fermented and fermented rooibos using chromatographic fingerprinting as a tool for untargeted chemometric analysis. Plant material preparation, extraction and high performance liquid chromatography-diode array detector (HPLC-DAD) analysis was performed as described by Walters *et al.* (2017). Plant material was divided into three sub-samples to produce green, semi-fermented and fermented rooibos. Green rooibos was prepared by drying shoots (without delay) at 40°C for 12 h in a cross-flow drying tunnel. Semi-fermented rooibos was prepared by shredding the plant material and drying it 1 h after shredding. Fermented rooibos was prepared by shredding the plant material, hydrating it with water, incubating at 38°C for 12 h followed by drying. The phenolic fraction was extracted using a 40% aqueous-acetonitrile solvent, a heat treatment at 100°C for 20 min and a 5 min sonication period. Fingerprints were constructed using the HPLC-DAD method reported by Walters *et al.* (2017) and were exported manually from the ChemStation software, transformed into comma delimited files before being imported into MATLAB. This process was tedious and repetitive as it had to be repeated for each fingerprint. Similar criticisms were raised by Schulze (2013), and in agreement with the recommendations suggested by the author, exportation should be automated or made simpler by means of a macro.

Pre-processing was performed prior to analysis of variance to remove any unwanted variation, *i.e.* variation unrelated to the treatment. The appropriate pre-processing techniques were applied to correct specified artefacts that were either related to the data or to the analytical instrument (Tistaert *et al.*, 2011). Peak alignment corrected retention time shifts (Jellema, 2009), signal enhancement eliminated the baseline and poor signal-to-noise ratios (Reis *et al.*, 2009), peak detection was performed to avoid working with exceptionally large data sets, normalisation was applied to remove the 'size effect' (differences in the overall concentrations of samples) (Bylesjo *et al.*, 2009) and centering and transformation removed unwanted variation between compounds within a sample (Van den Berg *et al.*, 2006). Elimination of the 'size effect' was investigated by two normalisation methods namely, probabilistic quotient normalisation (PQN) and the

pair-wise log ratio (PLR) approach. To determine which normalisation method was most suitable for the removal of the 'size effect', analysis of variance was performed using ANOVA-target projection. The underlying assumption of PQN, that the majority of the sample features should not differ, was not met. PQN typically works well for metabolomics or proteomics data, where the ratio of features that are changing to features that are not changing during treatment is small (Dieterle *et al.*, 2006; Bylesjo *et al.*, 2009). The final results of PLR was not expressed in terms of the original variables, but, instead in terms of the log ratios, which made it difficult to interpret the effect of the treatment on the detected peaks. Although PLR was used in a study on discrimination and biomarker discovery in arbitrary gas chromatography data attained from vegetable oil and meat samples, there is no mention of how compounds were discriminated based on the log ratios (Lehallier *et al.*, 2012). Due to PQN being incompatible with the data and the difficulty with interpretation of the final PLR results, a new approach called robust-PLR, proposed by Walach *et al.* (2017), was used to remove the 'size effect'. This method allowed for easier identification of compounds changing as a result of fermentation, and showed that all 56 detected peaks were significantly ($p=0.01$) different between the three treatments.

Considering that 56 compounds is an excessive number of markers for a single product, this number was reduced using a 30% change in peak area as a threshold. After implementing this threshold, the number of possible phenolic markers was reduced to 34 compounds. This is still a relatively high number of potential markers, but, it indicates that the number of phenolics examined in targeted analysis of rooibos fermentation is small in comparison to the number of compounds significantly changing.

It is important to note that a particular peak can be small and still show a 30% change in peak area as a result of fermentation (*e.g.* peak nr. 50, 55 and 56). This is relevant when considering quantitative analysis of these 'markers' as the likelihood of accurately quantifying compounds occurring in such small amounts is slim. With that being said, it should be highlighted again that all limitations of the chemometric methods should be considered in order to draw realistic and credible conclusions.

The second objective was to use kinetic modelling to broaden the understanding of rooibos phenolic reaction kinetics during elevated temperature and humid conditions (simulated fermentation). A temperature dependence experiment entailed monitoring the change in content of selected rooibos phenolics over time during a simulated fermentation at four temperatures between 37 and 50°C. Simulated fermentation was performed by incubating a small amount of hydrated, finely milled green rooibos plant material in a closed environment, while controlling the temperature. Preceding the temperature dependence experiment, two preliminary experiments were performed, namely an extraction protocol optimisation and an enzyme inactivation trial.

Plant material was prepared by finely milling shoots (leaves and stems) followed by sieving (fraction between 210 and 500 µm used) to improve homogeneity for mini-scale extraction and simulated fermentation. A standardised 40% aqueous acetonitrile was used as a solvent during the extraction protocol optimistion based on the recommendation by Walters *et al.* (2017).

For the extraction optimisation experiment, six treatments were investigated, including a control which entailed heating at 100°C for 20 min followed by a 5 min sonication period. Treatments 1-5 compared the

effects of different periods of sonication (*i.e.* 5, 10, 15, 20 and 30 min) without prior heating. None of the treatments differed significantly ($p<0.05$) from one another, indicating that no heat is required for extraction of the phenolic fraction of rooibos. As a result of the small particles attained after milling and sieving the plant material, sonication only needed to be applied for a short period (5 min). However, this may not hold true for samples with a larger particle size.

Based on findings in literature on black tea, it is understood that 'fermentation' is guided by two phases including enzymatic and chemical oxidation phases (Subramanian *et al.*, 1999; Stodt *et al.*, 2014). Since there is no previous evidence for the role of rooibos endogenous enzymes in 'fermentation', this theory was tested by applying a dry heat treatment at 170°C for 30 min to green rooibos plant material in an attempt to inactivate endogenous rooibos enzymes. Heat-treated and untreated (control) samples were compared based on their change in content (g.100 g^{-1} plant material) of aspalathin and nothofagin (major rooibos dihydrochalcones) during simulated fermentation. Polyphenol oxidising enzymes, such as polyphenol oxidase (PPO) and peroxidase (POD), are key elements in black tea fermentation and they are responsible for converting polyphenolic compounds to highly reactive *o*-quinones that partake in chemical oxidation (Subramanian *et al.*, 1999). Therefore, it was postulated that inactivation of the polyphenol oxidising enzymes would be detectable by significantly less degradation of aspalathin and nothofagin in the heat-treated samples in comparison to the control samples.

To determine the reaction rate constants for aspalathin and nothofagin degradation in heat-treated and untreated samples, their change in content over time was modelled according to four empirical reaction kinetics models, namely the zero-order, first-order, second-order and fractional conversion models. Measured data and estimated data (modelled data) were used to calculate the R^2 values and the best model was selected based on the highest R^2 value. Furthermore, comparisons were made between the effect of the dry heat treatment (170°C for 30 min) and the 'wet' heat treatment (simulated fermentation, wetted plant material incubated at 37°C for 6 h) based on the change in content of dihydrochalcones before and after the treatments.

The fractional conversion model best described the degradation observed during simulated fermentation since it allows for non-zero equilibrium concentrations. The fractional conversion model was used to estimate reaction rate constants (*k*'s) for aspalathin and nothofagin in control and heat-treated samples. Enzymes act by lowering the activation energy (energy barrier that molecules need to cross in order to react) for the reaction and reactions are, subsequently, faster if they are catalysed by enzymes (Van Boekel, 2008). To this effect, differences in the reaction rate constants were also used to portray enzymatic activity during rooibos fermentation.

The endogenous rooibos enzymes appeared to be at least partially inactivated after the dry heat treatment, as the extent of degradation after 6 h and the rate of the degradation of aspalathin and nothofagin in the heat-treated samples were significantly ($p<0.05$) lower than those of the untreated samples. The initial aspalathin and nothofagin contents between the heat-treated and control samples were compared to determine the effect of the dry heat treatment. The aspalathin content in the heat-treated samples was significantly ($p<0.05$) lower than that of the control samples, while the initial nothofagin contents showed no significant differences. Based on this observation it was concluded that nothofagin was more thermally stable than

aspalathin. Since nothofagin lacks a catechol group, which is present in aspalathin, it would be expected to be more stable under chemical oxidation conditions as experienced with the dry heat treatment.

It was, however, observed that aspalathin and nothofagin exhibited similar behaviour during the simulated fermentation (low temperature under moist conditions), which is evident from the similarity of their reaction rate constants and extent of degradation. The differences in behaviour of aspalathin and nothofagin during the dry and 'wet' heat treatment can be explained by the dependence of enzymatic reactions on water and the mechanism of polyphenolic oxidation via PPO. Water is an essential component of enzymatic reactions as it serves as a medium and it also facilitates the transportation of the substrates and reactants (Acker, 1969; Rezaei *et al.*, 2007). With respect to the mechanism of oxidation via PPO, monophenols (such as nothofagin) are converted into diphenols (such as aspalathin) before conversion into highly reactive *o*-quinones (McEvily *et al.*, 1992; Yoruk and Marshall, 2003). Therefore, it is reasonable to deduce that under conditions favouring PPO activity, nothofagin would be equally susceptible to degradation and that it would also follow the same reaction kinetics as aspalathin. This also means that nothofagin does not have its own enzymatic degradation pathway, as it is converted to aspalathin and follows degradation according to aspalathin oxidation, also yielding aspalathin oxidation products. If this is true, it would explain why even for a comprehensive 2D chromatographic analysis, no naringenin derivatives (nothofagin flavanone analogs), similar to the eriodictyol derivatives (aspalathin flavanone analogs), could be identified in rooibos (Walters *et al.*, 2017b).

In order to determine the temperature dependence of the reactions, green, untreated plant material was fermented at 37, 40, 43 and 50°C using the simulated fermentation procedure. Compounds were not modelled if they did not show a 10% change in content after 6 h of fermentation for at least one temperature. Of the 16 quantifiable compounds aspalathin, nothofagin, hyperoside, rutin, isoquercitrin and the aspalathin oxidation intermediate products ((*S*)/(*R*)-eriodictyol-6-C-β-D-glucopyranoside and (*S*)/(*R*)-eriodictyol-8-C-β-D-glucopyranoside) were selected for kinetic modelling using this criterion. In a similar manner to the enzyme inactivation trial, the degradation/formation of these compounds during simulated fermentation were modelled according to the four above-mentioned empirical reaction kinetics models. Based on the highest R^2 value, all reactions were best described by the fractional conversion model.

As expected, the reaction rate constants for all the reactions increased with an increase in temperature, however, it was peculiar to find that there was a lower extent of degradation (*i.e.* higher equilibrium concentration) at higher temperatures. Two possible explanations are offered, the first being that partial inactivation of polyphenolic oxidising enzymes was achieved at the higher simulated fermentation temperatures or the second being, that there was a limiting factor for the oxidation reaction. Simulated fermentation had to mimic the open-air rooibos fermentation process, while also strictly controlling the experimental conditions to reduce variation that may influence the reaction kinetic parameters. With this in mind, conditions for the simulated fermentation were chosen accordingly and a miniscule amount of moistened plant material (40 mg of plant material in 200 µL of water) was incubated in a relatively large vial (24 mL glass vials with screw caps). The vials were closed to prevent drying of the plant material and to minimise

heat loss and, although care was taken to ensure that there was a relatively a large head space, it is a possibility that oxygen may have been depleted, subsequently limiting the reaction.

The rate constant of the reactions increased with an increase in temperature, following the Arrhenius law. The activation energies and the pre-exponential factors for aspalathin and nothofagin were calculated based on the linearised form of the Arrhenius equation. The moderate activation energies (≥43 $kJ.mol^{-1}$) paired with the relatively high pre-exponential factors (≥3.33×10^5 min^{-1}) of aspalathin and nothofagin, indicate that their degradation is temperature dependent and will run relatively fast at high temperature and slower at lower temperature (Van Boekel, 2008). This is evident by the significant differences between the reaction rate constant at 37°C and 50°C for both aspalathin and nothofagin.

Plant material used in the small-scale fermentation in Chapter 3 was composed of 10 different fresh plant material batches and fermentation was performed directly on the plant material on a relatively large scale (>1 kg), in a forced-air oven, which limits the amount of control that can be exerted on conditions such as the temperature. This is in contrast to the simulated fermentation used in Chapter 4, where much smaller quantities of previously dried, milled and sieved plant material (<100 mg) from a single production batch were fermented under highly controlled conditions allowing for stricter temperature control. The extract preparation did not significantly differ (see above) and HPLC-DAD analysis methods used in Chapter 3 and 4 were identical, so in theory the percentage change in peak area after fermentation (Chapter 3) should loosely correspond to the percentage change in content after fermentation (Chapter 4). This comparison will give a general idea of the capabilities or limitations of the simulated fermentation method. Aspalathin will be used as an example since it is the most abundant phenolic compound in green rooibos.

The percentage change in peak area of aspalathin in samples fermented at 38°C for 12 h is ~85% in comparison to the 65% change in content observed for aspalathin during simulated fermentation at 37 and 40°C after 6 h (change in content calculated from the initial and equilibrium concentrations). These values can be directly compared if it is assumed that the larger scale fermentation had reached the equilibrium concentration after 12 h. The extent of degradation (*i.e.* change in peak area or content) at a higher temperature (40°C) during the simulated fermentation was much lower than the extent of degradation observed at a lower temperature (38°C) during fermentation of fresh plant material. Additionally, the equilibrium concentration of aspalathin was reached after only ~180 min of simulated fermentation at both 37 and 40°C. These observations reinforce the assumption that there might be a limiting factor to the oxidation reaction.

All things considered, new aspects of rooibos fermentation that had not been previously explored were covered in this study. Although the final number of identified marker compounds was large, this indicated that the amount of compounds targeted in studies of rooibos fermentation is small in comparison to the number of compounds showing changes. The use of advanced chemometric methods was fundamental to the untargeted analysis of rooibos phenolic compounds during fermentation, however, to ensure credible and realistic conclusions are drawn when applying these methods *a priori* information about the data is required. The importance of rapid drying directly after cutting in green rooibos preparation is demonstrated by the significant differences observed in the phenolic content between green and semi-fermented rooibos (rooibos that has been cut and dried one hour after treatment). Kinetic reaction parameters attained from rooibos

phenolic compounds exposed to elevated temperatures and humidity conditions can be used to construct rough guidelines for preventing loss of phenolics and browning of plant material during processing of green rooibos.

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