Cyclopia maculata – Source of Flavanone Glycosides as Precursors of Taste-Modulating Aglycones

by

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Declaration

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Abstract

The flavanone aglycones, hesperetin and eriodictyol, have been identified as potential taste modulators with reported sweetness-enhancing and bitterness-masking properties, respectively. Reduction of the sugar content of food products has become important in view of the global obesity epidemic. Taste modulators have shown potential to enhance the sweet taste of reduced-sugar foods without unfavourably affecting their flavour profile. On the other hand, bitterness-masking taste modulators are useful to mask the bitter taste of functional phytochemical ingredients. In the current study, *Cyclopia maculata* (honeybush) was investigated as potential source of hesperetin- and eriodictyol-enriched extracts. Hesperetin and eriodictyol were present mainly below the quantification limit in *C. maculata* plant material, including unfermented leaf and stem material, unfermented and fermented tea, as well as the fermented by-product (< 40 mesh and > 12 mesh). Conversely, their rutinoside and modulating-inactive derivatives, hesperidin and eriocitrin were present at substantially higher concentrations in the plant material. The stems and by-product were shown to be good sources of hesperidin, but not eriocitrin. The qualitative and quantitative phenolic profile of the by-product was similar to that of the stems. The tea processing by-product was therefore selected to optimise extraction of flavanone glycosides for subsequent de-glycosylation of the flavanone glycosides to aglycones.

The by-product was subjected to ultrasound-assisted extraction to investigate its potential as renewable source of the flavanone glycosides. Response surface methodology (RSM) was employed to optimise and study the individual and interactive effects of the process variables, namely ethanol concentration (% v/v), time (min), temperature (°C), and solvent:solid ratio (mL/g), on flavanone glycoside extraction. The hesperidin yield and content (of extract), as well as extract yield, increased with an increase in extraction time, temperature and solvent:solid ratio. Practical process restrictions limited global optimisation and only an optimum of 52.8% (v/v) ethanol for extract and hesperidin yield could be reached. Temperature was the parameter with the most significant effect ($p < 0.05$) on extraction efficiency among those studied. Practical process parameter values that were feasible for industrial application (52.8% (v/v) ethanol, 20 mL/g solvent:solid ratio, 60°C and 30 min) were selected for the preparation of a flavanone glycoside-enriched extract from the tea processing by-product.

The flavanone glycoside-enriched extract was subjected to acid-catalysed hydrolysis to de-glycosylate hesperidin and eriocitrin to hesperetin and eriodictyol, respectively. RSM was employed to optimise the acid hydrolysis process and to study the effect of the hydrolysis parameters (temperature (°C) and time (min)) on hydrolysis efficiency. At the maximum temperature (92.1°C) and corresponding optimum time (98.4 min) ca 80% conversion of hesperidin to hesperetin was achieved. Substantially more eriodictyol formed during acid hydrolysis than eriocitrin present in the initial extract owing to the de-glycosylation of unidentified glycosides with the same aglycone. Unidentified breakdown products imparting a red colour to the acid-hydrolysed extract were also observed. The total phenolic content of

the acid-hydrolysed extract was significantly higher ($p < 0.05$) than that of the unhydrolysed extract, indicating the formation of unidentified compounds with the ability to reduce the Folin-Ciocalteau reagent, although no significant difference ($p \geq 0.05$) between the antioxidant activities of these extracts, as assessed with the DPPH radical scavenging and ORAC assays, was observed. The potential of enzymatic bioconversion as an alternative to acid-catalysed hydrolysis was investigated using commercial hesperidinase. Bioconversion resulted only in de-rhamnosylation with ca 100% conversion of hesperidin to hesperetin-7-*O*-glucoside in an aqueous *C. maculata* extract at pH 4.0 and 40°C.

Uittreksel

Die flavanoon aglikone, hesperetien and eriodiktiol, is geïdentifiseer as potensiële smaakmoduleerders met berigte soetheid-versterkende en bitter-maskerende eienskappe, onderskeidelik. Vermindering van die suikerinhoud van voedselprodukte het belangrik geword in die lig van die wêreldwye vetsug-epidemie. Smaakmoduleerders het die potensiaal getoon om die soet smaak van voedsel met verlaagde suikerinhoud te versterk sonder om hul geurprofiel ongunstig te beïnvloed. Andersyds is bitter-maskerende smaakmoduleerders nuttig om die bitter smaak van funksionele fitochemiese bestanddele te maskeer. In die huidige studie is *Cyclopia maculata* (heuningbos) ondersoek as 'n potensiële bron van hesperetien- and eriodiktiol-verrykte ekstrakte. Hesperetien and eriodiktiol was hoofsaaklik teenwoordig onder die kwantifiseringsperk in *C. maculata* plantmateriaal, insluitend ongefermenteerde blaar- en stokmateriaal, ongefermenteerde en gefermenteerde tee, asook die gefermenteerde byproduk (< 40 maas en > 12 maas). Hierteenoor was hul rutinosiedes en modulerend-onaktiewe derivate, hesperidien and eriositrien, teenwoordig in aansienlik hoër konsentrasies in die plantmateriaal. Die stokmateriaal en byproduk is getoon om goeie bronne van hesperidien, maar nie eriositrien nie, te wees. Die kwalitatiewe en kwantitatiewe fenoliese profiel van die byproduk was soortgelyk aan dié van die stokke. Die tee-prosesseringsbyproduk is dus geselekteer om die ekstraksie van flavanoonglikosiede, voorafgaande hul de-glikosilering na aglikone, te optimeer.

Die byproduk is aan ekstraksie met behulp van ultrasoniese klank onderwerp om die potensiaal daarvan as hernubare bron van flavanoonglikosiede te ondersoek. Respons-oppervlak Metodologie (ROM) is gebruik om die individuele en wisselwerking effekte van die proses veranderlikes, naamlik etanolkonsentrasie (% v/v), tyd (min), temperatuur (°C), en oplosmiddel:vastestof verhouding (mL/g), op flavanoonglikosied ekstraksie te optimiseer en te bestudeer. Die hesperidienopbrengs en -inhoud (van ekstrak), sowel as die ekstrakopbrengs, het toegeneem met 'n toename in die ekstraksietyd, -temperatuur en oplosmiddel:vastestof verhouding. Praktiese prosesbeperkings het die globale optimisering beperk en slegs 'n optimum van 52.8% (v/v) etanol vir ekstrak- en hesperidienopbrengs kon bereik word. Temperatuur was die parameter met die mees beduidende effek ($p < 0.05$) op ekstraksie doeltreffendheid van dié wat bestudeer is. Praktiese prosesparameterwaardes wat haalbaar is vir industriële toepassing (52.8% (v/v) etanol, 20 mL/g oplosmiddel:vastestof verhouding, 60°C en 30 min) is geselekteer vir die voorbereiding van 'n flavanoonglikosied-verrykte ekstrak uit die tee-prosesseringsbyproduk.

Die flavanoonglikosied-verrykte ekstrak is aan suur-gekataliseerde hidrolise onderwerp om hesperidien en eriositrien na hesperetien en eriodiktiol, onderskeidelik, te de-glikosileer. ROM is gebruik om die suurhidrolise proses te optimeer en die effek van die hidrolise parameters (temperatuur (°C) en tyd (min)) op hidrolise doeltreffendheid te bestudeer. Ongeveer 80% omskakeling van hesperidien na hesperetien is behaal teen die maksimum temperatuur (92.1 °C) en ooreenstemmende optimum tyd

(98.4 min). Aansienlik meer eriodiktiol is tydens suurhidrolise gevorm as eriositrien wat in die oorspronklike ekstrak teenwoordig was, as gevolg van de-glikosilering van ongeïdentifiseerde glikosiede met dieselfde aglikoon. Ongeïdentifiseerde afbreekprodukte, wat 'n rooi kleur aan die suur-gehidroliseerde ekstrak gegee het, is ook waargeneem. Die totale fenoliese inhoud van die suur-gehidroliseerde ekstrak was beduidend hoër ($p < 0.05$) as dié van die ongehidroliseerde ekstrak, wat die vorming van onbekende verbindings met die vermoeë om die Folin-Ciocalteau reagens te reduseer aandui, hoewel daar geen beduidende verskil ($p \geq 0.05$) tussen die antioksidant-aktiwiteite van hierdie ekstrakte, soos bepaal met die DPPH radikaal blussings- en ORAC toetse, waargeneem is nie. Die potensiaal van ensiematiese bio-omskakeling as 'n alternatief vir suur-gekataliseerde hidrolise is ondersoek met behulp van kommersiële hesperidinase. Bio-omskakeling het slegs tot de-ramnosilering gelei met ca 100% omskakeling van hesperidien na hesperetien-7-*O*-glukosied in 'n *C. maculata* waterekstrak by pH 4.0 en 40°C.

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''It always seems impossible until it's done.'' – Nelson Mandela

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 with a chapter for elaborating a general discussion and conclusion. 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.

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CHAPTER 1

General Introduction

Honeybush, a traditional South African herbal tea, prepared from several *Cyclopia* spp., has grown in popularity during the past two decades. Demand for the product led to commercialisation of several *Cyclopia* species. One species of interest, currently under commercial evaluation, is *Cyclopia maculata*, a vigorous grower. This species has thin, needle-like leaves and thick stems. In nature the bush can grow up to 3 m tall (Joubert *et al.*, 2011).

Honeybush tea manufacture is accompanied by the generation of waste, comprising largely of stem material that does not meet the particle size requirements for retail products, i.e. loose tea and tea bags. The tea processing waste, which can constitute as much as 20% of production, currently has little value if not re-processed into smaller pieces for mixing with the sieved fraction. Whilst useful to increase bulk, the re-processed fraction is light coloured, as opposed to the characteristic dark brown of the "fermented" (oxidised) product. Alternatively, the coarse fraction could be used for extract production where physical appearance is not of concern. Globally, plant biomass waste ("by-product") from agricultural and food industries, is increasingly under consideration as an attractive renewable resource for a range of high value products such as polyphenols (Galanakis, 2012; Reis, *et al*., 2012; Wijngaard *et al.*, 2012). Recent research on *C. subternata* showed stems are a better source of the bioactive flavanone, hesperidin, than leaves (De Beer *et al*., 2012) and therefore merits investigation. Its recovery from citrus peel (a by-product of citrus processing) is well-documented and provides a good example of the recovery of a high value-added compound for the pharmaceutical industry from an agricultural by-product (Da Silva *et al*., 2013; Di Mauro *et al*., 1999; Garg *et al*., 2001).

Research to date on the value-adding potential of *Cyclopia* has concentrated on the plant material as a whole as source material for extract production (Joubert *et al*., 2003; Joubert *et al*., 2008). The xanthone, mangiferin, a major *Cyclopia* phytochemical, has been considered for extract enrichment (Joubert *et al*., 2003), while other bioactive compounds present in relatively high levels in *Cyclopia*, such as hesperidin (Joubert *et al.*, 2003; Joubert *et al*., 2006; De Beer & Joubert, 2010) have been largely ignored. Hesperidin, a flavanone glycoside, not only offers health-promoting properties that include antioxidant and vasoprotective activity (Garg *et al*., 2001; Valensi *et al.*, 1996), it is also the precursor of the aglycone, hesperetin, a patented taste modulator and sweetness-enhancer (Ley *et al*., 2008b; 2011a). Eriodictyol, the aglycone of eriocitrin, a minor flavanone glycoside of honeybush (De Beer & Joubert, 2010), is a bitter-masking compound (Kinghorn *et al*., 2010; Ley *et al*., 2005; 2011b).

In recent years, considerable progress has been made in the field of taste-modifying compounds and several flavonoids have been reported to enhance sweetness or to mask bitter taste without exhibiting any additional strong taste or flavour (Ley *et al*., 2002; 2005; 2008a; 2008b; 2011a; 2011b; Kinghorn *et al.*, 2010). Non-caloric compounds that could enhance sweetness without adding to the calorie intake are of great relevance to the food and beverage industries (Kinghorn *et al*., 2010), in light of the rising incidence of obesity and obesity-linked diseases such as type 2 diabetes, hypertension and cardiovascular disease, which is associated with high sugar intake (Block, 2013; Finucane *et al*., 2011;

Mollentze, 2006; Swinburn *et al*., 2004; WHO, 2003). However, the palatability of reduced-sugar foods is often sacrificed as these sweeteners may exhibit unpleasant bitter, astringent and/or metallic aftertastes or time-intensity profiles that are not characteristic of sucrose that result in a slow onset or unfavourable lingering aftertaste (Kinghorn *et al*., 2010; Kuhn *et al*., 2004; Ott *et al*., 1991; Schiffman *et al*., 1995). Therefore, research has concentrated on exploring alternative novel substances, which in low concentrations, could effectively enhance the sweet taste impressions of reduced-sugar foods, without negatively impacting on the remaining flavour profile (Bryant *et al*., 2008).

Hesperetin is present naturally in *Cyclopia* spp. at very low concentration (Joubert *et al.*, 2009). However, its levels in the extract could be increased through conversion of hesperidin to hesperetin. Several techniques exist to deglycosylate flavanone glycosides. These include acid-catalysed hydrolysis using strong mineral acids (Wingard, 1979) or organic acids (Hilmer *et al.,* 2008), as well as conversion using microbes (Manzanares *et al.,* 1997; Rajal *et al*., 2009) and enzymes (Da Silva *et al*., 2013; De Araújo, *et al*. 2013; Weignerová *et al*., 2012).

With the development of the `Green Chemistry' concept, environmentally benign extraction techniques have gained growing interest to allow for more environmentally sustainable extraction with high reproducibility, decreased extraction times, increased extraction yields, reduced solvent consumption and reduced temperature and energy input (Co *et al.,* 2012; Khan *et al.,* 2010; Mustafa & Turner, 2011). Numerous "green" extraction techniques such as sub-critical water extraction (Cheigh *et al.*, 2012) and microwave-assisted extraction (Inoue *et al*., 2010) have been explored for the recovery of flavanone glycosides from citrus processing by-products. Ultrasound-assisted extraction is one of the 'green' emerging technologies with low investment cost and energy. The mechanical effect of ultrasound during extraction is believed to accelerate the release of organic compounds contained within the plant due to cell wall disruption, easier access of the solvent to the cell content and increase in mass transfer rates (Chemat *et al*., 2011; Vinatoru *et al*., 1999). Ultrasound has been successfully applied for extraction of phenolic compounds from by-products from various food processes, including flavanone glycosides from citrus peel (Khan *et al.*, 2010; Londono-Londono *et al*., 2010; Ma *et al*., 2008a; 2008b).

Furthermore, in recent years Response Surface Methodology (RSM) has proven to be a popular optimisation technique for the optimum recovery of phenolic compounds from numerous food industry by-products (Khan *et al*., 2010; Pingret *et al*., 2012; Yang *et al*., 2010). Compared to the traditional, one-variable-at-a-time technique, RSM simultaneously optimises the levels of extraction parameters and includes the interaction effects among the variables studied (Bezerra *et al*., 2008; Dejaegher & Vander Heyden, 2011). RSM reduces the number of experimental runs required to evaluate multiple parameters and their interactions, thereby decreasing time and solvent consumption, and subsequently costs (Bezerra *et al*., 2008; Hibbert, 2012).

Therefore, the aim of this study was to explore the potential of honeybush tea processing by-product, in particular that of *C. maculata,* as renewable source material of valuable flavanone glycosides

to prepare a flavanone aglycone-enriched extract with potential taste-modulating properties. The focus was on hesperidin and hesperetin as hesperidin constitutes the major flavanone glycoside constituent of *C. maculata*. RSM was applied to optimise ultrasound-assisted extraction of the flavanone glycosides from *C. maculata* by-product, using an alcohol-water mixture, and their conversion to the taste-modulating aglycones using acid hydrolysis. Enzymatic bioconversion was also investigated as an alternative to acid hydrolysis for the de-glycosylation of the flavanone glycosides to their respective aglycones.

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CHAPTER 2

Literature review

In light of exploring the value-addition of honeybush tea processing by-product, in particular that of *Cyclopia maculata*, a brief background will be given of the honeybush industry and current research, followed by a review of the phytochemistry of honeybush with the emphasis on the flavonoids. Extraction and hydrolysis techniques of polyphenols will be reviewed as these respective techniques will be used for recovery of these valuable phytochemicals from tea processing by-products and for enhancing its health-promoting and taste-modulating potential. Review of taste modulation and phenolic compounds as sweetness-enhancing and bitterness-masking agents will provide insight into the potential of *C. maculata* extract as source of taste modulators.

2.1 Honeybush

2.1.1 Use as herbal tea

The traditional South African herbal tea, honeybush, is prepared from a number of *Cyclopia* spp. (Family: *Fabaceae;* Tribe: *Podalrieae*), which are endemic to the Cape fynbos biome. These long-lived perennials are well-adapted to the climate and soils of the coastal plains and mountainous areas of the Western and Eastern Cape Provinces of South Africa where they grow and can reach heights up to 3 m in the wild (Joubert *et al.*, 2011).

The historical use of *Cyclopia* spp., either as medicinal plant or as herbal tea, predates the 1800s; however, unlike rooibos (*Aspalathus linearis*), it was only 're-discovered' less than two decades ago when intense research on the cultivation, processing, composition and health-promoting potential of honeybush tea commenced (Du Toit *et al*., 1998; Joubert *et al.*, 2011).

This herbal tea provides the consumer with a caffeine-free antioxidant beverage, containing large quantities of health-promoting polyphenols (Joubert *et al*., 2009). It is generally consumed in its ''fermented" (oxidised) form since the high temperature oxidative process (''fermentation") is essential for development of the characteristic dark-brown leaf colour and pleasant sweet, honey-like flavour and aroma. There exists also the demand for the "unfermented" product (i.e. green plant material cut and dried without fermentation), both as herbal tea and source material for the preparation of extracts for the food, nutraceutical, and cosmetic industries (Joubert *et al.,* 2009; 2011).

The major phenolic compounds present in *Cyclopia* spp. quantitatively analysed to date are the xanthones, mangiferin and isomangiferin, and the flavanone, hesperidin (Joubert *et al.,* 2008b). The ubiquitously present mangiferin is particularly of interest as it displays various pharmacological effects, including immunoprotective, anti-inflammatory, cytoprotective and antigenotoxic activities (Vyas *et al*., 2012). Mango stem bark and leaves (*Mangifera indica L*.) are considered sources of mangiferin for production of a standardised mangiferin antioxidant extract (Anonymous, 2005). However, honeybush (particularly *C. genistoides* owing to its considerably high mangiferin content) is regarded as a sustainable alternative source of mangiferin for the production of mangiferin-rich antioxidant extracts (Joubert *et al.,* 2008b; 2011). Hesperidin, the major flavonoid in sweet orange and lemon, is an abundant and inexpensive by-product of citrus processing (Garg *et al*., 2001). This well-studied flavanone glycoside has been reported to possess a wide range of pharmacological properties including antioxidant, anti-allergenic, anti-carcinogenic, anti-hypotensive, anti-microbial, anti-inflammatory and anti-obesity properties (Bok *et al*., 1999; Chiba *et al*., 2003; Garg *et al*., 2001; Park *et al*., 2001). Known for its vasodilator property, hesperidin is widely used in combination with diosmin (another bioflavonoid) in the treatment of disease states associated with increased capillary permeability such as diabetes, chronic venous insufficiency, haemorrhoids and various ulcers (Garg *et al*., 2001). Therefore, in addition to mangiferin, honeybush could be regarded as a potential alternative source of hesperidin for the production of hesperidin-enriched therapeutic extracts.

2.1.2 Industry

Apart from the pleasant characteristic flavour profile, consumer interest in antioxidants and their beneficial properties has greatly contributed to increased consumption of rooibos and honeybush (Joubert *et al*., 2008a; 2011). Following the commercial success of rooibos tea, dramatic growth in the demand for honeybush tea over the past few years, both locally and internationally, is evident. In 2012, Germany, the U.S.A. and the Netherlands represented 43.8%, 25.7% and 13.5% of the export market, respectively (data supplied by Perishable Products Export Control Board). Export volumes have almost doubled from 2008 to 2011 (Fig. 2.1), however, supply still cannot meet demand, with shortages aggravated by severe drought and veld fires over the past few years (Joubert *et al*., 2011).

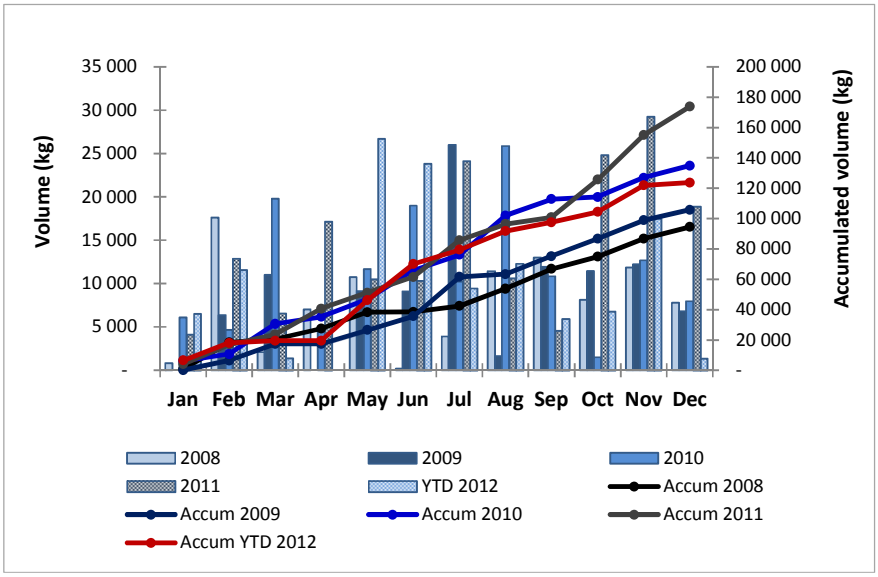


Figure 2.1 Honeybush tea export data (month to month comparison) for 2008-2012 (data from PPECB supplied by the South African Rooibos Council).

Honeybush tea processing has developed from primitive on-farm fermentation heaps (or baking ovens) and sun-drying delivering products of poor sensory and microbial quality, to factory processing, using temperature-controlled rotary fermentation and drying (Joubert *et al.*, 2011). Apart from export regulations dealing with pesticide residues, microbial content, and appearance of the tea, total polyphenol (TP) content and total antioxidant activity (TAA) are defined for extracts by manufacturers. Recently, a sensory wheel and lexicon were developed to assist in the sensory evaluation of fermented honeybush tea (Joubert *et al*., 2011; Theron, 2012). On-going research focusses on the development of species-specific sensory wheels and the development of a flavour kit.

Processed tea is mainly sold in bulk to the international market, although local customers can enjoy the packaged tea from on-farm and industrial processing facilities. To protect this indigenous

product from the threat of international trademarking (as in the case of the term "rooibos") and production delocalisation, the Agricultural Research Council (ARC) of South Africa took the initiative by trademarking the names 'Cape Herbal Tea', 'Cape Tea', 'Cape Honeybush Tea' and 'Cape Fynbos Tea' in 2006 (Joubert *et al*., 2011).

At present, wild-harvested *C. intermedia* comprises the bulk of production, with *C. subternata* and *C. genistoides* which are cultivated, contributing most of the remaining production. The commercial viability of other species such as *C. longifolia*, *C. sessiliflora* and *C. maculata* is still under investigation. Due to the limited supply, teabags with mixtures of different *Cyclopia* spp. (and even rooibos), are available on the supermarket shelf, with only a small percentage of green honeybush offered (Joubert *et al*., 2011). Waste material, not suitable for selling loose or in tea bag format, is generated from honeybush tea processing that can be used for the preparation of extracts (Joubert *et al*., 2009). Nonetheless, it is currently not utilised by the industry (D. Malherbe, Afriplex, Paarl, South Africa, 2013, personal communication). The percentage waste in terms of coarse (mainly the stem fraction) and fine (so-called "dust") plant material produced after the sieving step depends on the *Cyclopia* species. *Cyclopia* spp. with thick woody stems such as *C. maculata* yield a higher percentage of waste (Joubert *et al.*, 2011). Fine dust, whilst problematic in tea processing plants, is suitable for production of ethanol extracts (Malherbe, Afriplex, Paarl, South Africa, 2013, personal communication). The tea processing waste, which can constitute as much as 20% of production, are currently re-processed into smaller pieces and mixed with the sieved fraction (Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, 2012, personal communication). In this manner bulk is increased, although the practice is detrimental to the tea colour as the re-processed fraction is light coloured, as opposed to the characteristic dark brown of the "fermented" (oxidised) product. Using the coarse fraction for production of value-added extracts where physical appearance is not of concern, would be a more effective use of the material.

2.1.3 Value-addition

2.1.3.1 Exploring health-promoting properties

Tea flavonoids (from *Camellia sinensis*) play a key role in the average dietary intake of polyphenols in humans and numerous studies have confirmed that flavonoids can act as antioxidants at low concentrations relevant to physiological levels achievable through the diet (Joubert *et al.,* 2009). Similarly, rooibos has become more popular in recent years due to the potential health benefits of its polyphenols (Joubert *et al.,* 2009). The presence of known antioxidants mangiferin and hesperidin in honeybush underpins its value-adding potential.

Honeybush infusions demonstrated lower *in vitro* antioxidant activity compared to rooibos, which moved honeybush research in the direction of therapeutic uses not linked to antioxidant activity (Joubert *et al*., 2008a). In this regard the research on the phyto-oestrogenic (Mfenyana *et al*., 2008;

Verhoog *et al*., 2007a; Verhoog *et al*., 2007b), antimutagenic (Marnewick *et al*., 2000; Marnewick *et al*., 2003) and antimicrobial (Coetzee *et al*., 2008) properties of honeybush have shown promising results. Petrova *et al.* (2011) reported the *in vivo* potential of *C. intermedia* extracts to protect against UVB-induced skin damage via modulation of induced oxidative damage, inflammation and cell proliferation. The anti-diabetic potential of *Cyclopia* aqueous extract has also been demonstrated (Mose Larsen *et al*., 2008; Muller *et al*., 2011). Furthermore, Dudhia *et al.* (2013) recently demonstrated the potential of *C. maculata* and *C. subternata* aqueous extracts as anti-obesity agents through inhibition of adipogenesis *in vitro.* In addition, Pheiffer *et al*. (2013) reported that *C. maculata* stimulates lipolysis in mature 3T3-L1 adipocytes, thus providing further support for the anti-obesity effects of *Cyclopia* spp.

2.1.3.2 Extracts

Cultivated *C. genistoides* and *C. subternata* in particular, are attractive sustainable sources for preparation of mangiferin-enriched extracts as they are renewable, although *C. subternata* has a significantly lower mangiferin content (2.73 g/100 g dried green extract) compared to *C. genistoides* (9.55 g/100 g dried green extract) (De Beer & Joubert, 2010). Studies have indicated that the fermentation process is detrimental to the TP content and antioxidant activity of aqueous infusions and extracts (Du Toit & Joubert, 1999; Hubbe & Joubert, 2000). Joubert *et al*. (2008b) also showed that fermentation is accompanied by major losses in mangiferin and hesperidin. As a result of these detrimental effects of fermentation on composition and activity, the unfermented plant material is preferred for the preparation of antioxidant extracts for the nutraceutical and cosmetic markets (Joubert *et al*., 2008b). At this stage only a small quantity of extract is produced for food, cosmetic and toiletry products (Joubert *et al*., 2011). Food uses of extract include beverages, dairy products and snack bars. Due to the limited availability of honeybush, more than one specie is used for extract manufacturing (Joubert *et al.* 2011; Payne, 2012).

2.1.4 *Cyclopia maculata* explored

Cyclopia maculata (so-called “Vleitee” or “Genadendal tea”) naturally grows in the Overberg and southern Cape region of the Western Cape Province. This shrub is a vigorous grower (reaches heights up to 2 m within 1 year) forming thick stems (Joubert *et al*., 2011), which contribute to a large portion of tea processing by-products (depicted in Fig. 2.2). Until recently limited information on the chemical composition of *C. maculata* has been available. Joubert *et al.* (2003) quantified mangiferin, isomangiferin and hesperidin in unfermented plant material of which the coarse stems were removed before cutting and drying. In this case *C. maculata* had similar xanthone content to *C. intermedia*, but less than half its amount of hesperidin. Dudhia *et al*. (2012) demonstrated that hesperidin is the major compound of an aqueous extract of fermented *C. maculata*, while mangiferin was predominant in extract prepared from

unfermented plant material (leaves and stems). Schulze (2013), analysing a large number of aqueous extracts of unfermented *C. maculata*, showed mangiferin, isomangiferin and hesperidin to be the major compounds. Analysis of *C. subternata* leaves and stems indicated that the xanthones and hesperidin are predominant in the leaves and stems, respectively (De Beer *et al*., 2012).

Cyclopia maculata was one of the species subjected to recent sensory evaluation for the development of the honeybush flavour wheel (Theron, 2012). Interestingly, *C. maculata* was classed separately from the other species (*C. sessiliflora, C. intermedia, C. genistoides, C. longifolia* and *C. subternata*) evaluated, as the only specie that demonstrated woody, boiled syrup and cassia/cinnamon attributes. In addition, Theron (2012) studied the effect of fermentation temperature and time on the sensory characteristics of honeybush infusions. The results showed that 80°C/24 hrs were the optimum fermentation conditions for the sensory profile of *C. maculata*, since 90°C led to an increase in the negative sensory attributes, "hay/dried grass" and "green grass" (Theron, 2012). The TP content was not significantly affected by an increase in fermentation temperature (from 80 to 90°C); however, the TP content significantly decreased as the fermentation time (8, 16, 24 and 32 hrs) increased (Theron, 2012).

2.2 Phytochemistry of honeybush

2.2.1 Introduction

Tripoli *et al.* (2007) defines phytochemicals as constituents found in edible fruits and vegetables that, if ingested daily, may show potential for preventing chronic and degenerative diseases by modulating human metabolism.

Polyphenols exist as secondary plant metabolites that derive from the shikimate pathway and phenylpropanoid metabolism and exhibit a vast variety of structures in nature (Abad-García *et al*., 2009; Antolovich *et al.*, 2000; Macheix *et al.*, 1990). A convenient classification of plant phenols differentiates the number of constitutive carbon atoms in combination with the basic phenolic skeleton structure (C_6) (Antolovich *et al.,* 2000).

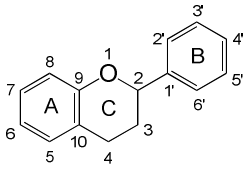
Flavonoids, a widespread family of phytochemicals that are highly diverse in chemical structure and biological function, are regarded as the single most important group of phenolic compounds with significant commercial interest (Abad-García *et al*., 2009; Park *et al.*, 1983). Strong interest in bioflavonoids increased over the past several decades, especially due to their recognised beneficial health-promoting properties and potential as nutraceuticals (Shahidi & Knack, 2004; Robards & Antolovich, 1997). Due to their specificity and ubiquity, they have also proven to be chemical markers for food authentication, for example naringin is used as a chemotaxonomic marker in distinguishing sweet orange from other citrus cultivars (Robards & Antolovich, 1997).

Figure 2.2 A) *C. maculata* bushes from a pilot plantation in Bereaville, Western Cape, South Africa, B) young shoots with needle-like leaves and C) coarse material (by-product of fermented tea) containing predominantly stems.

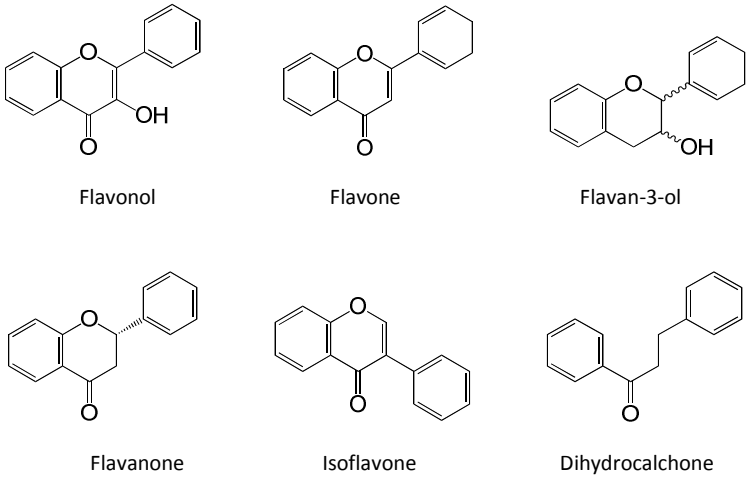
Flavonoids are characterised by a common benzo-γ-pyrone structure formed by a series of condensation reactions between a hydroxycinnamic acid and malonyl residue, giving rise to the basic C_6–C_3–C_6 flavone skeleton, in which the three-carbon bridge between the phenyl groups is commonly cyclized with oxygen (Abad-García *et al*., 2009; Benavente-García & Castillo, 2008; Robards & Antolovich, 1997; Tripoli *et al*., 2007). This group of compounds are divided into several classes that are differentiated according to the degree of unsaturation and oxidation of the three-carbon segment, the major ones being flavones, flavanones, flavonols, isoflavones, anthocyanidins, dihydrochalcones and flavan-3-ols (Robards & Antolovich, 1997; Tripoli *et al.*, 2007). The numerous combinations of multiple hydroxyl, methoxyl, *O*-glycoside and *C*-glycoside group substituents on the basic benzo-γ-pyrone structure have given rise to the more than 8000 identified flavonoids (Benavente-García & Castillo, 2008). Refer to Fig. 2.3 for the structures of different flavonoid classes.

Flavonoids generally occur as flavonoid *O*-glycosides, in which one or more hydroxyl groups of the aglycone are bound to a sugar (or sugars) by an acid-labile hemiacetal (glycosidic O–C) bond (Abad-García *et al*., 2009; Robards & Antolovich, 1997). Certain hydroxyl-group positions are more commonly glycosylated such as the 7-hydroxyl group in flavones and flavanones and the 3- and 7-hydroxyls in flavonols and flavan-3-ols (Abad-García *et al*., 2009). Additionally, although much less common, glycosylation may also occur via direct linkage of the sugar to the benzene nucleus, by an acid-resistant carbon–carbon bond, to form a flavonoid *C*-glycoside (Abad-García *et al*., 2009; Robards & Antolovich, 1997). The most common sugar moiety found in association with flavonoids is glucose, followed by galactose, rhamnose, xylose and arabinose whereas disaccharides such as rutinose and neohesperidose are also encountered (Iwashina, 2000; Markham, 1982).

The importance of phenolic compounds as potential therapeutic compounds is evident in the vast amount of research published on the extraction, quantitative and qualitative analysis, physicochemical characteristics and pharmaceutical properties of this group of phytochemicals. One group of phenolic compounds that has received intensive attention over the past two decades are those present in *Citrus* species. Interestingly, similar flavonoids have been found to be present both in the genus *Cyclopia* and *Citrus*. In the next section the focus will be predominantly on the major flavonoids, i.e. flavanones of *Cyclopia* spp.



Flavonoid skeleton



Flavonol Flavone Flavan-3-ol

Flavanone Isoflavone Dihydrocalchone

Figure 2.3 Structures of the various classes of flavonoids.

2.2.1.1 Honeybush flavonoids

Contrastingly to flavonoids such as flavonols, which are present in a wide range of foods, dietary flavanones are found nearly exclusively in citrus fruits and in certain aromatic herbs (Manach *et al.*, 2003).

Glycosylation of flavanones commonly occurs at position 7 either by a rutinose or neohesperidose, disaccharides formed by a glucose and rhamnose molecule. Neohesperidosides (naringin, neohesperidin and neoeriocitrin) comprise of a flavanone with neohesperidose (rhamnosyl-α-1,2-glucose), whereas rutinosides (hesperidin, narirutin and eriocitrin) consist of a flavanone and a rutinose (ramnosyl-α-1,6-glucose) (Tripoli *et al.*, 2007). Among the aglycone forms, naringenin, hesperetin, eriodictyol and isosakuranetin are the most common flavanones (Robards & Antolovich, 1997; Tripoli *et al.*, 2007). Refer to Fig. 2.4 for the structures of neohesperidosides and rutinosides.

Hesperidin is the main flavanone glycoside present in *Cyclopia* spp. (Joubert *et al*., 2011) and is an abundant and inexpensive by-product of citrus production (Da Silva *et al*., 2013). It comprises of a rhamnosyl-α-1,6-glucose, which is β-linked to the C-7-hydroxyl group of hesperetin, the aglycone of hesperidin (Horowitz & Gentili, 1969). The average hesperidin content of *C. genistoides* (2% of dried, green plant material (Joubert *et al*., 2006) is significantly higher than that of dried orange (0.03%)

(Peterson *et al*., 2006b). Aqueous extract of green *C. subternata* stems contains, 1.16-1.89% hesperidin compared to 0.34-0.74% eriocitrin (De Beer *et al*., 2012), whereas an aqueous extract of green *C. subternata* plant material (leaves and stems) contains 0.28-2.12% and 0.27-0.95% of hesperidin and eriocitrin, respectively (Schulze, 2013). A similar extract of *C. maculata* contained 1.44-2.69% and 0.25-0.69% of the respective compounds. Narirutin, another rutinoside, is a minor honeybush flavanone (Joubert *et al*., 2009). These two flavanones differ from hesperidin in their substitution of the B-ring, with eriocitrin containing a 3,'4' dihydroxyl group and narirutin a 4' hydroxyl group (Fig. 2.4). Their aglycones, eriodictyol and naringenin, respectively, were also identified in *Cyclopia* spp. at low concentrations (Joubert *et al*., 2011). The eriocitrin content of lemon was determined as 19.6 mg per 100 g dry weight, whereas 5.0 and 10.5 mg narirutin per 100 g dry weight was found to be present in orange and grapefruit, respectively (Peterson *et al*., 2006a; 2006b).

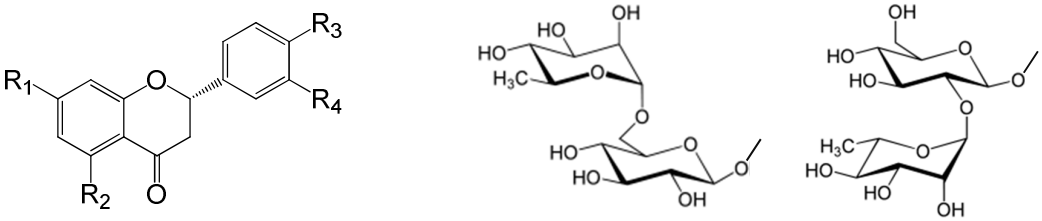
Other flavonoids identified in *Cyclopia* spp. include flavanones (naringenin-5-*O*-glucoside, eriodictyol-5-*O*-glucoside and eriodictyol-7-*O*-glucoside), flavones (luteolin, 5-deoxyluteolin, scolymoside and diosmetin), isoflavones (formononetin, a formononetin-diglucoside, afrormosin, calycosin, wistin, orobol, pseudobaptigenin, fujikinetin and isosakuranetin), flavonols (kaempferol glucosides), coumestans (medicagol, flemmichapparin and sophoracoumestan) and the flavan-3-ol, epigallocatechin gallate, as reviewed by Joubert *et al.* (2011).

2.2.1.2 Honeybush xanthones

Mangiferin, a *C*-glucoside xanthone with a basic C_6–C_1–C_6 skeleton (Pinto *et al*., 2005), is one of the major phenolic compounds present in *Cyclopia* spp. as previously mentioned. A well-known dietary source of mangiferin is mango (*M. indica*) (Robards & Antolovich, 1997; Vyas *et al.*, 2012). Comprehensive studies on the antioxidant, anti-inflammatory and anti-cancer properties of xanthones exist in which the xanthonoid structure with *C*-glucosyl linkage and polyhydroxyl groups is thought to contribute largely to the activities of mangiferin (Vyas *et al.*, 2012). The regio-isomer of mangiferin, isomangiferin, is present in substantially lower concentrations in *Cyclopia* spp. (Joubert *et al*., 2003; Joubert *et al*., 2008b; De Beer & Joubert, 2010; De Beer *et al*., 2012; Schulze, 2013).

2.2.1.3 Other honeybush phenolic compounds

Apart from xanthones and flavonoids, other phenolic compounds that were identified in *Cyclopia* spp. are the benzophenone, iriflophenone-3-*C*-glucoside, and dihydrochalcones, phloretin-3',5'-di-*C*-β-glucoside and 3-hydroxyphloretin-3',5'-di-*C*-hexoside (De Beer *et al*., 2012; Kokotkiewicz *et al*., 2012; Schulze, 2013). Furthermore, 3-hydroxyphloretinglycoside, an iriflophenone-di-*O,C*-hexoside, an eriodictyol-di-*C*-hexoside and vicenin-2 were tentatively identified in *C. subternata* for the first time (De Beer *et al*., 2012). In addition, *p*-coumaric acid and tyrosol were also identified in honeybush, as reviewed by Joubert *et al.* (2011).



Flavanone-*O*-glycoside [a] Rutinosyl (Rut) [b] Neohesperidosyl (Nh)

Compound name	R_1	R_2	R_3	R_4
Hesperidin	O-Rut[a]	OH	OMe	OH
Neohesperidin	O-Nh[b]	OH	OMe	OH
Eriocitrin	O-Rut[a]	OH	OH	OH
Neoeriocitrin	O-Nh[b]	OH	OH	OH
Narirutin	O-Rut[a]	OH	OH	H
Naringin	O-Nh[b]	OH	OH	H

Figure 2.4 Molecular structures of common flavanone rutinosides and neohesperidosides.

2.2.2 Potential of honeybush flavanones as nutraceuticals

2.2.2.1 Health-promoting properties

Biosynthesis of flavonoids in plants is involved in the response to biotic and abiotic stress (Petrussa *et al.*, 2013). Current interest in these compounds lies in their biological and pharmacological activities, amongst others anti-cancer, anti-atherogenic, anti-microbial and anti-inflammatory properties, as reviewed by Tripoli *et al*. (2007). These and other activities such as antioxidant, anti-viral hypoglycaemic, protection against bone loss and anti-tumour properties have been demonstrated both *in vitro* and *in vivo* (Cano *et al*., 2008; Espín *et al.*, 2007; Salas *et al*., 2011). Table 2.1 presents the potential health-promoting properties of well-known flavanones, also present in honeybush.

Flavanones are powerful radical scavengers that may help in many aging and degenerative events involving reactive oxygen species such as hydroxyl and superoxide radicals (Benavente-García *et al*., 1997; Tripoli *et al.*, 2007). This scavenging activity is attributed to the hydrogen-donating ability of their phenolic groups, which allows for the delocalisation of subsequent radicals produced over the flavonoid structure (Tripoli *et al.*, 2007). Three structural groups have been identified for flavonoids that are important for antioxidant activity: the ortho-dihydroxy (catechol) structure of the B-ring and the presence of both 3-and 5-hydroxyl groups (Bors *et al*., 1990a, 1990b). Flavanones are not grouped among the most potent antioxidant polyphenols in different *in vitro* assays (Rice-Evans *et al.*, 1996), as they lack the 2,3-double bond and the hydroxyl group at position 3 (Fig. 2.4). Glycosylation also decreased antioxidant activity as demonstrated for hesperidin and its aglycone, hesperetin (Williamson *et al.,* 1999; Londono-Londono *et al*., 2010). The xanthone, mangiferin, is much more effective as a hydrogen donor and superoxide radical scavenger than hesperidin and hesperetin (Joubert *et al*., 2003; Joubert *et al*., 2008a).

Hesperidin is the most highly consumed flavonoid (28.3 mg/day) in the diet, representing 30% of total flavonoid intake (Manach *et al.*, 2003). This well-studied flavanone glycoside has been reported to have antioxidant, anti-allergenic, anti-carcinogenic, anti-hypotensive, anti-microbial, anti-obesity and vasodilator properties (Garg *et al.,* 2001; Bok *et al*., 1999; Chiba *et al*., 2003; Park *et al*., 2001). It was successfully tested as a chemopreventive agent of variously induced tumours in rats and mice (Tanaka *et al*., 1994, 1997a-c; Yang *et al.,* 1997; Berkarda *et al*., 1998). Hesperidin also exhibits anti-inflammatory activity through its inhibition of the synthesis and biological activities of different pro-inflammatory mediators, as reviewed by Benavente-García & Castillo (2008). It is also known for improving vascular integrity and decreasing capillary permeability (Valensi *et al.,* 1996). The vasoprotective action of hesperidin can prevent microvascular leakage by inhibiting the enzyme, hyaluronidase, which has been described to control the permeability of capillary walls and supporting tissues (Beiler *et al.*, 1948). Furthermore, it has been demonstrated that hesperidin can decrease blood cell and platelet aggregation that may be advantageous in conditions of capillary permeability and fragility (Garg *et al.*, 2001). A micronized purified flavonoid fraction (450 mg diosmin plus 50 mg hesperidin) known as Daflon 500 is a

potent venotropic drug used in the treatment of venous insufficiency (Ramelet, 2001). The potential effect of hesperidin and hesperetin on ocular blood flow and vascular permeability renders these compounds as attractive options for the treatment of diabetic patients with proliferative diabetic retinopathy (Majumdar & Srirangam, 2009).

Eriocitrin has a high antioxidant activity when compared to hesperidin (Joubert *et al.*, 2008b). Its ability to inhibit lipid peroxidation *in vitro* was demonstrated by Miyake *et al*. (1997), and can be ascribed to the radical scavenging capacity of the *o*-dihydroxy-group on the B-ring. Additionally, eriocitrin reduced streptozotocin-induced (Miyake *et al*., 1998) and exercise-induced oxidative stress (Minato *et al*., 2003) in rats. Oxidative stress induced by streptozotocin leads to diabetes in rats as a result of pancreatic β-cell damage (Szkudelski, 2001).

Studies on various health benefits of narirutin such as anti-oxidant, anti-cancer, anti-inflammation and anti-lipidaemic properties have been reported (Abeysinghe *et al*., 2007; Funaguchi *et al*., 2007; Jung *et al*., 2006). Its aglycone, naringenin, demonstrated anti-atherogenic, hepatoprotective, nephroprotective, anti-ulcer, antioxidant, anti-inflammatory, antimutagenic, vasodilator, anti-thrombotic, and anticancer activities (Cui *et al.*, 2012).

2.2.2.2 Bioavailability

A significant drawback of many flavonoids is limited bioavailability, making them inefficient for dietary supplementation. In spite of limited bioavailability, sufficient evidence from an increasing number of *in vivo* studies and clinical trials support their use as therapeutic agents (as reviewed by Benavente-García & Castillo, 2008).

After ingestion hesperidin is absorbed across the gastrointestinal tract. Cumulative urinary recovery indicates bioavailability of less than 25% (Ameer *et al.*, 1996). Relative urinary excretion may be considered as an estimate of absorption efficiency and is expressed as percentage of the flavonoid intake (Manach *et al.*, 2003). The combination of poor water solubility, precipitation in an acidic environment, together with being substrates for the intestinal efflux protein, P-glycoprotein and intestinal and hepatic drug metabolizing enzymes CYP450, severely limit systemic bioavailability of hesperidin and hesperetin (Gil-Izquierdo *et al.,* 2003; Tsai *et al.,* 2004; Mitsunaga *et al.,* 2000; Ofer *et al.,* 2005). Hesperidin demonstrates poor transmembrane permeability and is supposedly absorbed mainly by the paracellular pathway, implying that intestinal tight junction proteins would limit intestinal absorption (Kobayashi *et al*., 2008; Serra *et al*., 2008). *In vivo* studies report that absorption of intact hesperidin and naringin does not occur. They are hydrolysed to their respective aglycones, naringenin and hesperetin, which are recovered in plasma, urine and bile as glucuronides and sulpho-glucuronides (Booth *et al.*, 1958; Hackett *et al*., 1979; Felgines *et al*., 2000).

Research has revealed that flavonoids with a rutinosyl moiety in position 7 are not a substrate for β-glucosidases of the human liver and small intestine (Day *et al*., 1998). Flavanone aglycones are

suggested to be absorbed faster and in higher amounts than their corresponding glycosides as reviewed by Tripoli *et al.* (2007). Therefore, glycosides with rhamnose are poorly absorbed compared to their aglycones and glucoside forms (Hollman *et al*., 1999). Nielsen *et al*. (2006) demonstrated that removal of the terminal rhamnose moiety from hesperidin to yield hesperetin-7-*O*-glucoside, increased its bioavailability in humans 3-fold. Furthermore, it showed that its absorption site is the small intestine and not the colon. In addition, Habauzit *et al.* (2009) demonstrated that the bioavailability of hesperetin-7-*O*-glucoside increased compared with hesperidin, which resulted in more efficient prevention of bone loss in adult ovariectomised rats.

Enzymatic deglycosylation of flavonoids is considered a good alternative for improving their bioavailability (Christensen, 2009; Erlund *et al.*, 2001; Hollman *et al*., 1999). Park *et al.* (2013) demonstrated that enzymatic modification such as glycosylation and de-rhamnosylation may improve bioavailability of hesperidin and narirutin. Glycosylated hesperidin and hesperetin-7-*O*-glucoside were found to be more easily absorbed than hesperidin, as glycosylation increased the solubility of hesperidin about 10,000 times (Park *et al*., 2013; Yamada *et al*., 2006). Alternatively, studies by Sansone *et al.* (2009) demonstrated the potential of hesperidin gastroresistant microparticles produced by spray-drying using cellulose acetate phthalate combined with enhancers such as cross-linked carboxymethylcellulose.

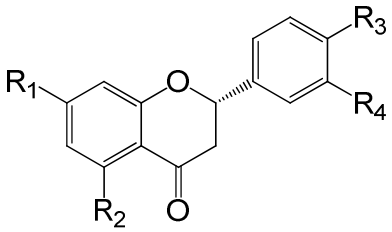
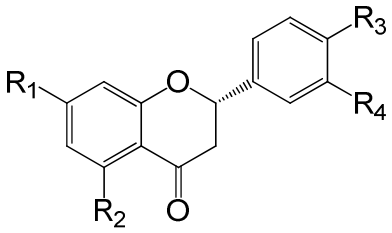
2.2.3 Physical and chemical characteristics of flavanones

2.2.3.1 Solubility and pH stability

Glycosylation has a profound effect on a flavonoid rendering it more water soluble, permitting storage of the flavonoid in the cell vacuole where they are commonly found (Robards & Antolovich, 1997). Although a flavonoid glycoside, hesperidin has an extremely low solubility in water, estimated to be 20 mg/L or less (Grohmann *et al*., 2000). Due to its low water solubility, hesperidin has been considered as one of the factors contributing to the cloudiness of orange juice (Kimball, 1991).

Majumdar & Srirangam (2009) reported that hesperidin demonstrated poor, pH independent, aqueous solubility. However, its solubility improved dramatically by complexation with 2-hydroxypropyl-β-cyclodextrin (HP-β-CD). In this complex hesperidin demonstrated adequate chemical and enzymatic stability in aqueous solutions and biological matrices, respectively (Majumdar & Srirangam, 2009). β-CD inclusion complexation also improved the aqueous solubility of the water insoluble flavanone neohesperidoside, naringin. The solubility of the naringin/β-CD complex (1:1 molar ratio) was 15 times greater in water at 37°C than that of free naringin (Cui *et al*., 2012).

Table 2.1 Potential health-promoting properties of major citrus and honeybush flavanones

Class	General structure	Flavonoid	Potential health-promoting properties	Reference
Flavanone glycoside		**Hesperidin** R_1 = *O*-Rutinose; R_2, R_4 = OH; R_3 = OCH_3	Antioxidant activity	Benavente-García & Castillo (2008) Wilmsen *et al.* (2005) Zhang *et al*. (2007)
			Anti-inflammatory activity	Benavente-García & Castillo (2008) Tripoli *et al*. (2007)
			Anti-allergic activity	Garg *et al*. (2001)
			Vasoprotective activity: improves the vascular integrity; decreases capillary permeability	Valensi *et al.* (1996) Garg *et al*. (2001)
			Anti-obesity activity	Bok *et al.* (1999); Chiba *et al.* (2003)
			Cholesterol-lowering activity	Park *et al.* (2001)
			Anti-microbial activity	Garg *et al.* (2001)
			Analgesic activity	Galati *et al*. (1994)
			Hypolipidaemic activity	Monforte *et al.* (1995)
			Anti-hypertensive activity	Galati *et al.* (1996)
			Diuretic activity	Galati *et al.* (1996)
			Regulation of bone metabolism	Habauzit *et al*. (2011); Horcajada & Coxam (2004)
		Eriocitrin R_1 = *O*-Rutinose; R_2, R_3, R_4 = OH	Antioxidant activity	Minato *et al.* (2003) Miyake *et al.* (1997)
			Lipid-lowering effect *(in vivo)*	Miyake *et al.* (2006)
		Narirutin R_1 = *O*-Rutinose; R_2, R_3 = OH; R_4 = H	Antioxidant, anti-cancer, anti-inflammation, anti-lipidaemic activity	Abeysinghe *et al.* (2007); Funaguchi *et al.* (2007); Jung *et al.* (2006)
Flavanone aglycone		**Hesperetin** R_1, R_2, R_4 = OH; R_3 = OCH_3	Antioxidant activity	Londono-Londono *et al.* (2010)
			Anti-inflammatory, hypolipidaemic, vasoprotective, anti-carcinogenic activity	Garg *et al.* (2001)
			Anti-cancer activity	Sivagami *et al*. (2012)
		Eriodictyol R_1, R_2, R_3, R_4 = OH	Antioxidant activity	Minato *et al.* (2003) Miyake *et al.* (1997)
			Indirect: bitterness-masking property	Ley *et al.* (2011b)
		Naringenin R_1, R_2, R_3 = OH; R_4 = H	Anti-atherogenic, anti-ulcer, antioxidant, anti-inflammatory, anti-mutagenic, vasodilator, anti-thrombotic, anti-cancer activity	Ghasemzadeh *et al*. (2010); Raja *et al.* (2012); Chen *et al.* (2012); Badary *et al.* (2005)

Grohmann *et al.* (2000) demonstrated that the solubility of hesperidin in water increased with increased temperatures. Significant acceleration of acid hydrolysis of hesperidin suspensions in water was observed at 120°C and higher (Grohmann *et al.*, 2000). The water solubility of naringin was also found to increase rapidly at temperatures higher than 50°C (Pully, 1936). Hesperetin is only slightly soluble in water, supposedly due to its crystalline state (Garg *et al*., 2001).

2.2.3.2 Heat stability

Xu *et al*. (2007) demonstrated that heat treatment of citrus peels at 120°C (heating time of 90 min) and 150°C (heating time of 30 min) led to degradation of hesperidin. Ho & Lin (2008) showed maximal extraction of hesperidin from citrus peel at 100°C after 30 min. Longer extraction was not beneficial, leading to degradation. On the other hand, Dhuique-Mayer *et al.* (2007) observed no significant decrease of hesperidin at 90°C in citrus juice, even after 240 min.

2.2.3.3 Sensory properties

Flavanone glycosides can be classified into bitter flavanone neohesperidosides and tasteless flavanone rutinosides. The point of attachment of rhamnose to glucose determines whether the compound is bitter or tasteless (Tripoli *et al*., 2007). Horowitz & Gentili (1969) reviewed the role of structure on the taste of phenolic glucosides. Hesperidin is essentially tasteless, while naringin is intensely bitter. Changes made at certain sites of flavanone neohesperidosides result in bitter, bitter-sweet, sweet, or tasteless products. On the other hand, corresponding modifications to flavanone rutinosides, generally resulted in tasteless compounds, unless the rhamnose is removed from the 6-position of glucose. Methylation or ethylation of one or more free phenolic hydroxyl groups on the A- or B-ring generally causes a decrease in bitterness of flavanones. The role of flavanones as taste modulators is discussed in Section 2.5: *Taste modulation and its application in the food industry.*

2.3 Extraction of phenolic compounds

2.3.1 Introduction to the '*Green revolution*'

The food industry is generating vast amounts of food processing wastes to keep up with increasing demand for food by a continuously growing world population, estimated at approximately 7.1 billion in 2012 (Anonymous, 2012d). Approximately one-third (1.3 billion ton/year) of the edible food portion produced globally for human consumption is lost, discarded or degraded in different stages of the food supply chain from production to consumption as reported by the Food and Agricultural Organisation (FAO) of the United Nations (Galanakis, 2012).

The large amount of global waste generated daily imposes a great threat to the environment; therefore, research is progressively more focused on utilising by-products (a term increasingly preferred by scientists over "waste" to emphasise their potential as ultimate substrates) to reduce their environmental impact and to explore their potential economic value (Galanakis, 2012). Apart from being used as fuel, fertiliser and animal feed, plant biomass waste from agricultural and food industries are also attractive renewable resources for chemical feedstock to produce phenolic precursors, polymer substitution, carbon fibre, glue and binders (Garrote *et al*., 2004; Kamm & Kamm, 2004; Tsubaki *et al*., 2010). Over the past two decades it is evident from literature that considerable research has been done on the exploitation ('valorisation') of by-products as a potential renewable source of valuable bioactive compounds owing to their inherent chemical complexity. It includes studies on by-products of rice, olive oil, tea, apple, tomato and citrus processing industries (Table 2.2).

The dramatic increase in citrus fruit consumption over the past few years, in particular, has contributed to the annual accumulation of an estimated 15.6 million metric tonnes of by-products in the form of peel, seed, cell and membrane residues, accounting for nearly half of the total fruit weight (Lin *et al*., 2013). Apart from their use for animal feed, molasses, pectins, essential oils and limonene as flavour compound or chemical building block, they are a rich renewable source of phenolic compounds, especially flavanone glycosides such as hesperidin, neohesperidin, naringin and narirutin. Their recovery as potential antioxidants has attracted considerable scientific interest (Lin *et al*., 2013). Tea residues are a major by-product in Japan and research has shown green, oolong and black tea residues to be potential sources of value-added products such as polysaccharides, polyphenols and caffeine (Tsubaki *et al*., 2008).

Extraction is regarded as the most important stage of the recovery process of micro- and macromolecules from by-product substrates (Galanakis, 2012). According to Wijngaard *et al.* (2012) the majority of waste material has not yet been explored in part due to the lack of suitable extraction methods of bio-active compounds. However, in recent times significant progress in extraction techniques has been made and numerous innovative extraction methods have emerged to optimise the extraction of bio-active compounds from by-products, which will be discussed in the following section.

With the development of the `Green Chemistry' concept, environmentally benign techniques have gained growing interest to allow for more environmentally sustainable extraction. High reproducibility, decreased extraction times, increased extraction yields, reduced solvent consumption and temperature and lower energy input are important considerations (Co *et al.,* 2012; Khan *et al.,* 2010).

2.3.2 Extraction solvents – Classical vs 'green'

Classical extraction methods make use of acid, alkali, solvents and supercritical fluids and are regarded as safe if the involved materials exist intrinsically in foods or are food grade (e.g. CO_2 gas) (Galanakis, 2012). For example, non-toxic ethanol and citric acid are preferred for use in the food industry compared to toxic methanol and corrosive hydrochloric acid, respectively, even though the last-mentioned solvents are

generally more efficient (Galanakis, 2012). Research has shown that the recoveries of total phenol content from citrus peels, obtained by using 95% (v/v) ethanol or 95% (v/v) methanol, were similar (Li *et al*., 2006a). For conventional extraction of less polar phenolic compounds (for analytical purposes), chloroform or dichloromethane are used as solvents (Ishida *et al*., 2001). To increase the polarity of the liquid phase during Soxhlet extraction, co-solvents are occasionally added, e.g. mixtures of isopropanol and hexane have been reported to increase extraction yield and kinetics, as reviewed by Wang & Weller (2006).

Commercial-grade hexane, a paraffinic petroleum fraction, is the most widely-used solvent to extract edible oils from plant sources; however, it mainly contains n-hexane, which has been listed as the most hazardous air pollutant by the US Environmental Protection Agency (Mamidipally & Liu, 2004). Due to environmental, health and safety concerns, alternative solvents such as isopropanol, ethanol, hydrocarbons and water are increasingly being used for classical extraction although potentially lower recovery yields and increased costs are implicated (Wang & Weller, 2006). In addition, Mamidipally & Liu (2004) investigated the use of n-limonene as an alternative solvent to hexane for the extraction of oil from rice-bran.

Hydro-distillation and steam diffusion are non-toxic techniques using only water, but these processes require relatively high energy consumption. In addition, the use of high temperature may be detrimental for heat labile compounds (Galanakis, 2012).

2.3.3 Conventional solvent extraction

Traditional extraction techniques include Soxhlet extraction, solid–liquid extraction and blending (Wang & Weller, 2006). Techniques such as Soxhlet extraction are generally well-established with wide industrial applications and demonstrate good reproducibility and reasonable recovery (Wang & Weller, 2006). Nonetheless, classical extraction methods pose several disadvantages that include the use of hazardous extraction solvents or extended extraction periods (Inoue *et al*., 2010). The long extraction time and consumption of large quantities of organic solvents required by organic extraction techniques such as maceration and Soxhlet extraction contribute to high energy consumption and general cost of these methods (Galanakis, 2012; Ma *et al*., 2008a). Volatilisation of organic solvents during the subsequent concentration step may result in environmental pollution (Ma *et al*., 2008a; Mustafa & Turner, 2011). Organic solvents may also leave unacceptable residues in extracts. Limits for solvents in herbal extracts and formulations are prescribed by ICH (International Conference on Harmonization, 1997) (Puranik *et al*., 2009). Food-grade solvents are water, ethanol and supercritical CO_2 (Wang & Weller, 2006).

Conventional extraction methods are generally performed at ambient temperature or at the boiling point of the solvent (Co *et al*., 2012). High temperatures and long extraction times may cause

overheating of the food matrix, ultimately leading to the degradation of sensitive, thermo-labile target compounds (Galanakis, 2012; Khan *et al*., 2010; Mustafa & Turner, 2011).

2.3.4 Emerging 'green' extraction techniques

There exists an increased need for environmentally friendly extraction techniques for optimum recovery of bioactive constituents in the shortest processing time at a low cost (Ma *et al*., 2008a). The application of the 'Green Chemistry' philosophy is to develop and encourage the use of novel extraction techniques that reduce and/or exclude the use of harsh organic solvents or production of hazardous materials, in order to preserve the natural environment and its resources (Mustafa & Turner, 2011). The potential of natural deep eutectic solvents (NADES) as green 'alternatives' for the extraction of phenolic compounds has been demonstrated (Dai *et al*., 2013). NADES are composed of primary metabolites common in living cells (e.g. choline, urea, organic acids, glucose and fructose) that are abundant in food and are thus considered inexpensive, biodegradable and safe. Certain NADES have shown a very high ability to solubilise both non-polar and polar compounds and have also proven to be able to dissolve macromolecules; therefore, NADES holds great potential as solvents in the extraction of valuable secondary metabolites for their use in the food and pharmaceutical industry (Dai *et al*., 2013).

2.3.4.1 Thermal extraction

Pressurised liquid (or solvent) extraction (PLE) is such an emerging environmentally friendly approach to obtain bioactive compounds and nutraceuticals from foods and herbal plants (Mustafa & Turner, 2011). PLE, also known as pressurised hot water or sub-critical water extraction (SWE) when water is used, involves extraction at elevated temperature (above the atmospheric boiling point of the solvent) and pressure, which allows for enhanced solubility and mass transfer of target compounds (Mustafa & Turner, 2011).

One of the advantages of pressurised hot solvents is that by merely changing the extraction temperature, the dielectric constant, diffusion rate, viscosity and surface tension of the solvent is changed, allowing for rapid and efficient extraction (Co *et al*., 2012). For the extraction of non-polar compounds, supercritical carbon dioxide (CO_2) can replace organic solvents such as chloroform and dichloromethane (Ollanketo *et al*., 2002). CO_2 is the most common supercritical solvent used in food applications as it is cheap, pure, readily available, non-toxic and easy to eliminate by simple expansion to common environmental pressure, which allows approval for food processing without declaration (Brunner, 2005). Examples of specialised industrial food applications include decaffeination of green coffee beans, production of hops extracts and extraction of flavours and aromas from herbs and spices (Brunner, 2005). Furthermore, PLE enables the use of water suitable for the extraction of polar, moderately polar and even non-polar organic compounds such as citrus flavanones (Cheigh *et al*., 2012;

Mustafa & Turner, 2011). In addition, Cheigh *et al.* (2012) demonstrated that SWE, compared to classical solvent extraction, is an extremely efficient and rapid technique for the recovery of hesperidin and narirutin from *Citrus unshiu* peel.

Although PLE technologies require the initial procurement of expensive equipment (Brunner, 2005) several advantages are associated with PLE. Not only does it improve the elevated temperature and pressure extraction yield, but it also decreases time and solvent consumption, provides protection for oxygen and light sensitive compounds, allows replacement of organic solvents with subcritical water, and ultimately leads to sustainable extraction (Cheigh *et al.*, 2012; Co *et al*., 2012; Mustafa & Turner, 2011). Microwave-assisted extraction (MAE) has received attention as an environmentally friendly extraction method for the extraction of valuable compounds, e.g. polyphenols and caffeine from green tea leaves as it allows the efficient use of microwave energy, a non-contact heat source (Chemat *et al.,* 2011). Only moderate initial investment is required for MAE compared to conventional solvent extraction (Galanakis, 2012; Tsubaki *et al*., 2010). The principle of MAE is the improvement in the extraction kinetics provided by heating (Biesaga, 2011). Extraction occurs as the result of changes in the cell structure of plant material caused by electromagnetic waves (Veggi *et al*., 2013). Dipolar compounds are directly heated by microwave-irradiation and extracted into a lower temperature solvent by destroying the cell wall (Inoue *et al*., 2010). The major advantage of heating by microwave-irradiation is that all of the sample fluid is heated, which allows the extraction solution (solvent and sample) to reach the desired temperature more rapidly, avoiding a thermal gradient caused by conventional heating (as reviewed by Biesaga, 2011). Therefore, MAE allows for more effective heating, faster energy transfer, quicker response to process heating control and faster start-up (Routray & Orsat, 2012; Veggi *et al*., 2013). An additional advantage is that microwave irradiation reduces the problem of overheating and could therefore minimise the degradation of heat-sensitive analytes. Furthermore, the use of closed systems reduces the risk of losses (Biesaga, 2011). MAE is, however, limited in terms of solvents as MAE efficiency is usually low when the solvent lacks a significant dipole moment for microwave energy absorption (Chemat *et al*., 2011).

Tsubaki *et al*. (2008; 2010) reported the effective use of MAE for the extraction of caffeine, polysaccharides and polyphenols from tea residues. In addition, Inoue *et al*. (2010) demonstrated the isolation of hesperidin from citrus peel (by-product from citrus processing) by MAE using aqueous ethanol solution as solvent.

Table 2.2 Studies on extraction techniques for the extraction of phenolic compounds from food by-products of plant origin

Industry	By-product source	Analytes/Target compounds	Extraction technique	Reference
Apple juice & cider	Apple pomace	Phenolic compounds	ULTRASOUND-ASSISTED	Pingret *et al*. (2012)
Apple juice & cider	Apple pomace	Phenolic compounds (chlorogenic acid, flavonols, phloretin glucosides)	SOLVENT	Wijngaard & Brunton (2010)
Citrus processing	Citrus peels	Flavanones (hesperidin, narirutin)	SUBCRITICAL WATER	Cheigh *et al*. (2012)
Citrus processing	Citrus peels - mandarin	Flavonoids (hesperidin)	SOLVENT	Ho & Lin (2008)
Citrus processing	Citrus peels - mandarin	Flavanone glycosides (narirutin, nobiletin, tangeretin, hesperidin); polymethoxylated flavones; phenolic acids	HOT WATER *(Infusion cooking)*	Xu *et al.* (2007)
Citrus processing	Citrus peels – lemon,grapefruit, mandarin, orange	Phenolic compounds	SOLVENT	Li *et al.* (2006a)
Citrus processing	Citrus peels – lemon, grapefruit, mandarin, orange	Phenolic compounds	AQUEOUS ENZYME-ASSISTED	Li *et al.* (2006b)
Citrus processing	Citrus: thinned immature fruit peels	Flavonoids (narirutin, hesperidin)	MICROWAVE-ASSISTED	Inoue, *et al.* (2010)
Citrus processing	Orange peels	Hesperidin	CAUSTIC	Di Mauro *et al.* (1999)
Citrus processing	Waste water ("yellow water") from orange juice processing	Hesperidin	CAUSTIC	Di Mauro *et al.* (2000)
Citrus processing	Orange peels	Flavanone glycosides (naringin, hesperidin)	ULTRASOUND-ASSISTED	Khan *et al.* (2010)
Citrus processing	Citrus peels – lime, orange and tangerine	Flavonoid fractions (including hesperidin, neohesperidin, hesperetin)	ULTRASOUND-ASSISTED	Londono-Londono *et al.* (2010)
Citrus processing	Satsuma mandarin peels	Phenolic acids & flavanone glycosides (narirutin, hesperidin)	ULTRASOUND-ASSISTED	Ma *et al.* (2008a)
Citrus processing	Penggan peel	Hesperidin	ULTRASOUND-ASSISTED	Ma *et al.* (2008b)
Marigold extraction (for carotenoids)	Defatted marigold (*Tagetes erecta* L.) extraction residues	Phenolic compounds (including flavonoids)	SOLVENT	Gong *et al.* (2012)
Olive Oil	Olive cake (solid waste)	Phenolic compounds (including hesperidin)	SOLVENT	Alu'datt *et al.* (2010)
Olive Oil	Olive tree leaves	Phenolic compounds (total phenolic content)	ULTRASOUND-ASSISTED	Şahin & Şamli (2013)
Rice milling	Rice bran (outer layer of brown rice)	Phenolic compounds	ULTRASOUND-ASSISTED	Tabaraki & Nateghi (2011)
Tea	Green, oolong, black tea residues	Polysaccharides, phenolic compounds, plant biopolyester	MICROWAVE-ASSISTED	Tsubaki *et al.* (2008; 2010)

2.3.4.2 Non-thermal extraction

Enzyme-assisted extraction has shown high catalytic efficiency and high specificity while using mild reactive conditions. The mechanism involves the weakening or breakdown of cell wall integrity by degrading enzymes, making the intracellular materials more accessible for extraction (Li *et al*., 2006b). A number of studies of enzyme-assisted extraction, e.g. polyphenol extraction from blackcurrant juice press residues (Landbo & Meyer, 2001) and polyphenols from rooibos (Pengilly *et al*., 2008; Coetzee *et al*., 2013), have been reported. However, according to Khan *et al.* (2010), enzyme-assisted aqueous extraction is limited due to problems of enzyme denaturation. Furthermore, Li *et al.* (2006b) demonstrated that the recovery of phenolic compounds from different citrus peels was not as effective compared to conventional solvent extraction. However, it can still be regarded as an environmentally friendly technique that preserves the maximum of the original efficacy (e.g. antioxidant activity) of the active target compound without the need for the use of harsh solvents (Li *et al*., 2006b).

Pulsed electric field (PEF) is another non-thermal food processing technique of high potential and the resulting accelerated mass transfer due to cell membrane breakdown has been applied for the enhanced extraction of phenols (Galanakis, 2012). PEF-assisted extraction induces a permeabilisation of the cytoplasmic membranes that facilitates the release of intracellular compounds from the cells (Puertolas *et al*., 2012). The major advantages of PEF are that no breakdown of the extracted compounds occurs as no heating is involved and the treatments may be applied in continuous flow at pilot plant and on an industrial scale (Toepfl & Heinz, 2011). In addition, this technique is an environmentally friendly and economical alternative to conventional extraction methods that require high solvent consumption, long extraction times and the prerequisite of a dried source material (Puertolas *et al*., 2012). PEF was demonstrated to increase the extraction rates and yields of different valuable compounds for the food industry (Puertolas *et al*., 2012). Luengo *et al.* (2013) demonstrated the potential of PFE treatment as a gentle technology to improve extraction of total polyphenols and flavonoids (naringin and hesperidin) from fresh orange peel.

2.3.5 Ultrasound-assisted extraction

Ultrasound-assisted extraction (UAE) is one of the 'green' emerging technologies with low investment cost and energy consumption that has found wide application in the food industry (Galanakis, 2012). Although it has been classified under non-thermal extraction techniques, it can also be used in combination with heating and referred to as 'thermosonication' (Chemat *et al*., 2011; Hossain *et al.*, 2012).

Ultrasound is defined as sound waves with a frequency beyond the limit of human hearing (~20 kHz). By adjusting its frequency, ultrasound can be applied to food processing, analysis and quality control (Knorr *et al.*, 2004). Low power (high frequency; > 100 kHz) ultrasound is used for non-invasive analysis and

monitoring (quality control) of the composition and physicochemical properties of food components and products during processing and storage. It is used to aid in ensuring high quality and safety, e.g. detection of honey adulteration and assessment of protein aggregation state and size (Awad *et al*., 2012). Contrastingly, high power (low frequency; 20 – 100 kHz) ultrasound is disruptive and causes mechanical, physical and chemical/biochemical changes through cavitation. High power ultrasound is applied to food processing operations (e.g. extraction, freezing, freeze-drying and concentration, defoaming and emulsification), preservation (e.g. inactivation of enzymatic activity for enhanced shelf life) and safety (e.g. inactivation of pathogenic bacteria on food contact surfaces) as reviewed by Awad *et al*. (2012). The simplicity, portability and low cost of ultrasound devices, i.e. high power sonication baths or ultrasonic immersion probes, make them valuable instruments in research laboratories, pilot plants and large food factories, especially as they can be modified to suit different applications in the food industry (Awad *et al*., 2012).

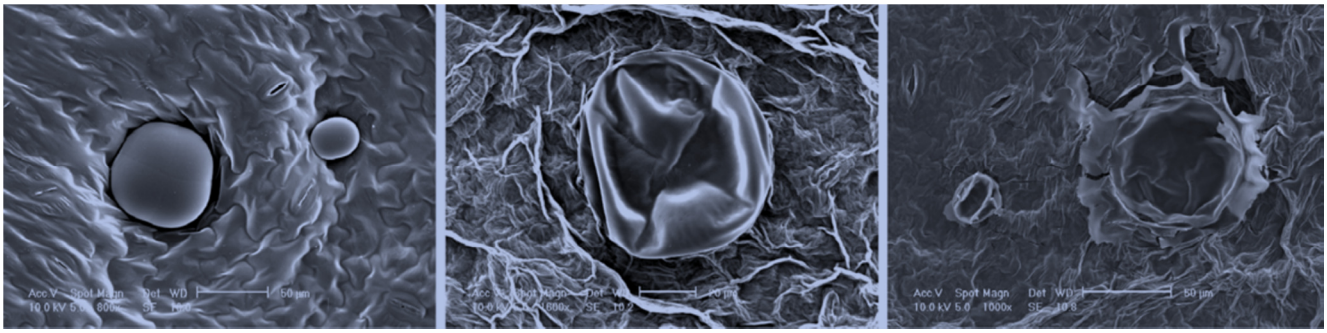


Figure 2.5 Ultrasound-assisted extraction of essential oil from basil indicating the collapse of a cavitation bubble and release of plant material (Chemat *et al*., 2011).

UAE is a major application in the food industry to aid in the extraction of numerous food components (e.g. herbal, oil and protein), as well as bioactive ingredients (e.g. polyphenols, anthocyanins, aromatic compounds, polysaccharides and functional compounds) from plant and animal resources (Vilkhu *et al*., 2008). The mechanical effect of ultrasound involves the action of higher intensities (low frequencies) to induce acoustic cavitation (so-called 'cavitation phenomenon') due to the generation, growth and collapse of large bubbles, resulting in the liberation of high amounts of highly localised energy, as depicted in Fig. 2.5. Consequently, this effect is believed to accelerate the release of extractable compounds from the disrupted plant cell walls, allowing for an increase in mass transfer and easier access of the solvent to the cell content (Chemat *et al*., 2011; Vinatoru *et al*., 1999). UAE has many advantages for the food industry, including reduced solvent consumption, avoidance of organic solvents (ultrasound is effective in 'Generally Regarded

as Safe (GRAS)' solvents) and reduced extraction time and temperature. Therefore, ultrasound allows for environmentally friendly extraction to be completed in minutes with high reproducibility and increased extraction yield under milder conditions (reduced processing temperature and pressure), which can potentially enhance the extraction of heat sensitive bioactive and food components, compared to the conventional extraction techniques (Galanakis, 2012; Vilkhu *et al*., 2008). In addition, there exists also the potential of using ultrasound to achieve simultaneous extraction and encapsulation of extracted components for protection (Vilkhu *et al*., 2008). Ultrasound has been successfully applied for extraction of phenolic compounds from various sources, indicating its potential industrial application (Table 2.2).

2.3.6 Extraction of honeybush polyphenols

Over the past decades numerous extraction techniques and their parameters for the recovery of phenolic compounds (including flavonoids) from plant material have been studied. Although basic concepts and extraction conditions could guide decision-making, differences in plant matrices would ultimately govern process conditions. To date limited research on extraction of polyphenols and specifically flavanone glycosides from *Cyclopia* spp. is available. From Table 2.3 it is evident that studies on the extraction and isolation of honeybush phenolic compounds are limited to classical solvent extraction. The focus was also on extraction for analytical purposes and compositional studies, and not on recovery of polyphenols in order to produce a food-grade extract. In the latter case other solvents should be used as previously mentioned (section 2.3.3). The food and pharmaceutical industries performed research work and obtained patents concerning extraction and purification of hesperidin from citrus in the 1940's and 1950's. These conventional methods were based on the alkaline treatment of citrus peel and precipitation of hesperidin from the subsequently acidified solutions, often followed by a crystallization step to increase the purity of the commercial product (Di Mauro *et al.*, 1999). Classical extraction methods gave reasonable recovery but posed numerous disadvantages resulting in the search for alternative, more environmentally friendly techniques, as discussed in the previous sections.

Table 2.3 Studies on the extraction and isolation of phenolic compounds from *Cyclopia* spp.

Species	Plant material	Analytes	Extraction technique	Extraction Solvent	Reference
C. intermedia	Fermented leaves and stems	Phenolic compounds (including mangiferin, hesperetin and hesperidin)	SOLVENT EXTRACTION	methanol or 70% acetone/water	Ferreira *et al.* (1998)
C. intermedia	Fermented leaves and stems	Phenolic compounds (including four flavanones)	SOLVENT EXTRACTION	methanol or 70% acetone/water	Kamara *et al.* (2003)
C. subternata	Unfermented leaves and shoots	Phenolic compounds (including hesperidin and mangiferin)	SOLVENT EXTRACTION	methanol or 70% acetone/water	Kamara *et al.* (2004)
C. subternata	Leaves	Mangiferin, isomangiferin	SOLVENT EXTRACTION	methanol	De Beer *et al.* (2009)
C. genistoides *C. intermedia* *C. maculata* *C. sessiliflora*	Unfermented plant material	Mangiferin, isomangiferin, hesperidin	SOLVENT EXTRACTION *(combined with sonication)*	methanol	Joubert *et al.* (2003)
C. subternata	Unfermented plant material	Mangiferin, hesperidin	SOLVENT EXTRACTION	water or 33% acetonitrile/water or 50/80/100% ethanol/water or 50/100% methanol/water or 70% acetone/water	Joubert *et al.* (2012)

2.3.7 Process optimisation techniques

2.3.7.1 Introduction

Experimental design and optimisation are tools used to systematically examine different types of obstacles that arise within the field of research, development and production. Optimisation procedures are followed to improve/optimise the performance of a system, process, product or analytical method to obtain the maximum benefit from it, i.e. the best possible response without increasing cost (Baş & Boyacı, 2007; Bezerra *et al*., 2008; Dejaegher & Van der Heyden, 2011; Lundstedt *et al*., 1998).

With the aid of optimisation techniques, the optimal settings for a number of factors and their level ranges in the experimental domain can be determined. Factors (or independent variables) are parameters that affect the responses or the outcome of a method or procedure, e.g. temperature, pH, reagents concentration and reaction time (Dejaegher & Vander Heyden, 2011). These factors and their range levels define the experimental domain (experimental 'area' investigated) in which the global optimum is potentially reached. The factors also may interact with each other, i.e. interaction can occur between two factors when the influence of one factor on the response is different at different levels of the second factor (Dejaegher & Vander Heyden, 2011), as was evident for temperature and solvent-solid ratio in UAE of citrus flowers (Yang *et al*., 2010).

The need to maximise the efficiency of scientific research and discovery, particularly to reduce waste and cost, has led to the use of smarter experiments that give the most information possible with minimum experiments performed (Hibbert, 2012). Although the classic concepts of experimental design have been in existence for more than 70 years, increased adoption of these optimisation methods in chromatography for separation of compounds in a given matrix and extraction of valuable phenolic compounds from various food sources was only evident in the past decade (Dejaegher & Vander Heyden, 2011; Hibbert, 2012; Leardi *et al*., 2009; Lai *et al*., 2013; Khan *et al*., 2010; Wijngaard & Brunton, 2010; Yang *et al*., 2010).

2.3.7.2 Simple univariate vs multivariate methods for optimisation

In most cases more than one factor needs to be optimised and multivariate optimisation strategies are used for the global optimisation of a process in which several factors are varied simultaneously; however, in the case of the optimisation of only one factor, a simple univariate procedure is performed. The so-called 'one-variable-at-a-time' (OVAT) approach is a classical procedure where only one factor at a time is varied, monitored and optimised, while the others are kept at a constant level (Bezerra *et al*., 2008).

However, the OVAT procedure poses many drawbacks as reviewed by Dejaegher & Vander Heyden (2011): OVAT does not take the interactive effects among factors into account; the number of experiments increases as the number of studied factors increases which leads to additional cost and solvent consumption; only local knowledge of the process is obtained as only a small part of the

experimental domain is examined (therefore the complete effects of the parameters on the process is not shown); and the global optimum might not be found.

In order to overcome the above-mentioned problems, the optimisation of various analytical and chemical procedures has been achieved by using multivariate statistical techniques (Fig. 2.6). Multivariate approaches are subdivided into sequential and simultaneous ones (Dejaegher & Vander Heyden, 2011). Sequential procedures may be applied when the experimental domain containing the optimum is *a priori* unknown; therefore, a few initial experiments may be conducted to identify the most influencing factors and to define the optimisation experiments to follow, although this approach is limited to the optimisation of only one response. Simultaneous approaches perform a predefined number of experiments, according to a well-defined experimental set-up, e.g. an experimental design. Experimental designs are experimental set-ups to simultaneously evaluate several factors at given number of levels in a predefined number of experiments. Different experimental designs can be distinguished by the model that is derived (linear or quadratic), constraints on factor levels, and the aim of the study, whether it is screening or optimisation of factors (Hibbert, 2012).

Experimental designs can be divided into screening designs (e.g. full factorial, fractional factorial, and Plackett–Burman designs where factors are typically evaluated at two levels), response surface designs and mixture designs. Response surface designs are used to find the optimal levels of the most important factors (which occasionally are selected from a screening design approach) and factors are examined at least at three levels. The optimal conditions are usually derived from response surfaces built with the design results. Mixture designs are response surface designs used when all factors examined are mixture-related, i.e. factors representing the fraction of a given component in a mixture, e.g. organic modifiers in a mobile phase in chromatography, or the excipients in a tablet or another pharmaceutical formulation (Dejaegher & Vander Heyden, 2011).

An advantage of experimental design is that one can obtain information using only a small number of factor levels; however, the choice of levels in a design is extremely important. Each factor level should be suitable and lead to useful information, i.e. the factor level values should not be too close or too far from each other to allow adequate variation in the response to be observed (Hibbert *et al*., 2012). For example, a central composite design combines a two-level factorial design with a star design and centre points, covering the factor space with more points near the centre than at the periphery (Fig. 2.7). This allows for a greater number of levels without performing experiments at every combination of factor levels (Hibbert *et al*., 2012).

One of the most relevant multivariate techniques used is response surface methodology, which will be discussed in the following section.

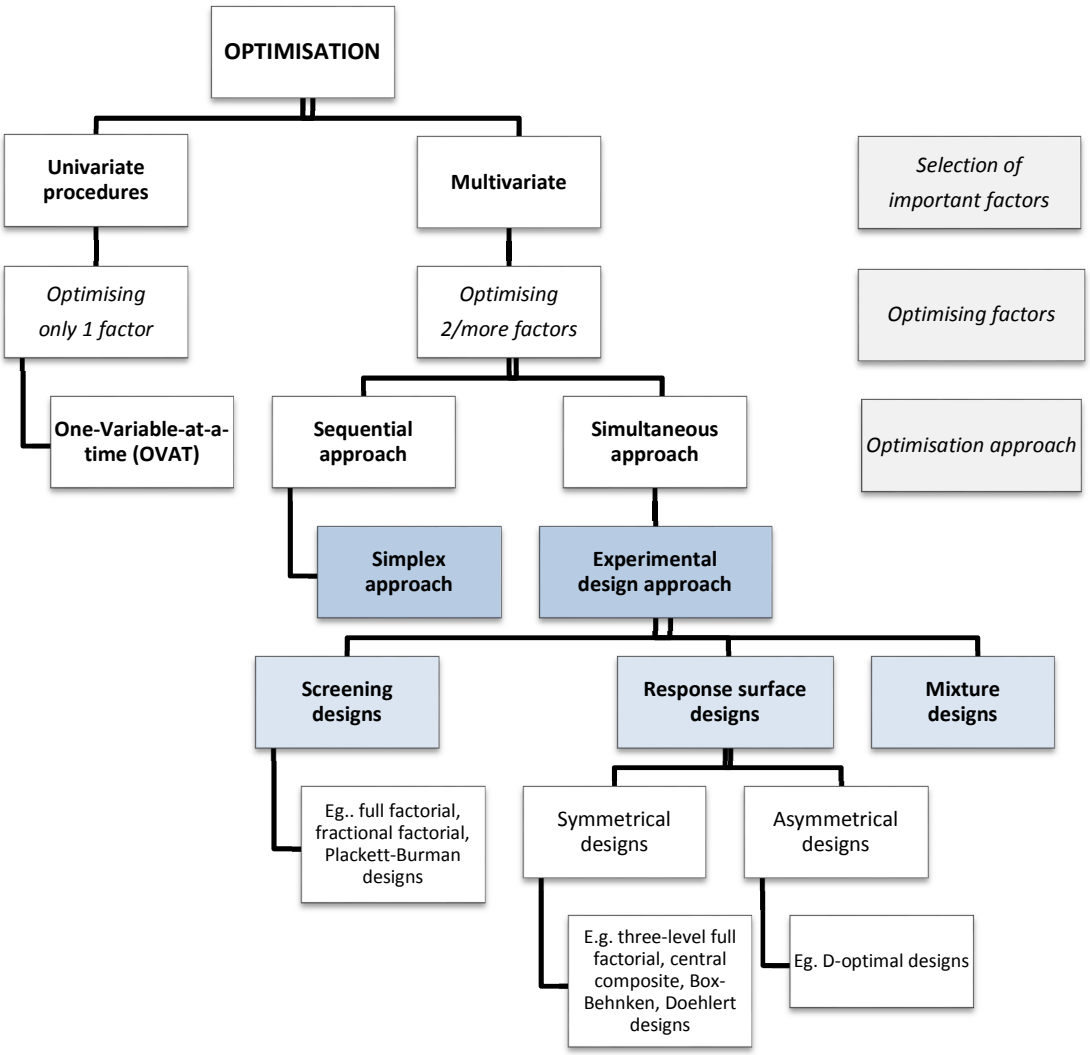


Figure 2.6 Optimisation strategies (adapted from Dejaegher & Vander Heyden, 2011).

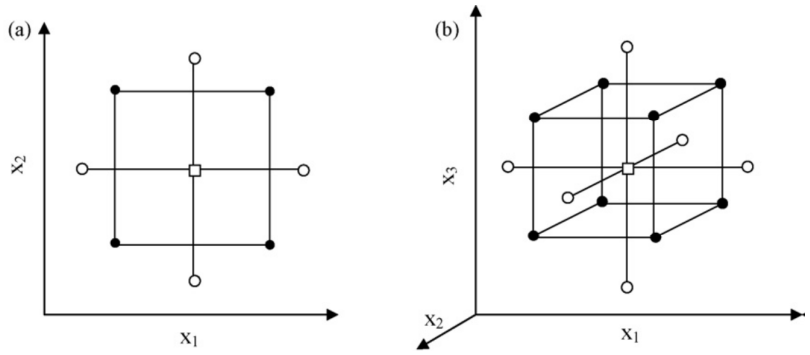


Figure 2.7 Central composite designs for the optimisation of (a) two variables and (b) three variables. (●) Points of factorial design, (○) axial points and (□) central point (Bezerra *et al*., 2008).

2.3.7.3 Response surface methodology as optimisation tool and its application

Response surface methodology (RSM) consists of a collection of mathematical and statistical techniques based on the fit of a mathematical model to the experimental data, obtained in relation to experimental design. Linear or quadratic polynomial functions are employed to describe the system evaluated and to explore the experimental conditions until its optimisation. It can be well applied when the response(s) of interest are influenced by several variables and the objective is to simultaneously optimise the levels of these variables to attain the best system performance (response) (Bezerra *et al*., 2008; Baş & Boyacı, 2007).

To apply RSM methodology, an experimental design has to be selected initially that will define which experiments in the experimental domain studied should be performed. Experimental designs such as three-level factorial, Box–Behnken, central composite, and Doehlert designs should be applied for quadratic response surfaces (Bezerra *et al*., 2008).

Stages in the application of RSM include: selection of independent variables that have major effects on the system via screening studies; delimitation of the experimental region (according to the objective of the study and the experience of the researcher); choice of the experimental design; performing of experiments according to the selected experimental domain; mathematic–statistical treatment of the obtained experimental data through the fit of a polynomial function; evaluation of the model's fitness; verification; obtaining the optimum values for each studied variable (Bezerra *et al*., 2008).

Using experimental results of a response surface design, a polynomial model (usually second-order) is constructed, describing the relation between a response (dependent variable) and the measured factors (independent variables). The model can be interpreted graphically (2-dimensional contour or 3-dimensional response surface plots) and/or statistically (analysis of variance, ANOVA). In Fig. 2.8 the contour plot shows the isoresponse lines as a function of the levels of two factors, whereas the response surface plot is the visualisation of the response in a third dimension, from which often the best or optimal conditions can be derived (Dejaegher & Vander Heyden, 2011).

RSM has important application in the design, development and formulation of new products, as well as in the improvement of existing product design. In addition, RSM has been used to optimise the extraction processes of polyphenols from various plant materials through several conventional (solvent/solid-liquid extraction) and novel 'green' methods (microwave-assisted, ultrasound-assisted and supercritical fluid extraction) (refer to Table 2.4).

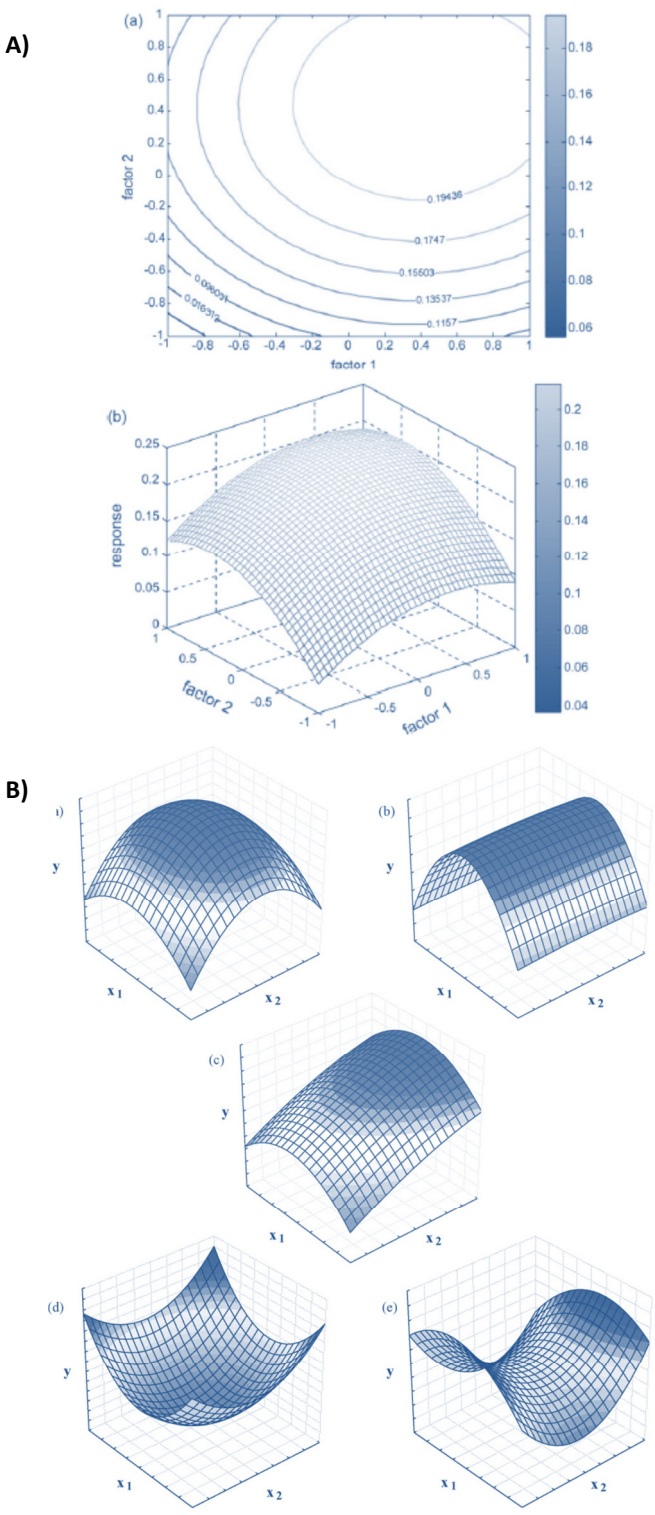


Figure 2.8 A) Example of a (a) contour and (b) response surface plot (Dejaegher & Vander Heyden, 2011); B) Examples of response surface plots as a functions of two factors showing (a) maximum response, (b) plateau, (c) maximum response outside the experimental region, (d) minimum response, and (e) saddle surface (Bezerra *et al.*, 2008).

Table 2.4 Studies on the optimisation of polyphenol extraction using RSM

Source	Analytes	Extraction technique	Solvent	Optimum Parameters	Recovery yield	Reference
Apple pomace	Phenolic compounds	ULTRASOUND-ASSISTED	Water	40°C, 40 min, 0.764 W/cm^2	Total phenolic content: 555 mg catechin equivalent/ 100 g dry sample	Pingret *et al.* (2012)
Apple pomace	Phenolic compounds	SOLVENT	acetone or ethanol	66% (v/v) ethanol , 80°C, 30 min or 65% (v/v) acetone, 25°C, 31 min	Total phenolic content: 2.37-6.35 mg gallic acid equivalent (GAE)/g dry sample	Wijngaard & Brunton (2010)
Citrus unshiu peels	Hesperidin and narirutin	SUBCRITICAL WATER	Water	160°C; 10 min	72 ± 5 mg hesperidin/g *C. unshiu* peel	Cheigh *et al*. (2012)
Citrus: thinned immature fruit peels	Hesperidin and narirutin	MICROWAVE-ASSISTED	ethanol	70% (v/v) ethanol; 140°C; 8 min	58.6 mg hesperidin/g plant material	Inoue *et al.* (2010)
Citrus flower (*Citrus aurantium* L.)	Flavonoids	ULTRASOUND-ASSISTED	Ethanol	51.2% (v/v) ethanol; 72.1°C; 51.9 min; 4:1 liquid:solid ratio	1.88% extract yield	Yang *et al.* (2010)
Gynura medica leaf	Flavonoids	SOLVENT	Ethanol	55% (v/v) ethanol; 92°C; 50:1 liquid:solid ratio	0.428 mg kaempferol-3-*O*-glucoside/g dry material	Liu *et al*. (2010)
Marigold - defatted (*Tagetes erecta* L.)	Polyphenols (flavonoids) correlated with antioxidant activity	SOLVENT	Ethanol	ABTS: 79.7% (v/v) ethanol; 74.2°C; 8.1 h DPPH: 89.3% (v/v) ethanol ; 81.5°C; 11.1 h	ABTS: 2.42 mmol Trolox equivalent (TE)/g dry basis DPPH: 1.86 mmol TE/g dry basis	Gong *et al.* (2012)
Orange peel	Flavanone glycosides	ULTRASOUND-ASSISTED	Ethanol	20% (v/v) ethanol; 40°C; 150 W	70.3 mg narirutin/100 g fresh weight 205.2 mg hesperidin/100 g fresh weight; 10.9% extract yield	Khan *et al.* (2010)
Olive tree leaves	Phenolic compounds	ULTRASOUND-ASSISTED	Ethanol	50% (v/v) ethanol; 50:1 liquid:solid ratio; 60 min	201.2 mg/g dry leaf 25.1 mg GAE/g dry leaf	Şahin & Şamli (2013)
Rice bran (outer layer of brown rice)	Phenolic compounds	ULTRASOUND-ASSISTED	Ethanol	65-67% (v/v) ethanol; 51-54°C; 40-45 min	Total phenolic content: 2.37-6.35 mg GAE/g dry sample	Tabaraki & Nateghi (2011)
Black Soybean sprouts	Polyphenols correlated with antioxidant activity	ULTRASOUND-ASSISTED	Water	29.19:1 liquid:solid ratio; 30°C; 32.13 min	67.6% of DPPH$^{\bullet}$-scavenging capacity	Lai *et al*. (2013)

2.4 Conversion of glycosides to aglycones

2.4.1 Chemistry of glycosides

Glycosides are present in numerous groups of valuable therapeutic compounds including antibiotics, alkaloids and flavonoids (Werner & Morgan, 2010). They are molecules that comprise of a sugar moiety, characteristically bound through its anomeric carbon to an aglycone (non-sugar) (Werner & Morgan, 2010). Phenolic OH-groups are in general good targets for biological glycosylation and many phenolic compounds are produced only as polyphenol glycosides (Kren & Martínková, 2001).

In general glycosides are more water soluble than their corresponding aglycone as the attached sugar moiety increases their hydrophilicity (Kren & Martínková, 2001). In certain circumstances the glycosidic residue is essential for their activity and in other cases glycosylation mainly improves membrane transport and pharmacokinetic parameters, such as distribution, excretion and the concentrations in the body fluids (Kren & Martínková, 2001). Flavonoid glycosides are recognised for their therapeutic properties and certain flavonoid glycosides are produced synthetically or via biotransformation, generally for pharmaceutical purposes (Kren & Martínková, 2001; Werner & Morgan, 2010). They are also recognised for their potential use as natural sweeteners (Werner & Morgan, 2010).

Refer to Section 2.2.1 for the phytochemistry of flavonoid glycosides.

2.4.2 Drivers for cleaving the sugar moiety

In the food and pharmaceutical industry, as well as the research laboratory there exist various different motives for the hydrolysis of the glycosidic bond in molecules, such as the synthesis of valuable chemicals, release of volatile compounds in fermented foods, de-bittering and clarifying of citrus juices, enhancement of therapeutic properties and bioavailability, and to aid structural elucidation and characterisation, as discussed in the following sub-sections.

2.4.2.1 Potential synthesis of valuable chemicals

Horowitz & Gentili (1969) reported that glycosidic flavonoids found in citrus peels could be converted to a new class of sweet compounds: for example, upon alkaline hydrogenation, the bitter neohesperidin (a flavanone-7-β-neohesperidose) was converted to an intensely sweet dihydrochalcone, neohesperidin dihydrochalcone (NHDC), that was found to be 1000 times sweeter than sucrose on a molar basis. Non-glycosidic dihydrochalcones such as hesperetin dihydrochalcone derivatives with simple 4-*O*-sulfoalkyl substituents display sweet taste properties that compare favourably with NHDC, as well as good water solubility properties (Seitz & Wingard, 1978). Hesperetin can be used as the basic building block for the development of potential sweeteners, and although this aglycone can be produced by total synthesis, a more

cost-effective preparation is the acid hydrolysis of the natural-occurring rutinoside, hesperidin (Seitz & Wingard, 1978).

Grohmann *et al.* (2000) demonstrated that through acid hydrolysis at elevated temperatures, hesperidin is a potential source of the valuable chemicals, rhamnose and hesperetin, and the chemical intermediate, hesperetin-7-*O*-glucoside. Rhamnose is a precursor for the synthesis of the flavour compound, furaneol, an important aroma component of fruits such as raspberry and strawberry, as well as processed foods (Maarse, 1991). Hesperetin has potential health-promoting properties (Garg *et al*., 2001) and has also demonstrated sweetness-enhancing properties (Ley *et al.*, 2011a). Through simple hydrogenation in alkaline media, hesperetin-7-*O*-glucoside can be converted to the intensely sweet hesperetin dihydrochalcone glucoside (Horowitz, 1986; Horowitz & Gentili, 1991).

2.4.2.2 Synthesis of volatile compounds and its release in fermented foods

A significant portion of aroma-precursors of tea, wine and other foods exist as disaccharide conjugates that become volatile after removal of the sugar moieties through enzyme-catalysed hydrolysis, which only then contribute to the flavour profile (Mazzaferro *et al.*, 2010; Minig *et al.*, 2011).

Various disaccharides exist in grapes, such as rutinosides, which are particularly abundant and have been found in several other fruits and plant material (Mazzaferro *et al*., 2010; Minig *et al*., 2011). Through enzymatic de-glycosylation, with the addition of commercially available enzyme preparations, aroma-precursors can be hydrolysed to release volatile compounds in a controlled manner (Genovés *et al*., 2005; Hemingway *et al*., 1999; Wang *et al.*, 2001). The potential use of these enzymes for biotechnological applications, such as aroma modulation in fermented foods (including wine), has been demonstrated (Mazzaferro *et al*., 2010).

It is important for analysis of plant-based foods that aroma precursors are readily available. Commercially available aromatic compounds are mostly acquired through extraction or chemical synthesis, although the building blocks required for synthesis are often unavailable or difficult to prepare. Glycosidase preparations have been shown to catalyse the synthesis of aroma precursors that can be used as standards for food analysis (Minig *et al*., 2011). For example, Minig *et al*. (2011) demonstrated that through significant hydrolysis of hesperidin (as rutinose donor), as well as transglycosylation in the presence of the aromatic compounds geraniol, nerol, or 2-phenylethanol (as alcoholic acceptor), their aroma-precursors were synthesised.

2.4.2.3 De-bittering and clarifying of fruit juices

Citrus flavonoids contribute to unpleasant characteristics of the fruit juices such as bitter taste and turbidity (clouding), and hesperidin in particular is responsible for clogging of steel pipes of citrus juice plants (Mazzaferro *et al*., 2010; Mazzaferro & Breccia, 2012). The addition of commercially available enzyme preparations allows for enzymatic de-glycosylation, hydrolysing these flavonoids and accordingly de-bittering and clarifying the juice (Mazzaferro *et al*., 2010). For example, hesperidinase is used to remove the bitter flavour in grapefruit juice through hydrolysis of naringin to naringenin, glucose and rhamnose, and to prevent the presence of hesperidin crystals in citrus products (Kim *et al*., 2009; Park *et al*., 2006; Rau *et al*., 1999).

2.4.2.4 Enhancement of therapeutic properties and bioavailability

Research has shown that enzymatic hydrolysis of specific glycosyl groups improved the bioavailability and biological properties of flavonoid glycosides.

This is evident in studies where enzymatic hydrolysis (or de-glycosylation) increased the anti-inflammatory activity of naringin (Amaro *et al*., 2009); improved the antioxidant activity of orange and lime juices to produce functional beverages (Da Silva *et al*., 2013); improved the protective effect (prevention of excessive lipid formation, protection of the antioxidant system and suppression of inflammation induction) against alcoholic liver disease of enzymatically modified citrus flavonoids (Park *et al.*, 2013); increased the anti-proliferative effect through synthesis of mono-glucosylated quercetin (quercetin-3-*O*-glucoside) from rutin (De Araújo *et al*., 2013); and increased the antimicrobial potency of citrus flavonoids (Mandalari *et al*., 2007).

A full or partial de-glycosylation step is critical for the absorption of flavonoids as reviewed by Crozier et al. (2009). Nielsen *et al.* (2006) demonstrated that the bioavailability of hesperidin in humans increased through its enzymatic conversion to hesperetin-7-*O*-glucoside. Park *et al*. (2013) also showed that enzymatic modification such as de-rhamnosylation can improve the bioavailability of hesperidin and narirutin.

2.4.2.5 Hydrolysis of glycosides as an aid to structural elucidation and characterisation

The use of hydrolysis (including acidic, enzymatic and alkaline) has been described as an aid to elucidate the structure of glycosides and the characterisation thereof, as reviewed by Antolovich (2000) and Robards & Antolovich (1997). It has been used to minimize interferences in subsequent chromatography and to simplify chromatographic data, especially when suitable standards are unavailable. Hydrolysis leads to a reduction in information, e.g. an extract that contains numerous *O*-glycosides of a single aglycone as well as the free aglycone will yield a single HPLC peak after acid hydrolysis. Therefore, acid or base hydrolysis can significantly simplify intricate HPLC profiles of complex phenolic-rich samples, simplifying interpretation and

quantification thereof (Antolovich, 2000; Robards & Antolovich, 1997). Additionally, Mazzaferro & Breccia (2012) developed an enzymatic–spectrophotometric technique to measure the concentration of hesperidin in orange juice samples by means of enzymatic hydrolysis of hesperidin, followed by the quantification of the resulting hesperetin.

2.4.3 Methods of conversion

2.4.3.1 Acid hydrolysis of glycosides

The rate of acid hydrolysis of glycosides is determined by the acid strength, the nature of the sugar and the location of attachment to the flavonoid nucleus, e.g. glycosides are cleaved rapidly while glucuronides resist acid hydrolysis, and *C*-glycosides usually remain intact (Antolovich & Robards, 1997). Due to its indiscriminate and more exhaustive nature, chemical hydrolysis has been favoured over enzymatic treatment for the structural elucidation of unknown flavonoids as it results in the reduction of information (Antolovich, 2000; Antolovich & Robards, 1997) as mentioned under 2.4.2.5. Different acids have been used for the hydrolysis of glycosides and patents have been developed for both strong mineral (Wingard, 1979), as well as organic acid (Hilmer *et al*., 2008) hydrolysis methods. Recently, hydrothermal hydrolysis of hesperidin catalysed by supercritical carbon dioxide has also been reported (Ruen-ngam *et al*., 2012).

2.4.3.1.1 Strong mineral acid as hydrolysis catalyst

The economic and generally applicable method involving regioselective alkylation and direct alkaline hydrogenation of hesperetin renders hesperetin dihydrochalcone derivatives as attractive candidates for potential development as food additives (Seitz & Wingard, 1978). However, as basic building block for these sweeteners, large quantities of highly pure hesperetin have to be readily available. As mentioned previously, a more cost-effective preparation is the acid hydrolysis of the naturally-occurring hesperidin (Seitz & Wingard, 1978). Acidic hydrolysis of hesperidin to hesperetin, rhamnose and glucose was first performed in the late 1800, employing ethanolic aqueous sulphuric acid at elevated temperatures (Tiemann & Will, 1881). However, due to the low solubility of hesperidin in ethanol, extended reaction times or pressure vessels are required (Seitz & Wingard, 1978).

Seitz and Wingard (1978) reported an improved method for the conversion of commercial hesperidin to high purity, crystalline hesperetin. It comprises of a purification step of the relatively crude hesperidin as starting material, isolated after the alkaline extraction of cut and pulped citrus peels, followed by a hydrolysis step, i.e. the sulphuric acid catalysed methanolic cleavage of the sugar residues. This process has been patented (Wingard, 1979). Requirements for this method include a starting hesperidin concentration in

alcohol preferably at 5-20% by weight, elevated reaction temperatures from 60-85°C and acid concentration 10-30 equivalents of acid per mole of hesperidin.

Grohmann *et al*. (2000) investigated the controlled (partial) hydrolysis of hesperidin to rhamnose and hesperetin-7-*O*-glucoside for the synthesis of valuable chemical intermediates such as dihydrochalcone sweeteners. The use of diluted sulphuric acid as catalyst significantly accelerated the hydrolysis of hesperidin-water suspensions at temperatures of 120°C and higher, which may be attributed to increased solubilisation of hesperidin in water at these high temperatures (Grohmann *et al*., 2000).

More recent efforts on hydrolysis of hesperidin has focussed on alternatives to acid hydrolysis catalysed by strong mineral acids, such as the use of organic acids as catalysts or enzymatic hydrolysis, as discussed in the following sections.

2.4.3.1.2 Organic acid as hydrolysis catalyst

Hilmer *et al.* (2008) demonstrated that surprisingly, organic acids are exceptional hydrolysis catalysts. A patent was developed for the method to release certain flavanone and dihydrochalcone aglycones using acid hydrolysis (Hilmer *et al*., 2008). The method claims the release of hesperetin, homoeriodictyol or eriodictyol from hesperidin, neohesperidin, eriocitrin, hesperetin-7-*O*-glucoside, eriodictyol-7-*O*-glucoside, homoeriodictyol-7-*O*-glucoside, homoeriodictyol-7,4”-di-*O*-glucoside or eriodictyol-7,4”-di-*O*-glucoside, as well as phloretin, 3-hydroxyphloretin or davidigenin from phloridzin, 3-hydroxyphloretin-2”-*O*-glucoside, davidigenin-2-*O*-glucoside or phloretin-2”-*O*-(β-D-xylosyl-(1-6)-β-D-glucoside) (Hilmer *et al*., 2008).

The hydrolysis steps require the suspension of e.g. hesperidin (preferably 10-30% by weight) in a solvent consisting of an organic acid with preferably two carbon atoms (e.g. citric acid, oxalic acid, lactic acid, acetic acid or mixtures thereof) in an amount which catalyses the acid hydrolysis in water, a heating temperature in the range of 125-145°C for 1-12 hrs, preferably at pH < 3 (Hilmer *et al.*, 2008).

Recently, carbonic acid was shown to catalyse the hydrolysis of hesperidin to hesperetin (Ruen-ngam et al., 2012). Hesperidin, dissolved in water, was subjected to elevated temperatures (110-140°C) in a high-pressure batch reactor followed by addition of liquefied CO_2 until the desired pressure of 25 MPa was reached. The resulting carbonic acid was shown to catalyse sequential cleavage of the rhamnose and glucose moiety, as well as direct cleavage of the glucosidic bond. The maximum hesperetin concentration was reached in 3 hrs.

2.4.3.2 Enzymatic de-glycosylation

The conventional chemical approach for de-glycosylation, such as acid hydrolysis, unavoidably leads to side reactions and undesirable by-products, due to the instability of flavonoids when kept at high temperatures and extreme pH values for long reaction times (Biesaga, 2011; Da Silva *et al*., 2013). In contrast, the biochemical (biotechnological) approach through enzymatic conversion by specific glycosyl hydrolases is regarded as far more advantageous due to the wide availability, high selectivity and low cost of enzymes, as well as the mild reaction conditions with fewer by-products (Da Silva *et al*., 2013; De Araújo *et al*., 2013). There has been significant scientific and industrial interest in enzymes for their role in modifying the structure of flavonoids and improving their physicochemical and biological characteristics (De Araújo *et al*., 2013).

2.4.3.2.1 Commercial enzymatic preparations of glycosyl hydrolases

Glycosyl hydrolases are an extensive group of enzymes that hydrolyse the glycosidic bond, and have been regarded as biotechnologically important due to their extensive application in the biotransformation of plant-based foods (Mazzaferro *et al*., 2010), as reviewed in Section 2.4.2.2.

The enzyme, α-L-rhamnosidase (E.C. 3.2.1.40), specifically splits off the terminal α-L-rhamnose from numerous natural glycosides, including hesperidin, naringin, rutin, quercitrin and terpenyl glycosides (Monti *et al*., 2004; Yadav *et al*., 2010). Currently there exist only two commercially available enzyme preparations with α-L-rhamnosidase activity, i.e. naringinase, obtained from *Penicillium decumbens*, and hesperidinase, obtained from *Penicillium* species or *Aspergillus niger* (Da Silva *et al*., 2013; Yadav *et al*., 2010; Rajal *et al*., 2009). Both preparations have also demonstrated significant β-D-glucosidase activity that catalyses the hydrolysis of β-D-glucosides, producing the corresponding aglycone and glucose (Yadav *et al*., 2010). Therefore, hesperidinase (hesperidin-α-1,6-rhamnosidase) expresses two enzymatic activities, namely α-L-rhamnosidase activity that cleaves hesperidin to yield rhamnose and hesperetin-7-*O*-glucoside, and β-D-glucosidase (EC 3.2.1.21) activity which subsequently splits hesperetin-7-*O*-glucoside to yield glucose and hesperetin (Kim *et al*., 2010, Furtado *et al*., 2012) (refer to Figure 2.9).

According to Furtado *et al.* (2012) certain aspects limit the use of enzymes as biocatalysts on an industrial scale, namely its instability and unsuitability for use in solvents, in the presence of denaturing agents and under extreme pH and temperature conditions. Another limitation is the low enzyme recovery yields and reuse in the soluble form. However, enzymatic immobilisation was shown to overcome these problems in large scale processes through obtaining a biocatalyst of which, compared to its free form, the activity and stability is not affected during the process. Advantages of enzymatic immobilisation include the protection from inactivation and denaturation in extreme conditions, subsequently forming a stable

microenvironment; potential to reuse the enzyme; facilitation of enzyme separation from the reaction medium; the capability to operate in continuous mode; prevention of processed product contamination; allowing the use of a higher enzyme concentration; and creating a greater reaction surface area (Polizzi *et al*., 2007; Nunes *et al*., 2010).

Other measures investigated to improve the bioconversion of hesperidin to hesperetin were the encapsulation and adsorption of hesperidinase immobilised in calcium alginate, κ-carrageenan and chitosan beads (Furtado *et al.*, 2012).

2.4.3.2.2 Bioconversion of flavonoids

Numerous successful studies on enzyme-catalysed hydrolysis of flavonoids have been reported and examples thereof will be briefly discussed in the following section to illustrate the various uses and potential applications of enzymes for bioconversion of flavonoids (as summarised in Table 2.5).

Park *et al*. (2006) investigated a commercially viable method for the production of the expensive flavonol, kaempferol, present in green tea. Previously they had established that a reasonable amount of kaempferol glycosides are present in green tea seeds. Park *et al*. (2006) reported an optimum enzyme combination of α-galactosidase and hesperidinase to prepare kaempferol (ca. 95% pure) through enzymatic hydrolysis of kaempferol-3-*O*-[2-*O*-α-D-galactopyranosyl-6-*O*-R-L-rhamnopyranosyl]-α-D-glucopyranoside and kaempferol-3-*O*-[2-*O*-α-D-xylopyranosyl-6-*O*-R-L-rhamnopyranosyl]-α-D-glucopyranoside. It was also demonstrated that the aglycone kaempferol had a higher antioxidant activity than the two glycosides.

Isoquercitrin (quercetin-3-*O*-glucoside), a scarce flavonol glycoside known for various biological activities, is the primary synthetic precursor for enzymatically modified isoquercitrin (EMIQ) that was recently approved as a new multiple food additive (Shimada *et al.*, 2010; *Salem et al*., 2010). However, as it constitutes only about 0.1% of plants, isolation of isoquercitrin is far less feasible than the highly abundant rutin (Wang *et al.,* 2012; Williams & Wender, 1953). Compared to conversion of rutin to isoquercitrin using acid (Wang *et al*., 2011), heat (Weignerová *et al.*, 2012) and microbes (Weignerová & Křen*,* 2010, Weignerová *et al.*, 2012; Manzanares *et al*., 1997), enzymatic hydrolysis has shown to have greater potential due to its high specificity, yield, and productivity (Weignerová & Křen, 2010). Wang *et al.* (2012) demonstrated the potential to produce isoquercitrin through hesperidinase-catalysed hydrolysis of rutin, by selectively removing the terminal rhamnose, rendering it an inexpensive and industrially feasible method. The optimum enzymatic reaction conditions were determined with 40°C as optimal temperature, 0.08 g/L as the optimal substrate concentration and 30 hrs as the optimum reaction time. The β-D-glucosidase activity was suppressed by adjusting the pH to 7.0. Furthermore, conversion of rutin to isoquercitrin was increased by the addition of certain metal ions, especially Zn^{2+}-ions ($ZnCl_2$).

De Araújo *et al.* (2013) also investigated the enzyme catalysed de-glycosylation of rutin for the synthesis of mono-glucosylated quercetin, isoquercitrin, as a good alternative for obtaining compounds with enhanced functional properties. Previously it has been reported that quercetin glycosides show higher solubility in water than quercetin (Aherne & O´Brien, 2002) and is better absorbed in comparison with rutin and quercetin (Arts *et al*., 2004). De Araújo *et al.* (2013) demonstrated that heated hesperidinase from *Penicillium* sp. (to inactivate the undesirable α-D-glucosidase activity) efficiently converted rutin to isoquercitrin. Furthermore, isoquercitrin exerted a more potent antiproliferative effect than quercetin or rutin on various cancer cell lines, indicating that it could be a promising functional derivative obtained by rutin de-glycosylation.

Da Silva *et al.* (2013) used hesperidinase, naringinase and α-D-glucosidase to catalyse the de-glycosylation of flavonoids in orange and lime juices. For example, after 4 hrs 60% of the hesperidin in the orange juice was converted to hesperetin by hesperidinase. The results showed that the antioxidant activity of the juices was enhanced by enzymatic hydrolysis, indicating the potential of the use of α-L-rhamnosidases to produce functional beverages.

Park *et al.* (2013) investigated the effects of orally administrated citrus flavonoids, hesperidin and narirutin, and their enzymatically modified versions to prevent alcohol liver disease through preventing excessive lipid formation, protecting the antioxidant system and suppressing inflammation induction in hepatocytes. Results indicated that their effectiveness can be improved through both hesperidinase-catalysed de-rhamnosylation and cyclodextrin-glucanotransferase-catalysed glycosylation resulting in hesperetin-7-*O*-glucoside and naringenin-7-*O*-glucoside, and glycosylated hesperidin and narirutin, respectively. Hesperetin-7-*O*-glucoside showed the highest absorption values indicating that de-rhamnosylation is an efficient tool for enhancing rapid absorption of flavonoids (Park *et al.*, 2013).

Furthermore, Kim *et al*. (2009) converted naringin and neohesperidin present in *Dangyuja* (*Citrus grandis* Osbeck) to their corresponding aglycones by naringinase and hesperidinase, and subsequently to their hydroxylated forms by *Aspergillus saitoi*. The results indicated that the conversion of these glycosides in *Dangyuja* extract through enzymatic and microbial treatment (fermentation) led to an increased antioxidant and anti-diabetic activity, showing potential use for the development of pharmaceutical foods to control the blood glucose level of diabetic patients.

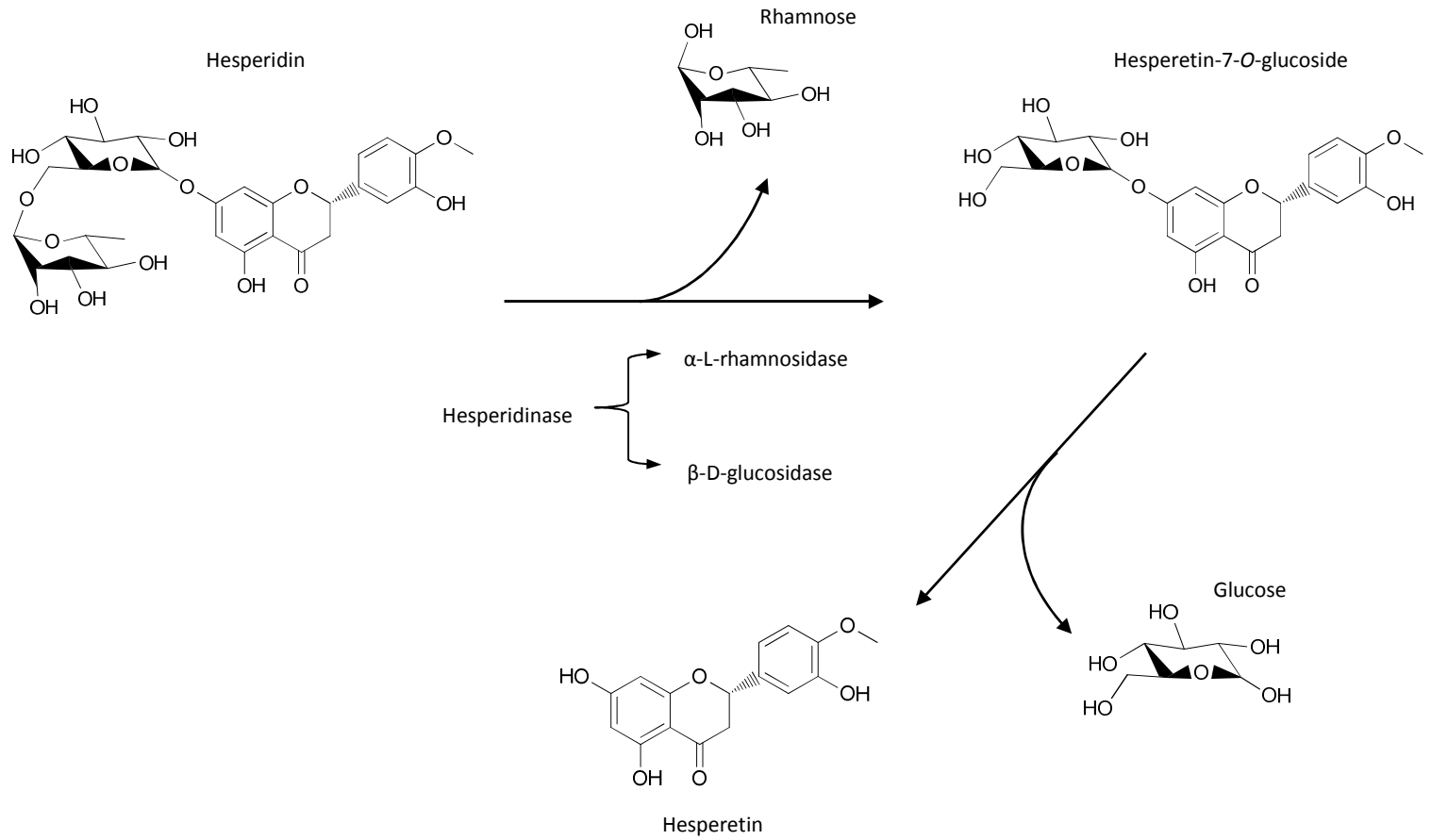


Figure 2.9 Hydrolysis of hesperidin to hesperetin, rhamnose, and glucose by hesperidinase expressing α-rhamnosidase and β-glucosidase activities (Furtado *et al.*, 2012).

2.4.3.2.3 Factors affecting bioconversion

Studies have shown that the hydrolysis parameters i.e. pH, temperature, substrate and enzyme concentration, agitation, time and metal ions/chelators affect the catalytic activity of hydrolases, as discussed in the following section.

Da Silva *et al.* (2013) evaluated the two crucial independent variables, pH and temperature, that significantly affect the enzyme activity of hesperidinase, naringinase and β-D-glucosidase. All the enzymes presented activity in acidic pH, however, at extreme pH values, such as pH 2.0, their activity was low. The highest (60°C) and the lowest (20°C) temperatures evaluated were also not favourable for enzymatic activities. This could be attributed to the fact that the energy provided by the high temperature causes certain intra-molecular attractions between polar groups, as well as hydrophobic forces between non-polar groups within the enzymatic protein structure to break, resulting in an altered active site unable to accommodate the substrate molecule (Lu *et al.*, 2006; Da Silva *et al.*, 2013). Furthermore, unlike psychrophilic (cold-active) enzymes that have the necessary conformational flexibility to the active site (owing to e.g. reduced hydrophobic interactions between subunits and lack of salt bridges), their mesophilic counterparts are less flexible resulting in lower activity at lower temperatures, as reviewed by Russell (2000). The results indicated that optimal conditions for the required conversions by hesperidinase, naringinase and β-D-glucosidase were pH 4.0 and 40°C (Da Silva *et al.*, 2013).

Similar to the findings of Da Silva *et al.* (2013), maximum hydrolytic activity of hesperidinase was in the pH range of 4.5 and 5.0, with maximum activity at pH 5.0 and 45°C, although the buffer at pH 4.0 allowed for a higher activity at 60°C (Furtado *et al.*, 2012). However, more pronounced enzyme deactivation was observed at temperatures above 60°C. Furtado *et al.* (2012) further investigated the effect of agitation type (horizontal shuttle vs orbital agitation), agitation velocity, substrate and enzyme concentration. Agitation achieved with an orbital shuttle type rotation at 150 rpm gave higher % bioconversion than at speeds of 100 and 200 rpm. Regardless of the low solubility of hesperidin in the reaction medium, enzymatic hydrolysis was performed with hesperidin in suspension (>0.1 mg/ml) and dissolved (≤0.05 mg/ml). The influence of a substrate concentration range of 0.05-0.4 mg/ml was evaluated and a nearly two-fold increase in enzyme activity was observed at 0.4 mg/ml compared to 0.05 mg/ml, indicating the capability of hesperidinase to degrade insoluble hesperidin (45°C in 0.02 M acetate buffer, pH 4.0). The influence of an enzyme concentration range of 5–15 mg/ml was evaluated (at 0.2 mg/ml substrate in 0.02 M acetate buffer, pH 4.0, 45°C and 150 rpm) and a significant increase in activity was observed from 12.5 to 15 mg/ml.

Wang *et al.* (2012) evaluated the effect of pH, temperature, time and metal ions and chelators on hesperidinase activity in the conversion of rutin to isoquercitrin. The optimal pH values for isoquercitrin and quercetin production were 6.0 and 5.0, respectively. The presence of β-D-glucosidase activity in hesperidinase is highly undesirable for the biotransformation of rutin to isoquercitrin, since conversion of

isoquercitrin to quercetin also takes place. However, for efficient control of the bioconversion process, control of pH instead of temperature was found to be more feasible, as high temperatures (usually 70°C) deactivates both β-D-glucosidase and α-L-rhamnosidase (Weignerová *et al*., 2012). Wang *et al.* (2012) reported that with increase in time, conversion of rutin to isoquercitrin increased linearly, and after 30 hrs, the isoquercitrin content reached a stable value. Furthermore, the effects of metal ions and chelators (at a final concentration of 1.0 mM to the reaction mixture) on enzyme activity were tested and it was found that most of the metal ions had either slightly stimulatory or inhibitory effects on the hesperidinase activity. The metal salts, $MnSO_4$ and $FeCl_3$, were strong inhibitors during the conversion of rutin. However, $ZnCl_2$, $MgSO_4$, $AlCl_3$, KCl, and Li_2SO_4 were found to increase the relative activities of hesperidinase, with $ZnCl_2$ demonstrating the highest effect.

2.4.3.2.4 Novel enzymatic preparation, α-rhamnosyl-β-glucosidase, and its application

Mazzaferro *et al.* (2010) screened for microorganisms that are able to use flavonoids as a carbon source and found one isolate, nominated *Acremonium* sp. SES201, to possess a disaccharide-specific hydrolase, i.e. α-rhamnosyl-β-glucosidase, which was able to cleave hesperidin to yield rutinose and hesperetin. Only intracellular traces of monoglycosidase activities (β-glucosidase, α-rhamnosidase) were produced. Studies on substrate preference revealed that the enzyme demonstrated high specificity toward flavonoid-7-*O*-rutinosides (such as hesperidin and eriocitrin), with no activity towards flavonoid-3-*O*-rutinosides, flavonoid-7-*O*-neohesperidosides and monoglycosylated substrates (Mazzaferro & Breccia, 2012). Although the commercially available naringinase and hesperidinase can be used for hesperidin de-glycosylation, they do not offer the required selectivity for hesperidin de-glycosylation in the presence of the several glycosides present in fruits, whereas α-rhamnosyl-β-glucosidase was demonstrated to be highly specific for the de-glycosylation of flavonoid-7-*O*-rutinosides in one single step (Mazzaferro *et al*., 2010).

Mazzaferro & Breccia (2012) developed a simple enzymatic–spectrophotometric method for the quantification of hesperidin in citrus-based foods as alternative to more expensive and time-consuming HPLC methods using the novel enzyme, α-rhamnosyl-β-glucosidase (DSM 24697) that can be applied in biochemical research, as well as by the citrus fruit industry. Upon addition of α-rhamnosyl-β-glucosidase, the glycosidic bond between the aglycone and sugar moiety was hydrolysed to yield hesperetin and rutinose, resulting in 100% conversion of hesperidin (Mazzaferro & Breccia, 2012). The enzyme was active over a broad pH range (>60% activity in the range 4.0–8.0).

Furthermore, Minig *et al.* (2011) also demonstrated that, in an aqueous medium, this unique enzyme was capable of transferring rutinose to other acceptors in addition to water, indicating its potential as biocatalyst for organic synthesis.

Table 2.5 Enzymatic conversion of flavonoid glycosides with commercial enzyme preparations

Substrate	Product	Enzyme	Reaction conditions			% Conversion	Reference
			Substrate concentration	Enzyme concentration	Time, temperature, pH, agitation		
Rutin	Quercetin-3-*O*-glucoside Quercetin	**Hesperidinase ex *Penicillium* spp. Naringinase ex *P. decumbens*** *(expressing maximum of α-L-rhamnosidase activity; β-D-glucosidase was inactivated via initial heating at 70°C for 30 minutes)*	4 mL of 1% m/v rutin solution (previously dissolved in 1 mL MeOH)	100 µL of 50 mg/L in 0.05 M acetate buffer	2, 4, 8, 12 hrs 40°C pH 4.0 130 rpm	ca 70% rutin conversion to quercetin-3-*O*-glucoside after 4 hrs	De Araújo *et al*. (2013)
Orange and lime juice (hesperidin and rutin used as standards)	Hesperetin Quercetin-3-*O*-glucoside Quercetin	**Hesperidinase ex *Penicillium* spp. Naringinase ex *P. decumbens* β-D-glucosidase ex *Aspergillus niger***	100 mL juice Standards: 10 mM of rutin/hesperidin solution in MeOH (15% v/v)	*Juice:* 1 mL enzyme solution (0.02 mg/mL in 0.1 M acetate buffer) *Standards:* 0.02 mg/mL in 0.1 M acetate buffer	2, 4, 8, 12, 16 hrs 40°C pH 4.0 130 rpm	60% conversion of hesperidin into hesperetin in orange juice after 4 hrs	Da Silva *et al*. (2013)
Hesperidin	Hesperetin	**Hesperidinase ex *A. niger***	0.2 mg/mL in 0.02 M acetate buffer	10 mg/mL	60°C pH 4.0 150 rpm	*Not reported*	Furtado *et al*. (2012)
Orange juice (~90% hesperidin content)	Hesperetin-7-*O*-glucoside	**Hesperidinase *Amano Concentrate***	1 L juice	100 mL enzyme solution in 10 µmol/L acetic acid buffer	4 hrs 70°C pH 3.5	*Not reported*	Nielsen *et al*. (2006)
Green tea seed extract (kaempferol glycosides)	Kaempferol	**Hesperidinase ex *Penicillium* spp. β-galactosidase ex *A. oryzae*** *(in combination)*	0.5 g in 8 mL 0.02 M sodium acetate buffer	2 mL (50 units/g of GTSE in final conc)	37°C pH 5.0 stirring *(rpm not reported)*	*Not reported*	Park *et al*. (2006)
Rutin	Isoquercitrin	**Hesperidinase ex *A. niger***	0.08 g/L	0.04 g/mL	30 hrs 40°C pH 7.0 120 rpm	ca 45% conversion	Wang *et al*. (2012)
Rutin	Isoquercitrin	**Hesperidinase ex *A. niger*** *(investigated use of "green" non-molecular solvents, ionic liquids, for increased solubility)*	0.2 g/L	0.05 g/mL [Bmim][BF_4]-glycine-sodium hydroxide buffer (10:90, v/v)	10 hrs *(reduced from 30 hrs using ionic liquids)* 40°C pH 9.0 120 rpm	93% conversion (91% yield isoquercitrin)	Wang *et al*. (2013)

2.5 Taste modulation and its application in the food industry

2.5.1 Global health concerns and its influence on the food industry

2.5.1.1 Obesity, a 21st century epidemic

In recent times, concerns over the control of food intake and its relation to health and disease have intensified with the recognition of obesity as a global epidemic. In 2008 1.46 billion adults worldwide were estimated to be overweight, and of these, 205 million men and 297 million women were obese (Finucane *et al*., 2011).

The World Health Organization (WHO) defines obesity as a disease in which excess body fat has accumulated to such an extent that health and well-being of the individual may be adversely affected. According to its criteria, obesity can be defined as a body mass index (BMI) of >30 kg/m^2 (Goedecke, *et al*. 2006; Mollentze, 2006; Rossouw *et al*., 2012). It has been found that obesity has many adverse health consequences including insulin resistance, type 2 diabetes, hypertension, coronary heart disease, osteoarthritis and cancer (Mollentze, 2006).

The obesity epidemic could be viewed as the result of dysfunctional ingestive behaviour over the past decades: meal sizes have increased and calorie-dense foods, high in fat and carbohydrates, are consumed easily and increasingly (Sprous & Palmer, 2010). Sugars that are used to sweeten food products are regarded as highly appetitive macronutrients, and numerous studies have shown that a large portion of the rising incidence of obesity can be explained by increased intake of sugars, particularly in sweetened beverages. These easily metabolised carbohydrates allow the blood sugar level to rise significantly causing the formation of fatty deposits, which ultimately leads to health problems such as excess weight and obesity (Sprous & Palmer, 2010).

Ironically, whereas undernutrition and communicable diseases were once the prevailing health threat in developing countries, it is now projected that non-communicable diseases, such as obesity-associated disorders, could be the cause of 7 out of every 10 deaths by 2020. Overweight and obesity growth rates in children and adolescents are increasing globally, with the highest to be found in Africa where the number of overweight or obese children has doubled since 1990 (Rossouw *et al*., 2012). Many middle- and low-income countries, such as South Africa, face the so-called double burden of disease, where overweight/obesity contribute to the burden of disease caused by undernutrition and communicable diseases. The co-occurrence of undernutrition and overweight/obesity in the same population or household, confirms malnutrition as a key contributor to the double burden of disease (Rossouw *et al*., 2012).

Diets rich in refined fats, oils and carbohydrates and physical inactivity are regarded as the two predominant causes of the alarming increase in overweight and obesity occurrence in developing countries (Rossouw *et al*., 2012). South Africa is following the global trend of this increase and disturbing statistics show that more than 60% of South Africans are overweight or obese and 25% of teens and 17% of children under the age of 9 years are overweight (Anonymous, 2012b). In addition, populations that

are in a rural-to-urban transition phase such as South Africa may experience an increase in overweight and obesity (Rossouw *et al.*, 2012).

As the high consumption of calorie-rich sugar-sweetened food is raising increased health concerns, it has long been the aim of the food and beverage industry to reduce the sucrose content of their products to the absolute minimum, without sacrificing their palatability. Sugar substitution through the addition of sweeteners has been the answer as these substances have no or only very low calorific values yet give simultaneously a strong sweet taste impression (Kinghorn *et al.*, 2010).

2.5.1.2 The sweetener industry

2.5.1.2.1 Quest for healthy sugar substitutes

The most commonly used natural sweetener in the world is sucrose, so-called table sugar, a disaccharide (α-D-glucopyranosyl-(1→2-β-fructofuranoside) that is produced from sugarcane and sugar beet (Kinghorn *et al*., 2010). The high daily intake of sucrose has been reported to be involved in the development of several health problems as mentioned in the previous section. Apart from major non-communicable diseases, the adverse effect of sugar is also notable in dental caries since many carbohydrates such as sucrose, lactose, glucose and fructose are broken down by certain bacteria in the oral cavity, to e.g. lactic acid which may attack the tooth enamel, which ultimately leads to tooth decay (Kinghorn *et al*., 2010).

Accordingly, there has been an increasing demand for new highly sweet, non-caloric, and non-cariogenic sucrose replacements by the food and beverage industry. The sweetener market presently accounts for approximately $1 billion in sales in the United States alone (Kinghorn *et al*., 2010).

With caloric-carbohydrate-sweetened beverages being scrutinised as major contributors to the obesity epidemic spreading through developed countries, low-calorie or diet drinks are becoming ever more popular, with more than 20% of new product launches positioned as low-calorie, reduced-sugar or sugar-free in 2010 (Johannes, 2011). In South Africa there is an awareness of health and energy intake among more affluent consumers and sugar-free or reduced kilojoules products are niche market, catering to less than 20% of the population (Anonymous, 2012c).

2.5.1.2.2 High-potency sweeteners

Sweet-tasting sucrose substitutes (synthetic or natural) can be categorized into two groups, namely: 'high intensity', 'low-calorie' or 'non-caloric' sweeteners, which are 50–100 to several thousand times more intensely sweet than sucrose and 'bulk' or 'reduced-calorie' sweeteners, such as certain monosaccharides, disaccharides, and polyols, which have approximately the same sweetness intensity as sucrose (Kinghorn *et al*., 2010; Magnuson, 2012).

The synthetic sweeteners acesulfame-K, alitame, aspartame, cyclamate, neotame, saccharin, and sucralose (trichlorogalacto sucrose) are currently available in the food and beverage industry (refer to Table 2.6). These sweeteners were submitted to comprehensive safety evaluation, metabolism and

stability testing before finally being approved by the Codex General Standard for Food Additives (GFSA) and an acceptable daily intake (ADI) for each sweetener was established by the WHO's Joint Expert Committee on Food Additives (JECFA) (Anonymous, 2012c; Kinghorn *et al.*, 2010).

With the interest in natural and clean-label foods, interest in more natural ingredients, including natural sweeteners extracted from source (e.g. agave nectar and monk fruit), are on the rise (Anonymous, 2012a; Anonymous, 2009). To date only a limited number of natural sweet-tasting plant-derived products have been launched commercially as sucrose substitutes. These include glycyrrhizin, mogroside V, phyllodulcin, rebaudioside A, stevioside, and thaumatin. The steviol glycosides, stevioside and rebaudioside A (so-called reb A) are extracted from the leaves of *Stevia rebaudianum*, and are probably the most well-known and published sweet glycosides with a sweetness intensity of 200-300 times greater than sucrose. Another important natural glycoside is glycyrrhizin, the flavouring agent of licorice (*Glycyrrhia glabra*), about 50 times as sweet as sucrose; however, it has been reported to induce hypertension at high doses (Temussi, 2007). The aglycone of NHDC represents an entire class of natural compounds that can be called isovanillyl sweet compounds since they contain the isovanillyl group (3-hydroxy-4-methoxyphenyl). The most representative natural isovanillyl molecule is phyllodulcin (Anonymous, 2012a; Magnuson, 2012; Martin, 2013; Kinghorn *et al.*, 2010; Temussi, 2007).

Naturally occurring 'bulk' or 'reduced-calorie' sweeteners that are commercially available as either foods or food additives include fructose and D-tagatose (monosaccharides), isomaltulose and trehalose (disaccharides); erythritol, mannitol, sorbitol, and xylitol (monosaccharide polyols), and lactitol and maltitol (disaccharide polyols) (Kinghorn *et al.*, 2010). Erythritol, regarded as one of the "big alternative" sweeteners for the beverage industry, is not fully metabolised and therefore results in a caloric contribution that is mostly negligible. This four-carbon sugar alcohol is about 60% less sweet than sucrose, while having a similar bulk density and can be well-paired with other high-potency sweeteners, particularly stevia-based sweeteners, and providing sugar mouthfeel and functionality, as well as flavour-masking properties (Anonymous, 2012a).

The best recognised proteins that exhibit a very strong, sweet taste are brazzein, monellin and thaumatin, however, these proteins have not been used as sweeteners thus far (Temussi, 2007).

2.5.1.2.3 Shortcomings of sweeteners

While non-nutritive, highly intensive sweeteners are very suitable for bringing sweetness into foods at low concentrations, they often show taste-related problems as a result of time-intensity profiles which are not similar to sugar (e.g. sucralose, stevioside, cyclamate), bitter and/or astringent or metallic aftertastes at high concentrations (e.g. acesulfame K, saccharin), or pronounced additional flavour impressions (e.g. glycyrrhetinic acid ammonium salt). Some of the sweeteners are very long-lasting in terms of their sweet effect and therefore give a strong sweet aftertaste (e.g. saccharin) (Kinghorn *et al*.,

2010; Kuhn *et al*., 2004; Ott *et al*., 1991; Schiffman *et al*., 1995) (refer to Table 2.6 for list of permissible sweeteners in South Africa).

It is therefore desirable to find novel substances which, in low concentrations, effectively enhance the sweet taste impressions of sweet substances, in particular the sweet taste impression of reduced-sugar foods, without unfavourably affecting the remaining flavour profile. Preferably, such enhancer would not produce sweetness on its own but would enhance the sweetness intensity of a reduced amount of sugar or sweetener (Bryant *et al*., 2008).

To overcome the numerous limitations of sweeteners, there exists an extensive search for these sweetener enhancers or taste modulators. The following section deals with taste modulation, following a brief summary of introduction of sweet taste perception.

2.5.2 Taste modulation

2.5.2.1 Sweet taste receptor

The sense of taste plays a key role in the selection of food and has evolved to serve as a main regulator and driver in feeding behaviour allowing for the detection of useful foods and avoidance or rejection of toxic substances (Temussi, 2007; Yarmolinsky *et al.* 2009).

Sweet taste plays a central role for humans since the majority of people react positively towards the sensation of sweetness. It is hypothesised that the role of sweet taste is to identify nutritive food sources rich in sugar for energy, even though many naturally sweet molecules have no nutritional value. Umami taste (a savoury taste stimulated by certain L-amino acids) has evolved to recognise foods that are rich in amino acids for protein synthesis. In contrast, bitter and sour tastes alert the organism to toxins and low pH respectively, to promote the rejection of harmful substances such as poisonous plants or spoiled/unripe fruits. Saltiness can either be attractive or repulsive, depending on the concentration of sodium and the physiological needs of the organism (Fujiwara *et al.*, 2012; Temussi, 2007, 2009; Yarmolinsky *et al.* 2009). At present, it has been established that the basic tastes (sweet, bitter, sour, umami and salty) are mediated by distinct groups of selectively tuned taste receptor cells. Interestingly, according to Yarmolinsky *et al.* (2009), since taste buds from all areas of the oral cavity contain cells that respond to these five modalities, contrary to popular belief, there exists no topographic map (i.e. a tongue map) of taste qualities on the tongue.

Sweet taste reception is mediated by only one sweet taste receptor, composed of two distinct subunits, T1R2 and T1R3, positioned at the surface (apical membrane) of taste receptor cells located in the taste buds (Fujiwara *et al.*, 2012; Kinghorn *et al.*, 2010; Servant *et al.*, 2010; Temussi, 2009). Fig. 2.10 depicts actives sites of the T1R2/T1R3 sweet receptor.

Insight into structure activity relationship (SAR) of sweet molecules revealed that the human sweet receptor has the capacity to accommodate extensive structurally diverse ligands, but with the presence of AH-B groups in which the AH group is a hydrogen donor and the B group an electronegative

group. According to this theory, the skeleton of all sweet-tasting compounds has a hydrogen bond donor (AH) and at close proximity, a hydrogen bond acceptor (B) such as oxygen or nitrogen, separated by a distance of 2.5–4.0 A°. These AH-B groups react with a corresponding AH–B pair on the membrane receptor (Kinghorn *et al.*, 2010; Kovacic & Somanathan, 2012; Temussi, 2007; Yarmolinsky *et al.*, 2009).

Table 2.6 List of permissible sweeteners in South Africa (Department of Health, 2012) and their maximum permissible amount (CODEX General Standard for Food Additives 192-1995)

Sweetener	**Maximum permissible amount** *(depending on the food application)*	**Reported shortcomings**	**Reference**
Acesulfame potassium	200-1000 mg/kg	Bitter and/or astringent or metallic aftertastes	Kuhn *et al*. (2004) Ley *et al*. (2008a)
Alitame	40-300 mg/kg	-	
Aspartame	300-10000 mg/kg	Not stable in all applications	Ley *et al*. (2008a)
Aspartame-acesulfame salt	200-1000 mg/kg	-	
Calcium cyclamate	250-3000 mg/kg	Time-intensity profile not similar to that of sugar	Ley *et al*. (2008a)
Calcium saccharin	80- 2500 mg/kg	-	
Cyclamic acid	250-3000 mg/kg	-	
Erythritol	Tba*	-	
Isomalt (Hydrogenated isomaltulose)	Tba*	-	
Lactitol	Tba*	-	
Maltitol	Tba*	-	
Maltitol syrup	Tba*	-	
Mannitol	Tba*	-	
Neotame	10-1000 mg/kg	-	
Polyglycitol syrup	Tba*	-	
Potassium saccharin	80- 2500 mg/kg	-	
Saccharin	80- 2500 mg/kg	Bitter and/or astringent or metallic aftertastes; long-lasting strong sweet aftertaste	Kuhn *et al*. (2004) Ley *et al*. (2008a)
Sodium cyclamate	250-3000 mg/kg	-	
Sodium saccharin	Tba*	Bitter with increase in concentration	Schiffman *et al.* (1995)
Sorbitol	Tba*	-	
Sorbitol syrup	Tba*	-	
Steviol glycosides	100-3500 mg/kg	Time-intensity profile not similar to that sugar	Ley *et al*. (2008a)
		Bitter with increase in concentration	Schiffman *et al.* (1995)
Sucralose (Trichlorogalactosucrose)	150-5000 mg/kg	Time-intensity profile not similar to that of sugar	Ley *et al*. (2008a)
Thaumatin	Tba*	Low heat stability	Ley *et al.* (2011a)
Xylitol	Tba*	-	

** To be advised*

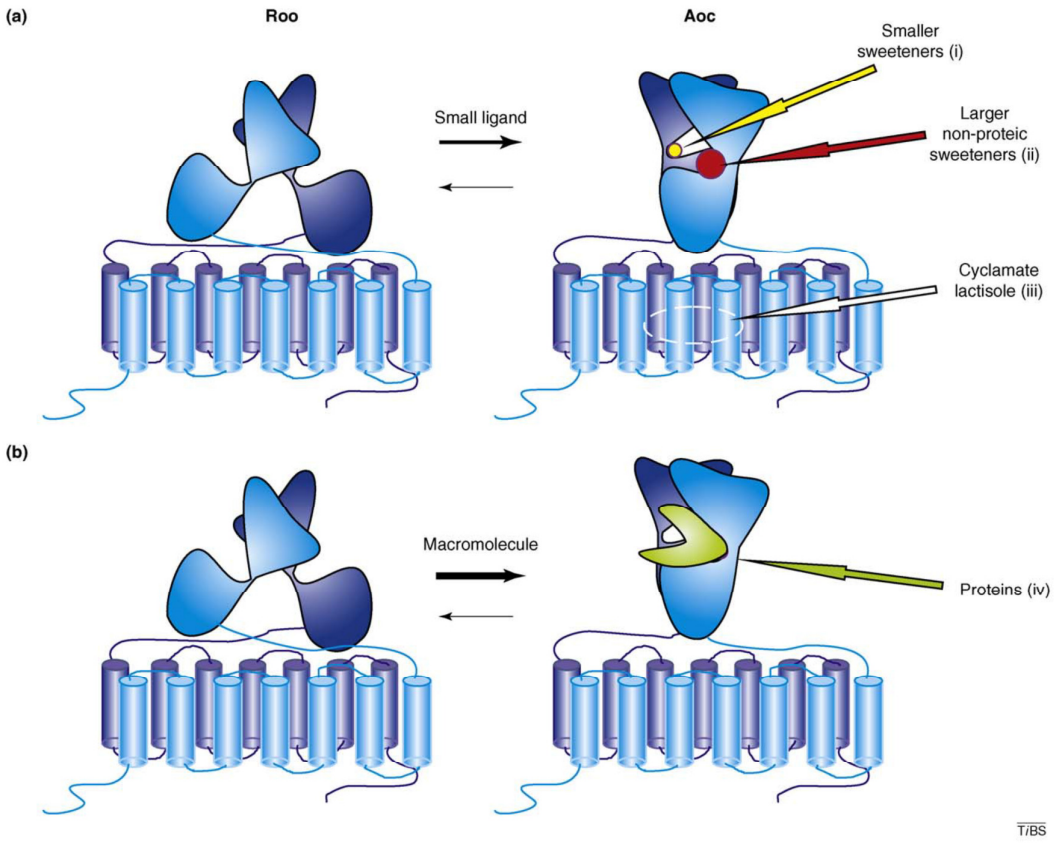


Figure 2.10 Active sites of the T1R2/T1R3 sweet receptor. (a) Binding of small-molecular-weight ligands transforms the inactive form (Resting open-open, Roo) into the complexed form (Active open-closed, Aoc). Smaller ligands, (represented by a yellow ball) are hosted by the closed cavity of T1R2 (i) of Aoc; larger ligands, represented by a red ball, are hosted by the open cavity of T1R3 (ii) of Aoc. The cyclamate site (iii) is indicated by the broken ellipse in the TMD. (b) Inactive form stabilised by protein complexation (iv) ('wedge' protein is shown in green) (Temussi, 2009).

2.5.2.2 Defining flavour modification: suppression and enhancement

The global tendency in modern food applications to improve the consumer's diet has given rise to new taste and flavour challenges in product formulation and reformulation (Servant *et al.*, 2011; Ley *et al.*, 2011b). The two main groups of processed food products that show extreme taste or flavour deficits are the so-called "*food minus*" foods that can be categorised as foods reduced in sugar, salt, fat or monosodium glutamate (MSG) and so-called "*food plus*" foods that include functional foods that are supplemented with healthy but unpalatable components including polyunsaturated fatty acids/oils and tea or grape skin polyphenols (Ley *et al*., 2011b).

Granting that certain "obvious solutions" in producing more healthy alternatives are used to improve these unpleasant taste profiles, it is generally recognised that these means often fail to meet the consumer acceptance (Ley *et al*., 2011b). These include using high potency sweeteners for sugar reduction and potassium chloride for sodium chloride reduction. Other solutions to counteract unpleasant tastes are the use of physical barriers such as (micro)encapsulation, emulsions and suspensions of fish oils and polyphenols or the use of strong masking flavours for soy, vegetable oil and pharmaceutical actives (Ley *et al*., 2008c; Ley *et al*., 2011b). There are also numerous options to "neutralise" the bitter taste of bitter tasting actives such as tea catechins, including the use of polysaccharides such as pectins or cyclodextrins for scavenging tastants or biotransformation of bitter molecules to non-bitter metabolites (e.g. naringin hydrolysis to naringenin) (Ley *et al.*, 2008c).

There exists an increasing demand for ingredients with flavour-modifying abilities that have minor or weak intrinsic taste and aroma characteristics at the intended dosage to produce healthier alternatives that cannot be distinguished from original products (Ley *et al*., 2011b; Servant *et al*., 2011). Ley *et al*., 2011b defines a "flavour modifier" as a compound that is able to influence the intensity or change the quality of the flavour or taste profiles of another flavour or taste compound and acts therefore as an indirect contributor.

2.5.2.3 Sweetener synergy and sweet taste modulation

Over the past decades intensive research was done to identify the ideal blend of zero-calorie sweeteners that would produce a taste profile closest to that of sugar (Servant *et al.*, 2011). Several publications on sweet-taste synergism in blends of sweeteners that elicit potentiation of sweet taste came to light. Because real synergistic effects (i.e., much more than additive) are difficult to define and to measure with sensory methods because of the nonlinear dose responses, both Kinghorn *et al.* (2010) and Servant *et al.* (2011) warned that most suggestions in the literature regarding "synergistic" sweet mixtures, need to be judged carefully. Apparent synergy in taste tests can only be perceived at lower sweetener concentrations, and at higher concentrations, mainly an additive effect or even sweetness suppression was observed (Servant *et al*., 2011).

Various psychophysical studies (tests for affinities, off-rates, solubility and component interaction by means of taste tests) have revealed that the addition of the NHDC or cyclamate to a sugar solution caused an over-additive enhancement of the perceived sweetness (Fujiwara *et al.*, 2012). Conversely, Servant *et al*. (2011) reported two independent studies that suggest that NHDC is not a taste enhancer in humans. However, Fujiwara *et al*. (2012) confirmed sweet-taste synergisms with receptor cell-based assays, which demonstrated greater synergism of NHDC compared to other sweeteners such as aspartame, saccharin and acesulfame K, whose effects were shown to be simply additive. Although sweetener blends remain popular in the industry, beneficial effects of sweetener blends are evidently not

the result of strong synergy between each of the components but rather due to their complementary flavour characteristics and physicochemical properties (Servant *et al*., 2011).

Recently positive allosteric modulators (PAMs) have shown to be a superior alternative to sweeteners or sweetener combinations in significantly enhancing sweetness at low concentration, while preserving the taste of sugar (Servant *et al*., 2011). Presently there is great focus in the area of high-throughput screening technologies on the prospect of discovering allosteric modulators that can favourably alter the functional relationship between taste receptors and their cognate ligands (Sprous & Palmer, 2010).

The identification of PAMs of the T1R2/T1R3 sweet receptor that could be used in conjunction with sucrose, fructose and glucose, to provide lower calories without changing any aspect of the desired sugar taste, will revolutionise the field of flavour development for sweetened products (Servant *et al*., 2011; Sprous & Palmer, 2010). In addition, it has been suggested that true sweetness enhancers could be used to improve the flavour profile of artificial sweeteners by lowering their levels to sub-threshold amounts for their off-tastes (Servant *et al*., 2011).

2.5.2.4 Recent discoveries of taste modulators

2.5.2.4.1 Sweetness enhancers

Considerable progress has been made in the development of new sweetness enhancers to gain synergistic sweet-enhancing effects.

Servant *et al*., 2010 discovered novel sweet-taste enhancers (SE-1, SE-2 and SE-3), which were PAMs for the sweet receptor dimer of hT1R2/hT1R3. These substances were also confirmed to enhance sweet-taste intensity in human sensory tests. However, compared to NHDC and cyclamate that increased the potencies of a range of sweeteners in sweet taste receptor cell-based assays, the enhancing effect of SE-1 was restricted to neotame, sucrose and sucralose (Fujiwara *et al*., 2012).

Ottinger *et al*. (2003) discovered the first non-volatile, sweetness-enhancing Maillard reaction product reported in the literature, namely alapyridine, which shows a sweet, as well as an umami or even general taste-enhancing effect. Numerous structural analogues of homoeriodictyol were evaluated for their sweetness-enhancing activity, for example certain isogingerdiones such as [2]-isogingerdione was found to exhibit sweet-enhancing effect by significantly and synergistically increasing the sweet taste of a 5% sucrose model solution by 27%, without showing any significant intrinsic sweetness (Ley *et al.*, 2008c). The very weak sweet-tasting phloretin, an aglycone of the bitter-tasting phloridzin, also exhibited significant strong sweet enhancing properties (Ley *et al.*, 2008d).

Other weak- or neutral-tasting sweet taste enhancers described in the literature include dihydromyricetin, trilobatin, combinations of 3-hydroxy- and 2,4-dihydroxybenzoic acid, certain bisaromatic amides, and naringin dihydrochalcone as reviewed by Ley *et al*. (2011b). The use of ethyl butyrate or pyridinium alanyl betaine as sweetener enhancers was demonstrated to increase the

perceived sweetness of a sugar containing product, without showing strong intrinsic sweetness (Ley *et al.*, 2008c).

Several flavonoids have also been reported to enhance sweetness or to improve taste in the patent literature (Kinghorn *et al.*, 2010). The flavanone hesperetin, the aglycone of hesperidin, has been proven as a sweetness-enhancing agent in various food applications (Ley *et al*., 2008b). Homoeriodictyol, apart from its bitterness masking properties, was found to exhibit a 6% sweetness-enhancing activity when present at 100 ppm in a 5% w/v sucrose solution (Ley *et al.*, 2008a).

Hesperetin and the salts thereof (Na^+, K^+, NH_4^+, Ca^{2+}, Mg^{2+}, Al^{3+} and Zn^{2+} in particular) were found to superproportionally increase the sweet taste impression of sweet-tasting substance at very low concentrations (<0.025% by weight) (Ley *et al.,* 2011a). Reichelt *et al.* (2010) also confirmed the flavour modulating activity of hesperetin. Fletcher *et al.* (2011), using a cell-based sweetness-enhancing assay, also demonstrated a sweetness-enhancing activity for hesperetin, sakuranetin and 3,2'-dihydroxy-4,4',6'-trimethoxychalcone. According to Kinghorn *et al.* (2010) hesperetin has low intrinsic sweetness while its flavour profile is described as slightly sweet, dry-dusty, balsamic and vanillic at 100 ppm in an aqueous solution (Ley *et al.*, 2005). Its glycoside, hesperidin, was reported to be tasteless (Ley *et al.*, 2005).

A patent for hesperetin as sweetness enhancer describes various applications in nourishment, consumption and even oral hygiene. Examples of the type of products included sugar-reduced soft drinks, sugar-reduced ice-cream, yoghurt, spray-dried and liquid flavour formulations, chewing gum and tooth paste (Ley *et al.,* 2008b). The sugar content (particularly sucrose, lactose, glucose, D-tagatose or fructose) was reduced in corresponding foods by up to 60% without reducing the sweet taste impression in the process (Ley *et al.,* 2011a).

To compare the sweetness enhancement produced by the addition of hesperetin with that of the hydroxyflavanones naringenin, eriodictyol, homoeriodictyol and eriodictyol-7-methylether, the respective sweetness of a 5% sucrose solution against solutions which contained both 5% sucrose and 100 ppm of these substances was determined by a group of experts (rating 0 [not sweet] to 10 [extremely sweet]). The results indicated that hesperetin showed significant sweetness enhancing activity (Ley *et al.,* 2011a). A 21% enhancement effect was seen for a reduced-sugar solution using 100 ppm hesperetin as sweetness enhancer (Ley *et al.,* 2011a). Hesperetin when added to a sugar-reduced soft drink formulation at 100 ppm significantly enhances sweetness by 33% (Ley *et al.,* 2011a).

2.5.2.4.2 Flavanone and other bitterness blockers

A few potent new bitterness masking molecules have been identified, for example, the flavanones, homoeriodictyol and eriodictyol. These compounds have significant bitter masking effects without exhibiting any additional strong taste or flavour (Ley *et al*., 2005).

Ley *et al.* (2006) reported the synthesis of structurally related hydroxybenzoic acid amides of benzylamines that were evaluated as masking agents toward bitterness of caffeine by sensory methods.

It was concluded that new amides may be alternatives for the expensive flavanones to create flavour solutions to mask bitterness of pharmaceuticals or foodstuffs.

2.6 Conclusion

Honeybush tea production is accompanied by the generation of by-product material, comprising largely of stem material that does not meet the particle size requirements for retail products. The by-product could be a potential renewable source of the flavanone glycosides, hesperidin and eriocitrin, which can be converted to the sweetness-enhancing taste modulator, hesperetin, and bitterness-masking taste modulator, eriodictyol, respectively. To our knowledge, no research is available on the preparation of hesperidin- and eriocitrin-enriched *Cyclopia* extracts and subsequent conversion of hesperidin and eriocitrin to hesperetin and eriodictyol, respectively, whilst present in the extract matrix. Various new emerging 'green' extraction technologies are available of which UAE has been demonstrated to be an environmentally-benign means to recover hesperidin from citrus processing waste material. Furthermore, RSM has been demonstrated to be an effective optimisation tool to optimise the extraction of various valuable phenolic compounds from food processing by-products. Conversion of hesperidin and eriocitrin to their respective aglycones, hesperetin and eriodictyol, to 'unlock' the taste-modulating ability of *Cyclopia* extracts could be done by acid-catalysed hydrolysis and enzymatic bioconversion. Both techniques have received attention for the conversion of flavanone glycosides to their respective aglycones and other flavanone derivatives. A flavanone aglycone-enriched extract from honeybush may have potential as a functional ingredient to reduce the sugar content of food products, such as iced teas, as well as mask the potential bitter taste of phytochemicals present in the extract. Therefore, in light of the afore-mentioned, the value-adding potential of honeybush tea by-product as source material for flavanone aglycone-enriched extracts should be investigated.

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CHAPTER 3

Optimisation of ultrasound-assisted extraction of flavanone glycosides from *Cyclopia maculata* tea processing by-product

3.1 Abstract

The phenolic content of honeybush sp. *C. maculata* was evaluated to determine its potential as source material for preparation of a flavanone glycoside-enriched extract with potential therapeutic and taste-modulating properties. Hesperidin was the major flavanone glycoside present in *C. maculata*, with another flavanone glycoside, eriocitrin, also present at relatively low concentrations. The taste modulators, hesperetin and eriodictyol, aglycones of hesperidin and eriocitrin, respectively, were present mainly below the quantification limit. The stems of unfermented plant material had a significantly ($p < 0.05$) higher hesperidin content than the leaves, while no significant ($p \geq 0.05$) difference between unfermented and fermented plant material for hesperidin was observed. Contrastingly, the leaves of unfermented plant material had a significantly ($p < 0.05$) higher eriocitrin content than the stems, while significantly ($p < 0.05$) more eriocitrin was present in the unfermented plant material. Fermented *C. maculata* waste material, a by-product from honeybush tea processing with high hesperidin content, was subjected to environmentally friendly ultrasound-assisted extraction to investigate its potential as renewable source of flavanone glycosides. Preliminary single factor experiments were performed to determine the effect of extraction parameters, namely ethanol concentration (0-96% v/v), time (30-180 min), temperature (30-60°C), and solvent:solid ratio (10-60 mL/g) on extraction efficiency. Response surface methodology was applied as optimisation tool to optimise these selected extraction parameters for maximum extract and hesperidin yield and to evaluate their effect on the responses studied. The average response values varied widely, from 10.83-20.57 g soluble solids (SS)/100 g plant material for extract yield, 0.68-2.00 g hesperidin/100 g plant material for hesperidin yield and 4.40-10.23 g hesperidin/100 g SS for hesperidin content of the extract, as a function of the parameter setting within the experimental domain. The extract and hesperidin yield, as well as hesperidin content of the extract, increased with an increase in extraction time, temperature and solvent:solid ratio in both single factor and RSM experiments. The hesperidin yield increased with increasing ethanol concentration up to 57.6% (v/v) ethanol in single factor experiments, whereafter a decrease was observed. On the other hand, the extract yield and hesperidin content of the extract decreased and increased, respectively, with an increase in ethanol concentration. From the experimental data, second order polynomial mathematical models were developed with high coefficient of determination values (R^2_{adj} = 0.8) for both hesperidin yield and hesperidin content of the extract, indicating good accuracy and predictive ability of the applied models. Only an optimum value for ethanol concentration (52.8% (v/v) for yield of SS and hesperidin from the plant material) could be calculated as the optimum values for the remaining independent variables were outside the experimental domain. Practical process parameter values that were feasible for industrial application (52.8% (v/v) ethanol, 20 mL/g solvent:solid ratio, 60°C and 30 min) were selected for the recovery of flavanone glycosides from *C. maculata* tea processing by-product.

3.2 Introduction

The traditional South African herbal tea, honeybush, is prepared from a number of *Cyclopia* spp., which are endemic to the Cape fynbos biome. With its historical use as medicinal plant or herbal tea that predates the 1800s, it provides the consumer with a caffeine-free beverage, known for its high phenolic content and antioxidant activity (Joubert *et al.*, 2009). Apart from its use as herbal tea, there is also a demand for honeybush as source material for the preparation of polyphenol-enriched extracts for the food, nutraceutical, and cosmetic industries (Joubert *et al*., 2009).

The major phenolic compounds present in *Cyclopia* spp. are the xanthones, mangiferin and isomangiferin, and the flavanone, hesperidin (Joubert *et al*., 2011). In light of the vast extent of studies on the health-promoting properties of mangiferin (including antioxidant, anti-inflammatory, anti-diabetic and hypolipidaemic properties, as reviewed by Vyas *et al*., 2012), it is clear why the focus of research on extracts from *Cyclopia* spp. over the past few years was predominantly on that of mangiferin-enriched extracts. Correspondingly, hot water honeybush extract with standardised mangiferin content is produced as a food ingredient for use in drinks, dairy products and food bars by the food industry (Joubert *et al*., 2011).

However, in addition to mangiferin, antioxidant phenolic compounds of interest that are present in aqueous *C. subternata* extracts are the flavanone glycosides, hesperidin and eriocitrin (De Beer & Joubert, 2010; De Beer *et al*., 2012). Also present in citrus, with hesperidin being the most studied citrus flavanone, these afore-mentioned flavonoids have received attention for their health-promoting properties (Garg *et al*., 2001; Minato *et al*., 2003; Miyake *et al*., 1997; 2006). Apart from its reported antioxidant, anti-allergenic, anti-carcinogenic, anti-hypotensive, anti-microbial, anti-obesity and anti-inflammatory properties, hesperidin is also known for improving vascular integrity and decreasing capillary permeability (Benavente-García & Castillo, 2008; Garg *et al*., 2001; Bok *et al*., 1999; Chiba *et al*., 2003; Park *et al*., 2001, Valensi *et al*., 1996). In addition, hesperidin is also a source of a valuable chemical, i.e. the taste modulator and sweetness-enhancer, hesperetin (Ley *et al*., 2008).

Cyclopia maculata grows in the Overberg area and its potential commercial viability is currently under investigation. To date limited research is available on *C. maculata* in terms of its chemical composition (Joubert *et al*., 2003; Schulze, 2013) and antioxidant activity (Hubbe & Joubert, 2000). Similar to the other commercialised species, the major phenolic compounds in *C. maculata* are also mangiferin, isomangiferin and hesperidin (Joubert *et al*., 2003; Schulze, 2013). Quantitative data for the eriocitrin content of *C. maculata* was only recently documented (Schulze, 2013). Approximately four times more hesperidin (1.782 g/100 g SS) was present in aqueous extracts from unfermented *C. maculata* plant material harvested from cultivated plants, compared to eriocitrin (0.436 g/100 g SS), whereas the hesperidin content (0.892 g/100g SS) of extracts from wild-harvested unfermented *C. maculata* plant material was approximately triple that of eriocitrin (0.334 g/100 g SS).

The millions of tons of food processing waste generated globally on a daily basis impose a great threat to the environment; therefore, research is progressively more focused on utilising by-products (a term increasingly preferred by scientists over "waste" to emphasise their potential as ultimate substrates) to reduce their environmental impact and to explore their potential economic value (Galanakis, 2012). Over the past two decades it has become evident in literature that a considerable body of research exists on the exploitation of different by-products (including rice, olive oil, tea, apple, tomato and citrus processing by-products) as a potential source of valuable bioactive compounds (Li, *et al*., 2006; Pingret *et al*., 2012; Şahin & Şamlı, 2013; Tabaraki & Nateghi, 2011, Tsubaki *et al.*, 2010). Citrus fruits make up the largest sector of global fruit production, with more than 100 million tons produced yearly, of which 34% are made into juices, resulting in high amounts of by-products, especially citrus peels (Ho & Lin, 2008). Hesperidin, extracted from citrus peel for the pharmaceutical industry, provides a good example for the recovery of high value-added compounds from by-products as abundant and inexpensive renewable resources (Da Silva *et al*., 2013; Di Mauro *et al*., 2000).

Honeybush tea processing by-product, comprising of plant material that does not meet particle size requirements, is generated as waste from honeybush tea processing (Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, 2012, personal communication). It represents the residues that remain after the sieving step, i.e. < 40 mesh (so-called "dust") and > 12 mesh (mainly stems). The tea processing by-product, which can constitute as much as 20% of production, currently has little value if not re-processed into smaller pieces for mixing with the sieved fraction. Whilst useful to increase bulk, the re-processed fraction is light coloured, as opposed to the characteristic dark brown of the "fermented" (oxidised) product. Alternatively, the coarse fraction could be used for extract production where physical appearance is not of concern (Joubert, 2012).

Cyclopia maculata plants are vigorous growers that can reach a height of up to 2 m within 1 year. The mature plant has thick stems, which may potentially contribute to a large portion of by-product material (Joubert *et al.*, 2011). Furthermore, the by-products generated will be largely from fermented plant material as most honeybush is consumed in this form. The value-adding potential of *Cyclopia* remains underdeveloped as demand for honeybush as a tea still exceeds supply (Joubert *et al.*, 2011). Therefore, the prospect exists to explore the potential of utilising honeybush tea by-products, and in particular, those from *C. maculata* as source for polyphenol-rich extracts.

With the development of the `Green Chemistry' concept, environmentally benign techniques have gained growing interest to allow for more environmentally sustainable extraction with high reproducibility, decreased extraction times, increased extraction yields, reduced solvent consumption and reduced temperature and energy input (Co *et al.,* 2012; Khan *et al.,* 2010). Ultrasound-assisted extraction (UAE) is one such method that has found popularity for the extraction of phenolic compounds. Khan *et al.* (2010) demonstrated the efficiency of UAE of hesperidin and naringin from orange peel (*Citrus sinsensis* L.), compared to conventional solvent extraction without sonication. Similarly, Hossain *et al.*

(2012) found extraction yields for marjoram (*Origanum marjorana* L.) using UAE to be significantly higher than conventional extraction. The mechanical effect of ultrasound during extraction is believed to accelerate the release of organic compounds contained within the plant due to cell wall disruption, easier access of the solvent to the cell content and increase in mass transfer rates (Chemat *et al*., 2011; Vinatoru *et al*., 1999). To our knowledge, ultrasound-assisted extraction has not been applied to extraction of flavanone glycosides from *Cyclopia* plant material before.

Response surface methodology (RSM) is the most popular optimisation technique used in recent years to improve the performance of systems and to increase the yield of processes without increasing its cost (Baş & Boyacı, 2007). It has been successfully applied for the optimisation of extraction processes for recovery of phenolic compounds from numerous food industry by-products (Khan *et al*., 2010; Pingret *et al*., 2012; Yang *et al*., 2010). Traditionally, the influence of one factor (extraction parameter) at a time on an experimental response is monitored, by changing only one parameter, while the other parameters are kept at a constant level. The disadvantage of this so-called one-variable-at-a-time (OVAT) technique are that it does not include the interaction effects among the variables studied and therefore does not represent the complete effects of the process parameters. In addition, the increase in the number of experimental runs required, leads to an increase in time and solvent consumption, and subsequently, increased cost (Bezerra *et al*., 2008). Previously, Maicu (2008) investigated the effect of time, mass:solvent ratio and solvent composition on solvent extraction efficiency for the optimisation of phenolic extraction from *C. subternata* using the OVAT technique; however, to our knowledge, to date the present study is the first where a RSM approach is used as an optimisation tool to simultaneously optimise the levels of several extraction parameters for *Cyclopia* spp., and specifically for honeybush tea processing by-products.

The first objective of this study was to identify *C. maculata* plant material with high flavanone glycoside content. The second objective was to determine the effect of solvent concentration, time, temperature and solvent:solid ratio on the extraction efficiency in terms of flavanone glycoside and extract yield from fermented *C. maculata* tea processing by-product through preliminary single factor UAE experiments. The final objective was to optimise the UAE parameters for recovery of flavanone glycosides from *C. maculata* by-product using RSM as optimisation technique.

3.3 Materials and methods

3.3.1 Chemicals

HPLC gradient grade acetonitrile was purchased from Merck Chemicals (Darmstadt, Germany), analytical grade acetic acid from Sigma-Aldrich (Sigma Aldrich, St. Louis, USA), and ethanol (96 and 99%) from Servochem (Cape Town, South Africa). Authentic reference standards with purity >95% were sourced from Sigma-Aldrich (hesperidin) and Extrasynthese (Genay, France: mangiferin, hesperetin, eriocitrin, eriodictyol, luteolin). Stock solutions of the phenolic standards were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) at ca 1 mg/mL and aliquots were frozen (-20°C) until analysis. L-Ascorbic acid was sourced from Sigma-Aldrich. An Elix water purification system (Millipore, Milford, MA, USA) was used to prepare deionised water, which was further purified to HPLC grade by means of a Milli-Q Académic water purification system (Millipore).

3.3.2 Plant material

3.3.2.1 Leaves and stems of unfermented plant material

Shoots were harvested randomly from 2-year-old seed-propagated *C. maculata* plants (n=20), grown in pots under standardised conditions at ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa (seeds for the pot trial were collected from a natural population, growing in marsh land near Riversdale, South Africa). The shoots of a single bush represented a sample.

The top part (ca 30 cm) of the shoots were cut off and dried whole at 30°C for ca 2.5 days. Final drying to a moisture content of <10% took place at 40°C for 6 hrs under forced convection using a temperature-controlled drying tunnel (Continental Fan Works CC, Cape Town, South Africa). The leaves (CmacL) and stems (CmacS) were separated and milled separately using a Retsch ball mill (Retsch GmbH MM301, Haan, Germany). Aqueous acetonitrile (33%, v/v) extracts of CmacL and CmacS samples were prepared for analyses (3.3.4).

3.3.2.2 Effect of fermentation

Individual plants (n=10) were harvested from a natural population, growing on the farm *Welgedacht* (Riversdale, South Africa). The shoots of each plant was shredded into small pieces (<4 mm) using a mechanised fodder cutter, before division into two sub-batches. The one batch, representing unfermented plant material (CmacU), was dried without delay in a drying tunnel at 40°C for 6 hrs and subsequently sieved using a mini-sifter (SMC, Cape Town) (200 g/30 s at 90 rpm). The fraction smaller than 1.4 mm was retained for milling. The other sub-batch was moistened to ca 60% moisture content and fermented at 90°C for 16 hrs (CmacF). After fermentation the plant material was dried, sieved and milled as described for the unfermented plant material. All the samples were first coarsely milled (<1 mm) using a Retsch mill (Retsch GmbH, Haan, Germany) whereafter it was finely milled, using a Retsch

ball mill. Aqueous acetonitrile (33%, v/v) extracts of CmacU and CmacF samples were prepared for analyses (3.3.4).

3.3.2.3 By-product from C. maculata tea processing

For quantification of the major phenolic compounds in the by-product of honeybush tea processing, different batches (n=10) of wild-harvested *C. maculata* plant material (*Romansrivier*, Riversdale, South Africa) were processed and milled as described for CmacF samples in 3.3.2.2. Honeybush tea processing by-product/waste material ($CmacW_i$) represents fermented plant material not meeting tea bag packaging requirements, i.e. < 40 mesh (so-called "dust") and > 12 mesh (mainly stems). Aqueous acetonitrile (33%, v/v) extracts of $CmacW_i$ samples were prepared for analyses (3.3.4).

Sub-samples of the above-mentioned 10 $CmacW_i$ batches were blended after coarse milling to form a single representative batch that was used for the optimisation of ultrasound-assisted extraction (3.3.3).

3.3.3 Ultrasound-assisted extraction (UAE) of *C. maculata* by-product

3.3.3.1 General

UAE experiments were carried out in an ultrasonic cleaning bath (Branson 8510, Branson Ultrasonic Corporation, Danbury, CT, USA) (20 L capacity) with a maximum input power of 930 W and digital temperature control of up to 69°C. Samples were sonicated at a constant frequency of 44 kHz.

3.3.3.2 Preliminary single factor experiments

Preliminary single factor experiments were performed on a $CmacW_i$ representative batch (3.3.2.3) to investigate the effect of solvent:solid ratio (mL/g), extraction time (min), extraction temperature (°C) and solvent composition (% ethanol, v/v) on flavanone glycoside extraction efficiency. In addition, it was used as a screening study to determine the optimum ranges of these extraction parameters (independent variables) to be used in design of RSM experiments, given practical limitations. For each single factor experiment, the other three parameters were kept constant at 50°C, 10 mL/g solvent:solid ratio, 57.6% (v/v) ethanol and 30 min where applicable (Table 3.1). The procedure for all UAE experiments was as follows: $CmacW_i$ plant material was weighed off in plastic 50 mL conical-shaped centrifuge tubes, solvent was added and samples were sonicated at the parameters specified for each experiment. After extraction, samples were cooled in crushed ice for 30 min and filtered through Whatman Nr 4 filter paper (Whatman International Ltd., Maidstone, UK), followed by preparation for HPLC analyses (3.3.4).

Table 3.1 Preliminary single factor experiment parameter values

Experiment	Units	Parameter range tested	Constant values
Effect of ethanol concentration	% v/v	0; 19.2; 38.4; 57.6; 76.8; 96*	50°C; 10 mL/g; 30 min
Effect of extraction time	min	30; 60; 90; 120; 150; 180	50°C; 10 mL/g; 57.6% ethanol
Effect of extraction temperature	°C	30; 40; 45; 50; 55; 60	10 mL/g; 30 min; 57.6% ethanol
Effect of solvent:solid ratio	mL/g	10; 20; 30; 40; 50; 60	50°C; 30 min; 57.6% ethanol

**Corresponding to 20, 40, 60, 80 and 100% of 96% ethanol*

3.3.3.3 RSM experiments

The same UAE experimental procedure, described for the preliminary single factor experiments (3.3.3.2), was used.

RSM was conducted to optimise the extraction parameters and a Central Composite Design (CCD) was employed for this experiment. Ethanol (solvent) concentration (X_1), extraction time (X_2), extraction temperature (X_3) and solvent:solid ratio (X_4) were chosen as the independent variables. The range and center point values of the four parameters were selected, based on the results of the preliminary single factor experiments. The coded levels and the de-coded (natural) values of the factors used in the CCD are shown Table 3.2.

The experimental design consisted of a combination of 26 randomised extraction conditions (including two replicates of the central point, designated by the coded value, 0) (Table 3.3). The experiment was conducted in triplicate (duplicate for eriocitrin) and the actual values (not mean) were stated as observed responses. The responses for the independent variables were selected as g soluble solids (SS)/100 g plant material (extract yield), g hesperidin (or eriocitrin) per 100 g plant material (yield) and g hesperidin (or eriocitrin) per 100 g SS (indication of enrichment) (Table 3.3).

Table 3.2 Independent variables and their levels used for Central Composite Design

		Levels				
Independent variable	**Symbol**	**-α**	**-1**	**0**	**1**	**α**
Ethanol concentration (% v/v)	X_1	43.2	48	52.8	57.6	62.4
Time (min)	X_2	75	90	105	120	135
Temperature (°C)	X_3	45	50	55	60	65
Solvent:solid ratio (mL/g)	X_4	20	30	40	50	60

Statistica data analysis software system (StatSoft, Inc. (2012), Version 11.0, www.statsoft.com) was used for the construction of the experimental design, development of the mathematical models, analysis of the experimental data and calculation of the predicted responses.

The behaviour of the system (UAE process) was explained by the following quadratic polynomial regression equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{1 \le i \le j}^{k} \beta_{ij} X_i X_j + \varepsilon \quad (1)$$

where Y is the response value (dependent variable), k is the number of variables, β_0 is the constant term (intercept), and X_i and X_j represent the levels of the independent variables. The terms β_i, β_{ii} and β_{ij} are the regression coefficients of the linear, quadratic and interaction parameters, respectively; i.e. they represent the linear, quadratic and cross-product effects of the four factors (X_1, X_2, X_3 and X_4) on the response values, respectively. The term ε represents the residual associated to the experiments.

Additional verification extractions (determined by Statistica 11.0) were subsequently performed to verify the validity of the experimental design.

3.3.4 Quantification of phenolic compounds by HPLC-DAD

3.3.4.1 Sample preparation

To quantify the major polyphenols of various plant material samples (3.3.2.1; 3.3.2.2 and 3.3.2.3) 33% (v/v) aqueous acetonitrile *C. maculata* extracts were prepared for HPLC analyses. Approximately 20 mg dried, finely milled plant material was weighed into a 4 mL Reacti vial (Sigma-Aldrich) in duplicate (n_{CmacL} = 20; n_{CmacS} = 20; n_{CmacU} = 10; n_{CmacF} = 10) and triplicate (n_{CmacWi} = 10). For extraction 3 mL 33% (v/v) aqueous acetonitrile was pipetted into each Reacti vial and vortex mixed. The Reacti vials were placed in a pre-heated Stuart block heater (Bibby Scientific Ltd, Stone, Staffordshire, UK) at 100˚C for 20 min, followed by sonication for 10 min in a sonicating bath after which extract samples were cooled in ice water to room temperature.

Prior to HPLC-DAD analysis, L-ascorbic acid was added to authentic reference standard solutions, ethanol extracts (3.3.3) and 33% (v/v) aqueous acetonitrile extracts at a final concentration of ca 5 mg/mL to prevent oxidative degradation of the phenolic compounds. The standard and extract mixtures were then filtered using 0.45 µm pore-size Millex-HV syringe filter devices with 4 and 33 mm diameters (Millipore), respectively. The injection volumes for the standards, ethanol extracts and 33% (v/v) aqueous acetonitrile extracts were 5-20 µL, 5-10 µL and 10 µL, respectively.

3.3.4.2 HPLC-DAD analysis

Analyses were performed on an Agilent 1200 series HPLC instrument that consisted of an in-line degasser, quaternary pump, autosampler, column oven and DAD, and controlled by Chemstation

software (Agilent Technologies Inc., Santa Clara, CA). A Zorbax Eclipse XDB-C18 column (150 × 4.6 mm, 5 µm, 80 Å) from Agilent Technologies was selected for separation of compounds with 2% acetic acid in HPLC grade water (v/v) (A) and acetonitrile (B) as mobile phases. Separation was carried out at 30°C with the following mobile phase gradient at a flow rate of 0.8 mL/min: 12% B (0-6 min), 18% B (7 min), 25% B (14 min), 40% B (19 min), 50% B (24 min) and 12% B (29-40 min), as described by De Beer & Joubert (2010).

UV-Vis spectra were recorded for all samples from 200 to 400 nm. Retention times and spectral characteristics of authentic reference standards were used for the identification of specific peaks, except isomangiferin that was identified based on relative retention time to mangiferin and spectral characteristics reported previously (De Beer & Joubert, 2010). Chemstation software was used for the integration of peak areas. The xanthones (mangiferin and isomangiferin) and flavone (luteolin) were quantified at 320 nm and the flavanones (eriocitrin, hesperidin, eriodictyol, naringenin and hesperetin) were quantified at 288 nm. For the quantification of compounds, a six-point calibration curve was set up for all the available authentic reference standards, with the exception of isomangiferin that was quantified using a response factor previously determined for isomangiferin relative to mangiferin.

3.3.5 Determination of SS content of ethanol extracts

The SS content of the extract filtrates from all preliminary single factor and RSM UAE experiments was determined gravimetrically by evaporating 5 mL aliquots (in duplicate) in pre-weighed nickel moisture dishes on a steam bath (Merck, South Africa) until dry. Samples were subsequently dried in a forced-air circulating oven at 100°C for 1 hr and the results were expressed as mg SS/mL extract. Theoretical extract yield was calculated from the SS content, extraction volume and mass of plant material extracted and expressed as g SS/100 g plant material.

3.3.6 Statistical analysis

The data for CmacL, CmacS, CmacU, CmacF and $CmacW_i$ (preliminary single factor UAE experiments) were subjected to univariate analysis of variance (ANOVA) using SAS® software (Version 9.2, SAS Institute Inc, Cay, USA) to determine whether differences between treatment means were significant. In circumstances where significant ($p < 0.05$) differences were found, least significant difference (LSD) of the Student's t test ($p = 0.05$) was calculated to compare treatment means. The Shapiro-Wilk test was executed to test for normality (Shapiro & Wilk, 1965). When significant non-normality ($p \leq 0.05$) was observed, outliers were identified and removed until the data were normally distributed. Principal component analysis (PCA) was performed using XLStat software (Version 7.5.2, Addinsoft, New York, USA) to visualise the relationship between the samples and their phenolic composition.

Statistica 11.0 was used for all data analyses of RSM experiments (3.3.3.3). ANOVA (95% confidence level) was carried out for each response variable in order to test the model significance and

suitability. The F-value in ANOVA is the ratio of the mean square error to the pure error obtained from the replicates at design center. The p-value expressed the significance of the different variables and a standardised Pareto chart presented the significant effects obtained from ANOVA for the different response values. The fitting efficiency of the data to the model was expressed by the correlation coefficient (R^2), adjusted R^2 (R^2_{adj}) and significance of the lack of fit (LOF).

Two-dimensional contour and three-dimensional response surface plots were developed from the calculated regression coefficients while holding one variable constant in the second order polynomial equation. These plots gave a graphical representation of the significant relationship linking the independent variables and responses studied.

3.4 Results and discussion

For this study *C. maculata* plant material and tea processing by-product was evaluated for its phenolic content, particularly that of flavanone glycosides, hesperidin and eriocitrin, as precursors of the taste modulators, hesperetin and eriodictyol, respectively. UAE was used to recover hesperidin and eriocitrin from *C. maculata* processing by-product and single factor experiments were performed to investigate the effect of four UAE parameters on the extract and flavanone glycoside yield. RSM was applied to optimise the extraction conditions for maximum recovery of extract and flavanone glycosides from the by-product plant material, and to study the effects of the UAE parameters on the yield.

3.4.1 Quantification of major phenolic compounds of *C. maculata* plant material

The extraction method used to compare the major phenolic compound content of different *C. maculata* plant material samples was based on a study by Joubert *et al.* (2012). In the latter study different extraction solvents (acetonitrile, ethanol, methanol and acetone) were evaluated to determine the optimum extraction conditions for HPLC-DAD analysis of the mangiferin content of dried, unfermented *C. subternata*. Extraction with 33% (v/v) aqueous acetonitrile or 50% (v/v) aqueous ethanol proved to be the most effective for the extraction of SS, total polyphenols and xanthones, however 33% (v/v) aqueous acetonitrile was significantly ($p < 0.05$) more effective for extraction of hesperidin than 50% (v/v) aqueous ethanol. Therefore, 33% (v/v) aqueous acetonitrile was selected as extraction solvent for the current study to evaluate phenolic content of plant material. The procedure was scaled down and conducted in a temperature-controlled heating block, allowing simultaneous heating of several samples.

Although the focus was on the flavanone glycosides eriocitrin and hesperidin, the extracts were characterised in terms of the full phenolic profile. The xanthones mangiferin and isomangiferin, the flavone, luteolin, and the flavanones, narirutin, eriodictyol, naringenin and hesperetin were also

quantified. Eriodictyol, luteolin, naringenin and hesperetin were mostly below the detection limit, while only a few samples contained eriodictyol, narirutin and hesperetin at very low quantities.

3.4.1.1 Leaves and stems of unfermented plant material

From Fig. 3.1.A it is evident that the xanthone content (mangiferin and isomangiferin) in dried, unfermented leaves was significantly higher ($p < 0.05$) than that of dried, unfermented stems. Although at much lower concentration than the xanthones, similar results were observed for eriocitrin. However, the hesperidin content of the stems was significantly higher ($p < 0.05$) than that of the leaves.

Fig. 3.2.A shows a PCA biplot of the individual leaves (n=20) and stems (n=20) samples and the content of the four major phenolic compounds mangiferin, isomangiferin, eriocitrin and hesperidin. From the PCA plot it is also clear that mangiferin, isomangiferin and eriocitrin associates more with the leaf samples, i.e. they are predominantly found in the leaf plant material, compared to hesperidin which was the predominant compound in the stems of the plant.

De Beer *et al.* (2012) similarly found that aqueous extracts from *C. subternata* stems contained higher hesperidin and lower mangiferin, isomangiferin and eriocitrin content than those from leaves. Stem extracts could thus be a valuable source of hesperidin and would be preferred if an extract high in hesperidin is the aim. Even after taking the lower yield into account, the stems were a better source of hesperidin (De Beer *et al.*, 2012).

3.4.1.2 Effect of fermentation

The mangiferin, isomangiferin and eriocitrin content of the unfermented plant material were significantly higher ($p < 0.05$) than in the fermented plant material (Fig. 3.1.B). Conversely, no significant difference ($p \geq 0.05$) between the hesperidin content of green plant material and that of fermented plant material was found. These findings were also evident from the PCA plot of the individual samples in Fig. 3.2.B where unfermented (n=10) and fermented (n=10) samples are separated along F1, while hesperidin is associated with F2. Hesperidin is therefore not significantly affected by the fermentation process (80°C/24 h or 90°C/16 h) as was the case for the other compounds. This could be ascribed to the heat stability of hesperidin at 90°C, as observed by Dhuique-Mayer *et al.* (2007) who found no significant decrease of hesperidin at 90°C after 240 min in a study on the thermal degradation of antioxidants. On the contrary, Joubert *et al.* (2008) reported almost 50% lower average hesperidin content for aqueous extracts from fermented C*. intermedia* (0.45 vs 1.12 g per 100 g aqueous extract) and *C. subternata* (0.27 vs 0.42 g per 100 g aqueous extract) compared to that from unfermented samples. Interestingly, no significant ($p \geq 0.05$) difference between the hesperidin content of aqueous extracts from fermented and unfermented *C. sessiliflora* was observed. In another study, Theron (2012) observed no significant ($p \geq 0.05$) difference in hesperidin content of infusions from *C. maculata* plant material fermented at 80°C and

90°C; however, the hesperidin content of infusions decreased significantly ($p < 0.05$) as the fermentation time increased from 16 hrs to 24 hrs.

3.4.1.3 By-product from C. maculata tea processing

As mentioned previously, the major honeybush tea processing by-product consists of fermented plant material that does not meet tea bag size requirements, and represents the residues after sieving, i.e. < 40 mesh (so-called "dust") and >12 mesh (mainly stems) (Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, 2012, personal communication).

From Fig. 3.1.C it is evident that hesperidin represented the most abundant compound in the by-product from fermented plant material. Mangiferin was present at significantly ($p < 0.05$) lower concentration than hesperidin. Furthermore, the other flavanone glycoside, eriocitrin, was present in very low amounts (ca 54 times less eriocitrin than hesperidin). In Fig. 3.3, the chromatogram of *C. maculata* leaves (A) and stems (B) are compared to that of *C. maculata* by-product (C). It is evident that the chromatogram of stems (B) and that of by-product (C) were very similar. These chromatograms give further visual confirmation that *C. maculata* by-product likely consists predominantly of stems with a high hesperidin content compared to xanthone content. Therefore, these results confirm that *C. maculata* by-product from tea processing could be a renewable source of hesperidin.

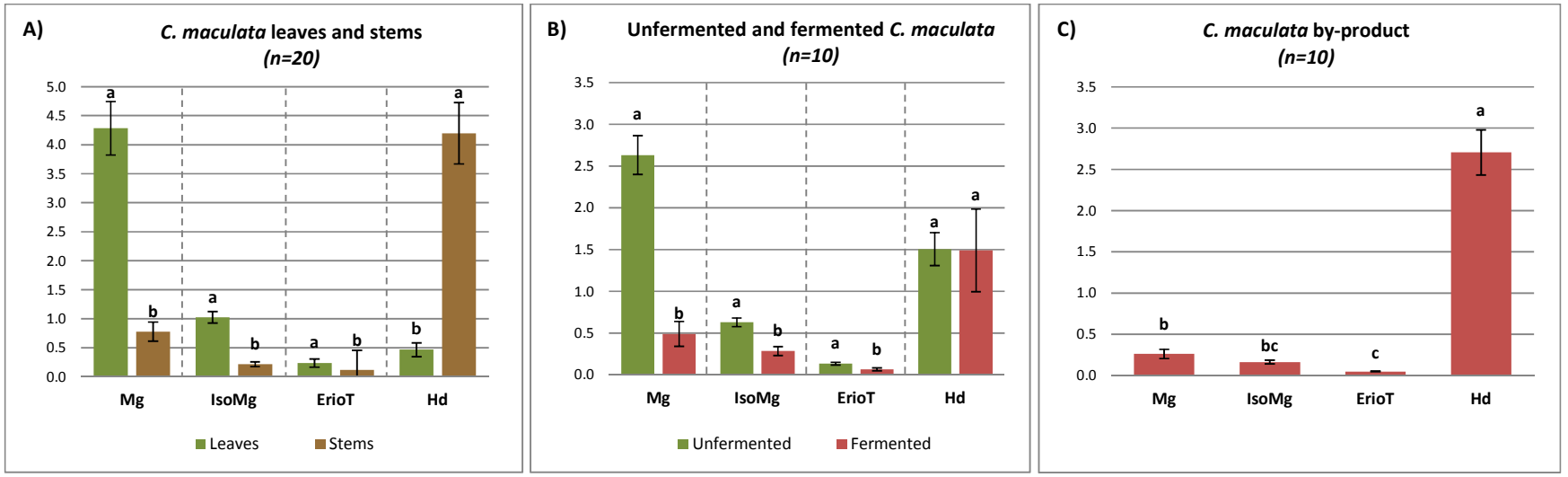

Figure 3.1 Average phenolic compound content (g compound/100 g plant material) of A) leaves vs stem, B) unfermented vs fermented plant material and C) *C. maculata* tea processing by-product ($CmacW_i$) (Mg = mangiferin, IsoMg = isomangiferin, ErioT = eriocitrin, Hd = hesperidin). *(Means with the same letter are not significantly different ($p \geq 0.05$). For A) and B) letters indicate significant differences between each individual compound in different plant material samples, as divided by the grey dotted line. For C) letters indicate significant differences between the different compounds).*

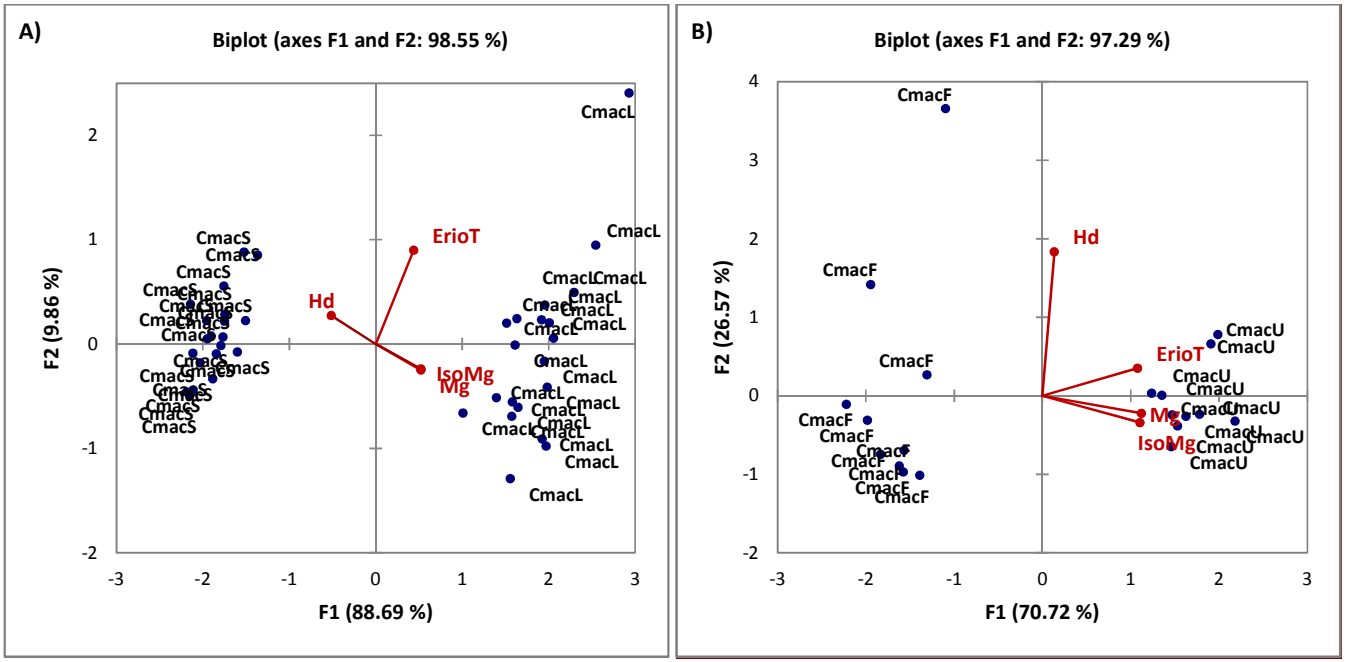

Figure 3.2 PCA biplots for the major phenolic compound contents of A) leaves (CmacL) and stems (CmacS) and B) unfermented (CmacU) and fermented (CmacF) *C. maculata* plant material (Mg = mangiferin, IsoMg = isomangiferin, ErioT = eriocitrin, Hd = hesperidin).

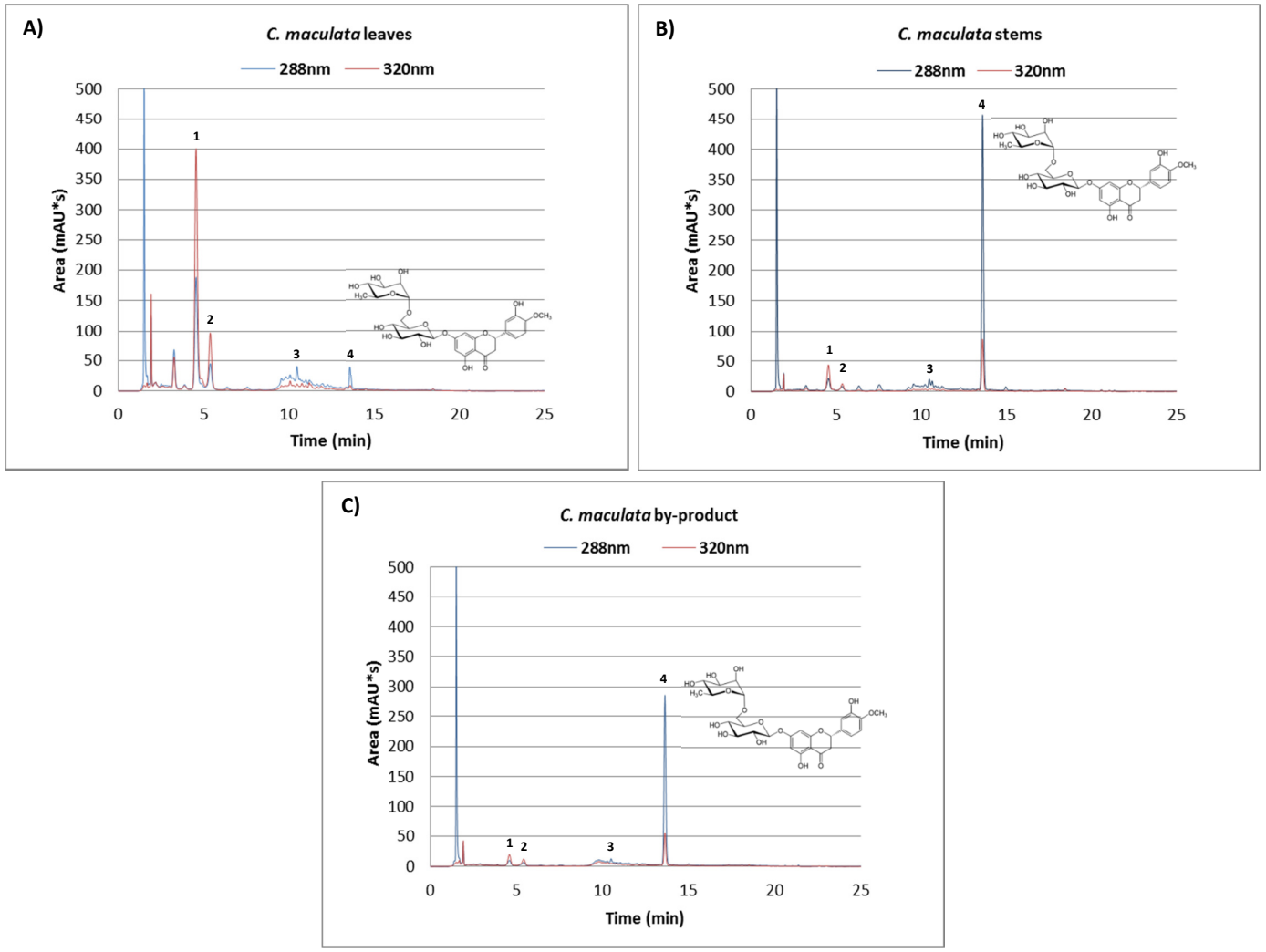

Figure 3.3 Chromatograms of *C. maculata* extracts from A) leaves (CmacL), B) stems (CmacS) and C) tea processing by-product (CmacW$_i$) (**1** = Mangiferin, **2** = Isomangiferin, **3** = Eriocitrin, **4** = Hesperidin).

3.4.2 Preliminary single factor experiments

Fermented by-product plant material from *C. maculata* ($CmacW_i$) (3.3.2.3) was selected for the preliminary single factor experiments, owing to its high hesperidin content as results in the previous section (3.4.1) have shown. As eriocitrin was present at very low concentrations the focus was to optimise the extraction of hesperidin with results obtained for eriocitrin shown in Addendum A.

Various studies have been performed on the effect of a number of extrinsic parameters on extraction efficiency during UAE with parameters such as frequency (Hz), power (W), ultrasonic intensity (W/cm^2), temperature (°C), time (min), ethanol-water ratio (% v/v) and solvent:solid ratio (mL/g) having the largest impact (Hossain *et al*., 2012; Khan *et al*., 2010; Pingret *et al*., 2012; Yang *et al*., 2010; Zhang *et al*., 2009). For this study four factors were chosen that may significantly affect the yield of the flavanone glycosides, hesperidin and eriocitrin, namely solvent composition (% ethanol, v/v), extraction time (min), extraction temperature (°C) and solvent:solid ratio (mL/g). A range of values for each variable was used in single factor experiments to determine their effect on extraction yield (g/100 g plant material) in terms of SS and hesperidin, as well as the hesperidin content of the SS (g /100 g) (Table 3.1).

3.4.2.1 Effect of solvent composition on extraction efficiency

The effect of solvent composition (0-96% ethanol, v/v) on extraction efficiency was evaluated while the other parameters were kept constant at 50°C, 10 mL/g and 30 min.

Water and ethanol are both food grade, non-toxic solvents, which made them suitable for this study (Li *et al*., 2006). However, the non-polar hesperidin is scarcely soluble in water (ca ≤ 20 mg/L) (Grohmann *et al*., 2000); therefore, ethanol/water mixtures were selected as more suitable for extraction of this major flavanone glycoside than pure water.

Extract yield was significantly ($p < 0.05$) affected by ethanol concentration, decreasing with increasing ethanol concentration (Fig. 3.4.A). The highest extract yield (12.6%) was observed at 19.2% (v/v) ethanol. Hesperidin yield, however, increased significantly with increasing ethanol concentration, reaching a maximum at 57.6%, followed by a significant decrease as the ethanol concentration increased further (Fig. 3.4.B). These effects caused the hesperidin content of the extract to increase significantly ($p < 0.05$) from 0 to 96% (v/v) ethanol (Fig. 3.4.C). This could be explained by the relative solubility of hesperidin and other compounds. Whilst the solubility of hesperidin increased up to 57.6 % (v/v) ethanol, whereafter it decreased, the solubility of polar solutes decreased with increasing ethanol concentration. A similar trend was observed for eriocitrin yield (Addendum A).

This trend is consistent with that reported by Yang *et al*. (2010). Ethanol was used as organic solvent for UAE of flavonoids from the flower of *Citrus aurantium* L. *var. amara* Engl. An increase in the flavonoid yield was observed as the ethanol concentration increased from 40 to 50% (v/v), followed by a decrease in yield when the ethanol concentration was further increased up to 80% (v/v). In addition, Şahin & Şamlı (2013) also reported an optimum total polyphenol yield from dried olive (*Olea europaea*)

leaves at an ethanol concentration of 50% (v/v). For conventional solvent extraction as preliminary experiment for RSM, Liu *et al*. (2010) reported an increase in total flavonoid compound from *Gynura medica* leaf with increasing ethanol concentration up to 45% (v/v), whereafter the yield decreased. On the other hand, Li *et al*. (2006) found that the total phenolic compound recovery from lemon peel increased with an increase in ethanol concentration up to 85% (v/v), followed by a decrease in recovery as the ethanol concentration increased further. Furthermore, Joubert *et al.* (2012) reported that, compared to 80 and 100% (v/v) ethanol, 50% (v/v) ethanol gave the highest recovery of total polyphenols, hesperidin and mangiferin from green *C. subternata* plant material.

These afore-mentioned results could be explained by the relationship between polarity and intermiscibility. When the polarities of the solvent and solute (target compound) are similar, the solute is easily dissolved from plant cells (Bazykina *et al*., 2002). In general, solvents with a lower ethanol concentration are suitable for extraction of polar flavonoids, whereas a higher ethanol concentration is more suitable for non-polar flavonoid extraction (Liu *et al*., 2010). The combination of water and ethanol as extraction solvents is regarded as ideal (Lang & Wai, 2001; 2006), since water is believed to act as the swelling agent of the plant matrix, whereas ethanol disrupts the bonding between the solutes and plant matrices. For this study, the polarity of 57.6% (v/v) ethanol was ideal to obtain an average hesperidin yield of 0.27 g/100 g plant material.

In the plant extract processing industry, 50% (v/v) ethanol is commonly used for commercial herbal extracts (D. Malherbe, Afriplex, Paarl, South Africa, 2013, personal communication). From Fig. 3.4 it is evident that although the hesperidin content increased with increased ethanol concentration, the extract yield decreased significantly. For example, at an ethanol concentration of 57.6% (v/v), one could produce a flavanone-rich extract with a high hesperidin content; however, the extract yield will be significantly less (10.3%) than at 19.2% (v/v) (12.6%). According to Malherbe (2013), from a cost perspective, obtaining a high yield of extract with a lower polyphenol content is more economical for the industry in terms of solvent consumption and plant material and solvent cost, than *vice versa*. However, for this study, a higher ethanol concentration of 57.6% (v/v) resulted in five times higher hesperidin content of the extract than 19.2% (v/v) and would therefore be more economical in this case.

From these results, the minimum (48%, v/v) and maximum (57.6%, v/v) ethanol concentration levels were selected for RSM experiments.

3.4.2.2 Effect of time on extraction efficiency

The effect of time (30-180 min) on extraction efficiency was evaluated while the other parameters were kept constant at 50°C, 10 mL/g and 57.6% (v/v) ethanol concentration.

From Fig. 3.5.A it is evident that the extract yield did not increase significantly after 90 min and reached a maximum of 9.4%. Both the hesperidin yield (Fig. 3.5.B) and the hesperidin content of the extract (Fig. 3.5.C) increased as extraction time (min) was increased. A similar trend was observed for

eriocitrin yield (Addendum A). Conversely, compared to the before-mentioned results, Yang *et al.* (2010) reported a significantly shorter optimum extraction time of 50 min, following a significant decrease in flavonoid yield as the time was increased. Similarly, Liu *et al*. (2010) reported an optimum extraction time of 30 min for maximum yield of total flavonoids.

For industrial application, too long extraction time would not be cost effective. According to Chemat *et al*. (2011), the industry currently performs UAE for a maximum of 60 min at a stage. Hot water honeybush tea extracts are currently produced by the industry at an extraction time of only 30 min (Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, 2012, personal communication).

To determine the optimum extraction time for all of the above-mentioned dependent variables combined, 90 and 120 min were selected as the minimum and maximum levels, respectively, for RSM experiments.

3.4.2.3 Effect of temperature on extraction efficiency

The effect of temperature (30-60°C) on extraction efficiency was evaluated while the other parameters were kept constant at 10 mL/g, 30 min and 57.6% (v/v) ethanol concentration.

From Fig. 3.6.A, B and C it is evident that the extract yield, hesperidin yield and hesperidin content of the extract, increased with increased temperature from 30 to 60°C. This could be ascribed to enhanced diffusion coefficients and flavonoid solubility with increasing extraction temperature (Liu *et al.* 2010). Molecular movement is increased by high temperatures, leading to increased solubility, allowing for flavonoids to dissolve from plant cells more effectively (Yang *et al.*, 2010). Grohmann *et al.* (2000) demonstrated that the solubility of hesperidin in water increased with increased temperatures. This was evident for both hesperdin and eriocitrin (Addendum A) yield and their content in the extract, which increased significantly with temperatures greater than 40°C (no significant increase from 30-40°C was observed). A maximum yield of 0.423 g hesperidin per 100 g plant material and maximum extract yield of 9.529 g SS per 100 g plant material was achieved at 60°C. Due to the limitations of the ultrasonic apparatus used for this experiment, temperature could not be set at > 69°C. Potentially, higher yields could have been achieved since hesperidin was found to be stable at 90°C, as reported by Dhuique-Mayer *et al.* (2007).

Similarly, Liu *et al*. (2010) demonstrated a significant increase in total flavonoid compound yield as the temperature was increased from 75 to 95°C. Yang *et al.* (2010) reported a steady increase in flavonoid yield from 30 to 70°C. The flavonoid yield decreased after 70°C, which was ascribed to oxidation and degradation of flavonoids at higher temperatures.

Nonetheless, it is important that cost implications, potential flavonoid degradation and flammability of ethanol should be considered before increasing extraction temperature. Furthermore, although the cavitation phenomena is directly proportional to an increase in temperature, too high temperatures (>50°C) can cause a decrease in shock waves, that may ultimately diminish the ultrasonic

effect (Lorimer & Mason, 1987). Therefore, Pingret *et al*. (2012) selected a temperature setting of 40°C for the UAE of polyphenols from apple pomace.

From the above-mentioned results, temperature levels of 50°C and 60°C were selected as the minimum and maximum, respectively, for RSM experiments.

3.4.2.4 Effect of solvent:solid ratio on extraction efficiency

The effect of solvent:solid ratio (10-60 mL/g) on extraction efficiency was evaluated while the other parameters were kept constant at 50°C, 30 min and 57.6% (v/v) ethanol concentration.

The results for the effect of solvent:solid ratio show an increased extract yield (Fig. 3.7.A) with increase in solvent:solid ratio which appears to start reaching a plateau at 40 mL/g with a maximum extract yield of 15.28% at 50 mL/g. Correspondingly, a significant increase in hesperidin yield with an increase in ratio from 10 to 50 mL/g was observed, before it appears to have reached a plateau (Fig. 3.7.B). A maximum hesperidin yield (1.35 g/100 g plant material) was observed at a ratio of 50 mL/g. Similar to the extract yield, the hesperidin content of the extract (Fig. 3.7.C) increased significantly as the solvent:solid ratio increased, but appears to have reached a plateau at 40 mL/g with a maximum hesperidin content of 9.02 g/100 g SS at 60 mL/g. Similar trends were observed for eriocitrin yield (Addendum A).

Similarly, both Yang *et al*. (2010) and Liu *et al*. (2010) also reported a significant increase in extraction efficiency with an increase in solvent:solid ratio, with an optimum solvent:solid ratio at 40 mL/g for flavonoids yield (% flavonoids yield of dry *Citrus aurantium* L. var. *amara* Engl flower material and mg kaempferol/g dry *Gynura medica* leaf material, respectively). However, Liu *et al*. (2010) also found no significant increase in flavonoid yield after 40 mL/g, whereas Yang *et al.* (2010) reported a significant decrease in yield after 40 mL/g.

This trend of increase in yield can be ascribed to the increase of concentration gradient of flavonoids that promotes greater dissolution of the flavonoids from the plant material (Liu *et al*., 2010; Yang *et al*., 2010). The decrease in flavonoid yield as the ratio increases can be ascribed to more impurities such as polysaccharides and protein that may have been solubilised from the plant material. When selecting a solvent:solid ratio, it is also important to consider not too high solvent:solid ratio's as it may lead to unfavourably high solvent consumption and higher solvent cost, as well as having a negative impact on the environment. Additionally it would mean higher volumes of solvent to be removed by evaporation with concomitantly high energy costs.

From these results, 30 and 50 mL/g solvent:solid ratio levels were selected as the minimum and maximum, respectively, for RSM experiments.

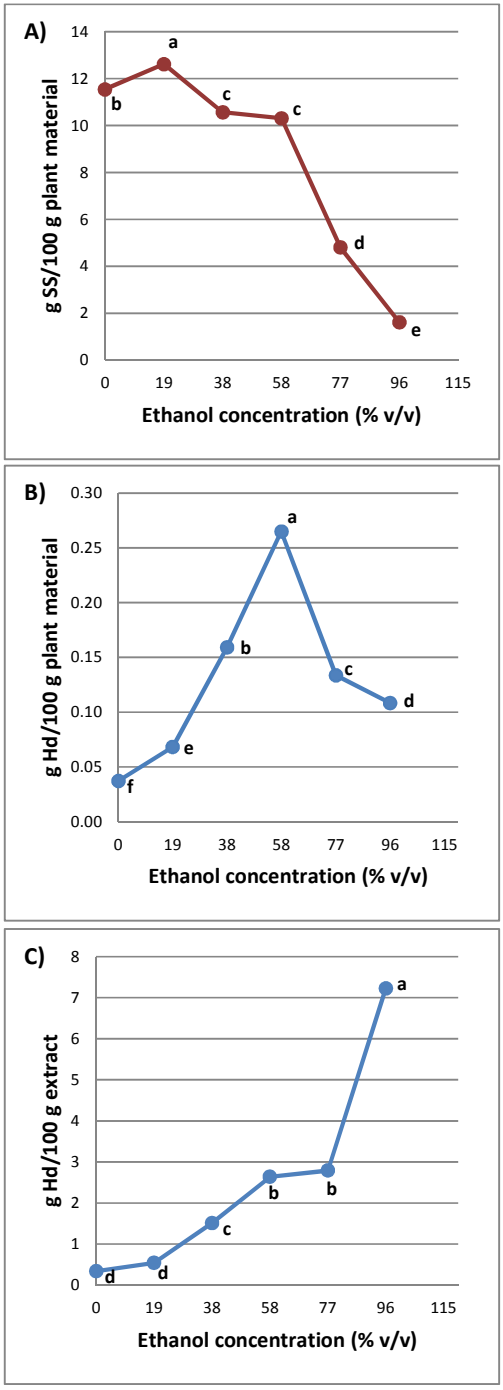

Figure 3.4 Effect of solvent composition (ethanol concentration, %v/v) on extraction efficiency of A) extract (SS) yield, B) hesperidin (Hd) yield and C) hesperidin (Hd) content of the extract. Constant parameters for each experiment were 50°C, 30 min and 10 mL/g solvent:solid ratio *(means with the same letter are not significantly different ($p \geq 0.05$)).*

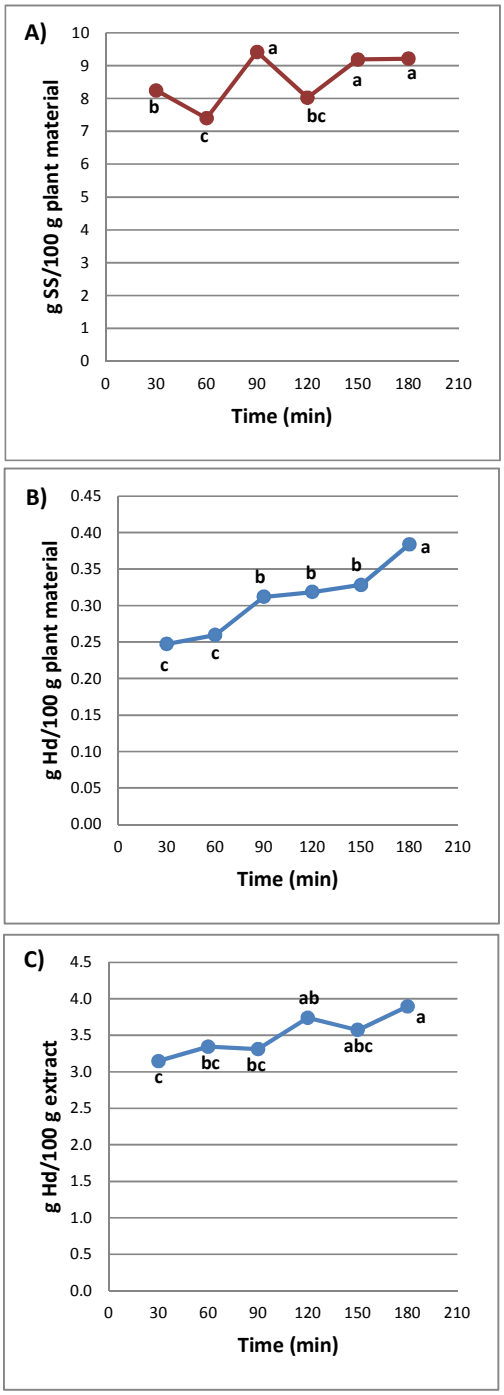

Figure 3.5 Effect of time (min) on extraction efficiency of A) extract (SS) yield, B) hesperidin (Hd) yield and C) hesperidin (Hd) content of the extract. Constant parameters for each experiment were 50°C, 10 mL/g solvent:solid ratio and 57.6% (v/v) ethanol *(means with the same letter are not significantly different ($p \geq 0.05$)).*

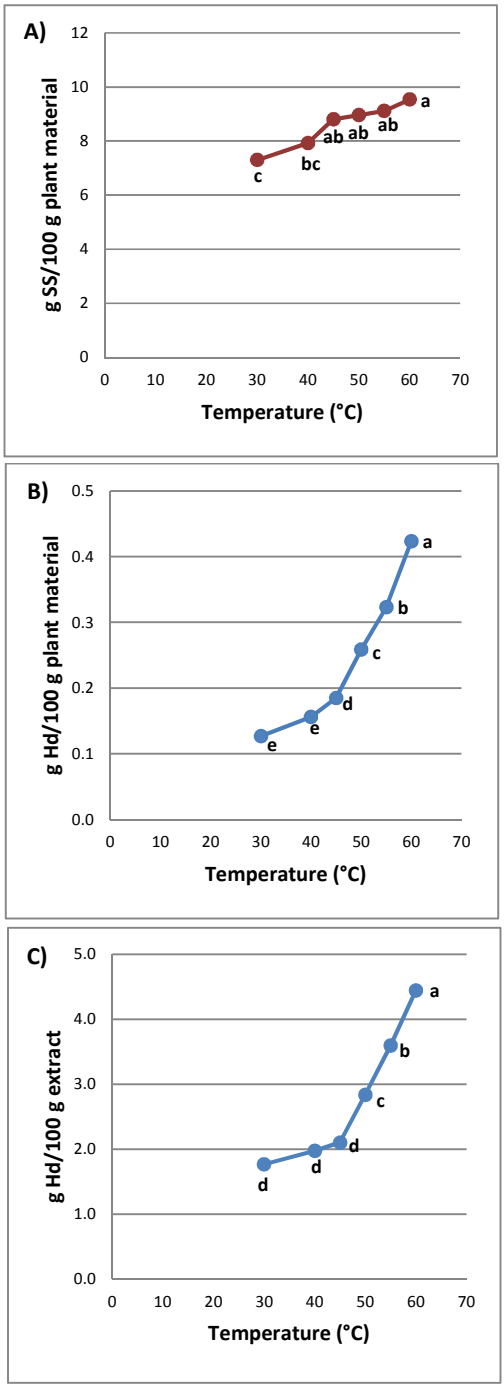

Figure 3.6 Effect of temperature (°C) on extraction efficiency of A) extract (SS) yield, B) hesperidin (Hd) yield and C) hesperidin content of the extract. Constant parameters for each experiment were 30 min, 10 mL/g solvent:solid ratio and 57.6% (v/v) ethanol *(means with the same letter are not significantly different ($p \geq 0.05$)).*

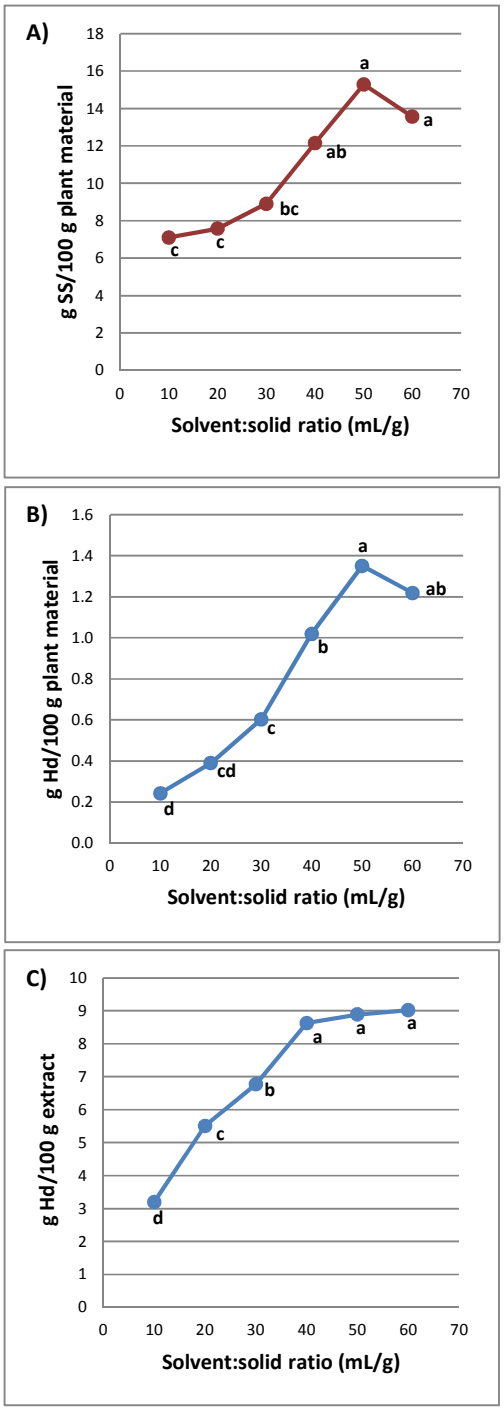

Figure 3.7 Effect of solvent:solid ratio (mL/g) on extraction efficiency of A) extract (SS) yield, B) hesperidin (Hd) yield and C) hesperidin (Hd) content of the extract. Constant parameters for each experiment were 50°C, 30 min, and 57.6% (v/v) ethanol *(means with the same letter are not significantly different ($p \geq 0.05$)).*

3.4.3 Response surface optimisation of flavanone extraction from *C. maculata* by-product

The hesperidin and eriocitrin content of *C. maculata* by-product ($CmacW_i$) as determined using 33% (v/v) aqueous acetonitrile (3.4.1.3), was 2.69 and 0.052 g/100 g plant material, respectively. The UAE of hesperidin and eriocitrin from *C. maculata* processing by-product was optimised using a RSM approach to evaluate the four selected extraction parameters, ethanol concentration (% v/v), extraction time (min), extraction temperature (°C) and solvent:solid ratio (mL/g), as well as the interaction between these variables. Based on the results from the preliminary single factor experiments (3.4.2), three levels for each variable were selected for the CCD and additional star points (-α and α) were calculated (Table 3.2).

The de-coded experimental parameters and the average response values obtained for each experimental run of the CCD are presented in Table 3.3. The average response values varied widely as a function of the parameter setting of the experiments, i.e. from 10.83-20.57 g SS/100 g plant material for extract yield, 0.67-2.00 g hesperidin/100 g plant material for hesperidin yield and 4.40-10.23 g hesperidin/100 g SS for hesperidin content of the extract. The range of hesperidin yield obtained were 25-74% of the yield obtained when analysing the plant material content using 33% (v/v) aqueous acetonitrile. Khan *et al*. (2010) reported a lower hesperidin yield of 0.21 g/100 g fresh orange (*Citrus sinensis* L.) peel (at optimum UAE parameters of 40°C, 30 min and 80% (v/v) ethanol), compared to the maximum hesperidin yield of 2.00 g/100 g *C. maculata* plant material. *Cyclopia maculata* by-product could therefore possibly be a better source of hesperidin than the orange processing by-product. However, the extraction conditions of these by-products were not the same and extraction yield could therefore not be compared directly.

Regression analysis and ANOVA were performed to evaluate the significance and suitability of the design.

3.4.3.1 Analysis of RSM data

RSM data analysis was conducted to determine the optimum parameter values (ethanol concentration, time, temperature, solvent:solid ratio) for hesperidin and eriocitrin extraction and to investigate the effect of these parameters on the responses. In light of the afore-mentioned, the second-order polynomial equation, analysis of variance (ANOVA), Pareto chart, response surface and contour plots and desirability profiling will be interpreted in the following section (RSM data analysis results for eriocitrin yield and content of extract are presented in Addendum A).

Regression equation

From the CCD results, all the response values for hesperidin, eriocitrin and extract yield, as well as hesperidin and eriocitrin content of the extract, were fitted as a function of ethanol concentration (% v/v), extraction time (min), extraction temperature (°C) and solvent:solid ratio (mL/g). Regression

equations of the response surfaces were obtained, for example for hesperidin (Hd) yield (g /100 g plant material):

$$Y_{Hd\ yield} = -6.764 + 0.293X_1 - 0.002X_1^2 - 0.038X_2 + 0.0001X_2^2 - 0.026X_3 + 0.0003X_3^2 + 0.003X_4 - 0.0002X_4^2 + 0.0001X_1X_2 - 0.0004X_1X_3 - 0.0004X_1X_4 + 0.0005X_2X_3 + 0.00004X_2X_4 + 0.0009X_3X_4 \quad (2)$$

The estimated coefficients (linear, quadratic and interaction) for the regression equations of all dependent variables are presented in Tables 3.4 and 3.5.

Analysis of variance (ANOVA)

For ANOVA the variation due to treatment (i.e. change in the combination of variables) is compared with the variation due to random errors inherent in the measurements of the produced responses. Consequently, one can evaluate the significance of the regression used to predict responses considering the experimental variance sources (Yang *et al.*, 2010). The ANOVA results for extract and hesperidin yield, as well as hesperidin content of the extract, are presented in Tables 3.4 and 3.5, respectively (see Addendum A for eriocitrin ANOVA results). The p-values of the parameter effects that were significant ($p < 0.05$) are highlighted in red.

The term R^2_{adj} represents the regression coefficient that is adjusted for the number of coefficients included in the model. Accordingly, one can compare models with different numbers of parameters (independent variables) and test the level of suitability to the regression coefficient (Pingret *et al.*, 2012). The ANOVA for hesperidin and eriocitrin yield gave R^2_{adj}-values of 0.80 and 0.77, respectively, and a R^2_{adj}-value of 0.79 for both hesperidin and eriocitrin content of the extract. Therefore, approximately 80% of the variability of the responses was explained, indicating a close agreement between experimental (observed) and predicted values and therefore a good accuracy and applicability of the applied models (Mirhosseini *et al*., 2008).

Conversely, the R^2_{adj}-value for extract yield (g SS/100 g plant material) was only 0.43, indicating a much lower correlation between the experimental and predicted values. Furthermore, the Mean Square (MS) residual of 4.28 for extract yield indicates a high value for unexplained experimental variation.

Furthermore, a so-called LOF-test is used as a more sensitive test of model fit, using the MS pure error (part of residual SS that cannot be predicted by any additional terms) as the error term. A model (regression equation) will fit very well to the experimental data should it show a significant regression and a non-significant LOF (Bezerra *et al*., 2008). No significance in LOF ($p > 0.05$) was found for four of the five models, i.e. hesperidin and eriocitrin yield and their content in the extract, which indicates that these models could be used to predict the responses. This further strengthens the reliability of these models (Gong *et al.,* 2012). However, as indicated by the R^2_{adj}-value for extract yield (Table 3.5) the significant LOF-value of 0.035 shows that this model will not be fitted as well to the experimental data as the other four models.

Table 3.3 Central Composite Design for independent variables and the average response values (n=3) for extract yield, hesperidin yield and hesperidin content of the extract

Run No.	X_1 Ethanol concentration (% v/v)	X_2 Time (min)	X_3 Temperature (°C)	X_4 Solvent:solid ratio (mL/g)	**g SS/ 100 g plant material**	**g Hesperidin/ 100 g plant material**	**g Hesperidin/ 100 g extract**
1	48.0	90	50	30	15.46 ± 1.41	0.68 ± 0.02	4.40 ± 0.50
2	48.0	90	50	50	16.14 ± 2.07	0.94 ± 0.15	5.82 ± 0.23
3	48.0	90	60	30	15.46 ± 1.81	1.18 ± 0.08	7.68 ± 0.42
4	48.0	90	60	50	20.57 ± 3.94	1.73 ± 0.47	8.36 ± 0.89
5	48.0	120	50	30	15.33 ± 0.62	0.71 ± 0.03	4.64 ± 0.10
6	48.0	120	50	50	16.79 ± 3.58	0.98 ± 0.12	5.93 ± 0.57
7	48.0	120	60	30	18.58 ± 1.71	1.46 ± 0.12	7.91 ± 0.93
8	48.0	120	60	50	20.05 ± 1.25	2.00 ± 0.10	10.00 ± 0.73
9	57.6	90	50	30	13.16 ± 2.54	0.74 ± 0.03	5.78 ± 1.07
10	57.6	90	50	50	15.49 ± 0.04	1.02 ± 0.03	6.60 ± 0.20
11	57.6	90	60	30	15.58 ± 2.57	1.41 ± 0.17	9.12 ± 0.53
12	57.6	90	60	50	17.12 ± 3.41	1.72 ± 0.35	10.07 ± 0.65
13	57.6	120	50	30	14.85 ± 1.96	0.87 ± 0.12	5.83 ± 0.25
14	57.6	120	50	50	17.88 ± 1.79	1.16 ± 0.05	6.50 ± 0.34
15	57.6	120	60	30	19.15 ± 1.37	1.57 ± 0.12	8.24 ± 0.84
16	57.6	120	60	50	19.25 ± 2.44	1.98 ± 0.48	10.21 ± 1.21
17	43.2	105	55	40	13.41 ± 0.54	0.93 ± 0.12	6.93 ± 1.06
18	62.4	105	55	40	14.19 ± 2.21	1.20 ± 0.16	8.49 ± 0.79
19	52.8	75	55	40	13.72 ± 0.28	1.15 ± 0.10	8.35 ± 0.60
20	52.8	135	55	40	19.28 ± 1.42	1.49 ± 0.09	7.76 ± 0.64
21	52.8	105	45	40	13.21 ± 3.16	0.67 ± 0.13	5.15 ± 0.99
22	52.8	105	65	40	18.81 ± 1.95	1.94 ± 0.48	10.23 ± 1.57
23	52.8	105	55	20	10.83 ± 2.31	0.70 ± 0.08	6.58 ± 1.13
24	52.8	105	55	60	18.54 ± 0.73	1.73 ± 0.34	9.33 ± 1.60
25 (C)*	52.8	105	55	40	15.81 ± 1.52	1.21 ± 0.11	7.68 ± 0.08
26 (C)*	52.8	105	55	40	16.92 ± 0.93	1.39 ± 0.09	8.20 ± 0.08

** Centre point parameter values*

Table 3.4 ANOVA of experimental results and estimated coefficients for quadratic polynomial models for hesperidin yield and hesperidin content of the extract *(p-values of the parameter effects that were significant (p < 0.05) are highlighted in red)*

	g Hesperidin/100 g plant material						g Hesperidin/100 g extract					
Parameter	**Regr. Coeff.[a]**	**SS[b]**	**DF[c]**	**MS[d]**	**F**	**p**	**Regr. Coeff.[a]**	**SS[b]**	**DF[c]**	**MS[d]**	**F**	**p**
Intercept	-6.7638						-64.2049					
(1)Ethanol concentration (L)	0.2930	0.2240	1	0.2240	5.1078	0.0280	1.1322	14.4054	1	14.4054	22.1523	0.0000
Ethanol concentration (Q)	-0.0023	0.1507	1	0.1507	3.4363	0.0693	-0.0064	1.1317	1	1.1317	1.7403	0.1928
(2)Time (L)	-0.0377	0.4942	1	0.4942	11.2687	0.0015	0.1207	0.0073	1	0.0073	0.0112	0.9160
Time (Q)	0.0001	0.0058	1	0.0058	0.1314	0.7184	-0.0003	0.1889	1	0.1889	0.2905	0.5922
(3)Temperature (L)	-0.0261	9.0703	1	9.0703	206.8154	0.0000	0.8582	164.4130	1	164.4130	252.8304	0.0000
Temperature (Q)	0.0003	0.0023	1	0.0023	0.0526	0.8196	-0.0061	1.2117	1	1.2117	1.8634	0.1780
(4)Solvent:solid ratio (L)	0.0030	3.1259	1	3.1259	71.2756	0.0000	0.0114	29.6672	1	29.6672	45.6215	0.0000
Solvent:solid ratio (Q)	-0.0002	0.0123	1	0.0123	0.2802	0.5988	-0.0009	0.3893	1	0.3893	0.5987	0.4425
1L by 2L	0.0001	0.0007	1	0.0007	0.0152	0.9023	-0.0026	1.6935	1	1.6935	2.6042	0.1125
1L by 3L	-0.0004	0.0051	1	0.0051	0.1155	0.7353	-0.0006	0.0111	1	0.0111	0.0171	0.8965
1L by 4L	-0.0004	0.0209	1	0.0209	0.4759	0.4933	-0.0014	0.2196	1	0.2196	0.3378	0.5636
2L by 3L	0.0005	0.0748	1	0.0748	1.7046	0.1973	0.0007	0.1286	1	0.1286	0.1977	0.6584
2L by 4L	0.0000	0.0021	1	0.0021	0.0473	0.8287	0.0009	0.8653	1	0.8653	1.3306	0.2539
3L by 4L	0.0009	0.0930	1	0.0930	2.1211	0.1512	0.0019	0.4145	1	0.4145	0.6374	0.4282
Lack of fit		0.2231	10	0.0223	0.5086	**0.8764**		10.4308	10	1.0431	1.6040	**0.1310**
Error (MS Residual)		2.3244	53	0.0439				34.4654	53	0.6503		
Total SS		15.9225	77					258.4153	77			
R^2						**0.8400**						**0.8263**
R^2_{adj}						**0.8045**						**0.7877**

[a] *Regression coefficients*
[b] *Sum of Squares*
[c] *Degree of Freedom*
[d] *Mean Square*
L = linear coefficient; Q = quadratic coefficient; L by L = interaction coefficient

Table 3.5 ANOVA of experimental results and estimated coefficients for quadratic polynomial models for extract yield *(p-values of the parameter effects that were significant (p < 0.05) are highlighted in red)*

Parameter	Regr. Coeff.[a]	SS[b]	DF[c]	MS[d]	F	p
Intercept	15.8142					
(1)Ethanol concentration (L)	1.2701	2.3405	1	2.3405	0.5473	0.4627
Ethanol concentration (Q)	-0.0157	6.8092	1	6.8092	1.5924	0.2125
(2)Time (L)	-0.6406	72.0311	1	72.0311	16.8453	0.0001
Time (Q)	0.0014	5.1465	1	5.1465	1.2036	0.2776
(3)Temperature (L)	-0.7676	126.8768	1	126.8768	29.6716	0.0000
Temperature (Q)	0.0076	1.9051	1	1.9051	0.4455	0.5074
(4)Solvent:solid ratio (L)	0.4677	121.2449	1	121.2449	28.3545	0.0000
Solvent:solid ratio (Q)	-0.0014	1.0223	1	1.0223	0.2391	0.6269
1L by 2L	0.0058	8.3346	1	8.3346	1.9492	0.1685
1L by 3L	-0.0031	0.2724	1	0.2724	0.0637	0.8017
1L by 4L	-0.0023	0.5588	1	0.5588	0.1307	0.7192
2L by 3L	0.0031	2.5547	1	2.5547	0.5975	0.4430
2L by 4L	-0.0015	2.4402	1	2.4402	0.5707	0.4533
3L by 4L	0.0009	0.1004	1	0.1004	0.0235	0.8788
Lack of fit		92.4742	10	9.2474	2.1626	**0.0350**
Error (MS Residual)		226.6299	53	4.2760		
Total SS		683.1439	77			
R^2						**0.5329**
R^2_{adi}						**0.4291**

[a] *Regression coefficients*
[b] *Sum of Squares*
[c] *Degree of Freedom*
[d] *Mean Square*

L = linear coefficient; Q = quadratic coefficient; L by L = interaction coefficient

Pareto chart

From the Pareto charts in Fig. 3.5 the significant effects of all parameters (linear and quadratic) and their interactions can be observed. The length of the individual bars is proportional to the absolute scale of the standard estimated effects and a negative value implies a negative parameter effect on the response value. The nominal magnitude of the statistically significant effects (95% of confidence interval) with regard to response value is represented by the red line on the chart.

From Fig. 3.5.A-C it is evident that the linear effect of extraction temperature (°C) had the most significant ($p < 0.05$) positive effect on all the responses. This could be ascribed to the increased solubility of the target compounds at higher temperatures, increased solvent diffusion rate and improved mass transfer rate of SS from plant material to solvent (Grohmann *et al*., 2000; Hossain *et al*., 2012). Similarly Zhang *et al*. (2009) and Pingret *et al.* (2012) reported temperature as the major contributing factor to the ultrasound-assisted recovery of oil from almond powder and polyphenols from apple pomace, respectively.

For extract, hesperidin and eriocitrin yield, the linear effect of solvent:solid ratio (mL/g) had the second most significant influence on the response values, followed by the linear effect of time (min) (refer to Addendum A for eriocitrin data). Furthermore, for the hesperidin content of the extract, the linear effect of solvent:solid ratio (mL/g) had the second most significant effect, followed by the linear

effect of ethanol concentration (% v/v), and *vice versa* for the eriocitrin content of the extract. Interestingly, when evaluating the effects on all response values, only hesperidin yield was significantly affected by the linear effects of all four extraction parameters. From Fig. 3.5.A it is evident that temperature (°C) and solvent:solid ratio (mL/g) had almost an equal significant effect on extract yield, followed by time (min).

The lack of significance of the interaction coefficients ($p \geq 0.05$) for all response values indicates that there were no significant interactions between the extraction parameters in the range evaluated in this study. Although no significant interaction between temperature and solvent:solid ratio was observed for hesperidin yield ($p = 0.15$), it was the interaction with the lowest p-value (Table 3.4). Similarly, no significant interaction between the extraction parameters for eriocitrin yield was observed (Addendum A). Conversely, Yang *et al.* (2010) found significant ($p = 0.02$) interaction between these two extraction parameters for UAE of flavonoids from the flower of *Citrus aurantium* L. var. *amara* Engl, i.e. the influence of temperature on the flavonoid yield was different at different levels of solvent:solid ratio.

Combined response surface and contour plots

Combined response surface and contour (at the base of each graph) plots for hesperidin yield and content (of the extract), and extract yield are shown in Fig. 3.6 and 3.7.

These response plots are a visualisation of the predicted model that is fitted to experimental data from which optimal conditions can be derived and refers to the relationship of a response to the values of one or more factors (Bezerra *et al*., 2008). The three-dimensional response surface plot represents the magnitude of the response value and reflects effects of two variables on a response value at a time, whereas the circular or ellipse shape of the contour lines on the two-dimensional contour surface plot show non-significant or significant interaction effect between the factors, respectively (Baş & Boyacı, 2007; Bezerra *et al*., 2008; Dejaegher & Vander Heyden, 2011; Lundstedt *et al*., 1998).

Similar to the Pareto charts, the response plots in Fig. 3.6 and 3.7 show the variables that had significant effect on hesperidin and extract yield and their interaction (or lack thereof). For example, in Fig. 3.6.B and 3.6.C the effect of temperature (°C) and ethanol concentration (% v/v) on hesperidin yield and its content in the extract are reflected. From the magnitude of the slope, it is evident that both of these two factors had a significant ($p < 0.05$) effect on the response values. In addition, the surface plots show that no optimum values for extraction were reached; however, they give a graphic indication of the direction of the optimum. The circular shape of the contour plots visualise that there was no significant interaction between temperature and ethanol concentration. Similar results for temperature (°C) and solvent-liquid ratio (mL/g) are depicted in Fig. 3.7.

From these graphs, it is evident that no global optimum for the different variables was reached, and that apart from ethanol concentration, the optimum values for temperature and solvent:solid ratio are outside the experimental domain. Response plots that were constructed for eriocitrin indicated similar trends (Addendum A).

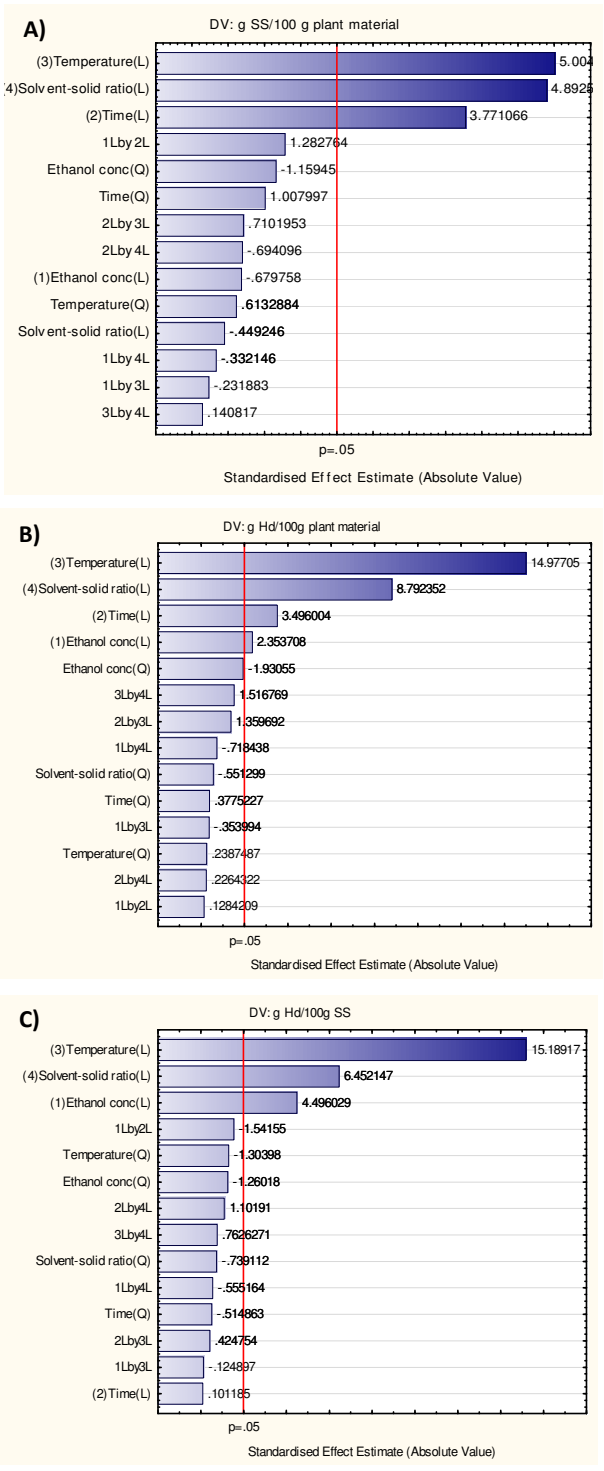

Figure 3.5 Pareto charts of standardised effect estimates (absolute value) for dependent variables (DV), A) extract yield, B) hesperidin (Hd) yield, and C) hesperidin (Hd) content of the extract, indicating significant ($p < 0.05$) effects of extraction parameters on each response value *(L = linear effect, Q = quadratic effect, L by L = interaction effect).*

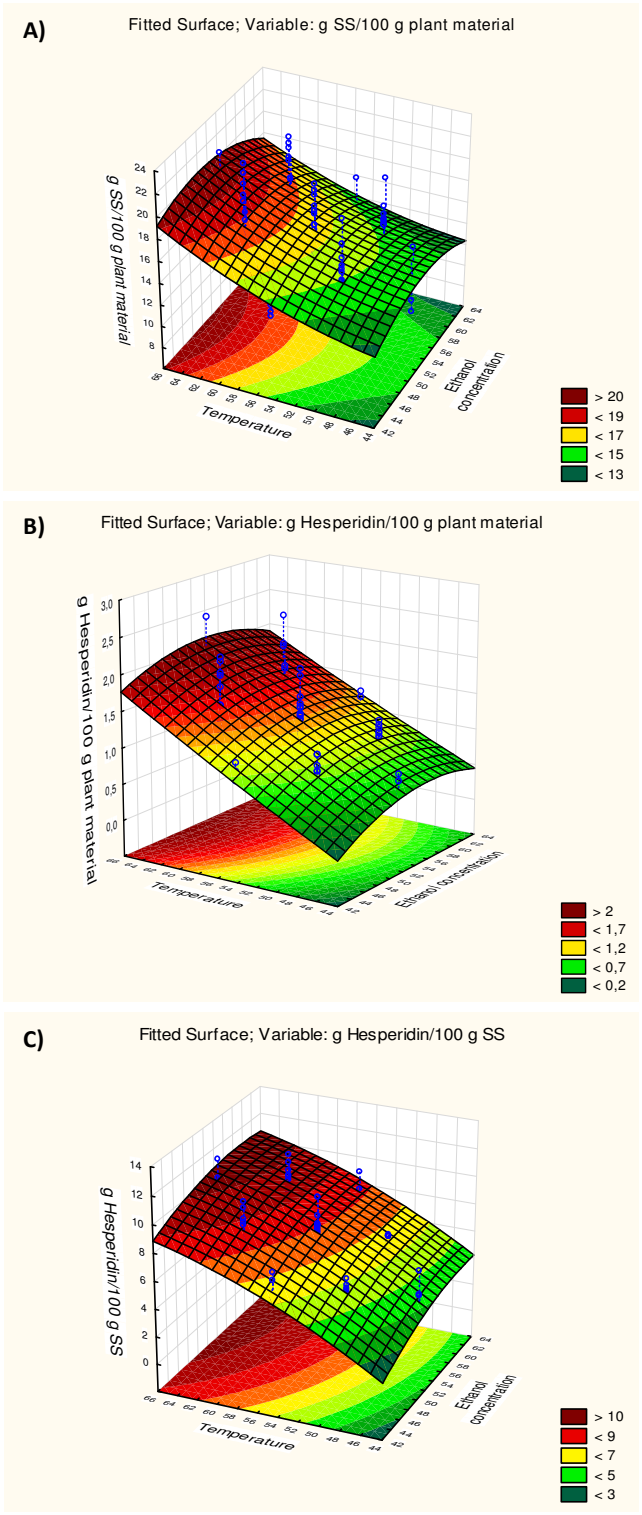

Figure 3.6 Combined response surface and contour plots for A) extract yield, B) hesperidin yield, and C) hesperidin content of the extract as a function of temperature (°C) and ethanol concentration (% v/v).

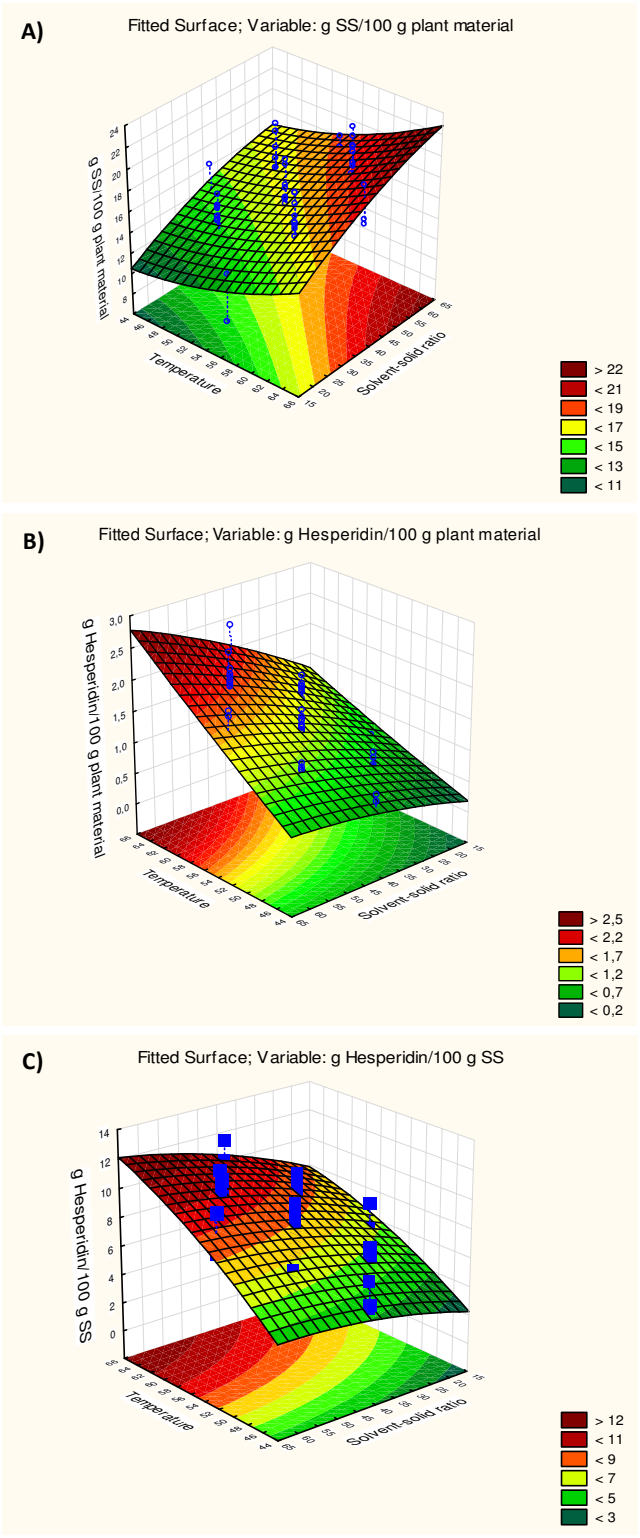

Figure 3.7 Combined response surface and contour plots for A) extract yield, B) hesperidin yield, and C) hesperidin content of the extract as a function of temperature (°C) and solvent:solid ratio (mL/g).

Optimisation of extraction parameters (desirability profiling)

In addition to the response plots, response and desirability profiling can be performed when analysing the experimental design. It is used to determine the levels of the independent variables (parameters) that produce the most desirable predicted responses on the dependent variables (response values). A prediction profile (Fig. 3.8-3.10) for a response value consists of a series of graphs (one for each parameter) of the predicted values for the response value at different levels of one parameter, keeping the levels of the other parameters constant at specified values (similar to response surface and contour plots). Confidence or prediction intervals (indicated in blue in Fig. 3.8-3.10) for the predicted values can also be shown to aid in the assessment of the reliability of prediction. The predicted values of the responses at different combinations of parameter levels are transformed into dimensionless individual desirability scores ranging between 0.0 and 1.0 in the desirability function. Thereafter, the overall desirability of the outcomes at different combinations of levels of the parameters can be computed, producing a series of graphs (one for each parameter) profiling the overall response desirability. This allows one to see at a glance how changes in the levels of each predictor variable influence not only responses on each dependent variable, but also the overall desirability of the responses.

Desirability profiling was conducted to produce a compound prediction profile graph that shows the prediction profiles for multiple dependent variables, i.e. hesperidin yield and content (of extract), as well as extract yield (Fig. 3.8). This allows one to see whether the levels of the independent variables that maximise responses for one dependent variable also maximise responses for other dependent variables.

It is evident from the compound prediction profile graph of hesperidin yield, content (of extract), and extract yield (Fig. 3.8) that only an overall desirability for ethanol concentration of 58.6% (v/v) for all three dependent variables was computed, as the optimum values for the other three parameters were outside the experimental domain (similar trends were observed for the single factor experiments results). In addition, the sharp slope of the desirability profile for temperature (°C), indicates the significant influence of temperature on all three dependent variables.

However, the individual desirability profiles for each dependent variable depicted that an optimum ethanol concentration value of 52.8% (v/v) was computed for extract yield (Fig. 3.9.A) and hesperidin yield (Fig. 3.9.B), whereas a value of 62.4% (v/v) was computed for hesperidin content (of extract) (Fig. 3.10). In addition, it was found that an optimum (or plateau) for time (105 min) was reached for hesperidin content (of extract) in individual desirability profiles (Fig. 3.9.B). Similarly, it was found that an optimum (or plateau) for time was reached for hesperidin content (of extract) in the preliminary single factor experiments. From the compound prediction profile graph of eriocitrin yield and content (of extract), as well as extract yield (Addendum A), no maximum desired parameter values were computed as all four optimum values were outside the experimental domain.

Nonetheless, no global optimum for the four extraction parameters was reached. Similarly, Khan *et al.* (2010) reported that the extraction efficiency for the UAE of hesperidin and naringenin from orange

(*Citrus sinensis* L.) peel increased as the extraction parameters, i.e. temperature, ultrasound power and ethanol concentration, were increased, resulting in optimum response values that were beyond the parameter limits selected. Therefore, Khan *et al*. (2010) selected the parameter values that corresponded to the maximum values chosen to define the experimental domain, i.e. temperature of 40°C, ultrasound power of 150 W and ethanol concentration of 80% (v/v), as the optimum conditions to determine further the predictability of the model. For this study, parameter values for the laboratory-scale preparation of a flavanone glycoside-enriched extract from *C. maculata* tea processing by-product were selected based on the feasibility of these values on industrial scale, as will be discussed in Section 3.4.3.3.

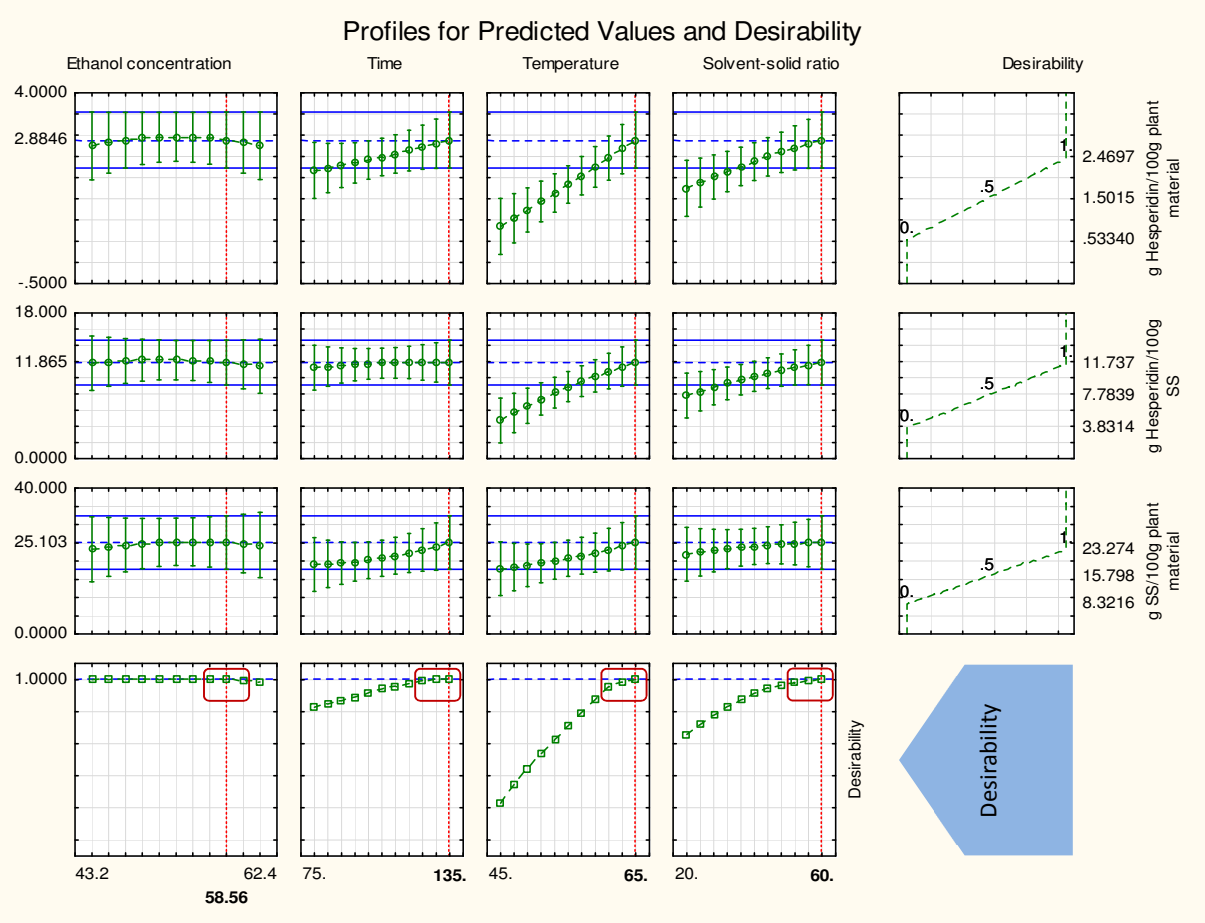

Figure 3.8 Compound prediction profile graph that shows the combined desirability profiles for dependent variables hesperidin yield and content (of SS), as well as extract yield *(red blocks indicate optimum/maximum parameter value combined for the responses)*.

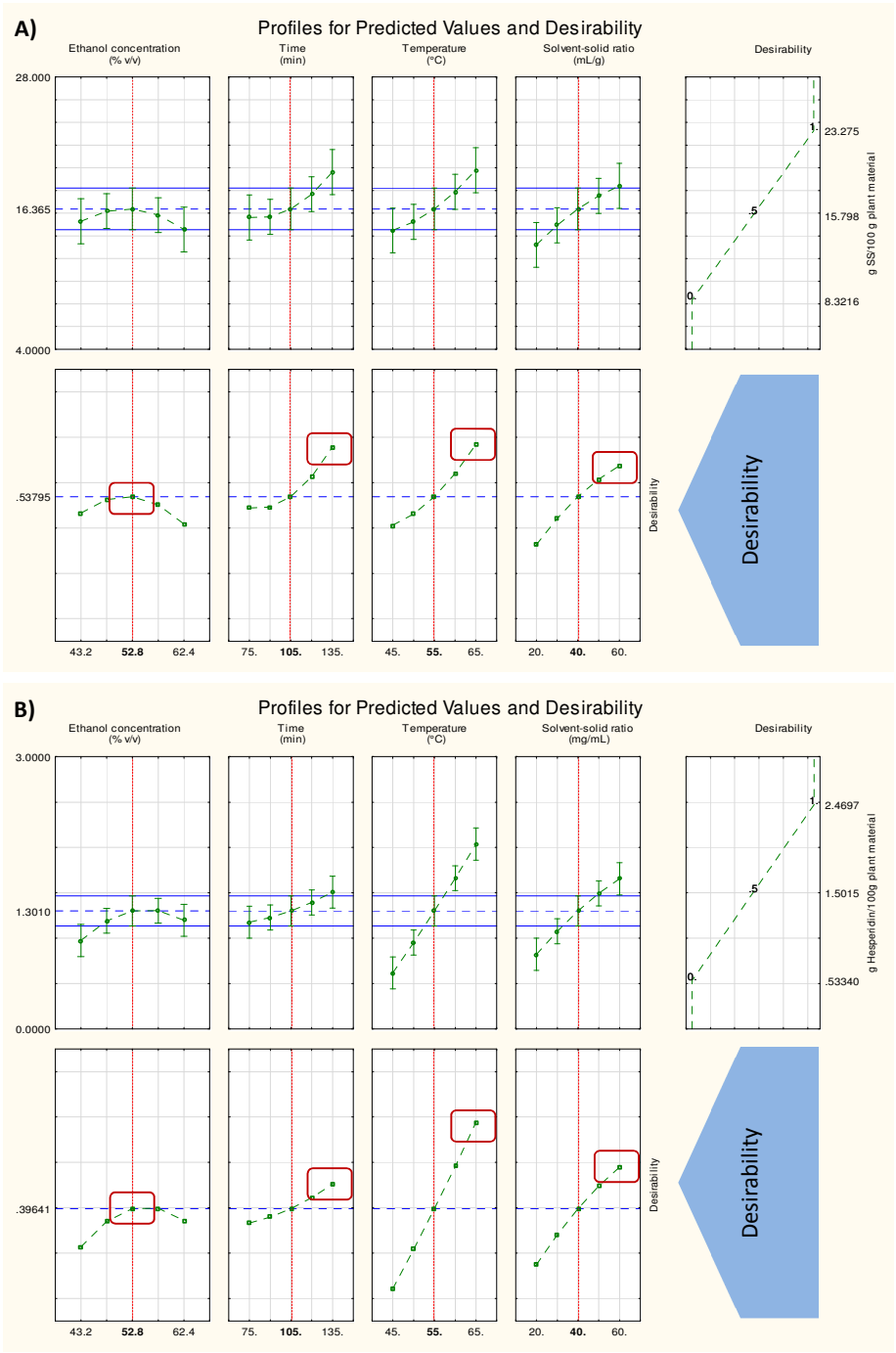

Figure 3.9 Individual prediction profile graphs that show the desirability profiles for each dependent variable, namely A) extract yield and B) hesperidin yield *(red blocks indicate optimum/maximum parameter value for the response)*.

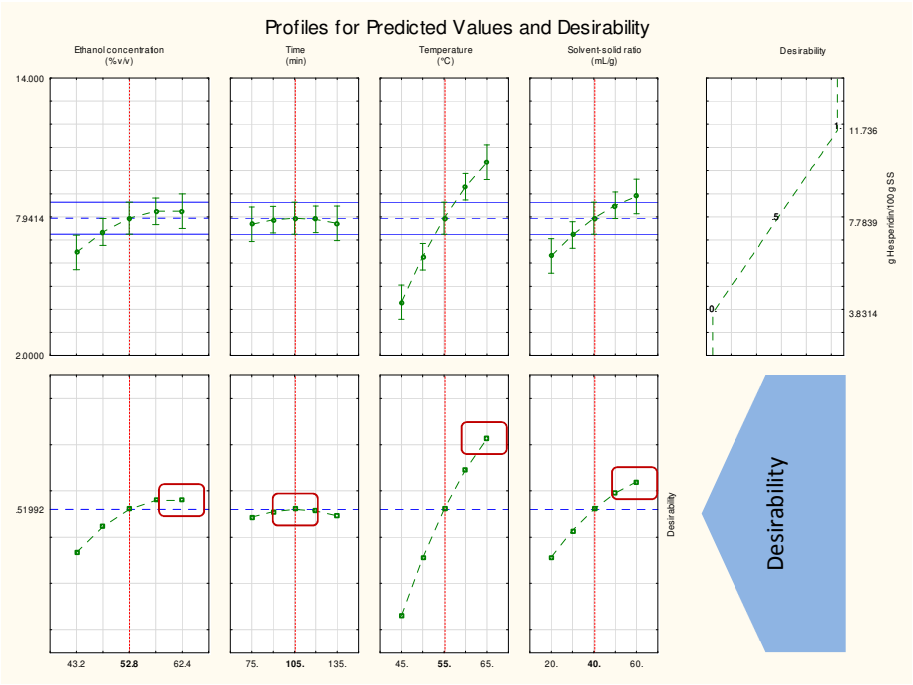

Figure 3.10 Individual prediction profile graph that show the desirability profile for hesperidin content of the extract *(red blocks indicate optimum/maximum parameter value for the response)*.

3.4.3.2 Verification of predicted value of the models

For this study, verification experiments were performed based on additional experimental runs to confirm how well data of new experiments will fit to the models computed in this study. The de-coded verification experiments and their responses obtained for each experimental run of the CCD are presented in Table 3.6.

To assess the fit of a model, values of explained variation (R^2) and predicted variation (Q^2) provide very good guidance (Lundstedt *et al*., 1998). Predicted variation (Q^2) consists of the fraction (0 < Q^2 < 1) of the total variation of the response that can be predicted in the model and is calculated by the following equation:

$$Q^2 = (SS - PRESS)/SS \quad (3)$$

where PRESS (predicted residual sum of squares) is determined through cross-validation (Lundstedt *et al*., 1998).

Thus, a small deviation between the experimental and the predicted residuals will give a low PRESS-value and a high value of predicted variation. According to Lundstedt *et al*. (1998), acceptable Q^2

values are dependent on the nature of the data evaluated, i.e. ≥ 0.5 for chemical and > 0.4 for biological data.

The ANOVA of the results for hesperidin yield and hesperidin content of the extract gave acceptable Q^2-values of 0.87 and 0.77, respectively, indicating a close correlation between actual and predicted values and demonstrating the validity of these afore-mentioned models. Similarly, eriocitrin yield and content of the extract gave acceptable Q^2-values of 0.65 and 0.59, respectively (Addendum A).

However, similar to the RSM findings (3.4.3.1), extract yield gave a much lower Q^2-value of 0.24. However, from Fig. 3.10.A and 3.10.B it is evident that, although the distribution of the predicted values vs actual values were similar for the RSM and verification experimental results, for the verification experiments, the predicted values shifted by a consistent factor, i.e. the actual values were smaller than predicted (over-estimation of predicted values). Similarly as depicted in Fig. 3.10 for extract yield, over-estimation of predicted values for hesperidin and eriocitrin yield was observed (plots not shown). Interclass correlation (ICC) is used to assess the consistency or reproducibility of quantitative measurements made by different observers (or experimental blocks in this case) measuring the same quantity. ICC (agreement) for the verification results takes the factor or standard error of measurement (SEM) of 1.2% into account resulting in a correlation coefficient of 0.24. However, when the SEM is not taken into account (ICC (consistency)), the correlation coefficient is rounded to 0.5, which is within the accepted range for fit of the model.

3.4.3.3 Practical optimum UAE parameters

From the RSM analyses (3.4.3.1) for the UAE of *C. maculata* tea processing by-products, apart from ethanol concentration (52.8% v/v for extract and hesperidin yield; 62.4% v/v for hesperidin content of the extract; 58.6% v/v for optimisation of all three parameters simultaneously), no global optimum for the extraction parameters were reached, since their optimum values were outside the experimental domain. Therefore, the maximum level for each independent variable, time (120 min), temperature (60°C) and solvent:solid ratio (50 mL/g) could be selected as optimum extraction parameter values. However, the parameter values 50 mL/g and 120 min are not feasible for industrial application due to high solvent consumption and cost, as well as high energy consumption and cost owing to extended heating periods. In addition, evaporation of large solvent volumes also adds to energy consumption. Therefore, below optimal extraction time and solvent:solid ratio could be selected to address these practical concerns. In light of the above-mentioned arguments, the following operating levels of the four UAE parameters were selected for laboratory-scale preparation of a flavanone glycoside-enriched extract from *C. maculata* tea processing by-products in the next research chapter (Chapter 4): 52.8% (v/v) ethanol, 20 mL/g solvent:solid ratio, 60°C and 30 min.

Table 3.6 Central Composite Design for verification experiments with average experimental (n=3) and predicted values for extract yield, hesperidin yield and hesperidin content (of extract)

Run No.	X_1 Ethanol concentration (% v/v)	X_2 Time (min)	X_3 Temperature (°C)	X_4 Solvent:solid ratio (mL/g)	**g SS/ 100 g plant material**		**g Hesperidin/ 100 g plant material**		**g Hesperidin/ 100 g extract**	
					Experimental	*Predicted*	*Experimental*	*Predicted*	*Experimental*	*Predicted*
1	52.8	105	55	40	20.16 ± 0.49	16.37	1.63 ± 0.16	1.30	8.08 ± 0.65	7.94
2	48.0	90	50	30	18.75 ± 0.93	13.21	0.87 ± 0.06	0.61	4.64 ± 0.11	4.89
3	57.6	120	50	30	17.80 ± 0.57	15.20	1.11 ± 0.11	0.86	6.24 ± 0.51	5.60
4	48.0	90	60	50	20.06 ± 1.37	18.82	1.79 ± 0.13	1.71	8.92 ± 0.56	8.99
5	57.6	120	60	50	20.53 ± 1.06	20.10	2.29 ± 0.35	2.01	11.14 ± 1.56	10.11
6	48.0	120	50	50	18.40 ± 1.50	16.63	1.12 ± 0.08	1.06	6.10 ± 0.07	6.42
7	57.6	120	50	50	17.79 ± 0.65	17.04	1.44 ± 0.16	1.16	8.10 ± 0.64	6.83
8	57.6	90	50	30	16.84 ± 1.75	12.38	0.91 ± 0.11	0.78	5.42 ± 0.32	6.33
9	48.0	90	60	30	20.77 ± 1.13	15.46	1.36 ± 0.14	1.17	6.61 ± 1.02	7.66

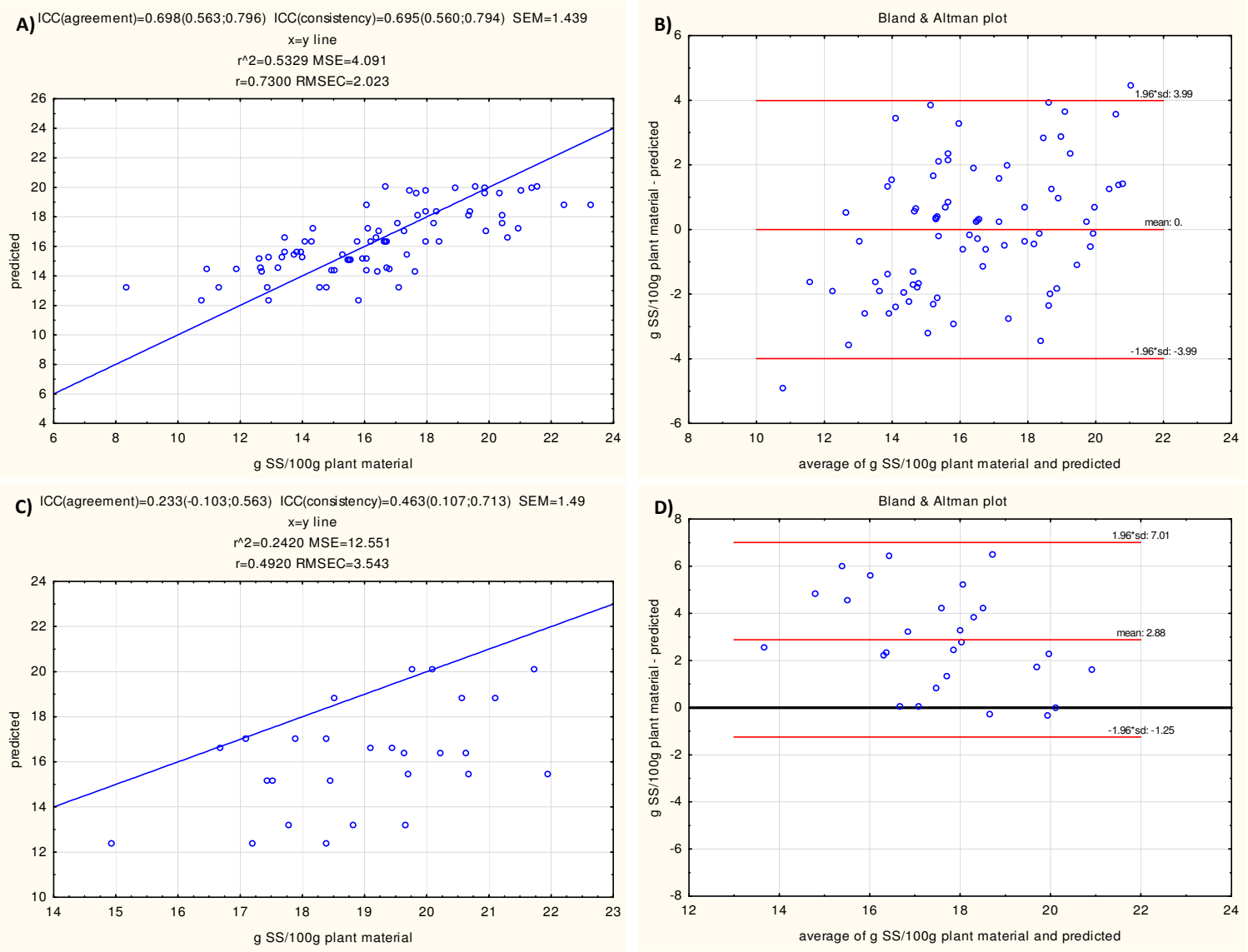

Figure 3.10 A) Scatter plot of correlation between experimental and predicted values and B) Bland & Altman plot for extract yield (g SS per 100 g plant material) for RSM experimental results, and C) scatter plot and D) Bland & Altman plot for extract yield for verification experimental results.

3.5 Conclusions

The phenolic content of the honeybush sp. *C. maculata* was evaluated to determine its potential as source material for flavanone glycoside-enriched extract. The xanthones and hesperidin were predominant in the leaves and stems, respectively. Fermentation had no significant ($p < 0.05$) effect on the hesperidin content. Analysis of the by-product confirmed that hesperidin is the major constituent and indicated that eriocitrin is present at a very low concentration. Therefore, the coarse plant material of *C. maculata* by-product comprises predominantly of hesperidin-rich stems. Honeybush tea processing by-products, particularly that of *C. maculata*, could be a potentially viable renewable source for the production of flavanone glycoside-enriched extracts with high hesperidin content for the nutraceutical and pharmacological industries. In addition, it could be a source of the taste-modulating compound, hesperetin, the aglycone derivative of hesperidin.

In this study UAE technology was explored as environmentally friendly extraction technique to recover hesperidin and eriocitrin from *C. maculata* tea processing by-product and RSM was applied to optimise the UAE process. Practical process and instrumental limitations restricted the selection of the experimental domain. Therefore, only an optimum for ethanol concentration (52.8% v/v for extract and hesperidin yield) could be determined. Temperature had the most significant effect on extraction efficiency of all responses studied. High correlation second-order polynomial regression models for hesperidin and eriocitrin yield and content (of the extract) were developed that could be employed to optimise flavanone glycoside extraction from *C. maculata* tea processing by-product by ultrasonic technology.

As no global optimum conditions for the UAE parameters could be determined in the present study, 52.8% (v/v) ethanol, 20 mL/g solvent:solid ratio, 60°C and 30 min were selected as industrially feasible process parameter values for subsequent preparation of flavanone glycoside-enriched extracts from *C. maculata* processing by-product.

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CHAPTER 4

Acid hydrolysis optimisation of flavanone glycoside-enriched *Cyclopia maculata* extract and the potential of enzymatic bioconversion

4.1 Abstract

The objective of this study was to explore the de-glycosylation of honeybush flavanone glycosides, hesperidin and eriocitrin, to their respective taste-modulating aglycones, hesperetin and eriodictyol. Hesperidin is the major flavanone glycoside present in *Cyclopia* spp. A flavanone glycoside-enriched *Cyclopia maculata* extract was subjected to acid-catalysed hydrolysis. The effect of temperature (60-100°C), heating time (15-90 min) and acid concentration (0.012-0.295 M HCl) on the hydrolysis efficiency of hesperidin and eriocitrin was investigated using single factor experiments. Conversion of hesperidin and eriocitrin to their respective aglycones increased with an increase in temperature, time and acid concentration. Response Surface Methodology was applied to optimise the acid hydrolysis process and to study the effect of the hydrolysis parameters on hydrolysis efficiency. An optimum time of 98.4 min for % hesperidin conversion was attained, but the optimum temperature value was outside the experimental domain. The maximum temperature (92.1°C) and its corresponding optimum time (98.4 min) were selected at which ca 80% hesperidin conversion was achieved. Significantly more eriodictyol formed during acid hydrolysis than eriocitrin present in the initial extract owing to the breakdown of other unidentified phenolic compounds. Temperature and time had a significant effect on % hesperidin conversion and significant interaction between these hydrolysis parameters was observed. The mathematical model showed satisfactory ability and accuracy (R^2_{adj} = 0.97) to predict % hesperidin conversion of *C. maculata* extracts. A change in colour from yellow to dark red-brown was observed during acid hydrolysis, which was attributed to unidentified hydrolysis by-products. The total phenolic content of hydrolysed extract was significantly higher than that of the unhydrolysed extract, indicating the release of compounds with the ability to reduce the Folin-Ciocalteau reagent. No significant difference between the antioxidant activities of these extracts was observed. The potential of enzymatic bioconversion as an alternative to acid-catalysed hydrolysis was investigated. Commercial hesperidinase was used as biocatalyst and de-rhamnosylation resulted in ca 100% conversion of hesperidin to hesperetin-7-*O*-glucoside without producing the aglycone, hesperetin, in an aqueous *C. maculata* extract at optimum pH 4.0 (40°C). Acid- and enzyme-catalysed hydrolysis showed potential for the production of *C. maculata* extracts enriched with valuable flavanone derivatives such as hesperetin (sweetness-enhancer and therapeutic agent) and hesperetin-7-*O*-glucoside (therapeutic agent).

4.2 Introduction

Studies have shown that the increase in calorie-dense foods over the past few decades, particularly the increased intake of sugars, for example in sweetened beverages, has resulted in the rising incidence of obesity, the global epidemic of the 21st century (Block *et al*., 2013; Rossouw *et al*., 2012). These easily metabolised carbohydrates allow the blood sugar level to rise significantly causing the formation of fatty deposits, which ultimately leads to health problems such as obesity and obesity-linked type 2 diabetes, cardiovascular disease and hypertension (WHO, 2003; Swinburn *et al*., 2004).

Therefore, non-nutritive, highly intensive sweeteners have been applied by the food and beverage industry to significantly reduce the sucrose content in their products (Kinghorn *et al*., 2010). However, the palatability of reduced-sugar foods are often sacrificed as these sweeteners may cause taste-related problems such as unpleasant bitter, astringent and/or metallic aftertastes (Kuhn *et al*., 2004; Ott *et al*., 1991; Schiffman *et al*., 1995). In addition, although the most commonly used non-nutritive sweeteners (aspartame, acesulfame-K, neotame, saccharin and sucralose) are approved by the U.S. Food and Drug Administration (FDA), consumers remain concerned about the health risk of artificial food additives (Gardner *et al*., 2012). Research has focussed on exploring novel substances which, in low concentrations, could effectively enhance the sweet taste impressions of reduced-sugar foods, without unfavourably affecting the remaining flavour profile (Servant *et al*., 2010). In addition to sweet taste modulation, on-going research exists to find solutions for masking/counteracting the bitter taste of unpalatable functional ingredients such as phytonutrients (Drewnowski & Gomez-Carneros, 2000; Ley *et al*., 2011b). In recent years, considerable progress has been made in the field of taste-modifying compounds and several flavonoids have been reported to enhance sweetness or to mask bitter taste in the patent literature (Ley *et al*., 2002; 2008; 2011a; Kinghorn *et al.*, 2010). For example, a patent was recently developed for the flavanone, hesperetin, as sweetness enhancer to be used in various applications such as sugar-reduced soft drinks, sugar-reduced dairy products and even chewing gum and tooth paste (Ley *et al*., 2008). Furthermore, flavanones such as eriodictyol have shown significant bitter masking effects without exhibiting any additional strong taste or flavour (Ley *et al*., 2005).

Although present at very low concentrations, hesperetin and eriodictyol have been identified in numerous *Cyclopia* spp. as reviewed by Joubert *et al.* (2009). Conversely, their rutinoside derivatives, hesperidin and eriocitrin, are present at significantly higher concentrations (Joubert *et al.*, 2009). Their potential to be hydrolysed to their respective aglycones has been investigated (Wingard, 1979; Da Silva *et al*. 2013). For example, Wingard (1979) has developed a patent for the acid-catalysed hydrolysis of hesperidin, the major citrus flavonoid. Several alternative methods for the transformation of flavonoids, including microbial (Manzanares *et al.,* 1997; Rajal *et al*., 2009) and enzymatic (Da Silva *et al*., 2013; De Araújo, *et al*. 2013; Weignerová *et al*., 2012) transformation techniques, have been investigated. Hydrolysis of glycosides has been widely used for different applications in the food and pharmaceutical industry, such as the synthesis of valuable chemicals (Horowitz & Gentili, 1969), release of volatile

compounds in fermented foods (Mazzaferro *et al.*, 2010; Minig *et al.*, 2011), de-bittering and clarifying of citrus juices (Mazzaferro *et al*., 2010, 2011; Mazzaferro & Breccia, 2012), enhancement of therapeutic properties (Da Silva *et al.*, 2013) and bioavailability (Nielsen *et al.*, 2006; Park *et al*., 2013), and to aid in the structural elucidation and characterisation of glycosides (Antolovich, 2000; Robards & Antolovich, 1997).

The objectives of this study were to investigate acid-catalysed hydrolysis of a flavanone glycoside-enriched extract (produced from fermented *C. maculata* processing by-products) for the conversion of hesperidin and eriocitrin to their aglycones, hesperetin and eriodictyol, respectively, and to optimise the hydrolysis process using Response Surface Methodology (RSM). An additional objective was to determine the effect of hydrolysis of the flavanone glycoside-enriched extract on its total phenolic content and antioxidant activity, by comparing the extract before and after hydrolysis. Furthermore, the potential of enzyme-catalysed hydrolysis (bioconversion) of honeybush flavanone glycosides to their aglycones was investigated.

4.3 Materials and Methods

4.3.1 Chemicals

Hydrochloric acid (HCl) (37%, m/m) (Merck, Darmstadt, Germany) and ethanol (99%, v/v) (Servochem, Cape Town, South Africa) were used for the acid hydrolysis experiments. For enzymatic hydrolysis, hesperidinase from *Penicillium* spp. was purchased from Sigma Aldrich (St. Louis, USA). Hesperidin (Sigma-Aldrich) and eriocitrin (Extrasynthese, Genay, France) authentic reference standards were used for validation of hydrolysis products in acid and enzymatic hydrolysis experiments. Mangiferin, hesperetin and eriodictyol authentic reference standards were sourced from Extrasynthese. Stock solutions of the phenolic standards were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) at ca 1 mg/mL and aliquots were frozen (-20°C) until analysis. L-Ascorbic acid was purchased from Sigma-Aldrich.

Sodium bicarbonate, gallic acid, Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), $DPPH^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) reagent, potassium phosphate (K-phosphate, KH_2PO_4), AAPH (2,2'-azobis-2-methyl-propionamidine dihydrochloride) and pH buffers were purchased from Sigma Aldrich. Folin-Ciocalteau reagent, fluorescein disodium and potassium hydroxide were sourced from Merck. Deionised water, solvents and purified water for HPLC analysis were the same as described in 3.3.7.

4.3.2 Plant material and preparation of extracts

4.3.2.1 Acid-catalysed hydrolysis

For all acid-catalysed hydrolysis experiments, freeze-dried hesperidin-enriched ethanol extracts (flavanone glycoside-enriched extract (FlvEE)) were used, prepared from fermented *C. maculata* processing by-products.

Samples of different batches of fermented *C. maculata* processing by-products were used originating from plant material harvested from *C. maculata* bushes located on different farms (*Esperanza*, Riviersonderend, *Middelplaas,* Greyton, *Romansrivier*, Riversdale and *Welgedacht*, Riversdale, South Africa). The shoots from each farm represented a sample. The shoots were shredded into small pieces (< 4 mm) using a mechanised fodder cutter before being divided into two sub-batches. Sub-batches were moistened to 60% moisture content and fermented at either 80°C for 24 hrs or 90°C for 16 hrs. After fermentation the plant material was dried in a temperature-controlled drying tunnel (Continental Fan Works CC, Cape Town, South Africa) at 40°C for 6 hrs and subsequently sieved using a mini-sifter (SMC, Cape Town) (200 g/30 s at 90 rpm) (Theron, 2012). The by-product fraction (<40 mesh and >12 mesh) of each batch was collected after sieving and coarsely milled (<1 mm) using a Retsch mill (Retsch GmbH, Haan, Germany). The milled by-product samples were blended to form a single representative batch ($CmacW_{ii}$) (ca 5.5 kg).

Replicate FlvEE batches (n=16) from *C. maculata* processing by-product ($CmacW_{ii}$) were prepared taking into account optimum values determined using RSM and practical aspects (as discussed in Chapter 3). The coarsely milled plant material ($CmacW_{ii}$) was weighed into 1 L screw-capped bottles and 52.8% ethanol (v/v) was added to a solvent-solid ratio of 20 mL/g (700 mL ethanol solution to ca 35 g plant material). Samples were sonicated in an ultrasonic cleaning bath (Branson 8510, Branson Ultrasonics Corporation, Danbury, CT, USA) at 60°C for 30 min and bottles were agitated at a 15 min interval. Directly after extraction, hot extracts were filtered consecutively through a standard kitchen sieve and Whatman Nr 4 filter paper, using a Buchner filter apparatus. The extract filtrates were subsequently evaporated *in vacuo* using a rotary evaporator (Büchi Labortechnik, Flawil, Switzerland) to remove the ethanol. Extracts were frozen and freeze-dried *in vacuo* using a VirTis Advantage Plus freeze-drier (SP Scientific, Warminster, PA, USA). The different batches of freeze-dried extract (FlvEE_1 to FlvEE_16) were milled (Fritsch ball mill, Fritsch GmbH, Idar-Oberstein, Germany) separately and the homogenous extracts were stored in sealed containers at 5°C until further analyses or hydrolysis experiments. The extract yield from the individual batches (n=16) was calculated as extract (g) recovered as a percentage of the initial amount of plant material (g) used (Addendum D).

4.3.2.2 Enzyme-catalysed hydrolysis

A fermented *C. maculata* aqueous extract, prepared on industrial scale (Dudhia *et al*., 2013), was used for all enzymatic hydrolysis experiments. *Cyclopia maculata* plant material was harvested on a farm from a

natural population (Riversdale area, Southern Cape), followed by shredding, fermentation, drying and sieving (particle size < 4 mm) by a commercial tea processor. Extraction of the plant material (300 kg) was performed in a stirred vessel with hot purified water that was pre-heated to ca 93°C. The mixture (10:1 solvent:solid ratio) was continuously stirred for 30 min, followed by coarse filtering through screen and bag filters, cooling, micro-filtration and reverse osmosis. A supplementary concentration step in a flow-through thin-film vacuum evaporator was performed on the semi-concentrated extract before spray-drying commenced. For the duration of the spray-drying process, the concentrate was exposed to an inlet temperature of 210°C and an outlet temperature of 95°C.

4.3.3 Acid hydrolysis

Preliminary single factor experiments were performed on extract FlvEE_5 to investigate the effect of temperature (°C), time (min) and acid concentration (M) on the hydrolysis efficiency to convert the flavanone glycosides, hesperidin and eriocitrin, to their respective aglycones, hesperetin and eriodictyol. For each single factor experiment, the other two factors were kept constant, where applicable (Table 4.1). The experimental design for Response Surface Methodology (RSM) experiments is described in 4.3.4. A concentration of 0.295 M HCl was used throughout for RSM experiments.

Table 4.1 Parameter values for preliminary single factor experiments

Experiment	Units	Parameter range tested	Constant values
Effect of temperature	°C	60; 70; 80; 90; 100	45 min; 0.295 M* HCl
Effect of heating time	min	15; 30; 45; 60; 90	90°C; 0.295 M* HCl
Effect of acid concentration	M	0.012; 0.030; 0.059; 0.148; 0.295*	90°C; 60 min

**Concentration in final reaction volume*

The following acid-catalysed hydrolysis procedure was performed for all preliminary single factor experiments (4.4.1), RSM experiments (4.4.2) and comparison of FlvEE and hydrolysed FlvEE (i.e. aglycone-enriched extracts (AgcEE)) (4.4.4):

Aliquots of approximately 12 mg of FlvEE_5 were weighed off in 4 mL Reacti vials (Sigma Adrich) and 2 mL ethanol was added, followed by vortex mixing. Fifty µL of concentrated HCl (12.1 M) or at a concentration as specified for each experiment was added to the ethanol suspension, followed by closing the vials using screw-caps with silicone/PTFE septa and vortex mixing. The vials were immediately heated for an allocated time period in a Stuart digital heating block (Bibby Scientific, Stone, UK), preheated to the specified temperature. Samples were vortex mixed every 15 min during the hydrolysis experiment. After hydrolysis, samples were cooled in ice water for 15 min. Subsequently, 0.8 mL DMSO was added and the samples were sonicated for at least 25 min to ensure complete dissolution of the hydrolysed extract.

Prior to HPLC analysis (4.3.7), 1.05 mL water was added to dilute the ethanol concentration for better peak shapes. All experiments were performed in triplicate. FlvEE_5 (control) samples were prepared as for the hydrolysed extracts except for the addition of the equivalent volume of water instead of 50 µL HCl and heated for an allocated time period in the heating block preheated to the specified temperature. However, FlvEE_5 (control) samples for RSM experiments were not heated since preliminary single factor experiments indicated that heating of the FlvEE_5 (control) in the absence of acid did not have a significant effect on the flavanone content of the FlvEE_5 (control) (as discussed in 4.4.1.1).

To confirm which acid hydrolysis products have formed, the afore-mentioned experiment was performed using hesperidin (ca 0.21 mg/mL; ca 2% v/v DMSO in final reaction volume) and eriocitrin (ca 0.017 mg/mL; ca 1.7% v/v DMSO in final reaction volume) authentic reference standards. Subsequently, the hydrolysed standard samples were subjected to HPLC-DAD (4.3.7) and LC-DAD-MS analysis (4.3.8). HPLC-DAD analyses of control and hydrolysed FlvEE_5 and flavanone standard samples were conducted on the day of preparation/hydrolysis. Aliquots of control and hydrolysed (80°C/125.4 min and 92.1°C/98.4 min) FlvEE_5 and standards were kept frozen (-60°C) until LC-DAD-MS analysis.

The conversion of hesperidin (Hd) and eriocitrin (ErioT) to their respective aglycones, hesperetin (Ht) and eriodictyol (ErioD), was expressed by the following equations:

$$Conversion\ of\ Hd\ (\%) = ([Ht]_f - [Ht]_0)/[Hd]_0\ \ x\ 100 \quad (1)$$

$$Conversion\ of\ ErioT\ (\%) = ([ErioD]_f - [ErioD]_0)/[ErioT]_0\ \ x\ 100 \quad (2)$$

where $[Ht/ErioD]_f$, $[Ht/ErioD]_0$ and $[Hd/ErioT]_0$ indicates the final hesperetin/eriodictyol concentration, initial hesperetin/eriodictyol concentration and initial hesperidin/eriocitrin concentration, respectively, expressed as mmol/100 g extract.

4.3.4 Experimental design

RSM experiments using a Central Composite Design (CCD) were conducted to optimise the hydrolysis parameters. Hydrolysis temperature (X_1) and hydrolysis time (X_2) were chosen as the independent variables. The range values of the parameters were determined from the single factor experiment results and the axial values were calculated using a Statistica data analysis software system (StatSoft, Inc. (2012), Version 11.0, www.statsoft.com). The coded levels and natural values of the factors used in the CCD are given in Table 4.2.

The experimental design consisted of a combination of 10 randomised experimental runs, including two replicates of the central point (0). The experiment was conducted in triplicate and the actual values were stated as observed responses. The response values for the independent variables were selected as % conversion of hesperidin and eriocitrin, respectively.

Table 4.2 Independent variables and their levels used for Central Composite Design

		Levels				
Independent variable	**Symbol**	**- α**	**-1**	**0**	**1**	**α**
Temperature (°C)	X_1	77.9	80	85	90	92.1
Time (min)	X_2	23.4	40	80	120	136.6

4.3.5 Enzymatic bioconversion

Approximately 8 mg of the commercial enzymatic preparation, hesperidinase (18 Units/g solid hesperidin-α-1,6-rhamnosidase activity and 7 Units/g solid β-glucosidase activity), was dissolved in 200 mL 0.1 M K-phosphate buffer. Hesperidinase solutions were prepared fresh on the day of analysis. The pH of the buffer was adjusted to 4.0 or 5.0 with 2 M HCl or 2 M KOH, respectively, as required.

The effect of methanol on enzyme inhibition/denaturation was evaluated to ensure the accurate quantification of hydrolysis products formed at a specified hydrolysis time. Twenty µL hesperidinase (0.04 mg/mL in K-phosphate buffer, pH 4.0) was added to 1 mL *C. maculata* aqueous extract solution (ca 2.36 mg/mL in K-phosphate buffer, pH 4.0), followed by the addition of 300 µL methanol. The same reaction solution was prepared for the control to which 300 µL 0.1 M K-phosphate buffer (pH 4.0) instead of methanol was added. The samples were vortex mixed and immediately prepared for HPLC-DAD analysis. The samples with and without methanol (control) were injected 2 min after addition of methanol or buffer and repeatedly injected every 91 min for 1200 min (ca 20 hrs) to evaluate the effect of methanol on the enzyme activity.

The effect of pH (4.0 vs 5.0) on enzyme activity was investigated. Bioconversion reactions were carried out in 4 mL Reacti vials that were placed in a pre-heated Stuart digital heating block (Bibby Scientific, Stone, UK) at 40°C for 6 hrs. Hydrolysis of the flavonoid glycosides was initiated by adding 20 µL of enzyme solution (pH 4.0 or 5.0) to 1 mL *C. maculata* aqueous extract solution (ca 2.36 mg/mL K-phosphate buffer, pH 4.0 or 5.0). For control samples 20 µL K-phosphate buffer instead of enzyme solution was used. The afore-mentioned experiment was performed on hesperidin (ca 0.014 mg/mL buffer; 0.5% (v/v) DMSO) and eriocitrin (ca 0.002 mg/mL buffer; 0.2% (v/v) DMSO) authentic reference standards to confirm the hydrolysis products that have formed in the hydrolysed *C. maculata* extract. The assays were performed in triplicate. Since the hesperidinase activity could not be completely stopped prior to analysis (as discussed in 4.4.5.2), each individual sample (extract and authentic standard) was prepared timely to ensure that hydrolysis commenced 6 hrs prior to its analysis (HPLC-DAD). Aliquots of control and hydrolysed (pH 4.0/40°C/6 hrs and pH 5.0/40°C/6 hrs) extracts and standards were kept frozen (-20°C) until LC-DAD-MS analysis. In the latter case exact timing of hydrolysis was less important as the focus was on compound identification and not quantification.

4.3.6 Comparison of flavanone glycoside- and aglycone-enriched *C. maculata* extracts

For the comparison of FlvEE and AgcEE, 16 batches of FlvEE were selected and individually hydrolysed as described in 4.3.3. The individual FlvEEs were prepared as for AgcEE except for the addition of 50 µL water instead of 50 µL HCl and no heating. Optimum time and temperature conditions were used as determined by RSM (3.3.4), namely 98.4 min and 92.1°C. Each FlvEE and its corresponding AgcEE were analysed for individual phenolic compound content (HPLC-DAD), colour, total polyphenol (TP) content and antioxidant activity. Colour measurements and HPLC-DAD analysis were conducted on the day of hydrolysis, whilst aliquots for TP and antioxidant assays were kept frozen (-20°C) until analysis.

4.3.7 Quantification of phenolic compounds by HPLC-DAD

Prior to HPLC-DAD analysis, L-ascorbic acid was added to authentic reference standard solutions, samples from acid hydrolysis experiments (4.3.3), samples from enzymatic hydrolysis experiments (4.3.5) and samples from FlvEE and AgcEE comparison (4.3.6) at a final concentration of ca 5 mg/mL to prevent oxidative degradation of the phenolic compounds. The standard and extract mixtures were then filtered using 0.45 µm pore-size Millex-HV syringe filter devices with 4 and 33 mm diameters (Millipore), respectively. The injection volumes for the acid hydrolysis experiments, FlvEE and AgcEE comparison, enzymatic hydrolysis experiments and standards were 10 µL, 10 µL, 50 µL and 5–20 µL, respectively.

The HPLC instrument, software and calibration curve used for analysis were as described in 3.3.7. A Gemini-NX C18 (150 × 4.6 mm; 3 µm; 110 Å) column from Phenomenex (Santa Clara, CA, USA) was used for the quantification of phenolic compounds with 2% acetic acid (A) and acetonitrile (B) as mobile phases. Separation was carried out according to Schulze (2013) at 30°C, using the following mobile phase gradient at a flow rate of 1 mL/min: 0-2 min (8% B), 2-31 min (8%-38% B), 31-32 min (38%-50% B), 32-33 min (50% B), 33-34 (50%-8% B), 34-44 min (8% B). UV-Vis spectra were recorded for all samples from 200 to 600 nm.

4.3.8 Identification of phenolic compounds and hydrolysis products by LC-DAD-MS

Liquid chromatography with diode array and mass spectrometry detection (LC-DAD-MS) analysis was performed on a Waters Acquity UPLC equipped with a binary pump, in-line degasser, autosampler, column oven and DAD detector (Waters, Milford, MA, USA). The system was coupled to a Synapt G2 Q-TOF system (Waters) equipped with an electrospray ionisation (ESI) source. The HPLC method of Schulze (2013), described in 4.3.7, was used as basis, but slightly adapted to accommodate difference in equipment configuration. The entire gradient was therefore delayed by 0.2 min, acetonitrile (solvent B) was increased by 0.5% at each interval of the gradient, and the column temperature increased to 32°C to obtain similar separation as on the Agilent HPLC system. An injection volume of 10 µL was used for both the standard and extract samples. UV-Vis spectra were recorded from 235 to 450 nm. The eluent was

split 3:2 prior to introduction into the ionisation chamber. MS^E data were acquired in negative mode using a collision energy ramp from 25 to 60 V. The MS parameters were as follows: desolvation temperature, 275 °C; nitrogen flow rate, 650 L/h; source temperature, 120 °C; capillary voltage, 2,500 V; cone voltage, 15 V. Data acquired were processed using MassLynx v.4.1 software (Waters). Peaks were identified by comparing the retention times, UV-Vis spectra and LC-MS spectra to those of authentic reference standards where available. LC-MS spectra for other peaks were compared to that of Schulze (2013), as well as spectra for hesperetin-7-*O*-glucoside and eriodictyol-7-*O*-glucoside formed during hydrolysis of hesperidin and eriocitrin authentic reference standards.

4.3.9 Colour measurement

Spectrophotometric measurements over the total visible spectrum of control (FlvEE) and hydrolysed (AgcEE) extract samples were carried out using a BioTek Synergy HT multiplate reader (BioTek Instruments, Winooski, USA). Following sample preparation for HPLC-DAD (4.3.6), 100 µL of FlvEE (ca 3.2 mg/mL) and 50 µL of AgcEE (ca 3.2 mg/mL) were transferred in triplicate into wells of a 96-well polystyrene flat-bottom microplate. Subsequently, 100 and 150 µL water was added to the FlvEE and AgcEE samples, respectively, and the well contents were thoroughly mixed for 30 sec at 1000 rpm using an Eppendorf MixMate (Merck). Absorbance of the 1:1 (FlvEE) and 1:3 (AgcEE) diluted mixtures were measured at 10 nm intervals ranging from 370 nm to 700 nm and Gen5 Secure software was used to obtain the data. Values for absorbance measurements at specific wavelengths (absorbance at 490 nm, A_{490}), as well as the integral of the absorbance spectrum, i.e. *Area under the Curve* (AUC), reflecting the "total colour" of the diluted sample across the wavelength range, were attained. All values for FlvEE and AgcEE were multiplied by 2 and 4, respectively, to obtain the absorbance and AUC values for the undiluted samples.

4.3.10 Determination of total polyphenol (TP) content

The TP content of FlvEE and AgcEE was determined according to the method developed by Singleton *et al.* (1999), adapted to a 96-well microplate format (Arthur *et al.*, 2011). The samples were defrosted at room temperature, and diluted (80 µL sample diluted with 920 µL water) to obtain absorbance values within the calibration curve range. Volumes of 20 µL of the assay control (water), standards (gallic acid standard solutions) and samples were transferred in triplicate into allocated positions of a 96-well polystyrene flat bottom plate. The Folin-Ciocalteau's reagent (10 x diluted; 100 µL) and sodium carbonate solution (7.5% w/v; 80 µL) were added to each well using a multi-channel pipette, and the contents mixed using an Eppendorf MixMate (0.3 min, 1000 rpm). After incubation of plates at 30°C for 2 hrs in a temperature-controlled incubation oven, the absorbance was measured at 765 nm using a BioTek

Synergy HT multiplate reader. A 6-point calibration curve ranging from 10 to 100 mg gallic acid/L was constructed and the results were expressed as g gallic acid equivalents (GAE)/100 g extract.

4.3.11 Determination of antioxidant activity

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

A modified version of the $DPPH^{\bullet}$ scavenging method by Rangkadilok *et al.* (2007) was used to determine the antioxidant activity of FlvEE and AgcEE extracts. The samples were defrosted at room temperature, and diluted (50 µL sample diluted with 950 µL water) to obtain absorbance values within the calibration curve range. The flask with $DPPH^{\bullet}$ reagent (5 mg $DPPH^{\bullet}$ in 50 mL methanol) was sonicated in an aluminium foil-covered sonication bath for 5 min. The $DPPH^{\bullet}$ concentration was adjusted by diluting with methanol to obtain an absorbance of ca 0.60 at 515 nm. The reagent was prepared fresh on the day of analysis and the flask was covered in aluminium foil and kept in the dark until analysis. Volumes of 30 µL of the assay control (MeOH), standards (Trolox standard solutions) and diluted samples were transferred into the assigned wells of a 96-well deep-well plate, followed by the addition of 270 µL $DPPH^{\bullet}$ reagent to each well using a multi-channel pipette. The plate was sealed with a silicone sealing mat and the contents were mixed (0.3 min, 1630 rpm, Eppendorf MixMate). After incubation in a dark cupboard for 2 hrs, 200 µL of the mixture in each well of the deep-well plate was transferred into the corresponding wells of a 96-well polystyrene flat-bottom plate and the plate was read at 515 nm, using a BioTek Synergy HT multiplate reader. A 6-point calibration curve ranging from 50 to 400 µM Trolox (MeOH solutions) was constructed and the results were expressed as µmol Trolox equivalents (TE)/g extract.

Oxygen Radical Absorbance Capacity (ORAC) assay

The ORAC assay as described by Huang *et al.* (2002) was modified by pipetting water (300 µL) in all outer wells of the plate to serve as a thermal barrier to improve reproducibility. The samples were defrosted at room temperature, and diluted (15 µL sample/10 mL water) to obtain absorbance values within the calibration curve range. Volumes of 25 µL of the blanks (water), standards (Trolox solutions) and diluted FlvEE and AgcEE samples were pipetted into their assigned positions in the remaining wells. For preparation of the fluorescein stock solution ca 3.1 mg fluorescein disodium was dissolved in 100 mL 75 mM K-phosphate buffer (ca 10.2 g KH_2PO_4 in 1 L deionised water, pH 7.4). The fluorescein working solution was prepared by diluting 100 µL of the stock solution in 100 mL 75 mM K-phosphate buffer. The fluorescein working solution (150 µL) was added to the wells containing samples and blanks, followed by incubation of the plate for 10 min at 37°C in place, using the heating facility of the BioTek Synergy HT multiplate reader. Volumes of 25 µL 153 mM AAPH solution (ca 0.414 g AAPH in 10 mL K-phosphate buffer) were automatically added to the applicable wells, using the dispenser of the BioTek Synergy HT multiplate reader. After shaking in place using the facility of the multiplate reader, reading commenced

at 1 min intervals for a total of 35 min and the net AUC was used for calculation. A 6-point calibration curve ranging from 5 to 30 µM Trolox was constructed and the results were expressed as µmol TE/g extract.

4.3.12 Statistical analysis

Data of single factor hydrolysis experiments, enzymatic hydrolysis experiments and comparison of FlvEE and AgcEEwere subjected to ANOVA using SAS® 9.2 (Version 9.2, SAS Institute Inc, Cay, USA), as described in 3.3.9 (Chapter 3). Statistica 11.0 was used to conduct all statistical analyses of RSM data (4.3.4), as previously described in 3.3.9.

4.4 Results and discussion

For this chapter an HPLC method with improved resolution (Schulze, 2013) was employed as separation and peak purities were critical to determine % conversion. In spite of this, several problems were encountered. Quantification of eriocitrin and one of its hydrolysis products, eriodictyol-7-*O*-glucoside, in the hydrolysed extract was not possible due to co-elution of the eriocitrin and eriodictyol-7-*O*-glucoside peaks with unidentified compounds in hydrolysed samples. In addition, a higher eriodictyol content than initial eriocitrin present (% eriocitrin conversion > 100%) was observed for most hydrolysis conditions, suggesting formation of eriodictyol from other compounds present in the extract. Therefore, the focus was on the optimisation of hesperidin conversion as main flavanone glycoside. Results obtained for eriocitrin conversion are given in Addendum B.

4.4.1 Single factor acid hydrolysis experiments

The effect of temperature (°C), time (min) and acid concentration (M) on hydrolysis of flavanone glycosides in a flavanone glycoside-enriched *C. maculata* extract was evaluated as the most likely factors playing a role. Liu *et al.* (2013) and Shatalov & Pereira (2012) selected these parameters in RSM optimisation studies on acid hydrolysis of soy whey waste water for isoflavone recovery and acid hydrolysis of giant reed (*Arundo donax* L.) into xylose for lignocellulosic feedstock bio-refineries, respectively.

A change in colour of the *C. maculata* extract was observed during hydrolysis, i.e. upon addition of HCl, the yellow FlvEE turned red-brown within a short time (< 5 min), which subsequently turned into dark red-brown when heat was applied (Fig. 4.1). This phenomenon was further investigated through colour measurements of hydrolysed RSM samples and LC-DAD-MS analysis (4.4.3.2).

Figure 4.1 RSM verification acid hydrolysis experiments performed in a temperature-controlled heating block. Reacti-vial with dark red-brown-coloured hydrolysed *C. maculata* extract after hydrolysis shown.

4.4.1.1 Effect of heating time on hydrolysis efficiency

Hydrolysis experiments were performed for heating periods of 15, 30, 45, 60 and 90 min, respectively, whereas temperature (90°C) and acid concentration (0.295 M HCl) were kept constant. Fig. 4.2 depicts the increase and/or decrease of hesperidin and its hydrolysis products at 90°C in the presence and absence (control) of 0.295 M HCl (in the final reaction volume). It is evident from Fig. 4.2.A-C that heating in the absence of acid did not have a significant effect on the hesperidin, hesperetin-7-*O*-glucoside and hesperetin content of the extract. Hesperidin was hydrolysed to hesperetin-7-*O*-glucoside within the first 15 min whereafter the hesperetin-7-*O*-glucoside content decreased to the initial amount present in the control sample (0.09 mmol per 100 g SS) (Fig. 4.2.B). The hesperetin content significantly ($p < 0.05$) increased over time whereas hesperidin significantly ($p < 0.05$) decreased over 60 min whereafter no significant decrease ($p \geq 0.05$) was observed. From the afore-mentioned results, it can therefore be interpreted that hydrolysis of hesperidin resulted in a step-wise splitting off of the sugar moieties, i.e. firstly the rhamnose, followed by the glucose molecule (refer to Fig. 4.3 for a schematic representation of the proposed hydrolysis reaction). The reaction seems to be completed in 90 min, as all hesperidin was depleted and % conversion of hesperidin reached a plateau (Fig. 4.2A and Fig. 4.4.A).

4.4.1.2 Effect of temperature on hydrolysis efficiency

Hydrolysis experiments were performed at 60, 70, 80, 90 and 100°C, respectively, whereas time (45 min) and acid concentration (0.295 M HCl) were kept constant. From Fig. 4.4.B it can be observed that with an increase in temperature, the % conversion of hesperidin increased. At temperatures below 80°C, less than 20% conversion of hesperidin was observed; however, the % hesperidin conversion increased almost 3-fold from 24 to 62%, when the temperature increased from 80 to 90°C. This could be ascribed to the increased solubility of hesperidin at higher temperatures as observed by Grohmann *et al*. (2000) who used sulphuric acid as catalyst for hydrolysis of aqueous hesperidin suspensions. No hesperidin was detected in samples hydrolysed at 90°C and 100°C, and as only ca 78% conversion was observed at 100°C, it may be assumed that degradation of the remaining 22% occurred.

Eriodictyol also showed an increase with increase in temperature (Addendum A). However, accurate quantification of eriocitrin in the hydrolysed samples was not possible as the UV-Vis spectrum showed that the eriocitrin peak was not pure. Furthermore, significantly more eriodictyol formed than the initial amount of eriocitrin (ca 0.566 mmol per 100 g SS) present in the control samples (Addendum B). This phenomenon will be explored in 4.4.3.

4.4.1.3 Effect of acid concentration on hydrolysis efficiency

Hydrolysis experiments were performed at HCl concentrations of 0.012, 0.030, 0.059, 0.148 and 0.295 M (in final reaction volume), respectively, whereas temperature (90°C) and heating time (60 min) were kept constant. It appears that acid concentration has a greater effect effect on eriocitrin conversion as an increase from ca 111% to 183% was observed when the acid concentration increased from 0.012 to 0.059 M HCl (Addendum B), whereas conversion of hesperidin only increased from ca 1.5% to 4.7% (Fig. 4.4.C). From Fig. 4.4.C it is evident that % conversion of hesperidin significantly increased with an increase in acid concentration, with ca 70% conversion of hesperidin at 0.295 M HCl. Very low quantities of hesperidin were detected in the samples after hydrolysis, using 0.295 M HCl, indicating that degradation of ca 30% had occurred. Therefore, it is evident that an increase in acid concentration will lead to an increase in % conversion until the flavanones are completely converted to their respective aglycones and other degradation products. The optimum acid concentration would therefore be the highest concentration to hydrolyse the flavanones in the shortest period of time. However, too high acid concentrations could be detrimental for phenolic compounds present in the extract as it may lead to a decrease in the health-promoting properties of the extract. Liu *et al.* (2013) reported the use of diluted HCl (1.37 M) for the recovery of isoflavone aglycones from soy whey waste water since too concentrated acid might destroy the structures of these compounds. Consequently, acid concentration (M) was not selected as independent variable in the experimental design (4.4.2) and fixed at 0.295 M for all RSM experiments.

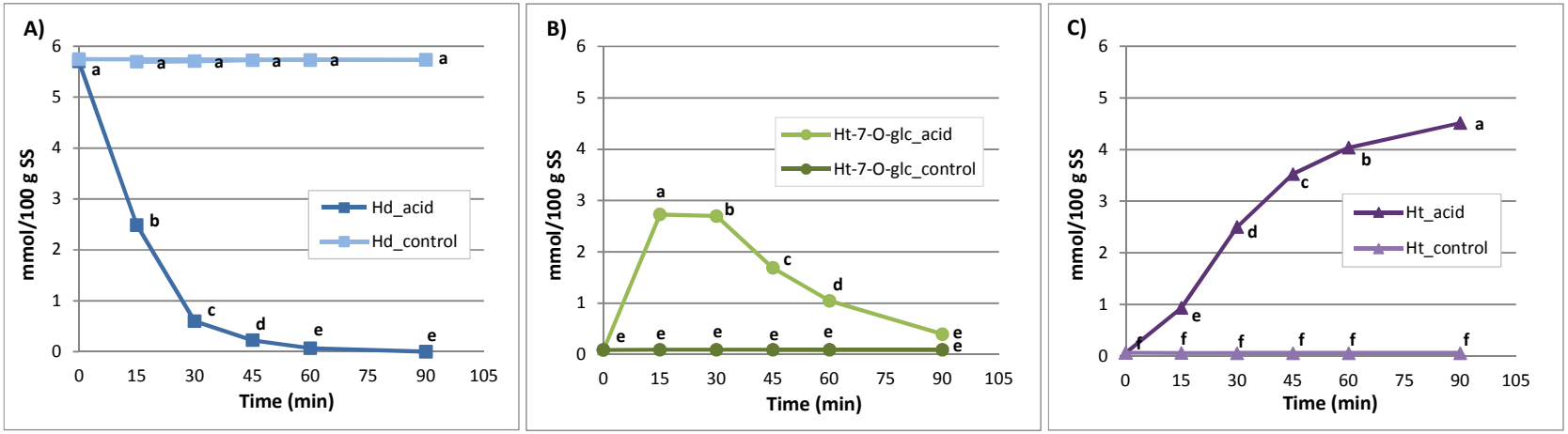

Figure 4.2 Hydrolysis of *C. maculata* extract (FlvEE_5): Conversion of A) hesperidin (Hd) to B) hesperetin-7-*O*-glucoside (Ht-7-*O*-glc) and C) hesperetin (Ht) over time. Constant parameters were 90°C and 0.295 M HCl. Control samples without acid were heated at 90°C *(means with the same letter in each sub-figure are not significantly different ($p \geq 0.05$)).*

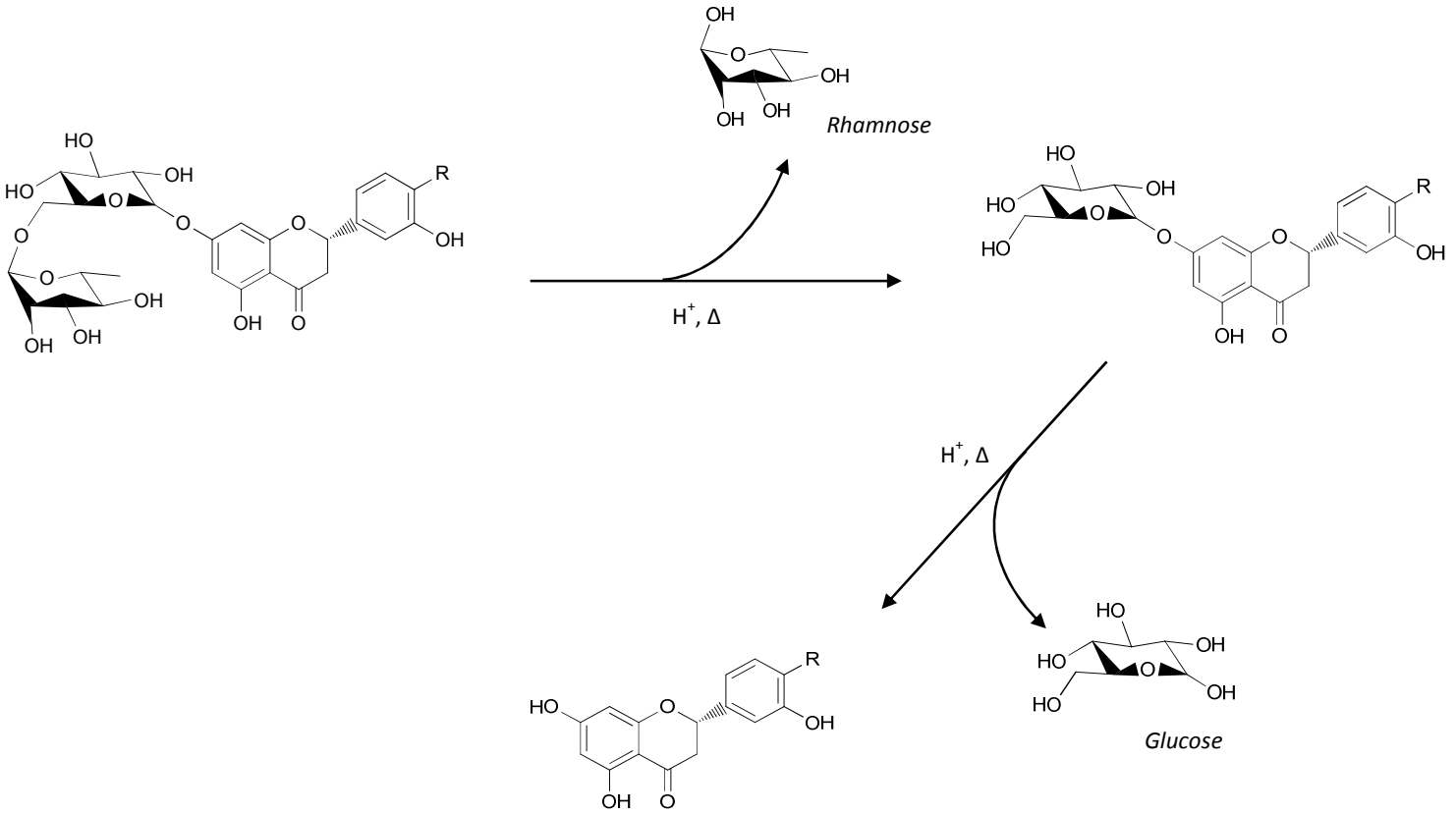

Figure 4.3 Hydrolysis reaction of hesperidin (R = OCH_3) and eriocitrin (R = OH) authentic reference standards as confirmed by LC-DAD-MS analysis.

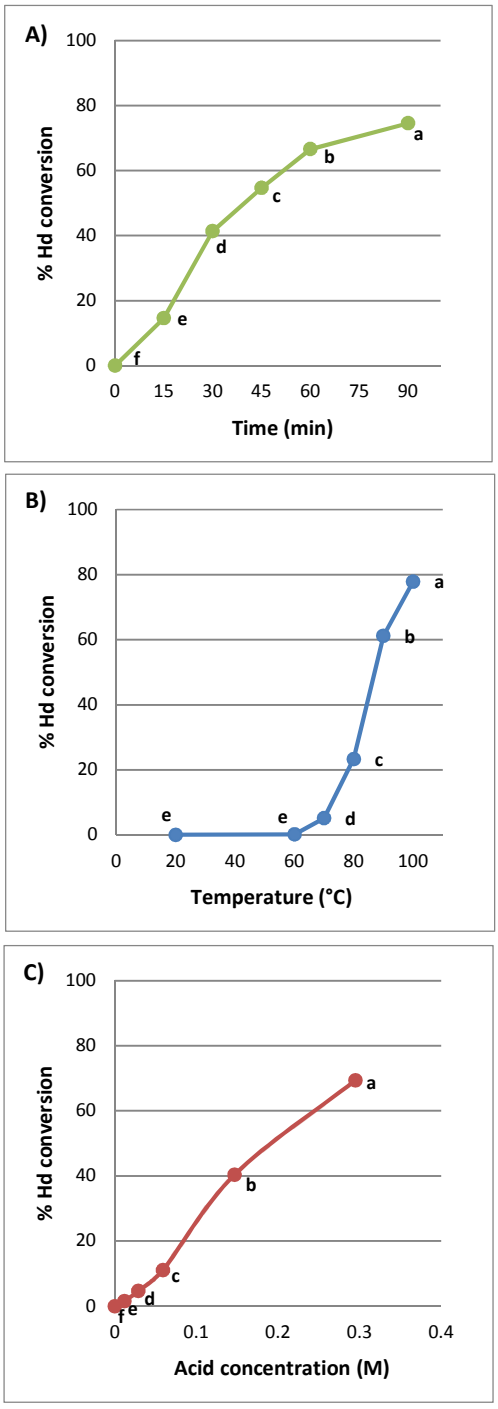

Figure 4.4 Hydrolysis of *C. maculata* extract (FlvEE_5) - Effect of A) heating time (min) B) temperature (°C) and C) acid concentration (M) on conversion of hesperidin (Hd) to hesperetin. Constant parameters were for A) 90°C and 0.295 M HCl, B) 45 min and 0.295 M HCl and C) 90°C and 60 min *(means with the same letter are not significantly different (p ≥ 0.05)).*

4.4.2 Optimisation of acid hydrolysis of hesperidin in *C. maculata* ethanol extract

The response of hesperidin to hydrolysis in a *C. maculata* extract with HCl as hydrolytic catalyst was modelled by applying RSM. The selected hydrolysis parameters, time (min) and extraction temperature (°C), as well as the interaction between these variables were evaluated. Based on the results from the preliminary single factor experiments (4.4.1), five levels for each variable were used for the CCD as given in Table 4.2. As discussed in 4.4.1.3 acid concentration (M) was not investigated as a hydrolysis parameter in the RSM experiments.

The response values for % hesperidin conversion to hesperetin varied from 19.2 (at 80°C for 40 min) to 74.1% (at 90°C for 120 min) (Table 4.3). Wingard (1979) reported ca 85% conversion hesperidin to hesperetin using ca 0.9 M sulphuric acid (H_2SO_4) as catalyst when a MeOH-H_2SO_4 solution was refluxed at ca 65°C for 7.5 hrs. According to this patent, temperatures from 60-85°C can be employed as higher temperatures may increase the production of resinous impurities, whereas lower temperatures results in a too slow reaction time. The reaction time is evidently inversely related to the temperatures employed (Wingard, 1979), which was also observed for the RSM results of the present study. Hilmer *et al.* (2008) reported a similar yield of ca 84% hesperetin when hydrolysed in water using ca 0.4 M citric acid; however, a significantly higher reaction temperature of 145°C (at ca 7.5 bar) for 8 hrs was used. The latter conditions could be attributed to the strength of the acid, with citric acid of lower strength than the mineral acid, HCl and H_2SO_4.

Table 4.3 Central Composite Design for independent variables and average response values (n=3) for % hesperidin conversion

Run No.	X_1 Temperature (°C)	X_2 Time (min)	% Hesperidin conversion
1	80.0	40	19.23 ± 1.65
2	80.0	120	54.97 ± 5.48
3	90.0	40	56.94 ± 1.03
4	90.0	120	74.11 ± 1.20
5	77.9	80	37.13 ± 1.07
6	92.1	80	72.67 ± 0.99
7	85.0	23	21.75 ± 0.84
8	85.0	137	64.94 ± 5.70
9 (C)*	85.0	80	62.20 ± 0.03
10 (C)*	80.0	80	60.16 ± 4.74

* *Centre point parameter values*

4.4.2.1 Analysis of RSM data

RSM data analysis was conducted to determine the optimum hydrolysis parameter values (time and temperature) for % hesperidin conversion to hesperetin and to investigate the effect of these parameters on the response. In light of the afore-mentioned, the second-order polynomial equation, analysis of variance (ANOVA), Pareto chart, response surface and contour plots and desirability profiling will be interpreted in the following section (results of RSM data analysis for % eriocitrin conversion are presented in Addendum B).

Regression equation

From the CCD results, the response value for % conversion of hesperidin to hesperetin was fitted as a function of hydrolysis temperature (°C) and heating time (min). Equation (3) represents the regression equation for the dependent variable, % conversion of hesperidin to hesperetin.

$$Y_1 = -1141.54 + 22.33X_1 - 0.1X_1^2 + 3.15X_2 - 0.01X_2^2 - 0.02X_1X_2 \quad (3)$$

The p-values of the intercept, linear, quadratic and interaction regression coefficients were significant ($p < 0.05$); therefore, each regression coefficient was included in the equation. The regression coefficients and their p-values are given in Table 4.4 with significant ($p < 0.05$) values highlighted in blue.

Analysis of variance (ANOVA)

The ANOVA results for % hesperidin conversion are presented in Table 4.4 and the regression coefficients and p-values of the parameter effects that were significant ($p < 0.05$) are highlighted in red. The ANOVA for % hesperidin conversion gave a high coefficient of determination (R^2) of 0.976. As mentioned previously, R^2_{adj} represents the regression coefficient that is adjusted for the number of coefficients included in the model. In this case, as only two parameters were evaluated, the R^2_{adj} value (0.971) differed only slightly from the R^2 value.

Therefore, the experimental and predicted values correlated well with each other, indicating good accuracy and predictive ability of the applied models. In addition, a 'lack-of-fit' (LOF) test was used as a more sensitive test of model fit and these values are presented in Table 4.4. It is evident that the LOF value was not significant ($p \geq 0.05$), indicating that the model could be used to predict the response, which further strengthens the reliability of the model.

Table 4.4 ANOVA of experimental results and estimated coefficients for quadratic polynomial model for % hesperidin conversion

Parameter	Regression Coefficient	p Regr. Coeff.	SS[a]	DF[b]	MS[c]	F	p
Intercept	-1141.54	0.00017					
(1)Temperature (L)	22.33	0.00106	4296.50	1	4296.505	469.2882	0.0000
Temperature (Q)	-0.10	0.00658	94.89	1	94.885	10.3639	0.0041
(2)Time (L)	3.15	0.00000	4870.15	1	4870.151	531.9450	0.0000
Time (Q)	-0.01	0.00000	963.45	1	963.454	105.2338	0.0000
1L by 2L	-0.02	0.00005	257.43	1	257.428	28.1177	0.0000
Lack of fit			65.01	3	21.671	2.3671	**0.0998**
Pure Error			192.26	21	9.155		
Total SS			10661.73	29			
R^2							**0.9759**
R^2_{adj}							**0.9708**

[a] Sum of Squares
[b] Degree of Freedom
[c] Mean Square
L= linear coefficient; Q=quadratic coefficient; 1L by 2L = interaction coefficient
Significant p-values ($p < 0.05$) of regression coefficients and parameter effects are highlighted in blue and red, respectively.

Pareto chart

The Pareto chart (Fig. 4.5) depicts the significant effects of the linear and quadratic parameters and their interactions (95% confidence interval is represented by the red line on the graph). From Fig. 4.5 it is evident that both the linear effect of hydrolysis time (min) and temperature (°C) had a significant ($p < 0.05$) positive effect on hesperidin conversion with the effect of time more pronounced. Similar results were observed for eriocitrin, although the effect of temperature was more pronounced (Addendum B). The significant effect of time and temperature on % conversion was also evident for the single factor experiments. The quadratic effect of time and temperature had a significant negative effect on % hesperidin conversion.

Significant interaction between temperature and time are indicated by the significant values for the interaction coefficient (1L by 2L). This implies that the influence of temperature on the response is different at different levels of time and *vice versa* (Dejaegher & Vander Heyden, 2011). The negative values indicate the inverse relationship of these parameters, e.g. as the temperature increases, less time is required to achieve the same response value.

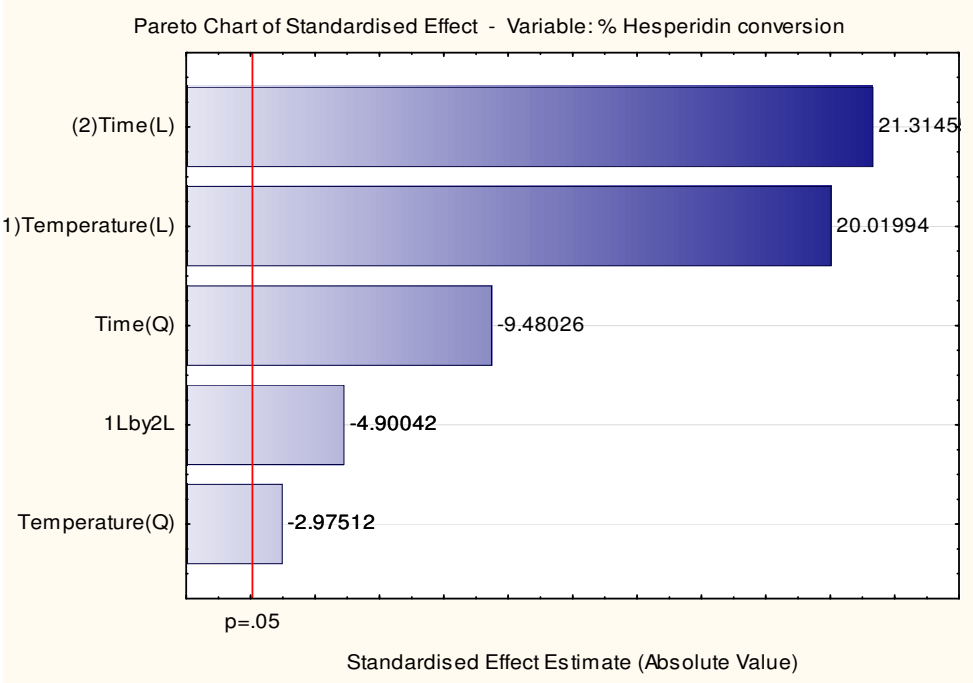

Figure 4.5 Pareto chart of standardised effect estimates for % hesperidin conversion showing the significant ($p < 0.05$) effects of extraction parameters on the response value.

Combined response surface and contour plot

The combined response surface and contour plot for % hesperidin conversion is shown in Fig. 4.6. As described previously, response plots are a visualisation of the predicted second-order polynomial model that is fitted to each set of experimental data to predict the optimal reaction conditions (Bezerra *et al*., 2008). The 3-dimensional surface plot shows that no global optimum for hydrolysis of hesperidin was reached, although an optimum for time of ca 110 min was reached for % hesperidin conversion. Additionally, the slope of the response surface plot depicts that an optimum was nearly reached for temperature, and although the optimum value for temperature was outside the experimental domain, it gives an indication that a plateau has been reached at 92.1°C. A similar combined response plot for % eriocitrin conversion is shown in Addendum B.

Similar to the Pareto charts, the response plot shows the significant interaction between the hydrolysis parameters, time and temperature. The ellipse shape of the contour lines on the 2-dimensional contour plot of both response values at the base of the surface plot indicates the significant interaction between these hydrolysis parameters. In addition, the contour lines are arranged densely, indicating that the two variables had a significant influence on the response values.

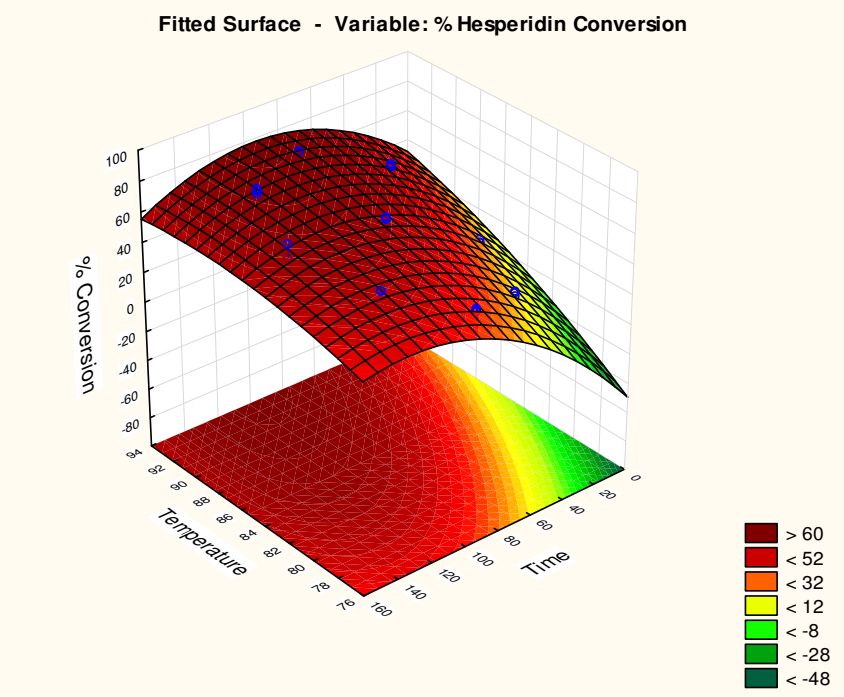

Figure 4.6 Combined response surface and contour plot for % hesperidin conversion as a function of time (min) and temperature (°C).

Optimisation of extraction parameters (desirability profiling)

Desirability profiling of the experimental data resulted in desirability graphs as depicted in Fig. 4.7. Similar to the use of the regression equations and response plots, the optimum (or most desired) parameter values for the different responses can be determined from the desirability graphs. From Fig 4.7 it is evident that only an optimum for time (108.5 min) for % hesperidin conversion was reached (a similar trend was observed for the single factor experiments). Conversely, as depicted in the response and contour plot, no optimum temperature was reached as the maximum temperature value was outside the experimental domain. However, it is evident that the temperature values for % hesperidin conversion are approaching a maximum. This could possibly be explained by the depletion of hesperidin over time as it is converted into hesperetin or deterioration of hesperetin as temperature increases to values above 92.1°C. Liu *et al*. (2013) reported significant degradation of the soy isoflavone aglycone, genistein, at 90°C.

Although the profile of desirability for % hesperidin conversion indicates an estimated optimum value of 108.5 min, a more accurate value of 98.4 min was obtained from the regression equation. Furthermore, as hesperidin is the main flavanone present in the *C. maculata* extract (FlvEE_5), its optimum time was used instead of 80 min for % eriocitrin conversion (desirability profiling for eriocitrin is shown in Addendum B). Therefore, 92.1°C (as maximum temperature value within the experimental domain) and 98.4 min were selected as optimum hydrolysis parameter values for the following hydrolysis experiments of the *C. maculata* extract. The model predicted a value of ca 76.6% hesperidin conversion at these selected parameter values.

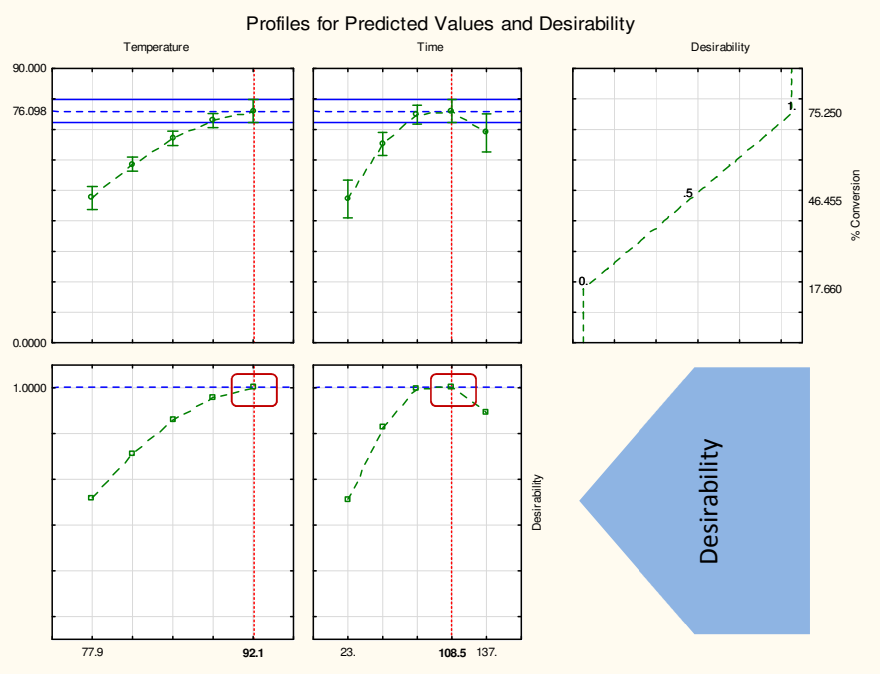

Figure 4.7 Prediction and desirability profiling for % hesperidin conversion into hesperetin *(red blocks indicate optimum/maximum parameter value for the response).*

4.4.2.2 Verification of predicted values of the models

Verification experiments were performed based on additional experimental runs to evaluate how well new experimental data fitted the constructed mathematical models (Table 4.5).

Similar to the verification results for flavanone extraction (Chapter 3), the distribution of the predicted values vs actual values was similar for the RSM and verification experimental results (data not shown). However, for the verification experiments, the predicted values shifted slightly by a consistent factor, i.e. the actual values were slightly lower than predicted. Interclass correlation (ICC) was used to take this factor (standard error of measurement) into account when calculating the correlation coefficient. Therefore, the ANOVA results for % hesperidin conversion gave an acceptable Q^2-value of 0.992 indicating a close correlation between actual and predicted value and demonstrating the validity of this model.

Table 4.5 Additional experimental runs for verification with average experimental (n=3) and predicted values for % hesperidin conversion to hesperetin

Run No.	X_1 Temperature (°C)	X_2 Time (min)	% Hesperidin conversion	
			Experimental	*Predicted*
1	85.0	80.0	64.59 ± 2.07	61.20
2	80.0	40.0	23.95 ± 0.56	18.12
3	80.0	120.0	60.95 ± 0.49	55.76
4	90.0	40.0	57.70 ± 0.46	54.08
5	90.0	120.0	80.06 ± 0.62	73.20
6	92.1	98.4	79.55 ± 0.57	76.63

4.4.3 Confirmation of acid hydrolysis products formed

4.4.3.1 Identification of major hydrolysis products by LC-DAD-MS

Extract and authentic reference standards, hesperidin and eriocitrin, were hydrolysed at two temperature/time conditions, i.e. 92.1°C/98.4 min and 80°C/125.4 min, representing harsh and milder conditions, respectively. The extract and standards were subjected to these conditions to compare their hydrolysis products, as degradation was indicated. Table 4.5 lists the compounds identified by LC-DAD-MS in the control and hydrolysed *C. maculata* extract, as well as the hydrolysed hesperidin and eriocitrin authentic reference standards. Hydrolysis of the hesperidin and eriocitrin standards showed that only hesperetin-7-*O*-glucoside and hesperetin, and only eriodictyol-7-*O*-glucoside and eriodictyol formed after hesperidin and eriocitrin hydrolysis, respectively (the hydrolysis reactions are depicted in Fig. 4.3).

Interestingly, conversion of the hesperidin and eriocitrin standards to their aglycones at the selected optimum conditions (92.1°C, 98.4 min) resulted in ca 83% and 75% conversion (Fig. 4.8), respectively, whereas ca 80% hesperidin (Table 4.5) and 460% “eriocitrin conversion” (data not shown)

was achieved in the hydrolysed *C. maculata* extract at the same hydrolysis conditions (92.1°C, 98.4 min). Fig. 4.8 depicts the % conversion of hesperidin and eriocitrin standards into their respective hydrolysis products at 80°C and 92.1°C, respectively.

From Table 4.6 and Fig. 4.8 it is evident that certain hydrolysis products that formed at 80°C were further hydrolysed (or degraded) at higher temperature. Eriodictyol-7-*O*-glucoside (directly eluted after eriocitrin) were detected in samples hydrolysed at 80°C, but not at 92.1°C, while a large peak for hesperetin-7-*O*-glucoside was observed at 80°C but only traces were detected at 92.1°C.

More than 100% eriocitrin conversion was observed for most of the conditions tested (Addendum B). A portion of this increase in eriodictyol may be attributed to the presence of eriodictyol-*O*-glucosides detected in the extract, as these compounds were not detected in the hydrolysed extracts as indicated in Table 4.6. However, the eriodictyol-*O*-glucosides could not be quantified due to their low concentration in the extract. Since these compounds were not present in large enough quantities to competely explain the increase in eriodictyol, other unidentified compounds must have contributed to this significant increase.

In addition, scolymoside (luteolin-7-*O*-rutinoside) was also hydrolysed as it was not present in the hydrolysed extracts (Table 4.6). However, no luteolin was detected in the hydrolysed extracts, presumably due to the low concentration of scolymoside in the FlvEE.

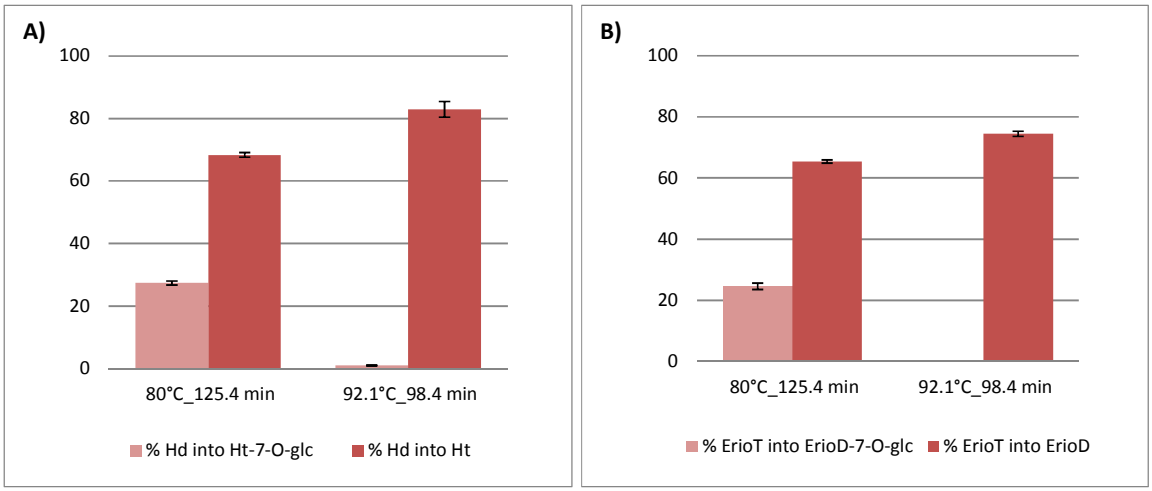

Figure 4.8 % Conversion of A) hesperidin (Hd) and B) eriocitrin (ErioT) authentic reference standards to their respective hydrolysis products: hesperetin-7-*O*-glucoside (Ht-7-*O*-glc) and hesperetin (Ht) and eriodictyol-7-*O*-glucoside (ErioD-7-*O*-glc) and eriodictyol (ErioD), respectively.

Table 4.6 Phenolic compounds identified (x) in control and hydrolysed *C. maculata* extract and hydrolysed hesperidin and eriocitrin authentic reference standards at 80°C (125.4 min) and 92.1°C (98.4 min) with 0.295 M HCl *(compounds are listed in order of elution)*

Compound	**Hesperidin standard** *Hydrolysed (80°C)*	**Hesperidin standard** *Hydrolysed (92.1°C)*	**Eriocitrin standard** *Hydrolysed (80°C)*	**Eriocitrin standard** *Hydrolysed (92.1°C)*	***C. maculata* extract** *Control*	***C. maculata* extract** *Hydrolysed (80°C)*	***C. maculata* extract** *Hydrolysed (92.1°C)*
Mangiferin					x	x	x
Isomangiferin					x	x	x
Apigenin-6,8-di-*C*-glucoside (vicenin-2)					x	x	x
Eriodictyol-*O*-glucoside (a)					x	-	-
Eriodictyol-*O*-glucoside (b)					x	-	-
Eriodictyol-7-*O*-rutinoside (eriocitrin)			-	-	x	-	-
Eriodictyol-7-*O*-glucoside			x	-	-	x	-
Luteolin-7-*O*-rutinoside (scolymoside)					x	-	-
Phloretin-3',5'-di-C-glucoside					x	x	-
Narirutin (naringenin-7-*O*-rutinoside)					-	-	-
Hesperetin-7-*O*-rutinoside (hesperidin)	x	-			x	x	-
Hesperetin-7-*O*-glucoside	x	x			x	x	x
Eriodictyol			x	x	x	x	x
Naringenin					x	x	x
Luteolin					-	-	-
Hesperetin	x	x			x	x	x

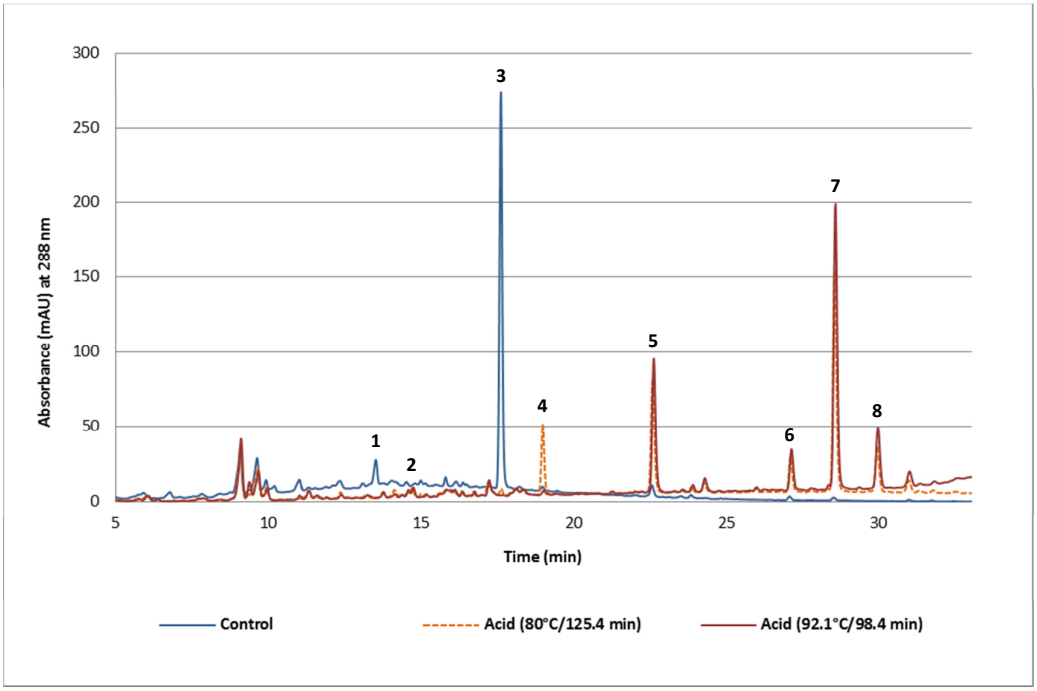

Figure 4.9 Chromatogram at 288 nm of control *(no heat and no acid)* and hydrolysed *(heat and acid) C. maculata* extracts: (**1**) eriocitrin, (**2**) eriodictyol-7-*O*-glucoside, (**3**) hesperidin, (**4**) hesperetin-7-*O*-glucoside, (**5**) eriodictyol, (**6**) naringenin, (**7**) hesperetin and (**8**) unknown.

From the chromatogram in Fig. 4.9 it is evident that the major phenolic compounds produced by hydrolysis of the flavanone-enriched *C. maculata* extract are eriodictyol **(5)** and hesperetin **(7)**, the aglycones of eriocitrin **(1)** and hesperidin **(3)**, respectively. Interestingly, naringenin **(6)** was also formed, with only traces detected in the control. However, no narirutin (naringenin-7-*O*-rutinoside) was detected in both control and hydrolysed samples. No other naringenin glycosides were detected in *C. maculata* previously (Schulze, 2013). The identity of another major hydrolysis product, compound **(8),** could not be determined. The peaks of the "intermediate" hydrolysis products, eriodictyol-7-*O*-glucoside **(2)** and hesperetin-7-*O*-glucoside **(4)**, are more visible for the extract hydrolysed at 80°C compared to that of the extract hydrolysed at 92.1°C.

4.4.3.2 Hydrolysis products contributing to red-brown colour formation

For this study a strong mineral acid, HCl, was used as hydrolysis catalyst. The conventional chemical approach for de-glycosylation, such as acid hydrolysis, unavoidably leads to side reactions and undesirable by-products, due to the instability of flavonoids when kept at high temperatures and extreme pH values for long reaction times (Biesaga, 2011; Da Silva *et al.,* 2013).

In this study, one of the side reactions of acid-catalysed hydrolysis was the formation of by-products that contributed to the dark red-brown colour of the hydrolysed *C. maculata* extract (Fig. 4.10) in contrast to the light orange/yellow-colour of the extract prior to hydrolysis. From the UV-Vis spectra, a substantial increase in absorbance over the entire visible wavelength range, but especially between 470-490 nm, is evident for the hydrolysed extract. At optimised hydrolysis conditions (92.1°C, 98.4 min) ca 80% conversion of hesperidin to hesperetin was achieved, after which no hesperidin was detected in the hydrolysed samples. However, no red-brown colour formed when the hesperidin authentic reference standard was hydrolysed under the same conditions, indicating that the red-brown "pigments" were formed from other extract constituents.

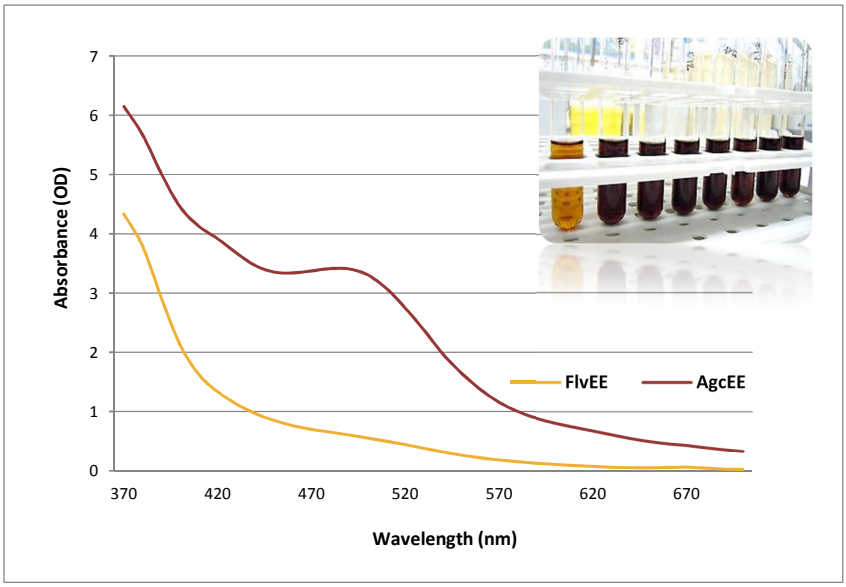

Figure 4.10 Spectra (370-700 nm) of flavanone-enriched (FlvEE) and hydrolysed (AgcEE) *C. maculata* extract. *(Photo insert depicts the filtered yellow-coloured FlvEE and dark red-brown-coloured AgcEE).*

A chromatogram of the hydrolysed extract at 490 nm (Fig. 4.11) was studied to attempt identification of peaks contributing to the observed UV-Vis spectral shift. A number of new peaks with UV-Vis maxima at ca 470-490 nm were observed in the hydrolysed extracts (Fig. 4.11). However, no tentative identification of these by-products could be made using LC-MS due to their low concentration in the hydrolysed extract.

One potential explanation for the red-brown colour of hydrolysed extracts is hydrolysis of proanthocyanidins. Proanthocyanidins (also known as condensed tannins) are colourless compounds and the term is used to describe flavan-3-ols that are polymerised to form dimers (2 subunits), oligomers (3-10 subunits) and polymers (>10 subunits) (Santos-Buelga & Scalbert, 2000; Souquet *et al.*, 1996). The

presence of the monomeric flavan-3-ol, (-)-epigallocatechin gallate, has been shown in *C. subternata* (Kamara *et al*., 2004). A fermented *C. intermedia* sample (ethyl acetate soluble fraction of a methanol extract) was also shown to contain flavan-3-ols by reaction with dimethylaminocinnamaldehyde although the nature of the flavan-3-ols (i.e. monomeric, dimeric, oligomeric or polymeric) was not determined (Marnewick *et al*., 2005). The content was determined as ca 0.02 mg catechin equivalents/mg SS, i.e. ca 14% of the TP content.

Natural proanthocyanidins, flavan-3,4-diols and condensation products of polymeric proanthocyanidins yield monomeric anthocyanidins when heated with alcoholic mineral acids (Jurd & Somers, 1970). This forms the basis of the detection of these compounds in plant extracts (Porter *et al*., 1986). Jurd & Somers (1970) demonstrated that monomeric and condensed natural leucoanthocyanidins yield both red anthocyanidins (λ_{max} ~550 nm) and yellow xanthylium salts (λ_{max} ~450 nm) when heated with butanol-HCl. Colour formation was very quick with the addition of one drop of concentrated HCl to a solution of 5,7,3',4'-tetramethoxyflavan-3,4-diol in warm butanol instantly resulting in a red colour.

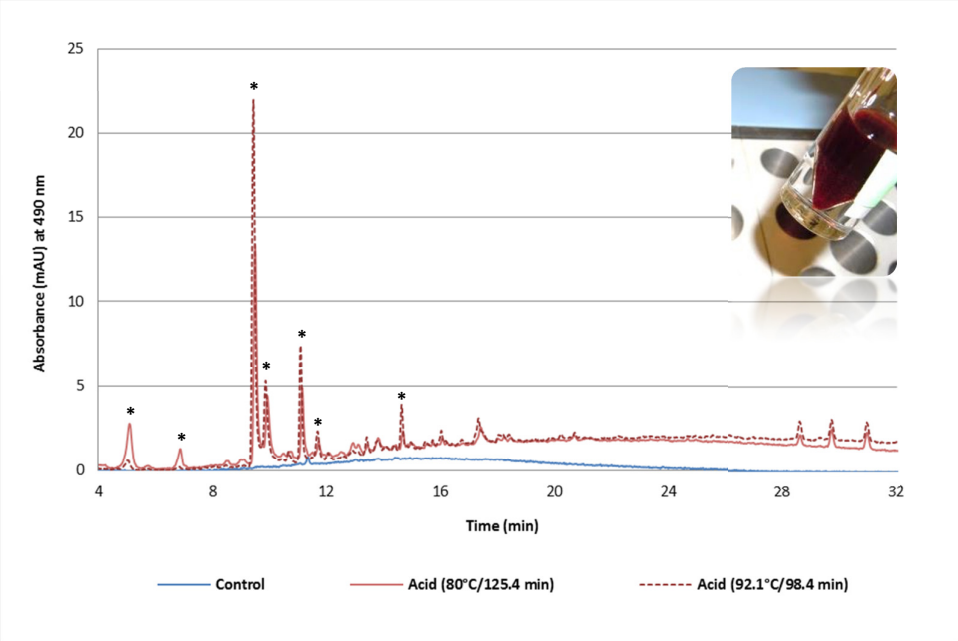

Figure 4.11 Chromatogram at 490 nm of *C. maculata* extracts hydrolysed at 80°C (125.4 min) and 92.1°C (98.4 min). No acid or heat was applied to the control sample. Peaks that could have contributed to the dark red-brown colour of hydrolysed extracts are indicated by ' * '. *(Photo insert depicts Reacti-vial with unfiltered red-brown-coloured hydrolysed extract).*

Spectrophotometric colour measurements were performed on the hydrolysed extracts of each CCD experimental run (4.3.2) and results were presented as Absorbance at 490 nm (A_{490}) and Integral (AUC) (Addendum C). As the hydrolysis parameters, temperature (°C) and time (min), increased, the A_{490}- and AUC-values increased, i.e. the red-brown colour intensity of the hydrolysed extract increased, which implies an increase in the formation of degradation by-products. Statistical analysis indicated good correlation between % hesperidin conversion and A_{490} ($r = 0.896$) and AUC ($r = 0.902$). However, this could just be an indication that the rate of conversion of hesperidin and the rate of formation of by-products from other compounds was similar.

4.4.4 Comparison of *C. maculata* extract before (FlvEE) and after hydrolysis (AgcEE)

For the comparison of the flavanone-glycoside-enriched and hydrolysed *C. maculata* extracts, each replicate flavanone glycoside-enriched tea extract (FlvEE) (n=16) and corresponding aglycone-enriched extract (AgcEE) (n=16) (4.3.6) were analysed for individual phenolic compound content, colour, total polyphenol content and antioxidant activity (Table 4.7).

Spectrophotometric colour measurements (4.3.9) showed that A_{490} of AgcEE (3.40) solutions was significantly higher than that of the FlvEE (0.60) at the same concentration. Additionally the AUC of AgcEE solutions (735.17) was significantly higher than that of the FlvEE (227.38). As discussed in 4.4.3.2, the increase in A_{490} and AUC for the hydrolysed extracts (AgcEE) may imply an increase in the formation of degradation by-products that could have contributed to the red-brown colour formation in AgcEE.

The TP content and antioxidant capacity of FlvEE and AgcEE were determined as described in 4.3.11 and 4.3.12. Although the difference between the TP content of the AgcEE (19.91 g GAE/100 g extract) and that of the FlvEE (18.39 g GAE/100 g extract) was small, the AgcEE had a significantly higher TP content. It is therefore further confirmation that although certain phenolic compounds such as hesperidin and eriocitrin were depleted during hydrolysis at 92.1°C, the hydrolysis products contributed to the higher TP value. It is possible that the aglycones (AgcEE) gave a higher response in the TP assay than the flavanone glycosides (FlvEE), i.e. due to hydrolysis (de-glycosylation) more free hydroxyl groups became available to be oxidised by the Folin-Ciocalteau reagent, which subsequently resulted in a higher response for the AgcEE.

Conversely to the TP content, no significance difference in the antioxidant activity between FlvEE and AgcEE was observed. Therefore, the chemical changes introduced had no effect as degradation of compounds resulted in the formation of other active compounds, for example, hesperidin and hesperetin-7-*O*-glucoside were converted to hesperetin, whereas eriocitrin was converted into eriodictyol. Table 4.7 indicates the significant decrease ($p < 0.05$) of flavanone glycosides and derivatives in AgcEE and the significant increase ($p < 0.05$) of aglycones in AgcEE.

Table 4.7 Average analysis results for the characterisation of flavanone glycoside-enriched extract (FlvEE) and hydrolysed aglycone-enriched-extract (AgcEE) *(means with the same letter are not significantly different (p ≥ 0.05))*

	Mg *(g/100 g extract)*	**IsoMg** *(g/100 g extract)*	**ErioT** *(g/100 g extract)*	**Hd** *(g/100 g extract)*	**Ht-7-*O*-glc** *(g/100 g extract)*	**ErioD** *(g/100 g extract)*	**Ht** *(g/100 g extract)*	**Conversion of Hd into Ht** *(%)*	**Conversion of ErioT into ErioD** *(%)*
FlvEE	1.029^{b} ± 0.026	0.721^{a} ± 0.010	0.265 ± 0.005	3.426 ± 0.178	0.046^{a} ± 0.004	0.064^{b} ± 0.001	0.014^{b} ± 0.001	*Na*	*Na*
AgcEE	1.327^{a} ± 0.022	0.591^{b} ± 0.012	*Nd*	*Nd*	0.040^{b} ± 0.007	0.647^{a} ± 0.012	1.378^{a} ± 0.078	80.39 ± 1.39	456.35 ± 11.37

	Colour *(AUC)*	**Colour** *(A_{490})*	**TP** *(g GAE/100 g extract)*	**TAA (DPPH)** *(µmol TE/g extract)*	**TAA (ORAC)** *(µmol TE/g extract)*
FlvEE	227.377^{b} ± 4.048	0.600^{b} ± 0.014	18.386^{b} ± 0.674	1364.118^{a} ± 36.034	3351.109^{a} ± 154.312
AgcEE	735.167^{a} ± 25.038	3.397^{a} ± 0.101	19.913^{a} ± 0.534	1349.986^{a} ± 30.916	3249.055^{a} ± 189.634

Nd = Not detected
Na = Not applicable
TAA = Total Antioxidant Activity
GAE = Gallic Acid equivalents
TE = Trolox equivalents
[Mg = mangiferin, IsoMg = isomangiferin, ErioT = eriocitrin, Hd = hesperidin, Ht-7-O-glc = hesperetin-7-O-glucoside, ErioD = eriodictyol, Ht = hesperetin]

Studies have shown that hesperetin is a relatively weak antioxidant since it does not display a catechol group (1,2-dihydroxybenzene), one of the critical structural determinants of strong phenolic antioxidants (Goupy *et al.*, 2003). Wilmsen *et al*. (2005) reported good DPPH$^{\bullet}$ quenching activity for hesperidin, as well as strong cellular antioxidant protection against the damaging effects induced by stressing agents, paraquat herbicide and hydrogen peroxide. However, mangiferin was shown to be substantially more active than hesperidin and other honeybush polyphenols, which suggests its importance as a potential major contributor to the *in vitro* and *in vivo* antioxidant activity of honeybush, as reviewed by Joubert *et al.* (2009). Therefore, the flavanones in both extracts, whether a glycoside or aglycone (particularly hesperidin and hesperetin), are expected to make a minor contribution to the overall antioxidant activity of the extracts. Londono-Londono *et al.* (2010) demonstrated that hesperidin was less active than its aglycone, hesperetin, indicating that the antioxidant activity decreases with glycosylation as similarly reported by Williamson *et al.* (1999).

Interestingly, results indicate that the mangiferin content of AgcEE (1.33 g per 100 g extract) was significantly higher than for FlvEE (1.03 g per 100 g extract). However, the mangiferin peak in the hydrolysed extract was not pure due to the formation of an unidentified compound at a similar retention time. Subsequently, it resulted in an over-estimation of the mangiferin content in the AgcEE; therefore, hydrolysis of the extract did not lead to an increase in mangiferin. Degradation on the other hand was expected, considering that fermentation of honeybush at a high temperature is accompanied by degradation of the xanthones (Joubert *et al.*, 2008; Theron, 2012). Conversely, the isomangiferin content of FlvEE (0.72 g per 100 g extract) was significantly higher than that of AgcEE (0.59 g per 100 g extract), indicating that isomangiferin degradation occurred during hydrolysis.

4.4.5 Enzymatic hydrolysis of *C. maculata* aqueous extract

Acid-catalysed hydrolysis as chemical approach for de-glycosylation of flavanone glycosides unavoidably leads to side reactions and undesirable by-products (Biesaga, 2011; Da Silva *et al.,* 2013), as was demonstrated in the previous section (4.4.3). Unidentified compounds were formed as by-products (4.4.3). Apart from the potentially undesirable red-brown colour caused by the unidentified by-products, the potential toxicity of these compounds is also of concern, especially in light of the application of the hydrolysed *C. maculata* extract (AgcEE) as food ingredient. Alternatively, the biochemical approach (bioconversion) through enzyme-catalysed hydrolysis by specific glycosyl hydrolases is regarded as far more advantageous due its milder reaction conditions with fewer by-products (Da Silva *et al*., 2013; De Araújo *et al*., 2013). Therefore, for this study, the potential of enzymatic bioconversion of flavanone glycosides in an aqueous *C. maculata* extract as alternative to acid hydrolysis was investigated. To ensure that the flavanone glycosides are solubilised and readily available for the hydrolase enzyme, an aqueous extract was selected instead of the FlvEE used for the acid hydrolysis experiments in 4.4.2 and 4.4.3. The commercially available hesperidinase from *Penicillium* spp., containing both hesperidin-α-1,6-

rhamnosidase and β-glucosidase activity was used as catalyst. Due to the low concentration of eriocitrin present in the aqueous *C. maculata* extract, the focus was on bioconversion of hesperidin as major flavanone glycoside. In addition, eriodictyol-7-*O*-glycoside could not be accurately quantified due to co-elution with another compound.

4.4.5.1 Inhibition of enzyme activity

In preliminary experiments (results not shown), several methods to inhibit (or degrade) the enzyme before HPLC analysis were explored to ensure the accurate quantification of hydrolysis products formed at a specified hydrolysis time; however, the addition of sodium carbonate, ethanol or methanol, as proposed in literature (Park *et al*., 2006; Wang *et al*., 2012; 2013; Workman & Day, 1982) did not effectively inhibit the enzyme activity.

Both De Araújo *et al.* (2013) and Da Silva *et al.* (2013) stopped the hydrolysis reaction by boiling the sample at 100°C for 30 min, followed by freeze-drying and storing at -80°C until analysis. However, this method could lead to the deterioration of phenolic compounds resulting in an extract that does not represent the hydrolysis metabolites accurately. Nielsen *et al.* (2006) froze the hydrolysed extract at -50°C, followed by freeze-drying and storing at 4°C until analysis. However, for this study, due to the small quantities at which experiments were performed, amounts of hydrolysed extract produced was not sufficient for freeze-drying.

Furthermore, Wang *et al*. (2012) stopped the bioconversion reaction by the addition of methanol, followed by centrifuging the crude hydrolysis products of rutin at 10 000 rpm for 10 min and filtering the supernatants before HPLC analysis. However, no coagulation of protein (enzyme) was visible after the addition of methanol or ethanol, possibly due to the low enzyme concentration of 0.0008 mg/mL in the final reaction volume compared to 0.25 mg/mL used by Wang *et al.* (2012). Another possible method to stop the reaction would be snap freezing with the aid of liquid nitrogen (C.J. Malherbe, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, 2013, personal communication); however, it was not available at the time of this study.

The effect of methanol on enzyme inhibition/denaturation after hydrolysis was investigated by injecting reaction solution samples (extract + hesperidinase) with added methanol and without methanol (control) ca every 91 min for 1200 min (ca 20 hrs). From Fig. 4.12 it is evident that during the bioconversion reaction (pH 4.0, room temperature) hesperetin-7-*O*-glucoside increased over time. However, although the conversion of hesperidin to hesperetin-7-*O*-glucoside seemed to have reached a plateau after 1200 min in the absence of methanol, no plateau was reached in the presence of methanol. Furthermore, more hesperetin-7-*O*-glucoside had formed after 1200 min in the absence of methanol (1.216 mmol per 100 g extract) than when present (0.592 mmol per 100 g extract). The hesperetin content of both reaction mixtures remained constant, showing no further de-glycosylation took place. More hesperetin was present in the reaction mixture containing methanol (0.319 mmol vs 0.100 mmol

per 100 g extract), which could be attributed to higher solubility of hesperetin in the presence of methanol. The % conversion of hesperidin to hesperetin-7-*O*-glucoside in the absence of methanol (ca 115%) was almost double that observed in the presence of methanol (ca 50%) after ca 20 hrs. A possible over-estimation of the hesperetin-7-*O*-glucoside peak may have led to % conversion values higher than 100%.

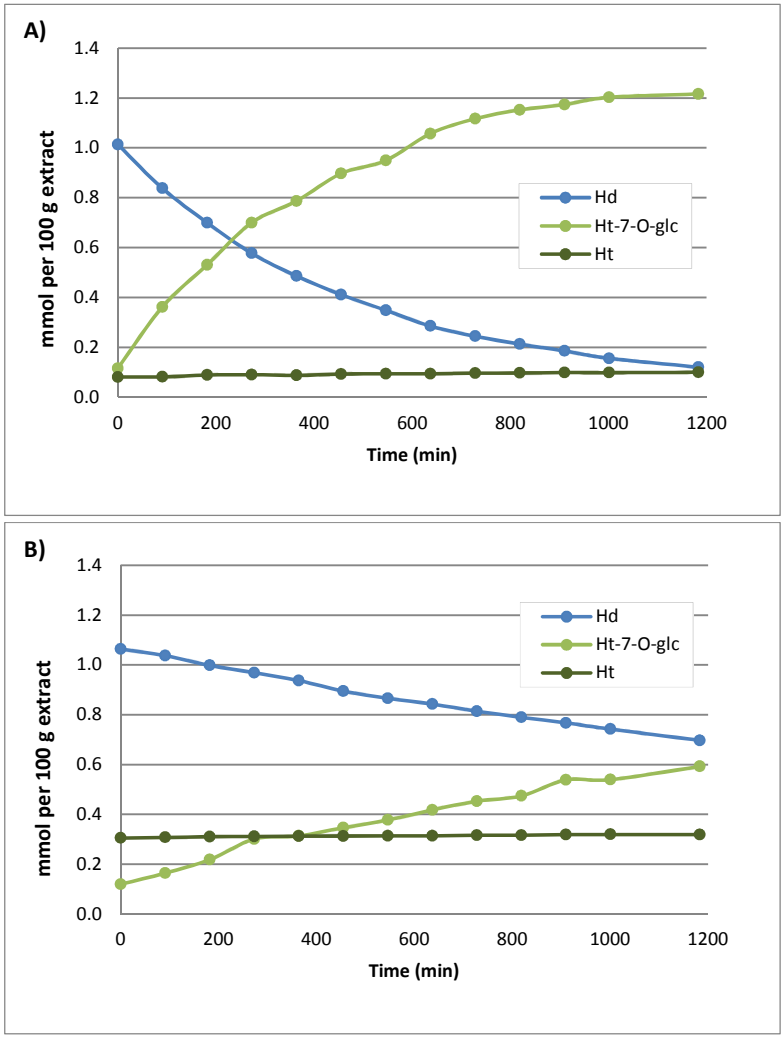

Figure 4.12 Effect of methanol on inhibition/degradation of hesperidinase: hesperidin (Hd), hesperetin-7-*O*-glucoside (Ht-7-*O*-glc) and hesperetin (Ht) content of hydrolysed extract at pH 4.0 in the A) absence and B) presence of methanol.

From the above-mentioned results it is evident that methanol had a significant effect on the activity of hesperidinase, and that it either partially inhibited the α-rhamnosidase catalytic ability or the enzyme was precipitated out. Furthermore, although the results indicate that methanol inhibited the enzyme activity by ca 50%, the addition of more methanol will negatively influence the peak shape during HPLC analysis, potentially resulting in less accurate quantification of the bioconversion metabolites. Due to the inability of methanol to completely inhibit/degrade the enzyme, each sample for the pH experiment (hydrolysis at pH 4.0 or 5.0 at 40°C for 6 hrs) (4.4.5.2) was prepared exactly 6 hrs prior to injection (HPLC analysis) as described in 4.3.5.

4.4.5.2 Effect of pH on hesperidinase-catalysed biotransformation of flavanone glycosides

Both pH and temperature are regarded as crucial independent variables that significantly affect the activity of hydrolases such as hesperidinase, naringinase and β-D-glucosidase (Da Silva *et al.*, 2013). The optimum conversion conditions for above-mentioned enzymes were reported as pH 4.0 and 40°C, while extreme pH values (e.g. 2.0) and temperatures (>50°C) were found to be unfavourable for the catalytic activity of these enzymes (Da Silva *et al*., 2013). The detrimental effect of high temperatures could be attributed to the fact that the energy provided by the high temperature causes certain intra-molecular attractions between polar groups, as well as hydrophobic forces between non-polar groups within the enzymatic protein structure, to break (i.e. denaturation). This results in a denatured protein with an altered active site which is unable to accommodate the substrate molecule (Lu *et al*., 2006).

Considering the afore-mentioned, for this study, only the effect of pH on enzyme hydrolysis of flavanone glycosides, was investigated. According to the hesperidinase supplier specifications, pH 4.0 (at 40°C) was the optimum for hydrolysis (industrial application). The reconstituted aqueous *C. maculata* extract (ca 2.36 mg/mL) had a pH value of ca 5.0. Therefore, hydrolysis of the extract was performed at pH 4.0 and pH 5.0, using K-phosphate buffer at the respective pH values. Furthermore, the extract was diluted to this concentration to investigate the potential of adding the enzyme directly to the extract as a processing step following extraction during the commercial production.

Similarly as for acid hydrolysis, enzyme hydrolysis was performed on hesperidin and eriocitrin standards, respectively, at the same conditions (pH 4.0, 40°C) as for the extract to confirm the identity of their hydrolysis metabolites in the extract. With the aid of LC-DAD-MS analysis (Table 4.8), peaks were identified and the presence of the hydrolysis metabolites was confirmed in the hydrolysed extract by comparing their retention times and LC-DAD-MS data to that of the hydrolysed flavanone standards (refer to insertions A and B in Fig. 4.13). Therefore, the bioconversion reaction of hesperidin and eriocitrin to hesperetin-7-*O*-glucoside and eriodictyol-7-*O*-glucoside, respectively, in which hesperidinase splits off only the terminal rhamnose-group, can be confirmed. No further de-glycosylation to the respective aglycones, hesperetin and eriodictyol, was observed.

From the chromatogram in Fig. 4.13 it is evident that the eriocitrin **(1)** and hesperidin **(3)** content in the hydrolysed extract decreased. An increase in peak area was observed for compound **(2),** confirmed to represent eriodictyol-7-*O*-glucoside, using LC-DAD-MS. The compound could not be quantified accurately using HPLC-DAD due to co-elution with an unidentified compound. Furthermore, an increase in compound **(4)** was observed, which was confirmed by LC-DAD-MS analysis as hesperetin-7-*O*-glucoside.

Park *et al.* (2013) demonstrated the de-rhamnosylation (pH 3.8, 40°C, 1 hr) of hesperidin and narirutin using hesperidinase (from *Penicillium* spp.) as catalyst, which resulted in the hydrolysis metabolites, hesperetin-7-*O*-glucoside and naringenin-7-*O*-glucoside, respectively, without producing their aglycones. Conversely, Da Silva *et al.* (2013) used hesperidinase (also from *Penicillium* spp.) to catalyse the de-glycosylation of flavonoids in orange juice (pH 4.0, 40°C, 4 hrs), in which ca 60% conversion of hesperidin to its aglycone, hesperetin, was reported. Wang *et al.* (2012) and De Araújo *et al.* (2013) reported that hesperidinase (from *Aspergillus niger* and *Penicillium* spp., respectively) could be used to only remove the terminal rhamnose from rutin for the successful production of isoquercitrin (quercetin-3-*O*-glucoside) by modifying the process conditions and the enzyme, respectively. Wang *et al.* (2012) demonstrated that by controlling the pH (which is more feasible than controlling the temperature) the bioconversion process can be controlled. The optimal pH values for hesperidinase to produce isoquercitrin and the aglycone, quercetin, were 6.0 and 5.0, respectively. Wang *et al.* (2012) also reported that while hesperidinase still had good α-L-rhamnosidase catalytic capacity at pH 7.0, it lost the β-D-glucosidase activity at this pH level. De Araújo *et al.* (2013), on the other hand, demonstrated that by pre-heating hesperidinase at 70°C for 30 min, the undesirable (for their study) β-D-glucosidase activity could be inactivated to efficiently convert rutin to isoquercitrin, instead of its aglycone, quercetin. However, in the present study, hesperidinase (from *Penicillium* spp.) was unable to convert hesperidin and eriocitrin, present in the aqueous *C. maculata* extract, to the desired taste-modulating aglycones at pH 4.0 or 5.0, although the enzyme was reported to have β-glucosidase activity according to the manufacturer.

Table 4.8 lists the phenolic compounds identified in the control and hydrolysed extracts and hesperidin and eriocitrin standards. It is evident that no eriodictyol was present in the control or formed during hydrolysis. Although hesperetin was present in both control and hydrolysed tea extract samples as confirmed by LC-DAD-MS, its concentration was too low to be accurately quantified.

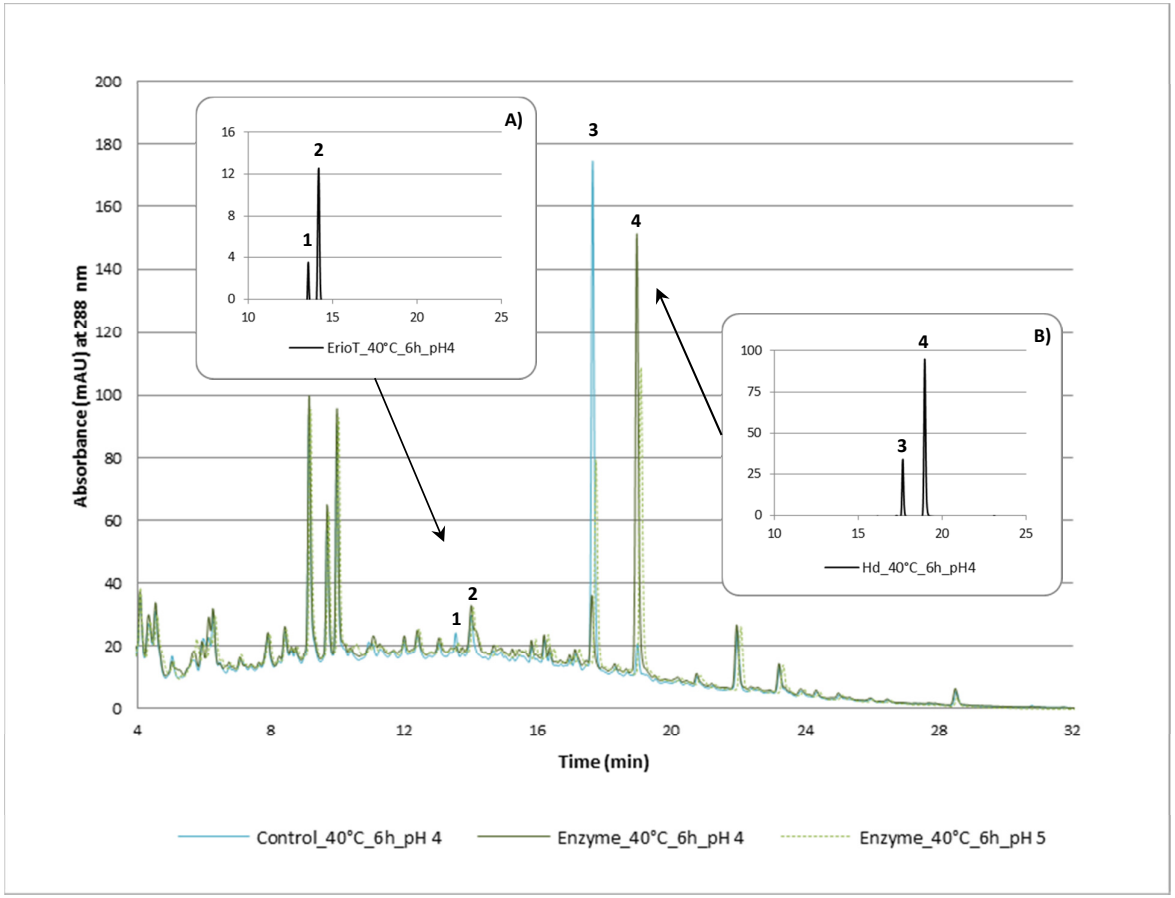

Figure 4.13 Chromatogram of control and bioconverted *C. maculata* aqueous extract, with the insertions indicating the enzymatic hydrolysis metabolites of A) eriocitrin standard and B) hesperidin standard, hydrolysed at the same conditions as for the tea extract [(1) eriocitrin, (2) eriodictyol-7-*O*-glucoside, (3) hesperidin and (4) hesperetin-7-*O*-glucoside].

Table 4.8 Phenolic compounds identified (x) in hydrolysed hesperidin and eriocitrin authentic reference standards and control and hydrolysed *C. maculata* extract *(compounds are listed in order of elution)*

Compound	**Hesperidin standard** *Hydrolysed (pH 4, 40°C)*	**Eriocitrin standard** *Hydrolysed (pH 4, 40°C)*	***C. maculata* extract** *Control (pH 4, 40°C)*	***C. maculata* extract** *Hydrolysed (pH 4, 40°C)*	***C. maculata* extract** *Hydrolysed (pH 5, 40°C)*
Mangiferin			x	x	x
Isomangiferin			x	x	x
Apigenin-6,8-di-*C*-glucoside (vicenin-2)			x	x	x
Eriodictyol-*O*-glucoside (a)			-	-	-
Eriodictyol-*O*-glucoside (b)			-	-	-
Eriodictyol-7-*O*-rutinoside (eriocitrin)		x	x	-	-
Eriodictyol-7-*O*-glucoside		x	-	x	x
Luteolin-7-*O*-rutinoside (scolymoside)			x	-	-
Phloretin-3',5'-di-C-glucoside			-	-	-
Narirutin (naringenin-7-*O*-rutinoside)			-	-	-
Hesperetin-7-*O*-rutinoside (hesperidin)	x		x	x	x
Hesperetin-7-*O*-glucoside	x		x	x	x
Eriodictyol		-	-	-	-
Naringenin			-	-	-
Luteolin			-	-	-
Hesperetin		-	x	x	x

Approximately 95% of hesperidin standard and 99% of eriocitrin standard were converted into their respective glucoside derivatives after 6 hrs (pH 4.0, 40°C). Hydrolysis of the hesperidin standard at pH 4.0 resulted in the formation of significantly more hesperetin-7-*O*-glucoside than at pH 5.0 (Fig. 4.14.A). Similarly for the extract, significantly more hesperetin-7-*O*-glucoside was formed at pH 4.0 than at pH 5.0 (Fig. 4.14.B). Approximately 110% conversion of hesperidin to hesperetin-7-*O*-glucoside was achieved at pH 4.0, compared to ca 75% at pH 5.0. Approximately 14% of the initial hesperidin content remained in the extract hydrolysed at pH 4.0, compared to ca 40% after hydrolysis at pH 5.0. Therefore, pH 4.0 was more suitable for bioconversion of hesperidin compared to pH 5.0. However, an over-estimation of hesperetin-7-*O*-glucoside peaks of the control and hydrolysed extracts resulted in these values.

Although enzymatic hydrolysis with hesperidinase did not produce the desired taste modulators, hesperetin and eriocitrin, as anticipated, the high level of conversion of the hesperidin and eriocitrin standards to their respective glucosides, hesperetin-7-*O*-glucoside and eriodictyol-7-*O*-glucoside, was achieved by the removal of the terminal rhamnose moiety. Studies have indicated that mono-glycosylated flavonoids could have enhanced bioavailability and therapeutic activity, compared to their respective rutinosides (Park *et al.*, 2013) and aglycones (Nielsen *et al.*, 2006). Therefore, enzyme-catalysed hydrolysis of honeybush tea could potentially be used to produce extracts with increased health-promoting activity and bioavailability. Enzymatic bioconversion deserves further attention and the use of other enzymatic preparations could be investigated in future studies.

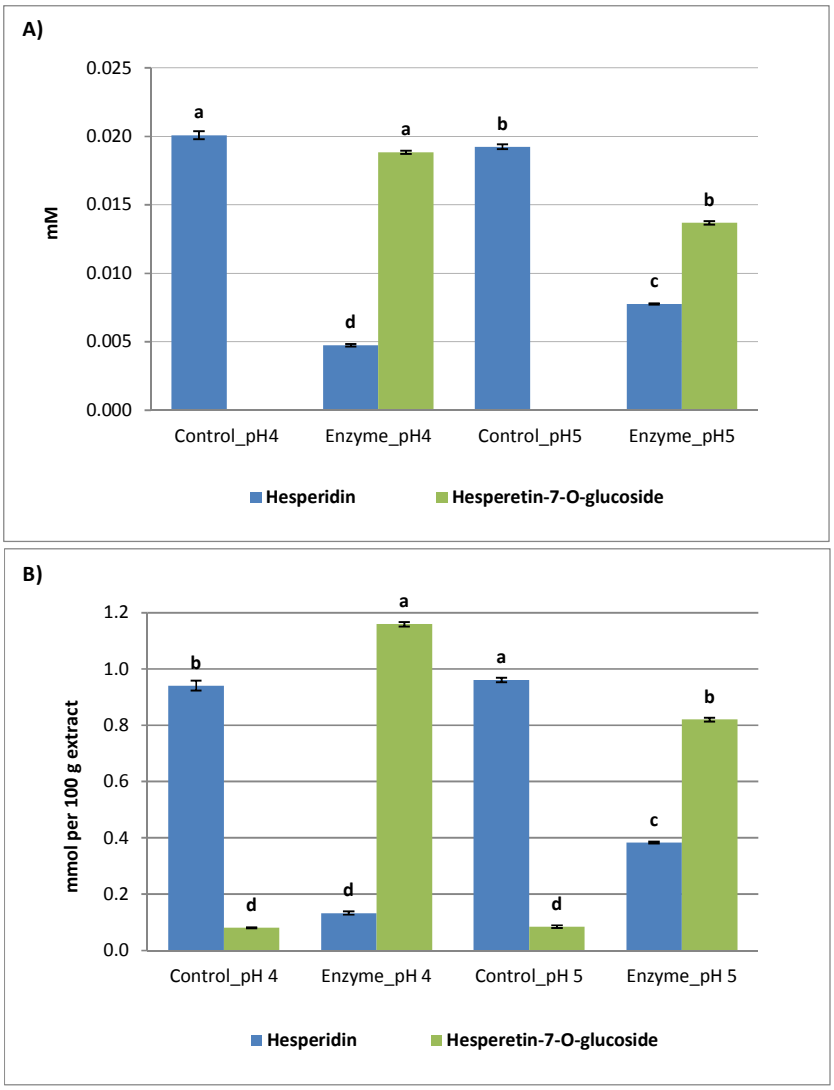

Figure 4.14 Hesperidin and hesperetin-7-*O*-glucoside content of the control and hydrolysed A) hesperidin standard (n = 3) and B) *C. maculata* tea extract (n = 3) at pH 4.0 and 5.0 (40°C, 6 hrs). Control samples without enzyme were heated at 40°C for 6 hrs. *(Means with the same letter are not significantly different (p ≥ 0.05); significant differences are shown separately for hesperidin and hesperetin-7-O-glucoside in blue and green, respectively).*

4.5 Conclusions

Acid-catalysed hydrolysis of flavanone glycoside-enriched *C. maculata* extracts achieved conversion of hesperidin and eriocitrin to their respective taste-modulating aglycones, hesperetin and eriodictyol. The % conversion of hesperidin and eriocitrin increased with an increase in temperature, heating time and acid concentration. Practical process limitations restricted the selection of the experimental domain. Therefore, only an optimum time of 98.4 min for % hesperidin conversion was obtained. A maximum temperature of 92.1°C and its corresponding optimum time of 98.4 min were selected as optimum parameters at which ca 80% hesperidin conversion was achieved. Both temperature and time had a significant effect on the responses studied and significant interaction between these hydrolysis parameters was observed. The colour change from yellow to dark red-brown with hydrolysis indicated formation of unidentified red-brown products, which could potentially be toxic. Before its use as food ingredient its toxicity needs to be evaluated. Enzymatic hydrolysis, as alternative, using hesperidinase as biocatalyst resulted in ca 100% conversion of hesperidin to hesperetin-7-*O*-glucoside, but no hesperetin was produced, contrary to expectation. Enzymatic bioconversion deserves further attention and the use of other enzymatic preparations such as α-rhamnosyl-β-glucosidase (ex *Acremonium* sp.) and β-glucosidase (ex *P. furiosus*) could be investigated in future studies.

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CHAPTER 5

General Discussion and Conclusions

Research has indicated that the increased intake of calorie-dense foods over the past few decades, particularly the increased intake of sugars, for example in sweetened beverages, has resulted in the rising incidence of obesity, the global epidemic of the 21st century (Block, 2013; Finucane *et al*., 2011; Rossouw *et al*., 2012; Sprous & Palmer, 2010). Many adverse health consequences are linked to obesity, including insulin resistance, type 2 diabetes, hypertension, cardiovascular disease, osteoarthritis and cancer (Mollentze, 2006; Swinburn *et al*., 2004; WHO, 2003). A substantial taxation (20%) on sugar-sweetened drinks has been proposed in an effort to reduce consumption of these beverages and to aid the fight against obesity (Block, 2013). Non-nutritive, highly intensive sweeteners are applied by the food and beverage industry to significantly reduce the sucrose content in their products (Kinghorn *et al*., 2010). However, the palatability of reduced-sugar foods is often sacrificed as these sweeteners may have unpleasant bitter, astringent and/or metallic aftertastes or time-intensity profiles that are not characteristical of sucrose that result in a slow onset or unfavourable lingering aftertaste (Kinghorn *et al*., 2010; Kuhn *et al.,* 2004; Ott *et al*., 1991; Schiffman *et al*., 1995). Artificial sweeteners are also gaining bad press (Strawbridge, 2012) so that the search for alternatives is increasingly relevant.

Therefore, research has focussed on exploring novel substances which, in low concentrations, could effectively enhance the sweet taste impressions of reduced-sugar foods, without unfavourably affecting their flavour profile (Servant *et al*., 2010). In addition to sweet taste modulation, on-going research exists to find solutions for masking/counteracting the bitter taste of phytonutrients used as functional ingredients (Drewnowski & Gomez-Carneros, 2000; Ley *et al*., 2011b). In recent years, considerable progress has been made in the field of taste-modifying compounds and flavonoids such as the flavanones, hesperetin and eriodictyol, have been reported to enhance sweetness or to mask bitterness, respectively (Ley *et al*., 2002; 2005; 2008c; 2011a; Kinghorn *et al*., 2010). Although present at very low concentrations, hesperetin and eriodictyol have been identified in numerous honeybush (*Cyclopia*) species as reviewed by Joubert *et al.* (2009). Conversely, their rutinoside derivatives, hesperidin and eriocitrin, are present at significantly higher concentrations (Joubert *et al.*, 2009). Their potential to be hydrolysed to their respective aglycones has been investigated (Wingard, 1979; Da Silva *et al*. 2013; Mazzaferro *et al*., 2010). In addition, hesperidin and eriocitrin have received attention for their health-promoting properties (Garg *et al*., 2001; Minato *et al*., 2003; Miyake *et al*., 1997; 2006), particularly the vasoprotective activity of hesperidin (Garg *et al*., 2001; Valensi *et al.*, 1996).

Many *Cyclopia* species are found in nature, but interest in commercialisation is currently limited to a few. One of the species under evaluation for commercial production, due to its vigorous growth (Joubert *et al*., 2011), are *C. maculata*. Infrequent harvesting and/or poor harvesting practices could lead to development of thick stems, which may potentially contribute to a large portion of herbal tea by-product. Until recently information on the chemical composition of *C. maculata* was limited to a study by Joubert *et al*. (2003). Schulze (2013), demonstrating the presence of 15 compounds, confirmed that

hesperidin and the xanthones, mangiferin and isomangiferin, are the major compounds in hot water extracts prepared from the unfermented and fermented plant material.

Therefore, the aim of this study was to explore the potential of honeybush tea, in particular *C. maculata,* as source material for preparation of a flavanone aglycone-enriched extract with potential therapeutic and taste-modulating properties. Furthermore, in light of the vast research on the exploitation (valorisation) of different food processing by-products as a potential source of valuable bioactive compounds (Galanakis, 2012; Reis *et al*., 2012; Wijngaard *et al.*, 2012), fermented *C. maculata* processing by-product was explored as a renewable source of valuable flavanone glycosides. Numerous emerging environmentally benign extraction techniques such as sub-critical water (Cheigh *et al*., 2012), microwave-assisted (Inoue *et al*., 2010) and ultrasound-assisted (Khan *et al*., 2010; Londono-Londono *et al*., 2010; Ma *et al*., 2008a; 2008b) extraction have been explored for the recovery of flavanone glycosides from citrus processing by-products. Acid-catalysed hydrolysis (Grohmann *et al*., 2000; Hilmer *et al*., 2008) and enzymatic bioconversion (Da Silva *et al*., 2013; De Araújo *et al*., 2013; Mazzaferro *et al*., 2010) techniques have received attention for the subsequent conversion of flavanone glycosides to their respective aglycones and other flavanone derivatives.

Recovery of hesperidin as abundant and inexpensive by-product of citrus processing is well-documented (Garg *et al*., 2001). Honeybush by-products, generated as waste from herbal tea processing, comprises of plant material that does not meet particle size requirements for retail products, i.e. loose tea and tea bags. The waste represents the residues that remain after the sieving step, i.e. < 40 mesh (so-called “dust”) and > 12 mesh (coarse plant material, comprising mainly pieces of stem) (Joubert, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, 2012, personal communication). Furthermore, by-products generated are largely from fermented plant material as most honeybush is consumed in this form. Honeybush tea manufacturing by-product (≤ 20% of production) at present has little value if not re-processed into smaller pieces for mixing with the sieved fraction. Whilst useful to increase bulk, the re-processed fraction is light coloured (as opposed to the characteristic dark brown of the “fermented” (oxidised) product), which could negatively impact the quality and appearance of the end product. Alternatively, the coarse fraction could be used for extract production where physical appearance is not of concern (Joubert, 2012).

In the present study, mangiferin, isomangiferin and hesperidin were confirmed to be the major compounds in *C. maculata* plant material. Hesperidin was the major flavanone glycoside present, with eriocitrin detected at relatively low concentrations. Their respective aglycones, hesperetin and eriodictyol, were present mainly below the quantification limit. In order to determine whether the by-product with its high stem content would be a good source of hesperidin, leaves and stems were separated and analysed. The xanthones and hesperidin were predominant in the leaves and stems, respectively. Similar results were observed for aqueous extracts from *C. subternata* leaves and stems (De Beer *et al*., 2012). Analysis of the by-product confirmed that hesperidin is the major constituent and

showed that eriocitrin is present at a very low concentration. From these results it was concluded that the coarse plant material of *C. maculata* by-product comprises predominantly of hesperidin-rich stems. The effect of fermentation was also considered in light of the decrease in hesperidin content found for other *Cyclopia* species (Joubert *et al*., 2008; De Beer & Joubert, 2010). Interestingly, no significant ($p < 0.05$) difference between the hesperidin content of unfermented and fermented plant material was observed, indicating that hesperidin is not affected by fermentation in the case of *C. maculata*.

The value-adding potential of *Cyclopia* remains underdeveloped as demand for honeybush as a tea still exceeds supply (Joubert *et al.*, 2011). The prospect, therefore, exists to explore the potential of utilising honeybush tea by-products, and in particular, those from *C. maculata* as source for polyphenol-rich extracts. Compared to unfermented honeybush plant material, currently sought-after for the production of xanthone-enriched extracts (Joubert *et al*., 2003), honeybush tea processing by-products could be a potentially viable renewable source for the production of flavanone glycoside-enriched extract with high hesperidin content for the nutraceutical and pharmacological industries. Furthermore, such an extract could be source material for hesperidin as precursor of the sweet taste enhancer, hesperetin. Although the production of fermented honeybush tea (and the processing by-products generated) is relatively small compared to that of the predominant indigenous South African herbal tea, rooibos (*Aspalathus linearis*), this study may form the basis for future studies on exploring value-addition of by-products from other *Cyclopia* spp. and even rooibos.

In this study ultrasound-assisted extraction (UAE) technology was explored as environmentally friendly extraction technique to recover hesperidin and eriocitrin from *C. maculata* tea processing by-product. Response surface methodology (RSM) was employed to optimise and study the individual and interactive effects of the process variables, namely ethanol concentration (% v/v), time (min), temperature (°C), and solvent:solid ratio (mL/g), on flavanone glycoside extraction. This is the first time that RSM have been used to optimise extraction of phenolic compounds from *Cyclopia*. Practical process restrictions limited global optimisation and only an optimum of 52.8% (v/v) ethanol for extract and hesperidin yield could be reached. Temperature had the most significant ($p < 0.05$) effect on extraction efficiency, which could be attributed to the increase in solubility of hesperidin at increased temperatures (Grohmann *et al*., 2000). Theoretically the increase in the rate of heat and mass transfer with increasing temperature is important, but since milled plant material (< 1 mm) was used, the solubility of hesperidin could be considered as the deciding factor. Potentially, higher yields through thermosonication at increased temperatures (> 60°C) could have been achieved, as molecular movement is increased by high temperatures, leading to increased solubility, that could allow for the extremely low water-soluble (20 mg/L) hesperidin to dissolve from plant cells more effectively (Grohmann *et al*., 2000; Yang *et al*., 2010). Hesperidin was found to be stable at 90°C (Dhuique-Mayer *et al.*, 2007). However, too high temperatures (> 50°C) can cause a decrease in shock waves that may ultimately diminish the ultrasonic effect (Lorimer & Mason, 1987).

The potential of UAE as extraction technique for the recovery of flavanone glycosides from *C. maculata* tea processing by-product has been demonstrated in this study. However, the application of other extraction techniques could result in higher extract and flavanone glycoside yield. Therefore, for future studies, other emerging "green" extraction techniques, such as sub-critical water extraction (SWE) (or pressurised liquid extraction) and microwave-assisted extraction (MAE) could be investigated for the preparation of flavanone glycoside-enriched *C. maculata* extracts. SWE (Cheigh *et al*., 2012) and MAE (Inoue *et al*., 2010) have received attention as efficient and rapid techniques for the recovery of hesperidin from *Citrus unshiu* peel. In addition, ultrafiltration could be explored for the concentration and purification of flavanone glycosides from *C. maculata* tea processing by-product through membrane technology (Shi *et al*., 2005). For example, Conidi *et al.* (2011) demonstrated that an integrated ultrafiltration-nanofiltration system could be potentially used for the recovery of the phenolic fraction (including hesperidin and narirutin) from bergamot (*C. bergamia* Risso) juice (by-product of essential oil production) in a suitable form to be used as a functional ingredient.

Since no global optimum conditions for the UAE parameters could be determined in the present study, process parameter values that were feasible for industrial application (52.8% (v/v) ethanol, 20 mL/g solvent:solid ratio, 60°C and 30 min) were selected to produce flavanone glycoside-enriched extracts (FlvEE_1-16) from *C. maculata* tea processing by-product for subsequent hydrolysis experiments.

Acid-catalysed hydrolysis of FlvEE from *C. maculata* tea processing by-product using the mineral acid, HCl, was investigated for the de-glycosylation of hesperidin and eriocitrin to their respective taste-modulating aglycones, hesperetin and eriodictyol. RSM was applied to optimise the hydrolysis process and to study the individual and interactive effects of the process variables, namely temperature (°C) and heating time (min). HCl-catalysed hydrolysis resulted in the conversion of hesperidin and eriocitrin to hesperetin and eriodictyol, respectively. The mathematical models showed satisfactory ability and accuracy ($R^2_{adj} \geq 0.9$) to predict % flavanone glycoside conversion in *C. maculata* extracts. Although an optimum time of 98.4 min for % hesperidin conversion was attained, the optimum temperature was outside the experimental domain (> 92.1°C). An increase in % hesperidin conversion at temperatures > 92.1°C would be expected owing to the increased solubility of hesperidin at higher temperatures (Grohmann *et al*., 2000); however, unfavourable degradation of flavanone glycosides and their hydrolysis products could occur at temperatures > 90°C (Dhuique-Mayer *et al.*, 2007). As no global optimum was reached, the maximum temperature (92.1°C) and its corresponding optimum time (98.4 min) were selected at which ca 80% hesperidin conversion to hesperetin was achieved. No hesperidin was detected in the extract after hydrolysis, indicating that degradation of ca 20% had occurred. Furthermore, significantly more eriodictyol, a bitterness masking compound, formed during acid hydrolysis than eriocitrin present in the initial extract owing to the breakdown of other unidentified phenolic compounds in the tea extract. In addition, the colour changed from yellow to dark red-brown with hydrolysis. These unidentified red-brown products could potentially be attributed to the hydrolysis of colourless

proanthocyanidins present in the *C. maculata* extract (FlvEE) (Marnewick *et al*., 2005). Hydrolysis of proanthocyanidins yield monomeric red anthocyanidins (λ_{max} ~550 nm) and yellow xanthylium salts (λ_{max} ~450 nm) when heated with alcoholic mineral acids (Jurd & Somers, 1970). However, no tentative identification of by-products could be made using LC-DAD-MS due to the low concentration of new peaks in the hydrolysed aglycone-enriched extract (AgcEE).

Other quality parameters used by the South African extract producers to ensure that extracts meet certain minimum standards, are total polyphenol content and antioxidant activity (Joubert & De Beer, 2012). The effect of acid-catalysed hydrolysis on these two quality parameters was therefore determined. The total phenolic content of the hydrolysed extract (AgcEE) was slightly, but significantly ($p < 0.05$) higher than that of the unhydrolysed extract (FlvEE), but no significant difference ($p \geq 0.05$) between the antioxidant activities (DPPH and ORAC assay) of these extracts was observed. Therefore, it could be concluded that the hydrolysis and degradation of compounds resulted in the formation of other active compounds with no net effect on antioxidant activity.

It is important to consider the potential toxicity of the afore-mentioned unidentified degradation products before evaluating the AgcEE as potential taste-modulating food ingredient. Any new natural product needs to be subjected to rigorous safety evaluation such as acute toxicity evaluation in mice and bacterial mutagenicity testing as a prerequisite to human tasting (Kinghorn *et al*., 2010). For example, the well-documented steviol glycosides (E960) have only recently been approved by the European Union (EU) as the first permitted natural non-caloric high potency sweetener (Anonymous, 2012; Commission Regulation (EU) No 1131/2011). Steviol glycosides (stevioside and rebaudioside A) are extracted from the leaves of *Stevia rebaudianum*, and have been the subject of biological and toxicological investigations for more than 50 years. Only in 2008, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) validated the safety of purified steviol glycosides for use as a food and beverage sweetener, which was supported by the European Food Safety Authority (EFSA) in 2010 (Anonymous, 2012). Therefore, for future research, stringent toxicological evaluation of AgcEE will be required before sensory evaluation of the extract by a trained human tasting panel may commence.

Once approved for human tasting, sensory evaluation of the hydrolysed extract (AgcEE) may be conducted. For samples with poor water solubility, as in this case AgcEE, samples may be solubilised with the aid of a very low ethanol concentration and further diluted with distilled water before tasting; however, caution should be taken to keep the quantity of ethanol to a minimum since ethanol has an inherent sweetness that may interfere with sensory evaluation (Kinghorn *et al*., 2010). Methods for detecting flavour modulation include sensory analysis and cell-based biological methods. Sensory evaluation using the human tongue as a detector is a critical step in the discovery of sweetness modifiers (Kinghorn *et al.*, 2010). Although cell biological screening techniques may strongly aid in the identification and development of specifically active compounds in the areas of taste modification (Kinghorn *et al*., 2010), the experienced tongue and nose of taste panels still play a key role in the assessment and

judgment of the complex aroma, taste, and chemosensory perception interactions, which allows the recording of a true taste profile (Ley *et al*., 2008b; Ley *et al*., 2011b).

Sensory evaluation results would determine whether the hesperetin- and eriodictyol-enriched AgcEE has a significant taste-modulating effect, and particularly a sweetness-enhancing effect. On the other hand, AgcEE could potentially exhibit strong adverse flavour profiles due its complex phenolic profile, which includes unidentified phenolic hydrolysis by-products. Ley *et al.* (2008c; 2011a) demonstrated that, hesperetin at 100 ppm significantly enhanced the sweetness intensity of a reduced-sugar solution by 21%. In another reduced-sugar soft drink formulation hesperetin (100 ppm) enhanced the sweetness intensity by 33% (Ley *et al.,* 2011a). Another aspect that needs to be considered in future is that the hesperetin content of acid-hydrolysed extract of *C. maculata* by-product could be too low to have a significant sweetness-enhancing effect in a sugar-reduced beverage such as iced tea. Based on the hesperetin content of the AgcEE (1.4 g/100 g extract), 7.1 g/L of the extract would be required to obtain a hesperetin concentration of 100 ppm. However, at such a high application concentration, the AgcEE may potentially impart a negative flavour and colour on reduced-sugar foods (e.g. iced tea). In addition, the cost implication of such a high application dosage should also be taken into account. Therefore, as previously discussed, alternative environmentally friendly extraction and/or "purification" techniques such as fractionation or ultrafiltration could be explored in future studies to produce hydrolysed AgcEE with higher hesperetin content. The application of a hesperetin-enriched extract as a constituent of a spray-dried flavouring could also be investigated. Flavouring formulations (liquid/spray-dried) that contained hesperetin were presented in patent literature for use as a food ingredient in various food and dental care applications (Ley *et al*., 2011a). Moreover, a spray-dried flavouring and sweetness-enhancing formula could enhance the solubility of hesperetin in a water-based product such as iced tea.

Acid-catalysed hydrolysis as chemical approach for de-glycosylation of flavanone glycosides unavoidably leads to side reactions and undesirable by-products (Biesaga, 2011; Da Silva *et al.,* 2013), as was demonstrated in the present study. The potential toxicity of these unidentified by-products is also of concern, especially in light of the application of the hydrolysed *C. maculata* extract (AgcEE) as functional food ingredient. Alternatively, bioconversion, using enzyme-catalysed hydrolysis by specific glycosyl hydrolases, is regarded as far more advantageous due to its milder reaction conditions with fewer by-products (Da Silva *et al*., 2013; De Araújo *et al*., 2013). Therefore, for this study, the potential of enzymatic bioconversion of flavanone glycosides in an aqueous *C. maculata* extract as alternative to acid hydrolysis was investigated.

For the present study commercial hesperidinase (*ex Penicillium* spp.) was used as biocatalyst and de-rhamnosylation resulted in ca 100% conversion of hesperidin to hesperetin-7-*O*-glucoside in an aqueous *C. maculata* extract at the enzyme pH optimum of 4.0 at 40°C. However, no hesperetin was produced, contrary to expectation as the manufacturer stated that the enzyme has both α-rhamnosidase and β-glucosidase activity. Previously, Da Silva *et al.* (2013) and De Araújo *et al.* (2013) demonstrated

hydrolysis of hesperidin and rutin, respectively, to their respective aglycones using hesperidinase (*ex Penicillium* spp.) from the same manufacturer at a similar pH and temperature. Enzymatic bioconversion deserves further attention and the use of other enzymatic preparations could be investigated in future studies. It is evident from literature that research is progressively more focused on exploring enzymatic hydrolysis of flavanone glycosides as a technique for the industrial production of valuable glucoside derivatives and aglycones. For example, the use of the novel enzymatic preparation, α-rhamnosyl-β-glucosidase, from *Acremonium* sp. with reported high specificity toward 7-*O*-linked flavonoid β-rutinosides (Mazzaferro *et al.,* 2010; 2011) could be investigated should it become available commercially. This could be a solution for the direct bioconversion of flavanone glycosides to their aglycones without the negative side-effects of acid hydrolysis. In addition, Shin *et al.* (2013) recently demonstrated the effective single-step conversion (de-glycosylation) of hesperidin to hesperetin in orange peel extract using a recombinant β-glucosidase from *Pyrococcus furiosus* (pH 5.5, 95°C) with a high productivity of 1.00 g/L/hr. Shin *et al*. (2013) reported a molar conversion yield of 100% compared to the highest previously reported conversion yield of 57% by hesperidinase from *Penicillium* sp. (pH 4.0, 40°C) (Da Silva *et al*., 2013).

Furthermore, although the hesperidinase-catalysed hydrolysis of the aqueous *C. maculata* extract did not result in the desired taste modulating aglycone, hesperetin, hesperetin-7-*O*-glucoside with improved bioavailability was formed. Several studies have shown that enzymatic de-glycosylation of flavonoids improves their bioavailability (Christensen, 2009; Erlund *et al.*, 2001; Hollman *et al*., 1999). Hesperetin-7-*O*-glucoside was found to be more easily absorbed than hesperidin, as it increased solubility (Park *et al*., 2013; Yamada *et al*., 2006). The potential of enzymatically hydrolysed *C. maculata* extract as functional food ingredient with enhanced bioavailability could also be further explored. The current study has provided insight into the value-addition potential of *C. maculata* extract, produced from honeybush tea processing by-product. Its value-addition potential focused on the extract as source of taste modulators for application as food ingredient. The ready-to-drink iced tea market is the second fastest growing category in the global soft drink market (Anonymous, 2011), and recent studies indicated that more and more consumers regard ice teas as too sweet, lacking the authentic taste of freshly brewed tea (Boukley, 2012). A hesperetin-enriched honeybush tea extract could potentially be applied as novel food ingredient for sugar-reduced beverages such as iced tea, as a starting point. Therefore, the findings from this study could form a basis for future studies to produce a natural food ingredient that could contribute to the quest to reduce the sugar content of food products in the global battle against obesity.

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Addendum A

Optimisation of ultrasound-assisted extraction of flavanone glycosides from *Cyclopia maculata* tea processing by-product: Results for eriocitrin

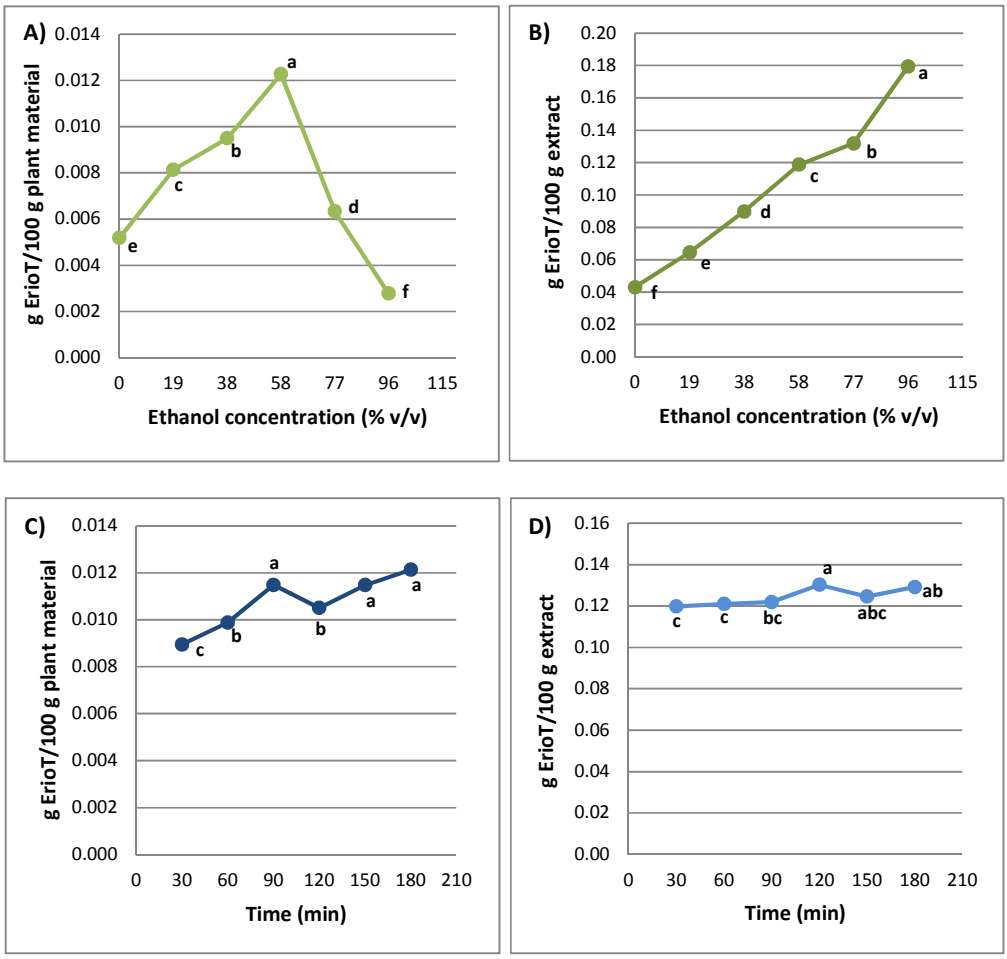

Figure A.1 Effect of solvent composition (ethanol concentration, %v/v) on extraction efficiency of A) eriocitrin (ErioT) yield and B) ErioT content of the extract, and effect of time (min) on extraction efficiency of C) ErioT yield and D) ErioT content of the extract. Constant parameters were 57.6% (v/v) ethanol, 50°C, 30 min and 10 mL/g solvent:solid ratio where applicable *(means with the same letter are not significantly different ($p \geq 0.05$)).*

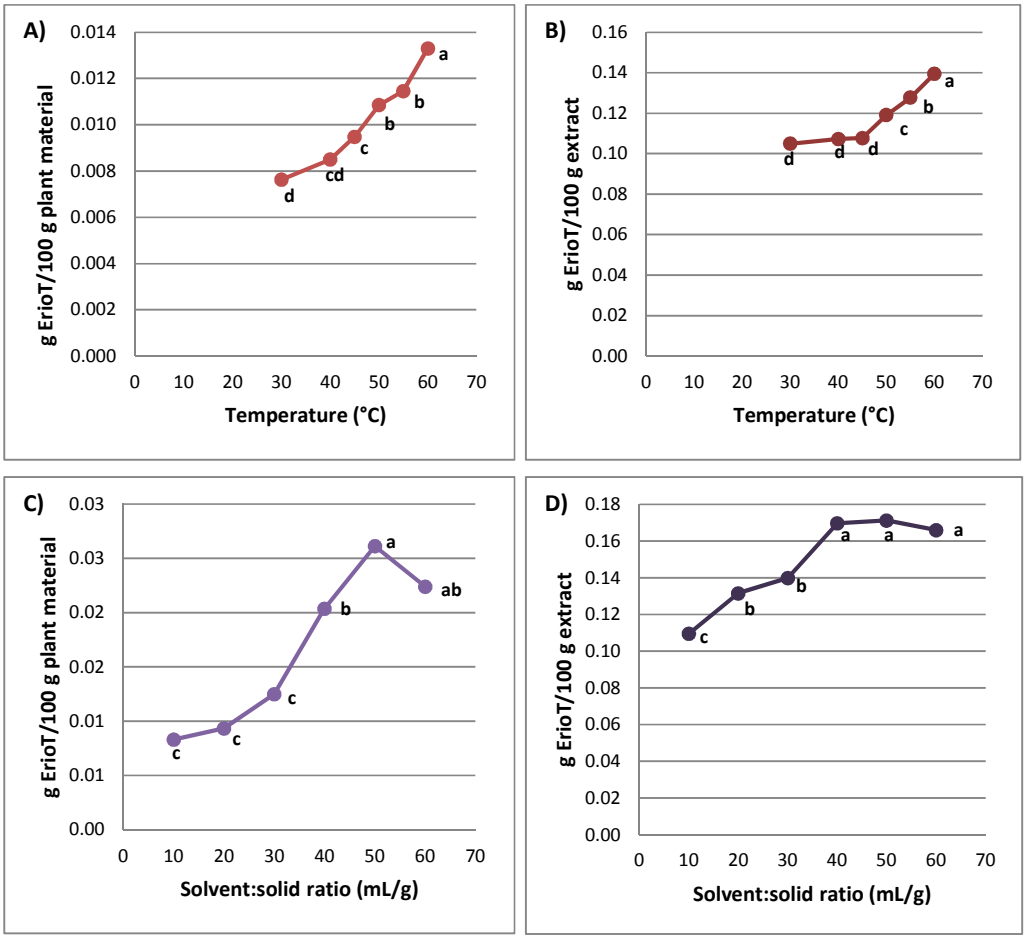

Figure A.2 Effect of temperature (°C) on extraction efficiency of A) eriocitrin (ErioT) yield and B) ErioT content of the extract, and effect of solvent:solid ratio (mL/g) on extraction efficiency of C) ErioT yield and D) ErioT content of the extract. Constant parameters were 57.6% (v/v) ethanol, 50°C, 30 min and 10 mL/g solvent:solid ratio where applicable *(means with the same letter are not significantly different ($p \geq 0.05$)).*

Table A.1 Central Composite Design for independent variables and the average response values (n=2) for eriocitrin yield and eriocitrin content of the extract

Run No.	X_1 Ethanol concentration (% v/v)	X_2 Time (min)	X_3 Temperature (°C)	X_4 Solvent:solid ratio (mL/g)	g Eriocitrin/ 100 g plant material	g Eriocitrin/ 100 g extract
1	48.0	90	50	30	0.0198 ± 0.0009	0.1247 ± 0.0071
2	48.0	90	50	50	0.0226 ± 0.0052	0.1379 ± 0.0077
3	48.0	90	60	30	0.0231 ± 0.0017	0.1591 ± 0.0004
4	48.0	90	60	50	0.0377 ± 0.0043	0.1647 ± 0.0146
5	48.0	120	50	30	0.0192 ± 0.0004	0.1277 ± 0.0027
6	48.0	120	50	50	0.0256 ± 0.0045	0.1385 ± 0.0022
7	48.0	120	60	30	0.0316 ± 0.0033	0.1634 ± 0.0038
8	48.0	120	60	50	0.0376 ± 0.0006	0.1823 ± 0.0126
9	57.6	90	50	30	0.0186 ± 0.0028	0.1422 ± 0.0171
10	57.6	90	50	50	0.0230 ± 0.0004	0.1483 ± 0.0026
11	57.6	90	60	30	0.0260 ± 0.0036	0.1791 ± 0.0074
12	57.6	90	60	50	0.0351 ± 0.0059	0.1895 ± 0.0034
13	57.6	120	50	30	0.0202 ± 0.0038	0.1406 ± 0.0024
14	57.6	120	50	50	0.0275 ± 0.0018	0.1479 ± 0.0053
15	57.6	120	60	30	0.0333 ± 0.0024	0.1798 ± 0.0015
16	57.6	120	60	50	0.0396 ± 0.0038	0.1926 ± 0.0057
17	43.2	105	55	40	0.0197 ± 0.0011	0.1502 ± 0.0050
18	62.4	105	55	40	0.0263 ± 0.0030	0.1766 ± 0.0095
19	52.8	75	55	40	0.0228 ± 0.0008	0.1671 ± 0.0019
20	52.8	135	55	40	0.0301 ± 0.0001	0.1591 ± 0.0163
21	52.8	105	45	40	0.0191 ± 0.0050	0.1380 ± 0.0049
22	52.8	105	65	40	0.0379 ± 0.0070	0.1959 ± 0.0103
23	52.8	105	55	20	0.0147 ± 0.0018	0.1511 ± 0.0146
24	52.8	105	55	60	0.0355 ± 0.0015	0.1882 ± 0.0002
25 (C)*	52.8	105	55	40	0.0247 ± 0.0031	0.1606 ± 0.0009
26 (C)*	52.8	105	55	40	0.0302 ± 0.0052	0.1759 ± 0.0170

** Centre point parameter values*

Table A.2 ANOVA of experimental results and estimated coefficients for quadratic polynomial models for eriocitrin yield and eriocitrin content of the extract *(p-values of the parameter effects that were significant (p < 0.05) are highlighted in red)*

	g Eriocitrin/100 g plant material						g Eriocitrin/100 g extract					
Parameter	**Regr. Coeff.[a]**	**SS[b]**	**DF[c]**	**MS[d]**	**F**	**p**	**Regr. Coeff.[a]**	**SS[b]**	**DF[c]**	**MS[d]**	**F**	**p**
Intercept	0.0292						-0.6203					
(1)Ethanol concentration (L)	0.0031	0.0000	1	0.0000	2.5304	0.1233	0.0132	0.0025	1	0.0025	31.1284	0.0000
Ethanol concentration (Q)	0.0000	0.0000	1	0.0000	1.8642	0.1834	-0.0001	0.0002	1	0.0002	2.9173	0.0991
(2)Time (L)	-0.0007	0.0002	1	0.0002	12.7952	0.0013	0.0023	0.0000	1	0.0000	0.1319	0.7193
Time (Q)	0.0000	0.0000	1	0.0000	0.0047	0.9459	0.0000	0.0003	1	0.0003	3.0732	0.0909
(3)Temperature (L)	-0.0034	0.0013	1	0.0013	106.3124	0.0000	0.0055	0.0146	1	0.0146	179.3939	0.0000
Temperature (Q)	0.0000	0.0000	1	0.0000	0.8706	0.3590	-0.0001	0.0001	1	0.0001	1.2512	0.2732
(4)Solvent:solid ratio (L)	0.0000	0.0008	1	0.0008	65.7748	0.0000	0.0010	0.0021	1	0.0021	25.9374	0.0000
Solvent:solid ratio (Q)	0.0000	0.0000	1	0.0000	0.2508	0.6206	0.0000	0.0000	1	0.0000	0.4687	0.4994
1L by 2L	0.0000	0.0000	1	0.0000	0.5282	0.4736	0.0000	0.0001	1	0.0001	0.8664	0.3602
1L by 3L	0.0000	0.0000	1	0.0000	0.0368	0.8493	0.0001	0.0001	1	0.0001	0.6871	0.4144
1L by 4L	0.0000	0.0000	1	0.0000	0.0799	0.7796	0.0000	0.0000	1	0.0000	0.2157	0.6461
2L by 3L	0.0000	0.0000	1	0.0000	1.4188	0.2440	0.0000	0.0001	1	0.0001	0.8885	0.3543
2L by 4L	0.0000	0.0000	1	0.0000	0.2447	0.6249	0.0000	0.0000	1	0.0000	0.3207	0.5758
3L by 4L	0.0000	0.0000	1	0.0000	2.2927	0.1416	0.0000	0.0000	1	0.0000	0.1710	0.6825
Lack of fit		0.0001	10	0.0000	1.1559	0.3608		0.0014	10	0.0001	1.6976	0.1328
Error (MS Residual)		0.0003	27	0.0000				0.0022	27	0.0001		
Total SS		0.0029	51					0.0234	51			
R^2						**0.8365**						**0.8474**
R^2_{adj}						**0.7746**						**0.7896**

[a] *Regression coefficients*
[b] *Sum of Squares*
[c] *Degree of Freedom*
[d] *Mean Square*
L = linear coefficient; Q = quadratic coefficient; L by L = interaction coefficient

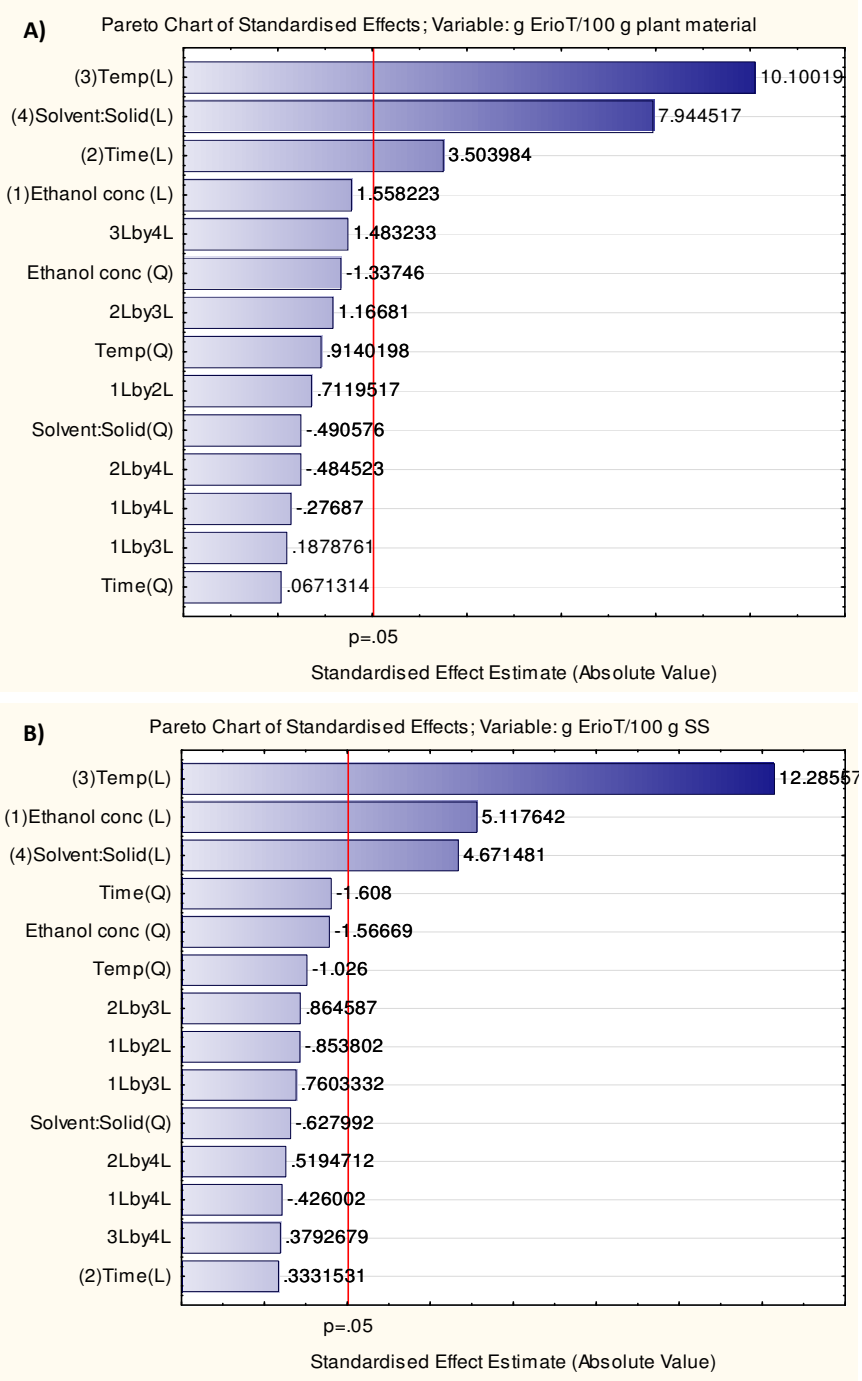

Figure A.3 Pareto charts of standardised effect estimates (absolute value) for dependent variables (DV), A) eriocitrin (ErioT) yield and B) ErioT content of the extract, indicating significant ($p < 0.05$) effects of extraction parameters on each response value *(L = linear effect, Q = quadratic effect, L by L = interaction effect).*

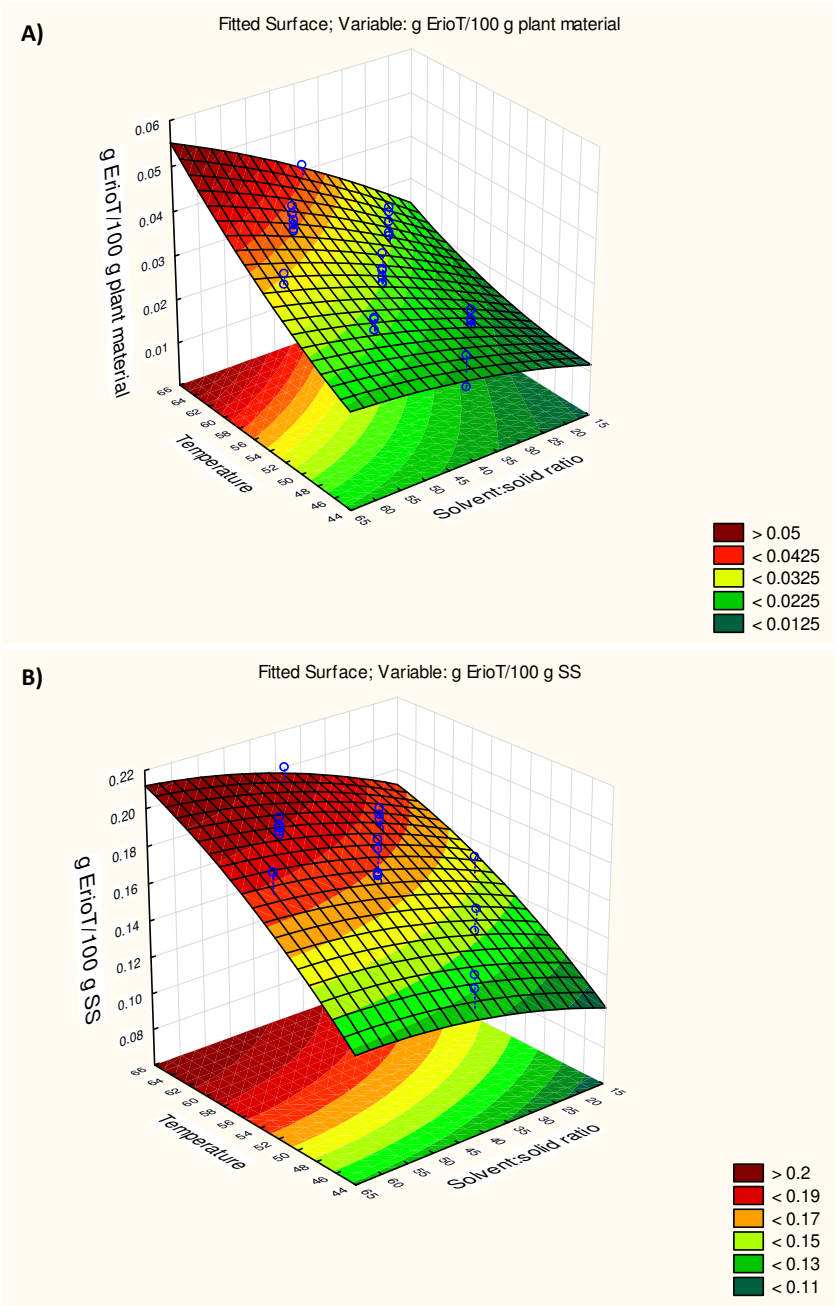

Figure A.4 Combined response surface and contour plots for A) eriocitrin yield and B) eriocitrin content of the extract as a function of temperature (°C) and solvent:solid ratio (mL/g).

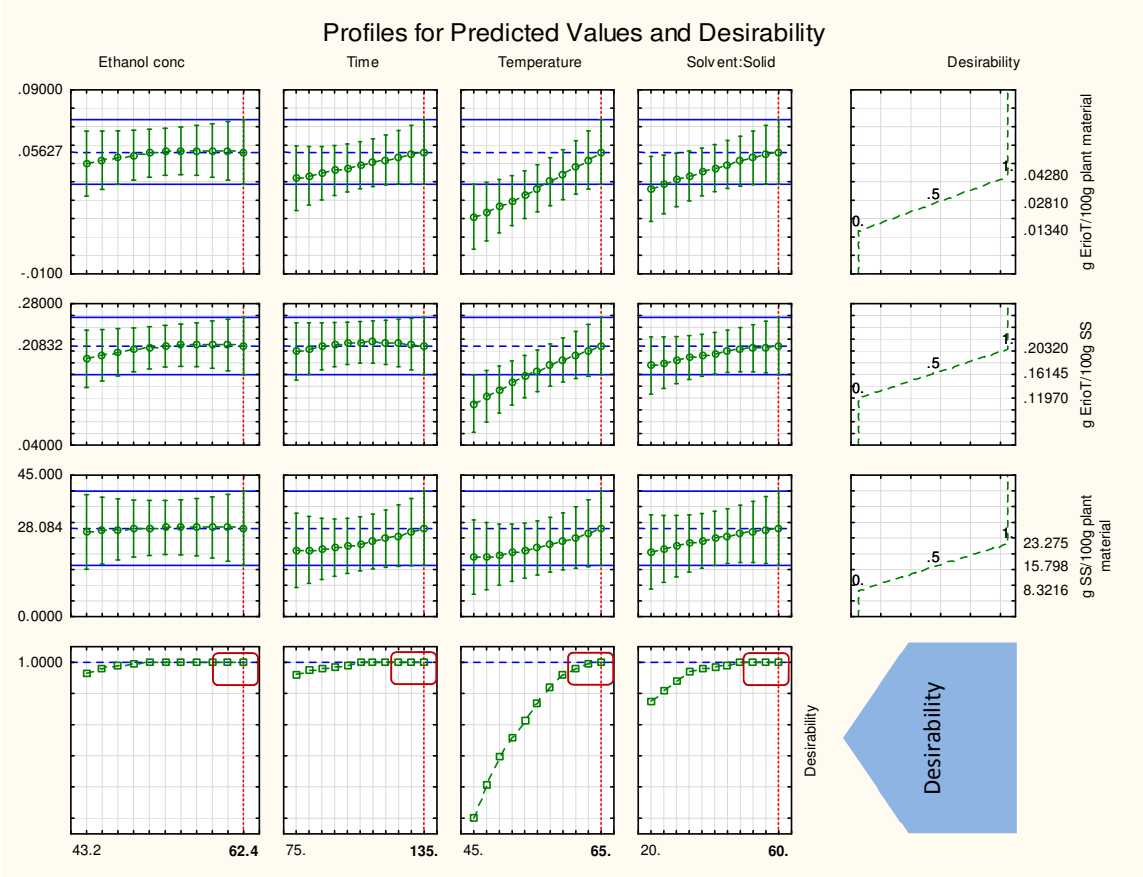

Figure A.5 Compound prediction profile graph that shows the combined desirability profiles for dependent variables eriocitrin yield and content (of extract), as well as extract yield *(red blocks indicate optimum/maximum parameter value combined for the responses)*.

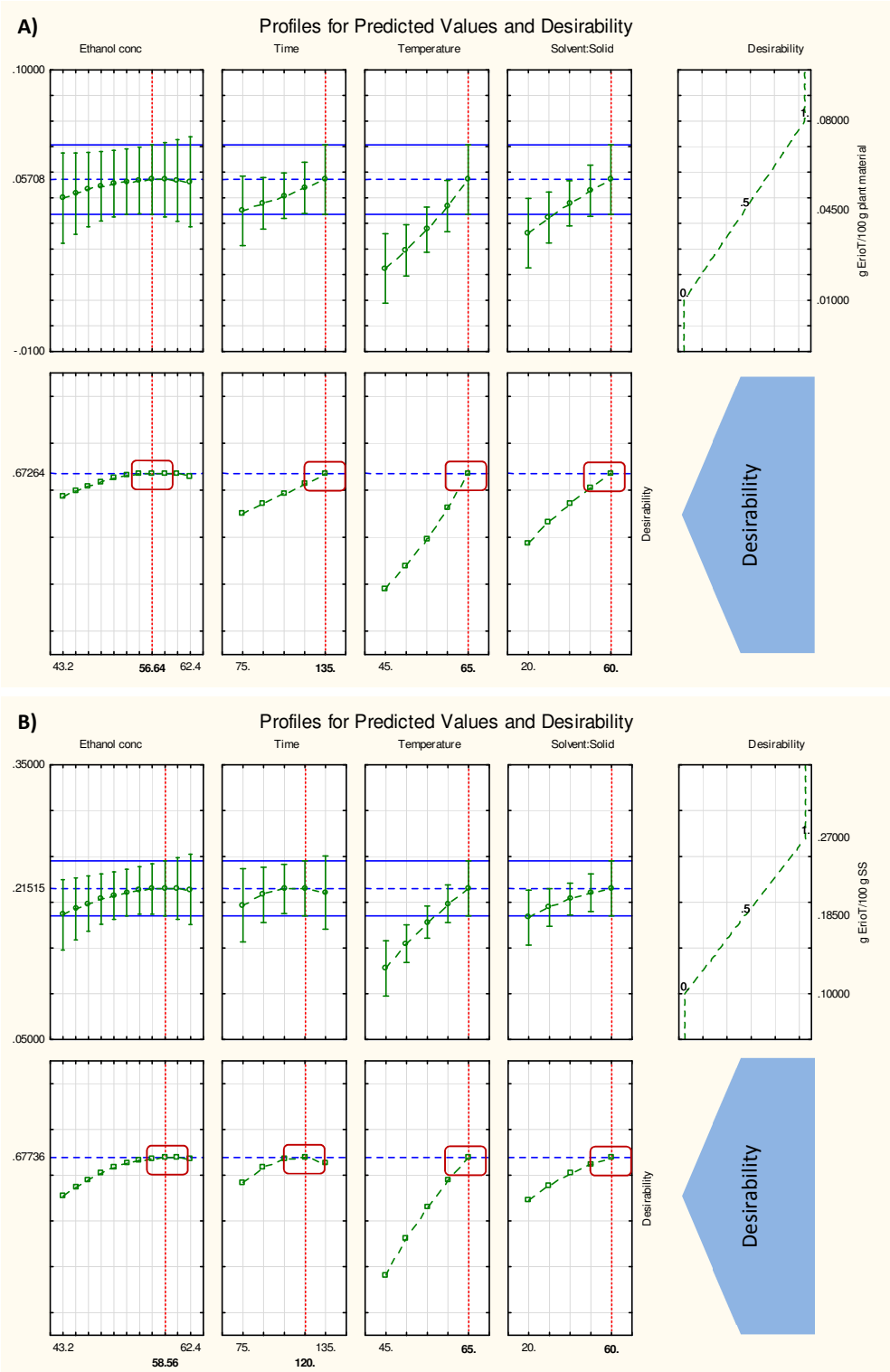

Figure A.6 Individual prediction profile graphs that show the desirability profiles for each dependent variable, namely A) eriocitrin (ErioT) yield and B) eriocitrin (ErioT) content (of extract) *(red blocks indicate optimum/maximum parameter value for the response)*.

Table A.3 Central Composite Design for verification experiments with average experimental (n=3) and predicted values for eriocitrin yield and eriocitrin content (of extract)

Run No.	X_1 Ethanol concentration (% v/v)	X_2 Time (min)	X_3 Temperature (°C)	X_4 Solvent:solid ratio (mL/g)	g Eriocitrin/ 100 g plant material		g Eriocitrin/ 100 g extract	
					Experimental	*Predicted*	*Experimental*	*Predicted*
1	52.8	105	55	40	0.0343 ± 0.0023	0.0274	0.1700 ± 0.0084	0.1680
2	48.0	90	50	30	0.0251 ± 0.0019	0.0167	0.1340 ± 0.0041	0.1304
3	57.6	120	50	30	0.0274 ± 0.0021	0.0211	0.1539 ± 0.0094	0.1398
4	48.0	90	60	50	0.0339 ± 0.0019	0.0346	0.1692 ± 0.0106	0.1725
5	57.6	120	60	50	0.0401 ± 0.0051	0.0406	0.1953 ± 0.0235	0.1938
6	48.0	120	50	50	0.0256 ± 0.0027	0.0248	0.1391 ± 0.0047	0.1446
7	57.6	120	50	50	0.0305 ± 0.0029	0.0266	0.1715 ± 0.0106	0.1520
8	57.6	90	50	30	0.0253 ± 0.0031	0.0175	0.1499 ± 0.0039	0.1467
9	48.0	90	60	30	0.0318 ± 0.0039	0.0234	0.1538 ± 0.0258	0.1583

Addendum B

Acid hydrolysis optimisation of flavanone glycoside-enriched *Cyclopia maculata* extract and the potential of enzymatic bioconversion: Results for eriocitrin

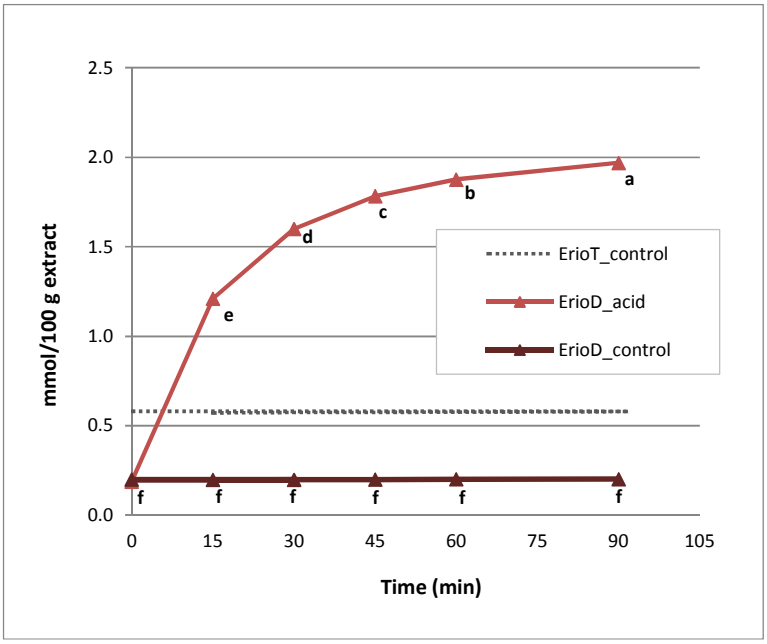

Figure B.1 Increase of eriodictyol (ErioD) over time with level of eriocitrin (ErioT) control indicated by grey dotted line. Constant parameters were 90°C and 0.295 M HCl *(means with the same letter are not significantly different ($p \geq 0.05$)).*

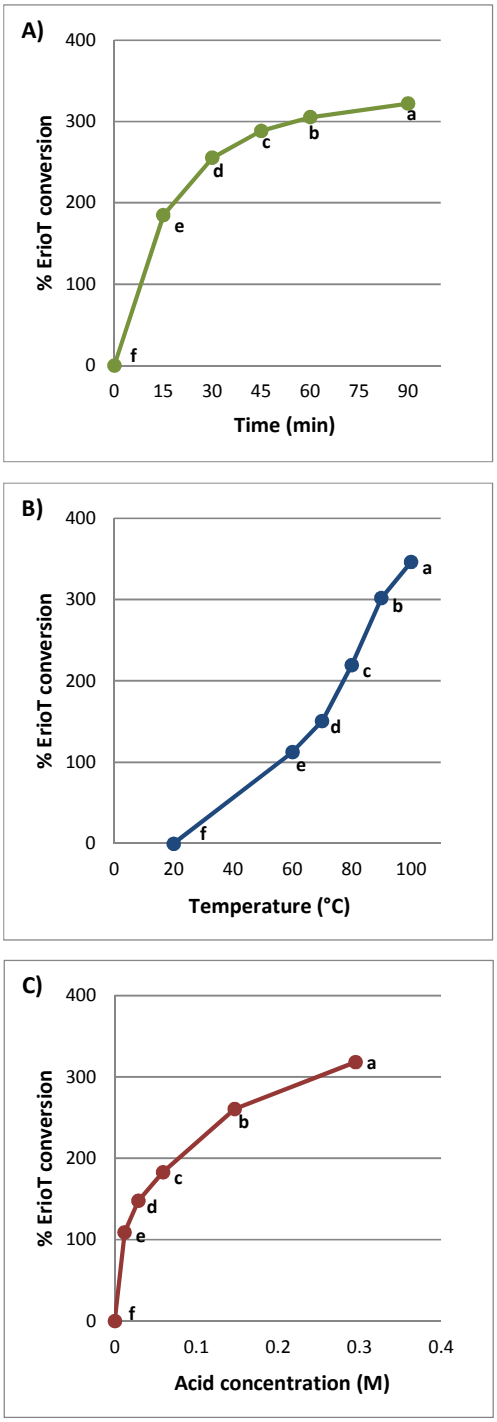

Figure B.2 Hydrolysis of *C. maculata* extract (FlvEE_5) - Effect of A) heating time (min) B) temperature (°C) and C) acid concentration (M) on conversion of eriocitrin (ErioT) to eriodictyol (ErioD). Constant parameters were for A) 90°C and 0.295 M HCl, B) 45 min and 0.295 M HCl and C) 90°C and 60 min *(means with the same letter are not significantly different ($p \geq 0.05$)).*

Table B.1 Central Composite Design for independent variables and average response values (n=3) for % eriocitrin conversion

Run No.	X_1 Temperature (°C)	X_2 Time (min)	% Eriocitrin conversion
1	80.0	40	205.99 ± 22.15
2	80.0	120	311.40 ± 34.36
3	90.0	40	334.59 ± 5.36
4	90.0	120	385.23 ± 8.85
5	77.9	80	279.23 ± 2.82
6	92.1	80	376.94 ± 5.76
7	85.0	23	240.98 ± 3.01
8	85.0	137	331.06 ± 31.87
9 (C)*	85.0	80	341.53 ± 1.87
10 (C)*	80.0	80	329.96 ± 27.20

** Centre point parameter values*

Table B.2 Additional experimental runs for verification with average experimental (n=3) and predicted values for % eriocitrin conversion to eriodictyol

Run No.	X_1 Temperature (°C)	X_2 Time (min)	% Eriocitrin conversion	
			Experimental	*Predicted*
1	85.0	80.0	402.19 ± 5.35	335.77
2	80.0	40.0	290.37 ± 3.95	216.93
3	80.0	120.0	385.30 ± 4.38	314.87
4	90.0	40.0	386.94 ± 2.89	329.25
5	90.0	120.0	453.33 ± 2.96	372.44
6	92.1	98.4	450.64 ± 3.74	391.53

Table B.3 ANOVA of experimental results and estimated coefficients for quadratic polynomial model for % eriocitrin conversion

Parameter	Regression Coefficient	$p_{Regr.\ Coeff.}$	SS[a]	DF[b]	MS[c]	F	p
Intercept	-1986.22	0.2114					
(1)Temperature (L)	36.74	0.3187	43474.92	1	43474.92	123.7980	0.0000
Temperature (Q)	-0.13	0.5330	155.20	1	155.20	0.4419	0.5134
(2)Time (L)	9.10	0.0012	30105.58	1	30105.58	85.7278	0.0000
Time (Q)	-0.02	0.0001	8099.50	1	8099.50	23.0639	0.0001
1L by 2L	-0.07	0.0241	2248.17	1	2248.17	6.4018	0.0195
Lack of fit			1935.07	3	645.02	1.8368	**0.1714**
Pure Error			7374.70	21	351.18		
Total SS			94104.61	29			
R^2							**0.9011**
R^2_{adj}							**0.8805**

[a] Sum of Squares
[b] Degree of Freedom
[c] Mean Square
L= linear coefficient; Q=quadratic coefficient; 1L by 2L = interaction coefficient
Significant p-values ($p < 0.05$) of regression coefficients and parameter effects are highlighted in blue and red, respectively.

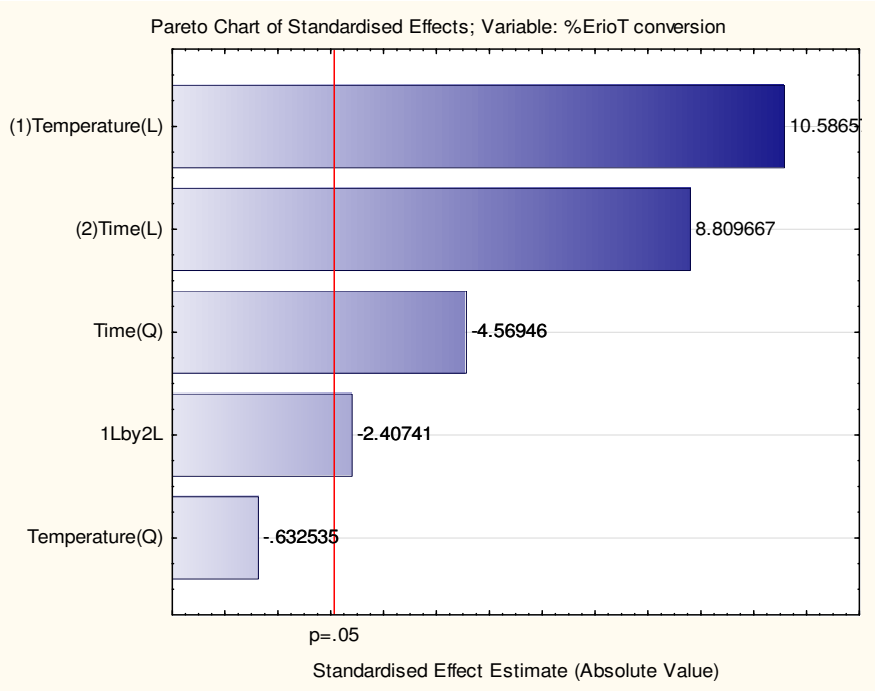

Figure B.3 Pareto chart of standardised effect estimates for % eriocitrin (ErioT) conversion showing the significant ($p < 0.05$) effects of extraction parameters on the response value.

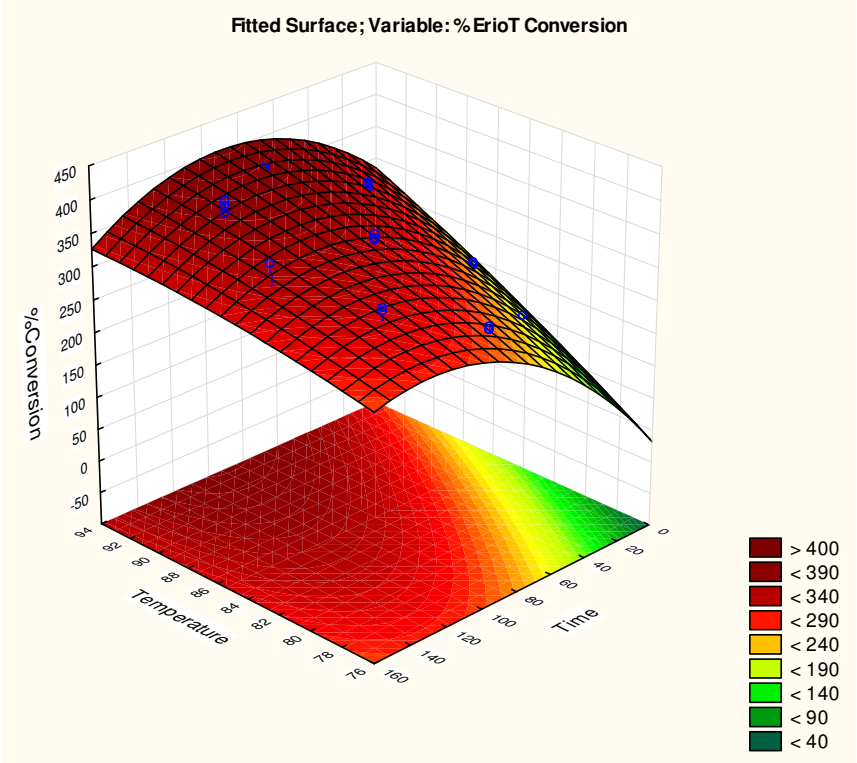

Figure B.4 Combined response surface and contour plot for % eriocitrin (ErioT) conversion as a function of time (min) and temperature (°C).

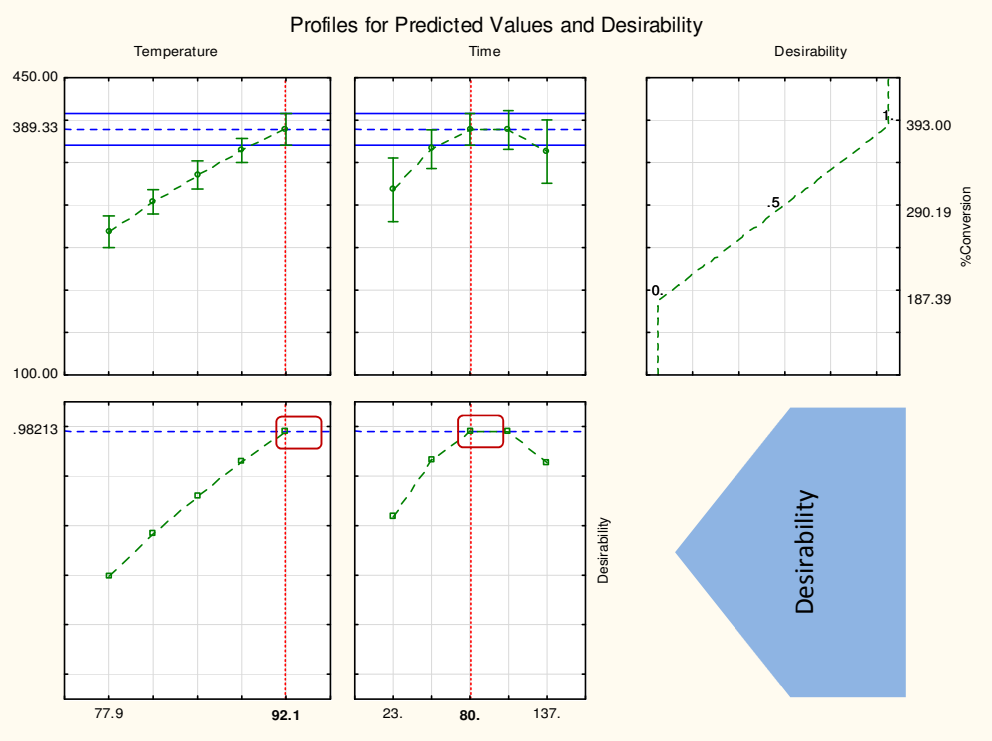

Figure B.5 Prediction and desirability profiling for % eriocitrin conversion to eriodictyol *(red blocks indicate optimum/maximum parameter value for the response).*

Addendum C

Spectrophotometric colour measurements of acid hydrolysed extracts of CCD (RSM) experimental runs

Table C.1 Average spectrophotometric colour measurement values of hydrolysed extracts of CCD experimental runs (n=3)

Run No.	Treatment	Integral (AUC)	Absorbance at 490 nm
Not applicable	Control (no acid/heat)	251.11 ± 30.94	0.67 ± 0.09
1	0.295 mM HCl, 80°C, 40 min	603.18 ± 13.53	2.62 ± 0.06
2	0.295 mM HCl, 80°C, 120 min	733.75 ± 11.85	3.29 ± 0.06
3	0.295 mM HCl, 90°C, 40 min	729.38 ± 35.59	3.24 ± 0.17
4	0.295 mM HCl, 90°C, 120 min	862.12 ± 20.78	3.93 ± 0.08
5	0.295 mM HCl, 77.9°C, 80 min	674.38 ± 16.10	2.98 ± 0.06
6	0.295 mM HCl, 92.1°C, 80 min	794.96 ± 41.85	3.60 ± 0.02
7	0.295 mM HCl, 85°C, 23 min	629.09 ± 18.82	2.73 ± 0.08
8	0.295 mM HCl, 85°C, 137min	779.64 ± 5.15	3.52 ± 0.01
9(C)*	0.295 mM HCl, 85°C, 80 min	747.46 ± 10.49	3.36 ± 0.05
10(C)*	0.295 mM HCl, 85°C, 80 min	749.38 ± 21.03	3.37 ± 0.10

** Centre point parameter values*

Addendum D

Extract yield of flavanone glycoside-enriched extracts (FlvEE) prepared from *Cyclopia maculata* tea processing by-product

Table D.1 Extract yield of flavanone glycoside-enriched extracts (n=16) prepared from *C. maculata* tea processing by-product

Sample	Replicate	Plant material extracted *(g)*	Mass of freeze-dried extract *(g)*	% Yield *(g extract per 100 g plant material)*
FlvEE_1	1	210.3279	32.0731	15.25
FlvEE_2	2	210.2025	31.9099	15.18
FlvEE_3	3	210.2440	27.6180	13.14
FlvEE_4	4	210.3054	35.6388	16.95
FlvEE_5	5	210.1312	35.7648	17.02
FlvEE_6	6	210.1610	36.9531	17.58
FlvEE_7	7	210.2412	37.2444	17.72
FlvEE_8	8	210.1636	32.8352	15.62
FlvEE_9	9	210.2194	36.0960	17.17
FlvEE_10	10	210.3409	37.4600	17.81
FlvEE_11	11	210.1705	34.1500	16.25
FlvEE_12	12	210.2547	33.4600	15.91
FlvEE_13	13	210.1099	37.4160	17.81
FlvEE_14	14	210.1501	37.0180	17.62
FlvEE_15	15	210.2013	34.7670	16.54
FlvEE_16	16	210.2267	37.2200	17.70
			Min	**13.14**
			Max	**17.81**
			Mean	**16.58**
			Std. deviation	**1.30**

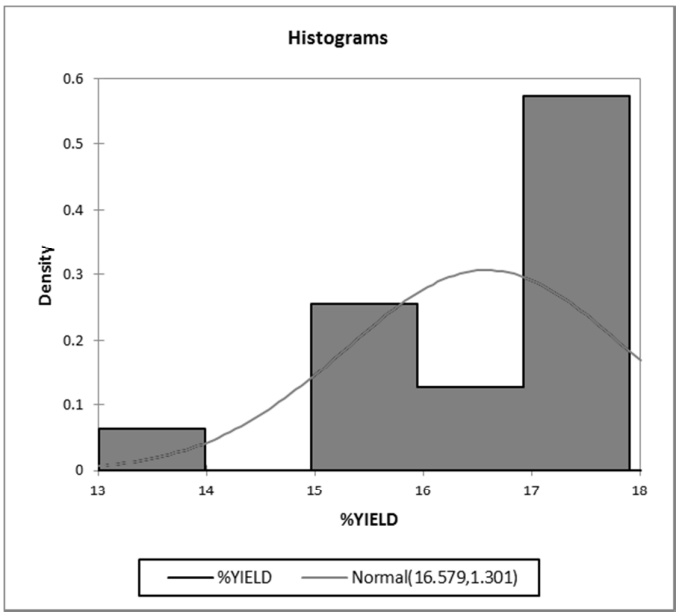

Figure D.1 Histogram and normal distribution for % yield of flavanone glycoside-enriched extracts (n=16).