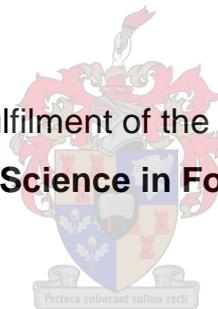


Development of a xanthone-enriched honeybush tea extract

by

Stephanie Cesa Bosman

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Department of Food Science
Faculty of AgriSciences
Stellenbosch University

Supervisor: Prof. E. Joubert
Co-supervisors: Dr. D. de Beer and Dr. G.O. Sigge

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Declaration

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Abstract

Cyclopia genistoides (honeybush) has been identified as an excellent resource for the production of a xanthone-enriched extract due to its high mangiferin content and successful cultivation. The predominant xanthone present in *C. genistoides* is mangiferin, a potent antioxidant proven to exhibit a wide range of bioactivities that contribute greatly to the health-promoting abilities of honeybush extracts. Isomangiferin, the regio-isomer of mangiferin and of comparable antioxidant capacity to mangiferin, is another valuable compound present in substantial quantities in *C. genistoides*. A xanthone-enriched extract would find possible application in functional food/beverage products that provide health benefits beyond basic nutrition. In the current study, the effect of ethanol (EtOH) concentration (0-100%, v/v), plant material size (milled vs. teabag fraction), extraction time (0-60 min) and elevated extraction temperatures on the extraction of xanthones from unfermented *C. genistoides* was investigated. Single factor experiments showed the best extraction efficiency, evaluated in terms of extract yield, xanthone yield and xanthone content of the extract, was achieved by extracting milled plant material with 20-60% EtOH (v/v) for 30 min at elevated temperatures (70°C). Response surface methodology (RSM) to evaluate the individual and interaction effects of process variables, namely EtOH concentration (0-100%, v/v) and temperature (0-70°C) was used to further optimise the extraction process. EtOH concentration was found to have the largest effect on extraction efficiency ($p < 0.05$), whilst temperature had a negligible effect. Optimal levels of EtOH concentration (40%, v/v) and temperature (70°C) for maximum extract and mangiferin yields were successfully achieved within the experimental domain, using 10 mL/g solvent:solid ratio and 30 min extraction time. Ultrafiltration (UF) was subsequently employed to facilitate further xanthone enrichment of the unfermented *C. genistoides* extract (40% EtOH, v/v). A series of laboratory scale membrane devices (centrifugal membrane units, stirred cell and tangential flow ultrafiltration (TFU) system) were used in an up-scale approach to determine the effect of membrane material (regenerated cellulose (RC) vs. polyethersulphone (PES)), molecular weight cut off (MWCO; 3 kDa, 10 kDa, 30 kDa), feed concentration (1% vs. 3% soluble solids (SS)) and operating parameters (transmembrane pressure (TMP) and feed flow rate) on membrane performance and permeate quality. The best performing membrane in terms of productivity and xanthone enrichment was the 10 kDa RC membrane when using an extract concentration close to that of industrially prepared extracts (3% SS). RSM was used to further optimise UF of unfermented *C. genistoides* through a 10 kDa RC membrane in the TFU system. The individual and interaction effects of TMP (0.82-2.04 bar) and feed flow rate (200-444 mL/min) on permeate flux, xanthone enrichment and the fouling index were investigated. The individual effects of TMP and feed flow rate had a significant effect on all measured

responses, while their interaction only affected average permeate flux and fouling index significantly. Optimal TMP and feed flow rate values of 2.04 bar and 444 mL/min, respectively, were selected within the experimental domain, restricted by equipment constraints. Validation of the combined protocol including ethanol-water extraction and UF using plant material from ten different unfermented *C. genistoides* batches resulted in enriched extracts containing 10.6-17.8% xanthone content. During UF, average mangiferin and isomangiferin enrichments of 20% and 22%, respectively, were obtained. Whilst no correlation was found between the feed concentration of the extracts, xanthone enrichment and fouling index, a strong linear correlation ($R^2 = 0.98$) was found between feed concentration and permeate yield.

Uittreksel

Cyclopia genistoides (heuningbos) is geïdentifiseer as 'n uitstekende bron vir die produksie van 'n xantoon-verrykte ekstrak weens sy hoë mangiferien-inhoud sowel as suksesvolle verbouing daarvan. Die oorheersende xantoon teenwoordig in *C. genistoides* is mangiferien, 'n kragtige antioksidant met 'n bewese wye reeks bioaktiwiteite wat grootliks bydra tot die gesondheidsvoordele van heuningbosekstrakte. Isomangiferien, die regio-isomeer van mangiferien met vergelykbare antioksidant-aktiwiteit as mangiferien, is nog 'n waardevolle verbinding teenwoordig in aansienlike hoeveelhede in *C. genistoides*. 'n Xantoon-verrykte ekstrak kan moontlik toegepas word in funksionele voedsel- of drankie-produkte, wat gesondheidsvoordele bo en behalwe die basiese voedsaamheid inhou. Die effek van etanol (EtOH)-konsentrasie (0-100%, v/v), plantmateriaal grootte (gemaal teenoor teesakkie-fraksie), ekstraksietyd (0-60 min) en ekstraksietemperatuur op die ekstraksie van xantone uit ongefermenteerde *C. genistoides* is ondersoek. Enkelfaktor eksperimente het getoon dat die beste ekstraksie-effektiwiteit, in terme van ekstrakopbrengs, xantoonopbrengs en xantooninhoud van die ekstrak, bereik is deur gemaalde plantmateriaal met 20-60% EtOH (v/v) vir 30 min by verhoogde temperatuur (70°C) te ekstraheer. Respons-oppervlak Metodologie (ROM) is aangewend om die individuele en interaktiewe effekte van die veranderlikes, naamlik EtOH-konsentrasie (0-100%, v/v) en temperatuur (0-70°C) te ondersoek asook om die ekstraksieproses verder te optimaliseer. EtOH-konsentrasie het die grootste effek op die ekstraksie-effektiwiteit gehad ($p < 0.05$), terwyl die effek van temperatuur onbeduidend was. Optimale vlakke van EtOH-konsentrasie (40% v/v) en temperatuur (70°C) vir maksimum ekstrak- en mangiferienopbrengs is binne die eksperimentele domein gevind, met die gebruik van 10 mL/g oplosmiddel:vastestof verhouding en 'n ekstraksietyd van 30 min.

Ultrafiltrasie (UF) is daarna gebruik om verdere xantoon-verryking van die ongefermenteerde *C. genistoides* ekstrak (40% EtOH, v/v) te fasiliteer. 'n Reeks laboratoriumskaal membraantoestelle (sentrifugale membraaneenhede, 'n geroerde selsistiem en 'n kruisvloei-ultrafiltrasie (KVVU) sistiem) is gebruik in 'n opskaleringsbenadering om die effek van die membraanmateriaal (geregenereerde sellulose (RS) vs. polyetersulfoon (PES)), molekulêre gewig afsnit (MWCO; 3 kDa, 10 kDa, 30 kDa), voerkonsentrasie (1% vs. 3% oplosbare vastestowwe (OV)) en operasionele parameters (transmembraandruk (TMD) en voervloei spoed) op membraanprestasie en permeaatkwaliteit te bepaal. Die membraan met die beste prestasie in terme van produktiwiteit en xantoon-verryking was die 10 kDa RS membraan wanneer gebruik met 'n ekstrakkonsentrasie na aan dié van die industrieel vervaardigde ekstrakte (3% OV). ROM is gebruik om die KVVU van ongefermenteerde *C. genistoides* deur 'n 10 kDa RS membraan verder te optimaliseer. Die

individuele en interaktiewe effekte van TMD (0.82-2.04 bar) en voervloei spoed (200-444 mL/min) op permeaatvloei, xantoon-verryking en die blokkeringindeks is ondersoek. Die individuele effekte van TMD en voervloei spoed het 'n betekenisvolle effek op alle gemete response gehad, terwyl hul interaksie net gemiddelde permeaatvloei en besoedelingsindeks beduidend beïnvloed het. Optimale TMD en voervloei spoed waardes van 2.04 bar en 444 mL/min, onderskeidelik, is geselekteer binne die eksperimentele domein, wat bepaal is deur die beperkings van die toerusting. Die geldigheid van die gesamentlike protocol, insluitende etanol-water ekstraksie en UF, is getoets deur plantmateriaal van tien verskillende ongefermenteerde *C. genistoides* monsters te gebruik. Dit het gelei tot verrykte ekstrakte wat 10.6-17.8% xantone bevat het. UF het onderskeidelik gemiddelde mangiferien- en isomangiferien-verryking van 20% en 22% gelewer. Geen korrelasie is gevind tussen die voerkonsentrasie van die ekstrakte en die besoedelingsindeks nie, maar 'n goeie liniêre korrelasie ($R^2 = 0.98$) is tussen voerkonsentrasie en permeaatopbrengs gevind.

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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

The functional food market continues to thrive as more educated consumers are selecting food products that provide health benefits beyond basic nutrition, i.e. functional foods (Jones & Jew, 2007). The consumption of functional foods allows consumers to conveniently prevent/manage specific conditions through the daily diet (Sloan, 2000). The evolution of functional foods has presented food manufacturers with an entirely new competitive market, with pressure to discover novel ingredients that could impart functional status to their products. Polyphenol-rich extracts offer good potential for functional food ingredients as epidemiological evidence has shown that selected polyphenols reduce the risk of chronic diseases (Nijveldt *et al.*, 2001; Williamson & Holst, 2008; Pandey & Rizvi, 2009; Joven *et al.*, 2013).

Honeybush (*Cyclopia* spp.) is one of the few indigenous South African plants that has been successfully developed into a commercial product within the past 100 years, partly due to the development of commercial cultivation and factory-based production (Joubert *et al.*, 2011). Of the 23 species of *Cyclopia*, only *C. genistoides* and *C. subternata* are currently cultivated in substantial quantities as they are fast growers that can be harvested annually (Joubert *et al.*, 2011). Since the 're-discovery' of honeybush in the mid-1990s (Joubert *et al.*, 2011), research has focused on the following aspects: characterisation of the phenolic composition and antioxidant activities of *Cyclopia* extracts (Ferreira *et al.*, 1998; Joubert *et al.*, 2003; Joubert *et al.*, 2008a; Joubert *et al.*, 2008b; Kamara *et al.*, 2003; Kamara *et al.*, 2004; De Beer & Joubert, 2010; De Beer *et al.*, 2012; Kokotkiewicz *et al.*, 2012; Kokotkiewicz *et al.*, 2013; Beelders *et al.*, 2014); optimisation of post-harvest processing (Du Toit & Joubert, 1999; Theron, 2012), sensory characterisation and evaluation of the tea (Theron, 2012); characterisation of volatile fraction, including aroma-impact volatiles (Le Roux *et al.*, 2012); testing alternative methods of polyphenol quantification for quality control purposes (Joubert *et al.*, 2006; Joubert *et al.*, 2012); and investigating the health-promoting properties of honeybush extracts (as reviewed by Joubert *et al.*, 2008c and Visser *et al.*, 2013). Other research not covered by these reviews indicated the anti-diabetic (Muller *et al.*, 2011), anti-obesity (Dudhia *et al.*, 2013; Pfeiffer *et al.*, 2013) and pro-apoptotic (Kokotkiewicz *et al.*, 2013) properties of honeybush. Due to the well-established therapeutic value of honeybush extracts, research is now moving in the direction of investigating the potential of honeybush extracts as functional food ingredients.

The predominant polyphenols present in *Cyclopia* spp. are the xanthone glucosides, mangiferin and its regio-isomer isomangiferin (Joubert *et al.*, 2003; Joubert *et al.*, 2008b). Although mangiferin is present in substantially higher quantities than isomangiferin, these two xanthenes have been shown to have similar antioxidant activity (Hubbe & Joubert, 2000; Malherbe *et al.*, 2014). The bioactivity of mangiferin has been well studied (as reviewed by Vyas *et al.*, 2012) and remains of interest, judging from the number of papers published

since. For isomangiferin, however, only its antioxidant activity has been demonstrated to date (Hubbe & Joubert, 2000; Malherbe *et al.*, 2014). Various antioxidant assays have shown that the xanthenes are the most active of all the major polyphenols present in *Cyclopia* (Hubbe & Joubert, 2000; Joubert *et al.*, 2008b) and the health-promoting properties of honeybush extracts, such as the antidiabetic (Muller *et al.*, 2011) and anti-obesity activities (Dudhia *et al.*, 2013), have been linked to their presence in the extracts. For the production of extracts where maximum polyphenol content is desirable, unfermented *Cyclopia* spp. should be used as fermentation has been proven to reduce the phenolic content of extracts (Joubert *et al.*, 2008b; De Beer & Joubert, 2010). *Cyclopia genistoides* has been identified as the species of choice for the development of a xanthone-enriched extract, not only due to its superior xanthone content compared to other *Cyclopia* spp. (Joubert *et al.*, 2003; Joubert *et al.*, 2008b), but also because of its sustainability made possible by successful cultivation (Joubert *et al.*, 2011).

The potential functional ingredient application of a mangiferin-enriched extract is largely attributed to the strong antioxidant activity of mangiferin that additionally show a vast array of pharmacological activities (Wauthoz *et al.*, 2007; Vyas *et al.*, 2012). The value of a mangiferin-containing extract is already well exploited in Cuba, where a standardised aqueous extract of mango (*Mangifera indica* L.) stem bark (Vimang[®]) with a mangiferin content of 10-20% is successfully sold as an antioxidant nutritional supplement to promote general health (Sánchez *et al.*, 2000; Núñez-Sellés *et al.*, 2002). Mangiferin-enriched extracts could also be marketed towards a specific bioactivity, fulfilling a new trend that has emerged in the antioxidant market (Becker, 2013).

Research to date has not only focused on the various bioactivities of mangiferin, but also on novel techniques for its extraction from various plant sources (Kim *et al.*, 2010; Fernández-Ponce *et al.*, 2012; Kulkarni & Rathod, 2014; Salomon *et al.*, 2014), as well as its recovery from mango processing by-products (Berardini *et al.*, 2005; Barreto *et al.*, 2008; Luo *et al.*, 2012). Novel techniques, offering several economic and environmental advantages over traditional techniques (as reviewed by Wang & Weller, 2006; Wijngaard *et al.*, 2012; Azmir *et al.*, 2013; Shah & Rohit, 2013) and the re-utilisation of food processing waste products are two different approaches of implementing 'green' chemistry. The concept of 'green' chemistry was established in order to develop more efficient and environmentally friendly processes, such as reducing energy requirements, operational times and chemical requirements (Clark, 2011). In terms of industrial extract production, this can be achieved by utilising an energy-efficient (novel) extraction technique or, alternatively, optimising the existing extraction procedure to achieve the best possible productivity and extract quality, whilst minimising energy consumption and, therefore, the carbon footprint of the process.

The increasing market value of natural polyphenols (Anon., 2013) has encouraged food ingredient manufacturers to investigate different techniques for their separation/purification from botanical extracts. One such technique is ultrafiltration (UF), which is particularly well suited to the production of high-value extracts destined for food, pharmaceutical or cosmetic use as it operates under mild temperature and pressure conditions, suitable for separating heat-labile, bioactive compounds (Galanakis *et al.*, 2010; Sun *et al.*, 2011). UF has successfully been used to process various waste streams (Lo *et al.*, 2005; Cassano *et al.*, 2011; Galanakis *et al.*, 2013; Conidi *et al.*, 2014) and other botanical extracts (Xu *et al.*, 2005; Conidi *et al.*, 2011; Sun *et al.*, 2011; Husson *et al.*, 2012; Kumar *et al.*, 2012), either to improve their overall quality or produce a high-value enriched fraction with possible functional food/nutraceutical application.

Response surface methodology (RSM) is a popular statistical tool that has been widely used for the optimisation of polyphenol extraction from various plant sources (Yang *et al.*, 2010; Prasad *et al.*, 2012; Lai *et al.*, 2013), including xanthone extraction (Zou *et al.*, 2013, Zou *et al.*, 2014), as well as the UF of botanical extracts (Cai *et al.*, 2012; Akdemir & Ozer, 2013; Baklouti *et al.*, 2013). RSM has several advantages as an optimisation technique compared to the one-variable-at-a-time (OVAT) approach as it allows simultaneous multivariate analysis, enabling the demonstration of inter-relationships between responses and identification of the most critical process parameters, which is not possible with the OVAT approach (Baş & Boyaci, 2007; Bezerra *et al.*, 2008). A further advantage is that it requires fewer experimental runs, ultimately resulting in a cost saving and reduced carbon footprint (Baş & Boyaci, 2007; Bezerra *et al.*, 2008; Dejaegher & Vander Heyden, 2011).

The purpose of this study was to develop an industrially practical process for the production of a xanthone-enriched honeybush extract, thereby broadening the research scope of the value-adding potential of honeybush. RSM was applied to optimise the extraction procedure of unfermented *C. genistoides* within a process space governed by current processing requirements of the South African plant extract industry, as well as subsequent enrichment of the extract using UF. Both processes were validated by performing the complete optimised procedure, from extract production to UF, using plant material from ten different *C. genistoides* batches.

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Chapter 2

Literature Review

2.1 Introduction

In recent years, consumers have become more educated about the food they eat and are making smarter food choices. There is a growing awareness of the link between diet and health with more consumers turning to 'self-medication' through food and beverage consumption as a means of prevention rather than cure (Crespo & Brazinha, 2010). Today, consumers no longer only want food that is healthy in terms of nutrition, but food that also imparts health benefits to their body (McKay & Blumberg, 2007). This trend, which has been around for years in traditional cultures where plants serve as both food and medicine, has redefined food quality not only in terms of nutritional value, but also in terms of the presence of bioactive compounds of a food product (Cassano *et al.*, 2008a).

The high market value and competitive nature of the nutraceutical industry coupled with increasing product demand encourages manufacturers to seek out novel plant sources or utilise those with a consistently high phenolic content for the production of their products (Crawford, 2012). On an agricultural level, plant breeders too are challenged to produce crops with the highest possible content of the target, bioactive compounds (Farnham *et al.*, 1999; Martin, 2013). It is for this reason that herbal teas, with their high content of easily extractable polyphenols and long history of regular use with no adverse side effects, have become a popular choice for the production of natural plant extracts (Joubert *et al.*, 2008a).

Honeybush (*Cyclopia* spp.) is a South African plant from which both 'fermented' (oxidised) and 'unfermented' tea and extracts are produced. These indigenous species offer a local, renewable source of the potent antioxidant, mangiferin (Joubert *et al.*, 2003). This compound is attracting the interest of scientists worldwide due to its vast range of medicinal properties (Vyas *et al.*, 2012). Currently, there is a great deal of research being done not only on the extraction of this valuable compound, but also on different delivery systems for incorporation into model food components as a stepping stone to the eventual production of a mangiferin-containing functional food ingredient (De Souza *et al.*, 2013).

Membrane filtration is an appealing technique for the recovery, concentration or fractionation of bioactive compounds from a variety of botanical extracts, ranging from waste streams to high-value pharmaceutical extracts. Membrane technology offers a 'greener' alternative to traditional separation techniques due to its mild operating conditions and therefore advantageous for the processing of heat-sensitive bioactive compounds (Galanakis *et al.*, 2010; Sun *et al.*, 2011).

This literature review aims to give a broad overview of the nutraceutical and functional food markets to date, as well as the theory and experimental techniques involved in the development of a natural xanthone-enriched honeybush tea extract with potential application as a functional food ingredient. Methods for quality control of polyphenol-rich plant extracts

will be briefly outlined within the context of their suitability for research and application by industry.

2.2 Nutraceuticals and functional foods

2.2.1 Background

Consumers of all ages are taking their health into their own hands as the link between diet and health is becoming more compelling. The market for food products and dietary supplements that provide 'prevention rather than cure' is booming. Not only are nutritional supplement sales on the rise, there is an increase in sales of health food products such as low-sugar and low-fat alternatives, foods that are high in fibre and those containing pre/probiotics. Although this provides lucrative opportunities for both food and pharmaceutical manufacturers, the result is a whole new range of food products whose classification can often be confusing to the un-educated consumer (Muratoglu, 2013).

The terms 'functional foods' and 'nutraceuticals' are buzz words in the food industry. These terms are both similar yet distinctly different at the same time. Nutraceuticals are natural, bioactive chemical compounds that are characterised by health-promoting, disease-preventing or medicinal properties (Arvanitoyannis & Van Houwelingen-Koukaliaroglou, 2005). They are generally taken like vitamins rather than eaten, but can be incorporated into foods or into dietary supplements to produce bioactive-enriched foods (BEFs). A current EU funded project aims, amongst others, at providing generic guidelines to small and medium-sized enterprises (SMEs) for providing health-promoting BEFs and for submitting convincing health claim dossiers to the European Food Safety Authority (EFSA) (<http://www.pathway27.eu/>).

The term functional foods was first introduced in Japan in the mid-1980s and refers to processed foods containing bioactive ingredients which impart health-promoting benefits in addition to providing nutrients (Siró *et al.*, 2008). The precise definition of functional foods varies from country to country. Functional foods differ from conventional foods as they contain nutraceutical or bioactive ingredients, for example cholesterol-lowering spreads, vitamin-enriched cereals and beverages, probiotic dairy products and eggs enriched with omega-3 fatty acids (Siró *et al.*, 2008). Bioactive compounds can be defined as 'extra nutritional' constituents that occur naturally in small quantities in plant products and lipid-rich foods (Kitts, 1994). Williamson & Holst (2008) re-defined polyphenols as 'lifespan essentials', stressing the need for a target intake value, based on the amount of polyphenols in the '5-a-day' diet. The 'extra nutritional' constituents are classified according to their chemical structure and health-promoting function (Kris-Etherton *et al.*, 2002). These bioactive

ingredients responsible for the functionality of functional foods include flavonoids, phytosterols, phytosterols, bioactive peptides and bioactive carbohydrates (Arvanitoyannis & Van Houwelingen-Koukaliaroglou, 2005).

It should be noted that functional foods and nutraceuticals are not intended to treat disease nor can they claim to. They are taken by healthy people as a preventative measure. On the other hand, medical foods are developed specifically for the treatment of disease and usually require a prescription. For example Axona[®], a milkshake type product consisting mainly of fractionated coconut milk is aimed at the treatment of Alzheimer's disease (Wang, 2012). Another example, Banatrol[®] Plus, a combination of natural banana flakes and prebiotics, helps relieve diarrhoeal symptoms and replenish the beneficial bacteria in the gut (Anon., 2013a).

In 2011 the global nutraceutical sales were estimated at US\$ 142 billion and predicted grow to as much as US\$ 207 billion by 2016 (Muratoglu, 2013). The three largest markets for functional food products are the United States, followed by Europe and Japan. Together, these three nations contribute over 90% of the total sales (Benkouider, 2005). Although the sale of functional foods and beverages is greatest in developed markets, the largest growth was experienced in emerging markets, such as China and Brazil. These countries experienced growth of US\$11.6 billion and US\$ 4.2 billion, respectively (Cowland, 2012). Despite the global economic slump, sales of herbal dietary supplements continue to grow, with an increase of 5.5% seen in the U.S. in 2012 (Anon., 2013b). It is evident from these sales figures that consumers of all types choose safe, natural, low-cost options to maintain their health and increase wellness (Anon., 2013b). The five top selling herbal supplements (excluding herbal teas) in 2012, according to SPINSscan Natural were flaxseed (*Linum usitatissimum*) oil, grass (wheat and barley; *Triticum aestivum* and *Hordeum vulgare*), turmeric (*Curcuma longa*) and concentrated curcumin extracts, aloe vera (*Aloe vera*) and spirulina/blue-green algae (*Arthrospira* spp.) (Anon., 2013b).

Although the sales figures continue to rise, many consumers still question the integrity and regulation of the health claims made by food manufacturers for their functional food products (Arvanitoyannis & Van Houwelingen-Koukaliaroglou, 2005). Legislation of functional foods and nutraceuticals will be discussed in section 2.2.4.

2.2.2 Phenolic compounds and their beneficial properties

Polyphenolic compounds are the most abundant secondary metabolites in plants and perform many functions including growth, pigmentation, reproduction and resistance to pathogens (Lattanzio *et al.*, 2006). Plants use secondary metabolites as their defence system (against pathogens or competing plants) and as signal compounds to attract pollinators or animals to disperse its seed (Kutan, 2001; Lattanzio *et al.*, 2006). As the name

implies, polyphenols consist of an aromatic group with one or more hydroxyl substituents (Wei *et al.*, 2010). There are over 8000 phenolic structures that have been discovered to date (Campos-Vegga & Oomah, 2013).

Polyphenols are classified into different groups according to the basic structure of their carbon backbone and the number of phenol rings they contain (Lattanzio *et al.*, 2006; Campos-Vega & Oomah, 2013). There are two major groups of phenolic compounds – flavonoids and non-flavonoids. Flavonoids are the most abundant polyphenols present in plant-derived foods (Kris-Etherton *et al.*, 2002). Flavonoids are categorised according to chemical structure into flavonols, flavones, flavanones, isoflavones, flavan-3-ols, anthocyanidins and chalcones (Manach *et al.*, 2004; Tsao, 2010).

Sources of polyphenols in the diet and our daily intake of these plant constituents are of interest as clinical and epidemiological evidence suggests that selected polyphenols reduce the risk of chronic diseases and are considered to be essential for humans to reach their full (genetically-determined) lifespan (Williamson & Holst, 2008). This interest in polyphenols is evidenced by databases such as Phenol Explorer, a freely available web-based database on the content of polyphenols in food, their *in vivo* metabolism and pharmacokinetics. It has recently been updated to include the effects of food processing on polyphenol contents in foods (Rothwell *et al.*, 2013). Major sources of dietary polyphenols are fruit, vegetables, cereals, legumes, chocolate, tea, coffee and wine (Pandey & Rizvi, 2009). These compounds have not only gained importance for their health-promoting properties, but they contribute to the sensory profile of food and beverages by impacting on the flavour, bitterness, astringency, odour, colour and oxidative stability of food and beverages. They have a tendency to complex with proteins which is responsible for astringency, haze formation in beverages, the inhibition of enzymes and reduced digestibility of proteins (Cheynier, 2005, Bandyopadhyay *et al.*, 2012).

As indicated at the beginning of this section, polyphenols are in demand by the nutraceutical industry as antioxidants due to their ability to scavenge free radicals *in vitro* (Nijveldt *et al.*, 2001; Frei & Higdon, 2003). Recently, more emphasis has been placed on their ability to modulate gene expression and cellular signalling pathways (as reviewed by Fraga *et al.*, 2010; Vauzour, 2012; Joven *et al.*, 2013; Maraldi *et al.*, 2014). Condition-specific application of polyphenols is coming to the fore. A recent paper by Bahadoran *et al.* (2013) reviewed the potential of polyphenols as nutraceuticals in the management of diabetes. According to a new market report by Transparency Market Research (<http://www.transparencymarketresearch.com>), the global polyphenol market was valued at US\$ 580 million in 2011 and is expected to reach US\$ 873.7 million by 2018 as worldwide polyphenol consumption is expected to nearly double.

On the negative side of polyphenol supplementation are safety concerns. These concerns are increasing as suppliers of supplements recommend daily flavonoid intakes in amounts that are many times higher than those doses which can normally be achieved from a flavonoid-rich diet (Egert & Rimbach, 2011).

The use of polyphenols in foods would limit their 'over-dosing' as they could have a negative impact on sensory properties, in particular by imparting a bitter taste, by increasing astringency and/or by changing the colour. Another consideration is the effectiveness of bioactive polyphenols in foods/ingredients/nutraceuticals which depends on the preservation of their stability, bioactivity and bioavailability (Thilakarathna & Rupasinghe, 2013). From a food technology perspective, encapsulation of polyphenols can successfully resolve both these limitations (Fang & Bhandari, 2010; Munin & Edwards-Lévy, 2011). Future research lies, amongst others, in novel food processing methods, food design and the understanding of the relations between bioactive ingredients release and the breakdown of food structures in the gastrointestinal tract (specifically aimed at microencapsulation development for bioactive compounds to meet specific needs of food applications) (Wang & Bohn, 2012).

Other challenges in the production of polyphenol nutraceuticals are the variability in bioactive content due to genetic and agronomic factors (Farnham *et al.*, 1999; Bruni & Sacchetti, 2009; Franz *et al.*, 2011; Martin, 2013), post-harvest handling, and subsequent processing or formulation steps (Irina & Mohamed, 2012; Tavares *et al.*, 2013; Harbourne *et al.*, 2013). Selection of plant species containing high levels of desired compounds and optimum harvest times (Joubert *et al.*, 2003; Joubert *et al.*, 2014) are practices that are followed by extract manufacturers, in addition to optimising extraction and other processing operations (Maicu, 2008; Orphanides *et al.*, 2013).

2.2.3 The antioxidant market

The 'antioxidant' category of nutraceuticals has gained considerable interest amongst food manufacturers because of the potential health benefits associated with many non-communicable diseases. This category encompasses a range of phytochemicals, including polyphenols and flavonoids, and well-known vitamins C and E, and beta-carotene (Daniells, 2009). The global market of antioxidants is increasing rapidly, not only because of the increased health risk in a constantly polluting environment, but due to an increased consumer education in terms of the health benefits of antioxidants (Becker, 2013). In addition to food, antioxidants find cosmetic and pharmaceutical applications (Crawford, 2012), leading to a great interest in the separation, purification and recovery of antioxidant compounds from natural sources (Ruby-Figueroa *et al.*, 2012).

There are four major consumer groups interested in a wide range of antioxidants and are therefore valuable to the nutraceutical industry. These groups include aging Baby

Boomers who are battling chronic diseases that occur later in life, women seeking anti-aging benefits, exercise enthusiasts who are at risk of increased build up of free radicals and children that would benefit from increased immune function (Becker, 2013). The Food Marketing Institute's 2011 'Shopping for Health' survey found that antioxidants are among the top five health components that U.S. consumers want in their food products. The success of a finished product, such as an antioxidant-enriched food or beverage, lies in the inclusion of one or more proven antioxidant ingredients with clear, easy-to-understand claims on the product's health benefits, competitive pricing and good distribution channels (Becker, 2013).

As consumers become more educated about antioxidants, it is no longer sufficient to simply claim their presence in a product. According to Paul Dijkstra, CEO of InterHealth Nutraceuticals, antioxidant products/ingredients that are marketed for a specific use are more successful, for example cranberry and urinary tract support, blueberries and pomegranates that support heart health and zinc that supports eye health. These associations give a more focused marketing message.

Today, antioxidant suppliers are faced with greater challenges than simply finding novel sources of the valuable compounds. The bioavailability of compounds is an area that still requires a great deal of research. Some manufacturers are, however, facing up to the challenge and shifting their emphasis to more bioavailable versions of traditional antioxidants, for example curcumin from powdered turmeric has a greater bioavailability and increased efficacy than plain curcumin (Shishu & Maheshwari, 2010). It is predicted that a trend will soon emerge in dosing antioxidants using standardisations based on scientific studies. Antioxidant specificity has been identified as another emerging trend. For example, the amino acid ergothioneine is a potent antioxidant not because of its ORAC value, but because of its ability to improve joint health due to its anti-inflammatory action (Benson *et al.*, 2012; Cheah & Halliwell, 2012).

The challenge faced by antioxidant suppliers and manufacturers apart from keeping with the trends of their competitors, is the need to provide effective, novel bioactive components that are clinically validated and appeal to both food manufacturers and consumers (Becker, 2013).

2.2.4 Legislation of functional foods and nutraceuticals

The increased consumer demand for functional foods is accompanied by a growing need for efficient regulation of both the manufacture and labelling of these products. Existing regulations are currently being revised worldwide in order to ensure that the consumers are appropriately informed about the use of these food products (Reis, 2011). The regulation of functional foods and ingredients varies from country to country but the common goal is to provide safe, effective, high-value products to the consumer.

Most of the controversy around the regulation of functional foods is the health claims. Health claims reveal a relationship between a nutrient or another substance in a food and a disease or health-related condition. They can be used on conventional foods or dietary supplements. They differ from nutritional claims, such as 'low fat', 'high fibre' and 'low calorie' (Arvanitoyannis & Van Houwelingen-Koukaliaroglou, 2005).

In the U.S., there are three different categories of claims that are allowed on food and dietary supplement labels: health claims, nutrient content claims and structure/function claims. Since functional foods and nutraceuticals are not intended to treat or cure any disease, they do not need FDA approval before going onto the market, yet they must comply with the GMPs launched by the FDA regarding their manufacture (Wang, 2012). One of the main focuses of the GMPs is the quality of the raw materials used, which ultimately determines the quality of the final product (Moloughney, 2013). In addition, the FDA released New Dietary Ingredient (NDI) notification regulations in 2011. For a new dietary ingredient to be marketed as such, it needs to undergo this notification process (Moloughney, 2013).

In Europe, the use of health or nutritional claims on foods is regulated by Regulation No.1924/2006 which came into effect in December 2006 (Siró *et al.*, 2008). These regulations define two types of health claims for foods: a function claim and a reduction of disease risk claim. The European Food Safety Authority (EFSA) is responsible for verifying the scientific evidence available to support food claims (Reis, 2011). Where plant food supplements (PFS) are concerned, the EFSA identified a considerable lack of knowledge in the area of product testing and efficacy and safety evaluation. To combat this, they launched the PlantLIBRA project (Plant food supplements: Levels of Intake, Benefit and Risk Assessment) which was expected to run from June 2010 - May 2014. This project aimed to develop, validate and disseminate data and methodologies for risk and benefit assessment of PFS, and to implement sustainable international cooperation (Bucchini *et al.*, 2011).

In Japan, there has been an established government regulation process for functional foods and beverages since 1991. It is called Foods for Specified Health Uses (FOSHU) (Siró *et al.*, 2008). Functional food products are a distinct group of products that are identified by the FOSHU symbol. The labelling of health claims on these foods is only possible when appropriate scientific evidence can substantiate the claim. Any manufacturer who applies to the government for approval under the FOSHU code for its product must provide all available publications and internal reports on the effectiveness of the product and/or its ingredients as including *in vitro* metabolic and biochemical studies, *in vivo* studies, and randomised controlled trials on Japanese people (Yamada *et al.*, 2008).

In South Africa, regulations regarding the health claims made on functional food products and nutraceuticals are still a work in progress. In March 2012, new regulations relating to the labelling and advertising of foodstuffs, No. R 146 of the Foodstuffs, Cosmetics

and Disinfectants Act, 1972 (more commonly known in industry as R.146) came into place. According to the South African Department of Health (D.O.H), these stringent regulations form Phase 1 which deals with the simpler aspects of food labelling such as ingredient lists and nutrient contents. Phase 2 saw the publication of draft regulations relating to the labelling and advertising of foods (R.429) by the D.O.H in May 2014. These regulations, currently still under discussion, aim to provide stricter regulations regarding the labelling and advertising of foods. As expected, the concept of nutrient profiling has been introduced as a screening mechanism to determine whether products are eligible for claims (Sunley, 2014).

Apart from regulation issues, food manufacturers will continue to be challenged to produce functional foods that are safe, affordable, convenient and tasty with substantiated health claims. Researchers still have a major role to play to support the growth in the use of functional foods and nutraceuticals. Aspects in particular that need more research is the stability of novel functional food ingredients in food systems, bioavailability and possible delivery systems (Pandey & Rizvi, 2009; Becker, 2013), and safety (Bucchini *et al.*, 2011; Egert & Rimbach, 2011).

2.3 *Cyclopia* spp. (Honeybush)

2.3.1 Background

In South Africa, the herbal tea industry is mainly comprised of the rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.) industries. Small quantities of other herbal teas, such as bush/Zulu tea (*Athrixia phylicoides*) are also consumed (Joubert *et al.*, 2008b). *Cyclopia* spp. forms part of the fynbos biome, growing in the Western and Eastern Cape provinces of South Africa. Although not as well established as the rooibos tea industry, the honeybush tea industry is rapidly expanding and there is a current struggle to meet the international demand for honeybush tea and its products (Joubert *et al.*, 2011). It developed from a very localised cottage industry product, when re-discovered in the 1990s (Du Toit *et al.*, 1998), to a global product. By 2011, it was sold in 25 different countries worldwide, the top importers being the Netherlands, Germany, United Kingdom and the United States of America (Joubert *et al.*, 2011). Most of the annual production is exported. This thriving export market has driven extensive research, conducted by the Agricultural Research Council (ARC), on the cultivation, processing and utilisation of honeybush tea to develop the industry. The growing popularity of honeybush tea worldwide is not only due to its unique taste, but also its health benefits, including antioxidant (Hubbe & Joubert, 2000; Joubert *et al.*, 2008c), antidiabetic (Muller *et al.*, 2011), antitumor (Marnewick *et al.*, 2005), antimutagenic (Marnewick *et al.*, 2000; Van der Merwe *et al.*, 2006), phytoestrogenic (Visser

et al., 2013) and anti-obesity (Dudhia *et al.*, 2013) activities. Honeybush tea is caffeine-free, low in tannins and has high polyphenolic content (McKay & Blumberg, 2007). Interestingly, the first references to this remarkable species were made as early as the 1700s. Honeybush was traditionally used for medicinal purposes such as digestive relief, laxative, sedative, to promote lactation and relieve skin irritations (McKay & Blumberg, 2007). In South Africa, the first branded honeybush tea product available on the market was 'Caspa Cyclopia Tea' in the 1960s (Joubert *et al.*, 2011).

There are 23 different species of honeybush of which six have commercial value at present (Joubert *et al.*, 2011). *Cyclopia subternata* and *C. genistoides* are the only species currently cultivated in substantial quantities (Joubert *et al.*, 2011). Small quantities of *C. intermedia* and *C. sessiliflora* are produced by cultivation. The bulk of *C. intermedia* is obtained from the wild. Cultivation trials of *C. longifolia* and *C. maculata* are still in progress (E. Joubert, Agricultural Research Council, Stellenbosch, South Africa, personal communication, 2014).

Traditionally, honeybush tea is fermented in order to be used as a herbal tea. The fermentation of the tea leaves is an oxidative process during which the leaves are exposed to temperatures above 60°C (Du Toit & Joubert, 1999). Although fermentation imparts the characteristic sweet flavour and attractive brown colour to the tea, it greatly reduces the polyphenol content (Table 2.1) and radical scavenging ability of aqueous extracts of the tea (Du Toit & Joubert, 1999; Joubert *et al.*, 2008c). It is for this reason that unfermented (green) honeybush, retaining higher levels of polyphenols (Table 2.1) and having higher antioxidant activity, was exploited as a product (Joubert *et al.*, 2011). Dried, unfermented plant material is thus the obvious choice for the production of xanthone-enriched honeybush tea extracts. In the next section the phenolic composition of *Cyclopia* spp., with the emphasis on the xanthone, mangiferin, will be discussed.

2.3.2 Phenolic composition

The phenolic composition of a number of *Cyclopia* species and their extracts has been both qualitatively and quantitatively studied (Ferreira *et al.*, 1998; Joubert *et al.*, 2003; Joubert *et al.*, 2008a; Joubert *et al.*, 2008c; Kamara *et al.*, 2003; Kamara *et al.*, 2004; De Beer & Joubert, 2010), showing interspecies variation. Major polyphenols present in all *Cyclopia* spp. to date are the xanthenes, mangiferin and isomangiferin, and the flavanone, hesperidin (Fig. 2.1). Recent investigations show that some species also contain high levels of benzophenones and dihydrochalcones (De Beer *et al.*, 2012; Schulze, 2013).

Of the species investigated to date for xanthone content of the plant material or extracts, i.e. *C. genistoides*, *C. maculata*, *C. subternata*, *C. sessiliflora* and *C. intermedia* (Joubert *et al.*, 2003; De Beer *et al.*, 2012), *C. genistoides* has the highest xanthone content

of species (Table 2.1). Variation in the xanthone content of *C. genistoides* has been shown to be dependent on harvest date, harvest interval and seed source (Joubert *et al.*, 2003; Joubert *et al.*, 2014). The study performed in 2003, which covered the period only from the end of March to mid-July, demonstrated that the levels of mangiferin in *C. genistoides* decreased progressively over this period. The most recent study confirms that mangiferin levels in *C. genistoides* leaves decrease from March to mid-July (start of autumn to mid-winter) and, most importantly, showed that summer is the best harvesting time for optimum xanthone levels (Joubert *et al.*, 2014). In addition, it was shown that plants produced from seeds sourced from the Cape Peninsula wild population as opposed to those from the West Coast and Overberg contain the highest xanthone content.

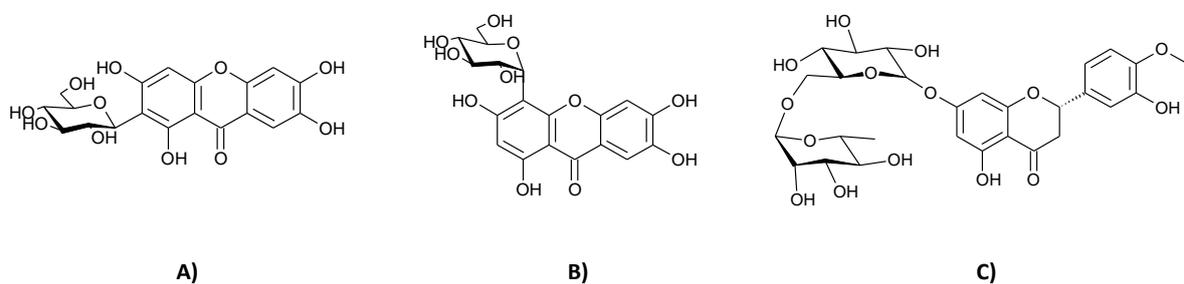


Figure 2.1 The chemical structures of the principal polyphenols in *Cyclopia* spp: mangiferin (A), isomangiferin (B) and hesperidin (C).

Table 2.1 Content (g/100 g) of major phenolic compounds in aqueous extracts of unfermented plant material of four *Cyclopia* spp. and effect of fermentation (Joubert *et al.*, 2011)

Compound	<i>C. genistoides</i>	<i>C. intermedia</i>	<i>C. sessiliflora</i>	<i>C. subternata</i>
Xanthones				
Mangiferin	9.55 ¹ (-83%) ²	4.35 (-97%)	4.67 (-87%)	2.73 (-98%)
Isomangiferin	2.72 (-59%)	1.40 (-81%)	1.69 (-54%)	0.86 (-83%)
Flavanones				
Hesperidin	0.71 (-56%)	0.62 (-47%)	0.74 (-49%)	0.62 (-61%).
Eriocitrin	Traces (-100%)	0.13 (-77%)	0.32 (-38%)	0.32 (-63%)
Eriodictyol glucoside	Nd ³	0.07 (-100%)	Nd	0.35 (-100%)
Flavone				
Scolymoside	Traces (-100%)	0.04 (-100%)	0.06 (-100%)	0.68 (-71%)

¹ average content in aqueous extract from unfermented tea; ² percentage decrease in content of extract with fermentation of plant material; ³ not detected

Initially, extract manufacturers utilised *C. subternata* (cultivated) and *C. intermedia* (wild-harvested) as they were the most abundant species available (Joubert *et al.*, 2008c). However, after the development of successful cultivation programmes of other valuable

Cyclopia species, manufacturers had a greater selection to choose from. In 2008, a study was done to determine the species with the best commercial potential based on the antioxidant activity and concentration of valuable polyphenols in both fermented and unfermented aqueous extracts. Species investigated were *C. genistoides*, *C. subternata*, *C. intermedia* and *C. sessiliflora* (Joubert *et al.*, 2008c). It was found that aqueous extracts of unfermented *C. genistoides* contained substantially more mangiferin (10.04%) than the other species tested. It also contained the highest isomangiferin content (1.73%) (Joubert *et al.*, 2008c). Isomangiferin is a regio-isomer of mangiferin, with the glucose moiety attached to R₁ instead of R₂ (Fig. 2.1). Currently, extract manufacturers are preparing extracts with a standardised mangiferin content from unfermented honeybush plant material. As extracts are still predominantly produced from *C. intermedia* and *C. subternata*, due to their availability, the standardised mangiferin content is low (<1%).

For the production of a xanthone-enriched extract, unfermented *C. genistoides* has been identified as the best species to use due to its high mangiferin content and its easy cultivation, making it a sustainable source of mangiferin (Joubert *et al.*, 2003; Joubert *et al.*, 2008c).

2.3.3 Mangiferin

Mangiferin was the first xanthone to be investigated for pharmacological purposes (Pinto *et al.*, 2005). Recent review papers provide a detailed summary of the bioactivities of mangiferin (Wauthoz *et al.*, 2007; Vyas *et al.*, 2012). Table 2.2 provides a list of bioactivities of mangiferin.

In India, mangiferin is recommended for the treatment of diseases such as arthritis, diabetes, hepatitis as well as cardiac and mental disorders (Sánchez *et al.*, 2000). Mangiferin has also been included in medical foods for the treatment of diabetes (Wada, 2007), as well as neurodegenerative diseases and aging symptoms (Matute *et al.*, 2007).

There are only a few mangiferin-containing supplements that are currently available on the international market. In Cuba, Vimang[®], a standardised aqueous extract made from mango stem bark, is used as an antioxidant nutritional supplement (Núñez-Sellés *et al.*, 2002; Mishra *et al.*, 2006). It contains 10-20% mangiferin, which is the main component of the supplement (Sánchez *et al.*, 2000; Núñez-Sellés *et al.*, 2002; Garrido *et al.*, 2004; Rodriguez *et al.*, 2006). Vimang[®] has been proven to be more active in preventing the production of reactive oxygen species (ROS) and oxidative damage in vivo than vitamin C, vitamin E and β - carotene (Sánchez *et al.*, 2000). Vimang[®] has been used in a clinical trial where its supplementation improves antioxidant status and reduced oxidative damage in HIV positive patients (Gil *et al.*, 2002). In India and Sri Lanka, Salaretin[®], a standardised extract

of *Salacia reticulata* containing ca 1% mangiferin (Anon., 2002), is used as an antidiabetic supplement and to treat obesity (Singh *et al.*, 2012).

Alternatives to mango tree bark as source of mangiferin are sought with the emphasis on mango by-products (peel and seed; Dorta *et al.*, 2014) and mango leaves (Ling *et al.*, 2009; Fernández-Ponce *et al.*, 2012; Zou *et al.*, 2013; Zou *et al.*, 2014). Given that the mangiferin content of mango leaves is ca 3.6% (Zou *et al.*, 2013), *Cyclopia* species, and in particular *C. genistoides* with similar levels in the plant material (Joubert *et al.*, 2003) and in excess of 7% in the leaves (Joubert *et al.*, 2014), offers a good source of this xanthone.

The focus on mangiferin as a bioactive constituent of *Cyclopia* extracts is merited given its relative antioxidant activity and contribution to the total antioxidant capacity of the extracts. Mangiferin has been shown to be one of the most active antioxidants of the *Cyclopia* polyphenols tested when evaluated in different antioxidant assays (Hubbe & Joubert, 2000; Joubert *et al.*, 2008c). It makes a substantial contribution to the antioxidant activity of *Cyclopia* extracts due to their high mangiferin contents (Joubert *et al.*, 2008a).

Table 2.2 Bioactivities of mangiferin

Bioactivity	References
Anti-allergic	Pinto <i>et al.</i> , 2005, Rivera <i>et al.</i> , 2006
Anti-diabetic	Miura <i>et al.</i> , 2001;
Anti-obesity	Zhang <i>et al.</i> , 2011a; Lim <i>et al.</i> , 2014
Antimutagenic	Hubbe & Joubert, 2000; van der Merwe <i>et al.</i> , 2006
Anti-tumour	Yoshimi <i>et al.</i> , 2001, Guha <i>et al.</i> , 1996
Anti-viral	Mishra <i>et al.</i> , 2006, Yoosook <i>et al.</i> , 2000
Analgesic	Dar <i>et al.</i> , 2005
Immunomodulator	Núñez-Sellés <i>et al.</i> , 2007; Mishra <i>et al.</i> , 2006
Antioxidant	Sato <i>et al.</i> , 1992; Dar <i>et al.</i> , 2005, Mishra <i>et al.</i> , 2006
Hepatoprotective	Das <i>et al.</i> , 2012
Gastro-protective	Carvalho <i>et al.</i> , 2007
Lipid peroxidase inhibition	Ling <i>et al.</i> , 2009; Pardo-Andreu <i>et al.</i> , 2005
Enhancement of cognitive memory	Pardo-Andreu <i>et al.</i> , 2010
DNA protection	Rodeiro <i>et al.</i> , 2012
Laxative	Ito <i>et al.</i> , 2012

2.3.4 Potential of a mangiferin-enriched *Cyclopia* extract as a functional food ingredient

It is now well known that foods and beverages rich in phenolic compounds have been associated with a reduced risk of several diseases. Consumers are making smarter food choices and are likely to choose healthier versions of the products they enjoy. This allows them to improve their diet without drastically changing it (Arvanitoyannis & Van Houwelingen-Koukaliaroglou, 2005). For example, popular ready-to-drink tea beverages (iced tea) could be enhanced by the addition of a polyphenol-enriched extract. Since consumers already

enjoy this type of beverage, they would consider buying a functional alternative, provided the price is reasonable and the product still tastes good (Becker, 2013).

Mangiferin ticks various boxes when it comes to its applicability as a functional food ingredient. Not only does it encompass many health benefits (Table 2.2), but is also non-toxic at high levels. In an acute test administration of 400 mg mangiferin per kg body weight was required to induce 50% mortality in mice (Jagetia & Baliga, 2005). A mangiferin-enriched extract could either be incorporated into a product that promotes general well being, or it can be marketed for a specific bioactivity, for example its antidiabetic activity (Miura *et al.*, 2001). Use of *Cyclopia* species, and more specifically *C. genistoides*, to produce such a mangiferin-enriched extract, has the benefit of the presence of several other phenolic constituents that could enhance the beneficial effects of the extract attributed to mangiferin. The inhibitory effect of the benzophenone C-glucosides and mangiferin on triglyceride accumulation in 3T3-L1 adipocytes (Zhang *et al.*, 2011a) indicated the potential for development of an anti-diabetes nutraceutical. Diabetes is the term used to refer to obesity-induced diabetes (Tharakan *et al.*, 2011). High levels of hesperidin in *Cyclopia* species and thus their extracts may also be beneficial as Bartoszewski *et al.* (2014) showed that mangiferin has an additive effect on the apoptotic properties of hesperidin in *Cyclopia* extracts.

Other uses for mangiferin or the mangiferin-enriched extract are not excluded, given the various applications for polyphenols in food. Green tea catechins, for example, have been proven to inhibit lipid oxidation in red meat, poultry and fish (Tang *et al.*, 2001) and various food grade oils (Chen & Chan, 1996; Chu & Hsu, 1999; O'Sullivan *et al.*, 2005).

Another application of green tea extract that is highly relevant from a food safety aspect is that it reduces acrylamide formation during heating (Zhang & Zhang, 2008). Flavone-C-glycosides of rooibos have been applied as colour enhancers and stabilizing agents in juice blends and beverages rich in cyanidin glycosides (Pacheco-Palencia & Talcott, 2010).

2.4 Extraction of bioactive compounds

The development in extraction techniques to obtain valuable compounds from plant materials and food processing waste is driven by an increasing interest in and realisation of the value of functional food ingredients (Toledo, 2007). Green tea and grape seed extracts are good examples of such ingredients. Their widespread usage is due to the fact that they are an excellent source of monomeric polyphenols that can be efficiently extracted at a low cost (Shi *et al.*, 2005). It is for this reason that the nutraceutical industry has focused a great deal of energy on optimising extraction procedures for green tea leaves and grape pomace, skins and seeds (Shi *et al.*, 2005). However, as the value and competitiveness of this industry

becomes clear, researchers and food manufacturers are compelled to investigate novel plant sources and different extraction techniques of bioactive compounds for the eventual production of value-added extracts/functional food ingredients. The following sections will address common extraction techniques of polyphenols from plant material and factors that affect extraction efficiency. A section will be dedicated specifically to the extraction of mangiferin. Given the movement towards 'green' processing, extraction techniques that would use less solvent and energy will also be highlighted.

2.4.1 Green extraction of natural products

In the food industry, extraction processes can be extremely energy intensive such as the extraction processes of sugar beet and sugar cane and the preparation of decaffeinated tea and coffee (Chemat *et al.*, 2012). It is common for an extraction process to require large quantities of solvents, energy and time to achieve a poor yield. As the environmental impact of extracting natural compounds becomes evident, there has been a great movement towards the development of more eco-friendly technologies.

The concept of 'green' chemistry and engineering was established in order to develop more efficient and environmentally friendly processes (Clark, 2011). In the food and nutraceutical industries, green chemistry can be divided into two broad categories. The first is to develop technologies that convert agricultural and food co-products/wastes into valuable resources such as biofuels and food ingredients, i.e. ensuring that 'today's waste is tomorrow's resource' (Clark, 2011; Chemat *et al.*, 2012). In this regard, extraction either on its own or in combination with subsequent separation methods have been used to improve the value of processing by-products such as olive leaves (Ahmad-Qasem *et al.*, 2013), olive waste waters (Russo, 2007; Cassano *et al.*, 2011), grape pomace (Díaz-Reinoso *et al.*, 2009), grape seeds (Santamaría *et al.*, 2002; Nawaz *et al.*, 2006), almond skins (Prodanov *et al.*, 2008) mango seeds and peels (Dorta *et al.*, 2012) and coconut shells (Rodrigues *et al.*, 2008).

The second concept of 'green' chemistry involves improving the sustainability of existing techniques by reducing the use of solvents derived from fossil fuels as well as their carbon footprint in terms of energy (Clark, 2011). When extracting natural products, there are a few important considerations to ensure the final product is destined to be natural and 'green'. Plant sources should be renewable (i.e. those that are being successfully cultivated) in order to prevent population depletion and fairly accessible to prevent excessive transport costs. Solvent-free techniques should be investigated or the use of greener solvents such as water or organic solvents that naturally biodegrade. The recent discovery of natural ionic liquids and deep eutectic solvents (NADES) composed of natural compounds is believed to offer many advantages that suggest their potential as green solvents for extraction.

Advantages associated with NADES are their sustainability, biodegradability, acceptable pharmaceutical toxicity profiles, and high solubilisation power of both polar and nonpolar compounds (Du *et al.*, 2009; Dai *et al.*, 2013a; Paiva *et al.*, 2014). NADES have recently been used for the extraction of polyphenols from green tea (Zhang *et al.*, 2014), green coffee beans (Paiva *et al.*, 2014), safflower (Dai *et al.*, 2013b) and *Chamaecyparis obtusa* leaves (Bi *et al.*, 2013). Although NADES show promising potential for the extraction of bioactive compounds from plant material, it is an area of research still in its infancy. The final extract produced should be free of contaminants and adhere to the applicable regulations, such as the ICH guidelines (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) which states the limits for residual solvents remaining in a product after manufacture according to their toxicity level (Puranik *et al.*, 2009; Chemat *et al.*, 2012). If a green extract is to be produced, it is important to not only look at the process itself, but also at the environmental impact of the whole supply chain, i.e. the life cycle analysis. This includes the growth and harvesting of plant material, the fate of the by-products/waste produced and the biodegradability of the products obtained (Mason *et al.*, 2011).

Energy consumption in the food industry is costly and large losses can occur throughout the food manufacturing process due to poor process design. Ideally, a system should be designed such that the maximum amount of work can be achieved using minimal energy consumption (heat and electricity). Here, thermodynamics plays a key role in analysing the energy transfers taking place at different unit operations as well as possibilities for heat exchange throughout the production process (Dincer & Cengel, 2001). One of the most energy-intensive processes in the food and pharmaceutical industry today is drying, accounting for between 15-20% of the total energy used in industry (Gilmour *et al.*, 2004). Drying is an important preservation technique for food ingredient and pharmaceutical products that also allows for reduced transports costs and increased consumer convenience, e.g. powdered milk (Djaeni *et al.*, 2013).

Reducing energy consumption throughout an extract manufacturing process can be approached in several different ways (Fig. 2.2). It can be minimised by avoiding unnecessary energy usage, recovery of liberated energy and recycling solvents when possible, optimisation or intensification of existing extraction processes, for example using microwave or ultrasound assisted extraction and ensuring proper equipment maintenance (Chemat *et al.*, 2012; Grobler, 2013). Ideally, the extraction process should consist of as few processing steps as possible. The greatest energy savings (up to 60%) can be made by replacing existing, energy intensive techniques with different, more sustainable technologies (Grobler, 2013). Techniques such as pinch analysis (Brouckaert & Buckley, 2000; Kemp 2011) and exergy analysis (Van Gool, 1992; Dincer & Cengel, 2001; Colak *et al.*, 2010) can be used to

identify where energy can be recovered and where the largest energy losses occur in a system, respectively. These analyses allow for the optimal utilisation of a resource or raw material in terms of energy consumption, which does not always translate to maximum productivity (Dincer & Cengel, 2001).

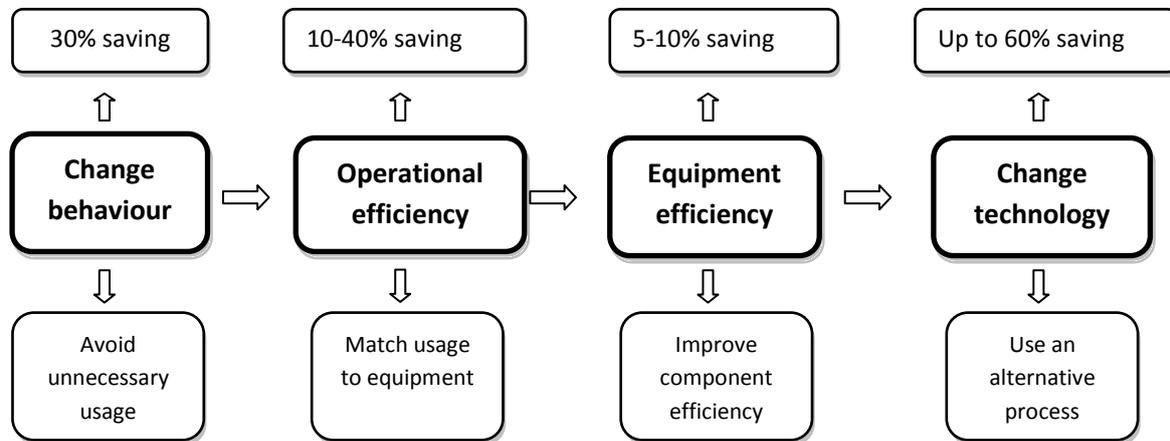


Figure 2.2 Different approaches to energy reduction in the food industry (Grobler, 2013).

2.4.2 Solid-liquid extractions

By definition, a solid-liquid extraction is the use of a liquid to selectively dissolve and remove a soluble fraction (the solute) from the insoluble, permeable solid matrix (Gertenbach, 2002; Takeuchi *et al.*, 2009). Plants consist of a network of passageways that allow the transport of nutrients and water through the plant whilst it is alive. Thus once dead, the plant material has a porous structure that facilitates solvent penetration and solute removal (Gertenbach, 2002). Solid-liquid extractions are encountered in everyday life, for example each time a cup of tea or coffee is made solid-liquid extraction takes place, and find many applications in the food and pharmaceutical industries. There is an increasing interest in the study of different extraction techniques of polyphenols from plant sources as the value of these compounds has become clear.

2.4.2.1 Novel vs. traditional techniques

Traditional methods of solid-liquid extraction include Soxhlet extraction, hydrodistillation and maceration with alcohol (Azmir *et al.*, 2013; Kadam *et al.*, 2013; Veggi *et al.*, 2013). These techniques are based on the extracting power of different solvents and the application of heat and/or mixing. The efficiency of these techniques relies predominantly on the choice of solvent, which is determined by the polarity of the target compounds (Azmir *et al.*, 2013). The use of conventional extraction techniques are often limited by various drawbacks, including overheating of the food matrix, high energy and solvent consumption, time consuming operation, reduction of bioactivity of the target compounds, poor extraction selectivity and the

use of harsh chemicals (Galanakis, 2012; Azmir *et al.*, 2013, Lai *et al.*, 2013). In addition, the large quantity of solvent used will need to be removed in downstream processing either before or during drying of the final product.

There has been increased research into alternative, novel extraction techniques of bioactive polyphenols from plant sources due to their economic and environmental advantages, such as reduced operational times, lower solvent and chemical requirements, increased yields and improved selectivity (as reviewed by Wang & Weller, 2006; Wijngaard *et al.*, 2012; Azmir *et al.*, 2013; Shah & Rohit, 2013). Ultrasound-assisted extraction (UAE) uses sound waves (20 kHz – 100 MHz) to induce cavitation in liquid medium which disrupts the plants cells, allowing easier penetration of solvent and recovery of target compounds (Wang & Weller, 2006; Shah & Rohit, 2013). However, ultrasound energy may have a deleterious effect on the active constituents of some medicinal plants due to free radical formation (Kawamura *et al.*, 1999). Microwave assisted extraction (MAE) works on a similar principle to UAE. Microwaves, in the form of electromagnetic radiation (300 MHz to 300 GHz), penetrate plant material and disrupt the weak hydrogen bonds in polar molecules due to dipole rotation of these molecules (e.g. water) (Wang & Weller, 2006; Shah & Rohit, 2013). Again, this facilitates the release of target compounds into the surrounding solvent. The disadvantages of MAE are that the efficiency is reduced when the target molecules are non-polar or volatile and additional filtration/centrifugation steps may be required to remove solid residue (Shah & Rohit, 2013). Enzyme-assisted extraction (EAE) is employed when the target bioactive compounds need to be released from the polysaccharide-lignin network to which they are bound (Azmir *et al.*, 2013). Plant material is pre-treated with enzymes such as cellulase, α -amylase and pectinase which break cell wall material and hydrolyse lipids and structural polysaccharides (Rosenthal *et al.*, 1996). Some of the limitations of EAE are that it is relatively expensive for processing large volumes of raw material, scaling up to industrial scale is complicated as the environmental conditions have a huge impact on enzyme functionality and that the extraction yields depend on the extent to which the enzyme can hydrolyze the plant material (Puri *et al.*, 2012). Supercritical fluid extraction (SFE) uses a solvent above its critical temperature and pressure. At these conditions, the solvent is in one phase but has the properties of both a gas and a liquid thereby increasing its solvation power (Wijngaard *et al.*, 2012; Azmir *et al.*, 2013). Carbon dioxide (CO₂) is the most common solvent used to extract non-polar molecules due to its low critical temperature (31.1°C) and pressure (7.4 MPa), safety, food-grade status and easy accessibility (Wijngaard *et al.*, 2012; Azmir *et al.*, 2013). Due to the low polarity of CO₂, the addition of a co-solvent such as ethanol (EtOH) or a chemical modifier is required to extract polar molecules (Azmir *et al.*, 2013). Although SFE is an attractive alternative for extracting heat sensitive compounds, the high cost and time-consuming operating conditions limits its application (Wijngaard *et al.*,

2012; Azmir *et al.*, 2013). In pulsed-electric field extraction (PEF), plant material is suspended between two electrodes and a transmembrane potential is created. This results in repulsion between charged molecules within the cells forming pores in weak areas of the membrane thereby increasing its permeability allowing easier extraction of target molecules (Wijngaard *et al.*, 2012; Azmir *et al.*, 2013). PEF is suitable for heat sensitive compounds as a moderate electric field can cause substantial membrane damage without a large increase in temperature (Azmir *et al.*, 2013). Drawbacks of this technique are that industrial-scale equipment is still in development stage and that it does not work with solid products (Han, 2007).

2.4.2.2 The extraction of mangiferin

The extraction of mangiferin (Table 2.3) has been more extensively studied than that of its regio-isomer, isomangiferin (Cafferty *et al.*, 1996; Berardini *et al.*, 2005; Barreto *et al.*, 2008; De Beer *et al.*, 2009; De Beer *et al.*, 2012). Mangiferin, a glycoside, is found in varying concentrations in a variety of plant families, including species of *Aquilaria* (Ito *et al.*, 2012), *Coffea* (Campa *et al.*, 2012), *Cyclopia*, *Mangifera*, *Phaleria*, *Salacia* and *Swertia* (as reviewed by Vyas *et al.*, 2012). The most abundant source of mangiferin is mango (*Mangifera indica* L.). Mangiferin is found in the peels, pulp and seeds of the fruit as well as in the leaves and bark of this plant. As xanthenes are secondary metabolites, they are found in higher concentrations in mango fruit peels, leaves and bark as their function is to help combat both biotic and abiotic stress (Luo *et al.*, 2012). Although the leaves are the best source of mangiferin, it is only recently that this source has been investigated (Ling *et al.*, 2009; Fernández-Ponce *et al.*, 2012; Bhuvanewari, 2013; Zou *et al.*, 2013; Zou *et al.*, 2014). Previously, the focus was on mango tree bark (Núñez-Sellés *et al.*, 2002; Garcia *et al.*, 2003; Garrido *et al.*, 2004; Wauthoz *et al.*, 2007).

Organic solvent extraction is the simplest extraction technique used for mangiferin (Joubert *et al.*, 2006; Barreto *et al.*, 2008; Ribeiro *et al.*, 2008; Ling *et al.*, 2009; Ito *et al.*, 2012), with increasing research being conducted on novel techniques such as SFE (Fernández-Ponce *et al.*, 2012; Prado *et al.*, 2013), MAE (Dorta *et al.*, 2013; Zou *et al.*, 2013; Kullu *et al.*, 2013; Ruiz-Montañez *et al.*, 2014), UAE (Joubert *et al.*, 2003; Kulkarni & Rathod, 2014; Ruiz-Montañez *et al.*, 2014; Zou *et al.*, 2014) and subcritical water extraction (SWE) (Kim *et al.*, 2010). Novel extraction techniques improve the mangiferin yield due to the addition of physical action upon the plant material which promotes the loss of compartmentalisation of the plant matrix and enhances extraction efficiency (Ruiz-Montañez *et al.*, 2014). Where mangiferin isolation or purification is required for further *in vitro* and *in vivo* testing, more exhaustive extraction techniques are typically employed coupled with subsequent partitioning techniques. Bhuvanewari (2013) isolated mangiferin from

Mangifera indica L. leaves by first removing fat from the samples with petroleum ether, followed by Soxhlet extraction with ethanol (EtOH) for 21 hours and subsequent organic solvent partitioning to obtain a yield of 1.2%. Luo *et al.* (2012) purified mangiferin extracted from the peels, pulp and seed kernels of different Chinese mango cultivars for testing in human umbilical vein endothelial cells. The mangiferin was extracted using organic solvent and purified in a two step process using macro-porous resin adsorption followed by high speed counter current chromatography (HSCCC). After starting with 20 g plant material, the final mangiferin recovery after HSCC was 74.3 mg with a purity of 99.1%. Kokotkiewicz *et al.* (2013) isolated xanthone and benzophenone derivatives from unfermented *Cyclopia genistoides* for subsequent testing in synovial cells from patients with rheumatoid arthritis. The phenolic compounds were extracted with methanol (MeOH) under reflux and mangiferin was obtained via multi-stage liquid-liquid partitioning using water, chloroform, ethyl-acetate and MeOH. From 100 g dried plant material, 850 mg mangiferin was obtained.

Table 2.3 Different extraction techniques of mangiferin from different plant sources for quantitative analysis

Plant species	Common name	Plant material ¹	Extraction Technique ²	Mangiferin Yield ³	Reference
<i>Mangifera indica</i> L.	Mango	Fruit peel	Acid-assisted extraction, resin adsorption, MeOH elution	11.2-1297.1 mg/kg DM	Berardini <i>et al.</i> , 2005
<i>Mangifera indica</i> L.	Mango	Fruit peel, seed kernels, bark, young leaves, old leaves	Soxhlet extraction with hexane, followed by MeOH	4.94-67.20 g/kg DM	Barreto <i>et al.</i> , 2008
<i>Mangifera indica</i> L.	Mango	Fruit pulp and peels, seed kernels	60% MeOH extraction	2.2-199 mg/kg DM	Ribeiro <i>et al.</i> , 2008
<i>Mangifera indica</i> L.	Mango	Leaves	EtOH extraction Aqueous extraction	9.1 g/100 g extract 7.1 g/100 g extract	Ling <i>et al.</i> , 2009
<i>Mangifera indica</i> L.	Mango	Leaves	SFE with CO ₂ and 20% EtOH/MeOH SWE	7.8-191.8 mg/100 g DM 1365.9 mg/100 g DM	Fernández-Ponce <i>et al.</i> , 2012
<i>Mangifera indica</i> L.	Mango	Leaves	LPSE with ethanol SFE with CO ₂	9.3 g/ 100 g DM 3.6 g/100 g DM	Prado <i>et al.</i> , 2013
<i>Mangifera indica</i> L.	Mango	Leaves	Aqueous UAE ⁷	31.1 mg/g ⁴	Kulkarni & Rathod, 2014
<i>Mangifera indica</i> L.	Mango	Leaves	MAE with 45% EtOH, 40% EtOH extraction	36.10 mg/g DM 15.24-25.17 mg/g DM	Zou <i>et al.</i> , 2013
<i>Mangifera indica</i> L.	Mango	Leaves	UAE with EtOH (20-60%)	47.12-58.06 mg/g DM	Zou <i>et al.</i> , 2014
<i>Phaleria macrocarpa</i>	Makhota Dewa	Fruit peel	SWE	2.17 mg/g sample	Kim <i>et al.</i> , 2010
<i>Aquilaria sinensis</i>	Agarwood	Leaves	EtOH extraction (20 – 100%) Aqueous extraction	4.06-5.97 mg/g extract 0.83-6.85 mg/g extract	Ito <i>et al.</i> , 2012

Plant species	Common name	Plant material	Extraction Technique	Mangiferin Yield	Reference
<i>Curcuma amada</i>	Mango ginger	Peeled rhizome	MAE with 80% EtOH	1.1156 mg/g ⁴	Kullu <i>et al.</i> , 2013
<i>Cyclopia intermedia</i> <i>Cyclopia maculata</i> <i>Cyclopia sessiliflora</i> <i>Cyclopia genistoides</i>	Honeybush	Shoots	UAE with 100% MeOH	1.691 g/100 g DM 1,626 g/100 g DM 1.040 g/100 g DM 3.607 g/100 g DM	Joubert <i>et al.</i> , 2003
<i>Cyclopia genistoides</i>	Honeybush	Shoots	MeOH extraction	0.7-7.21 g/100g PM	Joubert <i>et al.</i> , 2006
<i>Cyclopia subternata</i>	Honeybush	Stems Leaves	Aqueous extraction	0.373 g/100 g extract 0.817 g/100 g extract	De Beer <i>et al.</i> , 2012
<i>Cyclopia subternata</i>	Honeybush	Shoots	Organic solvent extraction	0.5-1.5 g/100 g PM	Joubert <i>et al.</i> , 2012

¹ Shoots = stems and leaves combined; ² MeOH = methanol; EtOH = ethanol; SFE = supercritical fluid extraction; CO₂ = carbon dioxide; SWE = subcritical water extraction; UAE = ultrasound assisted extraction, ³ DM = dry matter; PM = plant material; ⁴ Unit not specified

2.4.2.3 Extraction kinetics

Initially, the main purpose of studying the extraction of tea polyphenols was their role in the sensory quality of the tea due to the astringent and bitter sensory attributes associated with catechins and their oxidation products (Tounekti *et al.*, 2013). As the health benefits of tea polyphenols became clearer, the emphasis shifted to maximum recovery of bioactive phytochemicals and their possible food, cosmetic and pharmaceutical applications. For the extraction of phytochemicals to be economical, the largest possible quantity of target compounds should be removed from the plant material as quickly as possible. In order to perform such an extraction, an understanding of the kinetics involved is vital (Gertenbach, 2002).

The first important concept is equilibrium. When a solvent is added to plant material, an equilibrium concentration relationship develops between the solutes within plant material and the solutes dissolved in the solvent (Gertenbach, 2002). This relationship is present below:

$$K = \frac{C_e}{C_{dm}} \quad (2.1)$$

where K represents the equilibrium constant, C_e is the concentration of a given compound in the solvent and C_{dm} is the concentration of a given compound in the dry plant material. Ideally, K should be as large as possible as this indicates that a large quantity of the given compound will dissolve into the solvent. K is a function of the temperature and the type of solvent used, as well as the original content of the given compound in the plant material (Gertenbach, 2002). Equilibrium describes how much of a given compound will dissolve. Mass transfer describes the rate of dissolution of the given compound and how long it will take to reach the equilibrium concentration (Gertenbach, 2002).

The extraction of solutes from the plant particles to the bulk solution involves four stages. First the solvent penetrates and diffuses into the solid particles. The target compounds then dissolve into the solvent within the particle. Once dissolved, the target compounds diffuse through the plant matrix to the particle surface. Finally, the solute-rich solvent that builds up near the particle surface moves back into the bulk solvent (Gertenbach, 2002).

Previous studies established a first-order diffusion mechanism based on Fick's Law for the aqueous extraction of green and black teas (Spiro & Siddique, 1981; Spiro & Jago, 1982; Price & Spitzer, 1993; Price & Spitzer, 1994). It was shown that polyphenol extraction was increased with reduced tea particle size, longer steeping time, higher temperature and greater tea leaf amounts per volume of water. In addition, first order rate plots of kinetic data

were used to prove that the rate limiting step for the extraction of polyphenols from tea is the diffusion of constituents within the leaf.

This internal diffusion, therefore, determines the efficiency of the entire process and can be described according to Fick's Second Law (Fick, 1855) which states:

$$\frac{\delta C}{\delta t} = D \frac{\delta^2 C}{\delta x^2} \quad (2.2)$$

where C is the concentration of the solute or marker compound, t is time, D is the coefficient of diffusivity and x is the particle diameter. From this relationship, it is evident that the driving force for extraction is the concentration gradient between the amount of target compounds inside the plant particle and the amount at its surface. The extraction rate, $\frac{\delta C}{\delta t}$, can be increased by a larger concentration gradient or a smaller particle diameter (Gertenbach, 2002).

Many researchers have used a first-order mathematical model derived from Fick's Second Law to describe the solid-liquid extraction kinetics of polyphenols from various plant sources, including protopine extraction from *Fumaria officinalis* L. (Rakotondramasy-Rabesiaka *et al.*, 2010), polyphenol extraction from Jatoba (*Hymenaea courbaril* L. var *stilbocarpa*) bark (Veggi *et al.*, 2013), *Syzygium aromaticum* (cloves) and *Cinnamomum cassia* (cinnamon) (Radha Krishnan *et al.*, 2013), extraction of resveratrol and other phenolic compounds from milled grape canes (*Vitis vinifera*) (Karacabey & Mazza, 2008) and ethanolic extraction of soluble substances from rosehip seeds (*Rosa rubiginosa*) (Franco *et al.*, 2007).

2.4.2.4 Factors that influence extraction

Extraction parameters are chosen such that the equilibrium constant (K) and the extraction rate are as large as possible, resulting in an economical extraction process. From Equations 2.1 and 2.2, it is evident that there are four main factors that influence extraction:

Solvent composition

The type of solvent used will determine both the quantity and speed of extraction of the target compounds based on polarity. Mangiferin is typically extracted using hydro-alcoholic mixtures due to its low solubility in aqueous media (Wang *et al.*, 2007). By adding EtOH to water, the polarity is reduced allowing the extraction of less polar molecules according to the 'like dissolves like' concept (Cheok *et al.*, 2012; Tabaraki *et al.*, 2012). Water facilitates solvent penetration as it causes the plant material to swell, allowing an easier release of the target compounds (Gertenbach, 2002; Takeuchi *et al.*, 2009; Galanakis, 2012). For the

production of a food ingredient extract, an EtOH-water mixture has the advantages of being safe to work with, non-toxic, relatively cheap and has proven efficacy in extracting mangiferin from different plant sources (Ito *et al.*, 2012; Joubert *et al.*, 2012; Zou *et al.*, 2013, Zou *et al.*, 2014).

Temperature

The concentration equilibrium and extraction rate are both increased by higher temperatures, which improves the solubility of the target compound into the solvent. The temperatures used during extraction are limited by the boiling point of the solvent as well as the thermal stability of the target compounds (Gertenbach, 2002; Pinelo *et al.*, 2005; Takeuchi *et al.*, 2009). Examples are the formation of novel compounds during the extraction of grape by-products as a consequence of polyphenol polymerisation (Pinelo *et al.*, 2005) and epimerisation of flavan-3-ols (Wang & Helliwell, 2000). Mangiferin is typically extracted at temperatures below 100°C and has an anhydrous boiling point of 271°C (Vyas *et al.*, 2012).

Particle size

As is evident in Equation 2.2, a reduced particle size will cause an increase in extraction rate due to a shorter diffusion path within the plant matrix (Gertenbach, 2002; Takeuchi *et al.*, 2009). Industrially, it is important to ensure that the plant material is not ground too fine as it can cause blockage of the extraction equipment or increase the difficulty of filtering.

Solvent:solid ratio

Here, a compromise needs to be made. A higher solvent:solid ratio will increase the extraction rate due to an increased concentration gradient. However, a more dilute extract will require more intensive treatment further down the production line due to large volumes of solvent that must be removed (Gertenbach, 2002).

Apart from these four core parameters, the mechanical action experienced during extraction (such as pressure, shaking, stirring and more recently the exposure to ultrasound and microwaves) also has an effect on extraction. Any action that disrupts the cell membranes and facilitates the penetration of the solvent and subsequent dissolution of the target compounds improves the extraction efficiency (Gertenbach, 2002; Wang & Weller, 2006; Shah & Rohit, 2013).

2.5 Ultrafiltration

The first synthetic nitrocellulose ultrafiltration (UF) membrane was developed in 1906 by Bechhold (Baker, 2012). He was also the creator of the bubble test, a non-destructive technique used to measure the pore size of microporous membranes (Cheryan, 1998; Baker, 2012). It was Bechhold who came up with the phrase 'ultrafilter' (Baker, 2012). By the mid 1920s, nitrocellulose UF and microfiltration (MF) membranes had become more commonplace in laboratory applications (Baker, 2012). During World War II, membranes played an important role in determining the microbiological safety of water that had high contamination risks due to the severe destruction of cities caused by air raids (Cuperus & Smolders, 1991).

Up until the 1960s, membranes were predominantly used for isotope enrichments and in artificial kidneys (Cuperus & Smolders, 1991). They also had small scale laboratory applications in academic and medical fields. Their poor permeability performance limited their use in industrial applications, and it was only later in 1963, when Loeb and Sourirajan developed the first cellulose acetate anisotropic membrane, that this problem was resolved (Cuperus & Smolders, 1991; Baker, 2012). An anisotropic membrane is an asymmetric membrane that consists of a thin skin layer with tight pores (responsible for an effective separation) above a porous sublayer with wider pores which provides the mechanical strength and stability to the membrane. A phase-inversion process, named the Loeb-Sourirajan method in their honour, was developed and allowed a variety of porous and non-porous anisotropic membranes to be made from a wide range of polymers, the most popular being cellulose acetate (Cuperus & Smolders, 1991; Kayet & Matsuuri, 2011; Baker, 2012). This new membrane configuration dramatically improved the separation efficiency of UF membranes, which soon after became utilised on an industrial scale (Baker, 2012). In 1970, the first cheese whey ultrafiltration system was installed. The earliest UF systems consisted either of tubular or plate and frame modules. As the industry grew, the equipment became more cost effective to install. The UF industry then experienced massive growth and had become a well-established industry by the mid 1990s (Li & Chase, 2010; Baker, 2012).

Traditional separation techniques include evaporation, ion exchange, resin adsorption and solvent extraction. These methods can be tedious, inefficient and expensive with the additional complication of removing the added chemicals and traces of organic solvents used (Chhaya *et al.*, 2012). Membrane separations are an attractive alternative as they are performed under mild temperature and pressure conditions and do not involve any phase transitions, making them suitable for the separation and concentration of heat-sensitive, natural, bioactive compounds (Sun *et al.*, 2011). Other green advantages include low energy consumption, high efficiency, simple operation, affordable capital costs, easy upscaling and

minimal chemical waste as no extra solvent is required (Amanda *et al.*, 2000; Sun *et al.*, 2011; Ruby-Figueroa *et al.*, 2012). The main disadvantage of membrane processing is a phenomenon known as membrane fouling. This is discussed in detail in section 2.5.7. Excessive pre-treatment required by more sensitive membranes is another disadvantage and is dependent on the type of membrane used (Ruby-Figueroa *et al.*, 2012).

2.5.1 Principles of membrane processing

All membrane separation processes are based on the principle of selective permeability due to size exclusion – smaller compounds can permeate freely through the membrane whilst larger compounds are retained. Membrane separations are pressure-driven processes and are differentiated by the size and nature of the molecules they are able to separate (Fig. 2.3). The compounds retained by the membrane are collectively known as the retentate and those that are small enough to pass through the membrane, the permeate. Membrane separations can be classified into MF, UF, nanofiltration (NF), reverse osmosis (RO), dialysis, electrodialysis, pervaporation, gas permeation and membrane distillation (Cheryan, 1998; Li & Chase, 2010). The predominant processes used in industry are MF, UF, NF and RO (Sun *et al.*, 2011).

MF is used mainly as a clarification and sterilisation technique for various feed liquids as it removes suspended solids, bacterial skeletons and impurities from a solution (Cheryan, 1998; Wagner, 2001). It is a useful pre-treatment for subsequent membrane filtration techniques as it reduces ‘clogging’ of the membrane by removing the larger particles. UF allows the separation of high molecular weight (HMW) compounds, such as proteins and pectins, from low molecular weight compounds (LMW) such as sugars, salts and amino acids (Wagner, 2001). When dealing with the separation of components in natural plant extracts, UF is often used due to the mild nature of the process and the ability to separate HMW and LMW molecules (Pap *et al.*, 2012). UF is a pressure driven separation technique that allows better preservation of sensitive compounds. UF membranes are not classified according to their nominal pore size, but rather by molecular weight cut off (MWCO) expressed in Daltons (Da). This indicates that 90% of molecules with a molecular weight equal to or greater than the MWCO will be retained by the membrane (Cheryan, 1998; Shi *et al.*, 2005). UF membranes are capable of retaining compounds in the range 30-10,000,000 Da (Charcosset, 2012).

NF uses charged membranes to separate monovalent from multivalent ions in solution. It can also separate uncharged, dissolved molecules depending on their shape and size and the feed concentration (Wagner, 2001; Galanakis, 2012). NF membranes have a cut off value between UF and RO. RO is an effective concentration technique that removes water

from a solution, i.e. it performs a liquid/liquid separation. Only water can permeate through RO membranes, whilst all dissolved or suspended material is retained (Wagner, 2001; Galanakis, 2012). In summary, MF and UF are effectively sieve filtration techniques based mainly on particle size. The separations achieved by NF and RO, however, are not only governed by size, but also charge, solubility and diffusivity (Wagner, 2001).

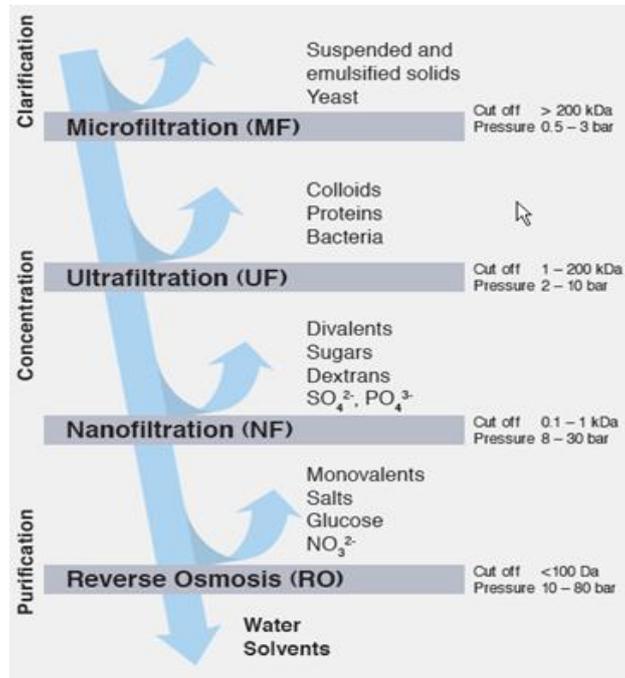


Figure 2.3 Characterisation of different membrane separation techniques (Anon., 2013c).

2.5.2 Types of membranes

Membranes can either be classified as inorganic or organic. Inorganic membranes are made from materials such as ceramics, metal oxides (titania, zirconia, alumina), carbon, silica (glass), metals and zeolite (Charcosset, 2012). Although inorganic membranes have greater chemical, thermal and mechanical stability than organic membranes, the high capital costs, brittleness and varying permeabilities of the membranes at mild temperature conditions can act as disadvantages (Charcosset, 2012).

Organic membranes are made by casting various polymers, such as cellulose acetate (CA), regenerate cellulose (RC), polysulphone (PS), polyethersulphone (PES) and polyvinylidene fluoride (PVDF). Different membrane materials and their industrial applications are shown in Table 2.4. Initially, CA was the most common membrane material used for UF, NF and RO applications due to the fact that it is cost effective and hydrophilic which reduced fouling (Cheryan, 1998). The disadvantages of CA are that it can be used as a carbon source by microorganisms and it has poor temperature and pH tolerance (Wagner, 2001). The predominant membranes used in industrial UF are RC, PVDF and PES

membranes. RC membranes are highly hydrophilic and have low protein binding properties, making them more suitable for protein concentration and pharmaceutical applications such as the production of expensive protein therapeutic drugs (Allegrezza *et al.*, 2010). Their strong resistance to pH and temperature make them suitable for many applications in food and dairy industries (Wagner, 2001). PES membranes are used when low protein binding is not as important and/or harsher cleaning chemicals are utilised (Allegrezza *et al.*, 2010). Their main disadvantage is their relative hydrophobicity, which can lead to increased membrane fouling (Zhao *et al.*, 2013). PES and PES-based membranes are widely used in industrial applications due to their high oxidative, thermal and hydrolytic stability and mechanical strength (Denis *et al.*, 2009; Zhao *et al.*, 2013). These applications include beer clarification (Van der Sman *et al.*, 2012), production of milk, cheese and whey products (Anekar & Rao, 2009; Paugum *et al.*, 2012) and water purification/waste water treatment (Abuhabib *et al.*, 2013). PVDF is a hydrophobic membrane material traditionally used to treat water due to its high oxidant resistance, although the more recently developed PES membranes are a cheaper alternative (Wenten, 2002).

Table 2.4 Membrane materials and their industrial applications (adapted from Wenten, 2002 and Kumar, 2012a)

Process	Industrial Applications	Membrane materials
Gas and vapour permeation	Hydrogen recovery, CO ₂ separation, ethanol dehydration	Cellulose acetates, polyetherimide, polysulphones, polydimethylsiloxanes, polyvinyl alcohol
Reverse osmosis	Drinking water, deionised water, dairy, fruit and vegetables, sugar, fruit juice, wine, beer, haemodialysis	Cellulose acetate, polyamides, polyimides, modified polysulphones
Nanofiltration	Drinking water, wastewater, sugar, tea	Polyacetonitrile, polyvinyl alcohol, polysulphones, silicone
Ultrafiltration	Drinking water, dairy, meat, fruit juice, sugar, wine, beer, tea, enzyme purification, haemodialysis	Polyacetonitrile, polyvinyl alcohol, polyvinylidene fluoride, polysulphones, regenerated cellulose, ceramics
Microfiltration	Wastewater, sugar, fruit juice, wine, beer, tea, fermentation broth	Polycarbonates, cellulose nitrate, carbon composites, modified polyethylene and polypropylene, ceramics

2.5.3 Membrane configurations for ultrafiltration

UF membranes are configured in such a way that surface area is maximised and fouling reduced by using a tangential flow design to reduce solute accumulation at the membrane surface (Nath, 2008). There are four main types of membrane configurations used for UF. These include the flat sheet membrane, tubular, spiral wound and hollow fibre membrane

(Shi *et al.*, 2005) (Fig. 2.4). Flat sheet membranes are most commonly used for research purposes due to the simple preparation methods involved and less complicated morphologies (Zhao *et al.*, 2013). They can be used as cassettes or plate and frame devices (Nath, 2008). Tubular membranes can either be polymeric or ceramic with the advantages of being able to handle viscous liquids, have a small replacement cost and are easily cleaned (Nath, 2008). However, their low surface area/volume ratio results in high floor space requirements (Cheryan, 1998). In the spiral wound configuration, many layers of flat sheet membranes are sandwiched between layers of mesh-like spacers and wrapped around a central core tube within a stainless steel or plastic cartridge cell (Cheryan, 1998; Nath, 2008). Hollow fibre configurations use a grouping of 50-3000 hollow tubes made from extruded fibres of membrane material in a cartridge shell made of stainless steel or plastic (Cheryan, 1998). The surface area of hollow fibre membranes and their ability to separate small particulates is greater than that of spiral wound membranes (Cheryan, 1998). For industrial purposes, hollow fibre membranes are the preferred choice as their larger surface/volume ratio makes them more cost effective (Zhao *et al.*, 2013). Whilst tubular membranes can handle solutions with high levels of suspended solids, the spiral wound and hollow fibre membranes require the pre-filtration of solutions containing large particulates and fibre and have high replacement costs (Cheryan, 1998).

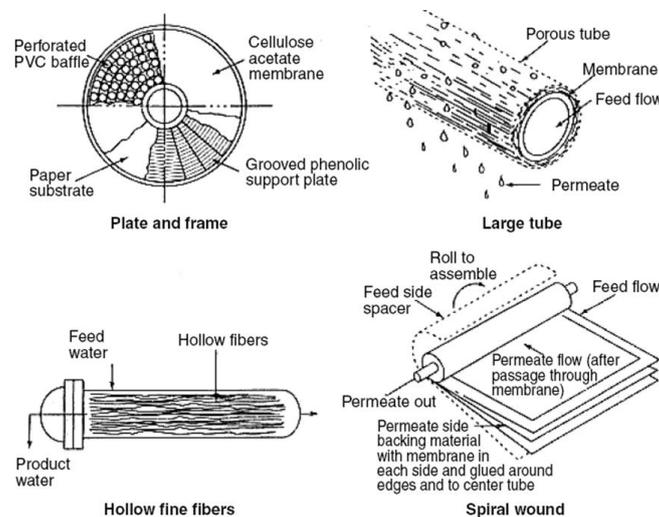


Figure 2.4 Schematic design of the four main membrane configurations (Sincero & Sincero, 2003).

2.5.4 Applications of ultrafiltration

2.5.4.1 Membrane processing of natural products

Membrane separations find a wide range of applications in the pharmaceutical, cosmetic and food industries. Membrane processing of liquids ranges from waste stream to high-value

pharmaceutical extracts. Its popularity as a separation technique is due to the fact that it is a more eco-friendly alternative to many traditional separation techniques (Galanakis *et al.*, 2010).

As natural products typically contain HMW molecules, MF, UF and NF are used. These pressure driven processes have the ability to separate compounds ranging from several nm to 1 μm (Li & Chase, 2010). They can either be carried out on their own or as a pre-treatment to other separation/concentration processes such as reverse osmosis (Pap *et al.*, 2012); addition of fining adsorbents (Rao *et al.*, 2011) and resin adsorption (Li *et al.*, 2005; Gu *et al.*, 2008), in order to concentrate or purify natural products from their source (Li & Chase, 2010).

The UF of plant/herbal extracts has not been as extensively studied compared to the use of UF in the fruit juice and wine industry. This separation technique is, however, of great interest to the food and drug industries as it avoids the thermal degradation of valuable compounds and can concentrate plant extracts using a lower energy consumption than other membrane technologies, such as RO, where a higher operating pressure is required (Cissé *et al.*, 2011). UF membranes can reject molecules that have molecular weights larger than 1000 g/mol, which is why they are successful in separating polyphenols from larger macromolecules, e.g. pectin, and fractionating different MW polyphenols (Nawaz *et al.*, 2006).

Table 2.5 provides details of a selection of studies using membranes in various applications such as clarification, fractionation and recovery of polyphenols and other valuable components from natural sources. Specific application of UF in the food industry will be highlighted in sections 2.5.4.2 and 2.5.4.3. In the latter section, the focus will specifically be on tea processing as an example of a polyphenol-rich beverage that contains valuable bioactives.

Table 2.5 Membrane processing of botanical extracts

Feed Solution	Compound/s	MWCO ¹ (kDa)	MF/UF/NF ²	Material ³	Application	Reference
Fruit Juices/ Wine						
Apple juice	Pectin, starch, polyphenols	10, 100	UF	RC	Clarification ⁴	Borneman <i>et al.</i> , 2001
Apple juice	Pectin, starch, polyphenols	15, 30	UF	ZrO ₂	Clarification ⁴	De Bruijn <i>et al.</i> , 2002
Kiwifruit juice	Pectin, proteins, fibre, polyphenols, organic acids	30	UF	CA	Clarification, recovery of bioactive compounds	Cassano <i>et al.</i> , 2008a
Blackcurrant juice	Pectin, anthocyanins, flavonols	100	UF	PES	Clarification	Pap <i>et al.</i> , 2012
Grape must	Pectin, proteins, polyphenols	100	UF	PS	Clarification	Cassano <i>et al.</i> , 2008b
Pinotage wines	Phenolics	10, 30, 50	UF	PES	Fractionation ⁴	De Beer <i>et al.</i> , 2006
Waste products/effluents						
Bergamot juice	Hesperidin, naringin, sugars, organic acids	0.45 100	NF UF	TiO ₂ PS	Clarification and subsequent concentration ⁵	Conidi <i>et al.</i> , 2011
Almond skin extracts	Low and high MW polyphenols	10, 30, 50	UF	RC	Fractionation ⁴	Prodanov <i>et al.</i> , 2008
Buckwheat bran extract	Total flavonoids	10	UF	RC	Purification	Yang & He, 2010
Defatted, milled grape seeds	Proanthocyanins of different MW	4 8, 20 200	UF UF MF	PES PS PVDF	Fractionation and subsequent purification ⁵	Santamaría <i>et al.</i> , 2002
Winery sludge	Pectin, phenolics, sugars	1 20, 100	UF	FP PS	Fractionation ⁴	Galanakis <i>et al.</i> , 2013
Poultry processing water	Proteins	30	UF	PS	Recovery	Lo <i>et al.</i> , 2005

Feed Solution	Compound/s	MWCO (kDa)	MF/UF/NF	Material	Application	Reference
Fermented grape pomace	Polyphenols	0.15-0.3 1, 350 1000	NF UF MF	PA/PS PA/PS TiO ₂	Concentration ⁴	Díaz-Reinoso <i>et al.</i> , 2009
Olive mill waste waters	Polyphenols	4 5 10 10	UF	PES RC RC PES	Concentration ⁴	Cassano <i>et al.</i> , 2011
Olive mill waste waters	Low molecular weight polyphenols, sugars, minerals	1 6 20, 80	UF	ZrO ₂ PES PS	Fractionation and subsequent purification ⁴	Russo, 2007
Olive mill waste waters	Pectins, polyphenols	2,10 25, 100	UF	PES PS	Separation and subsequent purification ⁴	Galanakis <i>et al.</i> , 2010
Olive mill waste waters	Polyphenols	8, 25, 100	UF	PS	Recovery ⁴	Gkoutsidis <i>et al.</i> , 2011
Orange press liquor (depectinised)	Proteins, fibre, flavonoids, phenolic acids	100	UF	PS	Clarification	Ruby-Figueroa <i>et al.</i> , 2011
Cork processing wastewaters	Polyphenols	5	UF	PES RC	Recovery	Acero <i>et al.</i> , 2005
Herbal extracts						
Roselle extract	Anthocyanins	1,2 5,20,30,50,150	UF	Thin film PES	Concentration ⁴	Cissé <i>et al.</i> , 2011
<i>Dendrocalamus latiflorus</i> leaf extract	Total flavonoids	50	UF	PS	Purification	Tang <i>et al.</i> , 2008
<i>Echinacea purpurea</i> herb juice extract	Phenolic acids	5 1, 5	UF UF	RC PES	Concentration ⁴	Hossain, 2005
<i>Radix astragalus</i>	Polysaccharides, saponins, flavonoids	10	UF	PS	Purification	Cai <i>et al.</i> , 2012
Rapeseed extract	Polysaccharides	3,8,12	UF	PES	Fractionation ⁴	Sun <i>et al.</i> , 2011

Feed Solution	Compound/s	MWCO (kDa)	MF/UF/NF	Material	Application	Reference
Teas						
Green tea	Tea polyphenols, free amino acids, proteins	10, 30, 50, 100	UF	RC	Clarification ⁴	Rao <i>et al.</i> , 2011
Green tea	(-)-Epigallocatechin gallate	5, 10, 30, 100	UF	PS	Purification ⁴	Kumar <i>et al.</i> , 2012b
Reconstituted black tea	Proteins, polysaccharides, polyphenols	30	UF	FP RC	Clarification	Evans & Bird, 2006
Black tea	Proteins, pectin, polyphenols, caffeine, minerals	25, 50, 100, 500	UF	PS	Clarification ⁴	Chandini <i>et al.</i> , 2012
Black tea	Proteins, polyphenols	40	UF	Ceramic	Clarification	Todisco <i>et al.</i> , 2002
Other						
<i>Grateloupia turuturu</i> (macro-algae) extract	R-phycoerythrin	300 10, 30, 100	UF	PAN PES,RC	Concentration ⁴	Denis <i>et al.</i> , 2009
Stevia extract	Stevioside, high and low molecular weight components	5 10, 30, 100	UF	Thin film PES	Clarification ⁴	Chhaya <i>et al.</i> , 2012
Corn extract	Xanthophylls: lutein, zeaxanthin	0.3	NF	PA/PS	Purification	Tsui & Cheryan, 2007
Wood hydrolysate	Galactoglucomannan	5, 10, 30	UF	RC	Purification ⁴	Al Manasarah <i>et al.</i> , 2012
Rutin-glucose binary feed	Rutin, glucose	1	UF	RC	Separation	Wei <i>et al.</i> , 2010

¹ molecular weight cut off; ² MF = microfiltration, UF = ultrafiltration, NF = nanofiltration; ³ RC = regenerated cellulose, ZrO₂ = zirconium dioxide, CA = cellulose acetate, PES = polyethersulphone, PS = polysulphone, TiO₂ = titanium dioxide, PVDF = polyvinylidene fluoride, FP = fluoropolymer, PA/PS = composite polyamide/polysulfone, PAN = polyacrylonitrile; ⁴ Membranes tested individually; ⁵ Membranes tested in combination

2.5.4.2 Membrane processing in the food industry

UF has been used to process different foods and beverages, including milk, dairy products, fruit and vegetable juices, wine, sugar, sweeteners and vegetable oils (Cheryan, 1998). Apart from dairy protein concentration, one of the major applications of UF is the processing of polyphenol-containing beverages, such as fruit juice, wine and beer. These beverages are easily susceptible to haze formation caused by the agglomeration of HMW molecules, such as proteins and pectins, with the polyphenols which substantially reduce product shelf life. UF prevents haze formation by effectively removing the HMW molecules, whilst maintaining the majority of the smaller sugars, acids and bioactive compounds. The end product is a clarified juice with the same sweetness, pH and health benefits (Galanakis *et al.*, 2010; Pap *et al.*, 2012). It eliminates the need for fining agents (bentonite, gelatine), enzymes (pectinase, amylase) and centrifugation prior to filtration – the clarification and fining are done in one step allowing a better yield of clarified juice in a shorter processing time (Cheryan, 1998). Furthermore, it provides microbiological stabilisation, clarification and concentration without the addition of extra chemical agents (Rao *et al.*, 2011). The clarification of juice on an industrial scale can involve methods such as the gelatine-silica sol treatment followed by vacuum filtration. These two methods combined have been shown to decrease the content of four major anthocyanins in blackcurrant juice by 19-29% (Meyer & Bagger-Jørgensen, 2002). Using UF as an alternative to traditional clarification methods ensures that the antioxidant profile of the juice remains the same (Galanakis *et al.*, 2010). Apart from the clarification of fruit juices, UF has been used either on its own, or in combination with other separation process to obtain fractions enriched in polyphenols with possible application as functional food ingredients/nutraceuticals. Examples include the UF of cranberry juice (Husson *et al.*, 2012), kiwifruit juice (Cassano *et al.*, 2008a), bergamot juice (Conidi *et al.*, 2011) and blood orange juice (Galaverna *et al.*, 2008).

In the past few years, a great deal of research has also been done on UF as a means of 'cleaning up' or recovering valuable compounds from various food waste streams (Table 2.5). Some of these include evaluating the use of UF to reduce the large quantities of polyphenols present in olive mill wastewaters (OMW) (Cassano *et al.*, 2011; Gkoutosidis *et al.*, 2011). The disposal of OMW is a huge problem in the Mediterranean area due to the large quantities produced, the high chemical oxygen demand (COD) values and the presence of phyto-toxic and antibacterial polyphenolics (Russo, 2007). Therefore it is not viable to dispose of these OMW on agricultural crops. Galanakis *et al.* (2010) used UF to produce a clarified pectin solution from OMW for possible application as a gelling agent in low fat meatballs. Another application that was investigated is the recovery of protein from poultry processing water (Lo *et al.*, 2005). This effluent contains approximately 35% protein

and requires extensive pre-treatment before disposal due to high COD and biochemical oxygen demand (BOD). UF was proven to retain nearly all the crude protein as well as substantially reduce the COD and BOD values (Lo *et al.*, 2005). UF was also proven to successfully reduce the tannic fraction, aromatic content, colour and COD value of cork processing waste waters (Acero *et al.*, 2005).

2.5.4.3 Application of ultrafiltration in tea processing

Investigations of the application of UF in tea processing focus mainly on its use as a possible clarification technique for cold tea beverages (Todisco *et al.*, 2002; Evans & Bird, 2006; Evans *et al.*, 2008; Chandini *et al.*, 2012), and the fractionation and clarification of polyphenols in green tea (Kawakatsu *et al.*, 1995; Rao *et al.*, 2011; Kumar *et al.*, 2012b) produced from *Camellia sinensis*, as discussed below.

Cold tea, more commonly known as iced tea, is generally produced from a spray dried tea powder to which sugars/sweeteners, lemon/peach juice, citric acid, colourants and stabilising agents are added (Todisco *et al.*, 2002). Although these teas have a satisfactory shelf life of 6-12 months, the inclusion of numerous additives does not satisfy consumers that are looking for a tea product with a high nutritional value (Todisco *et al.*, 2002). Thus, UF has been tested as a clarification technique to enhance the shelf life of natural, additive-free cold green (Rao *et al.*, 2011) and black teas (Todisco *et al.*, 2002; Chandini *et al.*, 2012). The main problem encountered in the production of additive-free tea beverages is the development of 'tea cream' during storage. Tea cream consists of monomeric/dimeric polyphenols, caffeine, metal ions and proteins which combine and precipitate during storage, affecting the visual appeal of the beverage as well as its colour and flavour (Evans *et al.*, 2008). Since tea cream composition is similar to that of the tea itself, removing the tea cream would result in loss of colour, flavour, taste and health promoting polyphenols (Chandini *et al.*, 2012). Chemicals and enzymes can be added to combat tea cream formation, but if a natural additive-free product is desired then the best solution would be to use a filtration technique such as UF for the primary extract to remove some of the larger compounds, e.g. proteins, that are responsible for tea cream formation (Rao *et al.*, 2011; Chandini *et al.*, 2012). Liang and Xu (2003) examined the size of black tea cream particles using a light scattering technique. They found that 84.4% of tea cream particles were below 1.03 μm and 7.5% were above 5.07 μm . This size difference could potentially allow a separation of some of the smaller polyphenols from the larger tea cream aggregates using a physical barrier, such as a membrane.

A number of studies have established that black tea clarified by UF is less prone to cream formation than unfiltered black tea. Chandini *et al.* (2012) found that black tea extract clarified using either MF or UF did not exhibit any signs of tea cream formation after 30 days

of cold storage. MF better retained the colour of the original tea than UF (Chandini *et al.*, 2012). Evans *et al.* (2008) showed that UF significantly removed haze with a simultaneous reduction in colour. UF was performed using different regenerated cellulose (RC) and fluoropolymer (FP) membranes separately to produce clarified black tea liquor with polyphenol transmission rates of approximately 90%. Todisco *et al.* (2002) proved that the UF of black tea through a 40 kDa ceramic membrane allowed the permeation of the most important polyphenols in the molecular mass range of 280-458 g/mol whilst maintaining the essential properties of tea. This study confirms the beneficial effect of UF on the stability of the tea in terms of polyphenol concentration and colour retention during storage. Further details are summarised in Table 2.5.

Table 2.6 Comparison of parameters of colour and polyphenol concentrations between permeate and initial tea infusion at different days of storage at -4°C (Todisco *et al.*, 2002)

Colour ¹	Sample ²	Time (days)			
		0	7	30	60
L*	IF	56	49	21	20
	P	59	61	62	62
a*	IF	14	16	18	21
	P	12	11	10	11
b*	IF	34	31	14	12
	P	36	36	36	36
Polyphenols (g/l)	IF	1.97	1.88	1.84	1.80
	P	1.74	1.73	1.74	1.72

¹ L = lightness, a = redness, b = yellowness, ² IF = initial feed (tea infusion); P = permeate.

In the case of green tea, Rao *et al.* (2011) found that fining adsorbents were more effective in clarifying green tea than UF as the tea became cloudy after one week of storage. A compromise may have to be made between the extended shelf life of such a product and whether or not the product is additive-free. Kawakatsu *et al.* (1995) reported the successful clarification of green tea using a MF/UF combination as a pre-treatment to concentration processes as well as to prevent the formation of tea cream in packaged drinks. However, the storage stability of the packaged product over time was not determined.

Apart from clarification, UF has been successfully used to fractionate and concentrate the major polyphenols in green tea. Kumar *et al.* (2012b) performed successful separation of (-)-epigallocatechin-gallate (EGCG) from (-)-epigallocatechin (EGC) based on their different solubility characteristics using a two step aqueous extraction followed by UF. The molecular weights of these compounds are 458.4 g/mol and 306.3 g/mol, respectively, similar to that of mangiferin, which suggests the potential of UF for enrichment of this compound in honeybush extracts. Different membrane sizes were tested (5, 10, 30 & 100 kDa) of which

30 kDa performed the best (Kumar *et al.*, 2012b). The EGCG-rich extract underwent MF as a pre-treatment before cross-flow UF through PS membranes and freeze-drying of the permeate. The freeze-dried permeate contained 90% polyphenols, of which 80% was EGCG. This extract would find many nutraceutical, pharmaceutical, cosmetic and functional food applications due to numerous studies that have linked EGCG with many health-promoting properties (as reviewed by Nagle *et al.*, 2010) as well as the ability of flavan-3-ols to extend the shelf-life of food products (as reviewed by Vuong *et al.*, 2011).

2.5.5 Selecting the appropriate membrane

In all membrane processes, an economical separation involves achieving the highest permeate flux possible whilst maintaining the lowest incidence of fouling. Since the success of UF is dependent on the membrane used, it is vital to consider all factors that influence the separation efficiency (Shi *et al.*, 2005). Membranes used for separation or purification processes must exhibit characteristics, such as high flux, high selectivity, mechanical stability, resistance to fouling and low cost (Baker, 2012).

Although UF is typically used to separate HMW compounds from LMW compounds, the separation efficiency of the membrane does not only depend on the MWCO and the MW of the feed components. The nature of the compounds to be separated, membrane chemistry and processing conditions also need to be taken into account (Santamaría *et al.*, 2002). Small-scale preliminary testing of a range of membrane sizes is recommended to determine the best MWCO to use. From literature it can be deduced that successful UF of polyphenols, present in various types of plant extracts, can be achieved using a 30 kDa membrane such as the clarification of kiwi fruit juice (Cassano *et al.*, 2008a), stevia extract (Chhaya *et al.*, 2012), green tea (Rao *et al.*, 2011; Kumar *et al.*, 2012b) and black tea (Evans & Bird, 2006) and for the concentration of proteins from macroalgae (Denis *et al.*, 2009) (Table 2.5).

The hydrophobicity of the membrane is very important as it directly affects membrane fouling. Different membrane materials are better suited to different feeds depending on the nature of the compounds. According to Evans and Bird (2006), hydrophobic membranes, e.g. PES, are better suited to reject the hydrophilic solutes in black tea. However, the opposite has also been reported, in that more hydrophobic membranes experience greater fouling during the UF of tea by attracting the hydrophobic solutes (Evans *et al.*, 2008). Hydrophilic membranes, such as RC membranes, are preferred for the concentration of proteins as they prevent protein-membrane interactions, which leads to increased fouling and a reduction in permeate flux (Charcosset, 2012). As hydrophilic membranes tend to swell in water reducing their mechanical strength, PES membranes may be favoured for use on an industrial scale due to their excellent mechanical strength (Denis *et al.*, 2009; Zhao *et*

al., 2013). The only way to ensure the best membrane is chosen is to perform small-scale test experiments using the feed solution to be processed (Cheryan, 1998).

2.5.6 Influence of parameters

In UF, the desired product can either be the retentate (e.g. concentration of proteins) or permeate (e.g. clarified fruit juices, wine, beer etc.). If the permeate is the desired product, its composition is the main factor that must be considered when choosing the optimum processing parameters (Chhaya *et al.*, 2012). The economic feasibility of UF for the processing of plant extracts is largely dependent on the yield which is measured in terms of permeated solids (Chandini *et al.*, 2012). The processing parameters are chosen such that the maximum permeate flux is obtained, combined with a good recovery of solids (yield) and as little membrane fouling as possible for high productivity as well as good permeate quality where necessary (Lo *et al.*, 2005; Chhaya *et al.*, 2012).

The film theory model, also known as the mass transfer model, is used to describe UF on a molecular level (Cheryan, 1998). This theory states that when a solution is filtered, solutes are brought to the membrane surface via convective transport which results in the build up of a concentration gradient (concentration polarisation). This concentration gradient causes the back diffusion of the solutes into the bulk solution. There are four major operating variables in UF that affect this process: transmembrane pressure (TMP), agitation of solutes near the membrane surface (i.e. stirring or cross-flow filtration), temperature and feed concentration (Cheryan, 1998). All these parameters directly affect the permeate flux and hence the efficiency of the UF process.

2.5.6.1 Permeate flux

Permeate flux is defined as the volume of liquid flowing through the membrane per unit area per unit time (Cheryan, 1998). It is one of the most important parameters used to evaluate the productivity of a pressure driven membrane filtration process (Evans *et al.*, 2008; Al Manasrah *et al.*, 2012). The influence of other important operating parameters is usually determined in terms of their effect on permeate flux (Fig. 2.5).

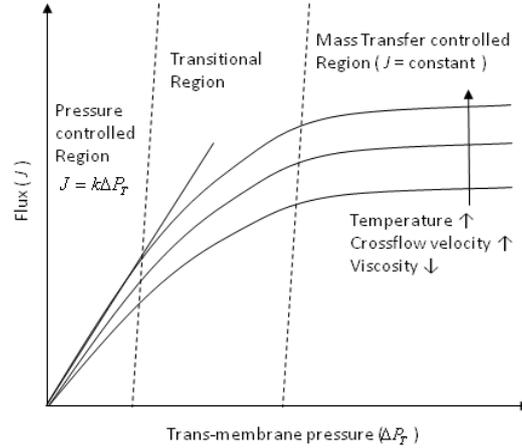


Figure 2.5 The effect of operating parameters on the permeate flux during ultrafiltration (Yoon, 2013).

2.5.6.2 Transmembrane pressure

During UF no permeation can take place in the absence of pressure (Todisco *et al.*, 2002). The pressure applied across a membrane is referred to as TMP. It is the average pressure difference between the permeate side and retentate sides, defined according to Equation 2.3 (Shi *et al.*, 2005; Wei *et al.*, 2010):

$$TMP = \frac{P_{in} + P_{out}}{2} + P_p \quad (2.3)$$

where TMP represents transmembrane pressure, P_{in} and P_{out} are the inlet and outlet pressures, respectively, and P_p is the pressure at permeate side (typically zero).

The TMP range used in UF is typically between 10-500 kPa (0.1-5 bar) (Charcosset, 2012). The applied pressure forces the solvent and some of the solutes through the membrane. The relationship between permeate flux and pressure is illustrated in Fig. 2.6. At low pressures (P_1), TMP and flux show a linear, reversible relationship. This is referred to as the pressure-controlled region. However, as the pressure increases (P_2) there is an increase in solute deposition on the membrane surface and the flux increases not linearly with pressure, but more slowly until the pressure reaches the critical pressure (P_3) where the concentration polarisation is enough for the solutes at the membrane surface to reach gel concentration, C_{gel} (Abdelrasoul *et al.*, 2013). As the pressure increases further (P_4), the flux becomes independent of pressure due to the solute depositions formed (Cheryan, 1998; Charcosset, 2012). The optimum TMP is indicated by the 'knee' of the flux curve, between P_2 and P_3 , at the onset of pressure independence (Cassano *et al.*, 2008b). At this TMP, a reasonable flux can be obtained whilst avoiding excessive aggregate formation. The flux so obtained is known as the critical flux, above which irreversible membrane fouling occurs (Charcosset, 2012).

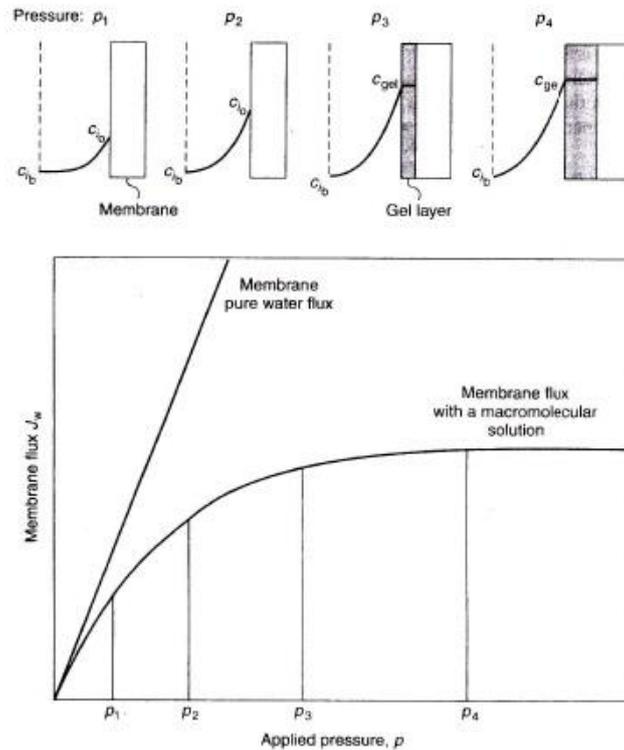


Figure 2.6 The effect of pressure on permeate flux (Abdelrasoul *et al.*, 2013).

2.5.6.3 Feed characteristics

The film theory model states that flux will decrease exponentially with increasing feed concentration, irrespective of the flow characteristics or temperature (Cheryan, 1998). As a general rule, the permeate flux decreases as the concentration of the feed solution increases (Chandini *et al.*, 2012; Charcosset, 2012). This is due to the fact that there are more solutes present that can accumulate at the membrane surface and form the concentration polarisation layer.

2.5.6.4 Temperature

Although temperature is not usually a controlled variable in industrial applications, it plays an important role in membrane fouling because of changes to the feed solution characteristics which can either increase/decrease solute deposits on the membrane (Cassano *et al.*, 2008b). One of the main advantages of membrane separations is the mild operating temperature conditions. From literature it can be seen that temperatures of 20-50°C are typically used for UF of plant extracts, depending on the nature of the feed and temperature limit of the specific membrane used. In industry, UF of juice is performed at 50-55°C to minimise bacterial/yeast growth (Cheryan, 1998), whereas the UF of beer is performed at 0-4°C to ensure product stability and prevent chill cloudiness (Finnigan *et al.*, 1989). CA membranes are most susceptible to damage by extreme temperatures and operate best below 30°C (Cheryan, 1998). PES membranes are more stable and can withstand

temperatures up to 75°C. The membranes with the greatest thermal stability are ceramic membranes which can withstand temperatures greater than 200°C (Cheryan, 1998). In general, higher temperatures of the feed solution will increase flux in both the pressure-controlled region and the mass transfer controlled region if no additional fouling is caused by chemical changes in the feed, such as protein denaturation, precipitation etc. (Cheryan, 1998). The effect of temperature in the pressure controlled region is due to its effect on fluid density and viscosity. It is best to operate at the highest temperature possible that will not have an adverse effect on either the feed solution or membrane (Cheryan, 1998). In the mass transfer-controlled region, an increase in temperature results in an increase of the back diffusion of solutes that have accumulated at the membrane surface (Sun *et al.*, 2011). Together with a reduced viscosity, the mass transfer coefficient is improved resulting in an increase in permeate flux (Cassano *et al.*, 2008b).

2.5.6.5 Feed flow rate and turbulence

Turbulence can be induced in the feed flow by increasing the cross flow velocity (Cheryan, 1998). It has a large effect on the mass transfer-controlled region of UF, where flux is independent of pressure and fouling is more extensive. A higher cross flow velocity can reduce concentration polarisation by 'sweeping away' solutes that have accumulated on/near the membrane surface (Zhao *et al.*, 2013). By increasing the cross-flow velocity, the cost of pumping energy will increase and it must be determined whether the increased cost is worth the benefits from a higher flux. However, a higher cross flow velocity can be cheaper than using a greater membrane area at lower velocities (Cheryan, 1998).

The effects of the operating variables (TMP, temperature, feed flow rate and solute concentration) on membrane fouling are interdependent and cannot be determined independently. It is necessary to consider their interactions if they are to be optimised (Ruby-Figueroa *et al.*, 2011).

2.5.7 Membrane fouling

2.5.7.1 Principles

The greatest problem encountered in membrane processing is the decline of permeate flux as a result of membrane fouling. Membrane fouling not only shortens the membrane life but directly affects the final product quality and economic feasibility of the entire process (Li & Chase, 2010; Wei *et al.*, 2010; Ruby-Figueroa *et al.*, 2011; Pap *et al.*, 2012). It results in unpredictable separation (Zhao *et al.*, 2013). The fouling of UF membranes is unavoidable when filtering natural plant extracts due to the fact that more than 90% of the components present are macromolecules larger than 50 kDa (Li & Chase, 2010). How the membrane

fouling is controlled will determine the success of the separation. Generally, membrane fouling is affected by three main factors: membrane material properties and surface chemistry, feed characteristics and operating parameters (Evans *et al.*, 2008; Susanto *et al.*, 2009).

Membrane fouling is a result of several coexistent yet independent phenomena (Jonsson *et al.*, 1996). There are many different methods available for the quantification of membrane fouling, including rate of flux decline, fouling time, final vs. initial permeate flux and fouling coefficients from logarithmic, exponential and linear empirical models (De Bruijn *et al.*, 2002). During UF of various extracts (including teas), membrane fouling has been determined using a 'resistors in series model' (Equation 2.4) (De Bruijn *et al.*, 2002; Todisco *et al.*, 2002; Evans & Bird, 2006; Cassano *et al.*, 2008b; Chhaya *et al.*, 2012).

$$R_T = R_M + R_F + R_{CP} \quad (2.4)$$

where R_T is the total resistance provided by the membrane, R_M is the intrinsic membrane resistance, R_F is the fouling resistance and R_{CP} is the resistance due to concentration polarisation. R_M of the clean membrane is calculated using pure water as feed before UF has taken place (De Bruijn *et al.*, 2002). R_F is due to pore blockage and cake formation on the membrane surface, i.e. a thin solute layer that sticks to the membrane surface. It is a result of physico-chemical solute-solute and solute-membrane interactions (Todisco *et al.*, 2002). This layer is incompressible and acts as a dynamic membrane layer, affecting the permeation of smaller solutes through the membrane as it reduces the MWCO (Cheryan, 1998). Fouling does not only include cake formation, but includes the adhesion of solutes within the membrane pores, causing a decrease in flux due to pore constriction (De Bruijn *et al.*, 2002; Li & Chase, 2010). This changes the membrane permeability and selectivity (De Bruijn *et al.*, 2002). Membrane fouling is irreversible and is dependent on the TMP, feed concentration and cross flow velocity.

R_{CP} builds up during UF. During membrane filtration, the solutes that are rejected by the membrane accumulate at the membrane surface via convective transport (Cheryan, 1998). This forms a concentrated layer of the particles at the membrane surface with a higher solute concentration than the bulk solution which forms a concentration gradient, known as concentration polarisation (Cuperus & Smolders, 1991; Cheryan, 1998). When the concentration reaches a certain point, a gel precipitation layer forms on the membrane surface and substantially reduces permeate flow (Ruby-Figueroa *et al.*, 2012). Gel precipitation is more predominant in protein and pectin containing liquids (Van den Berg & Smolders, 1990; Xie *et al.*, 2008) and can be controlled by adjusting the feed flow velocity,

feed concentration and TMP (Li & Chase, 2010). Any conditions that rapidly force solutes to the membrane surface, such as higher pressures and reduced cross-flow velocities, favour concentration polarisation and subsequent membrane fouling (Cheryan, 1998).

Different mathematical models are used to study membrane fouling depending on the predominant fouling mechanism (as reviewed by Song, 1998). They include concentration polarisation models, cake filtration models and blocking models. When the particle diffusion flux is dominant, the concentration polarisation model is used, while the cake filtration model is used when the accumulation of solids on the membrane surface is the dominant fouling mechanism. If the particle size of the feed constituents is smaller than that of the membrane pores, pore blocking can occur in which case the blocking model may be the most appropriate (Ruby-Figueroa *et al.*, 2011).

2.5.7.2 Prevention of membrane fouling

Membrane fouling can be reduced both by the setup of UF equipment and the operating parameters. UF can be operated in two different configurations, dead-end or tangential flow (also known as cross flow). In dead-end filtration, the direction of feed flow is in the same direction as the flow through the membrane (Fig. 2.7). This set up is mainly used for small-scale laboratory applications as membrane 'clogging' can easily occur and reduce the productivity of the process (Shi *et al.*, 2005, Zhao *et al.*, 2013). In tangential flow filtration, the feed flow is perpendicular to the flow through the membrane (Fig. 2.7). The feed is generally circulating at a higher speed than in dead-end filtration which, together with the direction of flow, displaces solutes that have accumulated near the membrane surface (Shi *et al.*, 2005, Zhao *et al.*, 2013). Industrialised or large-scale UF processes are always performed in cross-flow configuration as it facilitates the recirculation of retentate to the feed tank and is more economical due to the higher permeate flux involved (Cheryan, 1998).

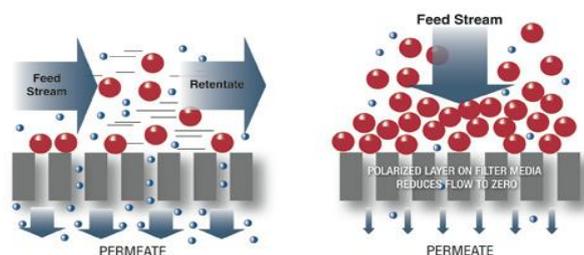


Figure 2.7 Tangential (left) versus dead-end (right) filtration configurations (Anon., 2013d).

Concentration polarisation is reversible and can be controlled by choosing the right operating parameters. Reducing the solid content in the feed will reduce fouling but may be counterproductive as a greater volume of solvent will need to be removed during subsequent concentration/drying processes. High pressures should be avoided as they can increase the

compaction of solutes at the membrane surface. Cassano *et al.* (2008b) found that during the UF of grape must, increasing the applied pressure decreased the solids and polyphenol content in the permeate. The TMP should thus be chosen so that a good permeate flux is obtained without excess fouling (Fig. 2.6) (Cheryan, 1998). At a high cross flow velocity, there is a fast, consistent circulation of rejected solutes in the retentate, preventing them from sticking to the membrane by shear force. De Bruijn *et al.* (2002) found that membrane fouling during UF of apple juice was greatly reduced by using a combination of high flow rate and low TMP. This resulted in an increased permeate flux.

Irreversible membrane fouling, such as pore blocking and cake formation, can only be removed by backflushing/backpulsing techniques or cleaning the membrane with chemicals (Li & Chase, 2010; Rao *et al.*, 2011). Backflushing involves reverse filtration by periodically reversing the direction of TMP or permeate flow which removes the solutes that had previously accumulated on the membrane surface (Charcosset *et al.*, 2012). Backpulsing works on the same principle, except the duration of reverse flow is shorter and more frequent (Charcosset *et al.*, 2012). Gas sparging is another technique used to prevent membrane fouling. In this technique, a bubble-induced secondary flow is generated which reduces concentration polarisation. This technique is most effective in tubular, hollow fibre and spiral wound membranes (Charcosset *et al.*, 2012).

Membrane surface modifications can also affect membrane fouling. As a general rule, hydrophilic membranes are usually less susceptible to membrane fouling than hydrophobic membranes (Li & Chase, 2010). Various researchers have increased the hydrophilicity (Reddy & Patel, 2008; Wang *et al.*, 2009; Zhao *et al.*, 2013) and hydrophobicity (Wu *et al.*, 1992; Jin *et al.*, 2008; Zhang *et al.*, 2011b) of membranes, depending on what is desired, by introducing polymeric groups to the membrane surface. Fouling causes the surface chemistry and hence the hydrophobicity of membranes to change. It was found that during UF of black tea, fouling of the polysulphone membranes resulted in an increase in hydrophilicity due to the deposition of negatively charged species on the membrane (Evans *et al.*, 2008).

There is a great deal of research being done on developing improved membrane systems with reduced fouling characteristics, improved selectivity and enhanced permeate flux (Li & Chase, 2010).

2.5.7.3 Fouling during ultrafiltration of tea

Membrane fouling during the UF of tea is unavoidable due to its chemical composition. Rao *et al.* (2011) found that after UF of green tea using RC membranes, a thin layer of yellow-green cake represented by tea polyphenols was present on the membrane. It was concluded that the protein and polyphenols present in the tea were the main foulants during

the UF process. Evans *et al.* (2008) found that the dominant fouling mechanism during the UF of black tea using hydrophilic RC membranes was also cake formation. They deduced this based on similar transmission readings for solids, polyphenols, caffeine and theaflavins irrespective of the different membrane pore sizes. They also found that more fouling occurred on rougher, more hydrophobic fluoropolymer membranes than on the RC membranes. A hydrophobic membrane surface would attract more hydrophobic substances present in tea, causing more extensive fouling (Evans *et al.*, 2008).

2.6 Drying of botanical extracts

Since most nutraceuticals/functional food ingredients are obtained in liquid form by means of extraction from botanicals, different drying techniques are used for final product formation. One such method is spray drying. It can be used as both a drying and an encapsulation technique and allows the rapid conversion of a liquid product to a final powder of even particle size in a single step (Galanakis *et al.*, 2012). Microencapsulation is a topic of great interest in the food industry as a means of delivering bioactive components into foods, thereby giving them 'functional' status. To be successful, research is required not only on the release of these compounds into media that mimic gastric fluid (De Souza *et al.*, 2009), but also to test their release into model food systems, such as a beverage (Wichchukit *et al.*, 2013).

The disadvantage of spray drying is the possible yield reduction that can occur due to the destruction of labile antioxidants, i.e. volatile low molecular weight phenols (Galanakis *et al.*, 2012). In some cases, freeze drying is preferred for sensitive constituents as the whole process is conducted under very low temperatures and vacuum conditions. However, freeze drying is both time consuming and energy intensive and is ultimately quite expensive (Galanakis, 2012). Compared to freeze drying, the cost of spray drying is 30-50 times less (Gharsallaoui *et al.*, 2007).

2.7 Response surface methodology

Optimisation of a process improves the performance of a system and increases yield without increasing cost (Baş & Boyaci, 2007). It can be approached using a univariate one-variable-at-a-time technique (OVAT) in which a single variable is optimised at a time. When more than one variable needs to be optimised, the OVAT approach is not ideal as it requires a great deal of experiments increasing time, expenses, and raw material and reagent consumption (Baş & Boyaci, 2007; Bezerra *et al.*, 2008). For simultaneous multivariate

analysis, response surface methodology (RSM) has become the most popular optimisation method used (Baş & Boyaci, 2007). RSM finds application in the extraction of phenolics from different botanicals. Some examples include the pressurised fluid extraction (PFE) of antioxidants from spruce (*Picea abies*) bark (Co *et al.*, 2012), the ultrasonic-assisted extraction of antioxidants from black soybean (*Glycine max var*) sprouts (Lai *et al.*, 2013), flavonoid extraction from the flower of *Citrus aurantium* (Yang *et al.*, 2010) and *Gynura medica* leaf (Liu *et al.*, 2010) and mangiferin extraction from mango (*Mangifera indica* L.) leaves (Zou *et al.*, 2013; Zou *et al.*, 2014).

2.7.1 Principles

RSM is an efficient, statistical tool used to analyse the effect of multiple experimental factors and their relationship on a particular response. It allows the optimisation of one or several responses, which are a result of the interaction of the independent experimental factors (Baş & Boyaci, 2007; Bezerra *et al.*, 2008; Dejaegher & Vander Heyden, 2011). Response refers to a measured or observed quantity that is to be optimised (Hibbert, 2012), for example extraction yield of total polyphenols (TP). A factor is an independent parameter that affects the response, for example extraction time, solvent composition and temperature are important factors when optimising the extraction of a substance. RSM is an effective alternative to testing one variable at a time, which does not account for the interaction between different factors. It provides a great deal of information with the fewest possible experiments, saving both time and money (Baş & Boyaci, 2007; Bezerra *et al.*, 2008; Dejaegher & Vander Heyden, 2011).

2.7.2 Experimental design

The first stage in an optimisation study using RSM is conducting preliminary trials to determine the independent parameters (factors) and their ranges to be tested. It is important to identify the factors that have major effects on responses. Typically only two or three factors are optimised. If more than three factors are chosen, a large number of experiments will be required and the entire response surface cannot be visualised making it difficult to determine the optimal conditions (Dejaegher & Vander Heyden, 2011). The next step is to select an appropriate experimental design. A full factorial design contains every possible combination of factors at the desired levels, i.e. there are L^k combinations of k factors at L levels (Hibbert, 2012). This can result in a great number of experimental runs needing to be performed if the factors have more than two levels, which is extremely time consuming. A central composite design (CCD) can be a better option as it can test the factors at five levels (as opposed to three) in fewer experiments. A CCD contains a two-level full factorial design at factor levels -1 and +1, a star design at factor levels 0, $-\alpha$ and $+\alpha$ and a centre point at

factor level 0 (Dejaegher & Vander Heyden, 2011). It can be used for the optimisation of two or three factors (Fig. 2.8). Selecting appropriate levels of factors, such as different solvent concentrations or temperatures, is crucial as it determines the success of the optimisation – factor levels should not be too close together or at the extremes of an experimental range (Hibbert, 2012).

2.7.3 Interpretation of results

After completion of the experimental design, the model equation is defined and the coefficients predicted (Baş & Boyaci, 2007; Hibbert, 2012). Response surface designs typically use a second order polynomial model to fit the data:

$$y = \beta_0 + \sum_{j=1}^k \beta_j X_j + \sum_{j=1}^k \beta_{jj} X_j^2 + \sum \sum_{i < j} \beta_{ij} X_i X_j \quad (2.5)$$

where β_0 , β_i , β_{ii} , and β_{ij} are regression coefficients for intercept, linear, quadratic and interaction terms, respectively, y is the response value (dependent variable) and X_i and X_j represent the level of the independent variables (factors). The term k represents the number of tested factors (Baş & Boyaci, 2007; Bezerra *et al.*, 2008; Dejaegher & Vander Heyden, 2011). The most reliable way to evaluate how well the generated model fits the data is to apply an analysis of variance (ANOVA). ANOVA compares the variation due to different treatments with the variation due to random errors inherent in the measurements of the responses which is represented by the coefficient of variation (R^2) (Bezerra *et al.*, 2008). In addition, ANOVA estimates the statistical significance of the parameters and their interactions on the measured responses. The validity of the generated model is subsequently tested by performing additional runs and comparing the experimental results to the predicted values.

The three-dimensional response surface plots (Fig. 2.9 a-c) and two dimensional contour plots (Fig. 2.9 d-f) are a graphical representation of the regression (predicted model) equation. They reflect the type of interaction (significant vs. negligible) between the tested factors and their relationship to the measured responses (Baş & Boyaci, 2007; Bezerra *et al.*, 2008; Tang *et al.*, 2011). The response surface plot shows the magnitude of a response value as a result of the combined effect of two factors at a particular time (Yang *et al.*, 2010). If the slope of the response surface plot is flat, then the response is not greatly affected by the change in factor levels (Fig. 2.9 a) while the opposite is valid if the slope is steeper giving it a rounded appearance, then the response value is greatly affected by the change in extraction conditions (Fig. 2.9 b & c). On the contour plots, if the rings are close together it means that the change in conditions greatly affected the response value. A circular contour

plot shows a non-significant interaction between factors (Fig. 2.9 e), whilst an elliptical contour plot represents significant interaction (Fig. 2.9 d & f) (Tang *et al.*, 2011; Hibbert, 2012). Standardised Pareto charts (Fig. 2.9 g & h) can also be used to show the significance of the linear, quadratic and interaction effects of the factors on the measured responses. The effect is significant if its corresponding bar crosses the vertical line at the $P=0.05$ level. The length of each bar is proportional to the absolute scale of the standard estimated effects. A negative value implies a negative parameter effect on the response value.

2.7.4 Process optimisation

For quadratic models, the stationary point or critical point of a response is regarded as the optimum value in the range of the tested parameters. It is calculated from the second order polynomial equation and can be characterised as the maximum, minimum or saddle point (Baş & Boyaci, 2007; Bezerra *et al.*, 2008). A clear global optimum is not always obtained using RSM and in some cases, it is sufficient to simply use the response surfaces to indicate an optimum region (Bezerra *et al.*, 2008). If several responses need to be optimised simultaneously, then a multicriteria methodology such as desirability profiling can be used. Since factors can have opposite effects on the measured responses, an optimal compromise has to be made (Bezerra *et al.*, 2008). The individual desirability function (d_i) for each response is specified by assigning predicted values a score ranging from 0 (very undesirable) to 1 (very desirable), from which an overall desirability function (D) can be obtained. In short, this method indicates the levels of factors that demonstrate maximum overall desirability (Bezerra *et al.*, 2008).

2.7.5 Advantages and disadvantages

As previously mentioned, RSM has the ability to generate a great deal of data from a small number of experiments and reduce energy, raw material and reagent wastage by optimising a particular process or analytical method (Djaegher & Vander Heyden, 2011). Unlike OVAT, RSM allows the interaction effect between parameters to be analysed, which is of great importance in biochemical processes due to the occurrence of synergistic, antagonistic or additive reactions (Baş & Boyaci, 2007; Bezerra *et al.*, 2008). One of the major limitations of RSM is fitting data to a second order polynomial model, which cannot account for all the curvature seen in the data obtained from different systems (Baş & Boyaci, 2007). This can be overcome by transforming data to another form, e.g. a logarithmic transformation, or by adjusting the range of independent parameters (factors). Although narrowing the range may increase the accuracy of the model equation, it reduces the possibility of determining a stationary point (Baş & Boyaci, 2007). Again, this highlights the critical importance of effective preliminary testing to allow for effective optimisation.

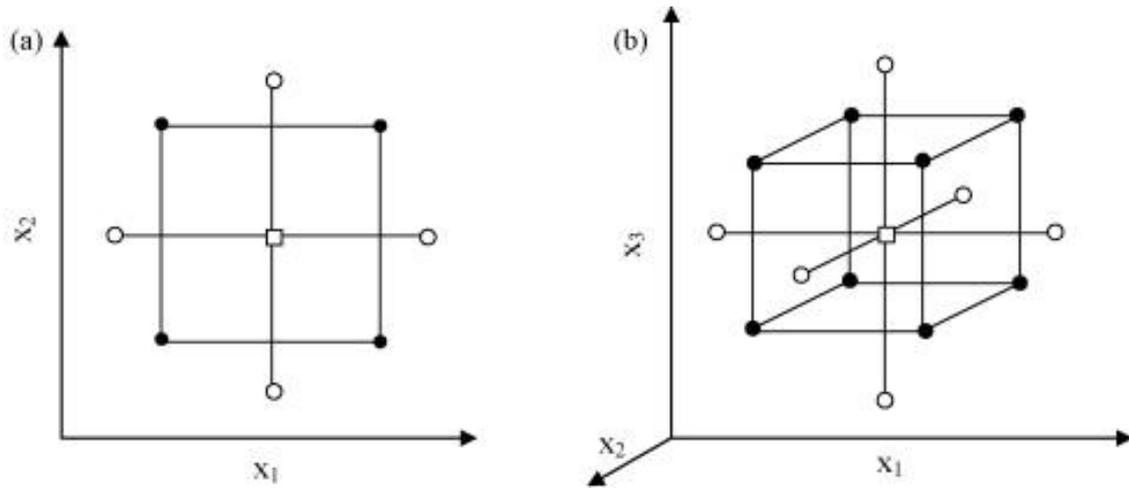


Figure 2.8 Central composite design (CCD) for the optimisation of (a) two variables and (b) three variables (Bezerra *et al.*, 2008).

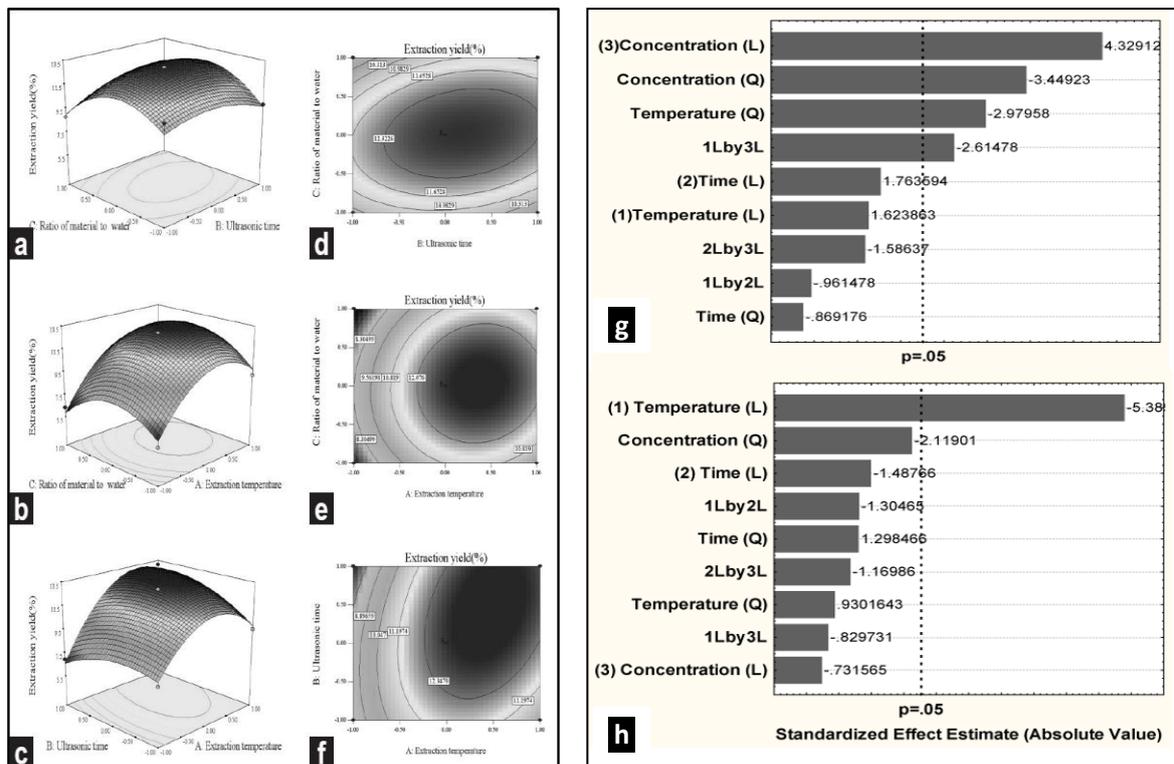


Figure 2.9 Response surface plots (a,b,c) and contour plots (d,e,f) obtained from the optimisation of polysaccharides from the root of *Limonium sinense* Kuntze (Tang *et al.*, 2011). Standardised Pareto charts for xylan conversion to xylose (g) and hydrolysis selectivity (h) in the optimisation of low temperature dilute sulphuric acid hydrolysis of the hemicellulose fraction of cardoon (*Cynara cardunculus* L.) (Shatalov & Pereira, 2011).

2.8 Methods for quality control and quantification

Standardisation of nutraceuticals and functional food ingredients is important so that consumers can be assured that the product will deliver what the label dictates. However, regulations and standardisation of such products is an area of dispute amongst scientists and of scepticism amongst consumers. Due to the complex nature of food and variability of the methods available to test the chemical composition and antioxidant profile of products, it is difficult to find one method to satisfy all requirements. The methodologies used must account for the chemical, physical and environmental conditions specific to a particular product (Seeram *et al.*, 2006).

Standardisation does not only refer to the testing of the final product, but entails the whole process, starting at the raw plant material (Garg *et al.*, 2012). Plant material is known to vary in terms of quality due to irregular chemical composition that is influenced by genetic factors, growing conditions and methods of harvesting, drying, storage, transportation and processing (Garg *et al.*, 2012; Kunle *et al.*, 2012) which can be minimised through the use of cultivated plants (Garg *et al.*, 2012). Marker compounds can be used to identify plant materials, set quality specifications for raw materials and standardise the preparation of botanical products during multiple stages of the production process (Chinou, 2011). Standardisation to fixed marker content guarantees this level in the product and assures both the manufacturer and consumer that the product will provide the claimed health benefit (Gertenbach, 2002; Garg *et al.*, 2012). For example, commercial green tea dietary supplements are chemically standardised to EGCG levels (the major antioxidant in green tea) and/or biologically standardised to antioxidant capacity (Seeram *et al.*, 2006).

The current problem facing extract manufacturers is that products do not always deliver the quantities of target compounds as depicted on the label (Seeram *et al.*, 2006). Although plant material variation is largely to blame, poor regulatory control in the nutraceutical industry allows manufacturers to cut corners. Standardised manufacturing practices together with reliable labelling information substantiated by both chemical and biological assays are the fundamental requirements for the successful quality control of botanical products (Seeram *et al.*, 2006). Standardisation carries an assurance of quality, efficacy, safety and reproducibility (Kunle *et al.*, 2012).

2.8.1 Quality evaluation of honeybush

In South Africa, rooibos and honeybush tea manufacturers need to comply with regulations in terms of pesticide residues and microbial contamination of the tea destined for export (Joubert *et al.*, 2008a). No regulations exist that stipulate minimum levels of bioactive compounds in the tea. Rapid screening methods as alternative to high performance liquid

chromatography (HPLC) such as near-infrared spectroscopy (NIRS) have been investigated for prediction of the mangiferin content of the raw plant material (Joubert *et al.*, 2006; Joubert *et al.*, 2012) - an essential step in the development of a standardised mangiferin-enriched extract. This method has yet to be implemented on an industrial scale. Furthermore, no regulations, stipulating minimum levels of compounds or activity, exist for tea extracts so that quality control procedures and standardisation are left to manufacturers. As total polyphenol content (TPC) and total antioxidant capacity (TAC) are universal parameters employed by both researchers and industry for evaluation of plant extracts, South African extract manufacturers use these parameters to standardise their tea extracts (Joubert *et al.*, 2008a; Joubert & De Beer, 2012). TPC is determined using the colorimetric Folin-Ciocalteu methods, while TAC are determined using the ABTS, DPPH and/or ORAC assays.

In the present study, HPLC analysis will be used to quantify individual compounds in *C. genistoides* extracts and permeates, specifically the mangiferin content, allowing industry to relate the data to current extract production procedures.

2.9 Conclusion

Consumers today are not only considering food as a means to satisfy their hunger and provide them with essential nutrients, but also to prevent metabolic diseases typically associated with poor nutrition. In this regard, functional foods and beverages continue to rise in popularity and food manufacturers who take advantage of this trend can benefit greatly due to the high market value associated with such products.

The health benefits associated with *Cyclopia genistoides* as well as its substantial mangiferin content give extracts from this species great potential as functional food ingredients. The optimisation and standardisation of extracting bioactives from this plant is essential for the successful production of a functional food ingredient on an industrial scale, not only in terms of energy efficiency but also quality consistency.

UF is an attractive approach to increase the value of plant extracts due to its mild operating conditions, simple operation and easy incorporation into the current extract production process used in industry.

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Chapter 3

Optimisation of xanthone extraction from unfermented *Cyclopia genistoides* using response surface methodology

3.1 Abstract

Single factor trials were performed as a preliminary investigation to determine the effect of ethanol (EtOH) concentration (0-100%, v/v), plant material size (milled vs. teabag fraction) and extraction time (0-60 min) on the extraction of xanthenes from unfermented (green) *Cyclopia genistoides*. Extraction efficiency was assessed in terms of yield of extract and xanthenes, as well as xanthone content of the extract. EtOH concentration had a significant effect on the extraction efficiency, which was greater when using 20-60% EtOH (v/v). The extract and xanthone yields were significantly ($p < 0.05$) greater when milled plant material was used. The optimal extraction time for milled plant material was 30 min, after which no significant ($p \geq 0.05$) increase in extract yield, xanthone yield and xanthone content of the extract was obtained. Central composite design (CCD) and response surface methodology (RSM) were applied to evaluate the individual and interaction effects of EtOH concentration (0-100% v/v) and temperature (40-70°C). A total of 30 experiments were performed and the optimised response values were extract yield, mangiferin yield and mangiferin content of the extract. Since isomangiferin exhibits the same trends as mangiferin during extraction and is present in substantially smaller quantities in *C. genistoides*, response values for isomangiferin yield and isomangiferin content of the extract were not evaluated. The average response values varied from 12.18-35.81 g soluble solids/100 g plant material for extract yield, 1.41-3.18 g mangiferin/100 g plant material for mangiferin yield and 8.55-13.04 g mangiferin/100 g extract for mangiferin content of the extract. EtOH concentration was found to have the largest effect on all measured responses ($p < 0.05$). The interaction effect between parameters had only a small, but significant effect ($p < 0.05$) on extract yield. Optimal levels of EtOH concentration (40%, v/v) and temperature (70°C) for maximum extract and mangiferin yields were successfully achieved within the experimental domain. From the experimental data, second order polynomial models for extract yield, mangiferin yield and mangiferin content of the extract were generated with R_{adj}^2 values of 0.92, 0.76 and 0.92 respectively. All models adequately fit the experimental data. Satisfactory intraclass coefficient (ICC) values indicate that the models obtained for extract yield and mangiferin content of the extract have good predictive capability for quality control purposes. Taking into account the experimental results obtained and industrial practicality, the optimised extraction conditions selected for maximum xanthone recovery from *C. genistoides* were 40% (v/v) EtOH, 10 mL/g solvent:solid ratio, 70°C and 30 min.

3.2 Introduction

Food and beverage manufacturers continually seek novel food sources or ingredients to meet the growing consumer demand for natural, additive-free, health-promoting products. Polyphenols have become a topic of increased interest in the health and wellness industry due to their vast array of health-promoting properties (Balasundram *et al.*, 2006; Wang & Bohn, 2012). One such compound that has attracted great interest due to its strong antioxidant activity and health-promoting activities including antidiabetic, anti-inflammatory and anticancer, is the xanthone, mangiferin (Vyas *et al.*, 2012; Chellan *et al.*, 2014; Wang *et al.*, 2014). A number of plants have been investigated as sources, amongst others *Mangifera indica* L. (mango), *Garcinia mangostana* (mangosteen) and many species of *Swertia* (Kulkarni & Rathod, 2014). In Cuba, Vimang[®], a standardised aqueous extract made from mango stem bark and containing 10-20% mangiferin, is used as an antioxidant nutritional supplement (Sánchez *et al.*, 2000).

Cyclopia spp., endemic to South Africa, and well-known for their use as the herbal tea, honeybush, have been shown to contain mangiferin. Levels in the plant material can vary greatly, depending on species. Of the commercialised species, *C. genistoides* contains the highest levels of mangiferin (Joubert *et al.*, 2011). This compound is one of the most active antioxidants of *Cyclopia* polyphenols tested (Hubbe, 2000; Hubbe & Joubert, 2000; Joubert *et al.*, 2008), making a significant contribution to the antioxidant activity of *Cyclopia* extracts (Joubert *et al.*, 2008). The other major xanthone present in *Cyclopia* spp. is isomangiferin, a regio-isomer of mangiferin and shown to have similar or higher antioxidant activity than mangiferin in on-line HPLC-DAD-radical scavenging assays (Malherbe *et al.*, 2014). Other compounds of interest in *C. genistoides* are the benzophenone iriflophenone-3-C-glucoside and the flavanone glycoside, hesperidin. Iriflophenone-3-C-glucoside has been proven to act as an α -glucosidase inhibitor (Feng *et al.*, 2011), which aids in the suppression of postprandial hyperglycaemia by delaying carbohydrate digestion (Xiao *et al.*, 2013). The health-promoting properties of hesperidin are well documented and include antioxidant, anti-obesity, anti-carcinogenic and anti-inflammatory activities (Garg *et al.*, 2001; Park *et al.*, 2001). Hesperidin, iriflophenone-3-C-glucoside, mangiferin and isomangiferin have recently shown *in vitro* pro-apoptotic activity in synovial cells from rheumatoid arthritis patients (Kokotkiewicz *et al.*, 2013).

The complex structure of mangiferin makes it difficult to synthesize chemically. Extracting mangiferin from a natural source is therefore the best possible alternative for its production (Kulkarni & Rathod, 2014). Organic solvent extraction is the most common extraction technique used for mangiferin, with more and more investigations being done on alternative 'novel' extraction techniques such as supercritical fluid extraction (Fernández-Ponce *et al.*,

2012), microwave-assisted extraction (Zou *et al.*, 2013) and ultrasound-assisted extraction (Ruiz-Montañez *et al.*, 2014).

Response surface methodology (RSM) is an effective statistical tool that has successfully been used to optimise extraction of polyphenols from various plant sources using various extraction techniques (Liu *et al.*, 2010; Yang *et al.*, 2010; Co *et al.*, 2012; Lai *et al.*, 2013; Lee *et al.*, 2013) including the microwave-assisted extraction of mangiferin from mango (*Mangifera indica* L.) leaves (Zou *et al.*, 2013). RSM allows the simultaneous optimisation of several responses, which are a result of the interaction of the controllable, independent experimental parameters (Bezerra *et al.*, 2008). It is an efficient alternative to testing one variable at a time, which does not account for the interaction between different factors. It generates a substantial amount of data with the fewest possible experiments, saving both time and money (Bezerra *et al.*, 2008). Typical parameters that are optimised for solvent extraction of polyphenols from plant material include extraction time, solvent composition, extraction temperature, particle size and solid:solvent ratio (Liu *et al.*, 2010; Yang *et al.*, 2010; Prasad *et al.*, 2012; Tabaraki *et al.*, 2012; Lai *et al.*, 2013).

As energy consumption in the food industry is costly, optimising the extraction process can reduce the required energy input by avoiding excessive solvent usage and extensive extraction times (Chemat *et al.*, 2012; Grobler, 2013). Extraction optimisation standardises the manufacturing processes affecting both the reproducibility of the process and final product consistency which contribute to the quality control of botanical products (Seeram *et al.*, 2006).

The objective of this study was to optimise the extraction conditions of xanthones from *C. genistoides* for the eventual production of a xanthone-enriched honeybush tea extract. The 'unfermented' (unoxidised) plant material was selected, as the 'fermentation' process, necessary for production of the characteristic herbal tea, substantially reduces xanthone levels (Joubert *et al.*, 2008; De Beer & Joubert, 2010). Preliminary trials, entailing single factor experiments, were performed to determine the effect of solvent concentration, plant material size, extraction time and elevated temperatures on the extraction efficiency. RSM was subsequently performed to further optimise the solvent concentration (% v/v) and extraction temperature (°C).

3.3 Materials and methods

3.3.1 Chemicals

Authentic reference standards with purity >95% were obtained from Sigma-Aldrich (St. Louis, MO, USA; hesperidin, iriflophenone-3-C-glucoside), Extrasynthese (Genay, France; mangiferin), PROMEC (Medical Research Council of South Africa, Tygerberg, South Africa; aspalathin) and Chemos GmbH (Regenstauf, Germany; isomangiferin). Ethanol (EtOH) used for sample preparation and extraction was analytical grade (Servochem, Cape Town, South Africa). HPLC gradient grade 'far UV' acetonitrile was purchased from Merck Millipore (Darmstadt, Germany) and acetic acid (>99.8%) was purchased from Sigma-Aldrich. Deionised water, prepared using an Elix (Merck Millipore) water purification system, was further purified to HPLC grade using a Milli-Q Academic (Merck Millipore) water purification system. Deionised water was used in the solvent preparation for all performed extractions.

3.3.2 Plant material

For the first two single factor experiments (preliminary trials), the shoots (stems and leaves) of *C. genistoides*, were harvested in January 2013 at the farm, Toekomst in the Bredasdorp district (Western Cape, South Africa). Upon arrival at ARC Infruitec-Nietvoorbij, Stellenbosch, the fresh plant material was mechanically cut into small pieces and dried at 40°C in a temperature controlled drying tunnel (Continental Fan Works CC, Cape Town, South Africa) with forced air circulation to <7% moisture content. The dried plant material was then sieved to obtain the fraction >16 and <12 mesh ('teabag' fraction, equalling >1.2 x 1.2 mm and <1.42 x 1.42 mm, respectively, according to rooibos grading standards). A sub-sample of the sieved fraction was coarsely pulverised using a Retsch mill (Retsch, GmbH, Haan, Germany) equipped with a 1 mm sieve. For the comparison of solvent efficiency at elevated temperatures *C. genistoides* shoots from a potting trial at ARC Infruitec-Nietvoorbij was used. It was harvested in May 2013, mechanically cut, dried and sieved according to the same method as described. For RSM experiments, plant material was obtained from Toekomst in July 2013. It was mechanically cut and then dried according to the same method and subsequently milled.

3.3.3 Single factor experiments

Before the development of response surface models, the effect of solvent composition, particle size and extraction time on the extraction efficiency, as well as the influence of elevated temperatures on the solvent efficiency, was evaluated. The solvent:solid ratio was kept constant at 10 mL/g (an industry standard) for all experiments.

3.3.3.1 *General extraction procedure*

The plant material (*ca* 7 g; milled) was weighed into 200 mL Schott bottles, 70 mL solvent mixture added (solvent:solid ratio of 10 mL/g) and the capped bottles, after brisk swirling to mix the content, were placed in a temperature-controlled water bath at 50°C (unless otherwise specified) for 30 min. The bottles were then removed, again briskly swirled and filtered immediately through a cotton pad (100% pure cotton, Dove[®], Lil-lets, Westville, South Africa) placed on top of Whatman no. 4 paper (Whatman International Ltd., Maidstone, England) directly into sealed volumetric flasks. The filtrates were cooled to room temperature and analysed for soluble solids (SS) content. Extract samples were diluted ten times using HPLC grade water and aliquots stored at -18°C for further analysis. The experiment was performed in triplicate on three consecutive days.

3.3.3.2 *Effect of ethanol concentration on extraction efficiency*

Six different deionised water/ethanol mixtures were prepared to give 0% (only deionised water), 20%, 40%, 60%, 80% and 100% EtOH (v/v). Extraction was carried out as described in section 3.3.3.1, except that in the case of 100% EtOH, 14 g milled plant material and 140 mL solvent mixture were used. Extraction was performed at 50°C for 30 min. The experiment was performed in triplicate on three consecutive days.

3.3.3.3 *Effect of plant material size and time on extraction efficiency*

For this experiment the teabag fraction and milled plant material were compared, extracted at 50°C using 60% EtOH (v/v) as extraction solvent as described in section 3.3.3.1. Sampling over time (60 min in total) was achieved by removing one bottle after 10 min intervals. The experiment was performed in triplicate on three consecutive days.

3.3.3.4 *Comparison of solvent efficiency at elevated temperatures*

The efficacy of two solvents, 60% EtOH (v/v) and 100% deionised water (v/v), were tested at 70°C and 90°C, respectively, over time with sampling at 10 min intervals for a total extraction time of 60 min as described in section 3.3.3.3. The teabag fraction was used for both experiments and each experiment was performed in triplicate on three consecutive days.

3.3.4 Response surface optimisation of xanthone extraction

A central composite design (CCD) consisting of 30 experimental runs which included 4 axial points, 20 factorial points and 6 central points was employed to optimise the independent variables (factors), EtOH concentration (% v/v) and extraction temperature (°C). The dependent variables (responses) were extract yield (g SS/100 g plant material), mangiferin yield (g/100 g plant material) and mangiferin content of the extract (g/100 g SS). The levels of each factor, presented in Table 3.1, were selected on the basis of the single factor

experimental results and industrial practicality. Milled plant material was used in a solvent:solid ratio of 10 mL/g for all experiments which were randomised and divided into three blocks of ten runs each. One block was performed per day for three days. Experiments were performed in triplicate.

Table 3.1 Factors and their levels used in the Central Composite Design

Factor	Symbol	Levels				
		- α	-1	0	1	α
Temperature (°C)	X_1	40	45	55	65	70
Ethanol concentration (% v/v)	X_2	0	20	50	80	100

Experimental data from the CCD were fitted to the following second order polynomial equation and regression coefficients were calculated:

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{1 \leq i < j}^k \beta_{ij} X_i X_j + \varepsilon \quad (3.1)$$

where β_0 , β_i , β_{ii} , and β_{ij} are regression coefficients for intercept, linear, quadratic and interaction terms, respectively. Y is the response value (dependent variable) and X_i and X_j represent the level of the independent variables (factors). The term k represents the number of tested factors and ε the residual associated with the experiments. The validity of the experimental design was subsequently determined by performing additional verification experiments.

3.3.5 Quantification of individual polyphenols by HPLC–DAD analysis

3.3.5.1 HPLC-DAD analysis

The quantification of the major phenolic compounds was conducted in duplicate by reversed-phase high performance liquid chromatography with diode array detection (HPLC-DAD) (De Beer *et al.*, 2012). Separation was performed using an Agilent 1200 series HPLC instrument (Agilent Technologies Inc., Santa Clara, CA, USA) consisting of an in-line degasser, quaternary pump, autosampler, column oven and DAD on a Gemini-NX C18 (150 × 4.6 mm; 3 μ m; 110 Å) column (Phenomenex, Santa Clara, CA, USA), temperature-controlled at 30°C. Mobile phases, i.e. 2% acetic acid (A) and acetonitrile (B), were used for gradient separation at a flow rate of 1 mL/min: 0–2 min (8% B), 2–27 min (8%–38% B), 27–28 min (38%–50% B), 28–29 min (50% B), 29–30 min (50%–8% B), 30–40 min (8% B). Ascorbic acid was added to extract samples and standard mixtures at a final concentration of 9.1 mg/mL to prevent oxidative degradation of the phenolic compounds. All samples, including standard

mixtures, were then filtered using a 0.45 µm pore size Millex-HV syringe filters (Millipore). The injection volume for the standards was 10-20 µL and for extracts 5-20 µL. UV-Vis spectra were recorded for all samples from 200-450 nm. Peaks were identified by comparison of retention time and UV-Vis spectra with those of authentic reference standards where possible. 3-Hydroxy-phloretin-di-C-hexoside and iriflophenone-di-O,C-hexoside were tentatively identified based on relative retention time and UV-Vis spectra from De Beer *et al.* (2012). The xanthenes mangiferin and isomangiferin were quantified at 320 nm, whilst all other compounds were quantified at 288 nm. 3-Hydroxy-phloretin-di-C-hexoside and iriflophenone-di-O,C-hexoside were expressed in aspalathin (3-hydroxyphloretin-3'-C-glucoside) and iriflophenone-3-C-glucoside equivalents, respectively. A seven point calibration curve was set up using a mixture of authentic standards.

3.3.5.2 Determination of soluble solids content of *C. genistoides* extracts

The SS content of all extract filtrates was determined gravimetrically (in duplicate) by evaporating 10 mL extract in pre-weighed nickel moisture dishes on a steam bath (Merck, South Africa) until dry. For 100% EtOH (v/v) extracts, 20 mL was used. The samples were subsequently dried at 100°C for 60 min in a forced-air circulating laboratory oven, cooled under desiccation, re-weighed and the results expressed as mg SS/mL extract. Theoretical extract yield was calculated from the SS content determined, taking into account the extraction volume (not the recovered extract volume) and mass of plant material used and expressed as g SS/100 g plant material.

3.3.5.3 Statistical analysis

Univariate analysis of variance (ANOVA) was performed on all data obtained in the single factor experiments using SAS[®] software (Version 9.2, SAS institute Inc, Cary, NC, USA) to determine whether differences between treatment means were significant. Least significant difference (LSD) of the Student's t test ($p = 0.05$) was calculated to compare treatment means where significant differences were found ($p < 0.05$). Levene's test was used to test for treatment homogeneity of variance. Where variances were not equal a weighted analyses of variance was used for the combined analyses.

Statistica 12.0 (Statsoft[®], Southern Africa Analytics (Pty) Ltd, Johannesburg, South Africa) was used to analyse all data generated by RSM (section 3.4.3). Statistical significance and suitability of the model, its factors and their interactions were determined at the 5% probability level ($p < 0.05$) using ANOVA. Standardised Pareto charts were employed to illustrate the significant effects obtained from the ANOVA for the different response values. The correlation coefficient (R^2), adjusted R^2 (R_{adj}^2) and the significance of the lack of fit (LOF) were used to evaluate the fitting efficiency of the data to the model.

The regression function generated for each response was graphically illustrated as two-dimensional contour and three-dimensional response surface plots. Desirability profiling was used to optimise the extraction parameters, while the intraclass correlation coefficient (ICC) was employed to evaluate the predictive ability of the model.

3.4 Results and discussion

As the value of natural antioxidants becomes clear, a great deal of research is being conducted on different extraction techniques of these compounds from novel sources. The optimisation of the extraction of xanthones from various plant types and parts has been studied, including mangosteen fruit (Zarena *et al.*, 2011; Cheok *et al.*, 2012; Zarena *et al.*, 2012), mango seeds (Dorta *et al.*, 2013), mango leaves (Zou *et al.*, 2013; Zou *et al.*, 2014) and agarwood leaves (Ito *et al.*, 2012). Joubert *et al.* (2012) investigated the extraction of mangiferin from *C. subternata* for analytical purposes. The solvent that was used to achieve optimum extraction is not suitable for production of a food-grade extract. Given this constraint, *C. genistoides* with its high xanthone content was selected for extraction optimisation.

The type of solvent used for extraction can have a significant effect on the extraction efficiency of polyphenols from complex samples due to its solvent polarity and the ability to overcome the required activation energy of extraction (Rakotondramsy-Rabesiaka *et al.*, 2007; Tabaraki *et al.*, 2012). Polyphenols are easily solubilised in hydro-alcoholic mixtures. Among the several alcohols commonly used for solvent extraction, EtOH was used in this study, specifically chosen as it is relatively cheap and possesses 'GRAS' status (Generally-Recognised-As-Safe) (Galanakis, 2012). Another consideration was the solvent:solid ratio. Although a large quantity of solvent increases the driving force for diffusion of solutes from the leaf matrix, excessive solvent usage requires more energy for its removal further down the processing line (Tabaraki *et al.*, 2012). The solvent:solid ratio used by an extract manufacturer of water-based honeybush extract served as guide and was kept constant at 10 mL/g for all extractions. Single factor experiments were first conducted to determine which extraction conditions would remain constant (time, plant material size, solvent:solid ratio) or be further optimised (EtOH concentration, temperature). RSM was applied to optimise these parameters for maximum extract yield, mangiferin yield and mangiferin content in the extract.

3.4.1 Single factor experiments

High extraction efficiency of bioactive compounds from natural plant sources allows for economical extraction on industrial scale. It is therefore necessary to understand how to

obtain the maximum quantity of target compound(s) from the plant material of interest by manipulating the extraction conditions whilst simultaneously avoiding extensive extraction times, unnecessary solvent wastage and excess energy consumption, following the principle of green chemistry. Apart from the xanthenes, mangiferin and isomangiferin, which are the focus of this study, other major polyphenols in *C. genistoides* were also quantified as part of a more comprehensive characterisation of the extracts. These compounds include the benzophenones iriflophenone-di-O,C-hexoside and iriflophenone-3-C-glucoside, the dihydrochalcone 3-hydroxy-phloretin-di-C-hexoside and the flavanone rutinoides, hesperidin. They present a range of polarities, with iriflophenone-di-O,C-hexoside most polar and hesperidin least polar, based on their relative retention times, using reversed-phase C18 separation. Where data for mangiferin and isomangiferin are presented in graphs, actual values are also presented in Addendum A, in addition to the values for the other compounds.

3.4.1.1 Effect of ethanol concentration on extraction efficiency of xanthenes

The effect of ethanol concentration on the extraction efficiency of xanthenes from *C. genistoides* at 50°C and extraction time of 30 min was investigated. In this experiment, solvent composition significantly ($p < 0.05$) affected the SS, and thus extract yield obtained from the plant material (Fig. 3.1 A). The best extract yields were obtained using 40% EtOH (v/v) (42.0%) and 20% EtOH (v/v) (41.7%) which did not differ significantly from each other ($p \geq 0.05$). Water was more effective than 80% EtOH (v/v), but less effective than 60% EtOH (v/v). The lowest extract yield was obtained using 100% EtOH (v/v) as solvent (12.5%). Apart from difference in polarity between water and EtOH, water will cause the plant material to swell, thereby increasing the surface area of the plant material in contact with the solvent (Dorta *et al.*, 2013).

Figures 3.1 (B) and 3.1 (C) show the extraction yield of mangiferin and isomangiferin. The same trend was observed for these xanthenes, with the highest yield achieved with an EtOH concentration between 20-60% (v/v) for both mangiferin (5.14-5.55%) and isomangiferin (1.51-1.54%). The differences within this solvent range for both compounds were not significant ($p > 0.05$). Again, it is evident that an EtOH/water mixture provides significantly better extraction efficiency of the xanthenes than 100% EtOH (v/v) or 0% EtOH (v/v) (deionised water) as solvents ($p < 0.05$).

For further insight into the effect of solvent composition on the recovery of SS from the plant material, the other major polyphenols were quantified. This data is presented in Addendum A. The maximum yield of iriflophenone-3-C-glucoside from the plant material was obtained for the range 0-60% (v/v), while most effective extraction of the di-O,C-hexoside derivative was achieved at 0% EtOH (v/v), although not significantly better than at 20% EtOH (v/v). Extraction at 40% EtOH (v/v) was only slightly less effective than at 20% EtOH

(v/v). 3-Hydroxy-phloretin-di-C-hexoside gave the best extraction over the range 20-60% EtOH (v/v). The most effective extraction of hesperidin was achieved at 80% EtOH (v/v). The data clearly showed that an EtOH/water mixture, instead of the pure solvents favours extraction of most of these compounds. Employing an EtOH/water mixture falling within the range 20-80% EtOH (v/v) would not only accommodate good extract yields, but also would achieve efficient extraction of all the polyphenols.

Figures 3.1 (D) and 3.1 (E) represent the solvent efficiency in terms of the composition of the extract. It is evident that increasing the EtOH content of the solvent resulted in a greater xanthone content in the extract. This is attributed to poor extraction of non-phenolic compounds, such as polysaccharides, denatured proteins, etc., due to poor solubility in solutions of higher EtOH concentration. A similar trend was also observed for the extraction of xanthones from dried, green *C. subternata* (Joubert *et al.*, 2012) and hesperidin from *C. maculata* waste material (Du Preez, 2014). Ito *et al.* (2012) found the mangiferin content of agarwood leaf extract could be increased by increasing the EtOH concentration from 20-80% (v/v), however, no indication of the effect of EtOH concentration on extract yield was given. Although 100% EtOH (v/v) resulted in an extract with the highest xanthone content (21.8%) ($p < 0.05$), it was accompanied by a very poor extract yield (12.5%) making the use of pure EtOH impractical for industrial application, considering the final application as a food ingredient and not a high value product such as a phytopharmaceutical. Industrial manufacture of extracts usually requires a trade-off between yield and content ('purity'), determined by the final application (D Malherbe, Afriplex, Paarl, South Africa, personal communication).

For further single factor experiments, 60% EtOH (v/v) was selected due to the good extraction efficiency of the xanthones from the plant material (7.1%), the high xanthone content of the extract (17.6%) and the high extract yield (40.2%). The EtOH concentration range to be tested in RSM experiments was identified as 0-100% (v/v), where the combined effect of temperature would be investigated.

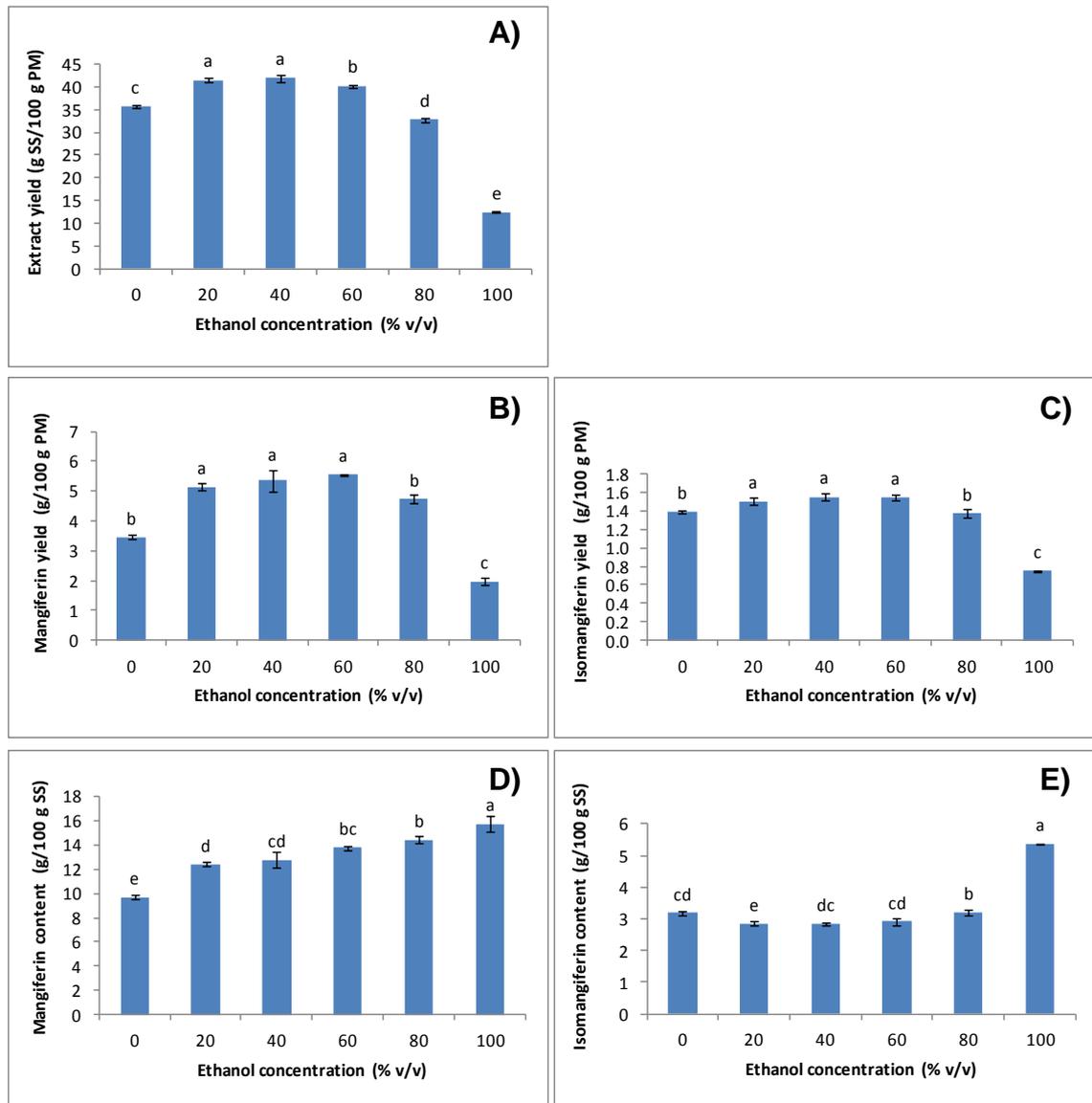


Figure 3.1 The effect of solvent composition (ethanol concentration, % v/v) on the extract yield (A), mangiferin yield (B), isomangiferin yield (C), mangiferin content of the extract (D) and isomangiferin content of the extract (E). Milled plant material was used at a solvent:solid ratio of 10 mL/g, extraction temperature 50°C and extraction time of 30 min (SS = soluble solids, PM = plant material).

3.4.1.2 Effect of plant material size and time on extraction efficiency

The effect of two different plant material sizes on the extraction efficiency of xanthenes from *C. genistoides* and the rate of extraction was investigated. It is evident from Fig. 3.2 (A) that the small particle size (<1 mm) results in significantly greater extract yield than the teabag cut (1.2-1.42 mm) at all time points ($p < 0.05$) and that steady state was reached at 20 min. The extraction of polyphenols from spherical plant particles is said to follow Fick's second law of diffusion (Fick, 1855; as reviewed by Gertenbach, 2002) which states:

$$\frac{\delta C}{\delta t} = D \frac{\delta^2 C}{\delta x^2} \quad (3.2)$$

where C is the concentration of the solute or marker compound, t is the extraction time, D is the diffusion coefficient and x is the particle diameter. A reduced particle diameter reduces the diffusion distance of the solute within the solid and increases the concentration gradient. This increases the extraction rate of target compounds ($\frac{\delta C}{\delta t}$) out of the leaf matrix as the diffusion through the particle to the particle surface is the rate-limiting step (Gertenbach, 2002, Cacao & Mazza, 2003). In addition, the surface area:volume ratio is greater allowing more solutes to diffuse into the surrounding solvent. It is for this reason that smaller particle sizes are preferred for industrial extraction processes to shorten extraction time even though additional energy is required to mill the samples (Luthria, 2008). When the coarse plant material was used, the extract yield increased significantly with increasing extraction time ($p < 0.05$), clearly indicating that diffusion of SS from the plant material was much slower due to the increased path length. After 60 min of extraction, the extract yield from the teabag fraction was only 63.6% of the yield achieved with milled plant material during a 20 min extraction period.

As expected from Fick's second law, the milled plant material also resulted in a higher xanthone yield than the teabag fraction for all extraction times (Fig. 3.2 B & C). The same trend was seen for all polyphenols quantified (Addendum A). After 60 min extraction, the teabag fraction delivered only 61.5% of the mangiferin and 59.4% isomangiferin yield obtained from the milled plant material during the same period. Similarly, it has been shown that the smallest particle size tested in studies on a variety of plant materials, i.e. sage and savoury (Gião *et al.*, 2009), grape skins (Bucić-Kojić *et al.* 2007) and pomegranate peel (Tabaraki *et al.*, 2012), favoured maximum extraction of polyphenols,

It is evident that particle size of the plant material and extraction time had no significant effect on the mangiferin and isomangiferin content of the extracts ($p < 0.05$) (Fig. 3.2 D & E). The mangiferin and isomangiferin contents of the extracts remained at ca 14% and 3.8%,

respectively. This indicates that the xanthenes extracted at the same rate as the majority of the other constituents. The same trend was seen for the other polyphenols quantified, except for hesperidin and iriflophenone-di-O,C-hexoside, where the maximum compound concentration in the extract was found after ten minutes of extraction followed by subsequent decreases with each time interval (Addendum A).

The optimum extraction times of polyphenols from various sources can differ. For example, Durling *et al.* (2007) found the optimum extraction time for phenolics from dried sage as three hours, whilst Nepote *et al.* (2005) found the optimum time to extract phenolics from peanut skins only ten min. Joubert *et al.* (2012) demonstrated that the yields of total polyphenols, mangiferin and hesperidin extracted from milled *C. subternata* were little affected by extraction time. Extended extraction times not only result in unnecessary cost but can also cause degradation of polyphenols (Liu *et al.*, 2010). In the current study, the extract yield from milled plant material reached steady state after 20 min (39.8%), showing that extraction times longer than 20 min are unnecessary as they do not significantly ($p \geq 0.05$) increase extract yield. The same trend was observed for mangiferin yield, while isomangiferin yield reached steady state after 30 min (Fig. 3.2 B & C). Therefore, an extraction time of 30 min would allow for optimum xanthone yield.

Given these results optimum extraction time of the milled plant material would thus be 30 min to minimise any xanthone degradation, while ensuring maximum xanthone yield and xanthone content of the extract. In the case of the teabag fraction, extraction should at least proceed for 50 min to obtain maximum extract and xanthone yields. The next step was therefore to investigate (3.4.1.3) whether increased temperature, as a major factor affecting diffusion (Gertenbach, 2002), could improve the extraction efficiency from the teabag fraction, using aqueous and ethanolic solvents.

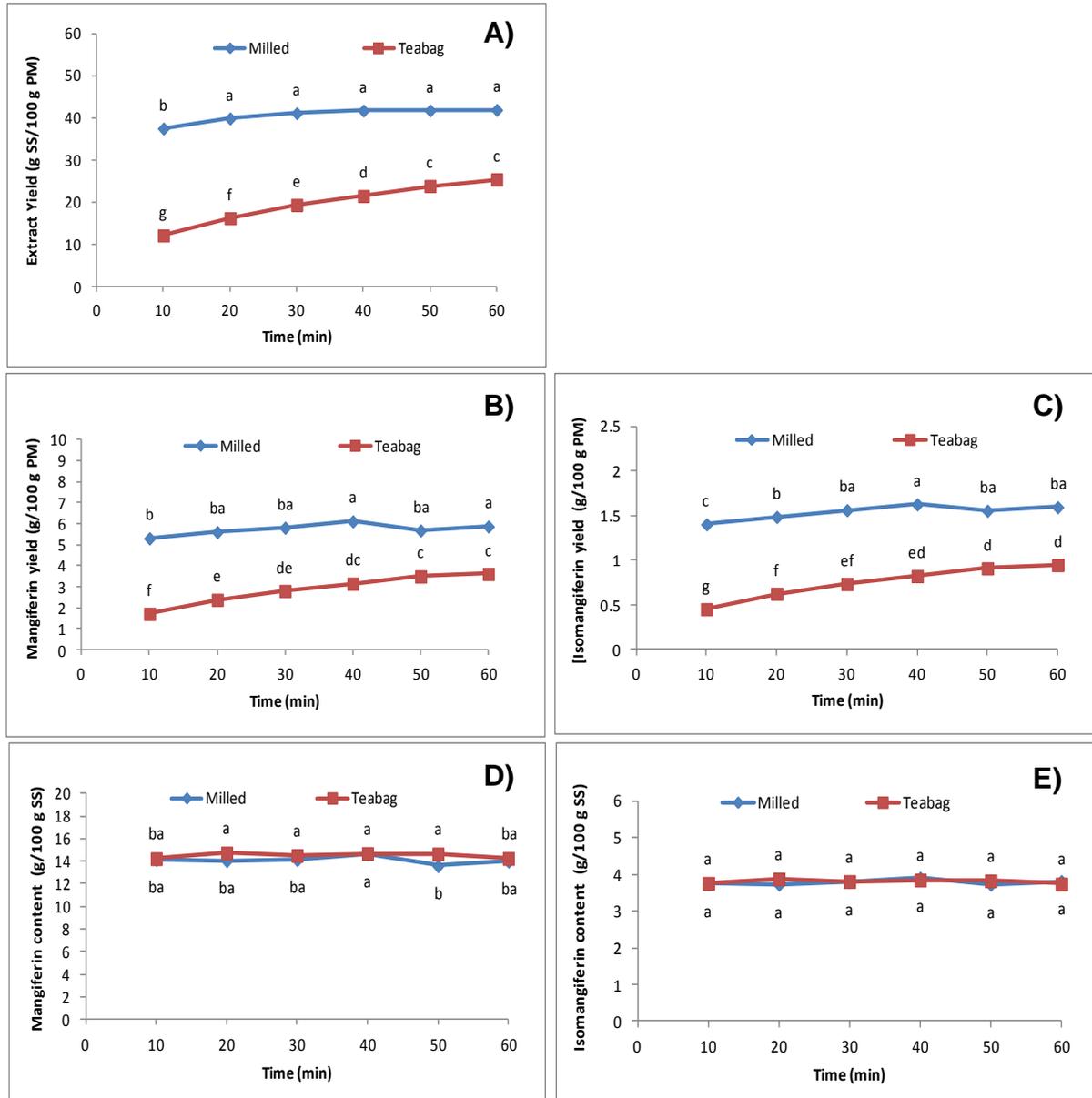


Figure 3.2 Effect of plant material size on the extract yield (A), mangiferin yield (B), isomangiferin yield (C), mangiferin content of the extract (D) and isomangiferin content of the extract (E). Extraction conditions comprised 60% ethanol (v/v) as solvent, solvent:solid ratio of 10 mL/g and extraction temperature of 50°C (SS = soluble solids, PM = plant material).

3.4.1.3 Comparison of solvent efficiency at elevated temperatures

The effect of elevated temperatures on the extraction efficiency of two different solvents on the teabag fraction was investigated. In this case aqueous extraction at 90°C was compared with 60% EtOH (v/v) at 70°C. The use of water at high temperature agrees with current industry practice to prepare food ingredient extracts from honeybush (Joubert *et al.*, 2011). During aqueous extraction at 90°C the extract yield increased significantly with each increasing time interval up to 50 min ($p < 0.05$) and ranged from 21.3-34.1% (Fig. 3.3 A). Use of 60% EtOH (v/v) as solvent limited the extraction temperature to 70°C. Under these extraction conditions extract yield ranged from 16.9-36.3% with no significant increases in yield after 50 min ($p > 0.05$). Between 20 and 30 min cross-over occurred with 60% EtOH (v/v) resulting in a greater extract yield than water (Fig. 3.3 A).

The difference in extraction efficiency of the two solvents became more pronounced when the mangiferin and isomangiferin yields are considered, with 60% EtOH (v/v) increasing the extraction of these compounds by 26.4 and 20.2%, respectively (Fig. 3.3 A & B). No significant increase in the extraction of these compounds occurred after 40 min, irrespective of solvents ($p \geq 0.05$), indicating that longer extraction times were unnecessary (Fig. 3.3 B & C). The maximum mangiferin and isomangiferin yields obtained for the aqueous extraction at 90°C were 2.8% and 0.9%, respectively. For the ethanolic extraction at 70°C, their maximum yields were 3.8% and 1.1%, respectively. Joubert *et al.* (2006) obtained a similar mangiferin yield (3.6%) from unfermented *C. genistoides* using methanol (MeOH) as extraction solvent at 65°C for 30 min. The values cannot be directly compared as different batches of plant material were used and are thus subject to variation in composition (Joubert *et al.*, 2006; Joubert *et al.*, 2014). MeOH, more effective than water or EtOH/water mixtures (Joubert *et al.*, 2012), however, cannot be used in the production of extracts intended for human consumption due to its toxicity.

As a different batch of plant material was used compared to that in 3.4.1.1, direct comparison of the obtained yields cannot be made. In 3.4.1.1, after 30 min extraction at 50°C, the aqueous extraction yielded 62.4% and 90.2% of the mangiferin and isomangiferin extracted by 60% EtOH (v/v), respectively (Fig. 3.1 B & C). In this experiment, after 30 min of aqueous extraction at 90°C, 75.3% and 80.9% of the mangiferin and isomangiferin, respectively extracted from the plant material by 60% EtOH (v/v) at 70°C, were achieved. It is evident that the extraction efficiency of water in terms of mangiferin extraction was improved by increasing the temperature from 50°C to 90°C. This is attributed to an improved extraction rate as a result of enhanced solubility of the xanthenes and higher diffusion coefficient (Al-Farsi & Lee, 2008; Ahmad-Qasem *et al.*, 2013).

The improved extraction of the xanthenes from the plant material with 60% EtOH (v/v) also produced extracts with higher xanthone contents (Fig. 3.3 D & E). The same trend was seen for all quantified polyphenols, except for iriflophenone-di-*O,C*-hexoside, the most polar compound of those quantified. Whereas iriflophenone-di-*O,C*-hexoside and iriflophenone-3-*C*-glucoside were, respectively, the third and fourth most abundant compounds in the extract prepared with water, the order was reversed with 60% EtOH (v/v) (Addendum A). The xanthone levels in both ethanolic and aqueous extracts remained fairly constant irrespective of the extraction time (Fig. 3.3 D & E) as were also found for the previous experiment (3.4.1.2), indicating once again that extraction rate of the various soluble compounds in the plant material in these solvents varied little. The maximum mangiferin and isomangiferin content in extracts prepared with 60% EtOH (v/v) was 11.1% and 3.3%, respectively. The corresponding contents for the water extract were 8.7% and 2.8%. It is therefore evident that extraction using a hydro-ethanolic mixture is more efficient than using water alone even at elevated temperatures. If water is to be used due to cost/practical implications, it should be used at high temperatures for improved extraction efficiency.

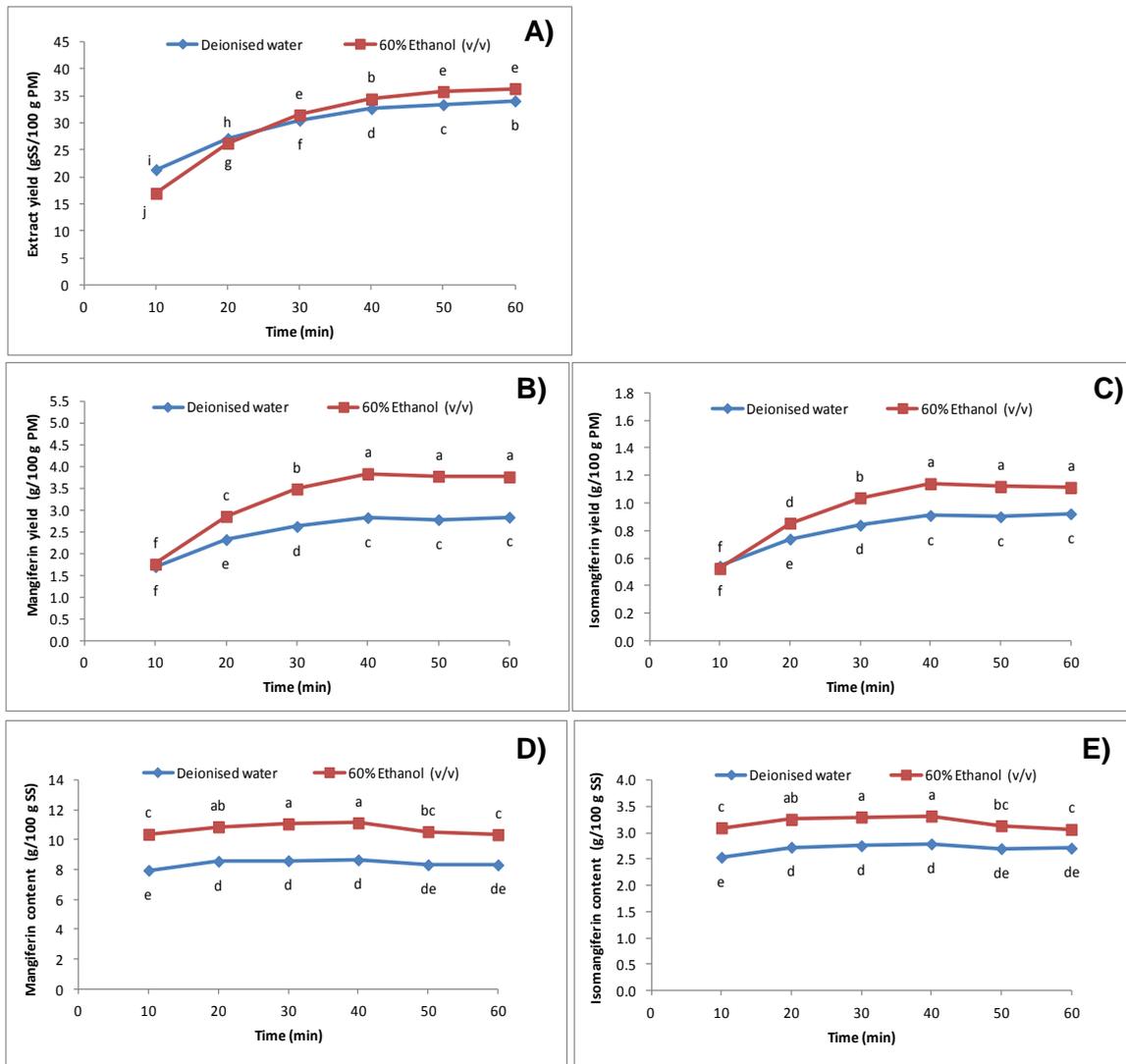


Figure 3.3 Effect of elevated solvent temperature ($^{\circ}\text{C}$) on extract yield (A), mangiferin yield (B), isomangiferin yield (C), mangiferin content of the extract (D) and isomangiferin of the extract (E). The ethanolic (60%, v/v) and aqueous extractions were performed at 70°C and 90°C , respectively. The teabag fraction was extracted for 30 min, using a solvent:solid ratio of 10 mL/g for both solvents (*SS* = soluble solids, *PM* = plant material).

3.4.2 Response surface optimisation of xanthone extraction

The single factor experiments were useful in determining the effect of particle size and showing that solvent composition is an important factor determining extraction of xanthenes from unfermented *C. genistoides*. For optimisation of the extraction of xanthenes from unfermented *C. genistoides* RSM was employed to evaluate the effect of temperature and EtOH concentration on the measured responses and to determine their interaction effects. A CCD was compiled and additional star points ($-\alpha$ and α) were calculated (Table 3.1). The extraction time was set at 30 min as a further increase in extraction time did not provide a substantial increase in extract or xanthone yield and the xanthone content of the extract remained constant, irrespective of extraction time (Fig. 3.2), as demonstrated with a single factor experiment. The solvent:solid ratio was also kept constant at 10 mL/g as for the single factor experiments. As isomangiferin is present in substantially lower quantities than mangiferin and followed the same trend in the RSM results, only the results obtained for mangiferin will be discussed in detail.

3.4.2.1 Analysis of RSM data

The results obtained from the CCD experiment are represented in Table 3.2. The response values varied from 12.18-35.81 g SS/100 g plant material for extract yield, 1.41-3.18 g mangiferin/100 g plant material for mangiferin yield and 8.55-13.04 g mangiferin/100 g extract for mangiferin content of the extract. The isomangiferin yield ranged from 0.52-3.18 g/100 g plant material and its levels in the extract from 2.29-4.23 g/100 g SS (Addendum A).

Using the experimental data, a regression analysis and ANOVA were performed to evaluate the suitability of the generated models. The significance of the linear, quadratic and interaction effects of parameters on each response was interpreted using standardised Pareto charts. Desirability profiling was used to determine the optimum extraction conditions for maximum response values. Verification experiments were performed to assess the predictive ability of the models.

Table 3.2 Central Composite Design and the response values for extract yield, mangiferin yield and mangiferin content of the extract

Run No.	Rep	X_1 Temperature (°C)	X_2		Extract yield ¹	Mangiferin yield ²	Mangiferin content in extract ³
			Ethanol concentration (% v/v)				
1	1	45	20		30.73	2.67	8.69
11	2	45	20		31.61	2.76	8.77
21	3	45	20		31.81	2.72	8.55
2	1	45	80		26.51	2.82	10.65
12	2	45	80		26.20	2.76	10.67
22	3	45	80		26.81	2.69	9.95
3	1	65	20		33.40	2.94	8.77
13	2	65	20		32.58	2.89	8.89
23	3	65	20		33.45	2.98	8.96
4	1	65	80		29.77	3.05	10.29
14	2	65	80		29.78	3.03	10.23
24	3	65	80		30.18	3.04	10.02
5	1	40	50		32.17	2.96	9.19
15	2	40	50		33.07	3.01	9.12
25	3	40	50		32.58	2.93	9.00
6	1	70	50		35.16	3.14	8.93
16	2	70	50		33.66	3.18	9.51
26	3	70	50		35.81	3.16	8.74
7	1	55	0		30.10	2.71	9.05
17	2	55	0		30.22	2.69	9.01
27	3	55	0		29.79	2.59	8.71
8	1	55	100		12.18	1.41	11.53
18	2	55	100		13.30	1.74	13.04
28	3	55	100		13.60	1.68	12.39
9(C) ⁴	1	55	50		34.06	3.09	9.04
19(C)	2	55	50		35.79	3.12	8.83
29(C)	3	55	50		34.93	3.03	8.72
10(C)	1	55	50		34.81	3.18	9.12
20(C)	2	55	50		34.84	3.02	8.72
30(C)	3	55	50		35.28	3.06	8.70

¹ g soluble solids/100 g plant material; ² g mangiferin/100 g plant material; ³ g mangiferin/100 g soluble solids; ⁴ (C) = Centre point value

Establishment of the regression equation

The response values for extract yield, mangiferin yield and mangiferin content of the extract were fitted as a function of EtOH concentration (% v/v) and temperature (°C). Using the regression coefficients generated by the ANOVAs, the prediction of each response variable can be obtained using the following quadratic regression equation to calculate, for example, mangiferin yield (g/100 g plant material):

$$Y = 2.3326 - 0.0016X_1 + 0.0001X_1^2 + 0.0284X_2 - 0.0004X_2^2 + 0.0001X_1X_2 \quad (3.3)$$

where Y is the mangiferin yield (g mangiferin/100 g plant material), X_1 is temperature ($^{\circ}\text{C}$) and X_2 is EtOH concentration (% v/v). Three second order polynomial equations were generated in total, one for each response. The estimated coefficients (linear, quadratic and interaction) for the regression equations of all responses are presented in Table 3.3-3.5.

Analysis of variance (ANOVA)

ANOVA was used to estimate the statistical significance of the parameters and interactions between them as well as the fit of the model to the data (Dejaegher & Vander Heyden, 2011). It compares the variance between different combinations of parameters (treatments) used with the variance due to random errors inherent in the measurements of the produced responses. The ANOVA results for extract yield, mangiferin yield and mangiferin content of the extract are presented in Tables 3.3, 3.4 and 3.5, respectively (see Addendum A for isomangiferin ANOVA results). The values of the parameter effects that were significant ($p < 0.05$) are highlighted in red.

The R^2 term measures the amount of variation around the mean that is explained by the model. The R_{adj}^2 is the R^2 term that has been adjusted for the number of terms included in the model and is useful to compare models with a different number of independent variables (Vázquez *et al.*, 2009; Martí-Calatayud *et al.*, 2010). The R_{adj}^2 is typically lower than the R^2 term. Guan & Yao (2008) reported that R^2 should be at least 0.8 for a model with a good fit. The R_{adj}^2 value for extract yield was 0.92, signifying that 92% of the variability of the responses was explained. The R_{adj}^2 values for mangiferin yield and mangiferin content of the extract were 0.76 and 0.92, respectively, indicating that 76% and 92% of the variation in the data were explained. Similar results were obtained for isomangiferin (Addendum A). These models, therefore, adequately represent the relationship between the responses and the independent variables tested (Tabaraki *et al.*, 2012).

To further test the suitability of the model, a LOF test was performed. This test compares the variability of the model residuals to the variability between observations at replicate settings of the factors. This is a more sensitive test of model fit than the R_{adj}^2 term as it uses the mean square (MS) pure error as the error term. Ideally, a model is well fitted to the data if it presents a significant regression and a non-significant LOF (Bezerra *et al.*, 2008). The models for extract yield and mangiferin yield exhibited a significant LOF ($p < 0.05$), indicating that there are unknown factors interfering with the test result. The model may be improved by identifying these factors and including them in the model (Tang *et al.*, 2011; Ruby-Figueroa *et al.*, 2011). For mangiferin content of the extract, a non-significant LOF was obtained ($p = 0.23$) indicating that this model is well-fitted to the data at the 95% confidence level.

Significance of parameters using standardised Pareto chart

Standardised Pareto charts show the linear, quadratic and interaction effects of each parameter presented in the ANOVAs for the dependant variables, i.e. the extract yield (Fig 3.4 A), mangiferin yield (Fig. 3.4 B) and mangiferin content of the extract (Fig. 3.4 C). The parameter effect is significant if its corresponding bar crosses the vertical line at the $p = 0.05$ level. The length of each bar is proportional to the absolute scale of the standard estimated effects. A negative value implies a negative parameter effect on the response value. The standardised Pareto charts for isomangiferin yield and isomangiferin content of the extract are presented in Addendum A.

Significant factors affecting all responses are the quadratic and linear effects of EtOH concentration. This is due to the change of solvent polarity with the change in EtOH concentration. The polarity of the EtOH-water mixture will increase with the addition of water, allowing more polar molecules to be extracted due to the 'like dissolves like' concept, i.e. solutes that can form hydrogen bonds are easily solubilised in solvents that can also form hydrogen bonds (Cheok *et al.*, 2012; Tabaraki *et al.*, 2012). For extract yield and mangiferin yield, the quadratic and linear effects of EtOH concentration had a negative effect on the response, whilst a positive effect was observed for the mangiferin content of the extract. The same trend was seen for isomangiferin (Addendum A). The single factor experiments (3.4.1.1) established that increasing EtOH concentration increases the mangiferin content of the extract due to the poor extraction of other compounds, especially non-phenolic compounds, at high EtOH concentrations. Solvent concentration has been found to be a significant factor in the extraction of xanthenes from mango seeds (Dorta *et al.*, 2013) and mango leaves (Zou *et al.*, 2013) using microwave assisted extraction; from mangosteen hull using conventional solvent extraction (as used in this study) (Cheok *et al.*, 2012) and mangosteen pericarp using ultrasonic assisted extraction (Yoswathana, 2013).

The linear effect of temperature had a significant, positive effect on the extract yield and mangiferin yield, indicating that an increase in temperature improves these yields due to its effect on extraction kinetics. Higher temperatures enhance mass transfer and thus the extraction rate due to the effect of temperature on vapour pressure, surface tension and viscosity of the liquid medium. In addition, higher temperatures can enhance the solubility of polyphenols and increase the diffusion coefficient thereby increasing the extraction rate (Al-Farsi & Lee, 2008; Ahmad-Qasem *et al.*, 2013). In previous RSM studies, temperature was found to have a significant effect on the extraction efficiency of polyphenols from grape pomace (Pinelo *et al.*, 2005), *Gynura medica* leaf (Liu *et al.*, 2010) and the flower of *Citrus aurantium* L. var (Yang *et al.*, 2010). It was also found to have a significant effect on the extract yield and hesperidin yield obtained with the ethanolic extraction of *C. maculata* by-

product (Du Preez, 2014). The quadratic effect of temperature was only significant for extract yield, where a small, negative effect was observed. Temperature had no significant effects on the mangiferin content of the extract. This lack of significant effects for temperature was also observed for isomangiferin (Addendum A). The interaction effect of EtOH and temperature was only significant for extract yield, where a small, positive effect was observed.

To summarise, temperature had a significant linear effect on extract and mangiferin yield, but not for the mangiferin content of the extract. In the latter case EtOH concentration showed a significant linear effect. For all parameters, EtOH concentration is the determining factor.

Table 3.3 ANOVA of experimental results for the polynomial quadratic equation for extract yield (*significant values ($p < 0.05$) are highlighted in red*)

Parameter ¹	Regr. Coeff. ²	SS ³	DF ⁴	MS ⁵	F	p
Intercept	12.642					
(1) Temperature (°C) (L)	0.532	25.912	1	25.912	76.337	0.000
Temperature (°C) (Q)	-0.005	3.251	1	3.251	9.577	0.005
(2) EtOH concentration (L)	0.329	417.348	1	417.348	1229.536	0.000
EtOH concentration (Q)	-0.005	538.759	1	538.760	1587.221	0.000
1L by 2L	0.001	2.025	1	2.025	5.967	0.024
Lack of fit (LOF)		70.761	3	23.587	69.489	0.000
Pure Error		7.128	21	0.3394		
Total SS		1181.693	29			
R ²						0.934
R ² _{adj}						0.920

¹ L = linear coefficient, Q = quadratic coefficient, L by L = interaction coefficient; ² Regression coefficients; ³ Sum of squares; ⁴ Degrees of freedom; ⁵ Mean square

Table 3.4 ANOVA of experimental results for the polynomial quadratic equation for mangiferin yield (*significant values ($p < 0.05$) are highlighted in red*)

Parameter ¹	Regr. Coeff. ²	SS ³	DF ⁴	MS ⁵	F	p
Intercept	2.333					
(1) Temperature (°C) (L)	-0.002	0.222	1	0.222	42.77	0.000
Temperature (°C) (Q)	0.000	0.001	1	0.001	0.174	0.681
(2) EtOH concentration (L)	0.028	0.816	1	0.816	157.124	0.00
EtOH concentration (Q)	-0.000	2.592	1	2.592	499.022	0.00
1L by 2L	0.000	0.003	1	0.003	0.579	0.455
Lack of fit (LOF)		1.003	3	0.334	64.354	0.00
Pure Error		0.109	21	0.005		
Total SS		5.622	29			
R ²						0.802
R ² _{adj}						0.761

¹ L = linear coefficient, Q = quadratic coefficient, L by L = interaction coefficient; ² Regression coefficients; ³ Sum of squares; ⁴ Degrees of freedom; ⁵ Mean square

Table 3.5 ANOVA of experimental results for the polynomial quadratic equation for mangiferin content of the extract (*significant values ($p < 0.05$) are highlighted in red*)

Parameter ¹	Regr. Coeff. ²	SS ³	DF ⁴	MS ⁵	F	p
Intercept	10.956					
(1) Temperature (°C) (L)	-0.086	0.004	1	0.004	0.038	0.847
Temperature (°C) (Q)	0.001	0.138	1	0.138	1.356	0.257
(2) EtOH concentration (L)	-0.091	23.879	1	23.879	234.102	0.000
EtOH concentration (Q)	0.001	9.400	1	9.400	92.152	0.000
1L by 2L	-0.000	0.150	1	0.150	1.467	0.239
Lack of fit (LOF)		0.471	3	0.157	1.540	0.234
Pure Error		2.142	21	0.102		
Total SS		37.715	29			
R ²						0.931
R ² _{adj}						0.916

¹ L = linear coefficient, Q = quadratic coefficient, L by L = interaction coefficient; ² Regression coefficients; ³ Sum of squares; ⁴ Degrees of freedom; ⁵ Mean square

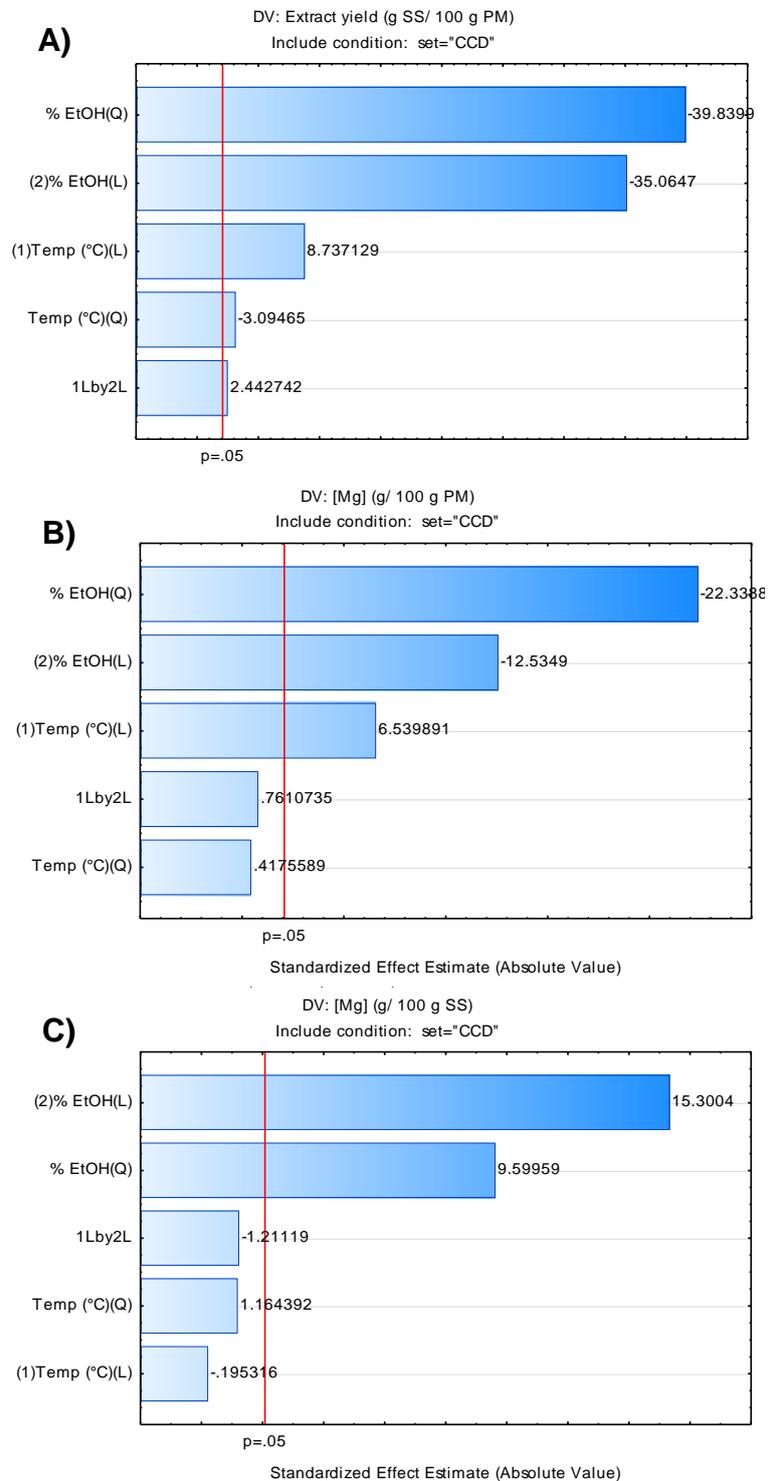


Figure 3.4 Standardised Pareto charts indicating the relationship of independent process variables on extract yield (A), mangiferin yield (B) and mangiferin content of the extract (C). (DV = dependant variable, L = linear effect, Q = quadratic effect, L by L = interaction effect).

Effect of parameters using combined response surface and contour plots

The combined three-dimensional response surface plots and two-dimensional contour plots for extract yield, mangiferin yield and mangiferin content of the extract, depicted in Figure 3.5 (A), (B) & (C), are graphical representations of the regression (predicted model) equations generated for each response. They illustrate the interaction between the two tested parameters and their relationship to the measured responses (Baş & Boyaci, 2007; Bezerra *et al.*, 2008; Tang *et al.*, 2011). These plots are drawn by maintaining one parameter value at the centre point whilst varying the other within the experimental range (Baklouti *et al.*, 2013). The response surface plot shows the magnitude of a response value as a result of the combined effect of two parameters at a particular time. The shape of the contour lines in the contour plots gives an indication of the significance of the interaction effect between factors. Although they are not as easily interpreted as the standardised Pareto charts, they perform a similar function. If the contour lines are elliptical then the interaction effect is significant, whilst circular lines indicate a negligible effect. Densely packed contour lines indicate that a change in extraction conditions has a prominent effect on the response values. It should be noted that although the plots give useful information about the model fitted, they may not be a true representation of the system as they represent contours of estimated response from a fitted model, rather than the true structure (Baş & Boyaci, 2007).

For extract yield, an optimum was obtained within the experimental domain. From visual inspection of the response surface (Fig. 3.5 A), maximum extract yield was obtained in the range 20-60% EtOH (v/v). As the concentration approached 0% (v/v) and 100% (v/v) on either side of this interval, lower extract yields were obtained. The same trend was observed in the single factor experiment (3.4.1.1). The plateau shape of the response curve relates to temperature, indicating that variation of its levels does not significantly affect the observed response (Bezerra *et al.*, 2008). The elliptical contour lines signify a prominent interaction between temperature and EtOH concentration. The fact that they are densely packed indicates that changes in these parameters greatly affected the extract yield.

An optimum for mangiferin yield was also obtained inside the experimental domain (Fig. 3.5 B). The response surface was near identical to that obtained for extract yield, with the optimum EtOH concentration between 20-60% (v/v) and a negligible effect of varying temperature on the response indicated by a plateau in the surface, which increased slightly towards 70°C. The densely packed contour lines indicate the prominent interaction effect between factors and their effect on the response. The same trend was observed for isomangiferin yield (Addendum A).

For the mangiferin content of the extract, an 'optimum' was obtained within the experimental domain (Fig. 3.5 C). However, this optimum relates to a minimum rather than a

maximum value indicated by the parabolic shape of the response surface (Bezerra *et al.*, 2008). Although no optimum was obtained for maximum mangiferin content in the extract, the response surface provides information regarding the region of maximum response. It is evident that as the EtOH concentration increased from the minimum point at ca 20% (v/v) to 100% (v/v), the mangiferin content of the extract increases. The effect of temperature is more difficult to deduce from the response surface with the minimum region being between 40-60°C. Therefore, to increase the mangiferin content of the extract, temperatures above this region should be used. Again, the elliptical contour lines are densely packed, indicating a prominent interaction effect between EtOH concentration and temperature and their effect on the response. The same trend was observed for isomangiferin content of the extract (Addendum A).

All the response surfaces show steep slopes relating to EtOH concentration and a plateau for temperature. This indicates that changes in EtOH concentration greatly affect the responses, whilst changes in temperature have a negligible effect.

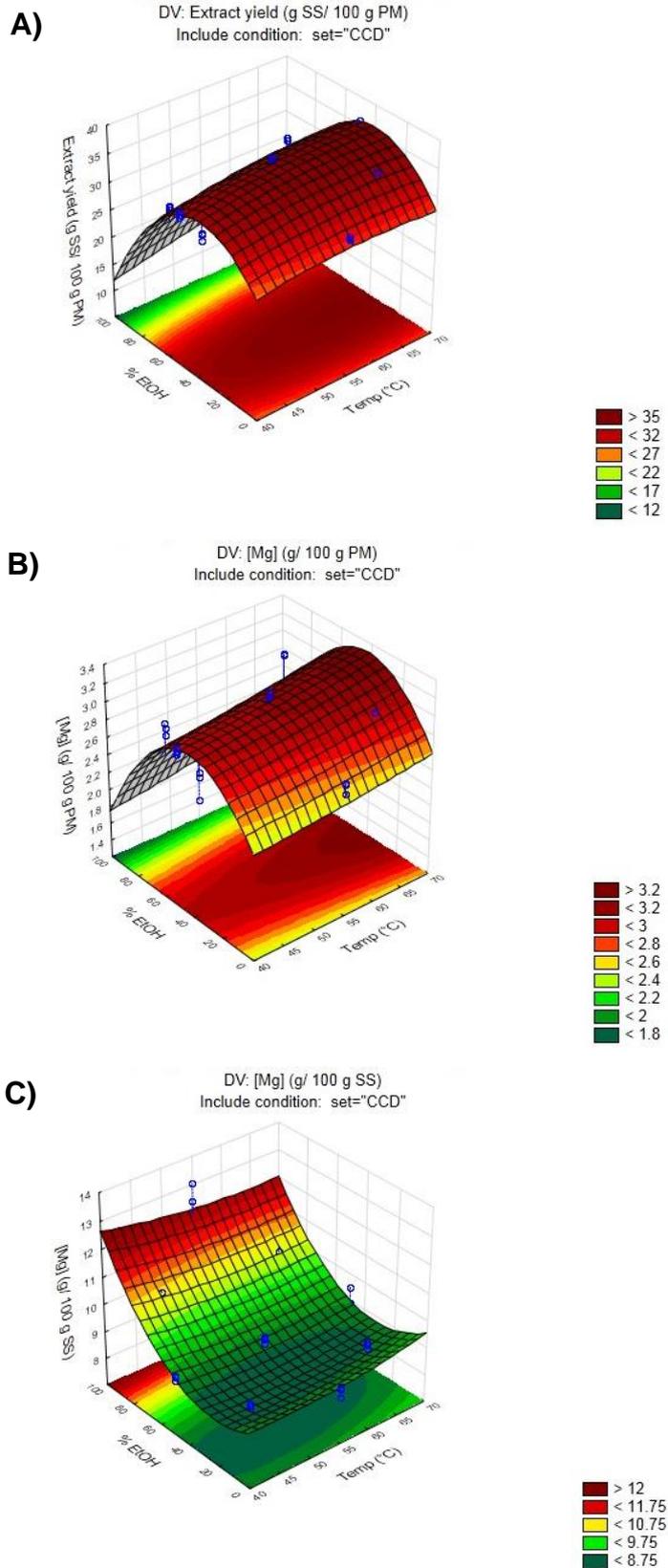


Figure 3.5 Combined fitted response surface plots for extract yield (A), mangiferin yield (B) and mangiferin content of the extract (C) as a function of temperature (°C) and ethanol concentration (% v/v). Experimental points are represented in blue. (DV = dependant variable).

3.4.2.2 Optimisation of extraction parameters (desirability profiling)

Desirability profiling identifies a desirability function for each response (dependant variable) and is specified by assigning predicted values a score ranging from 0 (very undesirable) to 1 (very desirable). When profiling the desirability of each response, a series of graphs is generated, one for each parameter tested. The overall desirability scores of a parameter are plotted whilst holding the levels of the other parameters constant at a specified value. The desirability profile indicates which level of the predictor variables (in this case temperature and EtOH concentration) produces the most desirable predicted responses on the dependant variables (extract yield, mangiferin yield and mangiferin content of the extract). In desirability profiling, responses can be maximised, minimised or held at a constant value. Confidence or prediction intervals (indicated in blue in Fig. 3.6-3.8) are used to aid in the assessment of the reliability of prediction.

In this study, extract yield and mangiferin yield were maximised and prediction profiles for these responses were compiled (Fig. 3.6 & 3.7). The desirability plot for mangiferin content in the extract is not included in desirability profiling due to the fact that this response was optimised in terms of a minimum rather than maximum value. The desirability plots for mangiferin content in the extract and isomangiferin are presented in Addendum A.

For the individual desirability profiles of extract and mangiferin yield, optimal EtOH concentrations of 40% and 60% (v/v), respectively, were calculated indicated by the apex of the desirability curves (Fig. 3.6 & 3.7). For temperature, optimum extract and mangiferin yields were achieved at 67°C and 70°C, respectively, indicated by a plateau in the desirability curve. Although higher temperatures may have improved these responses, the temperatures used did not exceed 70°C to ensure the boiling point of ethanol (78°C) was never reached. In both desirability profiles, the steeper slopes of the EtOH curves indicate that this factor had a greater effect on the extract and mangiferin yield than temperature – the same conclusion drawn from the standardised Pareto charts and response surface plots.

A compound prediction profile graph (Fig. 3.8) was compiled that shows the prediction profiles for extract and mangiferin yield. It indicates the level of the parameters tested that will simultaneously maximise these two responses. In this profile, extract and mangiferin yield were given equal weight. From these graphs it is evident that an EtOH concentration of 40% (v/v) and a temperature of 70°C are the optimum extraction conditions for maximum extract and mangiferin yield.

For this study, the optimum parameter values for the laboratory-scale preparation of a xanthone-enriched extract from unfermented *C. genistoides* were selected based not only on the desirability profiling of the responses, but also on literature findings, industrial application and practicality, as will be discussed in section 3.4.3.3.

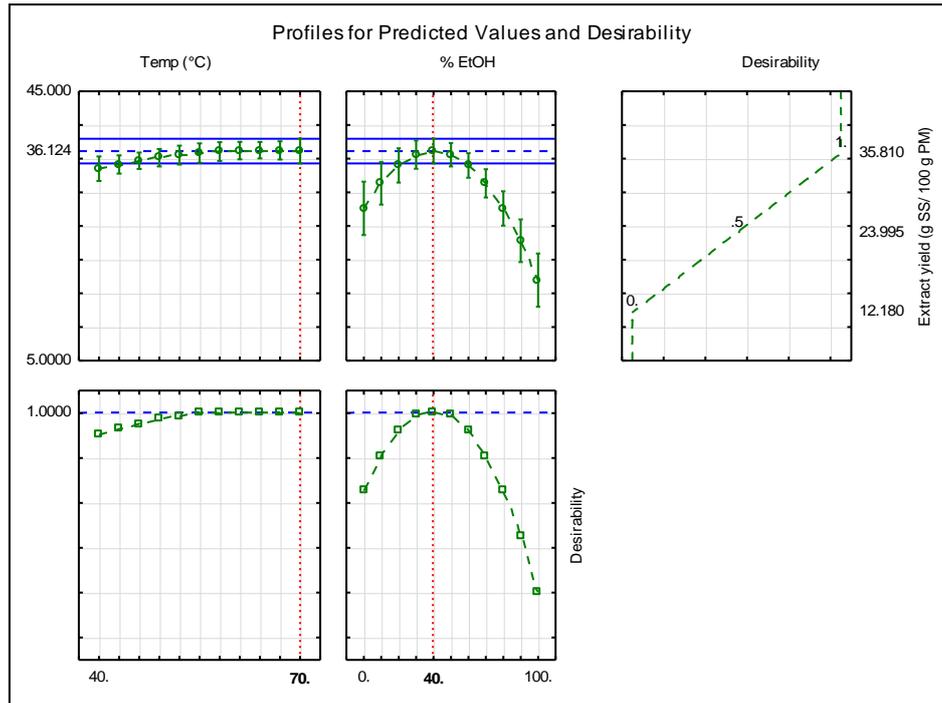


Figure 3.6 Individual prediction profile graph for extract yield with optimum ethanol concentration at 40% (v/v) and optimum temperature of 70°C for maximum response.

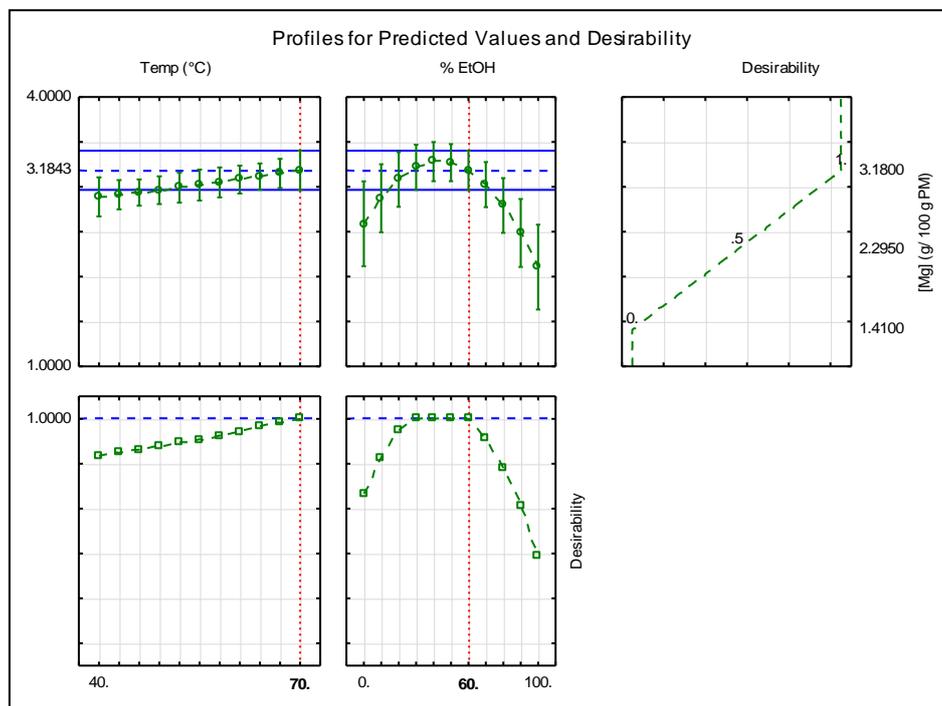


Figure 3.7 Individual prediction profile graph for mangiferin yield with optimum ethanol concentration at 60% (v/v) and optimum temperature of 70°C for maximum response.

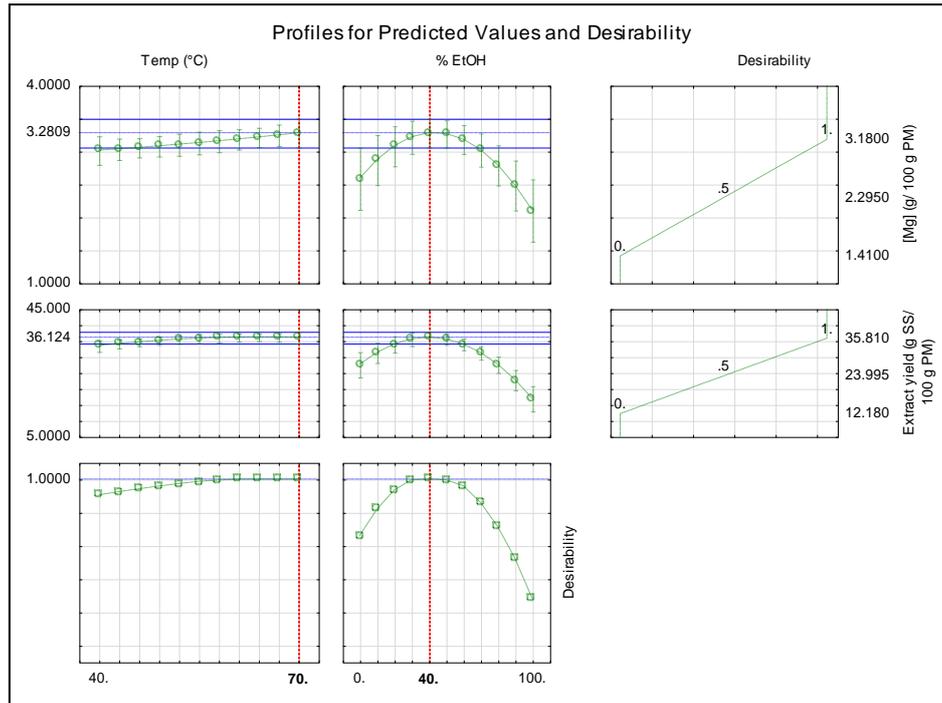


Figure 3.8 Compound prediction profile graph that shows the combined desirability profiles for extract yield and mangiferin yield with maximum responses obtained with ethanol concentration at 40% (v/v) and 70°C for maximising both responses.

3.4.2.3 Verification of predicted values of the models

Nine additional experiments were performed to verify the model, i.e. to assess how well these experimental results will fit the models already generated in this study. The parameters of each experimental run, as well as the response values obtained, are presented in Table 3.6. The ICC was used to evaluate how well the model fits the experimental data. The ICC statistic is a measure of the reliability of quantitative measurements. In this study, reliability refers to the reproducibility of the measurement when it is randomly repeated. ICC (agreement) takes into account the standard error of measurement (SEM), i.e. any bias that may have occurred. ICC (consistency) is a less sensitive statistic that excludes the SEM. Both values range from 0-1. As the values approach 1 the model fitting improves, i.e. if the value is close to 1, the model can be used to successfully predict the response values.

The distribution of the predicted values vs. the verification results are presented in scatter plots (Fig. 3.9). Both extract yield and mangiferin content of the extract displayed satisfactory ICC values. For extract yield, ICC (agreement) and ICC (consistency) values of 0.77 and 0.76, respectively, were obtained (Fig. 3.9 A). For mangiferin content of the extract, values of 0.75 and 0.80, respectively, were obtained (Fig. 3.9 C). These values indicate that the models have reasonably good predictive ability for use in quality assessment. However, if the models are to be used for large-scale cost/profit predictions, greater accuracy is required with ICC values as close to 1 as possible. Mangiferin yield displayed poor ICC (agreement) and ICC (consistency) values of 0.14 and 0.15, respectively (Fig. 3.9 B). This could be due to the fact that the small overall variation between different treatments (levels of factors) tested, resulted in a reduced margin for error. Although the model generated could successfully explain the relationship between parameters and their effect on the response (R_{adj}^2), the model has poor predictive ability which may be improved by incorporating more factors into the model as its significant LOF value suggests.

Bland-Altman plots are used to analyse the agreement between the predicted and observed values and the presence of bias within the measurements. The average of the observed and predicted values is displayed on the x -axis whilst the difference between the observed and predicted values is displayed on the y -axis (Fig. 3.10). Ideally, experimental results are scattered closely around the mean and between the 95% limits of agreement. This indicates a small difference between the experimental and predicted results. The position of the mean is an indication of bias. If no bias occurs within the quantitative measurements, then the mean will lie at 0 on the y -axis. In the Bland-Altman plots for all responses, the experimental points are scattered on either side of the mean and between the 95% limits of agreement. The means are slightly above/below 0 indicating a slight bias

due to random variation. For mangiferin yield and mangiferin content of the extract, a minor over-estimation was evident as the mean values are just above 0. This is also evident in the fact that the ICC (agreement) was slightly lower than the ICC (consistency) for both these responses. The scatter plots and Bland-Altman plots for the experimental data used to generate the prediction models are presented in Addendum A.

Table 3.6 Observed and predicted results obtained from the Central Composite Design experiment used for verification of the prediction model

Run No.	X_1 Temperature (°C)	X_2 Ethanol concentration (%, v/v)	Extract yield (g SS ¹ /100 g plant material)		Mangiferin yield (g mangiferin/100 g plant material)		Mangiferin content of the extract (g mangiferin/100 g SS)	
			<i>Observed</i>	<i>Predicted</i>	<i>Observed</i>	<i>Predicted</i>	<i>Observed</i>	<i>Predicted</i>
1	61	28	33.24	35.58	2.96	3.11	8.90	8.60
2	49	29	32.75	34.83	3.07	3.02	9.37	8.51
3	64	33	34.55	36.05	3.09	3.18	8.96	8.65
4	47	42	33.22	34.77	3.06	3.07	9.22	8.69
5	52	56	33.02	33.78	3.00	3.04	9.08	9.07
6	55	67	31.94	31.40	3.05	2.91	9.55	9.56
7	46	69	29.82	29.25	3.02	2.79	10.11	9.83
8	49	69	31.95	29.84	3.07	2.81	9.62	9.76
9	52	74	30.00	28.54	2.97	2.73	9.90	10.02

¹ Soluble solids

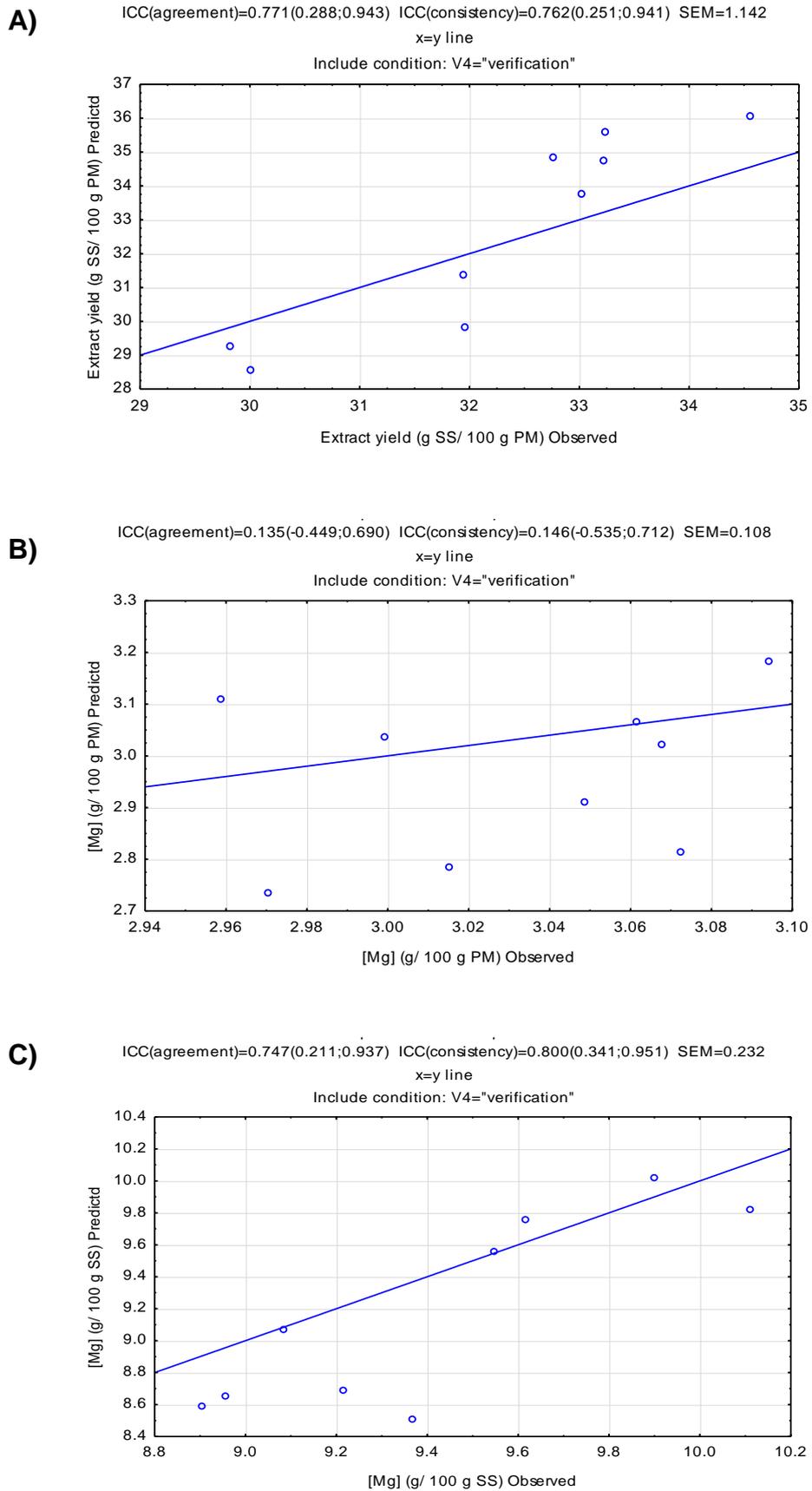
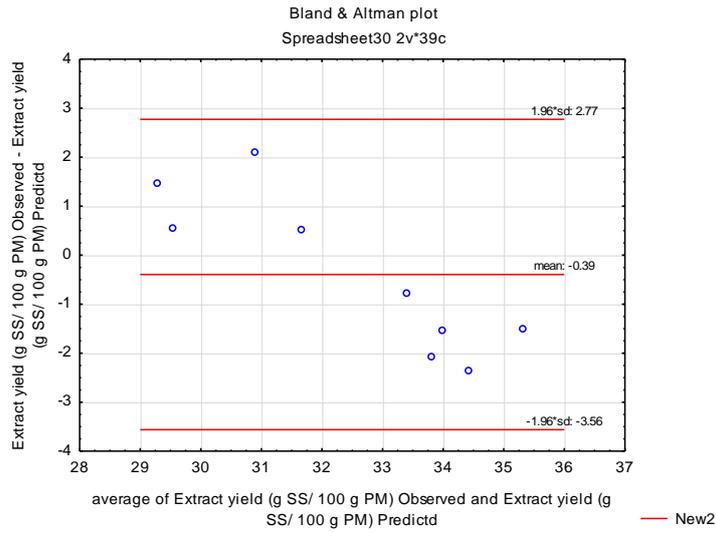
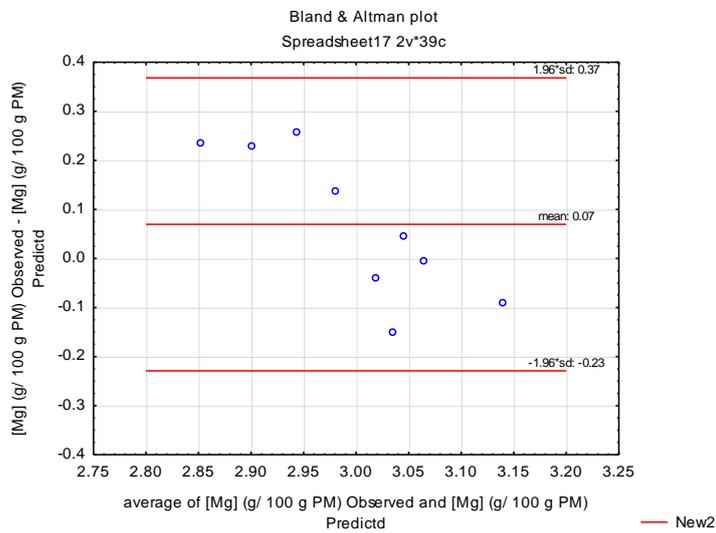


Figure 3.9 Scatter plots of the predicted vs. verification results for extract yield (A), mangiferin yield (B) and mangiferin content in the extract (C).

A)



B)



C)

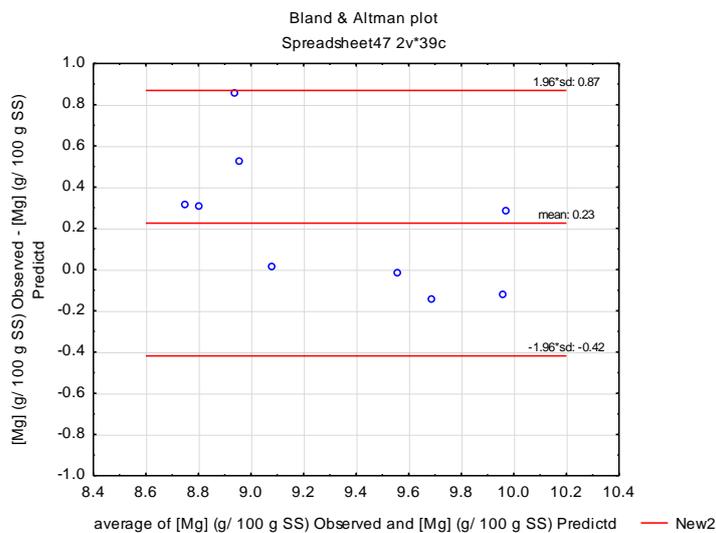


Figure 3.10 Bland-Altman plots for data from the verification experiments for extract yield (A), mangiferin yield (B) and mangiferin content in the extract (C).

3.4.2.4 Practical optimum extraction parameters

Solvent:solid ratio is an important factor that affects extraction (Prasad *et al.*, 2012), however, due to cost implication for industrial scale production, a solvent:solid ratio of 10 mL/g was selected for all extraction experiments as it is used for industrial preparation of hot water honeybush extracts (Joubert *et al.*, 2011). Single factor experiments further showed that maximum extraction is achieved after 30 min when using milled plant material and that the highest extract yield was obtained with 40% EtOH (v/v). Although the highest mangiferin yield was obtained using 60% (v/v) EtOH, the mangiferin yield obtained with 40% (v/v) EtOH did not differ significantly ($p \geq 0.05$). Using RSM to optimise extraction in terms of EtOH concentration and extraction temperature and to allow for interaction, desirability profiling showed an optimum of 40% EtOH (v/v) and 70°C to simultaneously optimise extract and mangiferin yield. These conditions would also provide an extract with satisfactory mangiferin content. In addition, maximum isomangiferin and iriflophenone-3-C-glucoside yields were obtained with 40% (v/v) EtOH (Addendum A). Similarly, Zou *et al.* (2013, 2014) found optimum EtOH concentrations for maximum mangiferin yield using conventional solvent extraction, MAE and UAE extraction of mango leaves to be 40%, 44% and 45% (v/v), respectively. The EtOH concentration of the extraction solvent is an important economical consideration for industrial application and the use of 40% EtOH (v/v) instead of 60% EtOH (v/v) would lower solvent requirements and thus production cost. Optimum extract yield is also of importance as it also directly impacts on production cost (D Malherbe, Afriplex, Paarl, South Africa, personal communication).

The highest temperature tested in the present study, 70°C, was selected as the optimum as extract and mangiferin yield increased linearly with temperature, although the effect of temperature was not as pronounced as that of solvent composition. Studies on other plant material (Yang *et al.*, 2010; Ito *et al.*, 2012; Zou *et al.*, 2014) provide evidence that elevated temperatures improve the extraction efficiency of EtOH/water mixtures when extracting polyphenols, including mangiferin, from botanicals. Since isomangiferin demonstrates identical trends to mangiferin during extraction, the factors chosen to optimise mangiferin extraction will automatically optimise isomangiferin extraction and thus xanthone extraction. The present study, to our knowledge, is the first to address optimisation of mangiferin extraction from *Cyclopia*, as well as isomangiferin extraction from plant material.

3.5 Conclusion

Preliminary experiments indicated that milled plant material extracted with an EtOH/water mixture for 30 min would allow for the best xanthone extraction. RSM successfully optimised the EtOH concentration (% v/v) and temperature (°C) to be used for maximum xanthone recovery and indicated that EtOH concentration had a greater effect than temperature on the extraction efficiency of the measured responses. The optimised extraction conditions for the preparation of a xanthone-enriched extract from *C. genistoides* are thus 40% EtOH (v/v), 10 mL/g, 70°C and 30 min based on the findings of this study and their industrial viability. Reliable second-order polynomial regression models were developed for the prediction of extract yield and mangiferin content of the extract as indicated by the ICC. These models would find future application in the industrial production of *C. genistoides* extracts as a method of standardisation and quality control.

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Chapter 4

Optimisation of ultrafiltration process parameters for xanthone enrichment of unfermented *Cyclopia genistoides* extract using response surface methodology

4.1 Abstract

This study investigated the potential of ultrafiltration (UF) for the enrichment of xanthenes in unfermented *C. genistoides* extracts (40% ethanol (EtOH), v/v). Laboratory-scale UF experiments were performed using centrifugal (3, 10, 30 kDa) and stirred cell (10, 30 kDa) membrane devices to determine the most suitable molecular weight cut off (MWCO), membrane material (polyethersulphone (PES) vs. regenerated cellulose (RC)) and feed concentration (1% vs. 3% soluble solids (SS)) for maximum productivity and xanthone enrichment. RC membranes of 10 kDa and 30 kDa were selected for further investigation due to their superior permeability and xanthone enrichment compared to PES membranes. Testing these membranes in a tangential flow ultrafiltration (TFU) system revealed that the 10 kDa RC membrane achieved better mangiferin enrichment with similar productivity and a lower fouling index compared to the 30 kDa RC membrane for both 1% and 3% SS feed concentrations. Further optimisation of UF was therefore undertaken with 3% SS unfermented *C. genistoides* extract (40% EtOH, v/v) through a 10 kDa RC membrane, using response surface methodology (RSM), to evaluate the individual and interaction effects of transmembrane pressure (TMP, 0.82-2.04 bar) and feed flow rate (200-444 mL/min). A Central Composite Design (CCD) consisting of 20 experimental runs was performed. The optimised responses were average permeate flux ($\text{kg/m}^2\cdot\text{h}$), mangiferin enrichment (%) and fouling index (%). The response values varied from 6.91-24.94 $\text{kg/m}^2\cdot\text{h}$ for average permeate flux, 13.1-26.4% for mangiferin enrichment and 3.7-14.9% for the fouling index. The individual effects of TMP and feed flow rate on all measured responses were significant, while their interaction only had a significant effect on average permeate flux and the fouling index. From the experimental data, regression models for average permeate flux, mangiferin enrichment and fouling index of the extract were generated with R_{adj}^2 values of 0.97, 0.95 and 0.65, respectively. The models obtained for average permeate flux and mangiferin enrichment adequately fit the experimental data, whilst the model for the fouling index only accounted for 65% of the observed variation. The optimal TMP and feed flow rate values of 2.04 bar and 444 mL/min were determined by visual inspection of the response surface plots. The validation of the optimised UF parameters on *C. genistoides* extracts (40% EtOH, v/v) of varying feed composition (6.8-11.8% mangiferin; 1.75-3% isomangiferin) resulted in average mangiferin and isomangiferin enrichments of 20% and 22%, respectively, resulting in extracts having 10.6-17.8% xanthone content.

4.2 Introduction

The incorporation of novel, health-promoting ingredients into food and beverage products is on the rise due to growing consumer demand for bioactive-enriched foods (BEFs) in response to increased incidence of metabolic diseases (Siró *et al.*, 2008). The growing awareness of the link between diet and health is causing consumers to turn to 'self-medication' through food and beverage consumption as a means of prevention rather than cure (Crespo & Brazinha, 2010). One category of nutraceuticals that has gained considerable interest amongst food manufacturers due to their potential health benefits associated with many non-communicable diseases is the 'antioxidant' category (Daniells, 2009).

Cyclopia genistoides (honeybush) extracts are a good source of potent antioxidants, especially the regio-isomeric xanthenes, mangiferin and isomangiferin. Evaluated in different antioxidant assays, mangiferin has been shown to be one of the most active antioxidants of the *Cyclopia* polyphenols tested (Hubbe & Joubert, 2000; Joubert *et al.*, 2008; Malherbe *et al.*, 2014). It encompasses many health-promoting properties including antidiabetic, anti-inflammatory and anticancer activities (Vyas *et al.*, 2012; Chellan *et al.*, 2014; Wang *et al.*, 2014). In addition to numerous health benefits, a mangiferin-enriched extract has good potential as a functional food ingredient due to the non-toxic nature of mangiferin even at high levels (Jagetia & Baliga, 2005). The application of such an extract in food/beverage products can promote either general well-being or a specific bioactivity, for example antidiabetic (Miura *et al.*, 2001) or anti-obesity (Lim *et al.*, 2014) activity, so that it also fulfils the new market trend for condition-specific antioxidant extracts (Becker, 2013).

Membrane separations, such as ultrafiltration (UF), are pressure-driven techniques growing in popularity for the fractionation/concentration of bioactive compounds in various feed solutions ranging from waste streams (Lo *et al.*, 2005; Cassano *et al.*, 2011; Al Manasrah *et al.*, 2012; Conidi *et al.*, 2014) to high-value pharmaceutical extracts (Hossain, 2005; Xu *et al.*, 2005; Yoon *et al.*, 2006). They offer a 'greener' alternative to traditional separation techniques, operating under mild temperature and pressure conditions suitable for separating heat-sensitive, bioactive compounds (Galanakis *et al.*, 2010; Sun *et al.*, 2011). The main disadvantage encountered during UF is membrane fouling, which reduces the productivity of the process, shortens membrane life and causes an unpredictable separation (Ruby-Figueroa *et al.* 2011; Pap *et al.*, 2012; Zhao *et al.*, 2013). The extent of membrane fouling occurring during UF can be reduced by manipulating the operating parameters, including transmembrane pressure (TMP), feed concentration, temperature and cross-flow velocity (Cheryan, 1998; Shi *et al.*, 2005).

As the beneficial bioactivities of polyphenols have become clearer, UF is increasingly applied to the production/recovery of polyphenol-enriched fractions for possible nutraceutical application (Cassano *et al.*, 2008a; Galaverna *et al.*, 2008; Conidi *et al.*, 2011; Husson *et al.*, 2012). Kumar *et al.* (2012) performed successful separation of (-)-epigallocatechin-gallate (EGCG) from (-)-epigallocatechin (EGC), using a two step aqueous extraction followed by UF. The molecular weights of these compounds are 458.4 g/mol and 306.3 g/mol, respectively, thus in the same range as that of mangiferin and isomangiferin (422.33 g/mol), suggesting the potential of UF for enrichment of this compound in honeybush extracts.

In this study, the use of UF was investigated for the enrichment of xanthenes in an extract of unfermented *C. genistoides* to develop a natural product suitable for nutraceutical and/or functional food applications. Laboratory-scale UF experiments were performed using centrifugal membrane units, a stirred cell device and a tangential flow flat sheet membrane cassette to evaluate the effect of membrane material, molecular weight cut off (MWCO) and operating parameters on membrane performance and permeate quality. Response surface methodology (RSM) was subsequently performed to further optimise UF operating parameters, using the tangential flow system. The latter system allows direct scale up to manufacturing scale. The optimised parameters were validated by UF of extracts made from 10 different batches of unfermented *C. genistoides* plant material of ranging phenolic composition to simulate industrial circumstances.

4.3 Materials and methods

4.3.1 Chemicals

Authentic reference standards for HPLC analysis, HPLC solvents and water purification have been described in Chapter 3 (section 3.3.1). Deionised water was used in combination with ethanol (EtOH) for the solvent preparation of all performed extractions and for the reconstitution of freeze-dried plant material. For cleaning of the Pellicon[®] 2 mini membrane cassette, deionised water, EtOH and a 0.1 N sodium hydroxide (NaOH) ($\geq 97\%$) (Sigma-Aldrich, St. Louis, MO, USA) solution (m/v) was used in sequence.

4.3.2 Plant material

For UF trials using centrifugal and stirred cell devices, *C. genistoides* shoots (leaves and stems) from a potting trial at ARC Infruitec-Nietvoorbij, Stellenbosch, harvested in May 2013, were used. The plant material was mechanically cut into small pieces, dried to <7% moisture content, sieved and milled to a powder as described in Chapter 3, section 3.3.2. For all optimisation experiments using the tangential flow ultrafiltration (TFU) system, *C. genistoides*

shoots were harvested in July 2013 on the farm, Toekomst, near Bredasdorp (Overberg, Western Cape, South Africa). For process validation following optimisation, 10 batches of *C. genistoides* were harvested on the farm, Koksrivier, near Pearly Beach (Overberg, Western Cape, South Africa) in May 2014. The plant material, randomly harvested at Toekomst and Koksrivier, were from commercial plantations, established with seedlings. In both cases the shoots were dried whole after harvesting at 40°C in a temperature controlled drying tunnel (Continental Fan Works CC, Cape Town, South Africa) with forced air circulation to a moisture content of <7%. After drying, the plant material was coarsely pulverised to a powder, using a Retsch mill (Retsch, GmbH, Haan, Germany) equipped with a 1 mm sieve.

4.3.3 Extract preparation

Optimum extraction conditions, determined for xanthone recovery from plant material (Chapter 3), were employed to prepare extracts for the different experiments. Briefly, extraction was performed at 70°C for 30 min, using 40% EtOH (v/v) and 10 mL/g solvent:solid ratio, followed by filtration of the warm extract. Extracts subjected to centrifugal ultrafiltration and TFU validation experiments following optimisation by RSM, were prepared on the day of experimentation. Bulk extract was prepared for stirred cell and single factor TFU experiments to minimise variation between the composition of the initial feed. The bulk extract, after filtration, was evaporated at 45°C *in vacuo* to remove EtOH using a Buchi R-215 rotary evaporator, V-700 vacuum pump and V-855 vacuum controller (Flawil, Switzerland). The remaining concentrated aqueous layer was subsequently freeze-dried *in vacuo*, using both a VirTis Genesis freeze-drier and a bench top VirTis Advantage Plus freeze-drier (SP Scientific, Warminster, PA, USA), to accommodate the large volume of extract produced. The freeze-dried extract was used to prepare reconstituted extracts in 40% EtOH (v/v) at the desired soluble solids (SS) content as required for the stirred cell and TFU experiments.

4.3.4 UF systems

Centrifugal UF was performed, using Amicon® Ultra centrifugal filter units with Ultracel® membranes (Merck, Millipore) of regenerated cellulose (RC), having MWCOs of 3, 10 and 30 kDa. Transmembrane flow was achieved by centrifuging the filled devices at 7500 x g, using a bench top Universal 320R centrifuge (Andreas Hettich GmbH, Tuttlingen, Germany) with a 12 piece fixed angle rotor (35°).

For dead end membrane filtration (i.e. flow of feed solution perpendicular to the membrane surface), the Amicon® 8200 Stirred Cell unit and Ultracel® UF membrane discs (diameter of 63.5 mm) with a pressure limit of 4.8 bar were employed. RC and polyethersulphone (PES) membrane discs of 10 kDa and 30 kDa had an effective filtration

area of 27.8 cm² when placed inside the stirred cell device. In this case nitrogen gas from a gas cylinder was used to maintain the necessary pressure gradient to force the feed solution through the membrane, while magnetic stirring (IKA-Werke GmbH & Co. KG, Staufen, Germany) was used to minimise concentration polarisation.

TFU was achieved, using a Pellicon[®] 2 Mini cassette holder and 10 kDa and 30 kDa Ultracel[®] RC membrane cassettes (0.1 m²) (Merck, Millipore). The pressure limit of the cassettes was ca 7 bar. When not in use, the membranes were stored in a 0.1 N NaOH solution at 4°C. The feed solution was fed through the TFU system using a 520S peristaltic pump, equipped with a 520 LoadSure element (peak operating pressure of 2 bar) (Watson Marlow Pumps, Cornwall, England). In-line pressure gauges were used to measure the inlet and outlet pressure of the TFU system. A schematic of the stirred cell and tangential flow TFU systems are depicted in Figures 4.1 and 4.2, respectively, whilst the actual experimental set up of each membrane system including the centrifugal membrane devices is given in Addendum B.

4.3.5 Assessment of membrane performance and permeate quality using different laboratory scale UF devices

Experiments were performed using the centrifugal and stirred cell devices to select the most appropriate membrane for xanthone enrichment before testing selected membranes, using the TFU system. Different MWCOs, membrane materials, feed concentrations and TMPs were investigated. Testing started with centrifugal devices (feed capacity of 4 mL; no stirring), followed by up-scaling to the stirred cell device (feed capacity of 200 mL), and finally to the TFU system (feed capacity of 80 mL-10 L).

4.3.5.1 Centrifugal UF

One hundred mL of *C. genistoides* extract was prepared according to the optimised extraction conditions (refer to 4.3.3) and the SS content of the extract standardised to 0.5% by diluting with 40% EtOH (v/v). The centrifugal tubes without the membrane inserts were weighed before filtration. The inserts were then placed inside the tubes, 4 mL of the diluted extract was pipetted into each membrane insert (3, 10 and 30 kDa) and centrifuged at 25°C until ca 100 µL retentate remained in the 30 kDa membrane insert, and 250-400 µL remained in the 3 kDa and 10 kDa membrane inserts. After the removal of the insert, the mass of the membrane tube and collected permeate was recorded. Permeate samples were diluted five times and aliquots stored at -18°C until HPLC analysis. Each membrane size was tested in triplicate. The content of individual phenolic compounds present in the permeate was calculated as a percentage of its concentration in the initial extract and the results expressed as mean ± standard deviation.

4.3.5.2 Stirred cell UF

This experimental set up is illustrated in Figure 4.1. The initial feed was prepared by reconstituting the freeze-dried extract with 40% EtOH (v/v) to the required concentration. For each UF experiment, 60 mL of feed was used. The stirred cell device was placed on top of a magnetic stirrer inside a temperature-controlled laboratory oven at 30°C and the device attached to a nitrogen cylinder (Afrox, Johannesburg, South Africa). Once the initial feed had reached 30°C, the magnetic stirrer was switched on to setting 4 and the required pressure applied. The permeate was collected through a tube into a series of separate 10 mL screw-cap glass vials, one for each 10 min time interval until the desired volume concentration ratio (VCR) of 6 was reached, i.e. a 10 mL retentate remaining. VCR is defined as follows:

$$VCR = \frac{V_F}{V_R} \quad (4.1)$$

where V_F is the volume of initial feed used and V_R is the volume of retentate remaining after UF. The mass of permeate collected during each 10 min interval was recorded and the permeate flux (J) calculated as follows:

$$J = \frac{M_p}{t \cdot A} \quad (4.2)$$

where M_p is the mass of permeate collected (in kg) at time t (in hours) and A is the membrane area in m^2 . The SS content of the permeate samples, collected at 10 min intervals, was determined and aliquots containing a 10 x dilution were stored at -18°C until HPLC analysis. The enrichment was expressed as the percentage increase of the compound concentration in the permeate relative to that in the initial feed, calculated on a SS basis. Sixteen experiments, representing different combinations of MWCO, membrane material, feed concentration and pressure as summarised in Table 4.1, were performed in triplicate and results expressed as mean \pm standard deviation.

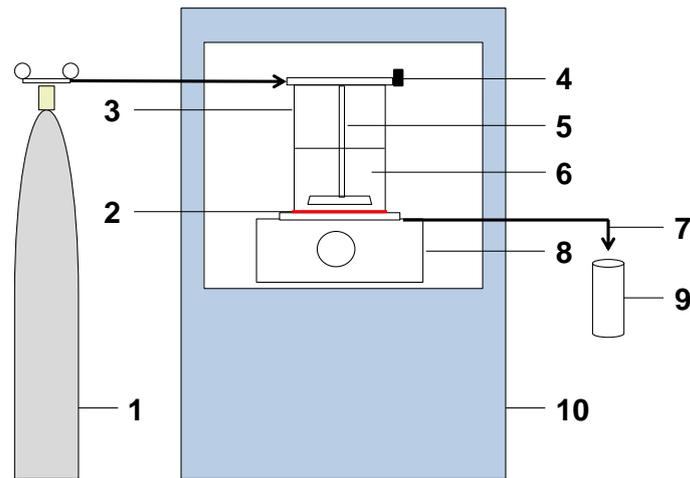


Figure 4.1 Schematic representation of the stirred cell ultrafiltration set up in batch concentration mode at 30°C: (1) nitrogen gas cylinder, (2) polymeric membrane disk, (3) filtration cell, (4) pressure release valve, (5) stirrer bar, (6) initial feed, (7) permeate flow, (8) magnetic stirrer, (9) permeate collection vial, (10) temperature controlled oven.

Table 4.1 Experimental conditions and membrane characteristics tested using stirred cell ultrafiltration of unfermented *C. genistoides* extract (40% ethanol, v/v)

Experiment	Membrane characteristics		Operating parameters	
	MWCO ¹ (kDa)	Material	Feed concentration (% SS ⁴)	Transmembrane pressure (bar)
1	10	PES ²	1	2
2	10	PES	1	4
3	10	PES	3	2
4	10	PES	3	4
5	10	RC ³	1	2
6	10	RC	1	4
7	10	RC	3	2
8	10	RC	3	4
9	30	PES	1	2
10	30	PES	1	4
11	30	PES	3	2
12	30	PES	3	4
13	30	RC	1	2
14	30	RC	1	4
15	30	RC	3	2
16	30	RC	3	4

¹ molecular weight cut off; ² polyethersulphone; ³ regenerated cellulose; ⁴ soluble solids

4.3.5.3 TFU

Batch concentration mode was employed for all TFU experiments. In this mode, the retentate was recycled to the feed tank and the permeate collected separately in order to assess the UF parameters and permeate quality (Fig. 4.2). The alternative is total recycle mode, where both the permeate and retentate are recycled back to the feed tank to assess membrane performance whilst keeping the volume and composition of feed constant. This

mode was used for initial pressure stabilisation of TFU runs and for membrane cleaning procedures.

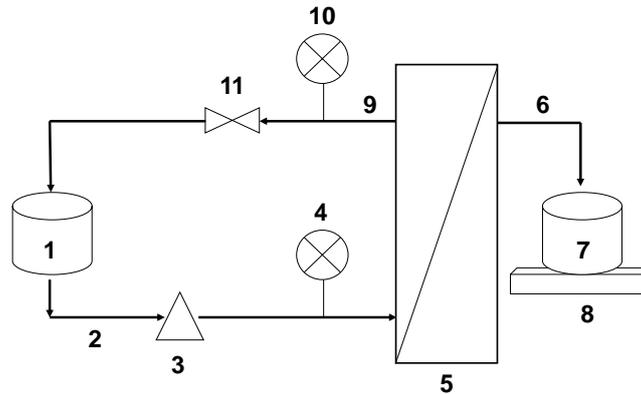


Figure 4.2 Experimental set up of the tangential flow ultrafiltration system in batch concentration mode: (1) feed container; (2) feed; (3) peristaltic pump; (4) manometer; (5) Pellicon[®] 2 Mini membrane cassette; (6) permeate; (7) permeate collection container; (8) digital balance; (9) retentate; (10) manometer; (11) retentate valve.

Measuring hydraulic permeability and fouling index

One litre of deionised water was flushed through the membrane at 260 mL/min with the retentate valve (11, Fig. 4.2) open to rinse the retentate side of the membrane. Another litre of deionised water was then flushed through the membrane at the same flow rate with an inlet pressure of ca 1.5 bar to rinse the permeate side of the membrane. After this, 2 L of deionised water was re-circulated through the system in total recycle mode at the same flow rate and inlet pressure for 10 min after which the initial water permeability was measured. This is done by recording the permeate flow rate (in mL/min) at four different transmembrane pressures (TMPs) ranging from 0.17-1.35 bar. The permeate flux is calculated according to Equation 4.2 using volume collected (in litres) rather than mass. The hydraulic permeability is given by the slope of the graph of permeate flux (L/m².h) versus TMP (bar) and is measured before UF, immediately after UF and after membrane cleaning. The fouling index can therefore be calculated by comparing the hydraulic permeability before and after UF according to the following equation:

$$Fouling\ Index = \left(1 - \frac{L_p^1}{L_p^0}\right) \times 100 \quad (4.3)$$

where L_p^0 and L_p^1 are the hydraulic permeabilities before and after treatment of unfermented *C. genistoides* extract, respectively. After membrane cleaning, between 80-100% of the initial hydraulic permeability was recovered, indicating an adequate cleaning procedure according to the manufacturer's specifications.

UF procedure

Freeze-dried bulk extract was reconstituted to the desired SS content in 40% EtOH (v/v) for preparation of 1.05 L initial feed. An aliquot of 50 mL was retained for SS and HPLC analyses and the mass of the remaining extract (1 L; initial feed) recorded. After the initial hydraulic permeability had been measured, air was flushed through the system to remove excess water. This was achieved by setting the pump at 260 mL/min and opening the retentate valve. The feed was then re-circulated in the system in total recycle mode at the selected feed flow rate (mL/min) and TMP. The TMP was set by partial closing/opening of the retentate valve. At each TMP, the permeate flow (mL/min) was recorded in batch concentration mode using a measuring cylinder until a steady flow rate was achieved. Two membrane sizes (based on MWCOs) were each tested at 5 TMPs in the range 0.3-1.7 bar and 3 feed flow rates, using two feed concentrations (Table 4.2). After testing all TMP x feed flow rate combinations with a particular feed, the same feed was then fed through the TFU system at a flow rate of 200 mL/min and TMP of ca 1.2 bar. The permeate flow was measured over time until a VCR of 5 was achieved. This was performed in order to test the effect of feed concentration and MWCO on the xanthone enrichment and membrane fouling.

Each membrane was tested in triplicate under the selected conditions and the results represented as mean \pm standard deviation. The SS content of the initial feed, permeate and retentate was determined and aliquots of each were diluted 10 times and stored at -18°C until HPLC analysis.

Table 4.2 Experimental conditions tested during the ultrafiltration of unfermented *C. genistoides* extract (40% ethanol, v/v) using Pellicon[®] 2 Mini membrane cassettes

Membrane characteristics		Operating parameters		
MWCO ¹ (kDa)	Material	Feed concentration (% SS ³)	Feed flow rate (mL/min)	Transmembrane pressure (bar)
10	RC ²	1, 3	200, 301, 401	0.3-1.7
30	RC	1, 3	200, 301, 401	0.3-1.7

¹ molecular weight cut off; ² regenerated cellulose; ³ soluble solids

Cleaning procedure

The cleaning procedure was performed according to the membrane manufacturer's recommendations. Once the hydraulic permeability of the membrane after UF had been measured, the membrane was rinsed with 2 L of 50% EtOH (v/v) at 260 mL/min with an inlet pressure of ca 1.5 bar. The retentate and permeate lines were directed to a waste container. After this, the system was flushed with 2 L of deionised water under the same conditions to prevent precipitation of the cleaning solution. Once complete, 2 L of 0.1 N NaOH solution was flushed through the system at a flow rate of 501 mL/min and an inlet pressure of ca 2 bar. The retentate and permeate lines were then redirected back to the cleaning solution

container and a further 1 L of fresh cleaning solution was re-circulated through the system for 60 min. The hydraulic permeability after cleaning was measured. According to manufacturer specifications, when repeatedly using the Pellicon[®] 2 mini membrane cassette, the hydraulic permeability measured after cleaning should not vary more than 10% from run to run, indicating an adequate cleaning procedure (Anon., 1998). When not in use, the membrane was stored in 0.1 N NaOH solution.

4.3.6 Optimising TFU of unfermented *C. genistoides* ethanolic extract using response surface methodology

4.3.6.1 TFU procedure

A feed concentration of 3% SS was selected and the initial feed (550 mL) prepared by re-constituting the freeze-dried extract in 40% EtOH (v/v). Of this feed, 500 mL was weighed and placed in a temperature controlled water bath set at 30°C to reach the operating temperature, whilst 50 mL was retained for SS and HPLC analyses. The initial water permeability before UF was measured after which air was flushed through the system as previously described (4.3.4.3). The temperature of the initial feed was monitored using an HI 9043 digital thermometer (Hanna instruments, Johannesburg, South Africa). Once at 30°C, the initial feed was re-circulated through the system in total recycle mode at the selected TMP for 2 min to allow the pressure to stabilise. The permeate line (6, Fig. 4.2) was then redirected to a beaker placed on top of a 1-decimal digital balance (Mettler PJ6000, Microsep, Cape Town, South Africa). The mass (g) of permeate collected was recorded every minute until ca 400 mL had been collected (VCR of 5). The permeate and retentate lines were then directed back to the feed container (1, Fig. 4.2) and air flushed to recover as much of the extract as possible. The hydraulic permeability after UF was measured. The mass and volume of the retentate and permeate were recorded. Samples were taken for SS and HPLC analyses (aliquots diluted 10 times and stored at -18°C). The membrane was then cleaned and the hydraulic permeability again measured (refer to 4.3.4.3).

4.3.6.2 Experimental design

A Central Composite Design (CCD) consisting of 20 experimental runs, which included 4 axial points, 12 factorial points and 4 central points, was used to optimise mangiferin and isomangiferin enrichment of the extract. The independent variables (factors) were TMP (bar) and feed flow rate (mL/min), while the dependent variables (responses) were permeate flux (kg/m².h), mangiferin enrichment (%) (or isomangiferin enrichment (%)) and fouling index (%). The levels of each factor were selected on the basis of single factor experimental results and equipment constraints (Table 4.3). Ten different TMP and feed flow rate combinations were tested in duplicate.

Table 4.3 Factors and their levels used in the Central Composite Design for optimising the ultrafiltration process parameters for the xanthone enrichment of unfermented *C. genistoides* extract (40% ethanol, v/v)

Factor	Symbol	Levels				
		- α	-1	0	1	α
TMP ¹ (bar)	X_1	0.82	1.00	1.43	1.86	2.04
Feed flow rate (mL/min)	X_2	158	200	301	401	444

¹ Transmembrane pressure

Experimental data from the CCD were fitted to the following second order polynomial equation and regression coefficients were calculated:

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{1 \leq i < j}^k \beta_{ij} X_i X_j + \varepsilon \quad (4.4)$$

where β_0 , β_i , β_{ii} , and β_{ij} are regression coefficients for intercept, linear, quadratic and interaction terms, respectively. Y is the response value (dependent variable) and X_i and X_j represent the level of the independent variables (factors). The term k represents the number of tested factors and ε the residual associated to the experiments. Nine additional verification experiments were performed to evaluate the predictive ability of the model by testing different TMP and feed flow rate combinations.

4.3.7 Validation of optimised UF parameters

Once the optimum UF conditions had been selected, it was decided to test the procedure on extracts made from 10 different batches of *C. genistoides* plant material to assess the variation of the obtained enrichment as affected by the xanthone content of the raw material used for extraction. This would give a better indication of the membrane performance when used in industry as it takes into account the natural variation in composition of the plant material. The TFU procedure and sample analyses were the same as used for the RSM TFU experiments (see 4.3.5.1). Each batch was tested in duplicate (20 experiments performed in total) and results expressed as mean \pm standard deviation.

4.3.8 Analysis of extracts - initial feeds and permeates

4.3.8.1 HPLC-DAD analysis

Quantification of the major phenolic compounds was performed in duplicate using reversed-phase high performance liquid chromatography-diode array detection (HPLC-DAD; refer to 3.3.5.1).

4.3.8.2 Determination of soluble solids content of *C. genistoides* extract

The SS content of all extracts, initial feed, retentate and permeate samples was determined as described in 3.3.5.2 with some minor adjustment for the stirred cell experiments. In the latter case 1.5 mL aliquots were evaporated in heat-resistant glass vials due to the small volumes recovered. Theoretical extract yield was calculated from the SS content determined, taking into account the extraction volume (and not the recovered extract volume) and mass of plant material used for extraction. Results were expressed as g SS/100 g plant material.

4.3.9 Statistical analysis

Univariate analysis of variance (ANOVA) was performed on all data obtained in the one factor at-a-time (OFAT) trials using SAS[®] software (Version 9.2, SAS institute Inc, Cay, USA) to determine whether differences between treatment means were significant. Least significant difference (LSD) of the Student's t test ($p = 0.05$) was calculated to compare treatment means where significant differences were found ($p < 0.05$). Levene's test was used to test for treatment homogeneity of variance. Where variances were not equal a weighted analysis of variance was used for the combined analysis.

Statistica 12.0 (Statsoft[®], Southern Africa Analytics (Pty) Ltd, Johannesburg, South Africa) was used to analyse all data generated by RSM (4.3.5). Statistical significance and suitability of the model, its factors and their interactions were determined at the 5% probability level ($p < 0.05$) using ANOVA. Standardised Pareto charts were compiled to illustrate the significant effects obtained from the ANOVA for the different response values. The regression function generated for each response was graphically illustrated as two-dimensional contour and three-dimensional response surface plots. The correlation coefficient (R^2), adjusted R^2 (R_{adj}^2) and the significance of the lack of fit (LOF) were used to evaluate the fitting efficiency of the data to the model. The predictive ability of the generated models, as indicated by the intraclass correlation coefficient (ICC), was determined by performing additional verification experiments.

4.4 Results and discussion

In this study different ultrafiltration systems were used to evaluate mangiferin and isomangiferin enrichment in 40% EtOH (v/v) extract of *C. genistoides*, starting with a small volume centrifugal device (4 mL), progressing to a stirred cell device (<200 mL) and ending with a tangential flow ultrafiltration system (≥ 500 mL). The systems not only differed in capacity, but the type of filtration, e.g. dead-end and tangential flow. Both the centrifugal and stirred cell devices made use of dead end filtration, but stirring of the feed in the stirred cell prevented concentration polarisation. Use of these devices was convenient to evaluate some UF parameters, in particular membrane type and MWCOs, as the membranes for the TFU system are expensive. The latter system, however, allowed process optimisation in a scaled-down format of the full-scale manufacturing process. Parameters obtained with the latter system are thus directly applicable in industry. Results obtained for mangiferin will be largely used to illustrate effects, however, similar trends were obtained for isomangiferin. These were mostly provided in Addendum B.

4.4.1 Assessment of membrane performance and permeate quality using different laboratory scale UF devices

4.4.1.1 Centrifugal UF

Figure 4.3 depicts the recovery of the major polyphenols, originally present in 40% EtOH (v/v) extract of unfermented *C. genistoides* (0.5% SS), in the permeate when using RC membranes of 3, 10 and 30 kDa MWCOs. From the experimental data, it is clear that the 30 kDa membrane recovered a significantly higher percentage ($p < 0.05$) of all quantified phenolic compounds in the permeate than the other membrane sizes tested. This is due to easier transmission through the larger pores of the 30 kDa membrane despite unavoidable membrane clogging during dead-end filtration. It can therefore be deduced that the smaller MWCO membranes had a greater retention of the phenolics in the retentate. The 3 kDa membrane resulted in the lowest recovery of compounds in the permeate, except for the two benzophenone glycosides, iriflophenone-3-C-glucoside and iriflophenone-di-O,C-hexoside, that were retained to the same extent by the 3 kDa and 10 kDa membranes ($p \geq 0.05$). The recovery of the di-O-C-hexoside was less than that of the mono hexoside. Overall the lowest recovery was observed for the dihydrochalcone glycoside, 3-hydroxyphloretin-3',5'-di-C-hexoside. Highest recoveries were observed for the xanthenes and iriflophenone-3-C-glucoside. From the data it is evident that MWCO and steric considerations alone are not responsible for the difference in permeation of compounds, but also factors such as membrane surface chemistry and fouling mechanisms which should be considered with regards to membrane selection (Evans *et al.*, 2008; Adams, 2012). Centrifugal UF

membranes have been used to separate low molecular weight (LMW) phenolic compounds (100-1000 g/mol) from high molecular weight (HMW) polymers such as proanthocyanidins (>1000 g/mol) in almond skin extracts (Prodanov *et al.*, 2008) and Pinotage wines (De Beer *et al.*, 2006). In both these studies, MWCOs of 30 kDa and 50 kDa showed a greater recovery of LMW phenols in the permeate than 10 kDa, whilst all these membrane sizes successfully retained polymeric substances likely to reduce permeate quality. Although this experiment was useful in assessing the effect of membrane size on the recovery of different major polyphenols present in *C. genistoides* ethanolic extract, focus of subsequent experiments was on the xanthenes. This is not only due to the fact that they are the most predominant polyphenols present in the extract, but also due to the interest in mangiferin as previously discussed.

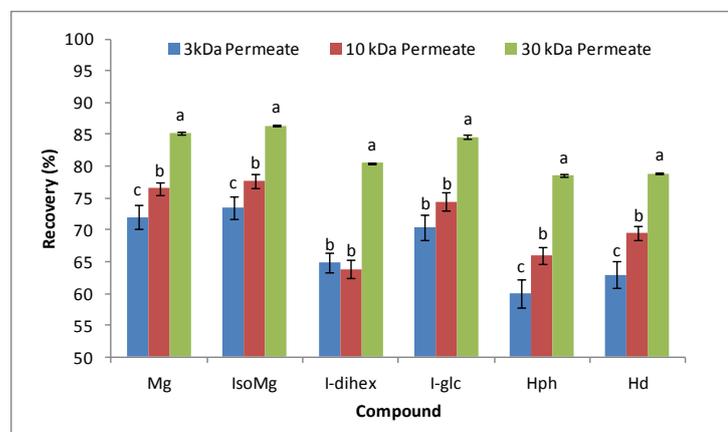


Figure 4.3 Recovery of major polyphenols in a 0.5% soluble solids (SS) unfermented *C. genistoides* extract (40% ethanol, v/v) after ultrafiltration using centrifugal regenerated cellulose membranes with molecular weight cut off values of 3, 10 and 30 kDa ($T = 25^{\circ}\text{C}$; Mg = mangiferin, IsoMg = isomangiferin, I-dihex = iriflophenone-di-O,C-hexoside, I-glc = iriflophenone-3-C-glucoside, Hph = 3-hydroxy-phloretin-3',5'-di-C-hexoside, Hd = hesperidin; mangiferin content of the feed = 10.83 g Mg/100 g SS; isomangiferin content of the feed = 3.2 g IsoMg/100 g SS; different letters for a compound indicate significant differences between values ($p < 0.05$); values represented as mean \pm standard deviation).

4.4.1.2 Stirred cell UF

Following centrifugal UF, a stirred cell device was used to further elucidate the effect of MWCO, but also to determine the effect of membrane type, TMP and feed concentration on membrane productivity and permeate quality. Two types of membranes (PES and RC) and two MWCOs (10 and 30 kDa) for each were tested.

Given the experimental set up and capacity of the stirred cell system, the membranes could be evaluated in terms of permeate flux under different conditions (initial feed concentration and TMP). The permeate flux over time was determined for each membrane in

batch concentration mode (Fig. 4.4). Permeate flux is important as it ultimately indicates the productivity of the UF process (Evans *et al.*, 2008; Al Manasrah *et al.*, 2012). Typically, the flux versus time curve is divided into two sections. The first section is a rapid decline in permeate flux due to the formation of concentration polarisation at the membrane surface. The second section reflects a smaller flux decline due to the achievement of a steady-state permeate flux (Cassano *et al.*, 2008b). The two sections are not easily distinguished in this study due to the fact that steady state permeate flux may not have been reached during the UF processing time, which was limited by the volume of the feed. Flux declined more or less linearly, but the RC membranes showed higher initial permeate fluxes accompanied by a more rapid flux decline than the PES membranes under all operating conditions (Fig. 4.4). This is in agreement with previous reports of Denis *et al.* (2009) and Cassano *et al.* (2011), showing that RC membranes had greater permeability than PES membranes during UF of a macro-algal extract and olive mill wastewaters, respectively. However, it is not the initial permeate flux, but rather the steady state permeate flux that will ultimately determine the productivity of the UF process.

The RC membranes took 50 min to reach a VCR of 6, whilst PES membranes took 70 min. A shorter processing time translates to economical savings on an industrial scale. The higher permeate fluxes obtained with the RC membrane (Fig. 4.4) is due to different membrane selectivity as a result of its hydrophilic nature (Cassano *et al.*, 2011). Different membrane selectivities were evident by visual inspection of the membranes after UF. The PES membrane had a thicker, fouling layer than the RC membrane and the colour was yellowish green compared to tan of the RC membrane (Fig. 4.5). PES membranes are hydrophobic and experience interactions with hydrophobic i.e. non-polar tea constituents, whilst RC membranes are hydrophilic and experience interactions with hydrophilic tea constituents. Ulbricht *et al.* (2009) found that PES membranes had the greatest adsorption of both polyphenols and polysaccharides to the membrane surface than other polymeric membranes tested during the microfiltration of wine. Similarly, Cassano *et al.* (2011) found that a 10 kDa PES membrane had a substantially greater fouling index than a 10 kDa RC membrane during UF of olive mill wastewaters. The adsorption of tea constituents on the membrane surface could also be due its roughness (Evans *et al.*, 2008). Evans *et al.* (2008) found that fluoropolymer (FP) membranes fouled more than RC membranes during the UF of black tea due to their increased hydrophobicity and surface roughness compared to RC membranes. It is important to note that although membrane fouling was not measured in this experiment, it has the ability to change membrane permeability and selectivity (De Bruijn *et al.*, 2002).

For further assessment of the membranes the average permeate flux (Fig. 4.6 A) rather than the steady state permeate flux was compared. This was to accommodate the

short process time due to the small feed volume preventing the onset of steady state permeate flux. The differences found between the average permeate fluxes obtained for 10 kDa and 30 kDa PES membranes for corresponding conditions were mostly insignificant ($p \geq 0.05$). The 30 kDa RC membrane achieved a significantly higher average permeate flux than the 10 kDa RC membrane ($p < 0.05$) under the same operating conditions. Conversely, Evans *et al.* (2008) reported similar average permeate fluxes for 10 kDa and 30 kDa RC membranes used during UF of black tea extract. For all membranes tested in the present study, except the 10 kDa RC membrane, average permeate flux was significantly increased when the pressure was doubled at constant feed concentration ($p < 0.05$). By increasing the pressure the driving force of solutes to the membrane surface is greater (Kumar *et al.*, 2012). Jayanti *et al.* (2010), also using a stirred cell device, demonstrated increasing permeate flux with increasing TMP (2.76-6.9 bar) during UF of green coconut water.

Increasing the feed concentration from 1% to 3% SS resulted in a significantly reduced permeate flux in most cases when all other factors remained constant. The only exception was the 10 kDa PES membrane operating at 4 bar where the reduction in permeate flux was not significant ($p < 0.05$) with an increase in feed concentration. Other studies have demonstrated a similar trend. An increasing feed concentration has reduced permeate flux during the UF of black tea, using a stirred cell device (Chandini *et al.*, 2012), as well as UF of grape juice (Kalbasi & Cisneros-Zevallos, 2007), *Ginkgo biloba* extract (Xu *et al.*, 2005), *Echinacea purpurea* extract (Hossain, 2005) and olive mill wastewaters (Gkoutsidis *et al.*, 2011), using tangential flow devices. For all pressure x concentration x MWCO combinations tested in the present study, a significantly greater permeate flux was achieved with the RC membranes, with the exception of the 10 kDa PES membrane using 3% SS feed and 4 bar ($p < 0.05$). The highest average permeate flux obtained with PES and RC membranes were 14.66 and 18.82 kg/m².h, respectively, using a 10 kDa membrane, 1% SS feed and 4 bar TMP. The reduction in permeate flux seen with the higher feed concentration is explained by the film theory, an engineering model that predicts flux decline according to mass transfer effects (Adams, 2012). According to this model, flux decreases exponentially with increasing feed concentration due to more blocking, both within the membrane pores and on top of the membrane surface (Cheryan, 1998).

Permeate yield is an important consideration as it determines the extent of enrichment and has direct economical implications. Permeate yield was represented as the percentage of permeated solids compared to the solids present in the initial feed (Fig. 4.6 B). The greatest permeate yield of 58% was achieved using a 30 kDa RC membrane, 1% SS feed and 2 bar TMP. For all membrane configurations tested, an increase in feed concentration from 1% to 3% SS significantly reduced permeate yield ($p < 0.05$). For the PES membranes, an increase in pressure significantly improved permeate yield, while the opposite was

observed for the RC membranes ($p < 0.05$), except for the 10 kDa RC membrane in combination with 3% SS feed, showing no significant effect for pressure ($p \geq 0.05$). Higher feed concentrations and pressures can promote membrane fouling. Based on the film theory, at pressures above 2 bar, employed in the present study, the flow of solutes is independent of pressure due to the solute depositions formed on the membrane (Cheryan, 1998; Charcosset, 2012). The ideal pressure for the 10 kDa RC membrane would therefore be closer to 2 bar to avoid a quick onset of membrane fouling and reduced productivity. On average, a significantly higher permeate yield for RC membranes was achieved with a MWCO of 30 kDa ($p < 0.05$), whilst for PES membranes, a slightly higher permeate yield was achieved with a MWCO of 10 kDa. This is a further confirmation that a larger pore size does not necessarily result in greater permeation as the larger pores can be more susceptible to blocking. This was observed during the UF of stevia extract, using a stirred cell device (Chhaya *et al.*, 2012) and the tangential flow UF of green tea using flat sheet membranes (Kumar *et al.*, 2012) where 30 kDa membranes allowed greater permeation than 100 kDa membranes.

The percentage enrichment of mangiferin and isomangiferin was calculated by comparing their content in the permeate and initial feed on a SS basis. The mangiferin enrichment ranged from 20.6-56.9%, whilst the isomangiferin enrichment ranged from 25.3-64.2% (Fig. 4.6 C & D). General trends were difficult to identify as each of the four membranes tested behaved differently under the investigated conditions, although similar trends were observed for mangiferin and isomangiferin enrichment.

Considering the PES membrane material, lowest mangiferin enrichment was observed for 10 kDa at 4 bar and 1% SS feed, followed by 30 kDa at 2 bar and 1% SS feed ($p < 0.05$). The highest enrichment was observed for 30 kDa at 4 bar and 1% SS feed ($p < 0.05$). For the RC membrane material, lowest mangiferin enrichment was obtained for 30 kDa and 1% SS feed, irrespective of TMP. The lowest values were not significantly different from the lowest enrichment obtained for the PES membrane type. Similarly, the highest mangiferin enrichment obtained for the RC membrane type (56.9% with 30 kDa, 4 bar and 3% SS feed) was not significantly different ($p \geq 0.05$) from the highest value obtained for the PES membrane type. The 10 kDa RC membrane was the only membrane that resulted in a greater mangiferin than isomangiferin enrichment for all operating conditions, again indicating that MWCO and steric considerations are not solely responsible for separation (Evans *et al.*, 2008). The 10 kDa RC membrane showed the most uniform xanthone enrichment under varying operating conditions.

From the stirred cell results it is evident that RC is the preferred membrane material as it allowed higher average permeate flux, permeate yield and xanthone enrichment compared to PES. It is, therefore, the more economical choice. Cassano *et al.* (2011) came to the

same conclusion during the UF of olive mill wastewaters, where RC membranes showed lower rejection towards polyphenols and higher permeate fluxes compared to PES membranes. In addition, RC performs better when using 3% feed concentration, close to that of undiluted extract produced by industry. A RC membrane was therefore selected for further testing using a tangential flow system. The effect of MWCO, feed concentration and pressure on process productivity and permeate quality were further investigated as the cross flow experienced in a tangential flow system would affect these parameters.

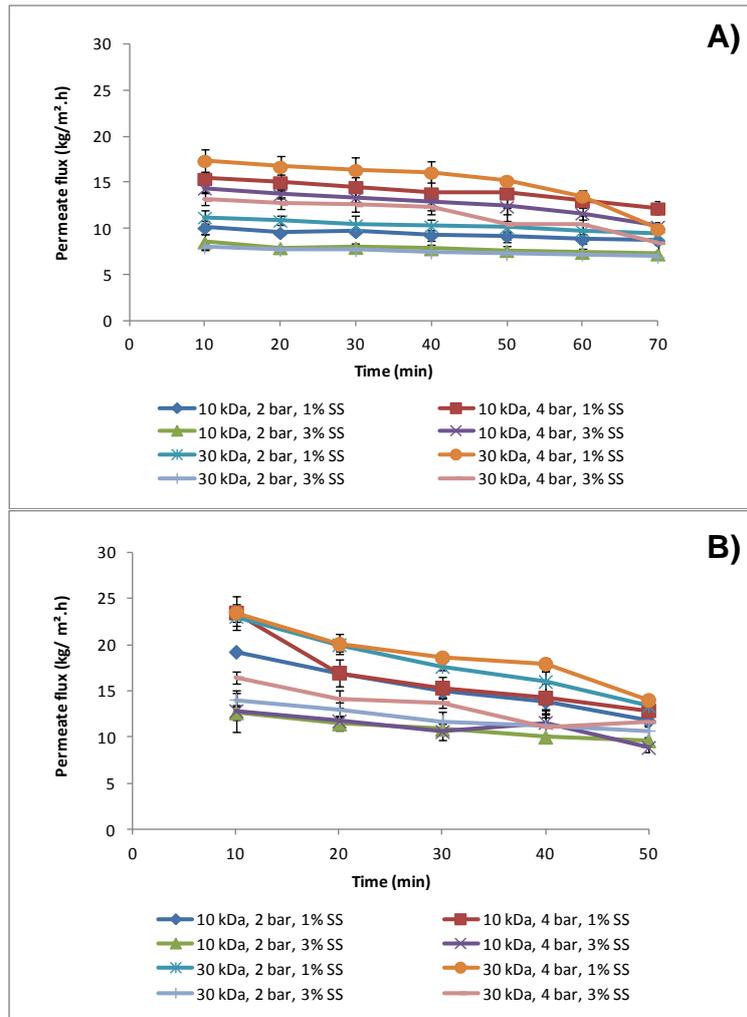


Figure 4.4 Permeate flux vs. time curves for unfermented *C. genistoides* extract (40% ethanol, v/v) through polyethersulphone (A) and regenerated cellulose (B) membranes under different ultrafiltration operating parameters using a stirred cell in batch concentration mode ($T = 30^{\circ}\text{C}$, $VCR = 6$; values represented as mean \pm standard deviation; SS = soluble solids).

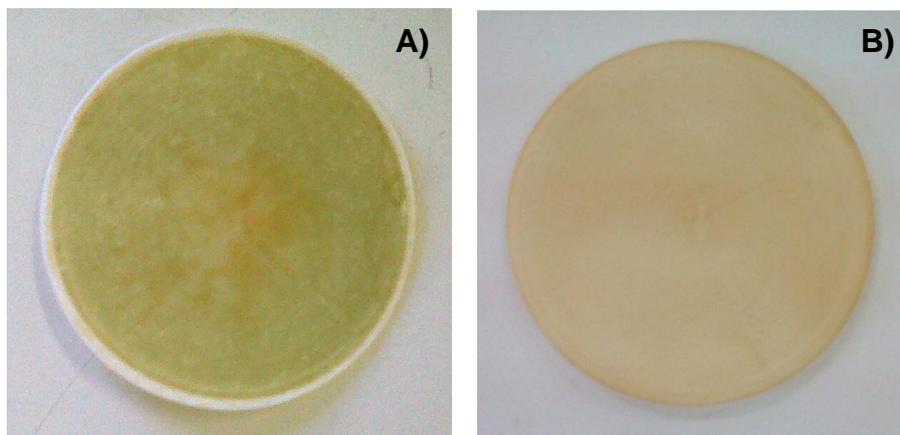


Figure 4.5 Polyethersulphone (A) and regenerated cellulose (B) membranes after ultrafiltration of unfermented *C. genistoides* extract (40% ethanol, v/v) using a stirred cell device in batch concentration mode ($T = 30^{\circ}\text{C}$; $VCR = 6$).

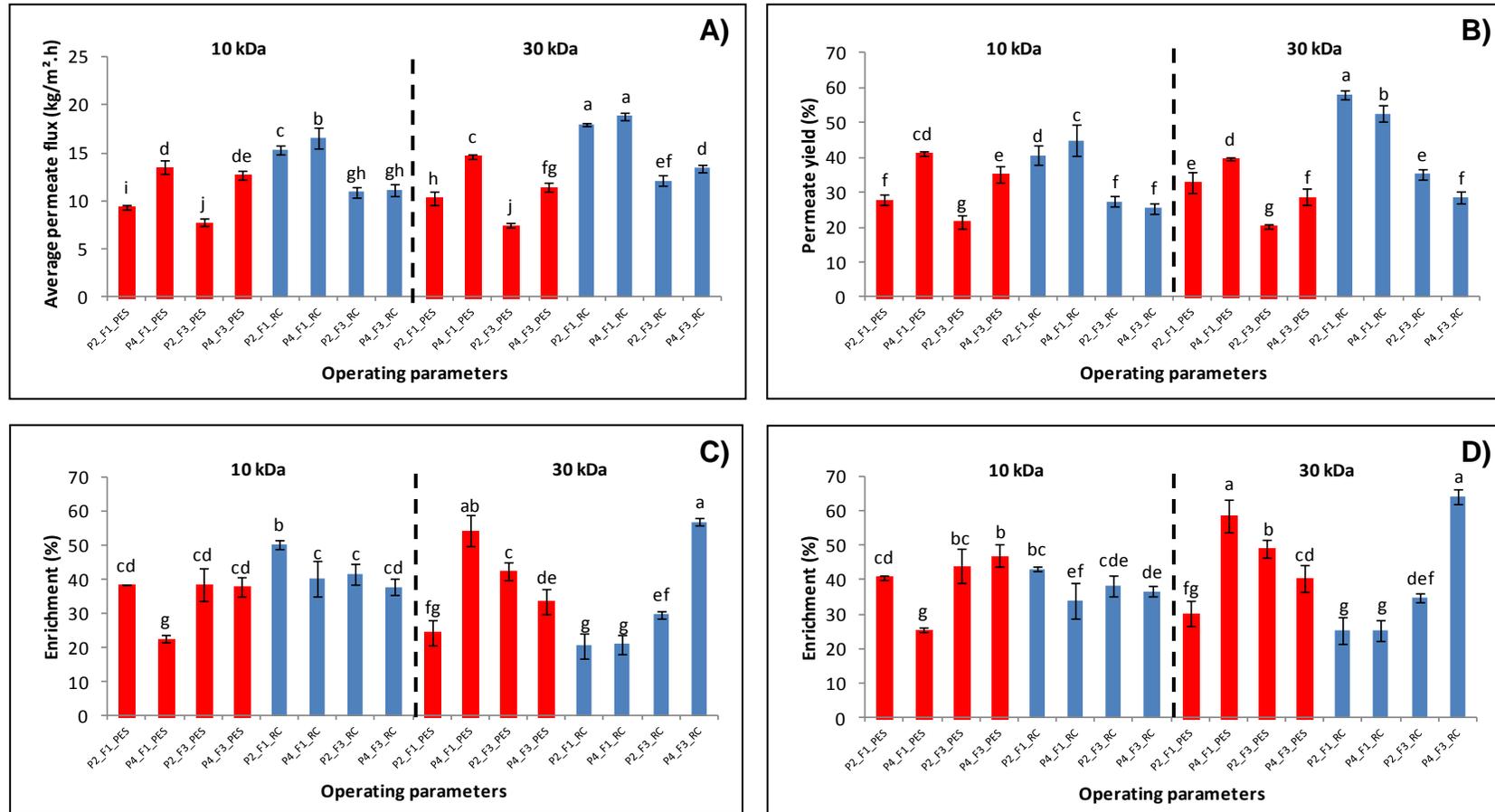


Figure 4.6 The effect of membrane material, molecular weight cut off (MWCO), pressure and feed concentration on the average permeate flux (A), permeate yield (B), mangiferin enrichment (C) and isomangiferin enrichment (D) during the ultrafiltration of unfermented *C. genistoides* extract (40% ethanol, v/v) using a stirred cell device in batch concentration mode ($T = 30^{\circ}\text{C}$; $VCR = 6$; average mangiferin (Mg) content of the feed = 10.80 g $Mg/100$ g SS; average isomangiferin ($IsoMg$) content of the feed = 3.26 g $IsoMg/100$ g SS; different letters indicate significant differences between measurements ($p < 0.05$); values represented as mean \pm standard deviation; P = transmembrane pressure (bar), F = feed concentration in % soluble solids (SS), PES = polyethersulphone (shown in red), RC = regenerated cellulose (shown in blue)).

4.4.1.3 TFU

Two RC membrane cassettes (10 kDa and 30 kDa) were tested under tangential flow conditions at room temperature using two feed concentrations (1% and 3% SS). The membranes were evaluated in terms of flux performance, permeate quality and fouling index as a preliminary, single factor experiment before further optimisation using RSM.

The effect of TMP on permeate flux at different flow rates and feed concentrations was determined in order to establish the point when limiting flux would be obtained (Fig. 4.7). The ideal TMP should be high enough to ensure a good permeate flux without excessive aggregate formation which reduces the productivity of the UF process (Cheryan, 1998). For both MWCOs, increasing TMP caused an increase in permeate flux at constant flow rate and feed concentration. This trend is explained by the film theory model and is commonly seen during the UF of botanical extracts, including green tea extract using a flat sheet 30 kDa polysulphone membrane (Kumar *et al.*, 2012). For the 10 kDa RC membrane, this relationship was predominantly linear, indicating that no limiting flux had been reached, i.e. the flux through the membrane remained pressure dependant for all tested TMP values (Cheryan, 1998; Abdelrasoul *et al.*, 2013). The same observation was made by Acero *et al.* (2005) during removal of phenolic compounds from water using a 5 kDa RC membrane.

For the 30 kDa membrane, there was a linear relationship between TMP and flux at lower TMP values for both feed concentrations (Fig. 4.7 B). However, as the TMP increased over 1 bar, the flux approached steady state, except for 1% SS feed at 401 mL/min, which remained linear. This indicates that the fouling that occurred under the latter conditions was not substantial enough to hinder the permeate flow (Acero *et al.*, 2005). The development of steady state conditions were most evident for the lowest feed rate as observed for 1% and 3% SS feed at 200 mL/min. The 3% SS feed reached a limiting flux at 20 L/m².h, attributed to more extensive fouling occurring at lower pressures. For other feed flow rate x feed concentration combinations tested with this membrane, a clear limiting flux would only be reached at higher pressures than those investigated.

Steady state indicates that a limiting flux is reached, i.e. the flux becomes independent of pressure due to the deposition of solutes on the membrane and is dependent only on mass transfer (Cheryan, 1998; Charcosset, 2012). The same trend for flux behaviour and TMP was observed during UF of other polyphenol-rich extracts, including grape must using a 100 kDa hollow fibre membrane (Cassano *et al.*, 2008b), and kiwifruit juice (Cassano *et al.*, 2008a) and depectinised black currant juice (Pap *et al.*, 2012) using 30 kDa and 100 kDa flat sheet membranes, respectively. Evans & Bird (2006) found that a limiting flux occurred at ca 3-4 bar during UF of reconstituted black tea (1%) when using four 30 kDa RC flat sheet membranes in series. It has been proposed that the relationship between permeate flux and

varying TMP is due to the MWCO of the membrane. A linear relationship is expected for lower MWCO values (<40 kDa), whilst the presence of a limiting flux can be expected for membranes with higher MWCOs (Brites & De Pinho, 2000; Minhalma & De Pinho, 2001).

For all flow rates tested, irrespective of the MWCOs of the membranes, higher permeate fluxes were obtained when using the lower feed concentration. This trend, explained by film theory, was observed with the stirred cell device (4.4.1.2). For a specific feed concentration, permeate flux was highest when the lowest flow rates were used. Similarly, De Bruijn *et al.* (2002) found that highest permeation was achieved with the lowest cross flow velocity tested during UF of apple juice. This result was unexpected as the film theory model states that permeate flux should increase with an increasing flow rate as the enhanced recirculation reduces concentration polarisation and enhances mass transfer (Cheryan, 1998). A number of studies on different feeds, including kiwifruit juice (Cassano *et al.*, 2008a), grape must (Cassano *et al.*, 2008b), green tea (Kumar *et al.*, 2012) and bamboo leaf extract (Tang *et al.*, 2008) showed behaviour consistent with the film theory. An increased flux at lower flow rates could simply be due to the fact that the convection of solutes towards the membrane was minimised thereby reducing the extent of concentration polarisation at the membrane surface allowing a greater permeate flux (Adams, 2012).

Following this experiment, it was decided to test the two membranes at a single pressure (ca 1.2 bar) and flow rate (200 mL/min) in order to investigate the effect of feed concentration and MWCO on average permeate flux, permeate yield, xanthone enrichment and fouling index (Fig. 4.8 A-E). For all experiments a VCR of 5 was achieved.

Feed concentration and MWCO had a significant effect on average permeate flux (Fig. 4.8 A) and permeate yield (Fig. 4.8 B). The highest average permeate flux (24.13 L/m².h) and permeate yield (69.4%), obtained using the 30 kDa membrane and 1% SS feed, were significantly higher than the other MWCO x feed concentration combinations tested ($p < 0.05$). As observed for the stirred cell, a significantly greater average permeate flux was obtained with the more dilute initial feed ($p < 0.05$). When 3% SS feed was used, MWCO did not affect permeate flux significantly ($p \geq 0.05$), indicating the same extent of membrane fouling for both membranes. Feed concentration had no effect on permeate yield when the 10 kDa membrane was used ($p \geq 0.05$), but for the 30 kDa membrane a 1% SS feed gave a higher permeate yield than the 3% SS feed ($p < 0.05$). A reduced feed concentration has also improved permeate yields during UF of grape juice (Kalbasi & Cisneros-Zevallos, 2007) and black tea (Chandini *et al.*, 2012), using membrane sizes of 10-1000 kDa and 500 kDa, respectively.

Large variation in mangiferin enrichment within a set of experimental conditions was observed (Fig. 4.8 C), making identification of maximum enrichment less obvious.

Approximately 28% enrichment was obtained using the 10 kDa membrane, irrespective of feed concentration ($p \geq 0.05$). For the 30 kDa membrane, 3% SS feed resulted in slightly greater mangiferin enrichment than 1% SS feed. The greatest isomangiferin enrichment (29%) was achieved using 10 kDa and 3% SS feed, although it was not significantly different from the other treatments ($p \geq 0.05$) (Fig. 4.8 D).

Fouling was the same for all treatments (ca 15%) ($p \geq 0.05$), except for 3% SS feed and the 30 kDa membrane, showing a significantly higher fouling index of 25.1% ($p < 0.05$) (Fig 4.8 E). This is attributed to easier clogging of larger pores, further enhanced by a higher feed concentration (Chhaya *et al.*, 2012; Kumar *et al.*, 2012). For both membranes, the fouling index increased with a higher feed concentration, although the difference was only significant for the 30 kDa membrane ($p < 0.05$). Evans *et al.* (2008) found that the dominant fouling mechanism during UF of black tea, using hydrophilic RC membranes, was cake formation. This is explained by the film theory model, where increasing feed concentration increases the level of concentration polarisation, which increases cake formation on top of the membrane resulting in flux decline (Marshall *et al.*, 1993). For both membrane sizes, a lower fouling index was associated with a higher average permeate flux and permeate yield, again expected from the film theory model.

Since this study did not aim to produce a high-purity xanthone-enriched extract intended for pharmaceutical use, factors other than xanthone enrichment (permeate flux, permeate yield and fouling index) need to be considered for their economical implications, such as final product (permeate) quantity and membrane life. After preliminary testing of the TFU system, it was evident that the 10 kDa membrane was able to achieve greater xanthone enrichment than the 30 kDa with only slight reduction in permeate yield, but a lower fouling index. For the subsequent optimisation trials, *C. genistoides* extract of 3% SS was used as it more closely mimics the concentration of the extract produced industrially. In addition, using a 1% SS extract would require dilution of the extract produced prior to UF and removal of this water further down the processing line. Although a more concentrated feed in combination with a smaller MWCO would result in reduced permeate flux, this could be avoided by investigating higher TMP x feed flow rate combinations than those tested in this trial. It is for these reasons that the 10 kDa RC membrane cassette was selected to further optimise the UF conditions (TMP and feed flow rate) for the production of a xanthone-enriched extract using a 3% SS *C. genistoides* extract (40% EtOH, v/v). Optimisation of these operating parameters was performed using RSM and is discussed in section 4.4.2.

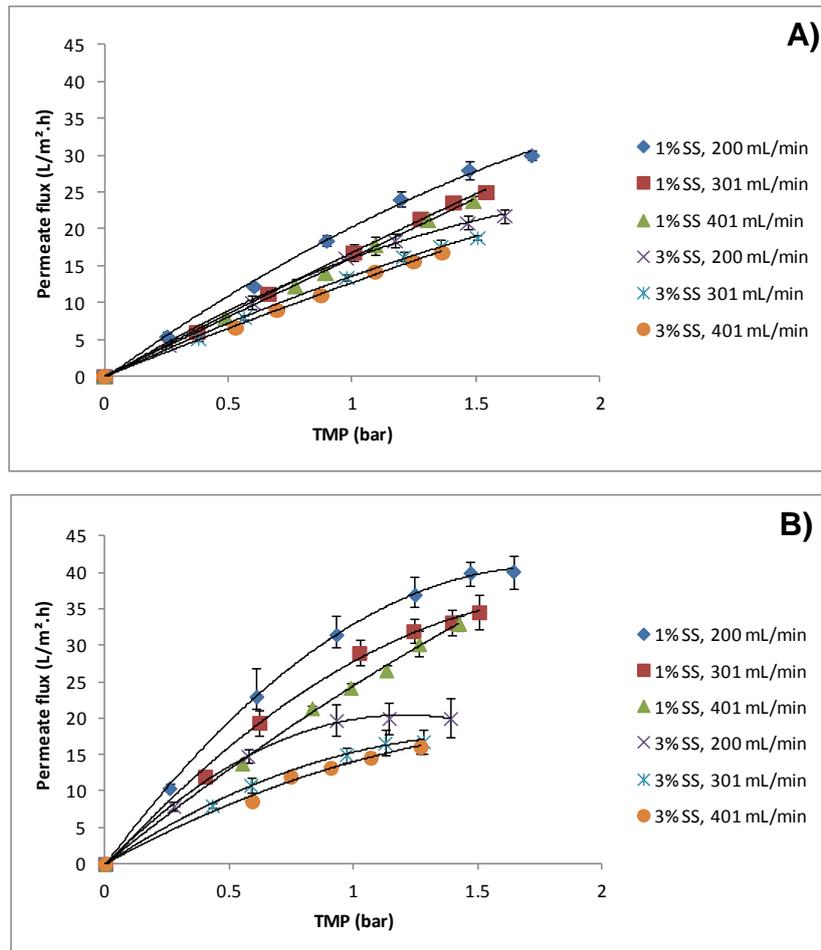


Figure 4.7 Effect of transmembrane pressure (TMP, bar) on permeate flux at different feed concentrations (% SS) and feed flow rates (mL/min) during tangential flow ultrafiltration of unfermented *C. genistoides* extract (40% ethanol, v/v) using 10 kDa (A) and 30 kDa (B) regenerated cellulose membranes in batch concentration mode ($T = 25^{\circ}\text{C}$; $VCR = 5$; values represented as mean \pm standard deviation; SS = soluble solids).

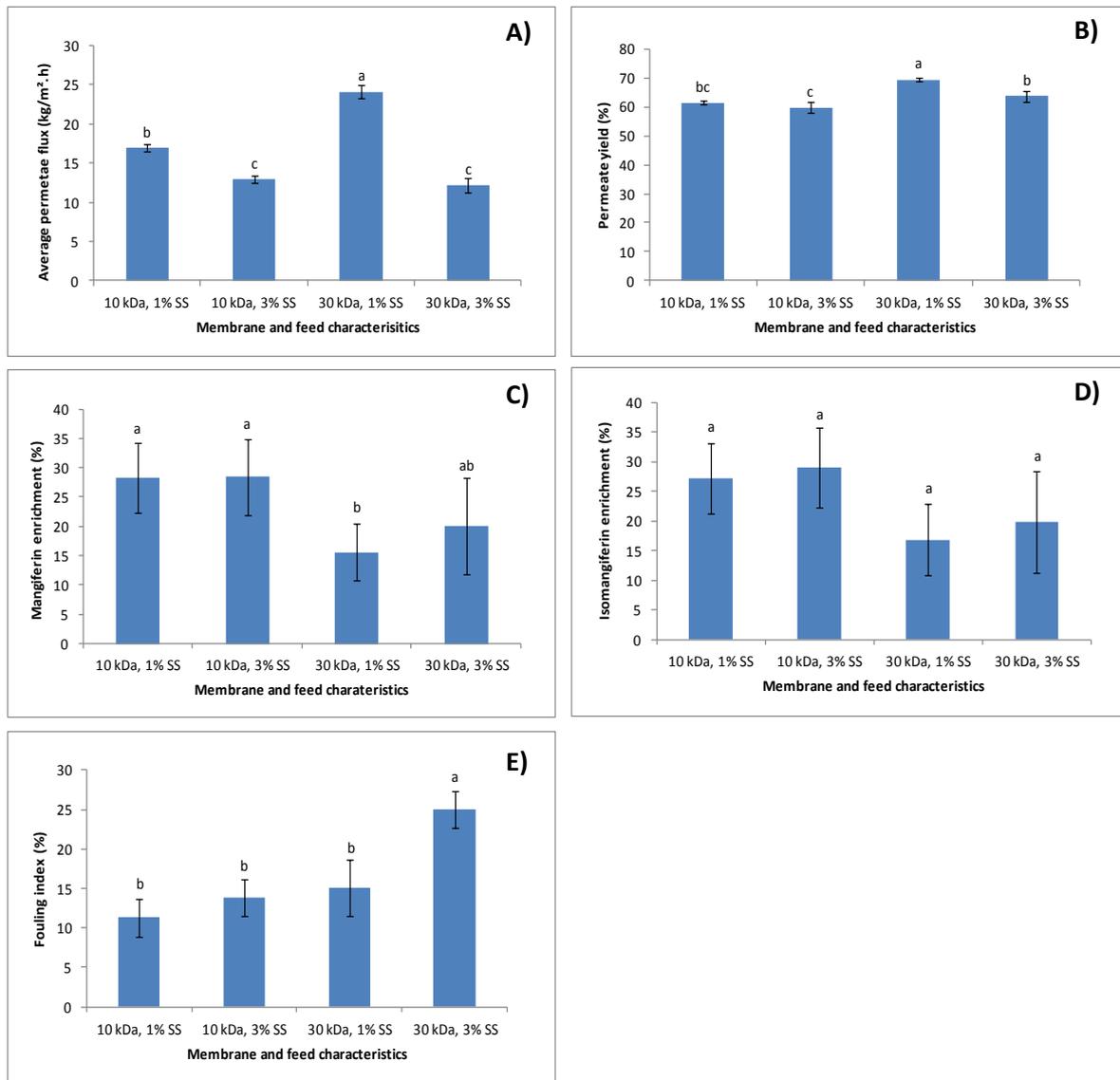


Figure 4.8 The effect of molecular weight cut off (kDa) and feed concentration (% SS) on the average permeate flux (A), permeate yield (B), mangiferin enrichment (C), isomangiferin enrichment (D) and fouling index (E) during ultrafiltration of unfermented *C. genistoides* extract (40% ethanol, v/v) using a tangential flow system in batch concentration mode ($T = 25^{\circ}\text{C}$; $VCR = 5$; $TMP = 1.2 \text{ bar}$; feed flow rate = 200 mL/min ; average mangiferin (Mg) content of the feed = $9.29 \text{ g Mg/100 g SS}$; average isomangiferin (IsoMg) content of the feed = $2.67 \text{ g IsoMg/100 g SS}$; different letters indicate significant differences between measurements ($p < 0.05$); values represented as mean \pm standard deviation; SS = soluble solids).

4.4.2 Optimisation of UF operating parameters using RSM

Previous UF experiments (4.4.1) were successful in identifying a membrane of suitable MWCO and material to use. Additionally, single factor experiments showed that TMP and feed flow rate have a significant effect on membrane performance and permeate quality. The next step was therefore to use RSM to establish the individual and interaction effects of TMP and feed flow rate on average permeate flux, xanthone enrichment and the fouling index during UF of unfermented *C. genistoides* extract. Experiments were carried out according to a CCD at levels stipulated in Table 4.3. The feed concentration used was standardised on 3% SS so that the results are more applicable to industry practice. As isomangiferin is present in substantially lower quantities than mangiferin in the feed and followed the same trend in the RSM results, only the results obtained for mangiferin will be discussed in detail. The RSM data pertaining to isomangiferin enrichment are presented in Addendum B.

4.4.2.1 Analysis of RSM data

The results obtained from the CCD experiments are presented in Table 4.4. The response values varied from 6.91-24.94 kg/m².h for average permeate flux, 13.1-26.4% for mangiferin enrichment and 3.7-14.9% for fouling index. Isomangiferin enrichment ranged from 13.7-27.9% (Addendum B). The average permeate flux and mangiferin enrichment values were comparable to those achieved in previous experiments (4.4.1.2 & 4.4.1.3). However, the stirred cell achieved greater xanthone enrichment on average than the tangential flow system, probably due to the fact that the permeation of other solutes in the extracts was less with the stirred cell device. Using the CCD experimental data, regression analysis and ANOVA were performed to generate the quadratic regression equations and determine whether they provide a good representation of the data. Guan & Yao (2008) reported that R^2 should be at least 0.8 for a model with good fit. The significance of the linear, quadratic and interaction effects of parameters on each response was interpreted using standardised Pareto charts. Response surface plots were used to select the optimum operating parameters for UF. Verification experiments were performed to assess the predictive ability of the models.

Permeate Flux

The R_{adj}^2 value of 0.97 (Table 4.5) indicates that the fitted model explained 97% of the variation in the response values for permeate flux. The LOF test is a more sensitive test of the suitability of the model and compares the variation of the model residuals to the variation between observations at replicate settings of the factors using mean square (MS) pure error 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). The significant p-value (0.0033) obtained for LOF (Table 4.5) indicates that a model which includes more factors may have a better fit. From the standardised Pareto chart for permeate flux (Fig. 4.9 A), it is evident that all parameter effects were significant and increased the permeate flux ($p < 0.05$). The linear effect of TMP on permeate flux was the greatest, followed by the quadratic and linear effects of feed flow rate, interaction effect between TMP and feed flow rate and the quadratic effect of TMP (Fig. 4.9 A). The second-order polynomial regression equation was obtained by fitting the response values for average permeate flux as a function of TMP and feed flow rate:

$$Y_1 = 37.8169 - 7.4235X_1 + 4.6303X_1^2 - 0.2183X_2 - 0.0003X_2^2 - 0.0248X_1X_2 \quad (4.5)$$

where Y_1 is the predictive permeate flux ($\text{kg/m}^2\cdot\text{h}$) for the UF process, X_1 is TMP (bar) and X_2 is feed flow rate (mL/min). The effect of TMP and feed flow rate on permeate flux are shown in the combined response surface and contour plot (Fig. 4.9 B). It is evident that no clear optimum was found within the experimental range which was restricted due to the pressure limits of the pump element. However, the response surface provides reliable information regarding the effects of the parameters and region of maximum response. An increase in TMP from 0.8 to 2.0 bar caused a linear increase in permeate flux for all values tested, as was also observed for the preliminary testing of the 10 kDa membrane (4.4.1.3). The same trend was seen in other UF studies on orange press liquors (Ruby-Figueroa *et al.*, 2011) and olive mill wastewaters (Akdemir & Ozer, 2013) within TMP ranges of 0.2-1.4 bar and 1-2 bar, respectively. Increasing the feed flow rate from 158 to 301 mL/min decreased the average permeate flux, whilst increasing the feed flow rate from 301 to 444 mL/min increased the average permeate flux. This is probably due to the fact that at flow rates above 301 mL/min, the scouring effect of cross-flow velocity is greater than the convective movement of molecules toward the membrane surface (Adams, 2012). Maximum response values would be obtained when a combination of the highest TMP and feed flow rate was used, i.e. 2.04 bar and 444 mL/min. Pap *et al.* (2012) found an optimum TMP of 2 bar for permeate flux of black currant juice when using a Pellicon[®] 2 Mini membrane cassette of the same area (0.1 m²). Similarly, a combination of high TMP and cross-flow velocity values gave the highest permeate fluxes when optimising UF of polyethylene glycol solution (Martí-Calatayud *et al.*, 2010) and olive mill wastewaters (Akdemir & Ozer, 2013). It is also evident from the response surface (Fig. 4.9 B) that the use of a low flow rate in combination with a high TMP could increase permeate flux, although not to maximum value. Similarly, De Bruijn *et al.* (2002) found that maximum permeation was obtained with a high TMP and low cross-flow

velocity during UF of apple juice. In the present study no critical TMP value was obtained, i.e. no TMP value above which permeate flux ceased to increase.

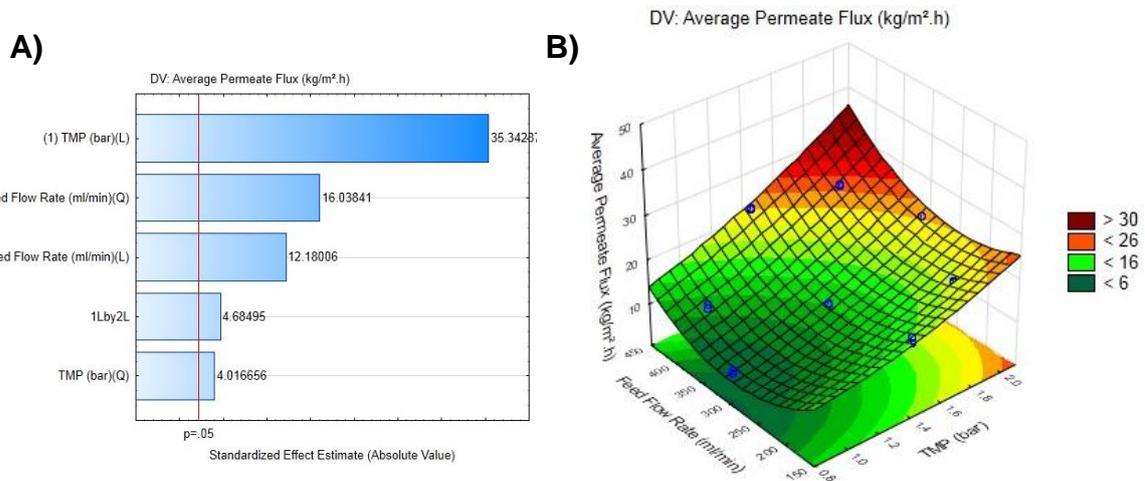


Figure 4.9 Standardised Pareto chart (A) and combined fitted response surface plot (B) for average permeate flux (kg/m².h) as a function of transmembrane pressure (TMP, bar) and feed flow rate (mL/min) (*DV = dependant variable, L = linear effect, Q = quadratic effect, L by L = interaction effect*).

Table 4.4 Central Composite Design and the response values obtained for average permeate flux, mangiferin enrichment and fouling index

Run no.	X_1 Transmembrane pressure (bar)	X_2 Feed flow rate (mL/min)	Average permeate flux (kg/m ² .h)	Mangiferin enrichment (%)	Fouling index (%)
1	1.00	200	8.45	13.09	6.05
11	1.00	200	9.10	14.75	5.54
2	1.00	401	10.77	16.53	8.01
12	1.00	401	9.86	18.11	6.72
3	1.86	200	18.96	21.02	9.35
13	1.86	200	19.21	22.22	10.68
4	1.86	401	24.90	23.11	6.84
14	1.86	401	24.94	23.05	5.92
5	0.82	301	7.33	15.19	3.65
15	0.82	301	6.91	15.15	4.55
6	2.04	301	22.97	24.62	11.00
16	2.04	301	20.72	26.43	9.28
7	1.43	158	17.19	16.18	13.29
17	1.43	158	16.08	16.74	14.90
8	1.43	444	22.59	18.56	7.14
18	1.43	444	22.70	19.29	6.52
9 (C) ¹	1.43	301	12.37	15.11	10.27
10 (C)	1.43	301	12.74	15.22	9.54
19 (C)	1.43	301	11.78	14.08	9.80
20 (C)	1.43	301	11.74	16.78	8.44

¹ (C) = centre point parameter values

Mangiferin enrichment

The R_{adj}^2 value of 0.95 (Table 4.6) indicates that the model explains 95% of the variation seen in the response values for mangiferin enrichment. The non-significant p-value (0.79) for LOF indicates that this model accurately describes the data at the 95% confidence level. The linear, quadratic and interaction effects of each parameter on mangiferin enrichment are shown in the standardised Pareto chart (Fig. 4.10 A). The linear effect of TMP on mangiferin enrichment was the greatest, followed by the quadratic effect of TMP, the linear effect of feed flow rate and the quadratic effect of feed flow rate. The interaction effect between TMP and feed flow rate on mangiferin enrichment was not significant ($p \geq 0.05$). The second-order polynomial regression equation for mangiferin enrichment is represented below:

$$Y_2 = 34.0274 - 27.2777X_1 + 13.5684X_1^2 - 0.0440X_2 - 0.0001X_2^2 - 0.0112X_1X_2 \quad (4.6)$$

where Y_2 is the predicted mangiferin enrichment (%) achieved from the UF process, X_1 is TMP (bar) and X_2 is feed flow rate (mL/min). The graphical representation of this model and the interaction effects between the tested parameters are illustrated in the combined response surface and contour plot (Fig. 4.10 B). As is evident from the parabolic shape of the response surface, the model produced an optimal minimum rather than maximum response, as expected. An increase in TMP from 0.8 to 1.0 bar caused a decrease in mangiferin enrichment, but when increased from 1.43 to 2.04 bar caused a linear increase in mangiferin enrichment. The initial decrease with increasing TMP is due to solid compaction acting as a dynamic membrane thereby reducing the MWCO (Cheryan, 1998). At pressures above 1.43 bar, the polyphenols in the solid layer were able to cross the membrane due to the increase in applied pressure. The same trend was observed for polyphenol rejection during UF of depectinised orange press liquors (Ruby-Figueroa *et al.*, 2012). The feed flow rates that allowed the highest level of mangiferin enrichment were the highest and lowest tested, 444 and 158 mL/min, respectively. Since the permeate yield remained fairly consistent for all experiments (ca 20-22%, data not shown), the increased enrichment would be due to increased mangiferin content in the permeate. Maximum enrichment at high flow rates is in accordance with the film theory model. At higher feed flow rates, the cross-flow velocity is greater which reduces the build-up of solutes at the membrane surface (reversible concentration polarisation) which prevents the onset of irreversible membrane fouling (Adams, 2012). A simultaneous maximum at low flow rates was unexpected and could simply be due to the fact that at constant pressure, decreasing the feed flow rate minimises convection towards the membrane surface causing a lesser extent of fouling (Adams, 2012). The effect of flow rate on mangiferin enrichment is therefore not a straight forward one. Similarly, Kumar *et al.* (2012) found that flow rate can have a different effect on polyphenols

during UF of green tea, depending on their nature. They found that increasing flow rate decreased the percentage (-)-epigallocatechin (EGC) whilst increasing the percentage (-)-epigallocatechin gallate (EGCG) in the permeate.

Given that the data fitted the model well, the response surface gives a good indication of the region of maximum response within the experimental domain, i.e. a high TMP in combination with either high or low feed flow rates.

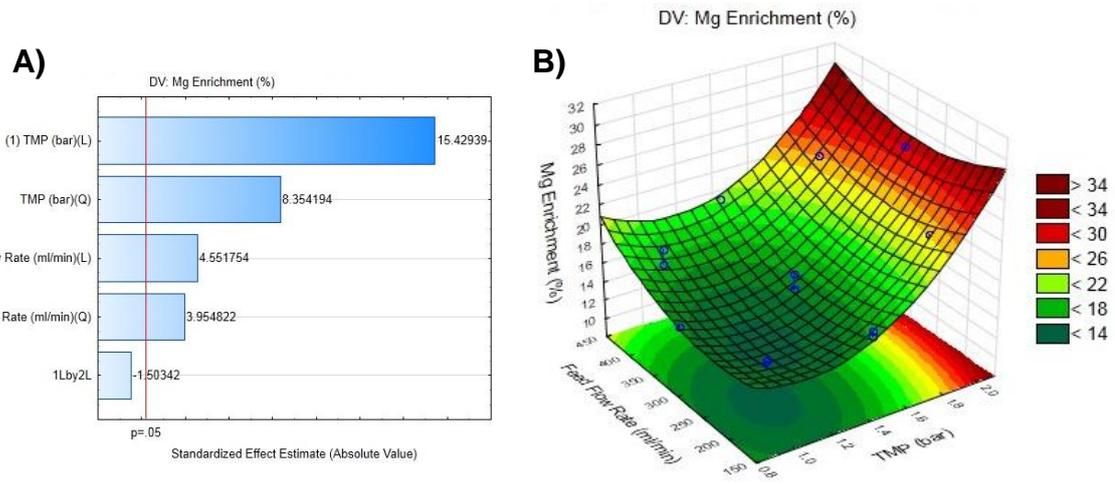


Figure 4.10 Standardised Pareto chart (A) and combined fitted response surface plot (B) for mangiferin enrichment as a function of transmembrane pressure (TMP, bar) and feed flow rate (mL/min) (DV = dependant variable, L = linear effect, Q = quadratic effect, L by L = interaction effect).

Fouling index

The R_{adj}^2 value of 0.65 indicates that the model only accounts for 65% of the variation seen in the response values for the fouling index. This, together with a significant p-value for LOF ($p = 0.0003$) indicates a poor fit for the model and that there are more factors at play that could contribute to the observed variation (Ruby-Figueroa *et al.*, 2011). From the standardised Pareto chart (Fig. 4.11 A), it is evident that the factors having the greatest effect on increasing and decreasing the fouling index are the significant linear effects of TMP and feed flow rate, respectively. In other studies TMP was also demonstrated to have a significant effect on the fouling index, by increasing fouling with increasing TMP during the optimisation of UF of *Radix astragalus* extract (Cai *et al.*, 2012) and depectinised orange press liquor (Ruby-Figueroa *et al.*, 2011). The quadratic effect of TMP and the interaction effect between TMP and feed flow rate on the fouling index were both significant. The negative quadratic effect of feed flow rate on the fouling index was not significant. The second-order polynomial regression equation obtained for fouling index is presented below:

$$Y_3 = 37.8169 - 7.4235X_1 + 4.6303X_1^2 - 0.2183X_2 - 0.0003X_2^2 - 0.0248X_1X_2 \quad (4.7)$$

where Y_3 is the predictive fouling index (%) for the UF process, X_1 is TMP (bar) and X_2 is feed flow rate (mL/min). This model is graphically represented in Figure 4.11 (B). As an increase in the fouling index causes a reduction in the efficiency of the UF process, it is desirable to minimise this response. Although no clear minimum was obtained, the response surface can give an indication of the region of minimum response and parameter effects. The fouling index increased linearly with TMP until 1.8 bar, after which it reached a steady state. Cassano *et al.* (2008b) showed that fouling increased with increasing TMP during UF of grape must, which in turn increased the rejection of polyphenols and solutes. The present study showed that at low TMP of 0.8-1.2 bar, an increase in feed flow rate did not cause a large increase in the fouling index. At TMP of 1.4-2 bar, the maximum fouling index was found when using the lowest feed flow rates tested. De Bruijn *et al.* (2002) showed that minimum fouling occurred using a combination of low TMP and high cross-flow velocity during the UF of apple juice, while Ruby-Figueroa *et al.* (2011) found that increasing the feed flow rate of orange press liquors increased the fouling index. The interactive effect of feed flow rate and TMP is clearly dependant on the type of extract, underlying the need to optimise UF parameters experimentally, and preferably, using a system that mimics the process eventually to be used by industry. For the present study on *C. genistoides* extract it is therefore evident that a combination of high TMP and low feed flow rates would result in a large fouling index. It can be reduced by using a low TMP, or if a high TMP is required, then it is best used in combination with a high flow rate which has the ability to reduce membrane fouling according to film theory (Cheryan, 1998; Adams, 2012).

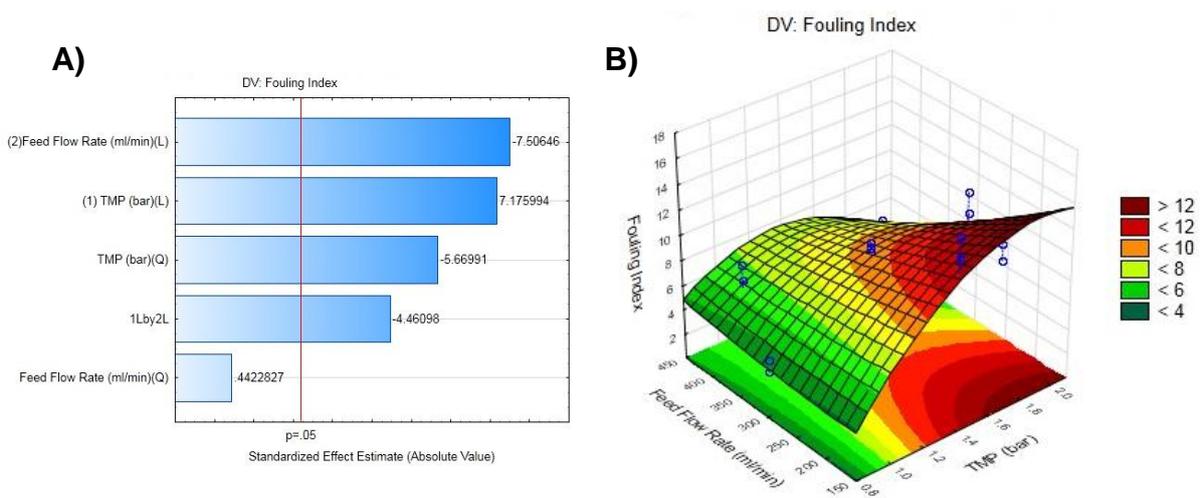


Figure 4.11 Standardised Pareto chart (A) and combined fitted response surface plot (B) for fouling index as a function of transmembrane pressure (TMP, bar) and feed flow rate (mL/min) (DV = dependant variable, L = linear effect, Q = quadratic effect, L by L = interaction effect).

Table 4.5 ANOVA of experimental results for the polynomial quadratic equation for average permeate flux (*significant values ($p < 0.05$) are highlighted in red*)

Parameter ¹	Regr. Coeff. ²	SS ³	DF ⁴	MS ⁵	F	p
Intercept	37.8169					
(1) TMP (bar) (L)	-7.4235	522.3784	1	522.3784	1249.1182	0.0000
TMP (bar) (Q)	4.6303	6.7470	1	6.7470	16.1335	0.0020
(2) Feed flow rate (mL/min) (L)	-0.2183	62.0413	1	62.0413	148.3539	0.0000
Feed flow rate (mL/min) (Q)	0.0003	107.5733	1	107.5733	257.2306	0.0000
1L by 2L	0.0248	9.17892	1	9.1789	21.9488	0.0007
Lack of fit (LOF)		10.6526	3	3.5509	8.4908	0.0033
Pure Error		4.6002	11	0.4182		
Total SS		722.2459	19			
R ²						0.98
R ² _{adj}						0.97

¹ TMP = transmembrane pressure, L = linear coefficient, Q = quadratic coefficient, L by L = interaction coefficient; ² Regression coefficients;

³ Sum of squares; ⁴ Degrees of freedom; ⁵ Mean square

Table 4.6 ANOVA of experimental results for the polynomial quadratic equation for mangiferin enrichment (*significant values ($p < 0.05$) are highlighted in red*)

Parameter ¹	Regr. Coeff. ²	SS ³	DF ⁴	MS ⁵	F	p
Intercept	34.0274					
(1) TMP (bar) (L)	-27.2777	197.6202	1	197.6202	238.0660	0.0000
TMP (bar) (Q)	13.5684	57.9353	1	57.9353	69.7926	0.0000
(2) Feed flow rate (mL/min) (L)	-0.0440	17.1985	1	17.1985	20.7185	0.0008
Feed flow rate (mL/min) (Q)	0.0001	12.9834	1	12.9834	15.6406	0.00225
1L by 2L	-0.0112	1.8763	1	1.8763	2.2603	0.1609
Lack of fit (LOF)		0.8540	3	0.2847	0.3429	0.7949
Pure Error		9.1312	11	0.8301		
Total SS		284.7403	19			
R ²						0.96
R ² _{adj}						0.95

¹ TMP = transmembrane pressure, L = linear coefficient, Q = quadratic coefficient, L by L = interaction coefficient; ² Regression coefficients;

³ Sum of squares; ⁴ Degrees of freedom; ⁵ Mean square

Table 4.7 ANOVA of experimental results for the polynomial quadratic equation for fouling index (*significant values ($p < 0.05$) are highlighted in red*)

Parameter ¹	Regr. Coeff. ²	SS ³	DF ⁴	MS ⁵	F	P
Intercept	-19.6296					
(1) TMP (bar) (L)	36.2422	34.8746	1	34.8746	51.4949	0.0000
TMP (bar) (Q)	-8.3178	21.7720	1	21.7719	32.1479	0.0001
(2) Feed flow rate (mL/min) (L)	0.0205	38.1606	1	38.1606	56.3470	0.0000
Feed flow rate (mL/min) (Q)	0.0000	0.13248	1	0.1325	0.1956	0.6669
1L by 2L	-0.0300	13.4774	1	13.4774	19.9003	0.0010
Lack of fit (LOF)		31.8197	3	10.6066	15.6614	0.0003
Pure Error		7.4497	11	0.6772		
Total SS		154.3101	19			
R ²						0.75
R ² _{adj}						0.65

¹ TMP = transmembrane pressure, L = linear coefficient, Q = quadratic coefficient, L by L = interaction coefficient; ² Regression coefficients;

³ Sum of squares; ⁴ Degrees of freedom; ⁵ Mean square

Practical optimum parameters

No desirability profiling was performed on the RSM data due to the nature of the model generated for mangiferin enrichment, represented in Figure 4.10 (B). The model has a negative curvature with a minimum reached around the centre point values, and then increasing on either side of the centre points. This indicates that maximising mangiferin enrichment can be done by either increasing or decreasing the control parameters, and therefore making optimisation of maximum mangiferin enrichment by desirability profiling unfeasible. Since the data fitted the model well the optimum parameters could therefore be selected by visual inspection of the response surface plots (Dejaegher & Vander Heyden, 2011). In UF, the processing parameters are typically chosen that the maximum permeate flux is obtained, combined with a good recovery of solids (yield), and as little membrane fouling as possible for high productivity and good permeate quality where necessary (Lo *et al.*, 2005; Chhaya *et al.*, 2012). The aim of RSM in the current study was to identify parameters that would simultaneously maximise mangiferin enrichment and average permeate flux, whilst minimising fouling. However, it is not always possible that the optimal conditions to maximise/minimise certain responses are the same. For example, Cassano *et al.* (2008b), when investigating UF of grape must, found that optimal conditions for permeate flux and minimum fouling differed, with higher feed temperature and TMP improving permeate flux, whilst lower temperatures and TMP reduced fouling. During UF of apple juice, the optimum conditions to minimise fouling were low TMP and high cross-flow velocity, whilst high TMP and low cross-flow velocity were ideal for optimum permeate flux (De Bruijn *et al.*, 2002). Akdemir & Ozer (2013) found, for UF of olive mill wastewaters, that a combination of high TMP and high flow rate favoured permeate flux, whilst maximum chemical oxygen demand (COD) removal was obtained using both a low TMP and low flow rate.

From analysis of the RSM data for UF of *C. genistoides* extract, it is evident that mangiferin enrichment was maximised at high TMP in combination with either high or low flow rates; permeate flux was maximised at a high TMP and a high flow rate and fouling was minimised using a low TMP in combination with any flow rate tested, or a high flow rate in combination with high TMP, if necessary. It was therefore decided to select the highest TMP (2.04 bar) and flow rate (444 mL/min) combination tested to maximise both mangiferin enrichment and permeate flux. Although a high TMP would increase the incidence of membrane fouling, it can be reduced by the 'self cleaning' effect of a high feed flow rate as is evident from the response surface (Fig. 4.10 B). Flow rates used in UF depend on the pump used, feed capacity and the scale of the operation, i.e. laboratory scale vs. industrial pilot plant. In the present study, the feed flow rate x TMP combinations were selected as to ensure the pressure limit of the pump element was not exceeded. The results obtained using

the Pellicon[®] mini cassette (0.1 m²) can be linearly up-scaled to larger membrane cassettes, such as 0.5 m² and 2.5 m², for use in pilot or manufacturing plants, respectively, to process volumes ranging from 1-1000 L (Anon., 2014).

Verification of predicted values of the models

Nine additional experiments were performed to verify the model. The parameters of each experimental run, as well as the response values obtained, are presented in Table 4.8. The ICC is used to assess the predictive capability of the models, as previously discussed (3.4.3.2). For all responses, low ICC values were obtained indicating that the models cannot be used to accurately predict values for mangiferin enrichment, permeate flux or fouling index. The observed values for all responses were higher than predicted by the model. Poor correlation between observed and predicted values was most probably due to human error as the control of fluctuating pressure during UF was done manually by adjusting the retentate valve. This was difficult due to the variation in pressure caused by the peristaltic nature of the feed flow. TMP has a significant effect on permeate flux and degree of fouling on the membrane and would therefore influence these responses. Results may have been improved if the actual pressure of each experimental run had been measured and a better control system employed. The distribution of the predicted values vs. the verification results are presented in scatter plots in Addendum B. Ideally, the experimental points are scattered along the 45° diagonal. Bland-Altman plots are used to evaluate the agreement between the predicted and observed values and the presence of bias within the measurements (refer to 3.4.3.2). If no bias occurs within the quantitative measurements, then the mean will lie at 0 on the *y*-axis. The predicted model produced a consistent under-estimation of response values evident from the observed experimental values lying below the 45° diagonal in the distribution scatter plots and the mean of the Bland-Altman plots being above zero. It was therefore decided to perform a subsequent validation experiment to test the UF performance at the selected 'optimised' conditions under simulated industrial circumstances, i.e. using extracts made from different *C. genistoides* batches to allow for natural variation in composition.

4.4.3 Validation of optimised UF parameters

The optimised UF parameters of 2.04 bar and 444 mL/min were tested on extracts made from 10 different batches of *C. genistoides* plant material, harvested from seedling plants, to assess the consistency or range of enrichment obtained with varying feed composition (Table 4.9). Each extraction was performed according to the previously optimised process

(Chapter 3) and the extraction efficiency measured in terms of extract yield, xanthone yield and xanthone content of the extract, is represented in Table 4.10.

For UF, the temperature of all extracts was 30°C. The feed concentration of the *C. genistoides* extracts ranged from 2.7-4.4% (mean = 3.4%). The average permeate flux for all 10 batches was 18.88 kg/m².h with no clear trend between the initial SS content of the feed and permeate flux. This is higher than the average permeate flux obtained for the 10 kDa RC membrane in the single factor trials using the stirred cell (10.93 kg/m².h) and TFU system (12.93 L/m².h), at the same pressure (2 bar) and feed concentration (3% SS). The higher permeate flux is a result of the increased flow rate (444 mL/min) which enhanced permeation through the membrane. The quadratic model generated by the CCD (Equation 4.5) predicted a much higher permeate flux of 33.9 kg.m².h at optimised conditions (2 bar, 444 mL/min). This could be due to the larger fouling index obtained than that predicted by the model (3.9%, Equation 4.7). Furthermore, feed flow rates, membrane size and material, nature of the feed and type of pump have an influence on the attained permeate flux (Wei *et al.*, 2010; Cassano *et al.*, 2011; Rao *et al.*, 2011; Chhaya *et al.*, 2012; Kumar *et al.*, 2012; Pap *et al.*, 2012). The permeate yield increased linearly with the SS content of the extract (Fig. 4.12) with an average value of 2.42 g/100 mL. No other linear relationships were apparent between the parameters measured (Table 4.9).

Since the verification trial indicated that the models exhibit poor predictive ability, this validation experiment would provide a better estimation of the expected enrichment under industrial conditions. The mangiferin enrichment obtained ranged from 12.4-23.6% (mean = 20%) and the isomangiferin enrichment ranged from 16.6-26% (mean = 22%). This is less than the enrichment obtained using 3% SS feed and 10 kDa PES and RC membranes in the stirred cell device operating at 2 bar, with enrichments of 38.7% and 41.8%, respectively. As previously stated, the lower enrichment obtained with the TFU is most probably due to an increased permeation of solids through the membrane as a result of tangential flow. Preliminary testing of the 10 kDa RC membrane in the TFU system using 3% SS feed (4.4.1.3) showed higher enrichment values of 28.41% and 29.04% for mangiferin and isomangiferin, respectively. The higher xanthone enrichment could be explained by the lower fouling index obtained in 4.4.1.3 as a result of using a lower TMP (ca 1.2 bar) and feed flow rate (200 mL/min). This, however, was accompanied by a smaller average permeate flux (12.93 L/m².h) than those achieved in the validation experiment (Table 4.9). A similar range of enrichment values were obtained for mangiferin (13.1-26.4%) and isomangiferin (13.7-27.9%) during RSM, albeit at varying TMP x feed concentration combinations. The models generated by the CCD (Equation 4.6 & 4.7) predicted a mangiferin enrichment of 28.2% at the optimised TFU conditions, accompanied by a fouling index 3.9%. The lower xanthone

enrichment achieved in the validation trial could again be attributed to a much larger fouling index than that predicted by the CCD.

From Table 4.9 it is evident that a greater xanthone content in the feed resulted in a greater content in the permeate, as expected. However, the % xanthone enrichment is dependent on both feed concentration and xanthone content in the feed. When comparing extracts 3 and 4; both had more or less the same SS content but differing mangiferin content, leading to different % enrichment. On the other hand, extracts 3 and 6 had the same mangiferin contents, yet the enrichment was greater for extract 6. Extracts 9 and 10 also had the same mangiferin content, yet extract 10 had substantially greater mangiferin enrichment, most probably due to a lower fouling index than obtained for extract 9. Where the isomangiferin content of the extracts were more or less the same, both similar (extracts 5 and 7) and different (extracts 3 and 4) enrichment values were obtained, again showing that feed composition alone is not responsible for enrichment. These results prove the inter-dependence of process parameters on permeate quality.

As previously stated, the fouling indexes obtained (Table 4.9) were substantially higher than those obtained during RSM with an average value of 22.1%, as well as during the preliminary testing of the TFU system (4.4.1.3). Generally, the extent of membrane fouling during UF is dependent on membrane material properties and surface chemistry, nature of the feed and operating parameters (Evans *et al.*, 2008; Susanto *et al.*, 2009). Since other parameters remained constant, the high values are attributed to differences in the composition of feed, clearly demonstrating variation in constituents responsible for fouling as no linear relationship was observed between feed concentration and fouling index. Other studies have shown that fouling can vary greatly, depending on the extract. Fouling observed during the UF of olive mill wastewaters through a 10 kDa RC membrane was 28.4% (Cassano *et al.*, 2011) and substantially less than the fouling seen during UF of *Radix astragalus* extracts (Cai *et al.*, 2012), artichoke wastewaters (Conidi *et al.*, 2014) and depectinised orange press liquor (Ruby-Figueroa *et al.*, 2011), using 10 kDa, 50 kDa and 100 kDa polysulphone membranes, respectively. The extent of fouling during UF can thus be unpredictable as it depends not only on the surface chemistry and pore size of the membrane, but also on the characteristics and chemistry of the feed solution, i.e. concentration, pH and salt content (Susanto *et al.*, 2009). Fouling can be controlled during UF by manipulation of operating parameters and effectively removed by proper cleaning of the membrane.

This study, to our knowledge, is the first to investigate the use of UF for processing of *Cyclopia* extracts, not only for analytical purposes, but for possible future application in the production of a value-added, xanthone-enriched extract.

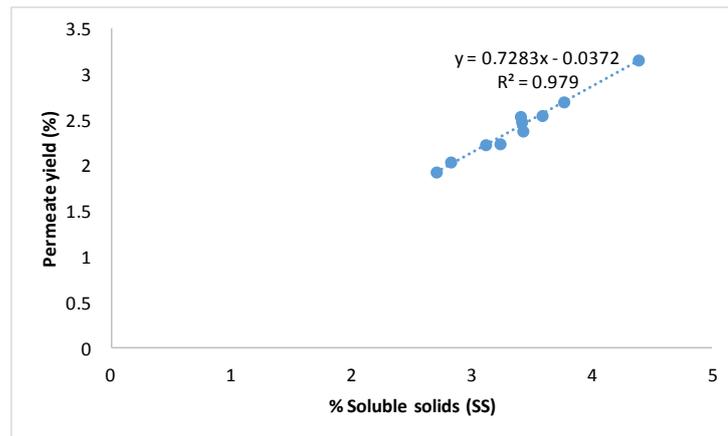


Figure 4.12 Permeate yield as a factor of % soluble solids of initial feed during the tangential flow ultrafiltration of extracts (40% ethanol, v/v) made from ten different unfermented *C. genistoides* batches under optimised conditions in batch concentration mode ($T = 30^{\circ}\text{C}$, $VCR = 5$).

Table 4.8 Observed and predicted results obtained from the Central Composite Design experiment used for verification of the prediction model

Run No.	X_1 Transmembrane pressure (bar)	X_2 Feed flow rate (mL/min)	Mangiferin enrichment (%)		Average permeate flux (kg/m ² .h)		Fouling index (%)	
			<i>Observed</i>	<i>Predicted</i>	<i>Observed</i>	<i>Predicted</i>	<i>Observed</i>	<i>Predicted</i>
1	1.2	230	18.28	13.81	14.54	10.03	13.02	8.94
2	1.2	301	17.55	14.14	16.07	9.34	9.94	8.28
3	1.2	371	16.45	15.62	16.61	11.98	7.38	7.75
4	1.5	230	16.97	15.84	16.41	13.26	13.83	11.00
5	1.5	301	29.53	15.94	16.45	13.10	12.49	9.71
6	1.5	371	20.97	17.18	19.53	16.26	6.45	8.55
7	1.7	230	18.37	18.56	17.88	15.88	14.76	11.55
8	1.7	301	20.94	18.49	16.47	16.07	15.90	9.82
9	1.7	371	22.29	19.58	19.06	19.58	9.70	8.24

Table 4.9 Experimental results of the validation of the combined optimised ethanol-water extraction and ultrafiltration protocol using extracts made from different batches of unfermented *C. genistoides* plant material (40% ethanol, v/v) in batch concentration mode ($T = 30^{\circ}\text{C}$, $VCR = 5$, values expressed as mean \pm standard deviation)

Extract no.	Feed concentration (g SS ¹ /100 mL)	Mangiferin content in initial feed (as % of SS)	Mangiferin content in permeate (as % of SS)	Mangiferin enrichment (%)	Isomangiferin content in initial feed (as % of SS)	Isomangiferin content in permeate (as % of SS)	Isomangiferin enrichment (%)	Permeate yield (g SS/100 mL)	Average permeate flux (kg/m ² .h)	Fouling index (%)
1	2.70 \pm 0.01	9.39 \pm 0.30	11.60 \pm 0.25	23.64 \pm 1.16	2.17 \pm 0.04	2.74 \pm 0.05	25.97 \pm 0.25	1.93 \pm 0.01	20.34 \pm 1.25	19.89 \pm 2.95
2	3.11 \pm 0.00	6.77 \pm 0.02	8.27 \pm 0.03	22.28 \pm 0.78	1.84 \pm 0.00	2.28 \pm 0.01	24.14 \pm 0.77	2.23 \pm 0.01	17.61 \pm 0.22	20.35 \pm 0.63
3	3.41 \pm 0.04	10.37 \pm 0.71	12.21 \pm 0.45	17.85 \pm 3.87	2.37 \pm 0.14	2.84 \pm 0.10	20.04 \pm 3.40	2.48 \pm 0.02	17.89 \pm 0.65	18.52 \pm 2.31
4	3.42 \pm 0.03	8.73 \pm 0.43	10.66 \pm 0.34	22.17 \pm 2.11	2.30 \pm 0.12	2.87 \pm 0.09	24.87 \pm 2.58	2.38 \pm 0.06	17.18 \pm 0.20	17.17 \pm 1.03
5	3.58 \pm 0.01	11.38 \pm 0.54	13.64 \pm 0.83	20.07 \pm 1.61	2.74 \pm 0.13	3.35 \pm 0.22	20.15 \pm 1.82	2.55 \pm 0.08	18.10 \pm 0.92	25.88 \pm 1.28
6	3.76 \pm 0.01	10.38 \pm 0.09	12.55 \pm 0.01	20.91 \pm 1.12	2.47 \pm 0.01	3.04 \pm 0.00	23.25 \pm 0.97	2.70 \pm 0.01	19.28 \pm 2.57	21.62 \pm 4.44
7	3.23 \pm 0.12	9.90 \pm 0.06	12.75 \pm 1.39	19.39 ² \pm 0.00	2.80 \pm 0.67	2.57 \pm 0.35	20.90 ² \pm 0.00	2.24 \pm 0.32	20.05 \pm 3.65	21.92 \pm 2.70
8	2.82 \pm 0.04	7.32 \pm 0.11	8.87 \pm 0.05	20.67 \pm 3.16	1.75 \pm 0.02	2.14 \pm 0.01	22.76 \pm 2.08	2.04 \pm 0.04	19.22 \pm 2.39	19.78 \pm 1.31
9	3.40 \pm 0.02	11.75 \pm 0.14	13.42 \pm 0.00	14.24 \pm 1.27	2.40 \pm 0.01	2.80 \pm 0.01	16.63 \pm 1.24	2.54 \pm 0.05	18.25 \pm 1.03	30.73 \pm 1.56
10	4.38 \pm 0.10	11.77 \pm 0.15	14.20 \pm 0.16	20.68 \pm 2.89	3.00 \pm 0.04	3.64 \pm 0.16	21.29 \pm 7.01	3.16 \pm 0.07	20.90 \pm 0.50	25.56 \pm 1.52

¹ soluble solids; ² n = 1 (outlier removed)

Table 4.10 Extraction parameters obtained using plant material from ten different unfermented *C. genistoides* batches for the validation of the combined ethanol-water extraction and ultrafiltration process (*values expressed as mean \pm standard deviation*)

Extract no. ¹	Extract yield ²	Mangiferin yield ³	Mangiferin content in the extract ⁴	Isomangiferin yield ⁵	Isomangiferin content of the extract ⁶
1	26.95 \pm 0.05	2.53 \pm 0.08	9.38 \pm 0.30	0.59 \pm 0.01	2.17 \pm 0.05
2	31.04 \pm 0.04	2.10 \pm 0.00	6.76 \pm 0.02	0.57 \pm 0.00	1.84 \pm 0.00
3	34.00 \pm 0.36	3.53 \pm 0.28	10.37 \pm 0.72	0.81 \pm 0.06	2.37 \pm 0.15
4	34.08 \pm 0.33	2.97 \pm 0.12	8.73 \pm 0.43	0.78 \pm 0.03	2.30 \pm 0.12
5	33.84 \pm 0.09	4.08 \pm 0.17	12.05 \pm 0.55	0.99 \pm 0.04	2.92 \pm 0.14
6	37.58 \pm 0.11	3.90 \pm 0.02	10.37 \pm 0.09	0.93 \pm 0.00	2.47 \pm 0.02
7	32.22 \pm 1.13	3.19 \pm 0.09	9.90 \pm 0.06	0.75 \pm 0.03	2.32 \pm 0.00
8	28.21 \pm 0.42	2.06 \pm 0.00	7.32 \pm 0.11	0.49 \pm 0.00	1.74 \pm 0.02
9	33.91 \pm 0.30	3.98 \pm 0.08	11.75 \pm 0.14	0.81 \pm 0.01	2.40 \pm 0.02
10	43.73 \pm 1.08	5.14 \pm 0.19	11.76 \pm 0.15	1.31 \pm 0.05	3.00 \pm 0.04

¹ extraction conditions: 70°C, 40% ethanol (v/v), 30 min, 10 mL/g; ² g soluble solids/100 g plant material; ³ g mangiferin/100 g plant material; ⁴ g mangiferin/100 g soluble solids; ⁵ g isomangiferin/100 g plant material ⁶ g isomangiferin/100 g soluble solids

4.5 Conclusion

This study confirmed that UF technology could be used to enrich xanthenes in an unfermented *C. genistoides* ethanolic extract. A series of UF experiments using different membrane devices identified the 10 kDa RC membrane to be the most suitable, which was subsequently employed in RSM experiments to optimise laboratory scale UF of the extract. TMP and feed flow rate were confirmed as having a significant effect on process parameters, i.e. average permeate flux, mangiferin enrichment and the fouling index. The optimised TMP and feed flow rate conditions were identified as 2.04 bar and 444 mL/min, respectively. The optimised parameters were tested on extracts of varying feed concentration as a result of employing different batches of *C. genistoides* plant material to mimic industrial circumstances. Average mangiferin and isomangiferin enrichments of 20% and 22%, respectively, were obtained. This xanthone-enriched extract would find possible functional food/beverage or nutraceutical application due to the vast array of health benefits associated with xanthenes. The easy incorporation of UF into existing processing lines, as well as its mild nature makes UF an attractive separation technique for the preparation of value-added honeybush extracts.

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Chapter 5

General Discussion, Recommendations and Conclusions

There is a growing demand for the delivery of antioxidants through functional foods and nutraceuticals as consumers shift to self-medication through 'positive' eating (Chen *et al.*, 2006; Kosaraju *et al.*, 2008; Sloan, 2012). Polyphenols are well recognised as natural antioxidants and have been linked to prevention of the development and progression of lifestyle-related diseases (Salah *et al.*, 1995; Nijveldt *et al.*, 2001; Scalbert *et al.*, 2005). In the field of functional food/ingredient development, there is currently an emphasis on the following aspects: testing alternative, more sustainable methods of polyphenol extraction from natural sources for ingredient application (as reviewed by Wang & Weller, 2006; Wijngaard *et al.*, 2012; Azmir *et al.*, 2013; Shah & Rohit, 2013); the effect of food microstructure on the bioavailability of nutrients (Parada & Aguilera, 2007); and the maintenance of bioactivity throughout food processing and subsequent passage through the gastrointestinal tract (McGhie & Walton, 2007; Kosaraju *et al.*, 2008).

Quantitative studies on the chemical composition of unfermented *Cyclopia genistoides* (Joubert *et al.*, 2003; Joubert *et al.*, 2008a; Joubert *et al.*, 2008b; De Beer & Joubert, 2010; Beelders *et al.*, 2014) have shown that this honeybush species is an excellent source of the xanthone C-glycoside, mangiferin, and its regio-isomer, isomangiferin. These xanthenes were shown to possess greater antioxidant activity compared to other polyphenols present in *C. genistoides* (Hubbe, 2000; Hubbe & Joubert, 2000; Joubert *et al.*, 2008b; Malherbe *et al.*, 2014). Mangiferin displays a wide range of health-promoting benefits including antidiabetic properties (as reviewed by Vyas *et al.*, 2012). To date, only antioxidant activity has been demonstrated for isomangiferin (Malherbe *et al.*, 2014). Extraction techniques previously investigated for xanthone recovery from various botanical extracts for quantitative purposes include conventional organic solvent extraction (Ito *et al.*, 2012), ultrasound assisted extraction (UAE) (Yoswathana, 2013; Zou *et al.*, 2014), sub-critical water extraction (Kim *et al.*, 2010), supercritical fluid extraction (SFE) (Fernández-Ponce *et al.*, 2012) and microwave assisted extraction (Zou *et al.*, 2013). Organic solvent extraction is the simplest extraction technique used for mangiferin (Joubert *et al.*, 2006; Barreto *et al.*, 2008; Ribeiro *et al.*, 2008; Ling *et al.*, 2009; Ito *et al.*, 2012), with increasing research being conducted on the aforementioned novel techniques (Zou *et al.*, 2013; Kulkarni & Rathod, 2014; Kullu *et al.*, 2014; Ruiz-Montañez *et al.*, 2014; Zou *et al.*, 2014). Vimang[®], an aqueous extract of mango (*Mangifera indica* L.) stem bark containing 10-20% mangiferin, is sold in Cuba as an antioxidant nutritional supplement. Salaretin[®], a standardised extract of *Salacia reticulata* containing ca 1% mangiferin (Anon., 2002), is sold in India and Sri Lanka as an antidiabetic supplement and to treat obesity (Singh *et al.*, 2012). Although extracts containing mangiferin have already achieved success as nutraceutical products, the incorporation of mangiferin-enriched extracts into food/beverage products has yet to take off.

The first aim of the current study was to optimise the extraction conditions of unfermented *C. genistoides* extract for the eventual production of a xanthone-enriched extract. Optimisation of extraction would not only allow maximum xanthone recovery from the plant material but will reduce the overall energy consumption of the extraction process according to 'green chemistry' principles. The effects of solvent composition, particle size, time and temperature on extraction efficiency were evaluated prior to process optimisation using one factor at-a-time (OFAT) principles. Apart from the xanthones, mangiferin and isomangiferin, which are the focus of the current study, other major polyphenols in *C. genistoides* were also quantified as part of a more comprehensive characterisation of the extracts. These compounds include the benzophenones, iriflophenone-di-O,C-hexoside and iriflophenone-3-C-glucoside, the dihydrochalcone glycoside, 3-hydroxy-phloretin-di-C-hexoside, and the flavanone rutoside, hesperidin.

Varying solvent composition had a significant effect ($p < 0.05$) on extraction yield, xanthone yield and xanthone content of the extract. The best extract and xanthone yields were achieved in the range 20-60% ethanol (EtOH) (v/v), whilst the poorest extract and xanthone yields were achieved with 100% EtOH (v/v). The polarity of the EtOH-water mixture increases with the addition of water, allowing more polar molecules to be extracted (Cheok *et al.*, 2012; Tabaraki *et al.*, 2012). In addition, higher EtOH concentration could increase the diffusion resistance due to the effect on other constituents present in the extract, such as protein coagulation and the extraction of lipids (Prasad *et al.*, 2012). It is therefore evident that an EtOH/water mixture rather than the pure solvents, even when compared at elevated temperatures, allowed for good extract yields, as well as an efficient extraction of all the quantified polyphenols, presenting a range of polarities. EtOH/water mixtures have been shown to efficiently extract mangiferin from different plant sources (Dorta *et al.*, 2012; Ito *et al.*, 2012; Zou *et al.*, 2013; Zou *et al.*, 2014). Methanol (MeOH) was shown to be more effective than water or EtOH/water mixtures for xanthone extraction from honeybush (Joubert *et al.*, 2012), however, it cannot be used in the production of extracts intended for human consumption due to its toxicity. A reduced particle size enhanced the extraction efficiency of all major polyphenols from unfermented *C. genistoides* as expected from Fick's second law of diffusion (Fick, 1855). Given the reduced particle size, an optimal extraction time of 30 min was obtained as longer extraction times were not beneficial, i.e. extraction reached steady state and prolonged heat exposure could enhance compound degradation.

The extraction of xanthones from unfermented *C. genistoides* plant material was further optimised using response surface methodology (RSM). Care was taken to ensure the process was in agreement with current industry practices as well as producing an extract safe for human consumption. This study represents the second instance where RSM has

been used to optimise the extraction of phenolic compounds from *Cyclopia*. Previously, Du Preez (2014) used RSM to optimise the UAE of hesperidin and eriocitrin from *C. maculata* tea processing by-product. The focus of RSM in the current study was mainly on mangiferin, the predominant xanthone in both fermented and unfermented *C. genistoides* extracts. RSM allowed the evaluation of the individual and interaction effects of temperature (°C) and EtOH concentration (% v/v) on xanthone extraction. The optimised conditions for maximum extract yield, mangiferin yield and mangiferin content in the extract were 40% EtOH (v/v), 70°C, 30 min and a solvent:solid ratio of 10 mL/g. These conditions allowed the simultaneous optimisation of isomangiferin extraction and are therefore suitable for production of a xanthone-enriched extract. For all measured responses, EtOH concentration was the determining factor as changes in temperature (40-70°C) showed a negligible effect. Similarly, Yoswathana (2013) found that EtOH concentration (0-95%, v/v) had a large effect on the recovery of xanthones from mangosteen hull using UAE, whilst increasing temperature (33-55°C) had a small effect. Prasad *et al.* (2012) reported a similar result with temperature (30-65°C) not significantly contributing to the extract yield obtained from the ethanolic (20-80%, v/v) extraction of brown mango (*Mangifera pajang*) pericarp. This could be due to the fact that although increasing temperature accelerates mass transfer and improves polyphenol solubility (Al-Farsi & Lee, 2008; Ahmad-Qasem *et al.*, 2013), it is accompanied by a reduction in solvent density and possible degradation of heat-labile constituents (Prasad *et al.*, 2012).

The complex structure of mangiferin makes it difficult to synthesise chemically, therefore the best alternative for its production is to extract it from its natural sources (Kulkarni & Rathod, 2014). To date, the mango (*Mangifera indica* L.) plant remains the most thoroughly researched source of mangiferin. The bark, leaves and fruit (pulp, peel and kernel) of different cultivars of this plant have been assessed for their mangiferin content (Berardini *et al.*, 2005; Barreto *et al.*, 2008; Ribeiro *et al.*, 2008; Dorta *et al.*, 2014). Although the mangiferin content of the components varies greatly amongst cultivars, the leaves are the best source of mangiferin in all cases. Research was initially directed towards investigating mango bark as a source of mangiferin (Núñez-Sellés *et al.*, 2002; Garcia *et al.*, 2003; Garrido *et al.*, 2004), but the focus has recently shifted to the leaves as this is a more sustainable source (Ling *et al.*, 2009; Fernández-Ponce *et al.*, 2012; Bhuvaneshwari, 2013; Zou *et al.*, 2013; Zou *et al.*, 2014).

The extraction of ten different batches of *C. genistoides* plant material performed in this study resulted in a greater mangiferin yield (2.06-5.14 g mangiferin/100 g dried plant material) than the extraction of mango pulp from various cultivars (0.0003-0.0019 g mangiferin/100 g dry matter) (Berardini *et al.*, 2005). This is due to the fact that the plant secondary metabolites (including xanthones) are found in higher concentrations in the fruit

peels, leaves and bark of the mango plant (Luo *et al.*, 2012). Although evident that mango bark is a good source of mangiferin (1.2 and 1.8 g mangiferin/100 g dry material) (Barreto *et al.*, 2008), the tree is not as easily cultivated or as quickly renewed as *C. genistoides*. Zou *et al.* (2013; 2014) looked at the MAE and UAE of mango leaves using aqueous EtOH as solvent. Using these novel techniques, they achieved optimal mangiferin yields of 5.8 and 3.6 g mangiferin/100 g dried plant material, respectively, similar to that achieved in this study. Kokotkiewicz *et al.* (2013) achieved a higher mangiferin yield from unfermented *C. genistoides* (8.5 g/100 g dried plant material) by extracting with MeOH under reflux followed by subsequent multi-stage liquid-liquid partitioning. However, this intensive extraction process was required to produce an extract suitable for subsequent *in vitro* testing and isolation of unidentified compounds. Joubert *et al.* (2006) obtained a similar mangiferin yield (3.6%) from unfermented *C. genistoides* using MeOH as extraction solvent at 65°C for 30 min, however, the values cannot be directly compared as different batches of plant material were used and are thus subjected to variation in composition (Joubert *et al.*, 2006; Joubert *et al.*, 2014). As previously stated, the toxicity of MeOH prevents its use in the development of an extract intended for human consumption, despite its efficacy in extracting xanthenes. The mangiferin yield obtained from the methanolic extraction of different cultivars of African *Coffea* leaves (0.03-16.4 g/100 g dry matter) (Campa *et al.*, 2012) overlapped with the range of mangiferin yield obtained in this study.

In the current study, the mangiferin content of the extracts obtained from ten batches of unfermented *C. genistoides* plant material (6.8-11.8%) were much greater than that obtained from the subcritical water extraction of *Mahkota Dewa (Phaleria macrocarpa)* leaves (2.2%) (Kim *et al.*, 2010) and the ethanolic extracts of agarwood leaves (0.5-0.7%) (Ito *et al.*, 2012). Compared to other plant sources of mangiferin and isomangiferin, *C. genistoides* offers the advantages of easy xanthone extraction and sustainability of the resource as it is cultivated and harvested annually (Joubert *et al.*, 2011).

Like all natural sources of mangiferin, there is currently a large variation in mangiferin content of *C. genistoides* due to harvest date, plant maturity at time of harvest, climate conditions and the cultivation area (Joubert *et al.*, 2003; Joubert *et al.*, 2006). Joubert *et al.* (2014) found the mangiferin content in *C. genistoides* leaves of different maturity to be 11.5% and 10.2%. Although the *Cyclopi*a leaves have higher mangiferin content than the stems (De Beer *et al.*, 2012; Du Preez, 2014), a combination of stems and leaves were used in this study to mimic current industry practice.

Once the extraction conditions were successfully optimised, xanthone enrichment of the optimised unfermented *C. genistoides* extract using ultrafiltration (UF) was subsequently investigated as a second aim of the study. When selecting an appropriate membrane for UF, it is commonplace to perform small-scale laboratory experiments to compare the membrane

performance before testing pilot plant/industrial scale systems which are substantially more expensive. This study used a similar up-scaling approach to that of Denis *et al.* (2009) for the UF of macro-algae extracts. Parameters used to evaluate membrane performance during UF of botanical extracts are dependent on the purpose of UF. Permeate flux is important for any UF application as it is an indication of the productivity of the process (Evans *et al.*, 2008; Al Manasrah *et al.*, 2012). The fouling index is another important consideration that not only affects the productivity and economic feasibility of UF, but also directly affects the final product quality, as well as causing an unpredictable separation (Li & Chase, 2010; Zhao *et al.*, 2013). Where the retentate is the desired product, e.g. protein/pectin/polysaccharide/polyphenol concentration (Cho *et al.*, 2003, Denis *et al.*, 2009; Conidi *et al.*, 2011; Al Manasrah *et al.*, 2012), the rejection (%) of the membrane towards compounds and retentate yield are important considerations. Where the permeate is the desired product, e.g. clarification/compound enrichment (Galanakis *et al.*, 2010; Rao *et al.*, 2011; Chandini *et al.*, 2012, Husson *et al.*, 2012; Tierny *et al.*, 2013), the recovery (%) of compounds, permeate quality and permeate yield are important considerations.

In the current study, the centrifugal membrane devices showed that molecular weight cut off (MWCO) values of 10 kDa or higher are best suited for processing unfermented *C. genistoides* extracts (40% EtOH, v/v) as they allow greater passage of desirable low molecular weight (LMW) polyphenols, namely the xanthenes (MW 422.33 g/mol), while retaining the other constituents, resulting in enrichment. The stirred cell device clearly showed that regenerated cellulose (RC) membranes perform better than polyethersulphone (PES) membranes during the UF of the *C. genistoides* extract due to the attainment of a greater permeate flux, permeate yield and xanthone enrichment. This is in agreement with the findings of a number of studies aimed at recovery of polyphenols from various types of extracts. Cassano *et al.* (2011) found for the UF of olive mill wastewaters, that 10 kDa RC membranes showed lower retention of polyphenols and higher permeate fluxes compared to 10 kDa PES membranes. Denis *et al.* (2009) also showed an increased permeability with 30 kDa RC rather than 30 kDa PES membranes during UF of macro-algae extracts. Hossain (2005) showed that RC membranes had a greater recovery (%) of polyphenols in the permeate compared to PES membranes during UF of *Echinacea purpurea* extract.

Both transmembrane pressure (TMP) and feed concentration influenced the permeate flux, permeate yield and xanthone enrichment during stirred cell UF due to their influence on concentration polarisation explained by film theory (Cheryan, 1998; Adams, 2012). Comparing the 10 kDa and 30 kDa RC membranes using a tangential flow ultrafiltration (TFU) system prior to RSM showed that the 10 kDa membrane allowed a greater xanthone enrichment and lower fouling index with only a slight reduction in permeate yield. Similarly, Rao *et al.* (2011) found that for the clarification of green tea using UF, the 10 kDa RC

membrane was more effective in removing protein/polyphenol-protein complexes whilst the 30 kDa RC membrane showed a better flux performance.

Although 3% SS feed for the *C. genistoides* extract was selected due to its industrial relevance, the optimal operating parameters of the TFU system, namely TMP and feed flow rate, needed further investigation to assess their combined effect on membrane productivity and permeate quality. RSM was employed for this purpose. The individual and interaction effects of TMP and feed flow rate (mL/min) on permeate flux (kg/m².h), xanthone enrichment (%) and fouling index (%) were investigated. Working within experimental constraints, the conditions that allowed for optimal membrane performance (permeate flux and fouling index) and permeate quality (xanthone enrichment) were 2.04 bar and 444 mL/min. Both parameters had a significant effect on all measured responses. TMP had the greatest effect on increasing permeate flux and xanthone enrichment, whilst feed flow rate had the greatest effect on reducing the fouling index. Similarly, Ruby-Figueroa *et al.* (2011) found that TMP had the greatest effect on increasing permeate flux during the UF of orange press liquors, but in another study on orange press liquors, Ruby-Figueroa *et al.* (2012) found that TMP had a significant effect on increasing the polyphenols retained by the membrane using a 100 kDa polysulphone (PS) membrane, the opposite to the finding of the current study on TMP and xanthone enrichment. This indicates that both the nature of the feed and characteristics of the membrane influence the separation process. Overall, the behaviour of the UF system expressed by RSM was in accordance with the film theory model and/or the results of other researchers investigating the UF of botanical extracts (De Bruijn *et al.*, 2002; Martí-Calatayud *et al.*, 2010; Ruby-Figueroa *et al.*, 2011; Cai *et al.*, 2012; Ruby-Figueroa *et al.*, 2012; Akdemir & Ozer, 2013). The poor correlation between the observed and predicted data was attributed to the manual control of the continuously fluctuating TMP. Since TMP was shown to have a significant effect on all measured responses, it is important to improve the accuracy of the measurement. This could be done by using an automatically controllable pressure regulator which would provide a fixed TMP throughout the UF process.

The extraction efficiency of the optimised extraction procedure on plant material from ten different *C. genistoides* batches was assessed using extract yield (26.95-43.73 g SS/100 g plant material), xanthone yield (2.56-6.46 g xanthonenes/100 g plant material) and xanthone content of the extract (8.60-14.97 g xanthonenes/100 g extract). The validation of the combined optimised extraction and UF parameters, using extracts from the ten different *C. genistoides* batches, saw an average permeate flux of 18.88 kg/m²h, permeate yield of 2.42 g/100 mL, mangiferin enrichment of 20%, isomangiferin enrichment of 22% and a fouling index of 22%. When compared to other studies that utilise 0.1 m² Pellicon[®] mini membrane cassettes at 2 bar, a higher flux was obtained during the UF of black currant juice using a 100 kDa PES membrane (Pap *et al.*, 2012), while a lower flux was seen during the UF of binary solution of

rutin and glucose through a 1 kDa RC membrane (Wei *et al.*, 2010). Similar enrichment values of 19% (Bazinet *et al.*, 2012) and 22% (Husson *et al.*, 2012) were obtained for the anthocyanin enrichment of cranberry juice using UF coupled with electrodialysis. Fu *et al.* (2007) also achieved different levels of enrichment for the isomeric compounds vitexin and isovitexin during the preparative separation of these compounds from pigeonpea extracts using macroporous resins. The fouling observed for *C. genistoides* extract during the validation experiment was similar to that seen in the UF of olive mill wastewaters through a 10 kDa RC membrane (Cassano *et al.*, 2011) and substantially lower than for the UF of various plant extracts using PS membranes (Ruby-Figueroa *et al.*, 2011; Cai *et al.*, 2012; Conidi *et al.*, 2014). When comparing the results of the validation experiment to the RSM data, a lower permeate flux, lower enrichment and higher fouling index was achieved for the *C. genistoides* extract. As no correlation was seen between increasing feed concentration and fouling index, the higher fouling index was most probably due to variation in feed composition of the fresh extracts as opposed to the reconstituted, freeze-dried extract. A higher fouling index explains the reduced permeate flux and xanthone enrichment obtained according to film theory (Cheryan, 1998, Adams, 2012).

In this study, UF resulted in a loss of ca 30% extract yield when comparing the SS content of the initial feed and permeate samples. Other researchers have also reported a reduction in extract yield as a result of UF (Wei *et al.*, 2010; Chandini *et al.*, 2012). Diafiltration is a possibility that could improve both the permeate yield and xanthone enrichment obtained during UF performed in this study. Diafiltration is typically used for solvent removal, buffer exchange or the recovery of LMW compounds such as anions, cations, sugars and salts (Lewis, 1996). Diafiltration can be carried out in continuous mode, where water is added to the retentate at the same rate that the permeate is removed, resulting in a constant volume and concentration of the retentate. Another alternative is discontinuous diafiltration, involving a sequential dilution of the feed prior to UF (Lewis, 1996). Chandini *et al.* (2012) found that diafiltration improved the recovery of solids, polyphenols and theaflavins during the stirred cell UF of black tea. Similarly, Cho *et al.* (2003) concluded that diafiltration improved the separation of flavonoids, polyphenols and carotenoids from pectin extracts during the microfiltration of mandarin peel extract. Al Manasrah *et al.* (2012) and Wei *et al.* (2010) found that diafiltration improved the separation of high molecular weight (HMW) and LMW compounds during the UF of spruce-sawdust extract and a mixture of rutin and glucose, respectively. The trade-off when using diafiltration is that it would require additional solvent that would need to be removed further down the processing line.

Another step that could also be investigated in future to further improve xanthone enrichment of unfermented *C. genistoides* extracts (40% EtOH, v/v) is the incorporation of a

separation step involving macroporous resins. In this case adsorption due to ion exchange between the functional groups of the target compound and the resin material leads to separation between constituents in the extract (Soto *et al.*, 2011). Resins have been used for the enrichment of flavone C-glycosides from various plant sources including pigeonpea extracts (Fu *et al.*, 2007), trolflowers (Sun *et al.*, 2013) and *Abrus mollis* extracts (Du *et al.*, 2012). In these studies, resins have achieved varying degrees of enrichment ranging from a 4 fold (Fu *et al.*, 2007) to 40 fold (Sun *et al.*, 2013) increase in flavone C-glycoside content in the refined extracts compared to the crude extracts. UF has successfully been used in combination with resin adsorption for the recovery of flavone C-glycosides from citrus peel wastes (Manthey, 2004), and other bioactive phenolics from distilled white grape pomace (Díaz-Reinoso *et al.*, 2010), green tea leaves (Li *et al.*, 2005) and persimmon pulp (Gu *et al.*, 2008). These findings suggest the potential of resin adsorption to further increase the extent of xanthone enrichment obtained in this study, although the industrial practicality and economical feasibility would need to be further investigated.

In addition to the xanthone content of the final extract, other quality parameters such as total polyphenol content and antioxidant activity should also be assessed as they are currently used by South African extract manufacturers for quality control purposes. Characterising the xanthone-enriched extract is merely the first step towards the eventual production of a functional food ingredient. The approval of the food status of an ingredient depends on the traditional use of the plant as food, the form in which it is presented to the consumer, its physiological functionality and the nature of its extraction (Wang & Bohn, 2012). When developing a functional food ingredient for incorporation into a food/beverage product, it is important to validate the bioavailability of the bioactive compounds in the ingredient by testing in *in vitro* and *in vivo* models; to develop a suitable food matrix taking into account the effect of processing on the bioactivity of the active ingredient/s; to educate the consumer on the health benefits of the product and to perform bioactivity testing and clinical trials to determine product efficacy in order to gain approval for health claims (Siró *et al.*, 2008). It is for these reasons that functional food development is mainly accessible to larger, well-established food manufacturers as they have the resources required for the product development and marketing of functional foods (Menrad, 2003). Adequate market research on the target consumer and their consumption habits would help to design a product that will suit their needs (Wang & Bohn, 2012). The sensory evaluation of the final product would need to be conducted to assess consumer acceptance of the final functional product. Any new natural product intended for human consumption is required to undergo thorough prerequisite safety evaluation procedures including acute toxicity testing in animals, for example mice, and bacterial mutagenicity testing before any sensory testing can be performed (Kingham *et al.*, 2010).

The simultaneous preservation of the bioactivity of the added active ingredient (both within a specific food matrix and the gastrointestinal tract) and of the desired sensory characteristics of a product is the major challenge in functional food development (Wang & Bohn, 2012). Microencapsulation, typically achieved via spray drying, could be utilised to retain the bioactivity of the final extract and minimise the negative sensory aspects associated with polyphenols, namely astringency, bitterness and colour adulteration (Fang & Bhandari, 2010; Munin & Edwards-Lévy, 2011; Wang & Bohn, 2012). Therefore, in future, investigation of microencapsulation using an appropriate carrier material and its effect on the antioxidant activity of the extract should be conducted before the final value-added extract can find application as a functional food/beverage ingredient. De Souza *et al.* (2013) studied the efficacy of four different pectin and chitosan formulations for mangiferin encapsulation. It was found that the formulations containing pectin gave a higher retention of mangiferin in the particles during the spray drying process. Other carrier materials typically used for the encapsulation of food ingredients include natural gums (gum arabic, alginates, carrageenan), proteins (milk or whey proteins, gelatins) and different blends of maltodextrins (Gharsallaoui *et al.*, 2007; Fang & Bhandari, 2010). Green tea extract (GTE), encapsulated with maltodextrin, was shown to be more effective than non-encapsulated GTE in protecting the cardiovascular system of rats subjected to high fructose-feeding (Jung *et al.*, 2013). The use of inulin as a possible carrier is currently a hot topic of research due to its nutritive and prebiotic properties (Stevens *et al.*, 2001; Saéñz *et al.*, 2009), which are particularly useful in the development of functional foods.

Research has shown that a mixture of phytochemicals in their natural food matrix is more effective in combating certain metabolic diseases compared to isolated compounds (Boileau, 2003; Yao *et al.*, 2010). Using a mangiferin-enriched extract could therefore potentially be more effective than using purified mangiferin as a functional ingredient as additive and synergistic effects of other polyphenols and nutrients present in the extract may play a role (Liu, 2003; Liu, 2004). A mangiferin-enriched extract would follow current market trends, offering the opportunity to be marketed for a specific bioactivity (Becker, 2013), such as its antiobesity (Lim *et al.*, 2014) or antidiabetic activities (Chellan *et al.*, 2014), both of which have been shown to be primary health concerns amongst consumers (Sloan, 2012).

This study confirmed the potential of ethanol-water extraction combined with ultrafiltration to produce an extract enriched in xanthone content that has possible future application as a functional food ingredient. Further studies are required on the antioxidant profile of the extracts produced via the proposed method for industrial relevance, as well as investigating the encapsulation of the extract to further contribute towards the development of a functional food ingredient. The enrichment of the extract could possibly be improved by the incorporation of a diafiltration step or subsequent enrichment using macroporous resins,

although the industrial viability would need to be considered. This study not only provides process parameters easily incorporated into current industrial extract processing procedures to manufacture a functional ingredient, but production of such an ingredient would add value to honeybush, in particular the species, *C. genistoides*.

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Addendum A

Optimisation of xanthone extraction from unfermented *Cyclopia genistoides* using response surface methodology

Table A.1 Effect of ethanol concentration on the extract yield, compound yield and polyphenol content of unfermented *Cyclopia genistoides* extract (values represent means \pm standard deviation)

Ethanol concentration % (v/v)	compound ¹ content ² (g/ 100 g soluble solids)						compound yield ² (g/ 100 g plant material)						Extract Yield ² (g SS ³ /100 g PM ⁴)
	Mg	IsoMg	I-dihex	I-glc	Hph	Hd	Mg	IsoMg	I-dihex	I-glc	Hph	Hd	
0	9.67 ^e \pm 0.32	3.89 ^c \pm 0.10	1.66 ^a \pm 0.05	3.18 ^b \pm 0.11	0.87 ^a \pm 0.04	0.36 ^c \pm 0.01	3.46 ^c \pm 0.12	1.39 ^b \pm 0.03	0.59 ^a \pm 0.02	1.14 ^a \pm 0.03	0.31 ^b \pm 0.01	0.13 ^d $<$ 0.01	35.68 ^c
20	12.39 ^d \pm 0.31	3.63 ^e \pm 0.11	1.39 ^{bc} \pm 0.03	2.86 ^c \pm 0.06	0.80 ^b \pm 0.02	0.30 ^d \pm 0.01	5.14 ^{ab} \pm 0.19	1.51 ^a \pm 0.07	0.58 ^{ab} \pm 0.02	1.19 ^a \pm 0.04	0.33 ^a \pm 0.01	0.12 ^d $<$ 0.01	41.47 ^a
40	12.78 ^{cd} \pm 1.16	3.71 ^{dc} \pm 0.09	1.33 ^c \pm 0.02	2.84 ^c \pm 0.05	0.79 ^b \pm 0.02	0.37 ^c \pm 0.02	5.36 ^a \pm 0.63	1.55 ^a \pm 0.07	0.56 ^{bc} \pm 0.02	1.19 ^a \pm 0.05	0.33 ^a \pm 0.01	0.16 ^b \pm 0.01	41.91 ^a
60	13.79 ^{bc} \pm 0.32	3.84 ^{cd} \pm 0.18	1.33 ^c \pm 0.06	2.92 ^c \pm 0.14	0.82 ^b \pm 0.05	0.45 ^b \pm 0.03	5.55 ^a \pm 0.06	1.54 ^a \pm 0.05	0.54 ^c \pm 0.02	1.17 ^a \pm 0.04	0.33 ^a \pm 0.02	0.18 ^a \pm 0.01	40.19 ^b
80	14.41 ^b \pm 0.47	4.20 ^b \pm 0.14	1.41 ^b \pm 0.03	3.21 ^b \pm 0.09	0.87 ^a \pm 0.02	0.43 ^b \pm 0.02	4.73 ^b \pm 0.25	1.38 ^b \pm 0.08	0.46 ^d \pm 0.02	1.05 ^b \pm 0.05	0.29 ^c \pm 0.01	0.14 ^c $<$ 0.01	32.75 ^d
100	15.74 ^a \pm 1.17	6.01 ^a \pm 0.02	1.62 ^a \pm 0.02	5.36 ^a \pm 0.03	0.72 ^c \pm 0.01	0.81 ^a \pm 0.03	1.96 ^d \pm 0.19	0.75 ^c \pm 0.02	0.20 ^e \pm 0.02	0.67 ^c \pm 0.02	0.09 ^d $<$ 0.01	0.10 ^e \pm 0.01	12.50 ^e

¹ Mg = mangiferin, IsoMg = isomangiferin, I-dihex = iriflophenone-di-O,C-hexoside, I-glc = iriflophenone-3-C-glucoside, Hph = 3-hydroxy-phloretin-di-C-hexoside, Hd = hesperidin; ² Different letters indicate significant differences ($p < 0.05$) between all measurements within a column, extraction temperature was 50°C, extraction time was 30 min and solvent:solid ratio was 10 mL/g; ³ Soluble solids; ⁴ Plant material

Table A.2 The effect of extraction time on the extract yield, compound yield and polyphenol content of unfermented *C. genistoides* extract using teabag fraction and milled plant material (*values represent means ± standard deviation*)

Extraction time (minutes)	Teabag Fraction												Extract Yield ² (g SS ³ /100g PM ⁴)
	compound ¹ content ² (g/ 100 g soluble solids)						compound yield ² (g/ 100 g plant material)						
	Mg	IsoMg	I-dihex	I-glc	Hph	Hd	Mg	IsoMg	I-dihex	I-glc	Hph	Hd	
10	14.24 ^{ba} ± 0.24	3.76 ^a ± 0.06	1.96 ^a ± 0.03	3.01 ^b ± 0.03	0.70 ^b ± 0.01	0.80 ^a ± 0.01	1.72 ^f ± 0.09	0.45 ^e ± 0.02	0.24 ^e ± 0.01	0.36 ^f ± 0.02	0.08 ^b < 0.01	0.10 ^e < 0.01	12.05 ^B
20	14.74 ^a ± 0.87	3.88 ^a ± 0.22	1.91 ^{ba} ± 0.11	3.22 ^a ± 0.21	0.71 ^b ± 0.04	0.68 ^b ± 0.02	2.38 ^e ± 0.14	0.63 ^f ± 0.04	0.31 ^f ± 0.02	0.52 ^e ± 0.03	0.12 ^e ± 0.01	0.11 ^f < 0.01	16.15 ^f
30	14.52 ^a ± 0.85	3.81 ^a ± 0.22	1.86 ^{ba} ± 0.10	3.25 ^a ± 0.17	0.71 ^b ± 0.04	0.59 ^c ± 0.01	2.80 ^{de} ± 0.17	0.74 ^{ef} ± 0.04	0.36 ^{fe} ± 0.02	0.63 ^{de} ± 0.03	0.14 ^{ef} ± 0.01	0.11 ^{ef} < 0.01	19.30 ^e
40	14.70 ^a ± 0.52	3.85 ^a ± 0.13	1.85 ^{bc} ± 0.05	3.33 ^a ± 0.09	0.71 ^b ± 0.02	0.56 ^{dc} ± 0.02	3.15 ^{dc} ± 0.17	0.82 ^{ed} ± 0.04	0.40 ^{de} ± 0.02	0.71 ^{dc} ± 0.04	0.15 ^{ef} ± 0.01	0.12 ^{ed} < 0.01	21.42 ^d
50	14.66 ^a ± 0.98	3.83 ^a ± 0.25	1.82 ^{bc} ± 0.09	3.32 ^a ± 0.20	0.71 ^b ± 0.04	0.52 ^{dc} ± 0.04	3.49 ^c ± 0.32	0.91 ^d ± 0.08	0.43 ^d ± 0.03	0.79 ^c ± 0.07	0.17 ^{ed} ± 0.01	0.12 ^d < 0.01	23.79 ^c
60	14.26 ^{ba} ± 0.15	3.74 ^a ± 0.05	1.76 ^c ± 0.03	3.28 ^a ± 0.04	0.70 ^b ± 0.01	0.49 ^{de} ± 0.01	3.61 ^c ± 0.05	0.95 ^d ± 0.02	0.45 ^{dc} ± 0.00	0.83 ^c ± 0.02	0.18 ^d < 0.01	0.12 ^{ed} < 0.01	25.33 ^c
Milled Plant Material													
10	14.16 ^{ba} ± 0.11	3.75 ^a ± 0.02	1.32 ^d ± 0.01	2.91 ^b ± 0.03	0.80 ^a < 0.01	2.91 ^e < 0.01	5.30 ^b ± 0.21	1.40 ^f ± 0.05	0.50 ^{bc} ± 0.01	1.09 ^b ± 0.03	0.30 ^c ± 0.01	0.17 ^c ± 0.01	37.43 ^b
20	14.06 ^{ba} ± 0.23	3.73 ^a ± 0.07	1.31 ^d ± 0.02	2.88 ^b ± 0.05	0.80 ^a ± 0.02	2.88 ^e ± 0.02	5.60 ^{ba} ± 0.19	1.49 ^b ± 0.05	0.52 ^{ba} ± 0.02	1.15 ^{ba} ± 0.03	0.32 ^{bc} ± 0.01	0.17 ^{bc} ± 0.01	39.83 ^a
30	14.14 ^{ba} ± 0.51	3.81 ^a ± 0.09	1.32 ^d ± 0.03	2.92 ^b ± 0.06	0.80 ^a ± 0.02	2.92 ^e ± 0.01	5.81 ^{ba} ± 0.25	1.56 ^{ba} ± 0.05	0.54 ^{ba} ± 0.02	1.20 ^{ba} ± 0.03	0.33 ^{ba} ± 0.01	0.17 ^{bc} ± 0.01	41.06 ^a
40	14.65 ^a ± 0.77	3.90 ^a ± 0.18	1.35 ^d ± 0.06	2.99 ^b ± 0.14	0.82 ^a ± 0.04	2.99 ^e ± 0.02	6.11 ^a ± 0.35	1.62 ^a ± 0.09	0.56 ^a ± 0.03	1.25 ^a ± 0.07	0.34 ^a ± 0.02	0.18 ^a ± 0.01	41.69 ^a
50	13.60 ^b ± 0.62	3.73 ^a ± 0.07	1.30 ^d ± 0.03	2.86 ^b ± 0.04	0.78 ^a ± 0.02	2.86 ^e ± 0.02	5.68 ^{ba} ± 0.33	1.55 ^{ba} ± 0.04	0.54 ^{ba} ± 0.01	1.20 ^{ba} ± 0.03	0.33 ^{ba} ± 0.01	0.17 ^{bc} ± 0.01	41.72 ^a
60	14.01 ^{ba} ± 0.74	3.81 ^a ± 0.26	1.33 ^d ± 0.08	2.93 ^b ± 0.16	0.81 ^a ± 0.04	2.93 ^e ± 0.02	5.87 ^a ± 0.37	1.60 ^{ba} ± 0.12	0.56 ^a ± 0.04	1.23 ^a ± 0.08	0.34 ^{ba} ± 0.02	0.18 ^{ba} ± 0.01	41.86 ^a

¹ Mg = mangiferin, IsoMg = isomangiferin, I-dihex = iriflophenone-di-O,C-hexoside, I-glc = iriflophenone-3-C-glucoside, Hph = 3-hydroxy-phloretin-di-C-hexoside, Hd = hesperidin; ² Different letters indicate significant differences ($p < 0.05$) between all measurements within a column, extraction temperature was 50°C, solvent used was 60% EtOH (v/v) and solvent:solid ratio was 10 mL/g; ³ Soluble solids; ⁴ Plant material

Table A.3 Effect of elevated temperatures of aqueous ethanol and deionised water on the extract yield, compound yield and polyphenol content of unfermented *C. genistoides* extract using teabag fraction (values represented as means \pm standard deviations)

Extraction time (minutes)	Deionised Water												Extract Yield ² (g SS ³ /100g PM ⁴)
	Compound ¹ content ² (g/ 100 g soluble solids)						compound yield ² (g/ 100 g plant material)						
	Mg	IsoMg	I-dihex	I-glc	Hph	Hd	Mg	IsoMg	I-dihex	I-glc	Hph	Hd	
10	7.96 ^e \pm 0.28	2.54 ^e \pm 0.08	1.33 ^a \pm 0.07	1.22 ^b \pm 0.05	0.75 ^{fe} \pm 0.02	0.64 ^e \pm 0.02	1.70 ^f \pm 0.10	0.54 ^f \pm 0.29	0.28 ^d \pm 0.02	0.26 ^g \pm 0.28	0.16 ^f \pm 0.05	0.14 ^h \pm 0.01	21.33 ⁱ
20	8.56 ^d \pm 0.05	2.73 ^d \pm 0.02	1.27 ^{ab} \pm 0.01	1.24 ^b \pm 0.01	0.80 ^d \pm 0.01	0.60 ^{fe} \pm 0.02	2.32 ^e \pm 0.03	0.74 ^e \pm 0.01	0.34 ^b $<$ 0.01	0.34 ^f $<$ 0.01	0.22 ^e $<$ 0.00	0.16 ^g $<$ 0.00	27.10 ^g
30	8.60 ^d \pm 0.15	2.75 ^d \pm 0.03	1.23 ^{bc} \pm 0.01	1.25 ^b \pm 0.02	0.80 ^d \pm 0.01	0.58 ^{fg} \pm 0.01	2.62 ^d \pm 0.07	0.84 ^d \pm 0.02	0.37 ^a $<$ 0.01	0.38 ^d $<$ 0.01	0.24 ^c $<$ 0.00	0.18 ^{gf} $<$ 0.00	30.47 ^f
40	8.66 ^d \pm 0.06	2.79 ^d \pm 0.03	1.18 ^{dc} \pm 0.03	1.23 ^b \pm 0.01	0.74 ^f \pm 0.01	0.56 ^{fg} \pm 0.01	2.82 ^c \pm 0.05	0.91 ^c \pm 0.01	0.38 ^a $<$ 0.01	0.40 ^{cb} $<$ 0.01	0.24 ^c $<$ 0.00	0.18 ^f $<$ 0.00	32.63 ^d
50	8.33 ^{de} \pm 0.14	2.70 ^d \pm 0.04	1.13 ^d $<$ 0.01	1.18 ^b \pm 0.01	0.69 ^g \pm 0.01	0.53 ^{fg} $<$ 0.01	2.78 ^c \pm 0.08	0.90 ^c \pm 0.02	0.38 ^a $<$ 0.01	0.39 ^{cd} $<$ 0.01	0.23 ^d $<$ 0.00	0.18 ^{gf} $<$ 0.00	33.35 ^c
60	8.32 ^{de} \pm 0.06	2.71 ^d \pm 0.02	1.12 ^{de} \pm 0.02	1.19 ^b \pm 0.01	0.66 ^g $<$ 0.01	0.52 ^g $<$ 0.01	2.83 ^c \pm 0.03	0.92 ^c \pm 0.01	0.38 ^a $<$ 0.01	0.41 ^{cb} $<$ 0.01	0.23 ^{de} $<$ 0.00	0.18 ^{gf} $<$ 0.00	34.06 ^b
60% EtOH (v/v)													
10	10.37 ^c \pm 0.09	3.09 ^c \pm 0.03	1.23 ^{bc} \pm 0.03	1.36 ^a \pm 0.02	0.79 ^{de} \pm 0.01	1.58 ^a \pm 0.06	1.76 ^f \pm 0.04	0.52 ^f \pm 0.01	0.21 ^e \pm 0.01	0.23 ^h \pm 0.01	0.13 ^g \pm 0.00	0.27 ^e \pm 0.01	16.94 ^j
20	10.87 ^{ab} \pm 0.62	3.26 ^{ab} \pm 0.19	1.17 ^{dc} \pm 0.05	1.35 ^a \pm 0.06	0.89 ^c \pm 0.05	1.37 ^b \pm 0.07	2.85 ^c \pm 0.20	0.85 ^d \pm 0.06	0.31 ^c \pm 0.02	0.35 ^e \pm 0.02	0.23 ^{dc} \pm 0.02	0.36 ^d \pm 0.02	26.22 ^h
30	11.07 ^a \pm 0.61	3.30 ^a \pm 0.18	1.12 ^{de} \pm 0.06	1.33 ^a \pm 0.07	0.94 ^{ab} \pm 0.04	1.26 ^c \pm 0.05	3.48 ^b \pm 0.18	1.04 ^b \pm 0.05	0.35 ^b \pm 0.01	0.42 ^b \pm 0.02	0.29 ^b \pm 0.01	0.40 ^c \pm 0.01	31.47 ^e
40	11.14 ^a \pm 0.23	3.32 ^a \pm 0.06	1.12 ^{de} \pm 0.04	1.33 ^a \pm 0.03	0.95 ^a \pm 0.01	1.27 ^c \pm 0.04	3.83 ^a \pm 0.10	1.14 ^a \pm 0.03	0.38 ^a \pm 0.01	0.46 ^a \pm 0.01	0.33 ^a \pm 0.01	0.44 ^a \pm 0.01	34.42 ^b
50	10.55 ^{bc} \pm 0.36	3.13 ^{bc} \pm 0.12	1.05 ^{fe} \pm 0.03	1.25 ^b \pm 0.04	0.90 ^{bc} \pm 0.03	1.17 ^d \pm 0.03	3.78 ^a \pm 0.13	1.12 ^a \pm 0.04	0.38 ^a \pm 0.02	0.45 ^a \pm 0.02	0.32 ^a \pm 0.01	0.42 ^b \pm 0.02	35.78 ^a
60	10.34 ^c \pm 0.41	3.06 ^c \pm 0.12	1.04 ^f \pm 0.06	1.23 ^b \pm 0.07	0.89 ^c \pm 0.05	1.13 ^d \pm 0.09	3.76 ^a \pm 0.17	1.11 ^a \pm 0.05	0.38 ^a \pm 0.02	0.45 ^a \pm 0.03	0.32 ^a \pm 0.02	0.41 ^{cb} \pm 0.03	36.33 ^a

¹ Mg = mangiferin, IsoMg = isomangiferin, I-dihex = iriflophenone-di-O,C-hexoside, I-glc = iriflophenone-3-glucoside, Hph = 3-hydroxy-phloretin-di-C-hexoside, Hd = hesperidin;

² Different letters indicate significant differences ($p < 0.05$) between all measurements within a column, aqueous extraction performed at 90°C, ethanolic extraction performed at 70°C, solvent:solid ratio was 10 mL/g; ³ Soluble solids; ⁴ Plant material

Table A.4 Central Composite Design and the response values for isomangiferin (IsoMg) content of the extract¹ and isomangiferin yield²

Run No.	Block	X_1 Temperature (°C)	X_2 Ethanol concentration (%, v/v)	g IsoMg/ 100 g soluble solids ¹	g IsoMg/ 100 g plant material ²
1	1	45	20	2.35	2.67
11	2	45	20	2.35	2.76
21	3	45	20	2.29	2.72
2	1	45	80	2.90	2.82
12	2	45	80	2.90	2.76
22	3	45	80	2.70	2.69
3	1	65	20	2.40	2.94
13	2	65	20	2.45	2.89
23	3	65	20	2.45	2.98
4	1	65	80	2.83	3.05
14	2	65	80	2.78	3.03
24	3	65	80	2.72	3.04
5	1	40	50	2.49	2.96
15	2	40	50	2.46	3.01
25	3	40	50	2.44	2.93
6	1	70	50	2.44	3.14
16	2	70	50	2.61	3.18
26	3	70	50	2.38	3.16
7	1	55	0	2.46	2.71
17	2	55	0	2.45	2.69
27	3	55	0	2.37	2.59
8	1	55	100	4.23	1.41
18	2	55	100	4.01	1.74
28	3	55	100	3.87	1.68
9(C) ³	1	55	50	2.46	3.09
19(C)	2	55	50	2.40	3.12
29(C)	3	55	50	2.40	3.03
10(C)	1	55	50	2.49	3.18
20(C)	2	55	50	2.37	3.02
30(C)	3	55	50	2.37	3.06

³ (C) = centre point parameter values

Table A.5 ANOVA of experimental results for the polynomial quadratic equation for isomangiferin yield and isomangiferin content in the extract (significant *p*-values (*p* < 0.05) are highlighted in red)

Parameter ¹	g Isomangiferin/100 g plant material						g Isomangiferin/100 g soluble solids					
	Regr Coeff ²	SS ³	DF ⁴	MS ⁵	F	p	Regr Coeff ²	SS ³	DF ⁴	MS ⁵	F	p
Intercept	0.4704						2.3455					
(1) Temperature (°C) (L)	0.0050	0.0224	1	0.0224	113.1162	0.000	0.0003	0.0016	1	0.0016	0.2205	0.6435
Temperature (°C) (Q)	-0.0000	0.0001	1	0.0001	0.3099	0.5836	0.0001	0.0007	1	0.0007	0.0945	0.7615
(2) Ethanol concentration (L)	0.0073	0.0264	1	0.0264	133.6080	0.000	-0.0120	3.9121	1	3.9121	549.8383	0.0000
Ethanol concentration (Q)	-0.0000	0.1361	1	0.1361	688.6989	0.000	0.0003	1.9022	1	1.9022	267.3504	0.0000
1L by 2L	0.0000	0.0000	1	0.0000	0.0422	0.8393	-0.0001	0.0192	1	0.0192	2.6985	0.1153
Lack of fit (LOF)		0.0397	3	0.0132	66.9140	0.000		0.6501	3	0.2167	30.4556	0.0000
Error (MS residual)		0.0042	21	0.0002				0.1494	21	0.0071		
Total SS		0.2678	29					7.1872	29			
R ²						0.84						0.889
R ² _{adj}						0.80						0.866

¹ L = linear coefficient, Q = quadratic coefficient, L by L = interaction coefficient; ² Regression coefficients; ³ Sum of squares; ⁴ Degrees of freedom; ⁵ Mean square

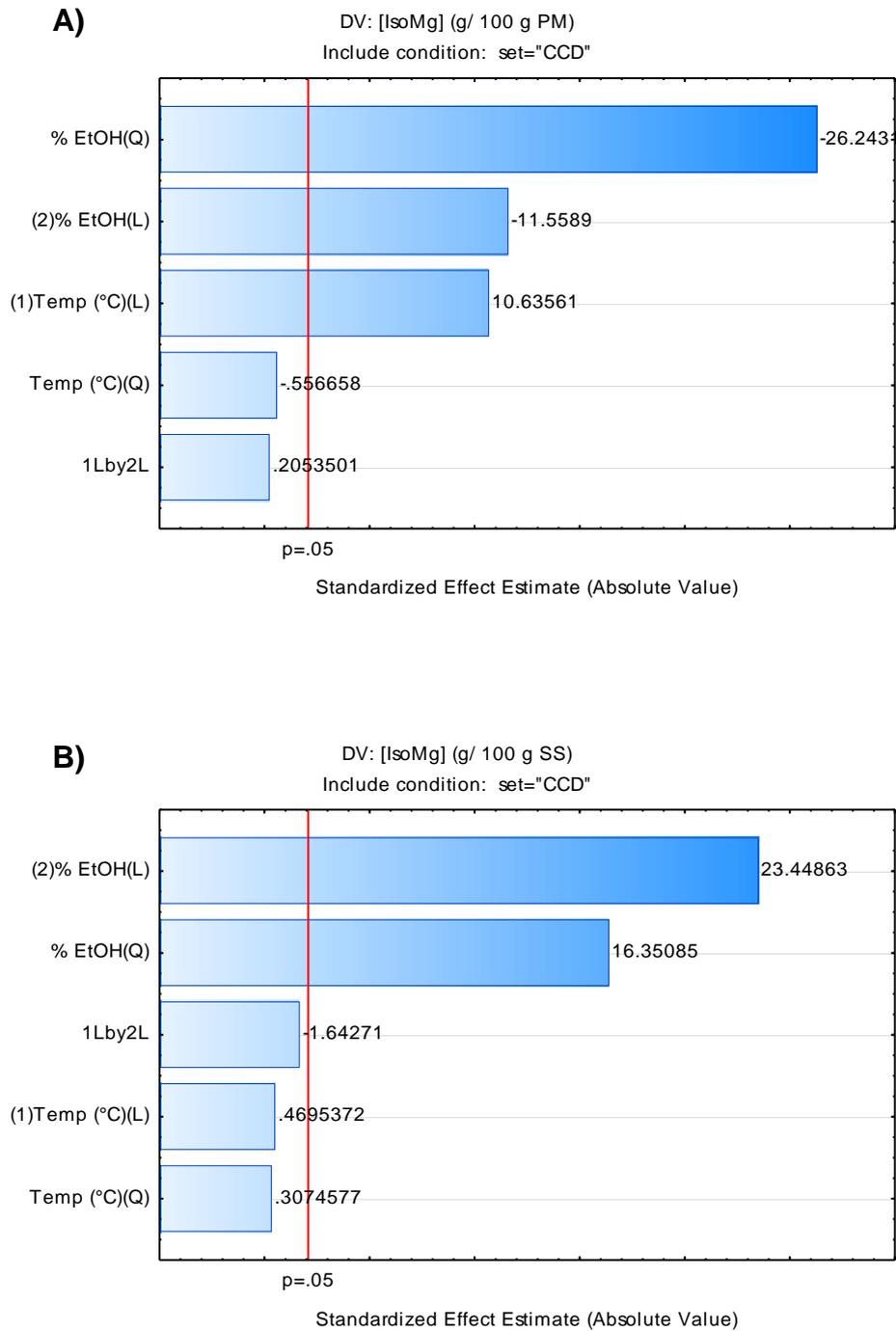
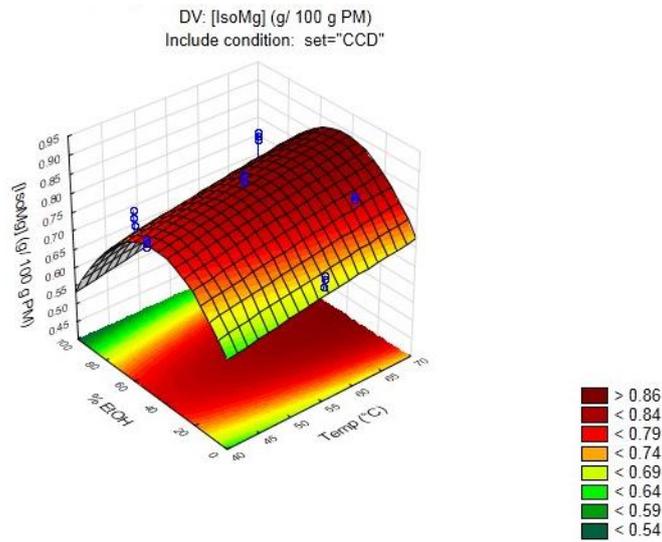


Figure A.1 Standardised Pareto charts indicating the relationship of independent process variables on isomangiferin yield (A) and isomangiferin content of the extract (B). (DV = dependant variable, L = linear effect, Q = quadratic effect, L by L = interaction effect).

A)



B)

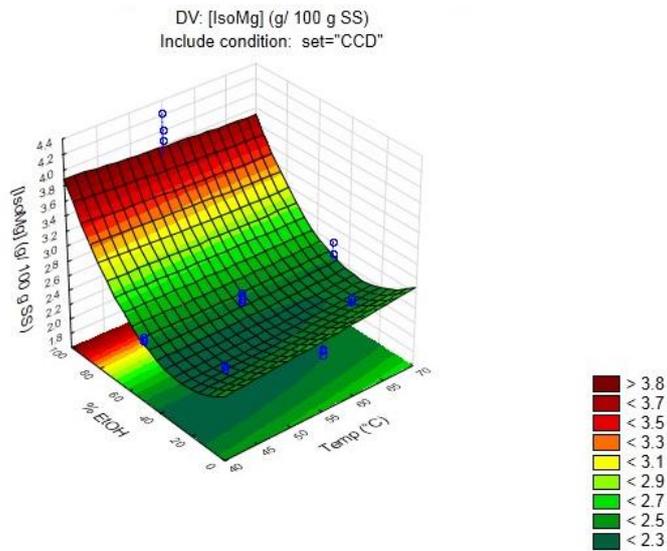


Figure A.2 Combined fitted response surface plots for isomangiferin yield (A) and isomangiferin content in the extract (B) as a function of temperature (°C) and ethanol concentration (% v/v). (DV = dependant variable).

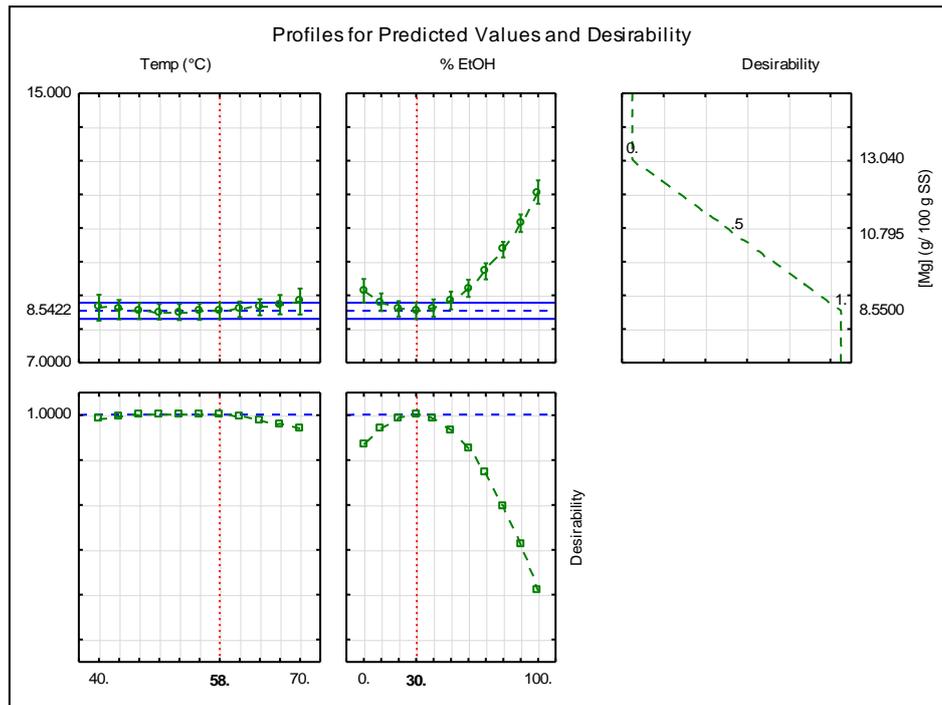


Figure A.3 Individual prediction profile graph for mangiferin content of the extract with optimum ethanol concentration at 30 % (v/v) and temperature at 58°C for minimum response.

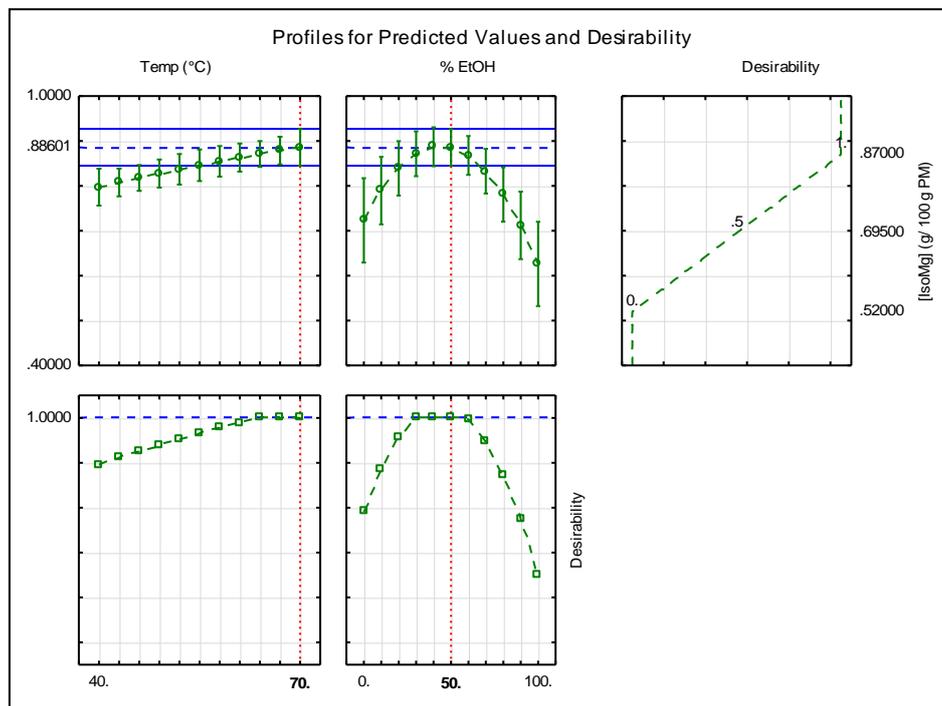


Figure A.4 Individual prediction profile graph for isomangiferin yield with optimum ethanol concentration at 50% (v/v) and temperature at 70°C for maximum response.

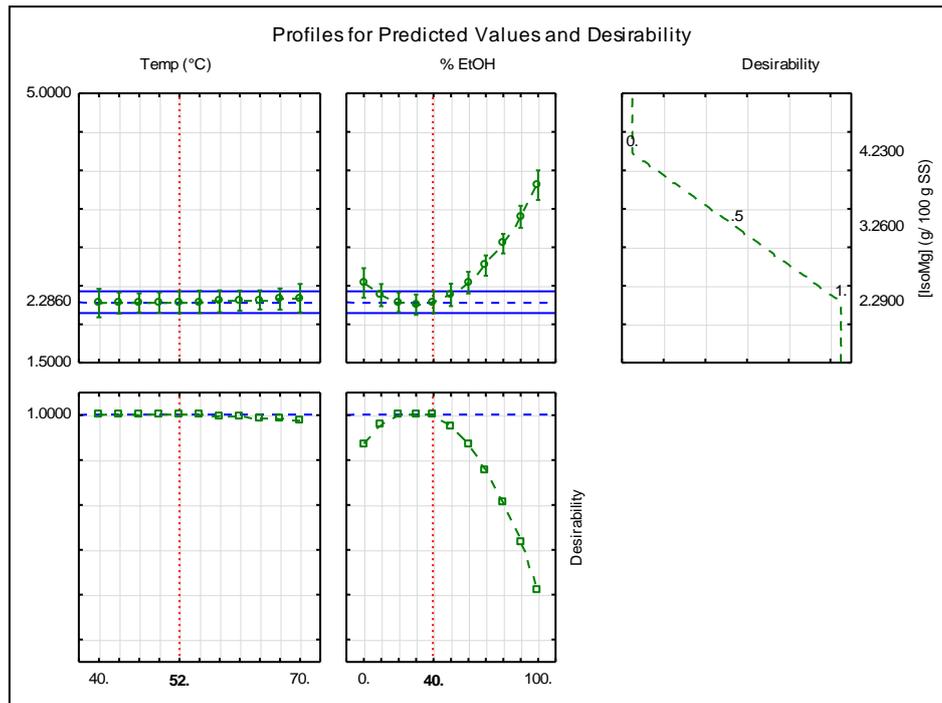


Figure A.5 Individual prediction profile graph for isomangiferin content of the extract with optimum ethanol concentration at 40% (v/v) and temperature at 52°C for minimum response.

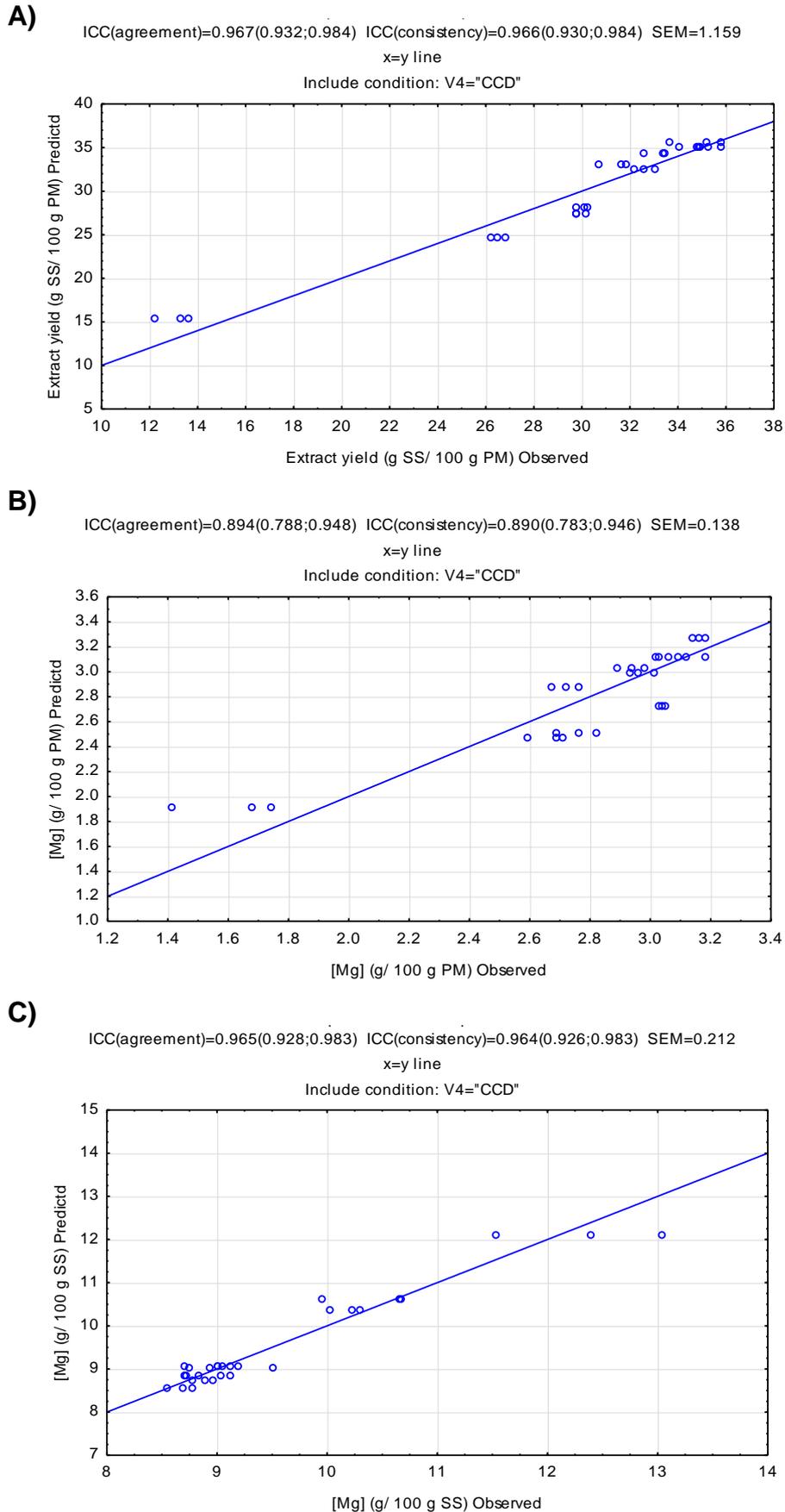


Figure A.6 Scatter plots of the predicted vs. experimental results for extract yield (A), mangiferin yield (B) and mangiferin content in the extract (C).

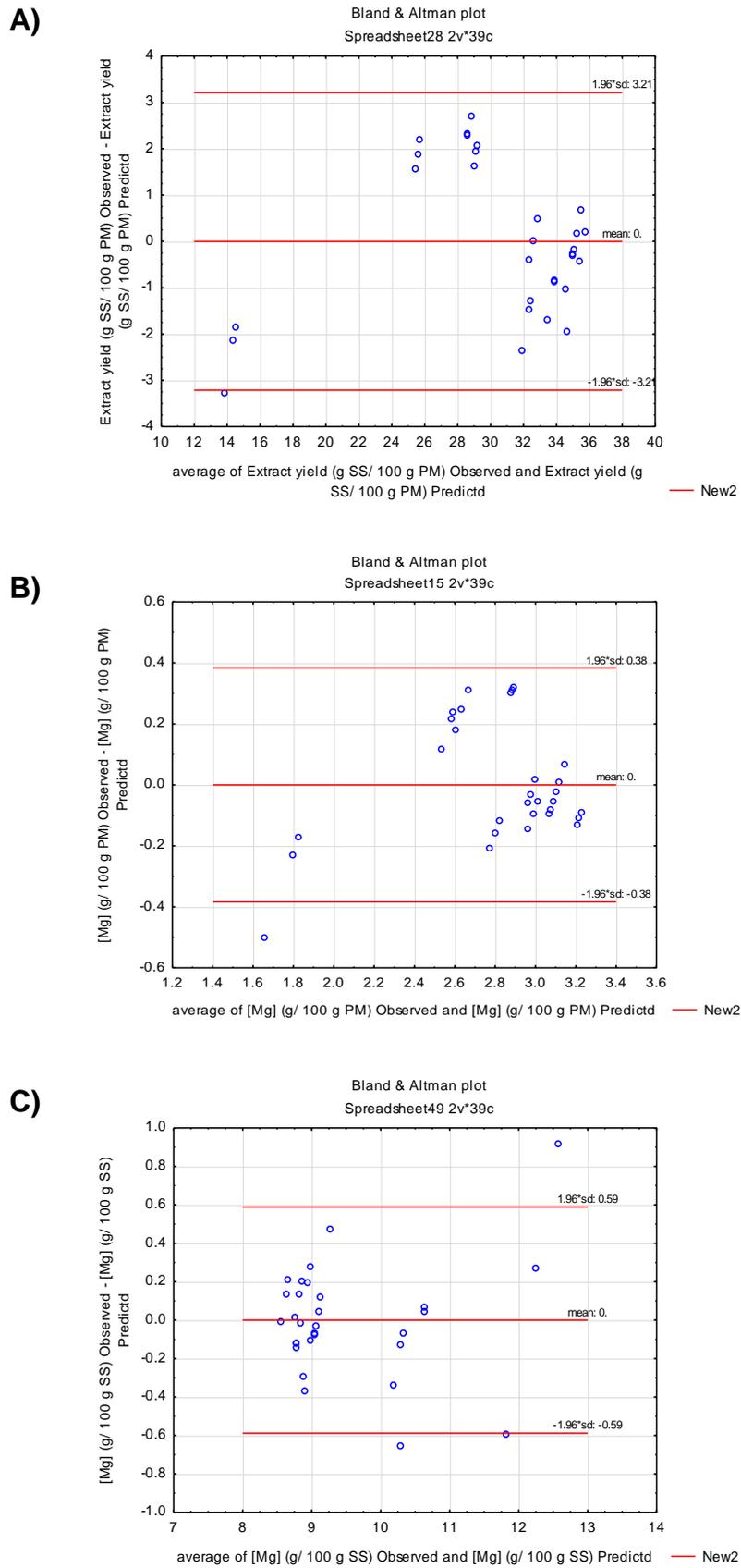


Figure A.7 Bland-Altman plots of the verification experimental data for extract yield (A), mangiferin yield (B) and mangiferin content of the extract (C).

Addendum B

Optimisation of ultrafiltration process parameters for xanthone enrichment of unfermented *Cyclopia genistoides* extract using response surface methodology

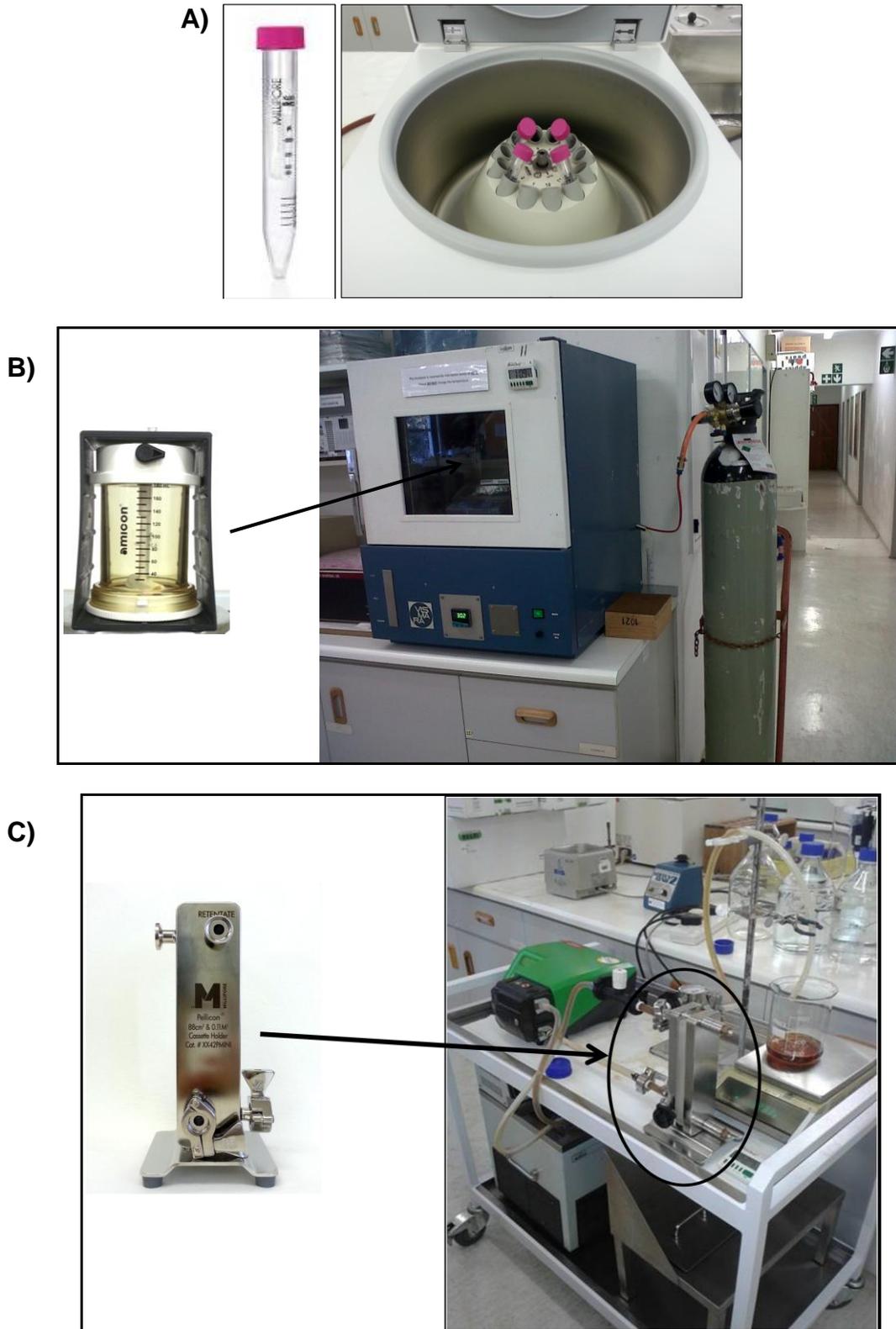


Figure B.1 Experimental set up of the centrifugal membrane (A), stirred cell (B) and tangential flow (C) ultrafiltration systems for the processing of unfermented *C. genistoides* extract (40% ethanol, v/v).

Table B.1 Central Composite Design and the response values obtained for isomangiferin enrichment

Run no.	X_1 Transmembrane pressure (bar)	X_2 Feed flow rate (mL/min)	Isomangiferin enrichment (%)
1	1.00	200	13.66
11	1.00	200	15.21
2	1.00	401	17.25
12	1.00	401	17.44
3	1.86	200	22.43
13	1.86	200	23.55
4	1.86	401	24.24
14	1.86	401	24.10
5	0.82	301	16.25
15	0.82	301	16.82
6	2.04	301	25.49
16	2.04	301	27.90
7	1.43	158	17.11
17	1.43	158	17.70
8	1.43	444	19.05
18	1.43	444	20.38
9 (C) ¹	1.43	301	15.68
10 (C)	1.43	301	15.90
19 (C)	1.43	301	14.23
20 (C)	1.43	301	17.76

¹(C) = centre point parameter values**Table B.2** ANOVA of experimental results for the polynomial quadratic equation for isomangiferin enrichment (significant values ($p < 0.05$) are highlighted in red)

Parameter ¹	Regr. Coeff. ²	SS ³	DF ⁴	MS ⁵	F	p
Intercept	38.19272					
(1) TMP (bar) (L)	-31.14084	221.34418	1	221.34418	198.30517	0.00000
TMP (bar) (Q)	14.96127	70.44048	1	70.44048	63.10856	0.00001
(2) Feed flow rate (mL/min) (L)	-0.05043	13.34987	1	13.34987	11.96032	0.00535
Feed flow rate (mL/min) (Q)	0.00012	14.31773	1	14.31773	12.82744	0.00431
1L by 2L	-0.01002	1.500994	1	1.50099	1.34476	0.27075
Lack of fit (LOF)		0.87358	3	0.29119	0.26088	0.85210
Pure Error		12.27798	11	1.11618		
Total SS		319.81658	19			
R ²						0.96
R ² _{adj}						0.94

¹ TMP = transmembrane pressure, L = linear coefficient, Q = quadratic coefficient, L by L = interaction coefficient; ² Regression coefficients;³ Sum of squares; ⁴ Degrees of freedom; ⁵ Mean square

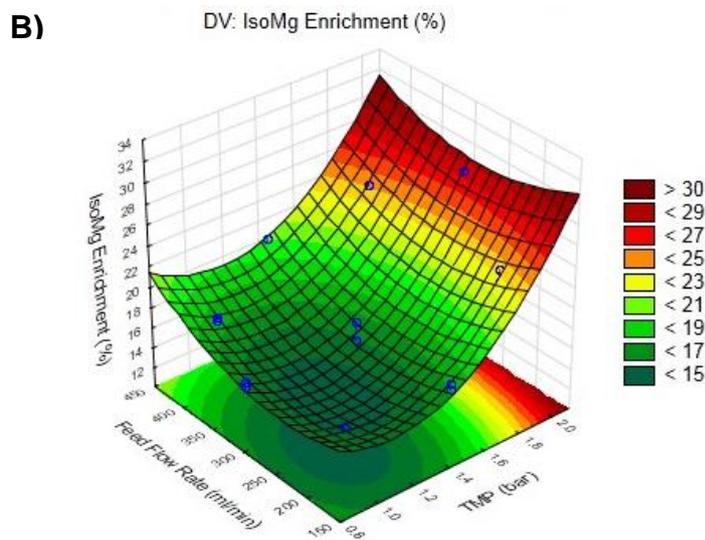
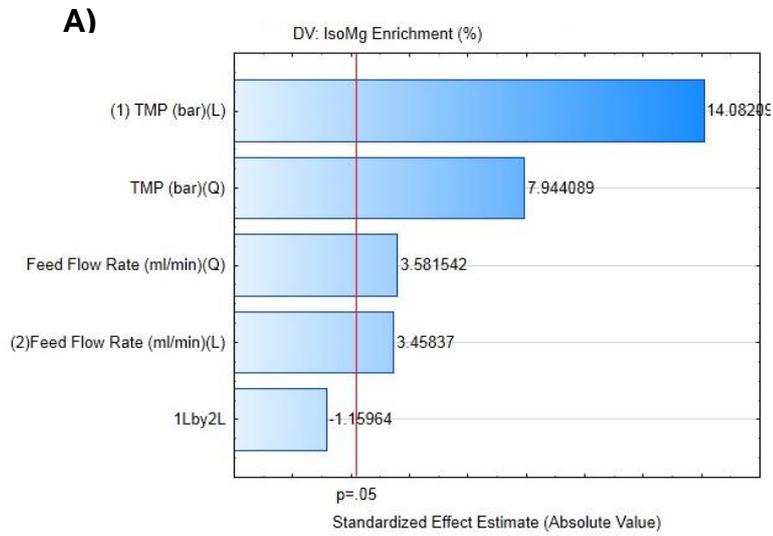


Figure B.2 Standardised Pareto chart (A) and combined fitted response surface plot (B) for isomangiferin enrichment as a function of transmembrane pressure (TMP, bar) and feed flow rate (mL/min). (DV = dependant variable, L = linear effect, Q = quadratic effect, L by L = interaction effect).

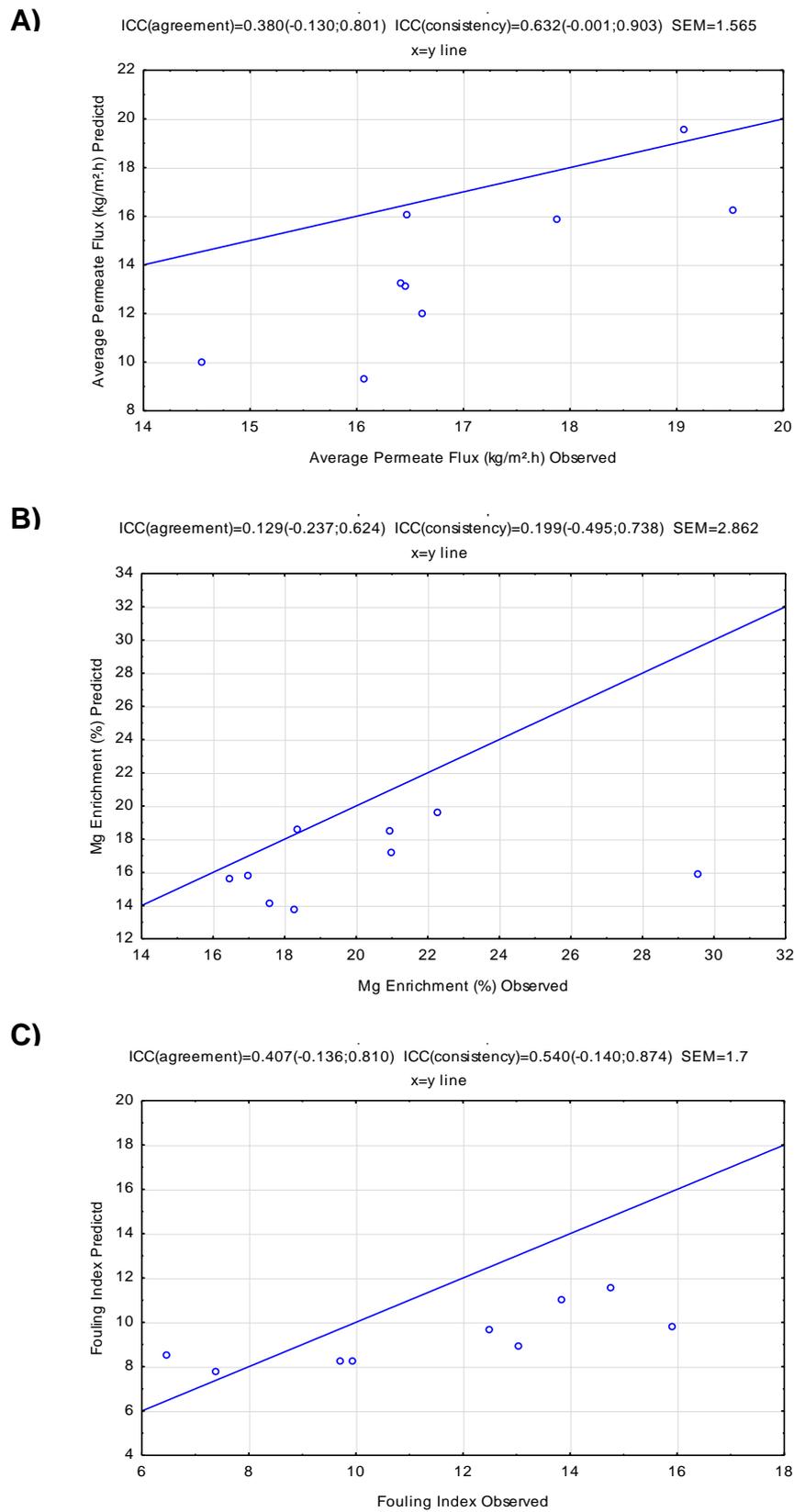
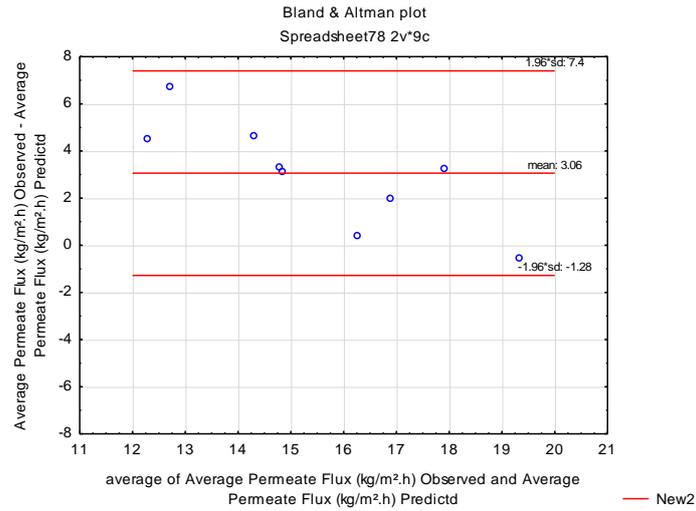
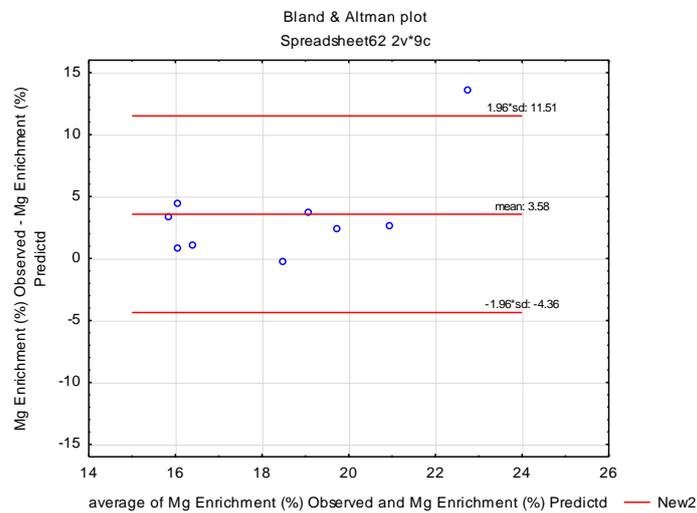


Figure B.3 Scatter plots of the predicted vs. observed verification results for average permeate flux (A), mangiferin enrichment (B) and fouling index (C).

A)



B)



C)

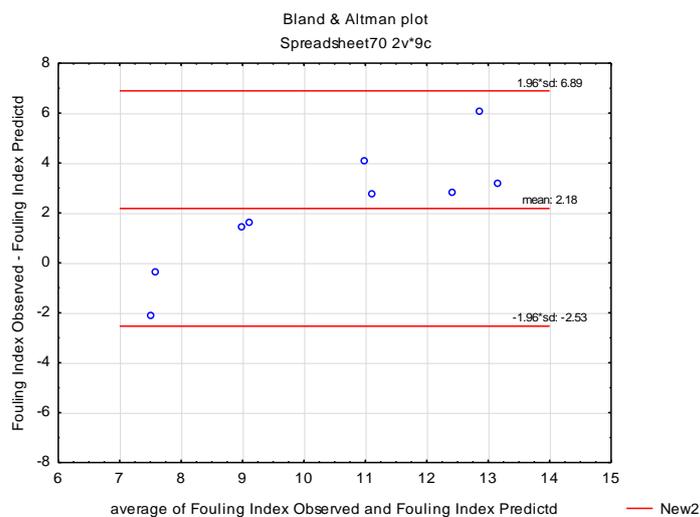


Figure B.4 Bland-Altman plots of the verification experimental data for average permeate flux (A), mangiferin enrichment (B) and fouling index (C).