UTILITY OF CRITICAL FLUIDS IN EXTRACTION AND ENCAPSULATION OF POLYPHENOLICS FROM BY-PRODUCT OF CIDER PRODUCTION

BY

SALIS IBRAHIM

A thesis submitted to
The University of Birmingham
for the award of a degree of
DOCTOR OF PHILOSOPHY

Supercritical Fluid Technology Research Group
School of Chemical Engineering
College of Engineering and Physical Sciences
University of Birmingham, United Kingdom

December, 2015

ABSTRACT

Efficacy of subcritical water in the recovery of the polyphenolic compounds from the apple pomace using a batch reactor system at 100-bar over a temperature range of 100-200°C for a residence time of 10–30 minutes was investigated. Organic solvent extractions using acetone and ethanol were carried out to serve as a baseline for comparison with the subcritical water extraction Subcritical water was efficient in solubilising the apple pomace, and extracting polyphenolics with high antioxidant activity. Maximum solubilisation of the apple pomace was achieved at 145°C for 30 minutes and total phenolic content and antioxidant activity at 200°C. Solubilisation, ORAC activity and total phenolic content of subcritical water extract were 28.20g/100g DW, 99285μmol TE/g DW and 49.86mg/g GAE DW of apple pomace respectively, compared to 19.20g/100g DW, 6260.27 μmol TE/g DW and 21.70mg/g GAE DW of acetone extracts of apple pomace respectively. Protocatechuic aldehyde was identified for the first time only in the subcritical water extract and to date has not been identified in solvent extracts of cider apple pomace.

Encapsulation of polyphenolic s of subcritical water extract using spray drying was explored. Particles/powders formed were derived from the naturally occurring carbohydrate polymers co-extracted with polyphenols. Addition of HPβ-Cyclodextrins (SWE+ HPβ-CD) to the directly encapsulated powder (SWE) significantly reduced hygroscopicity and improved antioxidant activity.

ACKNOWLEDGEMENTS

Firstly, I would like to thank the Ghana Education Trust Fund (GETFund) for the funding and support received for my PhD. Study at the University of Birmingham UK.

Special appreciation goes to my academic supervisor Prof. Liam Grover for his guidance and support in this research and related works.

My sincere appreciation goes to my industrial supervisor Dr. Steve Bowra for his priceless service, support, motivation and immense contribution offered me in the entire period of research and writing of this thesis.

I will also like to thank Dr. Regina Santos formerly of University of Birmingham for the supervising this research at the initial stages and also appreciate the love and care I received.

I grateful acknowledge the learning opportunities, help and support I have received from members of the supercritical fluid group especially Dr. Fabio Antas, Dr. Tiejan Lu, Dr Luis Roman, Edeh Ifeanyichukwu, Raitis Kalnins, Arielle Muniz De Barros and Muhammad Hazwan.

Finally I cannot express the love and support I have received from my family especially my lovely wife Ruhaimah Abdul- Bassit, who have endured patience and offered me encouragement throughout this PhD study. I could not have imagined having a better family.

TABLLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF EQUATIONS

STRUCTURE OF THE THESIS

The thesis has been structured in to 6 chapters and presented as follows. Chapter 1 reviews works related to apple pomace as a by-product of the food and drink industry, polyphenolics and related bioactivities, and applications of critical fluids both for extraction and encapsulation and the summary of the main objectives of the research. All analytical techniques used in the research are presented in chapter 2 as material and methods. Chapters 3 and 4 addresses the optimisation of organic solvent and subcritical water mediated recovery of polyphenolic compounds from the apple pomace using response surface methodology. Chapter 5 discusses the feasibility of encapsulating the polyphenolic fraction derived from apple pomace using naturally occurring polymers co-extracted with polyphenolics under the subcritical water mediated hydrolysis to improve oxidative stability and assess the impact on formulations and performance in for example skin care products. Overall conclusions and recommendations for future work are presented in chapter 6.

Chapter 1

Literature Review

1.1 Apples and Apple pomace

1.1.1 Apples

Apples (Malus domestica) have a long history and are extensively cultivated throughout the world. The fruits are appreciated for taste and flavour but also for their vitamins, antioxidants and dietary fibre (Brendan, 2010).

Apples are cultivated in moderate temperate regions of the world (Agrahari and Khurdiya, 2003, Joshi et al., 1996, Kaushal and Joshi, 1995, Kaushal et al., 2002). The global annual production in 2010 was approximately 70×10^6 tonnes covering a 4.8×10^6 hectares (FOA, 2011) thus making apples the 4th most cultivated fruit globally next to banana, oranges and grapes. See Table 1-1.

China contributes over 40% of the world's apple output. Within the European Union, the second largest producer in the world, Poland is the leading contributor. The United State contributes 6% of world output and is third in the international ranking. Iran, Turkey, Russian Federation, India and Chile are other large producers with Chile dominating production in Latin America which accounts for less than 6% of the world production.

Broadly, the leading producers of apple in the world are also the principal consumers. However there are regional and country variation in per capita consumption for example, Turkey, Canada, and New Zealand recorded higher consumption per head in 2007 (Brendan, 2010).

Each apple producing country at one time had their own local and regional cultivars as a result there are over 10,000 registered apple cultivars (Jules et al., 1996). However, due to expanding production and transport networks, and the need for commercial high quality products, only a select number of varieties are currently grown (Way et al., 1990). To date ten apple varieties dominate the market. These are ‘’McIntosh, Braeburn, Jonathan, Idared, Jonagold, Fuji, Granny Smith, Gala, Delicious and Golden Delicious (O’Rourke et al., 2003).

Table 1-1 Major Apple Producing Countries in the World (2009- 10).

Country	**Production (metric tonnes)**
China	29,857,163
USA	4,358,710
Poland	2,830,870
Iran	2,718,775
Turkey	2,504,490
Italy	2,208,227
France	1,940,200
India	1,777,200
Russian Federation	1,467,000
Chile	1,370,000
Argentina	1,300,000
Brazil	1,124,155
Germany	1,046,995
Others	15,113,739
World+	**69,819,324**

Source: Food and Agriculture Organization of United Nation FAO, website

Apples can be categorised according to use for example, culinary, desert and apple juice production. The different categories have desired characteristics in terms of texture and taste. Cider apples are distinct from the desert and culinary apples, due to the high content of tannin and their fibroid nature.

There are over seventy cider apple varieties reported in United Kingdom and approximately twenty three available in France (Johansen, 2000). The varieties are further categorised in to four groups according to acidity and tannins levels (Lea, 1990, Morgan and Richards, 1993).Sweet varieties are low in acidity and tannin and are the softest of the four groupings; common examples are court royal and sweet coppin. The Sweet apples are blended with the more strongly flavoured varieties in order to produce a commercially palatable cider.

Bittersweet varieties typically, Dabinett and Yarlington Mill, impart the distinct flavour associated with ciders produced in the United Kingdom which are low in acidity and high in tannin content. Palate-astringency and bitterness of these apples are attributed to high tannin content.

Brown’s apple and Crimson king are well-known sharp varieties of which acidity predominate over tannin content; however they are Scarce nowadays and are often substituted with culinary apples to balance flavour of cider.

The last category of cider apples is bittersharp of which Stoke red is an example, have moderate acidity and tannin contents (The National Association of Cider Makers UK, cider fruit research overview). Table 1-2 summarises the classifications.

Table 1-2 Classification of Cider Apples in England

Cider apple	**Acid** <**0.45**% (W/V)	**Acid** > **0.45**% (W/V)
Tannins < **0.2**%	Sweet	Sharp
Tannins >**0.2**%	Bittersweet	Bittersharp

Developed by Long Ashton research station (Lea, 1990, Morgan and Richards, 1993)

1.1.1.1 Principal Products from Apple processing

Of the total production of apples at least 71% of the fruit is eaten fresh, and approximately 20% is processed into apple juice and cider after fermentation. The balance is used to produce spirits, apple purées and other apple by-products (Joshi, 1997, Joshi and Attri, 1991, Kaushal et al., 2002). Various technologies are used to extract the juice from apples. A Continuous press method is used in large productions whereas simple vertical hydraulic presses are used in small production systems. Enzymes may be added to increase the extraction yield (Issenhuth, 2008). However the enzymes should not change the physical and chemical characteristics or standards related to the juice (Nogueira and Wosiacki, 2012).

Apple pomace is the residue left after juice or must extraction and constitutes between 20- 35% by weight of the original production feedstock. The amount of the pomace generated and its composition will depend on the variety of the apple and the techniques used in extracting the juice (Mahawar et al., 2012).

1.1.2 Apple Pomace

Approximately 9×10^6 tonnes of apple pomace are produced worldwide per the estimation of the amount of apples processed. The use and the amount of pomace vary according to country. China is the largest producer of apple pomace in the world with an annual yield of over 3 million tons since 2009 (Yue et al., 2012). In India at least 1 million tonnes of apple pomace every year is produced, of which approximately 10,000 tonnes is utilised (Shalini and Gubta, 2010). In Brazil, 80,000 tonnes of pomace are generated each year and primarily utilised as animal feed (Vendruscolo et al., 2008). At least 20,000 tonnes per annum of the pomace is generated in northern Spain, a region which accounts for a significant proportion of the global cider apples production (Diñeiro García et al., 2009).

There are two types of solid waste generated during the production of apple juice or cider. 1) apples which are rejected/ discarded during grading 2) the apple pomace residue as a result of pressing/juice extraction. The disposal of the solid wastes requires environmental consideration to forestall any pollution to the ecosystem. Apple pomace is a wet biomass and if dumped into landfills will create pollution problems because of its high chemical oxygen demand(COD) of 250-300g/kg which is prone to spontaneous fermentation (Mahawar et al., 2012). Hence, the increasing desire to develop alternative routes where by apple pomace will be utilised both adding value but avoiding disposal costs(Kennedy et al., 1999).

1.1.3 Basic Chemical Composition of Apple Pomace

Apple pomace is a heterogeneous biomass residue consisting of peels, discarded apples, seeds, core, stems and exhausted apple tissue (Kennedy et al., 1999). and as result contains water, sugar, small amount of protein at a low pH (Chantanta et al., 2008).

It is very difficult to find a standardised chemical composition of the apple pomace as it is highly dependent on the variety of apples used, the region of growth, the degree of ripening, agricultural practices and the extraction processes involved (Kennedy et al., 1999).

Furthermore the detail of the biochemical analysis reported in the literature is influenced by the research objective as illustrated in the following reports claimed apple pomace contained 26.4% dry matter, 4.0% proteins, 3.6% sugars, 6.8% cellulose, 0.3% ash, 0.42% acid and Calcium, 8.7 mg/100g of wet apple pomace. (Vasil’ev et al., 1976) While Carson et al (1994) went further and defined the fibre content of apple pomace suggesting it ranged from 11.6 to 44.5% and included cellulose, (12.0-23.2%), lignin (6.4 - 19.0%), Pectin (3.5 - 18%) and hemicellulose (5.0- 6.2%). The average dietary fibres (35.8%) and sugars (54.4%) this make up a total 91.2% of the pomace and the other constituents are lipids, proteins, and ash (Carson et al., 1994).

The Table 1-3 below is a compilation of compositional data sourced from a number of reports.

1.1.4 Potential Uses of Apple Pomace

Apple pomace is a potential source of carbohydrate, fibre, polyphenolics and pectin (Cetkovic et al., 2008, Guyot et al., 2003) which can find application in the food, feed, pharmaceutical, cosmetics, chemical, and biofuels sectors (Sato et al., 2010) The following offers a snap shot of some of the approaches and studies undertaken to enhance utility of apple pomace biomass.

1.1.4.1 Food sector

Efforts to utilise apple pomace residue to produce Jams and sauces has been reported by Kaushal and Joshi 1995, for which a technology was worked out for the preparation of apple press cake from apple pomace powder. The techniques involved; moulding, drying, crushing and fractioning and many bakery ingredients incorporating the pomace powder were also demonstrated. Similarly two types of toffee were produced by replacing soy meal with the pomace powder and the quality was not negatively affected (Shalini and Gubta, 2010).

Because of the large quantities of apple pomace produced during processing of apples it is important to explore a full range of products (Mahawar et al., 2012).

Table 1-3 Proximate composition of apple pomace (Devaravjan, 1997, Joshi, 1998, Joshi and Attri, 2006, Joshi and Pandey, 1999, Joshi and Sandhu, 1996, Parma, 2003).

Constituents	**Composition**	
	Wet weight basis	**Dry weight basis**
Moisture g/100g)	66.4-78.2	3.97-5.40
Acidity(% malic acid,w/v)	N/A	2.54-3.28
Total soluble solids(TSS,°B)	N/A	57.85
Totalcarbohydrate (mg/kg)	9.50-22.00	48.00-62.00
Glucose(mg/kg)	6.1	22.70
Frutose (mg/kg)	13.6	23.60
Sucrose (mg/kg)	N/A	1.80
Xylose (mg/kg)	N/A	0.06
pH	3.05-3.80	3.9
Vitamin C (mg/100g)		8.53-18.50
Soluble Proteins	N/A	3.29
Proteins (mg/kg)	1.03-1.82	4.45-5.67
Crude fiber(g/kg)	4.30-10.50	4.70-48.72
Fat[ether extract(%)]	0.82-1.43	3.49-3.90
Pectin (%)	1.50-2.50	3.50-14.32
Ash (mg/kg)	N/A	1.6
Polyphenol (%)	N/A	0.99%
Amino acids (%)	N/A	1.52
Minerals		
Potassium (mg/kg)	N/A	0.95
Calcium (mg/kg)	N/A	0.06
Sodium (mg/kg)	N/A	0.2
Magnesium (mg/kg)	N/A	0.02
Copper (mg/kg)	N/A	1.1
Zinc (mg/l)	N/A	15
Manganese(mg/l)	N/A	8.50-9.00
Iron (mg/l)	N/A	230
Calorific value (Kcal/100g)	N/A	295

N/A = non applicable

1.1.4.1.1 Pectin Production

Pectin is a structural heteropolysaccharide contained in the primary cell walls of terrestrial plants (Carpita and Gibeaut, 1993). Pectin is considered as a complex polysaccharide which consists of α1-,4-linked D-galacturonic acid, which are partly methyl esterified, and the side chain contains various neutral sugars, such as L-rhamnose, L-arabinose, and D galactose (Helene et al., 2012). According FAO and the EU, pectin must be composed of not less than 65% galacturonic acid units (United Nation Food and Agricultural Organization).Figure 1-1 an illustration of the types of Pectin structures

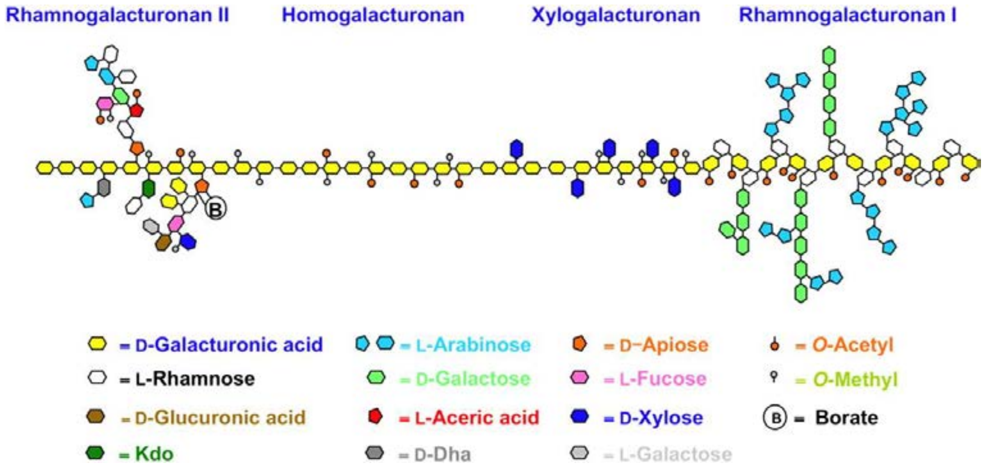


Figure 1-1 An illustration of the types of Pectin structures (Harholt et al., 2010)

Historically, pectin was cited first in1750 in an article published in England regarding the production of jelly from apples (Kertesz, 1951). Extraction processes started to develop around 1908 in Germany and rapidly advanced to the United States of America, of which a patient (US. Patent number 1082682) was issued to Douglas in 1913 (Douglas, 1913).

Pectins are categorised based on the extent of esterification into low methoxyl (LM) and high methoxyl (HM). Low methoxyl pectins are composed of between 25-50 % methoxylated carboxyl groups, and high methoxyl pectin contains between 50-80%. The characteristics of pectin portions are dictated by the structure of the molecules. Low methoxyl pectin form gel in the presence of cations like Ca^{2+} whereas high methoxyl pectins depends on high amount of co-solute in acidic medium to produce gel (Lundt et al., 2003, Voragen et al., 1995).

Presently the two main sources of pectin derived from plant materials are from citrus and apple peels. Other potential value resources are not explored due to unwanted properties (Marcon et al., 2005). Pectin is considered one of the essential components discovered in apples and capable of providing about 10% of a day fibre requirement. Interestingly, pectin is not broken down by digestive juices in the intestines and remains soluble. They help increase the amount of faecal substances in the intestines which aid their appropriate functioning. Pectin helps in retention of water and other residues in the intestines, promote removal of toxic substances onward with faecal matter, ensures good protection of mucus of intestines and aid in treatment of diarrhoea (Helene et al., 2012).

Pectins are primarily considered as health promoting food ingredients and catalogued alongside other food constituents. Global consumption of pectin annually has been estimated to be around 45000 tons with international market value of approximately €400 million (Savary et al., 2003).

In the food industry, pectin is used to enhance viscosity and act as safeguarding of colloids stability in foods and beverages (Helene et al., 2012). They are largely used during preparation of jellies and jams, fruit juice, confectionary food and baking industry as fillings. Pectins are used in stabilization of dairy products such as yogurts and mixture of milk and fruit juice because it is stable at acidic pH below 4.3 (May, 1990, Rolin and De Vries, 1990).

Apple pomace, on a dry weight basis contains approximately 10-15% pectin (Endreß, 2000). The production of pectin from apple pomace is regarded as more economic and realistic approach of utilization of the residue both from a commercial and environmental considerations (Fox et al., 1991).

The pomace has been used to recover pectin for several decades (Sharma et al., 1985). Normally for pectin recovery, dried apple pomace is usually employed in the extraction, by adding dilute mineral acids at relatively higher temperatures to ensure solubilisation of the insoluble pectic substances localised in the cell wall and middle lamella. The pectin is precipitated after concentrating, by adding ethanol. The precipitate is then dried, ground and sieved (May, 1990).

1.1.4.2 Feed Sector

Apple Pomace can be utilised as a component of animal feed formulations either fresh, dried and or after ensiling (Taasoli and Kafilzadeh, 2008).The apple pomace is highly palatable feed, medium in energy, but very low in protein content. When properly supplemented, it can replace up to about one-third of the concentrates in rations and 15-20% in complete feedlot rations. (Jane, 2007). Apple pomace has no fixed nutritive value and changes based on the varieties of apples, husbandry, maturity of fruit and the processes applied to extract the juice (Kennedy et al., 1999). The crude protein content of apple pomace varies from 19 to 65g Kg^{-1} on dry matter basis (Carson et al., 1994). However, due to the medium to low energy content after digestion compared to corn silage, apple pomace is considered as an average or poor quality animal fodder (Carson et al., 1994) The nutrient profile is shown in Table 1-4.

Moreover on a dry matter basis, the energy value of the ensiled apple pomace is about 80% that of corn silage and it is advisable to mix a dry absorbent material with the pomace at the time of ensiling in order to prevent excessive nutrient loss from seepage (Rust, 1991).

Table 1-4 Comparison of the nutrient profile for apples and apple pomace with Corn silage and corn, all nutrients expressed on DM basis

Constituent	**Apple**	**Apple pomace**	**Corn silage**	**Corn**
Moisture %	82.1	82.30	65	15
Dry matter%	17.9	27.70	35	86
TDN, %	69.7	63.40	72	90
ME, MJ/Kg	10.58	5.52	10.85	13.61
Fat, %	2.2	2.60	3.1	4.1
Crude protein,%	2.8	4.30	8.7	9.8
Crude fibre, %	7.3	38.2	19.5	2.3
Calcium,%	0.06	0.15	0.25	0.03
Potassium,%	0.78	0.58	1.14	0.44
Magnesium,%	0.28	0.07	0.18	0.12
Sulphur,%	0.06	0.11	0.12	0.11
Sodium,%	0.06	0.11	0.01	0.01
Phosphorus%	0.78	0.58	0.22	0.32

Source: Steven Rust and Dan Buskirk, Michigan state University, Department of Animal science (Buskirk and Rust, 2008). ME-metabolisable energy; TDN-total digestable nutrient

1.1.4.3 Chemical Sector

1.1.4.3.1 Citric acid production

Citric acid (2-hydroxyl-propane, 1-3-trix Carboxylic acid) with a molecular weight of 210.14 g/mol is considered among world's largest tonnage of fermentation products (Baei et al., 2008) with high demand (Hoseyini et al., 2011). It is white or colourless, odourless, crystalline solid. Approximately 70% of citric acid produced is utilised in the food industry, 12% in medicine and 18% in other industrial applications (Hang and Woodams, 1986).

The commercial production of citric acid is achieved by submerged fungal fermentation of molasses (Kapoor et al., 1982), The physical nature of apple pomace restricts it use in submerged fermentation, however if apple pomace is diluted one-quarter its volume with water submerged fermentation is feasible. However, the amount of citric acid recovered from diluted pomace solutions was low and therefore not economic (Hang and Woodams, 1986).

The production of citric acid using solid state fermentation process of apple pomace was developed using the fungus *Aspergillus niger* NRRL 567. The technique produced 90g of citric acid/kg of apple pomace by adding 3-4% methanol at 30^{o}C in 5days. Yields of citric acids of 77-88% were obtained if additional carbohydrate source was added (Hang and Woodams, 1986).

Song et al., (2003) suggested adding cellulose to supplement low reducing sugar content of apple pomace to effectively get higher yields of citric acid. The research of Song et al demonstrated that, the enzyme -cellulase effectively enhanced the yield of glucose and significantly influenced the production of citric acid from apple pomace using *Aspergillus niger* fungus. It was shown that, the celluloytic enzyme, cellulase A6 could yield about 170g glucose per 1kg of apple pomace over 12h reaction time with cellulase concentration of 20 U/g at pH=7 and 50^{o}C within the medium without alkali pre-treatment. When cellulase treated apple pomace was used as a liquid substrate with *Aspergillus niger*-C fungus selected yields reached 256g citric acid per 1 kg dried apple pomace at 35^{o}C in 3 days or 30^{o}C in 5 days with rotation speed of flask at 210 rpm. However three important problems were raised 1) The *Aspergillus niger* C selected needed to be grown well for the production of citric acid from apple pomace 2) yields of the product decreased during scale-up operations. Lastly extraction rate of citric acid was low and needed advance separation methods (Song et al., 2003).

An improved solid state fermentation for citric acid production from apple pomace residue was reported by Dhillon et al., (2011) where they evaluated the production of citric acid using *Aspergillus niger* strain NRR-576 from apple pomace complemented with rice husk. Moisture content and inducers (methanol and ethanol) concentration were optimised, by response surface methodology. They established that moisture and methanol positively influenced the yield of citric acid production using *Aspergillus niger* bred on the apple pomace. Higher yields of citric acid (342.41g/kg and 248.20g/kg dry substrate) from apple pomace by *Aspergillus niger* were achieved with 75% (V/W) moisture along with [3 % (V/W) methanol and 3 % (V/W) ethanol] as inducers. The efficiency of fermentations was 93.9% and 66.42% respectively based upon the total carbon consumption over 144h period of incubation. Normal fermentation on a tray was performed with the same optimised parameters and citric acid concentrations of 187.96g/kg dry substrate with 3% (V/W) ethanol, 303.34g/kg dry substrate with 3% (V/W) methanol were recorded respectively. Efficiency of fermentation of 50.8% and 82.89% in ordinary fermentation were achieved based on the amount of carbon utilised after 120h period of incubation (Dhillon et al., 2011). Therefore citric acid production using apple pomace as substrate could serve another alternative means of utilising apple pomace (Song et al., 2003)

However, the low content of reducing sugar in apple pomace (19.2%) makes the residue unsuitable for citric acid production directly and therefore a challenge in practical production (Song et al., 2003). However, fresh apple pomace is an acid-substrate with significant mean capacity which can facilitate microorganisms’ growth due to its high moisture content (Chaudhary et al., 1978, Roukas, 1991, Sanroman et al., 1994, Shankaranand and Lonsane, 1994, Shojaosadati and Babaeipour, 2002, Tran and Mitchell, 1995)

1.1.4.4 Biofuel sector

1.1.4.4.1 Ethanol Production

Bioethanol produced from biomass sources is regarded as an important biofuel because of the sustainable, environmental benefits (Fargione et al., 2008). Apple pomace is a bioactive biomass which poses problem with respect to disposal therefore can be seen as a cheap alternative feedstock for ethanol production. Ethanol has been produced from apple pomace using the Montrachet strain of *Saccharomyces cerevisiae* and more than 43g of ethanol was achieved from 1kg of apple pomace when fermented at 30^{o}C over a period of 24h giving a fermentation efficiency of 89 % (Hang et al., 1982).

Khosravi and Shojaosadati, 2003 described a process of ethanol production from apple pomace using *Saccharomyces cerevisiae* driven solid state fermentation. A moisture content of 75% (W/W), and starting sugar content of 26% (W/W), with 1% (W/W) content of nitrogen were the conditions applied. 2.5% (W/W) and 8% (W/W) of ethanol was produced without and with saccharification respectively. Their findings suggested production of ethanol by fermenting apple pomace could be a profitable means of producing biofuel at the same time reducing the amount apple pomace left as waste (Khosravi and Shojaosadati, 2003).

1.1.4.5 Biogas

Apple pomace has the potential to be used in anaerobic digestion plant to produce biogas, a renewable source of energy, which can be used directly in heating and cooking or used to drive electricity generation (Nicholas, 2012). The residue from the anaerobic digester represents a nutrient rich bio-fertiliser which can be recycled back onto arable land. Dried apple pomace can be utilised as solid fuel and burnt directly (Fischer, 1984), or converted to Charcoal briquettes which again can be used as solid domestic fuel (Walter and Sherman, 1976).

1.1.4.6 Recovery of Polyphenols

The preceding illustrated the potential to using the entire apple pomace biomass directly with little or no pre-treatment. However there is increasing awareness of the potential to add value to waste streams by applying what has become known as the biorefining concept.

Therefore the recovery of polyphenols, which are bioactive compounds, by fractionating the biomass aligns with biorefining and at the same time generates valuable feedstocks for food, feed, pharmacological or cosmetic purposes (Cetkovic et al., 2008, Y. Lu and Foo, 1997, Y. Lu and Foo, 2000).

Apples represent an important source of flavonoids in diets within Europe and the United States. Approximately 22% of polyphenolics consumed in the United states are derived from apples (Vinson et al., 2001). The phenolic compounds are widely distributed in the fruit and vegetables where they contribute to the colour and flavour. The major phenolic compounds found in apples include; Procyanidins, Epicatechins, Chlorogenic acids, Phloridzin and the Quercetin conjugates (Bhushan et al., 2008).

Flavonoids, and phenolic acids represent non-nutrient natural compounds derived from plants and are thought to offer protective effects of fruits and vegetables. It has been revealed that different polyphenolic compounds possess varied actions which could aid fight against diseases. Phytochemicals may impede proliferation of cancer cells, control immune and inflammatory response, and defend against oxidation of lipids (Hollman et al., 1997, Liu, 2003). A strong inverse relation exist between flavonoid intake and lung cancer development (Knekt et al., 1997), which results in a reduction in the risk of lung cancer associated with increased flavonoid consumption in younger people and in non-smokers. Potential functions of polyphenols on human body includes those shown is in Figure 1-2.

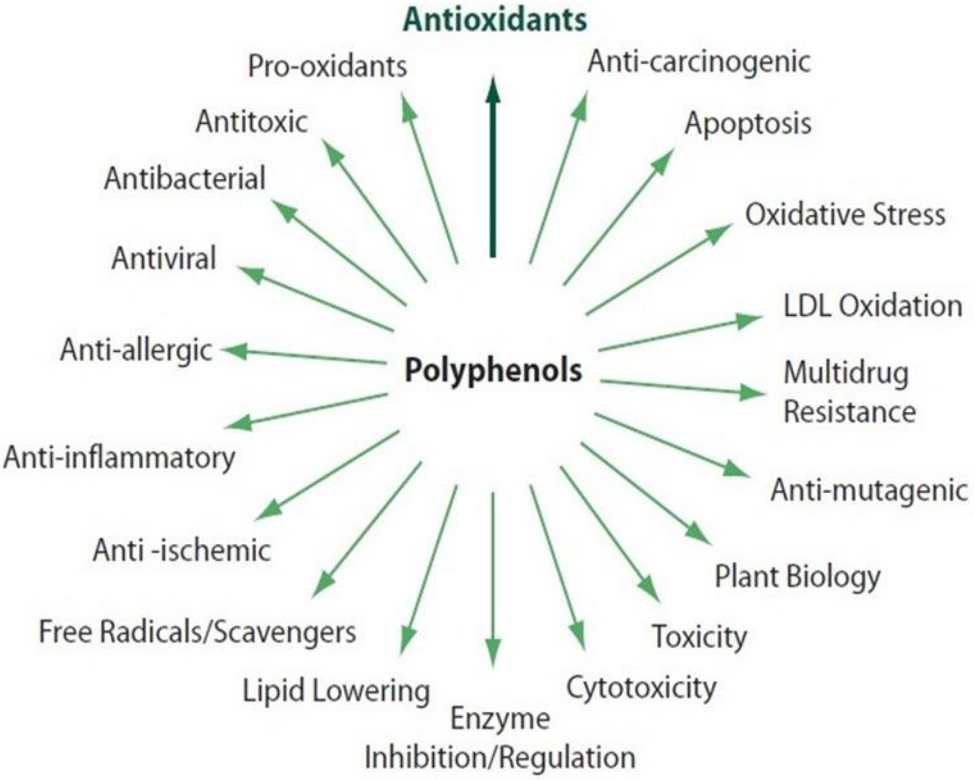


Figure 1-2 Benefits of Polyphenols on human health (Alexis, 2008).

Investigations have revealed that most of the phytochemicals are found in the peels (Serena et al., 2007). Therefore the polyphenolic content in the apple pomace will be greater than that in apple juice and varies among different cultivars of apples. The concentration of the polyphenolic compounds depends on several factors including storage of the apples, growth conditions, harvest time (Serena et al., 2007).

1.1.5 Classification of polyphenols

Polyphenols or Phenolic compounds represent one of the diverse and extensively distributed class of substances in the plant kingdom with over 8000 chemical structures discovered so far (Harborne, 1993).

The main group of polyphenolic compounds found in fruits and vegetables are classified as illustrated in Figure 1-3.

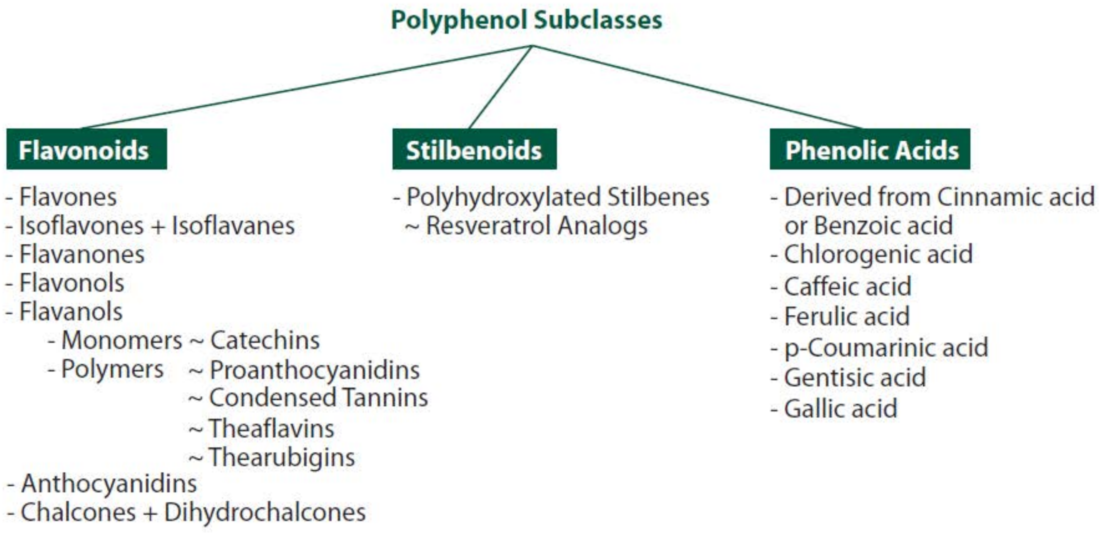


Figure 1-3 Classification of Polyphenols (Alexis, 2008).

Structures of Phenolic compounds are made up of one or more hydroxyl groups attached directly to an aromatic ring. Polyphenols on the other hand are compounds composed of at least one phenolic group attached to one or more aromatic rings (Crozier et al., 2006, Manach et al., 2004).The term polyphenols does not represent or refer to a polymer of simple phenols. Typically, polyphenols do not exist as free compounds in plants sources, but are found as glycoside or esters. They can vary from elementary compound like phenolic acid to extremely polymerized molecules as in tannins. Primarily, the sugar residues are glucose but occasionally can be galactose, rhamnose, xylose and arabinose linked to the hydroxyl groups directly or otherwise (Laura et al., 2013).

polyphenolic compounds are grouped into classes based on the number of carbon atoms in the molecule and also their chemical structure. They are classified as flavonoids and phenolic acids (Harborne, 1993).

1.1.5.1 Flavonoids

Flavonoids by far constitute the most diverse group of polyphenolic compounds in plants. Ordinarily they are discovered as glycosides derivatives and over 4000 flavonoids have been documented in plants (Harborne and Williams, 2000)). The list keeps growing because they can be many patterns of substitution of the ‘’hydroxyl, methoxy or glycosyl’’ groups of the primary structure, to form more complex ones (Véronique, 2005).

Flavonoids are soluble in water and the backbone molecule contains 15 carbon atoms. They have $C_6 - C_3 - C_6$ structure skeleton with ring A and B been phenolic in nature which are linked together by 3 grouped carbons as shown in Figure 1-4

They are sub-divided into 13 classes according to the extent of hydroxylation and the presence of a double bond between C_2-C_3 of the heterocycling pyron ring (A Scalbert and Williamson, 2000).

One of the 3-grouped carbons invariable is joined to a carbon of one of the aromatic rings, to yield a middle third ring C either directly or through an oxygen bridge as in Figure 1-5 Flavonoid structure.

, (http://www.phytochemicals.info/).

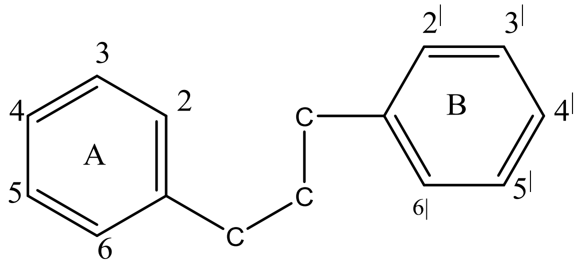


Figure 1-4 Generalised structure of Flavonoids.

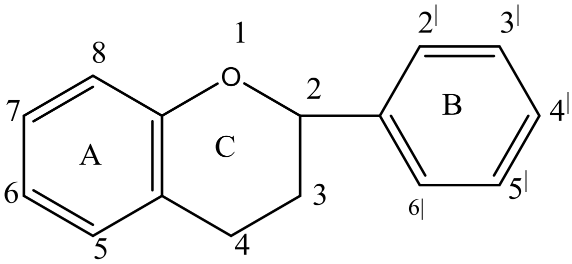


Figure 1-5 Flavonoid structure.

The variations in the chromate ring C as a result of hydroxylation pattern allow flavonoids to be classified further into six (6) sub-groups as; flavonols, flavanols, flavones, flavanones anthocyannins, isoflavones (A Scalbert and Williamson, 2000, Tsao and McCallum, 2009). Chalcones are still classified as a member of the flavonoids, although they lacked the heterocyclic ring C (Tsao and McCallum, 2009).

1.1.5.1.1 Flavonols and Flavones

Flanonols have double bond between $C_2 = C_3$ and contain OH group at position 3. They are characterized by complete unsaturated C ring that link the aromatic rings A and B in a single compound as in Figure 1-6.

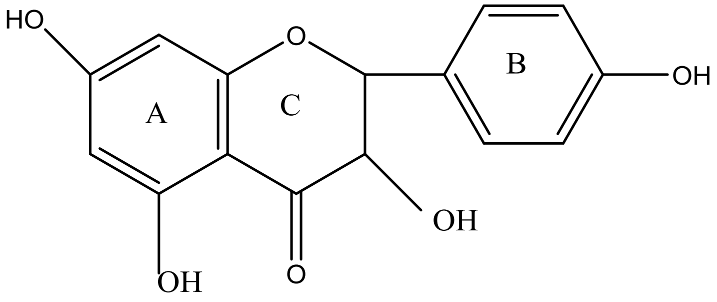


Figure 1-6 Flavonols structure.

Flavonols are abundant in diet with quercetin the most common flavonoids. They are usually found as O −Glycosides with at least 279 and 347 different glycosidic combinations for Quercetin and Kaempferol alone respectively (Tsao and McCallum, 2009). Onions, apples blueberries tea, curly kale and red wine are sources of flavonols (D'Archivio et al., 2007).

Dietary intake and bioavailability of flavonols are controlled by several factors including plant variety, growth, season, extent of ripeness, exposure to sun light, method of food preparation and processing (Aherne and O'Brien, 2002). Biosynthesis of flavonols is accelerated by light and they are accumulated around the outer tissues of fruits. Interestingly, depending on the exposure to sunlight, there can be differences in the amount of flavonoids among fruits on the same tree and even between different sides of a single piece of fruits (Cortell and Kennedy, 2006). Unlike flavonoids, Flavones are not widespread and have no OH – group attached to the position 3 of the C ring.

1.1.5.1.2 Flavanones

Flavanones have structure similar to Flavones only there is no double bond between C2 and C3. All three carbon chain atoms are saturated and an oxygen atom in the 4th position. They are glycosylated generally in C7 by disaccharides. Higher concentrations of Flavanones are obtained only from citrus species and lesser from tomatoes and some aromatic plants such as mint (D'Archivio et al., 2007).

1.1.5.1.3 Chalcones

Chalcones or dihydrochalcones have a linear C3 chain connecting the 2 benzene rings. There is a double bond in the C3-chain whereas all bonds are saturated in the dihydrochalcones as in Figure 1-7.

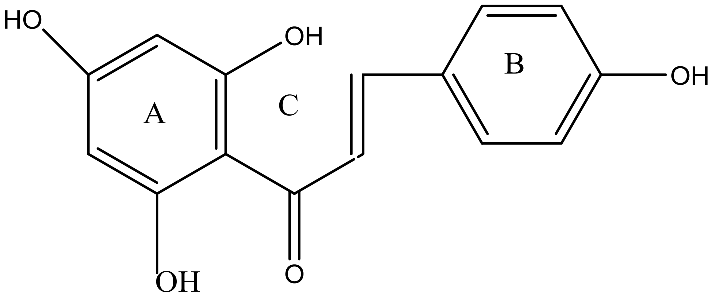
Typical example of dihydrochalcone is Phloridzin (Phloritin-2'-O-D-glucoside and commonly found in apple leaves and described as anti-tumour (Nelson and Falk, 1992).

1.1.5.1.4 Anthocyanidins

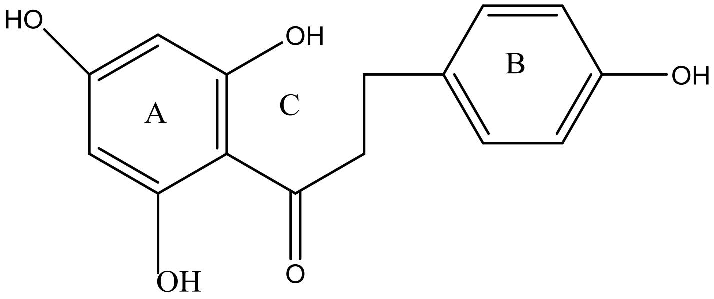
The plant pigments dominated by the water soluble anthocyanidins and are responsible for the colour of flowers, fruits, vegetables. Anthocyanins are mainly glycosides of anthocyanidins and are abundant in the skin except for some red fruits (e.g., pelargonidin, malvidin, cyanidins (Yildiz, 2010). Anthocyanidins have two double bonds in the C ring and also positively charged. Anthocyanin present large group of the colour pigments of plants with a sugar moiety attached primarily at C3 – position of ring C or at the 5 or 7 – position on aromatic ring A. Very rare glycosylation at the $3^1 – 4^1 – 5^1$ – position on the ring B have been reported(Mazza and Miniati, 1991). The sugar moieties may also be acylated by a range of aromatic or aliphatic acids.

1.1.5.1.5 Flavanols and Proanthocyanidins

Flavanols have structure similar to those of anthocyanidins with no positive charge on the oxygen atom. All the bonds in the ring 3- are saturated with hydroxyl group on the C3.



Chalcone



Dihydrochalcone

Figure 1-7 Structure of Chalcones and Dihydrochalcones.

Flavanols have two chiral centres on C2 and C3 due to the hydroxylation on the C3 which can possible yield four diastereomers. Catechin and Epicatechin are isomers with trans and cis configurations respectively. Each of the two flavan-3-ol have two stereoisomers as, (+)-catechin, (-)-catechin, (+)-epicatechin and (-)-epicatechin. They are commonly found in plants and are not glycosylated. Green tea, cocoa powder, red wine are example of sources of flavan-3-ol (Arts et al., 2000a, b).

Proanthocyanidins or condensed tannins are polymers of flavan-3-ol consisting up to 50 subunits resulting in formation of dimers and oligomers see Figure 1-8.

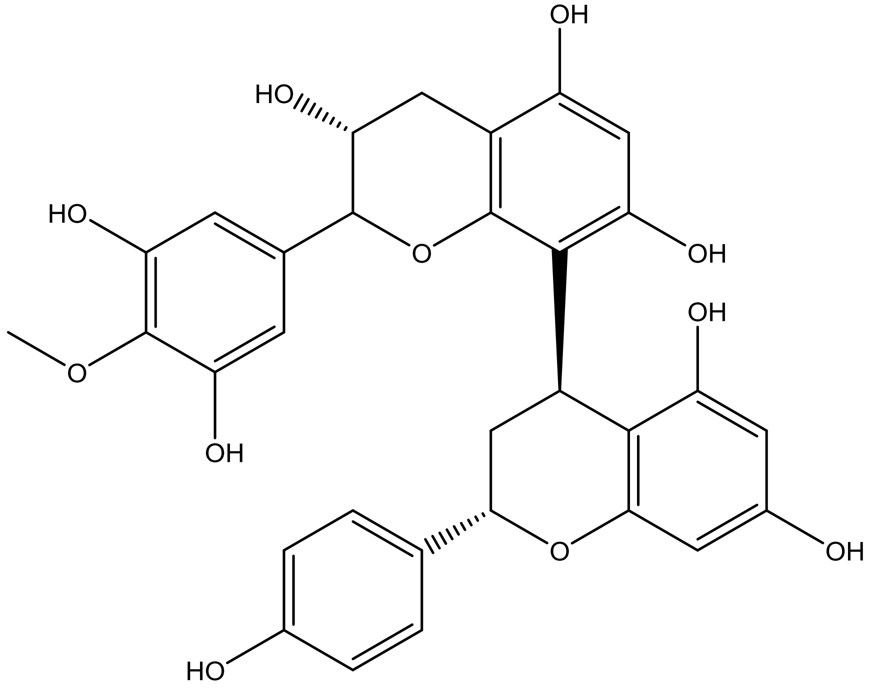


Figure 1-8 Simple Proanthocyanidins structure

Oligomeric Procyanidins (OPC) are made of chains of catechins, epicatechins and their Gallic acid esters and are water soluble (Porter, 1989). Proanthocyanidins oligomers can have different variations in their structures to form A-type oligomers due to the formation of a second interflavanoid bond by C-O oxidative coupling (Porter, 1988, 1989). The complexity of this type of conversion makes A type Proanthocyanidins oligomers as compared to the B type (Morimoto et al., 1985).

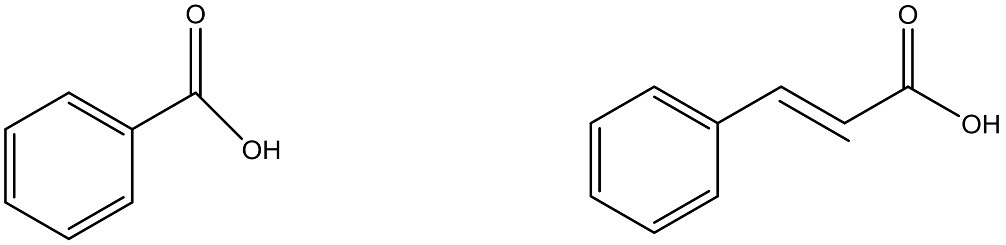
Proanthocyanidins are responsible for astringency in many fruits like grapes, apples, berries and some beverages such as, cider, wine tea, beer, and for the bitterness of cocoa. However it is important to note that astringency disappears when fruits are fully ripped (Rasmussen et al., 2005).

1.1.5.2 Phenolic Acids

Phenolic acids constitute non – flavonoids polyphenolic compounds which are further grouped into two main types, as benzoic acid and cinnamic acid derivatives (Manach et al., 2004). Free Phenolic acids are obtained from fruits and vegetables and are often in a bound form in grains and seeds and can be freed by acid or alkaline hydrolysis or treatment with enzymes (Adom and Liu, 2002, Chandrasekara and Shahidi, 2010, K.-H. Kim et al., 2006).

1.1.5.2.1 Hydroxybenzoic acids

They are characterized by the presence of the carboxyl group substituted on the Phenol. Examples are Protocatechuic acid, Salicylic acid, Vanillic acid and Gallic acid. Gallic and Protocatechuic acids are found very small concentrations in edible plants except for in blackberries and are usually not considered to be of high nutrition concern (D'Archivio et al., 2007). Benzoic aldehydes are formed when the substitution on the phenol is aldehyde instead of the carboxyl group. The general phenolic acid structures are shown in Figure 1-9.



Hydroxybenzoic acid

Hydroxycinammic acid

Figure 1-9 Typical phenolic acid structure

1.1.5.2.2 Hydroxycinnamic acid

Hydroxycinnamic acids are derived from cinnamic acids and comprised primarily of P-coumaric, Caffeic, Ferulic and Sinapic acids. They are usually not found in the free form but are glycosylated derivatives or esters of quinic, shikimic or tartaric acid (Manach et al., 2004). Chlorogenic acid found in many fruits and coffee in higher concentration is an ester of Caffeic and Quinic acids. Caffeic acid is the most abundant phenolic acid representing over 75% of the total of hydrocinnamates in most fruits. Hydrocinnamic acid are distributed in all parts of fruits with higher concentrations around the outer part which increases with increase in size but decreases in the course of fruit ripening (D'Archivio et al., 2007).

1.2 Extraction of phenolic compounds

The extraction process is an essential step towards ''isolation, identification'' and utilisation of polyphenolic compound and has therefore attracted increased research interest (Pinelo et al., 2007). The extracts are largely applied in, pharmaceutical, food and cosmetic industries because of their antioxidant properties.

Innovative methods have been explored to recover the polyphenolic compounds from natural sources to reduce the extraction time, the amount of solvent used, and improve yields and quality of extracts (L. Wang and Weller, 2006). The traditional solvent extraction methods are often time consuming and require large volumes of solvents (Luque de Castro and Garcia-Ayuso, 1998). Moreover, the procedure is laborious and produces low selectivity and extraction yield (Ibáñez et al., 2008). However simply substituting the solvent type usually results in low yields of recovery because of poor solute-solvent affinities and moreover the cost could be higher. Therefore advanced techniques of extraction with reduced recovery time and organic solvents consumption and increase pollution prevention are required. New extraction techniques like; Microwave assisted extraction, Ultrasound assisted extraction (UAE), Supercritical fluid extraction, Subcritical water (Pressurised hot water) extraction are perceived as efficient routes to recover phytochemical from plant matrices.

1.2.1 Ultrasound Assisted Extraction

Food and chemical industries have applied ultrasound technology for a variety of processes, as the approach is rapid, consumes less solvent produces a cleaner extract of higher yields while reducing the consumption of fossil energy (Pingret et al., 2012).

The ultrasound technique has been applied to recover aromas (Caldeira et al., 2004, Xia et al., 2006), pigments (Barbero et al., 2008, Chen et al., 2007) and antioxidant compounds including polyphenols (Khan et al., 2010, Ma et al., 2009, Pingret et al., 2012, Rodrigues and Pinto, 2007, Virot et al., 2010, Jing Wang et al., 2008). The acoustic cavitation generated in the organic solvent upon the passage of ultrasound radiation results in the rupture of cell walls therefore improving mass transfer between solute and solvent which accounts for the improved extraction yield (Ghafoor et al., 2009, Vinatoru, 2001).

Pingret et al., 2012, evaluated the impact of ultrasound assisted extraction of polyphenolic compounds derived from lyophilised apple pomace utilising an aqueous buffer as solvent at low temperatures. The intensity of the ultrasound, temperature and time of extraction were optimised using a response surface methodology to achieve optimal polyphenolic content. A parallel investigation comparing the ultrasound method and traditional maceration technique were conducted and evaluated in terms of antioxidant activity and kinetics of extraction. Ultrasonic treated samples yielded 30% increase in total phenolic content with higher antioxidant activity (Pingret et al., 2012).

1.2.2 Critical fluid

Critical state of a substance is that specific conditions of temperature, pressure or composition where liquid and gas converge, resulting in one single phase. It is the region where critical temperature (Tc) and critical pressure (Pc) intersect and no formation of liquid above the critical point (C) upon increasing pressure. Vapour pressure at the critical temperature is termed critical pressure. The phase diagram is shown in Figure 1-10.

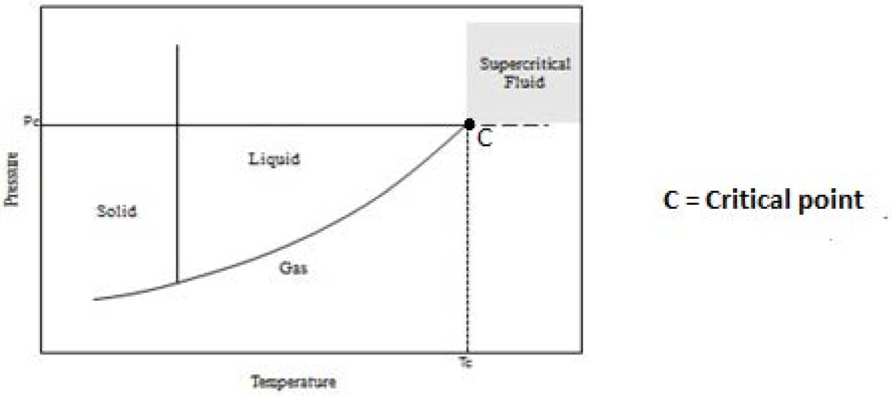


Figure 1-10 Phase diagram of Pressure-Temperature changes of water (Herrero et al., 2006)

The supercritical state is attained when pressure and temperature and of a substance is increased beyond the critical values. At this stage there is no clear difference between the liquid and the gas states, and the fluid is termed supercritical fluid. It is easier to control the physicochemical properties like diffusivity, density, viscosity, and dielectric constant by simply varying pressure or the temperature after even crossing phase boundaries (Sihvonen et al., 1999).

1.2.3 Supercritical Fluid Extraction (SFE)

Supercritical fluid extraction is among the more promising techniques able to overcome the drawbacks of using traditional solvent extraction (King, 2000). The extraction employs fluids in their supercritical state. Supercritical fluid has density similar to liquids and viscosities similar to gas. However its diffusivity is between those of liquids and gas (Herrero et al., 2006).

The solvating strength of a supercritical fluid correlates with density which can be manipulated by simply varying temperature or pressure (Del Valle and Aguilera, 1999, Raventós et al., 2002)., Which implies the solvent power of supercritical fluid can easily be manipulated. In addition supercritical fluids have superior coefficients of diffusion and inferior surface tension and viscosities compared to liquid solvents leading to a favourable mass transfer therefore allowing supercritical fluids to be a suitable solvent for extraction and separation processes (Sihvonen et al., 1999). The combined gas-like mass transfer and liquid –like solvating characteristics of supercritical fluids first lead analytical chemists to use them as chromatographic mobile phase and later as fluids capable of removing species from solids.

Many compounds can be used as fluids in supercritical technology including those listed in Table 1-5.

The most widely used supercritical fluid is carbon dioxide because of its modest critical pressure (72.9 atm) and temperature (31.2^{o}C). Carbon dioxide is considered a 'green solvent' as it is not toxic and is chemically inert. Moreover, when extractions are completed and the system depressurised, the carbon dioxide is eliminated because it is a gas at room temperatures and no residue of it is left in extraction products.

Therefore supercritical carbon dioxide is increasingly the solvent of choice in the food, pharmaceutical and nutraceutical sectors (Herrero et al., 2006, Luque de Castro et al., 1994).

Table 1-5 Critical Parameters of Selected Compounds

Solvent	**Critical Property**			
	Temperature °C	Pressure atm	Density g/ml	Solubility $\delta_{SFC}(cal^{-\frac{1}{2}}cm^{-\frac{3}{2}})$
Ethane	32.4	48.2	0.200	5.8
Ethene	10.1	50.5	0.200	5.8
Carbon dioxide	31.2	72.9	0.470	7.5
Methanol	-34.4	79.9	0.272	8.9
Water	101.1	217.6	0.322	13.5
Nitrous oxide	36.7	71.7	0.460	7.2
Sulphur hexafluoride	45.8	37.7	0.730	5.5
n-Butene	-139.9	36.0	0.221	5.2
n-Pentane	-76.5	33.3	0.237	5.1

(M Herrero et al 2006, Luque de Castro et al., 1994)

Although supercritical carbon dioxide is an excellent solvent and suitable for extracting temperature sensitive and non-polar phytochemicals, it is not able to effectively dissolve polar molecules like polyphenols because its polarity is too low. Modifiers or co-solvents are usually added to improve the efficacy of extraction. Modifiers are strongly polar compounds, and when added in smaller quantities, can produce considerable changes of solvating properties of supercritical carbon dioxide (Valcárcel and Tena, 1997). The modifier exerts it effects mainly in two ways: 1) by interacting with the analyte/matrix complex to promote rapid desorption into the supercritical fluid and 2) by enhancing the solubility properties of the supercritical carbon dioxide. The combination of modifier and high temperature is highly effective (Yang et al., 1995). Addition of modifiers decreases extraction time improves yield and allow mild operating conditions. However the introduction of modifiers can complicate the thermodynamics of the system and also introduces additional cost (Herrero et al., 2006).

There are two ways the modifier can be added to the system. It can be added to the sample in the extraction cell prior to the supercritical fluid extraction or be mixed with the CO_2. The latter is more effective since the modifier is continuously passed through the sample whereas in the former the modifier is swept from the extraction cell when the supercritical fluid starts to circulate through the sample. Normally two separate pumps are used in either situation. Alternatively a premixed fluid mixture of high purified gases can be prepared by suppliers, which eliminates the complexity and expense of operating two pumps in supercritical fluid extraction system. However this practice is not advisable because of the concentration shifts taking place during cylinder depletion (Via et al., 1994).

Of the many co-solvent including ethanol, methanol, acetone, acetonitrile, water, dichloromethane and ethyl ether, methanol is the most applied for it effective miscibility (up to 20%) with CO_2. Nonetheless ethanol could be preferred modifier in supercritical fluid extraction for food and nutraceutical applications because it is GRAS status (Hamburger et al., 2004, Lang and Wai, 2001).

1.2.4 Subcritical Water Extraction

Subcritical water extraction has emerged recently as a technique to substitute the conventional methods of extraction. Water is environmentally benign, non-toxic, easily accessible and inexpensive solvent for the extraction of bioactives from plant materials (Çam and Aaby, 2010). In addition it has been shown that subcritical water mediated extraction is efficient and can provide higher extraction yields from solid samples (Luque de Castro et al., 1999).

Subcritical water refers to liquid water at a temperature between the atmospheric boiling point and the critical temperature (374^oC) of water. Alternative terms include pressurised hot water (PHW), near critical water (NCW), hot compressed water (HCW), or superheated water (Meyer et al., 1999).

Application of Subcritical water as an extraction fluid was first reported by Hawthorne et al., 1994 for the extraction of some polar and non-polar analytes from soil samples (Hawthorne et al., 1994).

The most essential factor to consider in subcritical water procedure is the variation of it dielectric constant. At room temperature and normal pressure, water has high dielectric constant (ε) because of its highly polar nature with extensive hydrogen- bonded structure. So water is not considered a good solvent for extracting non –polar or organic compounds at room temperature (Teo et al., 2010). Water at room temperature has a dielectric constant of approximately 80 and decreases to 27 at 250°C, which is similar to that of ethanol (see Figure 1-11) therefore by modulating temperature and pressure water can perform like organic solvents solubilising low polarity analytes (Nieto et al., 2010).

Moreover the enhancement of extraction efficiency of subcritical water can be attributed to an improvement in the solubility and mass transfer effects as a result of the modification of physicochemical properties of water at elevated temperature, which also leads to disruption of surface equilibria (Ong et al., 2006). Therefore the increase in temperature can break the solute-matrix association as a result of van der Waal forces, hydrogen bonding, and dipole attractions of the solute molecules and other active sites in the matrix. The efficacy and impact of using subcritical water will be the subject of this thesis.

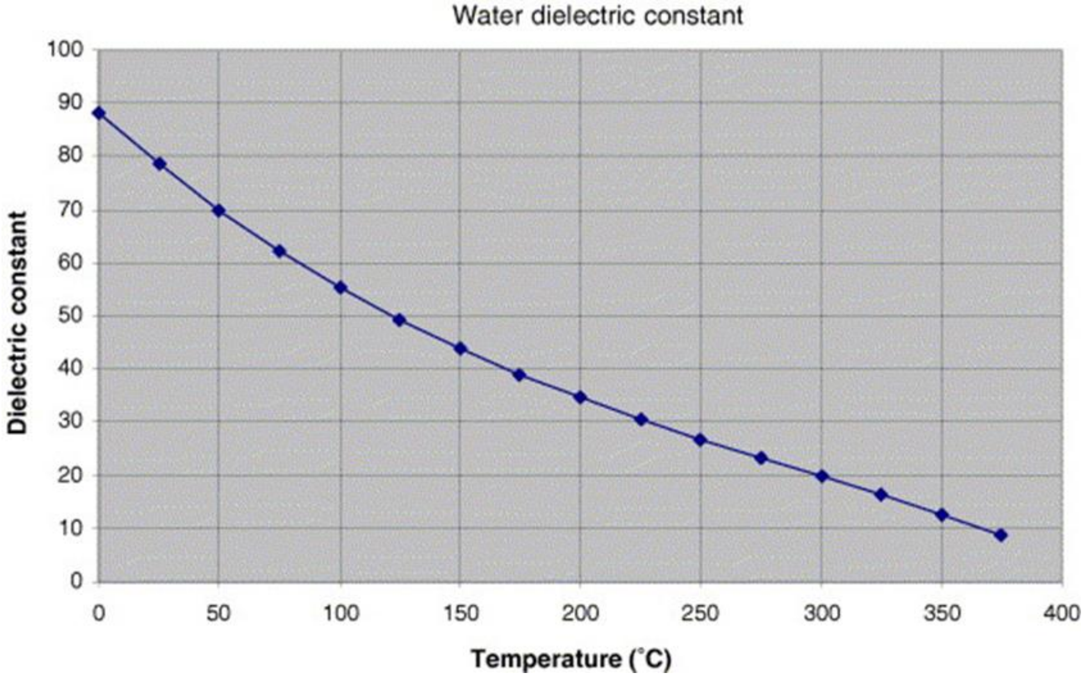


Figure 1-11 Variability of dielectric constant of water with temperature (Herrero et al., 2006)

Table 1-6 Dielectric constant of some common solvents (Mohsen-Nia et al., 2010)

Compound	**Dielectric constant** **20**℃
Water	79.99±0.04
Methanol	33.30±0.02
Ethanol	25.02±0.02
Acetone	21.30±0.02

1.3 Antioxidant properties of Phenolic Compound

Antioxidants can protect materials susceptible to oxidation by delaying, inhibiting, or preventing their damage thereby reducing oxidative stress (Ames et al., 1993). They are regarded as good protectors of tissues prone to ROS damage (Wu-yang Huang et al., 2012). As discussed above antioxidant compounds possess anti-inflammatory, anti-atherosclerotic, anti-proliferative, anti-tumour, anti-mutagenic, anti-carcinogenic, antibacterial or antiviral activities (Ratnam et al., 2006, Shuang et al., 2010). Currently, polyphenolic compounds have been shown to be strong antioxidants in vitro and demonstrated further effective antioxidant activity compared with Carotenoids, Vitamins C and E (Rice-Evans et al., 1997, Rice-Evans et al., 1995). There is a reported inverse relationship between the risk of oxidative related illness like cancer and cardiovascular diseases and the consumption of fruits and vegetables partly attributed to polyphenols (Hollman and Katan, 1999, Augustin Scalbert et al., 2005a, Augustin Scalbert et al., 2005b). Unfortunately, intervention studies with humans have not shown a clear benefit positively confirming the findings of epidemiological studies (Dimitrios, 2006). However, many reviews on effectiveness of polyphenolic compounds in humans have been published in classified nutritional journals, suggesting polyphenols may show some health benefits (Kroon et al., 2004). Furthermore, it is also very clear that, data from bioavailability studies are accumulating (Manach et al., 2004).

Apples are common fruit in many diets, and represent a significant source of bioavailable polyphenolic compounds (Escarpa and Gonzalez, 1998).

1.4 Determination of Total Antioxidant Capacity

The composition of polyphenolic compounds, coupled with diverse chemical properties across the plant materials has made the isolation and investigation of antioxidant properties of individual phenolic compounds very ineffective and costly. Hence, the need to explore acceptable and fast methods of determination of antioxidant capacities of extracts (Dai and Mumper, 2010).

Interestingly, there is no simple or single acceptable standard method to accurately quantify antioxidant capacities of food substances. The need for a standardized method according to Prior et al., 2005, will eliminate inconsistencies and provide proper interpretation of antioxidant capacity, and the standardised assay will allow;

1. Appropriate controls for assay application.
2. Considerable comparison between market products
3. Discrepancies between or within products to be regulated
4. Regulatory issues and health claims to be provided based on quality standards

(Prior et al., 2005).

1.4.1 Selection of Methods and Development

Well established methods are normally given priority over new ones because the strengths and weaknesses of the existing ones are already known and fewer efforts are required to address any shortcomings. Nonetheless, newer methods may possess some potential or may even be better (Prior et al., 2005).

Any new method should however meet the following classical criteria;

1. Explain or measure the chemistry actually occurring in potential application.
2. Employ biological relevant radical source.
3. Must not be too complex.
4. Make use of well-defined chemical mechanism and end points.
5. Instrumentation should not be difficult to come by.
6. Acceptable reproducibility within-run- and between –days.
7. Adjustable for assays of both hydrophilic and lipophilic antioxidants with different radical sources.
8. Adaptable to high-throughput analysis for routine quality control analysis

(Prior et al., 2003).

Methods for measuring antioxidants capacity in vitro have been reviewed by Cao and Prior, (1999) and classified into two groups according to the chemical reactions involved. Electron transfer (ET-based assays) and the hydrogen atom transfer (HAT) (D Huang et al., 2005). Irrespective of the mechanisms involved, the final or end results should be equivalent, but may differ in terms of their kinetics and side reactions (Prior et al., 2005). The major factors that determine the mechanism and efficacy are the bond dissociation energy (BDE) and Ionization Potential (J. S. Wright et al., 2001). Therefore, an integrated protocol is required to incorporate the different properties of polyphenolic compounds, which possess potentially several different reaction routes.

1.4.2 Hydrogen Atom Transfer Assays- HAT

Mechanism of hydrogen atom transfer assays involves the simple donation of hydrogen atom by the antioxidant to inhibit free radicals as in;

$$X^{\cdot} + AH \rightarrow XH + A^{\cdot}$$

Hydrogen atom transfer (HAT) based reactions are dependent on the bond dissociation energy (BDE) of the group in the antioxidant donating the hydrogen and also its ionization potential (IP) (J. S. Wright et al., 2001) which in turn are influenced by solvent, pH and the presence of reducing agents like metals which are capable of complicating the assays. HAT based reactions are very rapid and are completed within seconds to few minutes (Prior et al., 2005).

Hydrogen atom transfer methods (HAT) include:

1. Oxygen radical absorbance capacity (ORAC) assay
2. Lipid peroxidation inhibition capacity (LPIC) method
3. Total radical trapping antioxidant parameter (TRAP)
4. Inhibition oxygen uptake (IOC)

5. Crocin bleaching Nitric oxide radical inhibition activity
6. Hydroxyl radical scavenging activity by p-NDA (p-butrisidunethyl aniline)
7. Scavenging of H_2O_2 radicals
8. ABTS radical scavenging of superoxide radical formation by alkaline (SASA).

(Badarinath et al., 2010).

The mechanism of many of the assays outlined above involve in a system of competition where the antioxidants and substrates battle for thermally generated peroxyl radicals from the decomposition of azo- compounds (D Huang et al., 2005). ORAC and TRAP assays are good examples that met most of the requirements for a screening assay outlined in the previous paragraphs (Prior et al., 2005).

1.4.2.1 Oxygen Radical Absorbance Capacity (ORAC) Assay

The ORAC assay measures the inhibition of free peroxyl radical damage by antioxidant to a fluorescent probe. The peroxyl radicals are generated by the decomposition of 2, 2^1-azobis (2-amidinopropane) dihydrochloride (AAPH) as in

$$R - N = N - ROO \rightarrow N_2 + 2ROO^{\cdot}$$

$$ROO^{\cdot} + Probe(fluorescent) \rightarrow ROOH + oxidized\, probe(loss\ of\ fluorescence)$$

$$ROO^{\cdot} + AH \rightarrow ROOH + A^{\cdot}$$

$$ROO^{\cdot} + A^{\cdot} \rightarrow ROOA$$

The fluorescence intensity is measured every minute under physiological conditions (pH 7.4, 37°C) to obtain a kinetic curve of fluorescence decay. The net area under the curve (AUC) calculated by subtracting the AUC of blank from that of the sample or standard over time, as compared to Trolox, a water-soluble analogue of α-tocopherol (Cao et al., 1995b, D Huang et al., 2005). Originally B- phycoerythrin (BPE) was the protein source which later found to have short comings (Sanchez-Moreno, 2002).as its reactivity with the peroxyl radical was not stable (Cao and Prior, 1999) and complicate the observation of the fluorescent molecules when exposed to light (Niki, 2002). Additionally there are reports of its binding with some polyphenols especially Proanthocyannidins leading to inaccurate ORAC values (Prior et al., 2005).

In recent times new fluorescent probes like FL; 3^l, 6^l-dihydroxy-spiro [isobenzofuran-[3H], 9^l[9H]-xanthen]-3-one (Ou et al., 2001) or dichlorofluorescein (H_2DCF-dA; 2^l,7^l-dichlorodihydrofluorescein diacetate) are preferred because they are less reactive and consistent (Ishige et al., 2001).ORAC assay can be automated (D Huang et al., 2002). COBAS FARA II analyser was the original automated equipment, whose production has been discontinued by the manufacturers (Cao et al., 1995a). The equipment in recent times had undergone some better improvements through innovations and with some fluorescent probes (Ou et al., 2001, Prior et al., 2003). Excellent results were achieved using multichannel liquid handling system coupled with a fluorescence microplate reader with either 96-or 48- well format (D Huang et al., 2002, Ou et al., 2001). The coefficient of variation of the assay was lower in the 48- well format compared with the 96-well format (i.e. 4-5%, compared to 4-10% in 96- well format) (Prior et al., 2003). It is very important to monitor temperature very closely throughout the plate as ORAC reactions is temperature sensitive and therefore incubating the reaction buffer before reacting with AAPH improves the variability between determinations (Prior et al., 2005). Reproducibility of the assay can be affected by slight variations in temperatures of the external wells of the microplate (Lussignoli et al., 1999).

1.4.2.2 Total Radical Trapping Antioxidant Parameter (TRAP)

Reaction mechanisms under TRAP are similar to ORAC assay and results are also reported in Trolox equivalents. TRAP has been demonstrated in human plasma sample. The lag-time induce by plasma was compared with that induced by Trolox within the same plasma sample. The rate of peroxidation induced by AAPH $2,2^1$-azinobis- methyl-propanidamide hydrochloride was monitored through the loss of fluorescence of the protein R-phycoerythrin (R-PE) (Ghiselli et al., 1995). Another reaction probe used was ABTS ($2,2^1$-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (Bartosz et al., 1998).

1.4.3 Electron Transfer- Based Assays (E-T)

Electron transfer assays measure the capacity of antioxidants to transfer an electron to reduce free radicals, carbonyls and metallic compounds (J. S. Wright et al., 2001).

$$X^{\cdot} + AH \rightarrow X^{-} + AH^{\cdot +}$$

$$AH^{\cdot +} \overset{H2O}{\longleftrightarrow} A^{\cdot} + H_3O^{+}$$

$$X^{-} + H_3O^{+} \rightarrow XH + H_2O$$

$$M(III) + AH \rightarrow AH^{+} + M(II)$$

The E-T based assays include:

1. Trolox equivalent antioxidant capacity (TEAC)
2. Ferric reducing antioxidant power (FRAP)
3. DPPH free radical scavenging assay
4. Copper (II) reduction capacity
5. Total phenols by Folin-Ciocalteu
6. N, N-dimethyl-p-Phenylenediamine (DMPD) assay (Badarinath et al., 2010).

Both electron transfer and hydrogen atom transfer almost occur simultaneously in all samples, however pH of medium and antioxidant structure determine the balance (Prior et al., 2005). The reactivity of electron transfer assays are based on deprotonation (Lemanska et al., 2001) and ionization potential (J. S. Wright et al., 2001) of the reactive functional group and therefore are pH dependent. Increasing pH generally decreases the ionization potential values and hence increased in electron donating ability with deprotonation (Prior and Gu, 2005, Prior et al., 2005). ET- assays measure the capacity of an antioxidant in reduction of an oxidant probe, which changes colour when reduced (D Huang et al., 2005). The reaction is completed when the colour change stops and degree of colour change is proportional to the concentration of antioxidant. Electron transfer methods are very sensitive to ascorbic and uric acids, which are very important in maintaining plasma redox tone and also capable of detecting reducing polyphenolic compounds (Prior et al., 2005) The antioxidant power estimated using ET-assays is not dependent on the kinetics but on the percent decrease of product formed. If the half- life of the $AH^{\cdot+}$ is long enough very sufficient, secondary reactions may result in interference within the assay (Sartor et al., 1999).

The oxidising probes used are 2,2^{1}-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS\cdot+$) in TEAC, Fe^{3+}(2,4,6-tripyridyl-s-triazine)2Cl_3 in FRAP and bis(neocuproine) $Cu^{2+}Cl_2$ in CUPRAC assays, respectively. TEAC method is practically, simple, reproducible, and inexpensive (Awika et al., 2003). Most importantly, the TEAC assay can be adapted to different media allowing the assessment of antioxidant capacity of plant extracts in terms of hydrophilicity and hydrophobicity, since the reagent is soluble in both aqueous and organic solvent media (Re et al., 1999). As opposed to TEAC assay, FRAP assay measures the reduction capacity of $Fe^{3+}\ to\ Fe^{2+}$ of water-soluble antioxidants in acidic pH such 3.6.(Pulido et al., 2000).

1.5 Bioavailability and Bioefficacy of Polyphenols

The in vitro evaluation or assessment of the biological activity of polyphenols employs cultured cells as tissues which are exposed to aglycones or polyphenol rich-extracts. Unfortunately polyphenols exist in food sources as esters, glycosides or polymers and therefore human plasma and tissues are not exposed in vivo to free polyphenols forms and concentrations (D'Archivio et al., 2007, Roupas and Noakes, 2010). Furthermore, studies have shown that the polyphenol undergo several modifications during digestion and therefore the forms registered in the blood and tissues are neither aglycones (except for green tea catechins) nor the forms in dietary sources (Roupas and Noakes, 2010). Enzymes in the intestines or gut microbiota interact with these compounds thereby changing their forms (Aron and Kennedy, 2008, D'Archivio et al., 2007, Saura-Calixto et al., 2007). In one such in vitro analysis, as high as 58% portion of polyphenol was released when treated with a digestive enzyme emphasizing the forms reaching the blood and tissues are different from those appearing in food (Saura-Calixto et al., 2007). Therefore action in the human body may be attributed to other metabolites and not polyphenolic compounds in their original form (Visioli et al., 2009).

It is very important that concentration of polyphenols should be identical to those appearing in plasma. Concentrations of polyphenolic in an apple- rich meal are in the range 0.1-10 μmol/L which is far higher than those in plasma and tissues and therefore bioavailability data and the understanding of the metabolism in human should be taken into consideration before making conclusions as the benefit/impact of polyphenolic in general (Kroon et al., 2004). Regrettably, literature on bioavailability issues of phytochemicals of whole food is not very extensive. Bioavailability' is the proportion of the nutrient that is digested, absorbed, and metabolised through normal pathways (D'Archivio et al., 2007). As a result it is important to distinguish between how much of a particular nutrient is present and how much of it is bioavailable (Srinivasan, 2001). It is a known fact that polyphenols are not totally absorbed in the small intestines because of the sugar residue and its position. Although flavonoids can be absorbed in the gastrointestinal tract, their absorption is incomplete and levels of circulation are low (Biedrzycka and Amarowicz, 2008). A study on bioavailability of polyphenols from alcoholic cider in human was done using volunteers (DuPont et al., 2002).

One hour after consuming 1.1 litres of apple cider, no quercetin was registered in their plasma, while low levels of 3^1-methylquercetin and 4^1- methylquercetin were observed. Caffeic acid which was rapidly absorbed could not be traced in the plasma after 1.5 hrs. Catechin, epicatechin and phloridzin were not seen in the plasma and according to the researchers it could be that their concentrations were low in the cider. The concentrations of hippuric acid, which is a type of carboxylic acid, and phloretin a dihydrochalcone both increased in the volunteers' urine, after the consumption of the cider, however, no traces of quercetin, catechin and epicatechin in urine were registered (DuPont et al., 2002).

Another study on bioavailability in humans revealed that the amount of quercetin from onions accumulated in plasma to higher levels compared those from apples. The differences were attributed to bioavailability of the conjugates of quercetin in the different foods. Onions have more quercetin aglycons and glycosides whereas apples contain quercetin monoglycosides and quercetin rutinoside which may be less bioavailable (Hollman et al., 1997). Indeed it is becoming very clear that findings of in vitro studies may not necessary reflect in vivo effects. Quercetin, rutin, epicatechin have been shown to have high antioxidant capacity, but their contribution to the total effect in aqueous extract are low. There are situations where positive antioxidant effect in vitro turned not to be reproducible in vivo in human studies and direct antioxidant effects of polyphenols in vivo are questionable. As previously mentioned, concentrations in blood are low compared with other antioxidants, and extensive metabolism after ingestion lowers their antioxidant activity (Hollman et al., 2011). Moreover the observed increase in antioxidant effect of some polyphenols in apples after ingestion may be due to a metabolic effects of fructose on urate and not flavonoids (Lotito and Frei, 2004). Therefore antioxidant effect in vivo is open to many interpretations and must be done with caution (Roupas and Noakes, 2010). However there are other findings that point to the fact that polyphenols might exert other biological effects, such as inhibitions, and cell receptors (Kong et al., 2000, Spencer et al., 2003, Wiseman et al., 2001) and several other functions as shown in Figure 1-2.

1.6 The role of Antioxidants in Cosmetics

The human skin is the first line defence and therefore subjected to a range of biotic an abiotic pressures .The skins defence mechanism, generates reactive oxygen species (ROS) which in turn causes damage to DNA, proteins and destabilises membranes of epidermal cells resulting, in early or premature aging of the skin (Mariethoz et al., 1998). Approximately, half of all skin damage or disorders is attributable to free radicals induced by UV radiation (Harman, 2002, Herrmann et al., 1993).

Interestingly, living tissues fight to balance the effects of the ROS by using internal antioxidative enzymes and nonenzymatic antioxidants. Antioxidative enzymes comprises superoxide dismutase, superoxide reductase, catalase, glutathione peroxidase whereas nonenzymatic antioxidants are vitamin E, vitamin C, glutathione, ubiquinone (Shindo et al., 1994). For instance, when ROS are induced within the living cells, the endogenous antioxidants are utilised. Over time the endogenous defence mechanisms are stressed, and with increasing loads of reactive oxygen species, stimulate the aging process (Fuchs et al., 1989). The equilibrium can be restored by supplying exogenous antioxidants (Pouillot et al., 2011).

External antioxidants like ascorbic acid (vitamin C), tocopherol (vitamin E), have been demonstrated to inhibit peroxidation and chain reactions within lipid membranes in topical applications. Similar results were reported for trace elements and some polyphenols. Vitamin E is a known powerful liposoluble antioxidant which reacts with free radicals to form the stable tocopheryl radical, that stops the chain reaction (Pouillot et al., 2011). In vitro and in vivo analysis demonstrated the effect of vitamin E in preventing damages by reducing cell burn induced damage and decreasing the redness of the skin when prone to both UVA and UVB radiations (Streilen and Gilchrist, 1995). Vitamin C exhibited better protection against UVA or UVB when it was mixed in sunburn cream than cream alone (Darr et al., 1996). Therefore, incorporating vitamin C in to after–sun products was worked well against UV-induced reactive oxygen species (Cabelli and Bielski, 1983).

There is a growing concern that common synthetic produced preservatives may have hazardous effects (Krishnakumar and Gordon, 1996) which coupled to consumer demand for natural ingredient has driven increased interest in natural antioxidants and other active ingredients obtained from plants (COELHO et al., 2001). However topical formulations incorporating extracts derived from plant sources may be restricted due their physical and chemical properties, which might affect stability of the product and overall performance (Cardoso et al., 2011).

Several plant extracts have been investigated for their antioxidant activity and utilised in the cosmetic industries. Proanthocyanidins from grape seeds extract has been shown to be potent antioxidants with free radical scavenging activities greater than vitamin C and E (Bagchi et al., 2000, Vinson et al., 1995) Mittal et al., 2003 demonstrated photo protection by inhibiting UV- radiation- induced oxidative stress with grape seed extract (Mittal et al., 2003). Therefore topical application of proanthocyanidins from grape seed seems to enhance the sun protection factor in human (Afaq et al., 2003).

The green tea catechins are main antioxidant ingredients of green tea extracts and are made up of up epicatechin-derivatives, namely epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin gallate (EGCG). kaemferol, myricetin and quercetin are other constituent flavonoids in the extract (Graham, 1992). The antioxidant- rich extracts can be utilised either orally or topically (Katiyar et al., 2000).

Phloridzin (Phloretin) is a major phenolic glycoside found in apple trees and has a characteristic bitter taste that contribute to the original flavour of cider (Whiting and Coggins, 1975). However since its isolation from the bark of apple trees in 1835 by De Konnick (Petersen, 1835), Phloridzin has gained some attention as an ingredient in pharmaceutical applications and also a subject for human physiology research (Baldisserotto et al., 2012).

Phloridzin and derivatives exhibited high in vitro antioxidant activity (Ridgway et al., 1996), memory enhancement in humans (Boccia et al., 1999), inhibit peroxidation of lipids (Rezk et al., 2002), prevention of bone loss (Puel et al., 2005), and growth retardation of certain types of cancer cells (Veeriah et al., 2006). A phenolic extract rich in phloridzin was claimed to exert photo- genoprotective effects and free radical scavenging mediated the anti-ultraviolet protective role of phloridzin (Gaudout et al., 2006, Salles et al., 1995). Unfortunately phloridzin has a poor stability in finished formulations as the aglycone is much easier to handle which is not the natural form derived from plants (Vertuani et al., 2011). Moreover previous reports about the dihydrochalcone presented promising dermo-cosmetic applications as potential antifungal agents with extracts from apple species (Hunter and Hull, 1993).

1.7 Encapsulation of Polyphenols

During the last decades encapsulation has found many applications in the food, cosmetic and pharmaceutical industries in order to: (i) safeguard the active ingredient from degrading through lessening its activity to its surroundings; (ii) reduce rate of transfer of encapsulate to immediate environment due to evaporation; (iii) prevent alteration of the physical properties of starting material to ensure simpler manipulation; (iv) modify rate of release active ingredient (v) cover-up undesired flavour or taste of the active ingredient; (vi) reduce concentration of active substance to required levels and ensuring uniform distribution within the carrier; (vii) to ensure components of the mixture are separated and do not react with one another. (Desai and Park, 2005) in (Fang and Bhandari, 2010). Encapsulation techniques of bioactive ingredients generally involve 3 steps: (i) Creation of a wall over the active ingredient to be encapsulated; (ii) Prevention of unwanted gradual loss; (iii) ensuring that unwanted substances are eliminated (Gibbs et al., 1999, Mozafari, 2006).

The coated materials otherwise known as; packaged material, actives, fill, internal phase or payload usually made up of natural material or a mixture. Likewise, the coating materials can also be made from pure or modified polysaccharides and occasionally includes sugars, proteins, gums, lipids and synthetic polymers. They are also called carrier, shell, packaging material, wall material, membrane or capsule (Gibbs et al., 1999, Mozafari, 2006).

1.7.1 Methods of Encapsulation

Encapsulation techniques can be divided into three classes as chemical processes; physicochemical techniques; and physical processes.

The chemical processes involves; chemical reaction and cross-linking of molecules concerned and include; in situ polymerization, interfacial polycondensation, interfacial cross-linking, interfacial polymerization (Munin and Florence, 2011).

Physicochemical processes includes; hot melts coating, spray- cooling, ionic gelation, simple or complex coacervation, and solvent evaporation- extraction.

Physical processes comprised; freeze and spray drying, spheronisation, fluid bed coating, centrifugal extrusion, and procedures employing critical fluids. The focus of this review will be on encapsulation techniques applied to polyphenols.

1.7.1.1 Spray Drying

Spray drying as an encapsulation technique is widely applied in the food industry because it is continuous operation, economical and flexible and delivers good quality, stable, dried particles (Desai and Park, 2005). The technique employs special equipment that generates particles from a solution containing homogenised active ingredients and coating agents (Giunchedi and Conte, 1995, Madene et al., 2006, Patel et al., 2009, Vehring, 2008). Initially the dispersed active ingredient within the coating agent is atomised by allowing it through a nozzle with the aid of a compressed gas system. Hot process nitrogen or air is delivered to meet with the atomised sample in order to rapidly evaporate the solvent from the droplets. The particles formed whose properties are strongly influenced by geometry of nozzle and viscosity of the feed, drop to the bottom of the drying chamber and a cyclone or filter bag is used to recover the powder from the exhaust (Munin and Florence, 2011). Spray dried particles are spherical with average size of particles ranges 10- 100µm (Gibbs et al., 1999). Spray dried technology however requires the carriers used are water soluble (Desai and Park, 2005).

Maltodextrins are commonly used as carrier for encapsulating polyphenols as well us flavours (BR Bhandari, 2004). Maltodextrins were able to protect the degradation of heat sensitive anthocyanins from the high temperatures of the process gas, thereby preserving the integrity of anthocyanins during encapsulation (Ersus and Yurdagel, 2007, Robert et al., 2010). Maltodextrins can blend with gum arabic as coating substances (Fang and Bhandari, 2010). A composition of gum arabic with Maltodextrins and in a ratio of 2:3 had been utilised for procyanidins encapsulation from grape seed extract (Lianfu Zhang et al., 2007). The active ingredient and coating material were mixed in a ratio 3:7 W/W, and amount of solid in feed was 20% W/V. Efficiency of encapsulation was close to 89% and the stability of the product was clearly enhanced. Similarly epigallocatechin gallate (EGCG) has been successfully encapsulated within Maltodextrins as was shown to be efficacious in reducing carcinogenic cell development (Rocha et al., 2011).

Chitosan, a linear polysaccharide has also been employed as a coating substance during spray drying of extracts from olive (Kosaraju et al., 2006). The polysaccharide was loaded with 27% of phenolic compounds and surface morphology of the microspheres produced was very smooth, FTIR spectroscopy revealed the antioxidant was physically incorporated into the Chitosan.

Extract from soybean rich in polyphenols, have been stabilised within a composite mixture of maltodextrin, starch and silica (tixosil 333) (Georgetti et al., 2008). The results revealed a slight deterioration of the polyphenol encapsulated with tixosil 333, and the antioxidant activity was not significantly affected. Therefore inclusion of the bulking agent amid drying stage ensures efficacy and stability of the dried powder.

Carrageenan, a sulphated linear polysaccharide derived from red algae has been used as a carrier and was effective in preserving the antioxidant activity of variety of extracts rich in polyphenolic compounds(Krishnaiah et al., 2009a, Krishnaiah et al., 2009b).

An emulsion of sodium caseinate- soy lectithin (Protein –lipid) was another wall material used to stabilise polyphenols. The emulsion was added during spray drying of extracts of apple grape seed and olive leaf. The particle size distribution analysis and observations from light microscopy showed that, morphology of micronized particles were all spherical with uniform size distribution (80%, 6-60μm). Antioxidant activity after the encapsulation exercise was significantly retained as verified by radical scavenging studies (Kosaraju et al., 2008).

1.7.1.2 Freeze Drying

Freeze drying also termed lyophilisation or cryodesiccation is the preferred dehydration method for many temperature sensitive and unstable substances (Fang and Bhandari, 2010). The process of freeze drying requires first freezing the substance and then decreasing the pressure of chamber and applying sufficient heat to cause water frozen within the material to sublime (Oetjen, 2004). Freeze drying is an encapsulation technology suitable for water-soluble perfumes or fragrances and aromas of natural origin, as well as drugs (Desai and Park, 2005). A pomace sample containing anthocyanins and maltodextrin DE20 was stable for two months when freeze dried and stored at 50^0C/0.5 (Delgado-Vargas et al., 2000).

Extract of polyphenol- rich bakeapple (*Robus chamaemorus*) was subjected to freeze drying using two forms of maltodextrins (DE5-8 and DE18-5) as packaging materials. Encapsulated product showed good stability over long periods of time and offered an effective protection of the polyphenols against reaction with surrounding air during storage, while maintaining its antioxidant activity or slightly improving it (Laine et al., 2008).

Nonetheless there is another school of thought that freeze-drying as an encapsulation technique could not enhance bioavailability or stability of products (Fang and Bhandari, 2010). Antioxidant activity of the polyphenol after storage did not improve when extract of Hibiscus anthocyanins was encapsulated in black yeast by freeze drying. The freeze drying encapsulation did not exhibit any significant differences in their properties and no benefit in terms of antioxidant activity was achieved (Gradinaru et al., 2003).

1.7.1.3 Molecular Inclusion Encapsulation

Molecular inclusion is extensively realised by employing Cyclodextrins as a carrier substances. Cyclodextrins are cyclic polysaccharides consisting of six, seven or eight residues of glucose. The glucopyranose units are linked by α (1-4) glycosidic bond. They are labelled as α, β, ϒ- Cyclodextrins, with the β- Cyclodextrins as the most widely used (Pagington, 1986).

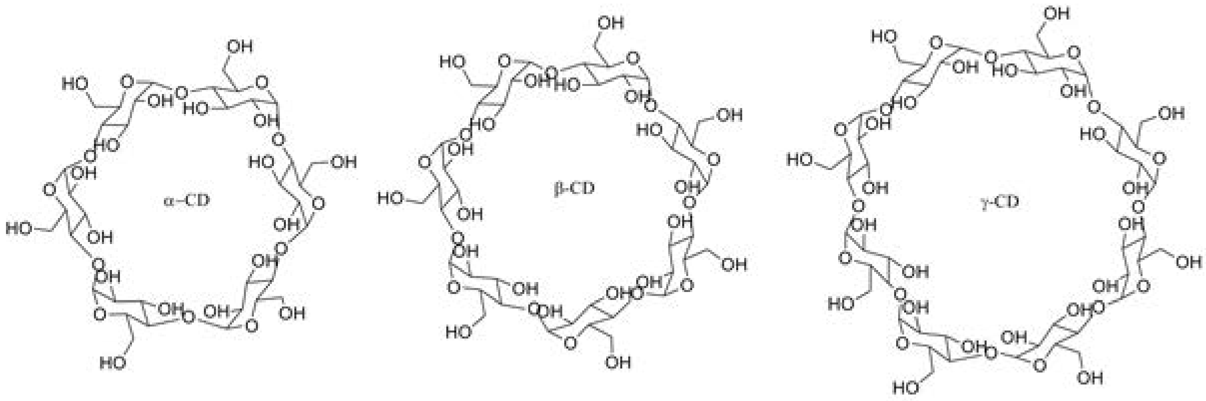


Figure 1-12 Molecular structure of the three forms of Cyclodextrins; six membered alpha cyclodextrin (α-CD), seven member beta cyclodextrin (β-CD) and eight member gama cyclodextrin (γ-CD) (Olsson and Westman, 2013).

The internal cavity of the cylindrical shaped Cyclodextrins exhibit hydrophobia, whereas the external surface is hydrophilic therefore Cyclodextrins are capable to accommodating diverse guests molecules including less polar ones (B. R. Bhandari et al., 1999, Dziezak, 1998). A wide range of phytochemicals with poor water solubility have been encapsulated within cyclodextrins. For example Resveratrol trapped in β- Cyclodextrin and Maltosyl-β-Cyclodextrin (Lucas-Abellán et al., 2007), extract of Olive leaf in β-Cyclodextrins (Mourtzinos et al., 2007), Kaemferol, Myricetin and Quercetin in 2-hydroxylpropyl-β-Cyclodextrins (Mercader-Ros et al., 2010), Rutin in β-Cyclodextrins (Haiyun et al., 2003), Hesperetin in hydroxylpropyl-β-Cyclodextrins (Tommasini et al., 2005). The encapsulation of phytochemicals with Cyclodextrins resulted in improved solubility in water as well as their antioxidant activities. The increase in antioxidant capacity may be the result of protection of the polyphenolic compounds against fast free radicals oxidation (Mercader-Ros et al., 2010) leading to higher solubility in biological matrices (Haiyun et al., 2003).

However the encapsulation capacity of phenolic compounds depends on the wall material or the carrier. Smaller molecules with higher hydrophobicity are attracted more to the Cyclodextrins in for example hesperetin was more efficient compared to hesperidin based on their relative Cyclodextrins affinity (Tommasini et al., 2005). Also different carrier substances influence encapsulation efficiency for the same active ingredient (Fang and Bhandari, 2010). For example hydroxylpropyl-β-Cyclodextrins produced highest encapsulation efficiency for curcumin compared to other Cyclodextrins forms (Tang et al., 2002, Tomren et al., 2007). Furthermore, the order of the affinity of Myricetin and Quercetin to β-Cyclodextrins forms was hydroxylpropyl-β-Cyclodextrins > maltosyl-β-Cyclodextrins > β-Cyclodextrins, thus showing efficacy of modified or transformed Cyclodextrins (Lucas-Abella´n et al., 2008).

Encapsulating polyphenolics within Cyclodextrins has also been shown to protect thermal degradation. A flavonoid rich extract encapsulated in the β-Cyclodextrin exhibited stability against thermo-oxidation and offered effective protection of polyphenolics against light when exposed to ultra violet rays at λ= 254 nm.(López-García et al., 2010).

The insertion of Ferulic acid within the lipophilic interior of α-Cyclodextrin was studied based on modelling and simulation technology. The result showed the encapsulation of ferrulic acid increased the photo-stability and also regulated the release of Ferulic acid and can safely be included in cosmetic formulations to offer protection against radiation emitted by the sun (Anselmi et al., 2008).

1.7.1.4 Particle formation process with Supercritical fluids

The application of supercritical fluids to support encapsulation processes are an alternative to traditional particle formation technology and have received tremendous attention over the last ten years (Cocero and Martin, 2008, Jung and Perrut, 2001, Shariati and Peters, 2003). Supercritical fluid technology offers new and interesting route for particle formation which avoids many of the drawbacks of the traditional methods (Fages et al., 2004). Supercritical carbon dioxide is the most widely utilised fluid in supercritical fluid encapsulation technology processes because of relatively low or moderate critical parameters. In addition supercritical carbon dioxide is inexpensive, non-flammable and non- toxic, and are generally recognised as safe (Cocero et al., 2009). The supercritical fluid can be separated from the product by simply depressurisation (Miguel et al., 2006).

Supercritical fluid based particle formation techniques can be classified into 3.groups.

(i) Rapid Expansion of Supercritical Solution (RESS).

(ii) Supercritical Anti-solvent (SAS).

(iii) Particles from Gas Saturated solutions (PGSS) (Munin and Florence, 2011).

1.7.1.4.1 Rapid Expansion of Supercritical Solution (RESS)

The Rapid Expansion of Supercritical Solution (RESS) process consists of saturating supercritical fluid with a solute(s) and depressurising rapidly the solution using a nozzle of selected aperture into a low pressure chamber, resulting in rapid crystallisation of the solute into very small particles (Jung and Perrut 2001). The pressure drop as a result of the process creates very high jet velocity to atomise the droplets (Debenedetti et al., 1993).

Many researchers have applied the RESS to produce single- component particles with uniform particle size distributions (Chang, 2006, Matson et al., 1987, McHugh and Krukonis, 2013, Mohamed et al., 1989, Petersen et al., 1986, Tom and Debenedetti, 1991).

The concept has been applied in wide varieties of substances for example; dyes, polymers, inorganic substances, and in pharmaceuticals (Knez and Weidner, 2003). Different particle size and morphology can be produced from the primary material by carefully varying process parameters to influence supersaturation and rate of nucleation (Knez and Weidner, 2003). Evaluation of solubility data is very important in designing the RESS process as it provides the process conditions necessary to investigate the feasibility of the process for particle formation (Jong-Hyun Kim et al., 1996). Thus experiments to model solubility of solutes in the supercritical fluid are conducted or simple make use of published data if available (Knez and Weidner, 2003).

Rapid Expansion of Supercritical Solution process can also be utilised in the production of composite substances. This is achieved by first dissolving both the payload and the carrier in the supercritical fluid and precipitate both substances simultaneously (Cocero et al., 2009). Host molecules like Cyclodextrins can be used to achieve microencapsulation by the RESS process (Fages et al., 2004).

The RESS is a fairly simple process and requires only the use of one capillary nozzle for the expansion process (Chang, 2006). Very fine and controllable nanometre size particles are generated which are free from solvents (Knez and Weidner, 2003).

Limitations of the RESS process

The fundamental setback of the RESS process is the low solubility of many substances in supercritical carbon dioxide. The limitation is even worse for co-precipitation applications which requires both substances to be soluble in the supercritical carbon dioxide (Cocero et al., 2009). However it is possible to overcome this limitation by the use of alternative organic supercritical solvents such as, triflouromethane or chlorofluoromethane (Pestov et al., 2003), or organic co-solvents can be employed with supercritical carbon dioxide to improve the solubility of the target ingredient. Several pharmaceuticals and proteins were encapsulated by using RESS with modifiers/co-solvents (Mishima et al., 2000). Since RESS precipitation is extremely rapid, there is a difficulty in controlling loading and particle morphologies of composites as both criteria are very sensitive to the chain of ‘’supersaturation’’ which in turn rely on operation parameters and therefore is another potential limitation of RESS (Yeo and Kiran, 2005). Particle formation processes utilising coating substance that are sufficiently soluble in supercritical carbon dioxide would be simple, clean and is a very promising application (Cocero et al., 2009).

1.7.1.4.2 Supercritical Antisolvent process (SAS)

The supercritical fluid is utilised as a solvent under RESS process, which revealed several notable drawbacks. These limitations could be avoided by using the supercritical fluid as an antisolvent in micronisation processes. Supercritical antisolvent process was first proposed in the 1920 (Zsigmondy and Bachmann, 1918) and further developed in the 1980’s (Gallagher et al., 1989). Supercritical antisolvent processes are practicable for solutes whose solubilities are poor in supercritical fluid where the latter being utilised as an antisolvent to cause solute nucleation from solutions. Initially the active ingredient to be encapsulated is mixed with a solvent usually organic and later contacted with the supercritical fluid.

However the solvent chosen should be miscible with the supercritical fluid at an appreciable level. At the same time the solute should not be soluble in the supercritical fluid at operating conditions. After intensive mixing, to saturate the liquid solution with the supercritical fluid, causing the reduction in density and the solvating strength of the organic solvent, thereby removing it from solution. Solute concentration increases to supersaturation and finally micronised nano size particles are nucleated. The rate nucleation, growth rate, shape, particle size and, particle size distribution can be controlled by changing the temperature, pressure and mass transfer as they influence the supersaturation ratio. Particle size distribution from this process is non- homogeneous. The organic solvents normally used in such processes for pharmaceutical applications include; Dimethyl sulphur oxide (DMSO), ethanol, acetone and ethyl acetate. Excess solvent is removed after particle formation by continuous flow of pure antisolvent which is the supercritical fluid. Nonetheless this continuous stripping with the SCF is not able to remove all residual solvent because of the strong affinity between the solute and the solvent. Some solute may even be extracted if care is not taken. However, concentrations of solvent residue not exceeding 5000ppm are permitted in pharmaceutical applications (Fages et al., 2004, Knez and Weidner, 2003, Munin and Florence, 2011).

Supercritical antisolvent process can be applied to generate composites by concurrent precipitation of the active ingredient and coating material together simultaneously or by encapsulating previously formed solute particles in a suspension of the walling material and precipitating (Yulu Wang et al., 2004). Supercritical antisolvent method is best for non-soluble organic substances like proteins (Whitaker et al., 2005).

Under co-precipitation utilisation, a situation where both active ingredient and coating material are insoluble in the same solvent, then it is appropriate to prepare two separate solutions with each substance and then co-precipitate by the antisolvent process (Cocero et al., 2009).

1.7.1.4.3 Particles from Gas saturated Solutions (PGSS)

Particles from gas saturated solutions allow formation of substances that are insoluble in the supercritical fluid, but can absorb a substantial amount of the gas that either swells the substance or decrease melting point (Knez and Weidner, 2003). PGSS technique for particle formation is good for substances that take in supercritical fluids at higher concentrations (Cocero et al., 2009). The increased gas concentration in the liquid phase gives indication of a decrease of melting point, viscosity as well as interfacial tension, thereby allowing substances to be sprayed for which under normal circumstances would have been very difficult to form particles by spraying (Weidner, 2009).

The solute which is to be micronised (in molten state) is pumped into a pressure resistant pipe, and pressurized gas is then allowed into the static mixer to meet the solute. After an intensive mixing, the mixture is then depressurised through a nozzle and fine droplets are formed by the Joule Thompson effect. As the gas rapidly cools down, it removes the heat from the molten solute droplets, and solidification time vary between 10mS and 100mS (Weidner, 2009). PGSS defer from RESS and Supercritical antisolvent technique because PGSS does not depend on solvent strength but rather utilises other properties of the supercritical fluids (Weidner et al., 1994a, Weidner et al., 1994b).

The PGSS process is more applicable for particle formation of polymeric substances for encapsulating the active ingredient within the substance (Knez and Weidner, 2003). In this application, the incorporation of the active substance into the polymer is strongly assisted by effect of swelling of supercritical fluid. Interestingly, about 100 or more substances have been powdered using PGSS process (Weidner, 1996).

Production of particles from PGSS has been feasible in the laboratory, and pilot scale production for both continuous and discontinuous modes can be employed. In the laboratory scale, the active ingredient to be powdered is introduced into an autoclave and then pressurized. Subsequently an appreciable concentration of the gas is mixed with the solute, after which system is depressurized through a nozzle and partially received in spray tower connected to a cyclone and the gas escapes through the vent. Normally few kilograms of powdered substance are obtained in the laboratory scale operation. Larger quantities are produced from continuous process which can be a pilot plant (Weidner, 2009).

The PGSS process has been demonstrated in pilot- scale for example surface-active components, waxes, resins, some polymers and pharmaceuticals. Improved micronized powders were produced compared to powders generated by traditional encapsulation techniques (Knez and Weidner, 2003). The benefits of the PGSS technique over the conventional methods for micronisation include:

- The process is adaptable and, applicable to many substances;
- Low pressures are used;
- Solvent free powders are produced;
- Minimum amount of gas is consumed;
- Capable working with highly viscous and sticky products;
- Different particle morphologies are produced;
- Process can be scaled up. (Knez and Weidner, 2003).

Supercritical fluid can also be employed in the established spray drying process (Reverchon, 2002, Sievers et al., 2003). The role of the supercritical fluid is to cause the disintegration of liquid-solute mixture to generate very fine droplets (Weidner, 2009). In this process the extracts can be preheated in a vessel and transported by the aid of a piston pump into a static mixer. The SCF is held high pressure tank at vapour pressure and is collected from the tank in the form of liquid using a diaphragm pump. The flow rate of the fluid varied between 6 to 10 kg/hr which can be measured by Coriolis flow meter. The fluid is heated through a heat exchanger and pumped through a non-return valve to the static mixer to contact the solution to be dried like similarly to PGSS process. Hence the modified process is termed PGSS- drying. Subsequently the gas is depressurised, and fine droplets are formed. The spray conditions can be manipulated to allow the solvent escape alongside the gas. The temperature of the spray tower can be varied to ensure the mixture is superheated under pressure so as achieve a temperature not less than the dew point within the gas and solvent binary system. This implies that the gas and solvent results in a uniform phase being removed from the spray. However it is very important to have an idea about the solubility of the solvent in the supercritical fluid under spray tower conditions(Meterc et al., 2008, Weidner et al., 2000).

1.8 Objectives

1.8.1 General Objectives

The overall objective of this thesis is to develop an understanding of the utility of subcritical water to support efficient selective extraction of polyphenolics from the by-products of cider production and evaluate extracts in terms of antioxidant activity and the impact of encapsulation of the polyphenolics on overall stability and bio-availability.

1.8.1.1 Specific research objectives

The specific research objectives are as follows:

- Develop an advanced understanding of the impact of process operating parameters on subcritical water mediated extraction of polyphenolics from apple pomace;
- Develop a basic understanding of the efficacy of polyphenolics as a complex mixture with respect to antioxidant activity; and

- Evaluate the feasibility of encapsulating the polyphenolic fraction derived from apple pomace using naturally occurring polymers co-extracted with polyphenolics under the subcritical water mediated hydrolysis to improve oxidative stability and assess the impact on formulations and performance in for example skin care products.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Apple Pomace

6.5 kg of apple pomace made up of 7 apple varieties (Michelin, Dabinett, Yarlinton Mill, Chisel Jersey, Brown Snout, Vilberie and Harry Masters Jersey) was collected from Universal Beverages Limited (UBL), Ledbury a subsidiary company of Bulmers, UK on the 8th of November, 2012. The material was transported to the University of Birmingham and partitioned into 0.7 kg aliquots using freeze bags before storing at -20ºC until further use.

2.2 Chemical Reagents

All chemicals and standards used where purchased at the highest grade of purity from supplies indicated in the methods.

2.2.1 Methods

2.2.1.1 Dry weight content of apple pomace

The dry weight and moisture content of the apple pomace obtained from Bulmers were determined using AOCS (American Oil Chemist Society) standard procedure and expressed as the percentage of total wet weight of the apple pomace. Apple pomace was very heterogeneous comprising, seeds, peel, apple flesh and calix, therefore was thoroughly mixed to ensure replicate samples were representative of the pomace population. Evaporating dishes (Pyrex glass, Fisher Scientific) were dried for 24h in a bench top Laboratory convention oven (STATUS International, UK) set at 103±3°C. The evaporating dishes were cooled in the desiccator and their weight accurately determined. 10g ±0.03 of the pomace was sampled and placed in the evaporating dish using electronic semi micro balance (Mettler PM 4600 Delta Range) before transferring into the convection oven. Weight loss of pomace was monitored every 24hours until there was no change in weight. Dried pomace was allowed to cool in a desiccator for at least 30 minutes to return to ambient conditions before weighing again. To ensure good results gloves were worn throughout and all measurements were done in triplicates or more.

Calculations:

The change in the weight of the apple pomace during the drying process was used to determine the moisture content expressed as a percentage (% moisture content) of wet weight as received as shown in Equation 2-1and Equation 2-2

Equation 2-1 Moisture Content Determination of Apple pomace

$$\%\,Moisture \quad = \left[\frac{(weight\ dish + wet\ pomace) - (weight\ dish + dry\ pomace)}{(weight\ dish + wet\ pomace) - (weight\ empty\ dish)}\right] X100$$

The percent Dry weight content of the apple pomace was calculated as follows;

Equation 2-2 Dry Matter Content Determination of Apple pomace

$$\%\,Dry\ Apple\ pomace = 100 - \%\,Moisture\ content\ of\ apple\ pomace$$

2.2.1.2 Preparation of freeze dried apple pomace

Apple pomace was freeze dried using a vacuum freeze dryer (Model number EQ03 by Vacuum and Industrial products). The pomace sample previously stored at -20°C was used. To set up the freeze dyer all valves of the instrument were closed and the refrigerator was switched on to allow temperature to cool to -50°C. Samples were loaded and the Pirani gauge turned on and waited for some few minutes before opening the isolation valve for pressure to reach 670mmHg. The pump was kept cooled by keeping a standing fan by the equipment. Samples were dried when vacuum pressure was 4 Mbar. Prior to withdrawing the dried sample from the dryer, the isolation valve was closed, the vacuum pump switched off, and chamber drained. Samples were removed and placed in desiccator for at least 30 minutes to return to ambient conditions. Moisture and dry weight content of the freeze dried were determined as in section 2.2.1 and milled (1 minute) into a powder using a domestic Moulinex blender 530 (KEMAEU, France). Apple pomace powder was packed in dark plastic bags and stored at room temperature for subsequent use.

2.2.1.3 Homogenisation of wet apple pomace

Apple pomace was a heterogeneous source of biomass as stated above. In an attempt to minimise batch to batch variability, wet apple pomace was first homogenised (for 2 minues) using a Moulinex domestic blending machine before material was sampled for further experimentation. Homogenised apple pomace was sampled for dry weight determination as in section 2.2.1 and the remaining bulk stored in freezer bags at -20°C for further use.

2.2.2 Solvent extraction of polyphenolics from apple pomace

Extractions were done in an orbital shaker incubator MaxQ 4000 (Thermo Scientific). Known weight (according to Solid-to-solvent ratio) of the freeze dried apple pomace powder was placed in 100ml Duran bottle and solvent (Acetone or Ethanol) was added based on the solid -to - solvent ratio desired, and placed inside the incubator. A thermometer was attached to the inside of the incubator to confirm temperature conditions. Extraction time and shaking (150rpm) were set on the equipment. To prevent any loss of solvent during extraction, the lid of the Duran bottle was screwed tight before extraction begun. Shaking of incubator automatically stops when extraction time has elapsed. Content of Duran bottle after extraction were immediately transferred into 50ml centrifuge tube and centrifuged at 4000g for 10 minutes using the Juan C4 I centrifuge. The volume of supernatant were determined using a 25ml glass measuring cylinder and clean extract subsequently transferred into a 50ml blue cap tube for further investigations. Extractions at higher temperatures (60°C and 85°C) were carried out similarly in water bath equipment Grant OLS200 (England).

2.2.3 Subcritical water extraction of polyphenolics from apple pomace

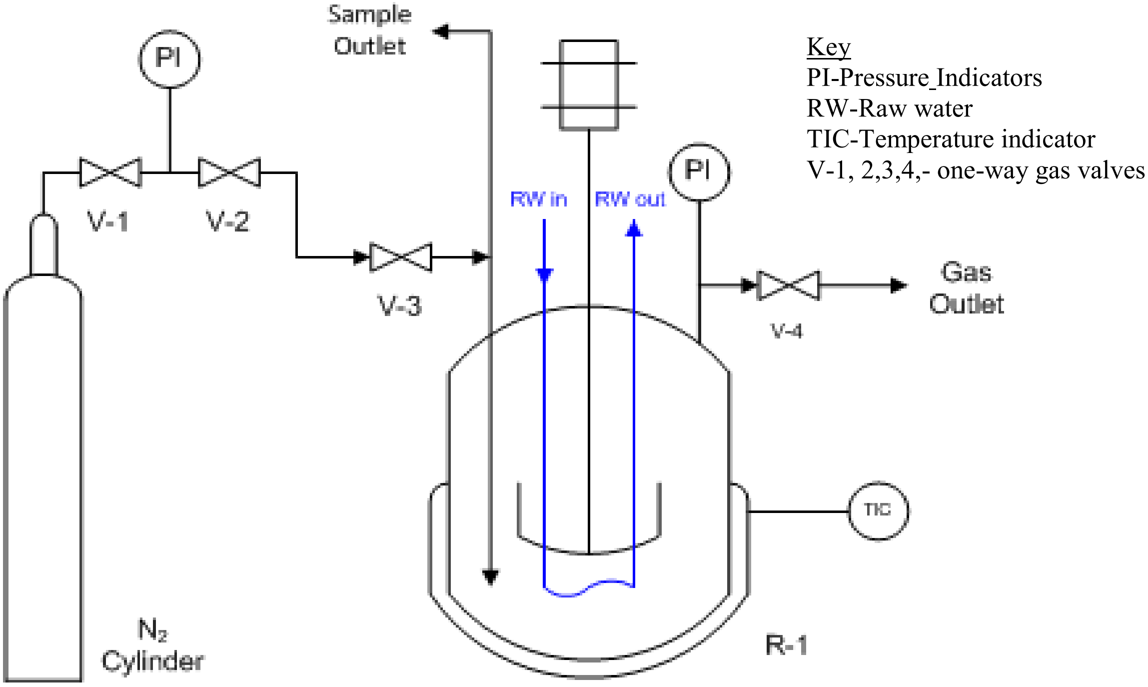
2.2.3.1 Materials and Equipment

Wet homogenised apple pomace was used in subcritical extraction equipment, Parr instrument model (5521), which was a stainless steel reactor vessel of internal volume 300ml with 2.5inch diameter and with a heating jacket. The vessel was connected with a temperature and pressure sensors whose readings were obtained from digital display of the meters. Magnetic stirrer (1240rpm) with integrated cooling system was attached to help enhance mass transfer. A back pressure regulatory valve was used to control pressure inside the vessel. A cylinder containing Nitrogen gas was connected and used to pressurize the vessel. The setup is shown in Figure 2-1.

2.2.3.1.1 Operating Procedure of the subcritical water Reactor

Wet homogenised apple pomace was loaded into the vessel according to the solid- to-solvent ratio (water) desired. The reactor vessel was sealed and placed inside the heating jacket. All valves into the reactor were closed. Checks were made to ensure the cooling systems to the vessel and stirrer were working. The stirrer mechanism to the mixer was connected and switched on. Gas purge valve (V-4) in Figure 2-1 was opened followed by gas valve (V-1) to set a maximum pressure of 100bar by opening gas regulator (V-2). Gas valve

(V-3) was then opened slowly to allow the Nitrogen gas into the reactor, while closing gas valve (V-4) slowly to purge the vessel within 10-15 seconds. Initial extraction pressure inside the vessel was set to 50 bar and the gas valve (V-3) closed. The desired extraction temperature was set on the controller and the heating switched on. Once the reaction was finished, the gas valve (V-1) was closed and the heating and stirring turned off. Cooling system to the reactor was turned on and vessel quickly removed from the heating jacket into an ice bath to allow the vessel to cool temperature below 50°C.The vessel was depressurised to release all gasses by carefully opening valve (V-4). The gas line was also depressurised by slowly opening the gas inlet valve (V-3). Gas regulator valve (V-2) was closed and the vessel opened to collect sample.



<u>Key</u>
PI-Pressure_Indicators
RW-Raw water
TIC-Temperature indicator
V-1, 2,3,4,- one-way gas valves

Figure 2-1 Setup for the Subcritical water Extraction of Apple pomace.

Processing of Extract

All extracts were transferred into a 500ml Beckman centrifuge bottles and centrifuged at 4000g for 10 minutes at a temperature 4℃ using the Beckman J2-20 centrifuge equipment. Supernatant were further filtered under normal pressure using Fisherbrand filter paper (QL 100). The volumes of the filtrates were measured using a 250ml measuring cylinder and transferred into 150ml plastic bottles and stored at -20℃ for further analysis. Solid residue were collected into freezer bags and also stored at -20℃.

2.2.4 Determination of biomass solubilisation

1ml of clear supernatant extract was pipetted using the 1000μl pipette into pre-weighed dried Eppendorf tube and reweighed to get the weight of tube and wet sample. Dry solid content of extract per millilitre were determined as in section 2.2.1. With total volume of extract already recorded, the total weight of solids (in grams) was determined and the fraction in terms of dry weight of the starting material was obtained using Equation 2-3.

Equation 2-3 Fraction of Solids Solubilised

$$Fraction\ of\ solids\ solubilised$$

$$= weight\ \left(\frac{solids\ (g)}{ml}\right) \times (\frac{Total\ volume\ of\ extract(ml)}{weight\ of\ starting\ material\ (g)}$$

2.2.5 Total Phenolic Content Determination

Total Phenolic Content (TPC) of all extracts were determined using Folin-Ciocalteu (FC) micro-scale method described by (Waterhouse, 2001).

Background

Phenolic compounds in the extracts were oxidized by the Folin Ciocalteus's reagent (FC-reagent) composed of a mixture of phosphotungnstic acid $H_3PW_{12}O_{40}$, and

Phosphomolybdic acid $H_3PMo_{12}O_{14}$. The acids were reduced to a mixture of blue oxides of tungsten, W_8O_{23} and Molybdenum Mo_8O_{23} in the presence of the phenols (V.L.; Singleton and Rossi, 1965b). The blue pigment has maximum absorption in the region of 765nm which was proportional to concentration of phenolic compounds initially present. Usually the development of the colour takes some time and can be facilitated by moderately warming the sample. Excessive heat will cause the colour to disappear (V. L. Singleton and Rossi, 1965a). The Folin Ciocalteu's method measures total phenolics in addition to other oxidisable substances within the sample matrix and can over estimate the value (V.L.; Singleton et al., 1999, V.L.; Singleton and Rossi, 1965b).

Total phenolic content determined was expressed in terms of Gallic acid equivalent (GAE). Gallic acid is so chosen because of its availability in pure form and relatively less expensive compared to other alternatives.

2.2.5.1 Chemicals and Equipment

Gallic acid $C_7H_6O_5$, 2,2,2 Trichloroacetic acid, 99.5% (TCA), Folin & Ciocalteu's Phenol reagent were procured from Sigma-Aldrich (Dorset, UK), Sodium carbonate Na_2CO_3 from J.T. Baker (Holland), Absolute ethanol (Fisher Scientific, UK), Clifton unstirred water (Nickel Electro Ltd, England), Miximatic Vortex equipment (JENCONS Scientific), Promega Glomax Microplate Spectrophotometer, Sigma k30 Laboratory centrifuge (SciQuip Ltd, UK).

2.2.5.1.1 Trichloroacetic Acid, TCA 100% (W/V) Preparation

30g of TCA solid was weighed using a precision balance (Mettler PM 4600 delta Range) into a 100ml glass beaker and 13.620ml of distilled water was measured using a 25ml measuring cylinder to measure 13ml and a 1ml micro pipette to measure 0.620ml. The TCA solution was transferred into 80ml Duran bottle and stored at 4^{o}C.

2.2.5.1.2 Sodium Carbonate Solution

200g of anhydrous Sodium Carbonate was weighed into 100ml glass beaker and 800ml of distilled water added. The mixture was stirred continuously with heating using Clifton magnetic stirrer hot plate to completely dissolve the solid and brought to boil. The solution was left to cool and additional sodium carbonate solid added and allowed to stand for 24hours. Precipitated crystals were filtered off and the solution transferred into a 1000ml volumetric flask. Distilled water was added to the mark and the Sodium carbonate solution was stored at room temperature which can be stable indefinitely.

2.2.5.1.3 Gallic Acid Stock Solution (5g/l)

0.5g of Gallic acid solid was weighed using (Mettler PM 4600 balance into 100ml volumetric flask and 10ml of absolute ethanol was added to aid dissolution. Distilled water was then added to the mark and transferred into 100ml Duran bottle and screwed tightly and stored at 4^{o}C which can be stable up to 2 weeks.

2.2.5.1.4 Gallic acid Standards for Calibration Curve

Standard concentrations of 50, 100, 150, 250 and 500mg/l of Gallic acid were prepared by adding 1, 2, 3, 5 and 10ml of the stock Gallic acid solution into five separate 100ml volumetric flasks. Distilled water was added to the mark to produce the resulting standard concentrations which were freshly prepared for calibration curve each time analysis was to be made.

2.2.5.1.5 Precipitation of Proteins in extracts (Sivaraman et al., 1997)

Apple pomace contains about 4.0% proteins (Vasil'ev et al., 1976) and may be co-extracted to interfere with the Folin- Ciocalteu's reagent (Singleton and Rossi, 1965). The proteins were precipitated out by measuring 80µl of the polyphenolic rich extract sample into 2ml Eppendorf tube, followed by 120µl of the 100% (W/V) TCA solution. The mixture was thoroughly mixed by using Miximatic Vortex equipment and kept under -20°C for 5minutes, and then 4°C for 15minutes. Sample was then centrifuged at 15000g, at 4°C for 15minutes using the Sigma k30 Laboratory centrifuge with the rotor 12154-H. Supernatant was transferred to clean new 2ml Eppendorf tube for subsequent analysis. Any protein precipitate formed was discarded (Sivaraman et al., 1997).

2.2.5.2 Microscale Procedure for Determination of Total Phenolic Content

20µl of sample, standard and blank was pipetted into a test tube followed by addition of 1.58ml of distilled water. 100µl of the Folin- Ciocalteu's phenol reagent was then added and shaken to mix. 300µl of Sodium Carbonate was then added and mixed thoroughly by Miximatic Vortex. Samples were left for 30 minutes at 40°C in the Clifton Unstirred water bath. After the incubation period, 300µl of the resulting solutions were pipetted into 96 Well F/B microplate and absorbance of standards/sample and blank were read at 750nm. The actual wavelength for the absorbance measurement should have been 765nm but Promega Glomax Microplate spectrophotometer could only be read at 750nm. All measurements for sample/standard/ blank were in triplicates. Concentrated samples were diluted where necessary and the dilution factor accounted for when determining final concentration of samples.

Calculation of Concentration of Samples

The variations of average absorbance with concentration of standard Gallic acid were used to construct a standard curve typically as in Figure 2-2

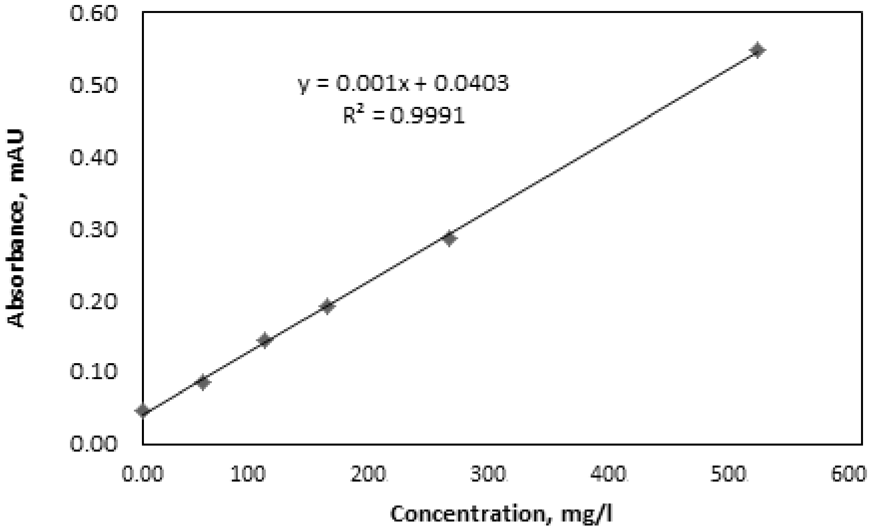


Figure 2-2 Calibration curve of standard Gallic acid

From the standard curve, the equation relating absorbance (y) and total phenolic content (x) is given by;

$$y = 0.000984x + 0.040299 \ \ (R^2 \ = 0.9991)$$

Where y is concentration in mg/l Gallic acid, and x is absorbance in mAU. Therefore the concentration of sample was calculated in terms of Gallic acid equivalent (GAE) as;

$$x = \frac{(y - 0.040299)}{0.000984}$$

Converting from mg/l GAE of Total phenolics to mg/g dry weight apple pomace is as in .

Equation 2-4.

Equation 2-4 Expressing Total Phenolic Content in milligram per gram Apple pomace

$$GAE(mg/g) = \frac{GAE\left(\frac{mg}{l}\right) \times Total\ Extract\ volume, ml}{Sample\ dry\ weight(g) \times 1000}$$

2.2.6 Experimental Design

Design of experiments for solvent extraction and subcritical water extraction techniques were divided into two sections. Preliminary or screening experiments and the actual design by response surface methodology. Screening experiments were conducted to identify effects of dependent variables and their limits leading to the actual design.

2.2.6.1 Screening Experiments under organic Solvent Extraction

The screening experiments involved one factor at a time (OFAT) executed by changing one experimental factor at a time whilst keeping the other factors constant. Solvent type, solvent concentration, solid- to- solvent ratio, temperature and extraction time were considered in the OFAT experiments.

2.2.6.1.1 Solvent Selection

The extractive capabilities of ethanol and acetone were investigated for consideration in an overall response surface design for optimization of polyphenolics from the apple pomace. Acetone and Ethanol were considered because they are generally recognized as safe (GRAS). Aqueous forms of the solvents were used, specifically 60% (V/V) for the investigation. Conditions applied were, fixing the loading ratio (solid: solvent) at (1:15, or 6.67%) (Suarez et al., 2010), fixing the extraction time of 3 hours and at temperature (25℃) and polyphenolics were extracted per the procedure for solvent extraction enumerated in section 2.2.2 above. Solvent extract with the highest Total phenolic value expressed in mg/g GAE (Gallic acid equivalent) dry weight was selected.

2.2.6.1.2 Effect of Extraction time

Recovery of polyphenolic compounds were done by varying the extraction time from 60, through 360 minutes while maintaining temperature at 25℃ using a solid to solvent ratio of 1:20 with the best solvent chosen above. Total phenolic content of extracts were evaluated according to Folin Ciocalteu's procedure and best time selected based on the highest TPC in mg/g GAE dry weight basis.

2.2.6.1.3 Effect of solid-to-solvent ratio

Using 60% (V/V) best solvent, at temperature 25℃ for 60minutes, the impact of loading was studied between 1 - 8% (W/V). Best solid-to-solvent in terms of total phenolic content (mg/g) GAE dry weight basis was selected for further screening.

2.2.6.1.4 Effect of Solvent concentration

With the best solvent selected and the best solid-to-solvent ratio, and a fixed extraction temperature at 25℃ and for 60minutes extraction time, phenolic compounds were extracted by varying the concentration of solvent from 20% (V/V) through 100% (V/V). Solvent concentration with highest total phenolic content in mg/g DW GAE was used for subsequent investigation.

2.2.6.1.5 Effect of temperature

The boiling points of solvents were taken into consideration before setting the upper limits of temperature in the experiment to avoid any loss of solvent by evaporation. Ethanol has a boiling point of 78℃ and acetone 56℃. Extractions were done below the boiling points.

2.2.7 Experimental Design for Optimization of Solvent Extraction

The results of the preliminary experiments under solvent extraction was used with the aid of Stat-Ease design software 7.0 to design an experiment using the central composite rotatable design using four design parameters as shown Table 2-1.

2.2.7.1 Screening Experiments under subcritical water extraction

Screening exercise involved preliminary investigation into the nature or form of the apple pomace used, and the impact of extraction parameters such as solid-to-solvent ratio, temperature and residence time on the overall recovery of antioxidant compounds under sub-critical water mediated hydrolysis.

Table 2-1 Experimental design by Central Composite Rotatable using 4 factors under acetone extraction

Standard order	**Run order**	**Factor 1**	**Factor 2**	**Factor 3**	**Factor 4**
		A:Acetone conc. %	**B: Temp. °C**	**C:Solid/solvent ratio %**	**D:Time min**
11	1	40.0	60.0	1.0	90.0
21	2	60.0	35.0	1.0	60.0
13	3	40.0	10.0	8.0	90.0
9	4	40.0	10.0	1.0	90.0
28	5	60.0	35.0	4.5	60.0
7	6	40.0	60.0	8.0	30.0
18	7	100.0	35.0	4.5	60.0
5	8	40.0	10.0	8.0	30.0
25	9	60.0	35.0	4.5	60.0
17	10	20.0	35.0	4.5	60.0
1	11	40.0	10.0	1.0	30.0
15	12	40.0	60.0	8.0	90.0
19	13	60.0	10.0	4.5	60.0
22	14	60.0	35.0	11.5	60.0
16	15	80.0	60.0	8.0	90.0
23	16	60.0	35.0	4.5	5.0
6	17	80.0	10.0	8.0	30.0
26	18	60.0	35.0	4.5	60.0
14	19	80.0	10.0	8.0	90.0
4	20	80.0	60.0	1.0	30.0
24	21	60.0	35.0	4.5	120.0
12	22	80.0	60.0	1.0	90.0
8	23	80.0	60.0	8.0	30.0
30	24	60.0	35.0	4.5	60.0
29	25	60.0	35.0	4.5	60.0
20	26	60.0	85.0	4.5	60.0
10	27	80.0	10.0	1.0	90.0
2	28	80.0	10.0	1.0	30.0
3	29	40.0	60.0	1.0	30.0
27	30	60.0	35.0	4.5	60.0

2.2.7.1.1 Nature of sample

Wet and freeze dried homogenized apple pomace were investigated with solid-to-solvent ratio (1-8%) under subcritical water mediated hydrolysis using procedure described in 2.2.3 under a fixed temperature of 150°C for a residence time of 20 minutes. The nature of the sample that yielded higher total phenolic content in mg/g DW GAE was selected and used thereafter.

2.2.7.1.2 Temperature

Now using the 1% (solid/solvent) loading and a fixed residence time of 20 minutes, extraction for phenolic compounds was targeted at 100°C, 160°C and 200°C using procedure described in section 2.2.3. Temperature condition with the highest value of total phenolic expressed in mg/g dry weight of Gallic acid equivalent was selected.

2.2.7.1.3 Residence time

Residence time (holding time at defined temperature of extraction) for 0, 10, 20, 30 and 60 minutes were investigated with 1% loading and best extraction temperature. Extractions were done using procedure described in section 2.2.3. The residence time that yielded highest total phenolic content in mg/g dry weight Gallic acid equivalent (GAE) was selected.

2.2.8 Experimental Design for Optimization of Subcritical water Extraction

Depending on the outcome of the screening experiments, the nature of the apple pomace, limits for loading (solid/solvent) ratio, temperature and residence time were then used with the aid of Stat-Ease design software 7.0 to design the central composite rotatable design for the experiment as shown Table 2-2.

Table 2-2 Experimental design by Central Composite Rotatable using 3 factors under subcritical water extraction

		Factor 1	**Factor 2**	**Factor 3**
Standard	**Run**	**A:Solid/Solvent ratio %**	**B:Temp ℃**	**C:Residence time min**
6	1	8.0	100.0	30.0
11	2	4.5	66.0	20.0
9	3	0.5	150.0	20.0
18	4	4.5	150.0	20.0
2	5	8.0	100.0	10.0
3	6	1.0	200.0	10.0
4	7	8.0	200.0	10.0
12	8	4.5	234.1	20.0
15	9	4.5	150.0	20.0
20	10	4.5	150.0	20.0
14	11	4.5	150.0	37.0
13	12	4.5	150.0	3.0
1	13	1.0	100.0	10.0
7	14	1.0	200.0	30.0
17	15	4.5	150.0	20.0
8	16	8.0	200.0	30.0
16	17	4.5	150.0	20.0
5	18	1.0	100.0	30.0
10	19	9.5	150.0	20.0
19	20	4.5	150.0	20.0

2.2.9 Separation, Identification and Quantification of Phenolic Compounds by High Performance Liquid Chromatography (HPLC)

Reverse Phase high performance liquid chromatographic (HPLC) procedure previously described by (Schieber et al., 2001b) was used for the separation of the Phenolic compounds from the aqueous acetone and the subcritical water mediated hydrolysis extracts. The compounds were separated based on their respective affinities between the mobile and the stationary phase. Identifications were done using their respective retention times and spectra data of respective standards. Quantification of the phenolic compounds made use of their chromatographic peak areas at maximum absorbance. Major polyphenolic compounds were identified and quantified.

2.2.9.1 Reagents and Chemicals

All reagents and chemicals used were either HPLC or analytical grade and will be stated when necessary. Phenolic standards comprising, Chlorogenic acid (≥ 95%), (-) Epicatechin (≥ 90%), ± Catechin hydrate, Phloridzin dihydrate (≥ 99%), Procyanidin B2 (≥ 90%), Quercetin -3-β-D-glucoside (≥ 90%), Quercetin-3-D-galactoside (≥ 97%) Phloretin, Chromasolv for HPLC water purchased from Sigma-Aldrich (UK). Solvents such as Acetonenitrile, methanol and glacial acetic acid were obtained from Fisher Scientific (UK).

2.2.9.2 Instrumentation and Operation

Separation of phenolic compounds in extracts were performed using the Agilent 1100 series HPLC value system with DAD-UV detector (Agilent Technologies- Germany), supplied with a solution Chemstation software for both online and offline sections. Prodigy 5µm ODS3 100A, C18 (250 x 4.6 mm I.D) column from Phenomenex (Torrance, CA, USA) was the stationary phase with a guard column operated at 40°C.

The mobile phase consisted of 2% (V/V) of the glacial Acetic acid in water as eluent A. Eluent B was made with 0.5% of Acetic acid in 50:50 (V/V) of Water and Acetonitrile. Eluent C was (100%) Acetonitrile. The gradient solvent systems programmed for the separation with a flow rate of 1ml/min were as follows: beginning with 10% of B and increasing the gradient to 55% B in 50 minutes. Further increased from 55% B to 100% B was done in 10 minutes and finally decreased from 100% B to initial 10% B in 5 minutes. Eluent C was used in reconditioning the column under isocratic flow by pumping 100% C for

10 minutes, and 10% B also for 10 minutes. Volume of all samples injected at a time was 10µl and phenolic compounds were monitored at 280nm (flavanols), 320nm (Hydrocinnamic acid) and 370nm (Flavonols). Retention times and Spectra data were collected.

2.2.10 Determination of Concentration of Phenolic Compounds

Concentrations of phenolic compounds were derived from calibration curves of corresponding pure standards. Five different concentrations of the phenolic standards were each prepared from a stock solution and analysed on the HPLC system using the method described in 2.2.9 above. Calibration curves were obtained by plotting areas under peaks against concentrations.

2.2.10.1 Typical Calculation of concentration of Chlorogenic acid

Stock solution of 1mg/ml of standard Chlorogenic acid was prepared by weighing 0.05g of the solid into a 50ml volumetric flask and making it up to the mark with HPLC water. 5ml of 1mg/ml of the stock solution was pipetted using the 5ml pippete into a 50ml volumetric flask and adding HPLC grade water to the mark to yield a solution of concentration 0.1mg/ml. The solution was transferred into a 50ml red cap tube covered with aluminium foil to prevent oxidation from light. 10, 100, 200, 300, 500 µl of the 0.1mg/ml of the Chlorogenic acid solution were pipetted into separate HPLC vials and adjusting total volume to 1ml using HPLC water with 1000µl micropipettes. The solutions were thoroughly mixed to give final concentrations of 1, 10, 20, 30 and 50µg/ml. 1ml of the 0.1mg/ml (100µg/ml) was also pipetted into the vial to result in six different concentrations of samples for the calibration curve. Autosamplar was loaded with the Samples and were analysed by the HPLC method described in section 2.2.9.

The calibration curve for standard Chlorogenic acid is shown in Figure 2-3 below;

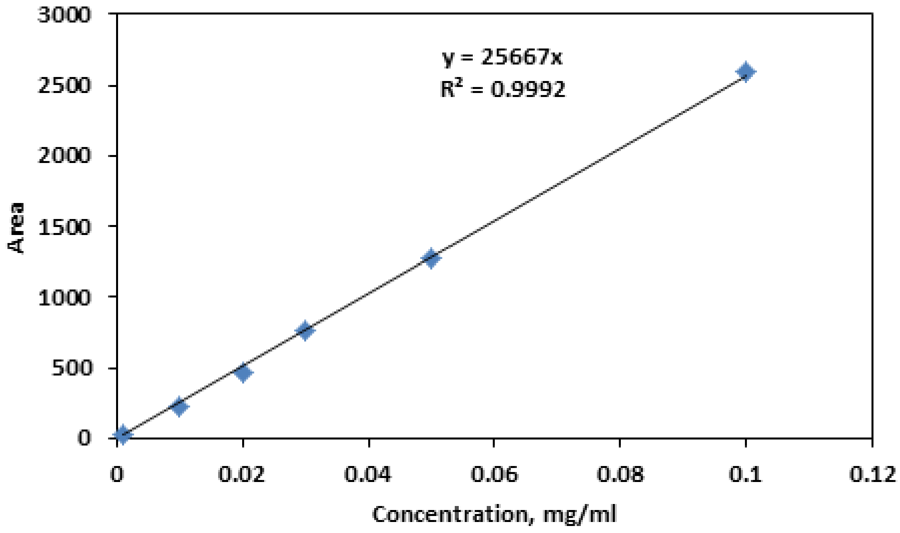


Figure 2-3 Calibration curve of standard Chlorogenic acid

The regression equation for Standard Chromogenic acid is

$$y = 26612x$$

Where y represent peak area, and x is Concentration of standard.

$$Amount\ of\ sample\ (interpolated) = \frac{y}{26612}$$

The purity of the standard used is taken into consideration and corrected. It can be used as a multiplier. Purity of Chlorogenic acid used ≥95%, therefore the multiplier will be

$$M = \frac{1}{0.95} = 1.0526$$

And amount of Chlorogenic acid in sample = $\left[\left(\frac{y}{26612}\right) \times 1.0526\right]\ mg/ml$

Conversion of mg/ml to $mg/g\ DW$ of apple pomace used is

$$Amount\left(\frac{mg}{g}DW\right) = \frac{(Amount\ \left(\frac{mg}{ml}\right) \times (Volume\ of\ extract, ml)}{Dry\ weight\ of\ apple\ pomace\ used, g}$$

Similar procedure was used to construct calibration curves for other phenolic standards and their concentrations derived from the graphs.

2.2.11 Estimation of Antioxidant Activity in-Vitro by ORAC Assay

Determination of antioxidant activity of phenolic extracts by ORAC assay was described by Huang and co-workers with modifications (D Huang et al., 2002).

The fundamental principle of the assay described the inhibition of the oxidation of a protein target by reactive oxygen species of a test sample relative to standard water soluble-like vitamin E (Trolox). The source of reactive oxygen species was a peroxyl radical ($ROO^{\cdot}$) generated by thermal decomposition of $2,2^{|}$-azinobis- methyl-propanidamide dihydrochloride, AAPH at 37℃, which quenches fluorescence signal overtime. Substances with antioxidant properties restrained and produced a more stable signal with extent of inhibition dependent on the capacity of the antioxidant. The inhibition was by hydrogen transfer mechanism and results were reported as ORAC values which obtained by comparing the area under the curve of the intensity of the fluorescence overtime of samples and Trolox relative to the blank.

2.2.11.1 Materials and Methods

$2,2^{|}$- azinobis- methyl-propanidamide dihydrochloride, AAPH, Phosphate buffer powder, Sodium Fluorescein salt were obtained from Sigma-Aldrich (UK), PHMP-4 Microplate thermoshaker was used to preheat and incubate samples at 37℃ prior to reading. Fluorescence measurements were done using the Promega Glomax Microplate reader using 96 Well black- microplate (single invitros use only).

2.2.11.1.1 Phosphate Buffer 75mM, (pH $= 7.4$)

All solutions were prepared using the phosphate buffer (pH=7.4). Buffer powder (P7994-1EA) from Sigma-Aldrich was reconstituted with 3.8litres of deionised water to produce 0.1M stock buffer solution. 75mM of pH 7.4 was prepared by diluting 750ml of the 0.1M Stock buffer solution with deionised water in a 1000ml volumetric flask to the mark.

2.2.11.1.2 Sodium Fluorescein Solution

A stock solution of 6.64µM of Sodium Fluorescein was made by weighing out 25mg of the salt into 100ml volumetric flask and diluted with phosphate buffer (75mM, pH=7.4) to the mark. Resulting solution was transferred into a clean 100ml Duran bottle and covered with an aluminium foil to prevent oxidation and stored at 4°C. Working fluorescein concentration of 300nM was freshly prepared by diluting 900µl of the stock fluorescein solution (6.64µM) to 20ml using 75mM phosphate buffer and covered with a foil and stored at 4°C prior to use (Jumbu, 2014) .

2.2.11.1.3 Trolox Standard Solution

Standard stock solution of Trolox (0.02M) was prepared by weighing out 0.250g into 10ml of absolute ethanol to facilitate dissolution and then transferred into a 50ml volumetric flask and diluted with Phosphate buffer (pH 7.4). Trolox working concentration of 25µM was prepared by measuring out 62.5µl of the 0.02M standard Trolox into a 50ml volumetric flask and further diluted with the buffer (pH 7.4). Concentrations such as 12.5µM, 6.25µM and lower were simply prepared by diluting 25µM with phosphate buffer (pH 7.4).

2.2.11.1.4 AAPH Solution

AAPH solution was freshly prepared each day of analysis by dissolving completely 0.414g of the solid in 5ml of 750mM phosphate buffer (pH, 7.4) and incubated at 37°C prior to application.

2.2.11.1.5 Assay Procedure

150µl of the fluorescein working solution was added in quadruplicate into a 96 well microplate using a multichannel pipette designated as control, blank, sample and Trolox. 25µl of blank/sample/Trolox were added into the respective wells. 50µl of buffer (pH 7.4) was added only into the control wells. The content of the microplates were thoroughly mixed using the thermoshaker at 1000rpm for 3 minutes and left to incubate at 37°C for 30minutes. 25µl of previously incubated AAPH solution was then added to the blank/sample/Trolox wells using a multichannel pipette and thoroughly mixed using the thermoskaker at 1000rpm for 20 seconds. Microplate was immediately placed into the Promega Microplate reader to record the fluorescence decay per minute for 45 minutes.

Analysis of Data

Data analysis was done using area under the curve of blank/sample/TROLOX in Microsoft Excel 2010 as shown Equation 2-5.

Equation 2-5 Area under the fluorescence decay curve

$$AUC \ = 0.5 + \frac{f_1}{f_0} + \cdots \frac{f_i}{f_0} + \cdots \frac{f_{44}}{f_0} + 0.5 \left(\frac{f_{45}}{f_0}\right)$$

Where f_0 is the fluorescence reading at time 0 minutes, and f_i is the reading at the ith minute. A typical decay curve of antioxidant standard and sample at different concentrations is shown in Figure 2-4 and normalised to 100%.

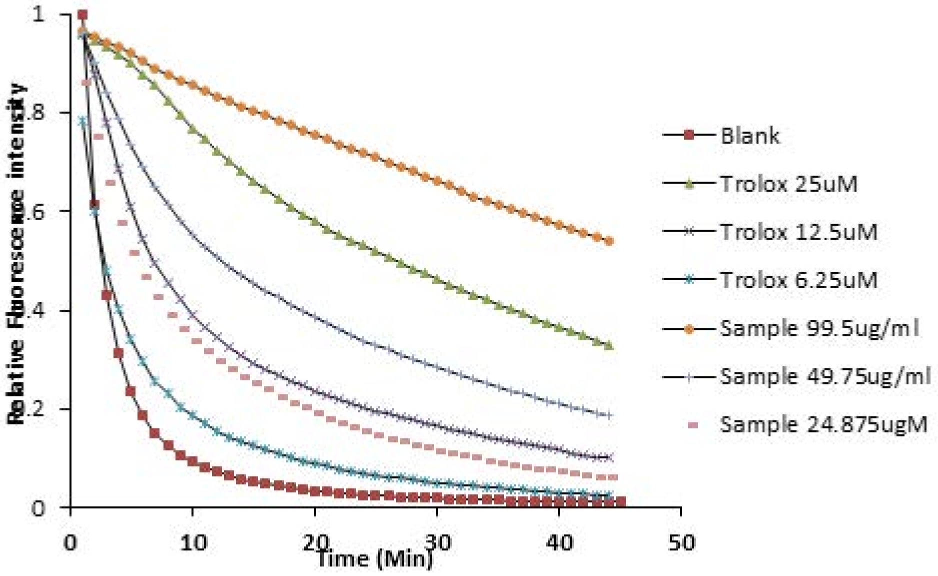


Figure 2-4 Typical decay curve of Trolox and sample

2.2.11.1.6 Calculation of Relative ORAC Value

Relative ORAC value is calculated using Equation 2-6 and expressed in µmol TE/ g DW.

Equation 2-6 Calculation of Relative ORAC value

$$Relative\ ORAC\ value$$

$$= \left[\left(AUC_{sample} - AUC_{blank}\middle|AUC_{Trolox} - AUC_{blank}\right)\right]\left(Molaity\ of\ Trolox\middle|Molarity\ of\ sample\right)$$

$$Relative\ ORAC\ value = \frac{Slope\ of\ sample}{Slope\ of\ Trolox}$$

2.2.12 Preparation of the inclusion complex

Procedure previously described by Gioxari et al.,2010 was adopted with slight variations (Kalogeropoulos et al., 2010). The inclusion complex was prepared in a mixture of Subcritical water extract and HP-β-cyclodextrin in a mass ratio (4:1). 56.5g of HP-β-Cyclodextrins was mixed with 500ml of subcritical water extract of the apple pomace containing 0.0283g/ml of dry extract in 1000ml Erlenmeyer flask covered with aluminium foil. Mixture was stirred continuously by magnetic stirring on a Clifton hot plate Stirrer for 4hours at room temperature. Inclusion complex mixture was transferred into 1000ml plastic container and stored at 4°C until further applications. Prior to storage, 5ml were pipetted into 15ml plastic vials and frozen at -20°C for 24hrs and then freeze dried according the procedure previously described under section 2.2.1 .Dried solid lumps were broken using glass rod and stored in a desiccator for future analysis.

2.2.13 Spray Drying

Laboratory scale spray dryer (Model- SS07, Labplant Ltd, UK) was used to prepare powders. All glassware were fitted to the unit and inlet temperature were set to 200°C and left to warm up for 15 minutes before setting the actual spray temperature. Inlet and outlet temperatures were set at 170°C and 84°C respectively. Sample feed was delivered at (3.6ml/min) using a variable speed peristaltic pump into a 0.5mm two- fluid- stainless spray nozzle with air flow rate 180g/min. The subcritical water extract containing 0.0283g/ml dry solids was used to identify spray condition. Inclusion complex previously prepared in section 2.2.12 was also sprayed at the same condition. Feeds were continuously stirred during spraying and dry powders were separated by a cyclone and collected into an insulated sample collection bottle. Total weight of powders was recorded, and portion sampled for analysis and bulk packed in seal polyethylene bags and stored in a desiccator for further investigation.

2.2.14 Determination of Density of Powder

Gas displacement technique was employed to determine powder density using an equipment AccuPyc II 1340 gas Pycnometer by Micrometrics Instruments Corporation. A 1cm^3 volume capacity cup whose weight previously determined using the microbalance SART 1702 (Germany) was then filled with portions of powder and reweighed to obtain accurate weight of power. Powder in the cup was sealed in the instrument compartment and helium gas admitted to serve as displacement medium and expanded within the internal volume of powder. Solid phase volume of powder was computed from the changes in pressure during filling of sample chamber and that of the discharge empty chamber. Data were analysed using VI.05 software and density of powder was determined by dividing average volume into powder weight.

2.2.15 Particle Size Measurement

HELOS/RODOS/VIBRI dispersing system (Sympatec GmbH, Clausthal-Zellerfel Germany), a laser diffraction equipment was used to determine particle size and distribution of powders. The setup consisted of HELOS (Helium-Neon-Laser optical system), a dry powder dispersion system RODOS, and a vibrating feeder VIBRI. The feeder line has an inlet funnel connected to the dispersion line which produces the dispersed aerosols beam. Operating pressure of the entire setup was between 2- 3 bar air pressure. Powder samples were manually filled into the vibrating feeder through the funnel to an approximate 1cm^3 high. Different measuring ranges of laser sensors were provided by interchangeable objectives R1 (0.1-35µm), R2 (0.1-875µm) and R3 (0.9-175µm) selected based on the density value of powder. During operation, the powder passes through a plough scraper on a roller that removes excess sample and finally deliver them into the dispersing line. Operations were controlled by software WINDOX 5 for evaluation of particles size and other analysis.

2.2.16 Hygroscopicity Test

0.5g of encapsulated dried powders (SWE and SWE+HPβ-CD) were weighed using a microbalance instrument GR-202 (A&D Scientific Laboratory suppliers) and spread uniformly on a glass petri dish. Three (3) replicates samples of each powder were kept at 23℃ in an incubator model SI- 600R (Medline Scientific). 300ml saturated Sodium Chloride solution was placed inside the incubator to provide approximately 75.5% relative humidity and left for 7 days. Samples were weighed after the 7 days and Hygroscopicity HG determined according to Equation 2-7.

Equation 2-7 Calculation of Hygroscopicity of powder

$$HG = \frac{\Delta m/(M + M_i)}{1 + \frac{\Delta m}{M}}$$

Where Δm was the increase in weight of powder after equilibrium. M was the initial mass of powder and M_i was the free water content of powder prior to exposure to the humid environment (Caparino et al., 2012, Jaya and Das, 2004, Sablani et al., 2008, Tonon et al., 2008).

2.2.17 Scanning Electron Microscopy

Environmental Scanning Electron Microscopy (XL 30 ESEM FEG Philips Netherlands) was used to observe the morphology of the powders generated from freeze and spray dried processes. Samples were spread on ESEM-stub covered with sticky carbon tape, and sputter coated under high vacuum with Gold using EMSCOPE SC 500 Gold sputter coater. All samples were scanned at a voltage of 15kV using XL 30 ESEM FEG electron microscope and images captured for several magnifications at separate location on the sample.

2.2.18 Fourier Transform Infrared Spectroscopy (FT-IR)

Molecular characterization of powders by FTIR analysis was performed on a Jasco FT-IR 6300 infrared spectrometer. Resolution of 4 cm^{-1} and 32 scan were used in a range between 4000 and 600 cm^{-1}. Higher resolution gave more detailed (peaks), however, included more noise; increasing the number of scans decreased the noise, but increased significantly the time spent in each sample analysis by the spectrometer.

A background was performed before each sample to scan the environment and subtracted from the sample spectra to avoid any interference in the results..

2.2.19 Stability studies

1.5ml of subcritical water extract and subcritical water extract with HPβ-cyclodextrin were measured into separate 2ml caped Eppendorf tubes and placed in an upright position on a test tube rack. All samples were stored at 65^{o}C in a drying cabinet by Fisons Scientific instruments UK. Stability assessment in terms of antioxidant activity of all samples were determined every 7days for 35days by Folin Ciocalteu method. All measurements were done in triplicates and samples were not protected from external light.

2.2.20 Statistical analysis

All results were expressed as mean± standard error (at least 3 replicates). One-way Analysis of variance (ANOVA) testing was conducted at 95% confidence interval with either Stat-Ease design expert software, XLstat and will be stated when applied.

Chapter 3

Optimisation of Organic Solvent Mediated Extraction of polyphenolic compounds from apple pomace using Response Surface Methodologies

3.1 Introduction

Recent interest by researchers and nutraceutical manufacturers in polyphenolic compounds from fruit and vegetable sources have grown because of their potential antioxidant properties (Manach et al., 2004). Extracts of these phytochemicals have found applications in food, pharmaceutical and cosmetic industries (Cetkovic et al., 2008, Hunter and Hull, 1993, Lu and Foo, 1997, 2000, Vinson et al., 2001).

Recovery of the polyphenolic compounds from plant sources was regarded as the number one step towards their application and could be extracted from either fresh or dried samples (Dai and Mumper, 2010). Apple pomace a by-product from apple juice and cider production is a source of polyphenolic compounds whose isolations are routes towards adding value to the residue (Ignat et al., 2011). The polyphenolics fall into two classes, non-extractable (NEP) and extractable (EP). Simple polar phenolic compounds such as hydrocinnamic acids and other flavonoids are easily extracted using organic solvents and therefore EPs dominate the scientific literature. Arranz et al., 2010, demonstrated that, non-extractable polyphenols are a major part of dietary polyphenols, therefore suggesting that solely reporting extractable polyphenolics will underestimate the nutritional value of fruits and vegetables. Non-extractable polyphenols constitute polymeric polyphenols for example proanthocyannidins and cell wall bound polyphenolics (Arranz et al., 2010, Saura-Calixto, 1998). Non extractable polyphenolics found in extracts may account for the variation of total phenolic content of apple pomace reported in the literature (Bai et al., 2010, Reis et al., 2012, Suarez et al., 2010, Wijngaard and Brunton, 2010).

The main objective of the present study was to explore selectivity of aqueous organic solvents towards polyphenolics and at the same time optimise process parameters to maximise the recovery of polyphenolic compounds from apple pomace so as to serve as a baseline for comparison with novel Subcritical water mediated extraction considered in Chapter 4. Solubilisation based on the biomass (apple pomace) loading was investigated to understand the relationship between soluble solids and total phenolic content recovered. The yields and content of polyphenolic compounds extracted from the apple pomace using organic solvents has been shown to be influenced by process parameters including solvent type, solvent concentration, temperature, sample to solvent ratio, and extraction time (residency time).

Polyphenolic extraction was optimised by applying design of experiments (DoE) and surface response methodology which are, multivariate statistics techniques to assess interrelationship between process parameters (Çam and Aaby, 2010, Pinelo et al., 2007, Silva et al., 2007). Response Surface Methodology (RSM) was first described by Box et al.,1950 (Gilmour, 2006), and is a collection of mathematical and statistical techniques employed to improve system performance for maximum benefits (Bazerra et al., 2008), by fitting a polynomial equation to observed data from within a designed experiment. The technique was able to predict the behaviour of a response based on the set of independent variables (Bazerra, 2008). Response surface methodology has important application in process development, formulation and design of contemporary products in addition to established ones. The technique is widely applicable in chemical and biochemical processes for different objectives (Bas and Boyaci 2007). Comprehensive description of design of experiments by response surface methodology can be obtained from (Box and Draper, 2007, Gunst, 1996, Myers et al., 2009).

In summary the aim of the current chapter was to extend the understanding of the behaviour of polyphenolics derived from apple pomace during organic solvent mediated extraction.

3.2 Results and Discussion

3.2.1 Sample preparation

Correct and appropriate sample preparation is a very important initial step to ensure reproducible data is obtained therefore maximum care was employed to minimise errors so as to achieve quality results. Apple pomace as received was very heterogeneous and composed of peels, seeds, apple flesh and calix, and therefore was thoroughly mixed to ensure replicate samples were representative of the pomace population.

The mean dry weight content of the apple pomace sample received estimated using the standard protocol described in section 2.2.1 as 27.7±0.3 g/100g wet weight with average moisture content of 72.3g/100g of wet apple pomace. The value obtained for the apple pomace moisture content falls within the 21.8-33.6g/100g reported in literature (Joshi and Attri, 2006, Kennedy et al., 1999, Sun et al., 2002). Measured dry weight of 27.7±0.3g/100g may include some amounts of moisture from the atmosphere and the freezer, but these sources were considered insignificant as compared to the nature of the apple pomace as received.
However the differences in the measured values in the experiments were attributed largely to the heterogeneous nature of the apple pomace with pieces of rejected apples finding their way

into samples. Apple pomace represents a substrate with high moisture content and the analysis confirms the fact if left into the environment may result into a spontaneous fermentation due to the high chemical oxygen demand, thereby causing environmental pollution. The pomace can be stabilized by reducing the water content through drying. Furthermore Vasil'ev et al (1976) reported dry matter (DM) content of apple pomace to be 26.4g/100g of fresh weight apple pomace (Vasil'ev et al., 1976). The variations in the reports can also be attributed to the several varieties of apples occurring in nature and their composition which depends on the region of growth, the degree of ripening, agricultural practices and the extraction processes involved (Kennedy et al., 1999).

Mean dry weight content of the apple pomace after freeze drying was 28.3±0.6g/100g fresh weight. Freeze drying method was chosen to stabilize apple pomace residue so as to retain the active ingredients (polyphenolics). There was high probability that quality of the apple pomace sample was assured after freeze drying. Freeze dried samples are thought to be free from biological and chemical activities (Luthria, 2006).

3.2.2 Screening Experiments under organic solvent extraction

Five factors were considered in the screening experiments to identify levels of influencing or important parameters to be used in an overall experimental design. Selection was based on the factors which resulted in highest total phenolic content expressed in mg/g GAE DW.

3.2.2.1 Organic Solvent selection

Efficiency of solvents in extraction of bioactive compounds depends strongly on the type of the plant material and has been reported in the literature (Dai and Mumper, 2010). The controlling parameter in addition to the nature of the plant matrix is the polarity of the solvent. Polar solvents have higher efficiency to solubilize polar ingredients (Liyana-Pathirana and Shahidi, 2005). Phenolic compounds are polar and therefore soluble in highly polar solvents. Solvents such as methanol, ethanol and acetone have been employed to recover polyphenolics at room temperature from plant sources. Many researchers have used these solvents particularly with varying concentrations to extract polyphenolics from apple pomace (Cetkovic et al., 2008, Diñeiro García et al., 2009, Foo and Lu, 1999, Lu and Foo, 2000, Schieber et al., 2001b, Suarez et al., 2010). Aqueous acetone (60% (V/V) showed better recovery of phenolic compounds than corresponding aqueous ethanol as shown in

Figure 3-1.

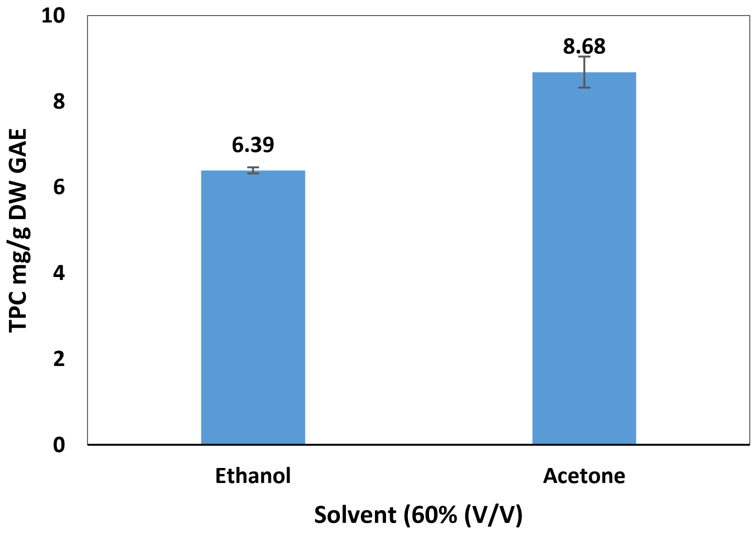


Figure 3-1 Solvent type selection for maximum total phenolic content (TPC,mg/g GAE DW) apple pomace using 60%(v/v) Ethanol and 60%(v/v) acetone with solid to solvent ratio of 6.7% at 25°C for 360 minutes extraction time

This results agreed with reports that, aqueous acetone extract polyphenolics better than aqueous ethanol (Heinonen et al., 1998, Kahkonen et al., 2001, Suarez et al., 2010). Acetone is regarded as a strong hydrogen bond breaker than ethanol (Hellström and Mattila, 2008).Total phenolic content of the ethanolic extract was approximately 26% less than acetone extracts. Massini et al., reported 20% less phenolic content in ethanol extract than acetone using apple pomace (Laura et al., 2013). The difference could be as a result of differences in the cultivars and also extraction methods. Therefore acetone was preferred solvent for the conventional solvent extraction throughout this investigation.

3.2.2.2 Effect of extraction time

The results of the effect of extraction time on total phenolic content of apple pomace under screening experiment described in section 2.2.6.1.2 is presented. Total phenolic content at the 60 minutes was 12.08 mg/g GAE DW whereas for 120 minutes and 360 minutes were respectively 11.21 mg/g GAE DW and 11.26 mg/g GAE DW as shown in Figure 3-2.

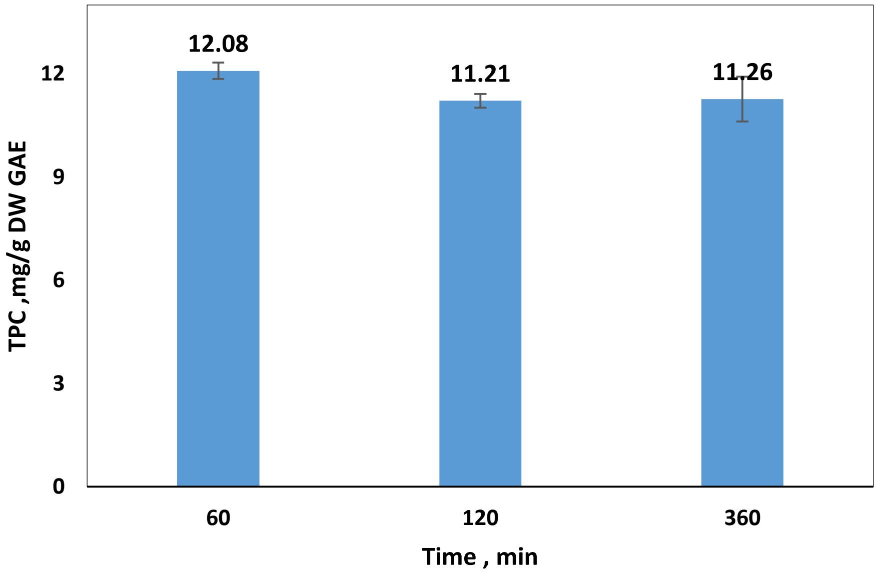


Figure 3-2 Effect of Extraction time (minutes) on Total phenolic content (mg/g GAE DW) of apple pomace using 60%(v/v) acetone concentration, 5%.solid-to-solvent ratio at 25°C

From the results it was shown there was no advantage of extracting beyond 60 minutes. The decrease in total phenolic content (mg/g GAE DW) could be as a result of oxidation of the phenolic compounds due to exposure of surrounding light during the extended time, or reaction with dissolved oxygen in water (Silva et al., 2007). Phenolic compounds may also be degraded or formation of non oxidisable components which were not reactive with the Folin reagent. Therefore 60 minutes was considered maximum extraction time for subsequent investigations. Further scanning using 60% (V/V) acetone at 25℃ confirms 60 minutes extraction time better than 90 minutes as shown in Figure 3-3.

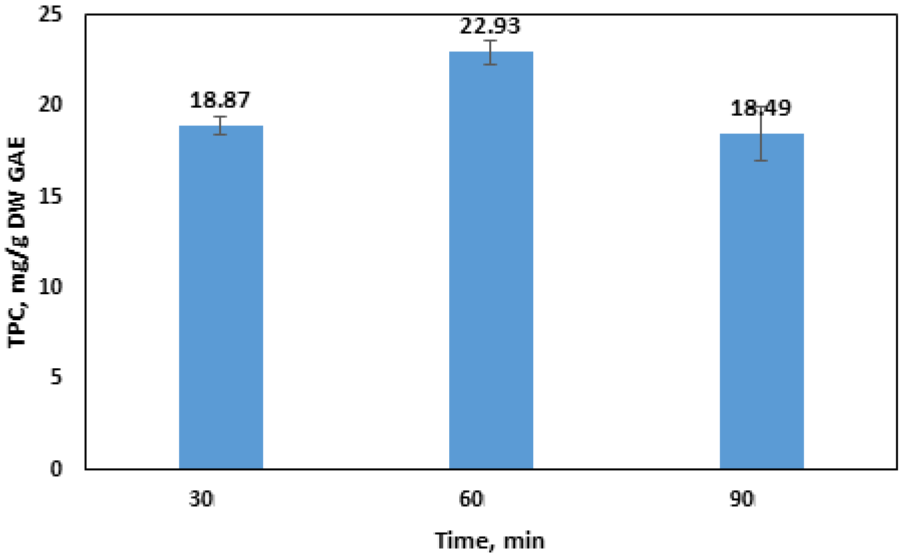


Figure 3-3 Effect of Extraction time (minutes) on Total phenolic content (mg/g GAE DW) of apple pomace using 60%(v/v) acetone concentration, 1%. solid-to-solvent ratio at extraction temperature 25°C.

3.2.2.3 Effect of solid-to-solvent ratio

The impact of solid-to-solvent ratio on total phenolic content from the freeze dried apple pomace powder using 60%(V/V) acetone for an extraction time of 60 minutes, is shown in Figure 3-4.

From the graph it shows that total phenolic content decreases as the ratio of solid to solvent increases. Total phenolic content of 1% was 22.93 mg/g GAE DW and for 8 % was 9.69 mg/g GAE DW. The results showed that better mass transfers were observed in the lower loadings thereby giving higher amounts of total phenolic content. Solvent was able to touch almost every sheet of particle in lower solid-to-solvent ratio than higher loadings. See Figure 3-4.

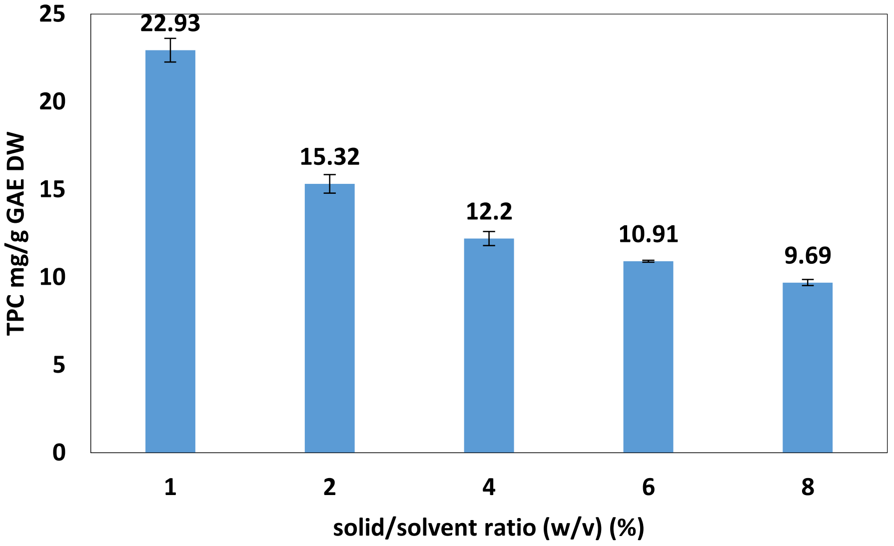


Figure 3-4 Effects of solid-to-solvent ratio %(w/v) on Total phenolic content (mg/g GAE DW) of apple pomace using 60%(v/v) acetone concentration, at temperature 25°C for 60 minutes extraction time

Higher loading ratios may result to incomplete extraction due to insufficient amount of solvent (Cruz et al., 2013). Therefore 1% solid-to-solvent ratio was considered as the lower limit and the 8% as the higher limits to study how the influence of other parameters will impact total phenolic content. One of the most important goals of industry is to optimise operating cost to achieve higher output and increasing loading rate (Klein-Marcuschamer et al., 2011).

3.2.2.4 Effect of Solvent Concentration

The effect of the acetone concentration on the polyphenolic recovery is shown in Figure 3-5.

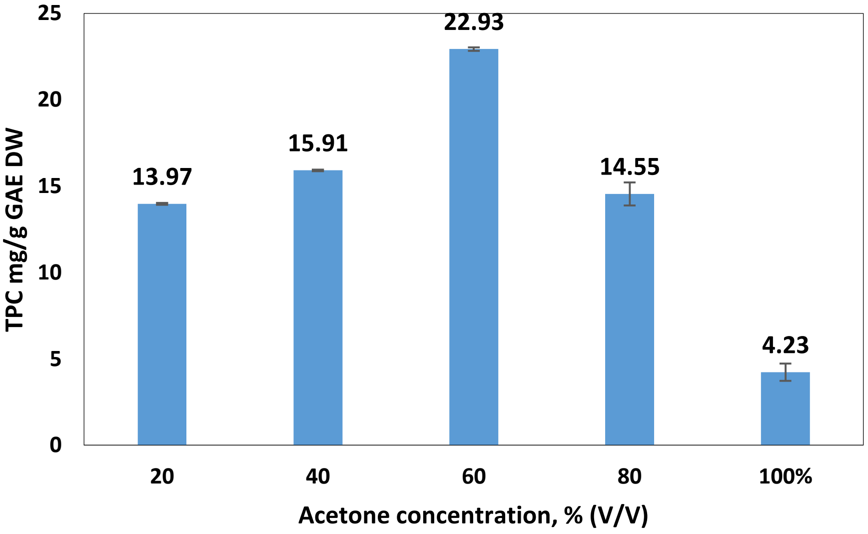


Figure 3-5 Effect of acetone concentration %(v/v) on Total phenolic content (mg/g GAE DW) of apple pomace using 1% solid -to -solvent ratio at temperature 25°C for an extraction time of 60 minutes

From the graph total phenolic content increases from 20% (V/V) acetone concentration to 60% (V/V) and decreases thereafter. 60%(V/V) acetone showed the best solvent concentration in this comparison with total phenolic content of 22.93 mg/g GAE DW, suggesting that addition of water improved the polarity of acetone and hence its efficiency. However, too much water couldn’t enhance recovery of phenolic compounds as shown in 20 % (V/V) acetone concentration recording approximately 14 mg/g GAE DW. Total phenolic content decreased above 60% (V/V) acetone concentration and to a low of 4.23 mg/g GAE DW at 100% (V/V). The results showed that pure or total acetone demonstrated poor recovery of antioxidant compounds. Similar results of poor efficacy of pure acetone in extracting polyphenolic compounds have been reported (Chen et al., 2007, Liyana-Pathirana and

Shahidi, 2005). Hence 40% (V/V) and 80% (V/V) were used as the lower and upper limits in designing the experiment.

3.2.2.5 Effect of Temperature

Total phenolic content of extracts of the screening experiments did not vary significantly from 25℃ to 45℃ suggesting that temperature has minimal effect on the extraction of polyphenolic compounds within this range. It is known that increasing temperature generally enhances solubility and diffusion rates which promote effective mass transfer to results in higher recovery. Increasing temperature lowers the surface tension and viscosity of solvent which enhances its percolation around sample matrixes resulting in higher yields of extract (Dai and Mumper, 2010). However this was not observed for the selected (solid/solvent) ratio of 1% where the solid matrix would have been well saturated with more than enough solvent and changes in temperature would have very minimal effect on overall recovery of phenolic compounds. The ANOVA results shows that there was no difference between the total phenolic content of extracts at 25℃, 35℃ and 45℃ ($p<0.05$). Extraction temperatures were lower than boiling point of acetone and may explain why slight variation of total phenolic content shown in Figure 3-6 below Therefore temperatures of 10℃ and 60℃ were employed as lower and upper limits in designing the experiment.

3.2.3 Experimental Design by Response surface methodology (RSM)

Following the results from the screening experiments, it was realised that acetone concentration, temperature, solid-to-solvent ratio and time were identified to have an impact on total phenolic content. The lower and upper limits of these variables were defined and Design Expert 7.0.0 (Stat-Ease Inc Minneapolis, USA),was used based on the Central

Composite Rotatable Design (CCRD) to generate 30 experimental runs, consisting of 16 trials for factorial points, 8 runs for axial points (2 for each of the 4 factors) and 6 replicates run around the centre points. This was shown in Table 2-1 for actual design and Table 3-1 for design summary under solvent extraction.

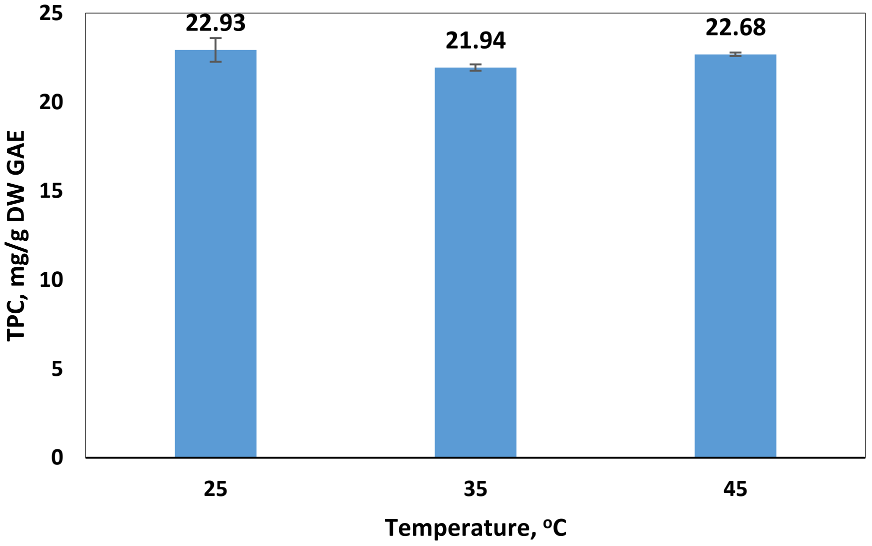


Figure 3-6 Effect of temperature (°C) on Total Phenolic content (mg/g GAE DW) of apple pomace using 60%(v/v) acetone concentration, 1% solid to solvent ratio for 60 minutes extraction time.

Table 3-1 Design summary under solvent extraction

Factor	Name	Units	Low	High	Mean	Std. Dev
A	Acetone Concentration	%	40	80	60	17.89
B	Temperature	°C	10	60	35.83	20.90
C	Solid/Solvent	%	1	8	4.62	2.99
D	Time	min	30	90	60.17	26.47

Extractions of phenolic compounds were done based on the extraction procedure described in section 2.2.2 and total phenolic content quantified according to the protocol outlined in section 2.2.5 . Several other responses were considered and modelled as such.

3.2.4 General Consideration for Model Selection

Several options including linear, two factor interaction and polynomial models were investigated for selection of most appropriate one that will be able to depict real time response of the surface. Statistical analysis of variance (ANOVA) was performed on the data using the Stat-Ease design Expert. For a given model to fit well, it should be significant and the lack of fit insignificant. Additionally, the models were compared based on adjusted R^2 and predicted R^2. It is very important not to measure the success of the regression analysis based on the coefficient of determination R^2 alone, which is the calculation of the ''variation explained by the model relative to the mean (overall, average of response)''. As the statement goes '' don't let R^2 value fool you'' (Hair et al., 1995). The quadratic model was appropriate among the rest and follows a generalised second order polynomial equation as in Equation 3-1.

Equation 3-1 Generalised second order polynomial equation

$$y = \beta_0 + \sum_{i=1}^{4} \beta_i \, x_i + \sum_{i=1}^{4} \beta_{ii} \, x_i^2 + \sum \sum_{i<j=1}^{4} \beta_{ij} \; x_i x_j$$

In building a good model, the simplest one is most preferred and to achieve this, may require eliminating outliers and transformation of the selected quadratic models.

3.2.5 Model Selection for Total Phenolic Content under solvent extraction

Total phenolic content ranged from 6.99 mg/g DW GAE to 22.55 mg/g DW GAE with mean phenolic content of 12.42 mg/g DW GAE. By subjecting the experimental data to multiple regression analysis, at 95% confidence interval, a transformed quadratic equation excluding outliers in the analysis was selected. The model was based on most influential design parameter solid-to solvent ratio which varies with Total phenolic content as a single factor as in Figure 3-7.

The transformed generalised second order polynomial model is as in Equation 3-2;

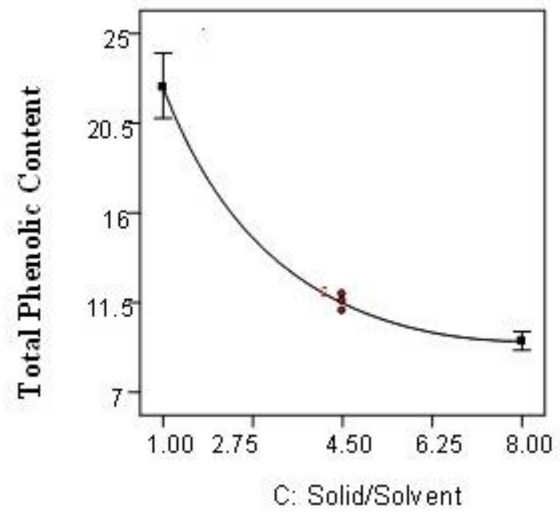


Figure 3-7 Variation of Total phenolic with Solid/Solvent ratio of apple pomace using 60%(V/V) acetone concntration, at temperature 25°C for 60 minutes extraction time.

Equation 3-2 Transformed generalised Polynomial Equation

$$\frac{1}{\sqrt{y}} = \beta_0 + \sum_{i=1}^{4}\beta_i x_i + \sum_{i=1}^{4}\beta_{ii}x_i^2 + \sum\sum_{i<j=1}^{4}\beta_{ij}x_i x_j .$$

Where, β_0 β_i β_{ii} and β_{ij} are the respective coefficient of regression for intercept, linear, quadratic and interaction terms. x_i and x_j are coded design variables. Results for the Analysis of variance (ANOVA) for the response surface of the reduced quadratic model are shown in Table 3-2.

Solid/solvent which was the most significant factor among the independent variables within the selected range has probability values for both the linear and quadratic terms ($p < 0.0001$). Acetone concentration was significant only in the quadratic term ($p< 0.0001$). Interaction between solid-to-solvent ratio and time was significant ($p=0.0346$).

Table 3-2 ANOVA for Response Surface Reduced Quadratic Model for TPC under Solvent Extraction

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p value**
Model	0.051	7	7.217×10^{-3}	49.70	< 0.0001
A *Acetone Conc*	2.649×10^{-4}	1	2.649×10^{-4}	1.82	0.1945
B *Temperature*	1.357×10^{-3}	1	1.357×10^{-3}	9.35	0.0071
C *Solid/solvent*	0.042	1	0.042	288.76	< 0.0001
D *Time*	1.054×10^{-4}	1	1.054×10^{-4}	0.73	0.4060
CD	7.659×10^{-4}	1	7.6659×10^{-4}	5.27	0.0346
A^2	4.392×10^{-3}	1	4.392×10^{-3}	30.86	< 0.0001
C^2	4.481×10^{-3}	1	4.481×10^{-3}	30.86	< 0.0001
Residual	2.468×10^{-3}	17	1.452×10^{-4}		
Lack of Fit	1.263×10^{-3}	12	1.053×10^{-4}	0.44	0.8886
Pure Error	1.205×10^{-3}	5	2.410×10^{-4}		
Cor Total	0.053	24			

However, temperature effects was significant (P=0.0071) within the selected range contrary to observation in the optimization of antioxidant compounds from apple pomace by response surface methodology (Wijngaard and Brunton, 2010). A response surface methodology using a Box-Behnken design for the optimization of polyphenols from apple pomace by micro-wave assisted extraction method by Bai and co-workers revealed that, total phenolic content followed a normal quadratic and depended on the solid to solvent ratio in addition to microwave power, solvent concentration and extraction time (Bai et al., 2010).

Range of solid to solvent ratio was between $1{:}10$ and $1{:}30$ in their investigation compared to a ratio of $1{:}12.5$ to $1{:}100$ (8% to 1% W/V) in this research. This justifies a transformed quadratic model as suitable, simple and meaningful which was highly leveraged by the total phenolic content, and significantly depended on the loading of the biomass. Extraction time was not significant within the range (P=0.4060) consistent with results of the one-to-one factor experiments but generally cannot be accepted. The contribution of the centrifugation process to this phenomenon cannot possibly be ruled out as it may be compensating for the overall extraction time. From the regression analysis it shows that selected model was highly significant (P<0.0001) and lack of fit is insignificant (p> 0.1). Additionally, the predicted R^2 value of 0.9096 reasonable agrees to the adjusted R^2 value of 0.9342 with a low coefficient of variation of 4.07% suggesting a high level of precision and reliability of measured values as shown in Table 3-3.

Table 3-3 Adequacy Level for Response Surface Reduced Quadratic Model for TPC under Solvent Extraction

Std. Dev.	0.0.12	R^2	0.9534
Mean	0.30	R^2_{Adj}	0.9342
C.V. %	4.07	R^2_{Pri}	0.9096
PRESS	4.790×10^{-3}	$Precision_{adeq}$	22.133

Adequate precision defined as ‘’the ratio of signal to noise ratio’ ’of this analysis was 22.133 which was good as a ratio greater than 4 is usually preferred. Hence this model can be used to navigate the design space. Final estimates for the intercept, linear and quadratic terms at 95% confidence interval are shown in Table 3-4.

Table 3-4 Coefficients Estimates terms for Reduced Quadratic Model for TPC under Solvent Extraction

Factor	**Coefficient Estimate**	**df**	**Standard Error**	**95% CI Low**	**95% CI High**	**VIF**
$Intercept$	0.30	1	4.075×10^{-3}	0.29	0.30	
A	-3.523×10^{-3}	1	2.609×10^{-3}	-9.028×10^{-3}	1.981×10^{-3}	1.02
B	-8.598×10^{-3}	1	2.812×10^{-3}	-0.015	-2.664×10^{-3}	1.03
C	0.056	1	3.283×10^{-3}	0.049	0.063	1.03
D	2.487×10^{-3}	1	2.919×10^{-3}	-3.671×10^{-3}	8.646×10^{-3}	1.05
CD	7.529×10^{-3}	1	3.278×10^{-3}	6.126×10^{-4}	0.014	1.04
A^2	0.013	1	2.361×10^{-3}	8.004×10^{-3}	0.18	1.02
C^2	-0.028	1	4.965×10^{-3}	-0.038	-0.017	1.05

A= acetone concentration (%v/v), B=Temperature (°C), C= solid-to- solvent ratio, (%w/v) and D= extraction time (minutes).

Coefficient estimates for acetone concentration was -3.523x10^{-3} of post ANOVA analysis at 95% confidence and a Variance inflation factor (VIF) of 1.02. Variance inflation factor is an estimation of how much of the variance of a coefficient is ‘’inflated’ ’because of linear dependence with other predictors. This factor has a lower bound value of 1 with several recommended upper bound values reported in literature. Maximum level of 10 was recommended (Hair et al., 1995), 5 (Rogerson, 2001), 4 (Park and Jackson, 2008) and 2.5 (Allison, 2012). Lower levels of VIF are desired because higher levels constitute a problem and affect results in multiple regression analysis (Allison, 2012). Variance inflation factor of acetone concentration of 1.02 means the coefficient was larger than a factor 1.02 than would otherwise be if there were no intercorrelations between total phenolic content, solid-to solvent ratio, temperature and time. Variances of all coefficients in the model were not very much inflated as the highest value of 1.05 in the model was just close to the minimum VIF of 1.0.

Negative values of any coefficient suggest a certain maximum value beyond which total phenolic content will decrease significantly. Therefore increasing, acetone concentration, solid-to-solvent ratio and temperature arbitrary may not favour overall yield of polyphenolic compounds in extract. Higher loading of solids relative to solvent reduced effective mass transfer and higher temperature may lead decomposition of polyphenolic compounds originally accumulated around low temperatures as shown in Figure 3-8.

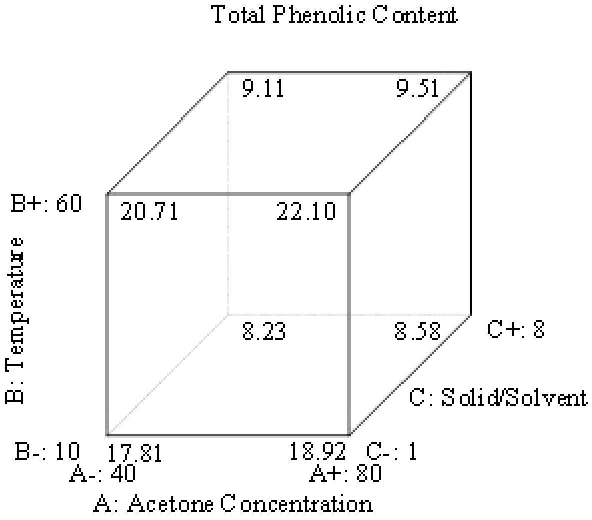


Figure 3-8 Variation of total phenolic content (mg/g GAE DW) with temperature (°C), acetone concentration (%(v/v) and solid-to solvent ratio (%(w/l) of apple pomace at a fixed extraction time of 60 minutes.

Degradation of phenolic compounds under high temperatures in solvent extraction has previously been reported (Hismath et al., 2011, Pacheco-Palencia et al., 2009).

The final equation in terms of actual factors is shown in Equation 3-3.

Equation 3-3 Model Equation for TPC under Acetone Extraction

$$\frac{1}{\sqrt{TPC}} = +0.33175 - 4.07195 \times 10^{-3}A - 3.43929 \times 10^{-4}B + 0.031902C - 2.39748 \times 10^{-4}D + 7.17020 \times 10^{-5}C\,D + 3.24648 \times 10^{-5}A^2 - 2.25179 \times 10^{-3}C^2$$

Where A,B,C,D and TPC represent Acetone concentration, temperature solid/solvent ratio, extraction time and total phenolic content respectively.

Case statistics report which is an evaluation of the model showing the experimental (actual) values of total phenolic content and the predicted are shown Table 3-5 below.

This model was only an approximation and does not represent the actual and can’t be relied upon predictions outside the limits of the design factors. It was only good enough to give an idea or direction (Shari, 2013).

Analysis of Response Surface Plots for Total Phenolic Content

Three dimensional plots (3D) showing the effects of two independent factors whilst keeping other two at mean values on the total phenolic content are represented in Figure 3-9 below. Total phenolic content increases as acetone concentration, temperature increases initially and decreases significantly when acetone concentration was above 70% with increasing solid-to solvent ratio.

3.2.5.1 Optimization of Process and Verification of Model for Total Phenolic Content

Optimal conditions were obtained using the transformed quadratic model with the objective to achieve maximum total phenolic content from apple pomace. The conditions for the optimization were set using the numerical optimization and the design expert generated series of solutions with varying desirability.

Table 3-5 Actual total phenolic content vs predicted (mg/g)GAE DW

Standard order	**Sample**	**Actual TPC (mg/g) GAE DW**	**Predicted TPC (mg/g) GAE DW**
1	40-10-1-30	17.47	17.36
2	80-10-1-30	17.73	18.90
3	40-60-1-30	19.58	18.90
5	40-10-8-30	8.90	8.65
6	80-10-8-30	8.90	8.65
7	40-60-8-30	9.90	9.18
8	80-60-8-30	9.87	9.18
9	40-10-1-90	21.68	17.36
11	40-60-1-90	19.25	18.90
12	80-60-1-90	22.55	20.66
14	80-10-8-90	8.74	8.65
15	40-60-8-90	8.30	9.18
16	80-60-8-90	9.36	9.18
17	20-35-4.5-60	8.14	8.65
18	100-35-4.5-60	8.46	8.65
19	60-10-4.5-60	10.13	11.11
20	60-85-4.5-60	14.03	14.79
21	60-35-1-60	15.32	18.90
22	60-35-11.5-60	9.27	9.77
23	60-35-4.5-5	6.99	10.41
24	60-35-4.5-120	10.95	10.41
25	60-35-4.5-60	13.31	10.41
26	60-35-4.5-60	11.92	10.41
27	60-35-4.5-60	11.08	10.41
28	60-35-4.5-60	11.57	10.41
29	60-35-4.5-60	9.69	10.41
30	60-35-4.5-60	11.85	10.41

Sample 80-60-8-90 reads as; 80%(v/v) acetone concentration at 60^{o}C for 8% solid-to-solvent ratio for 90 minutes extraction time.

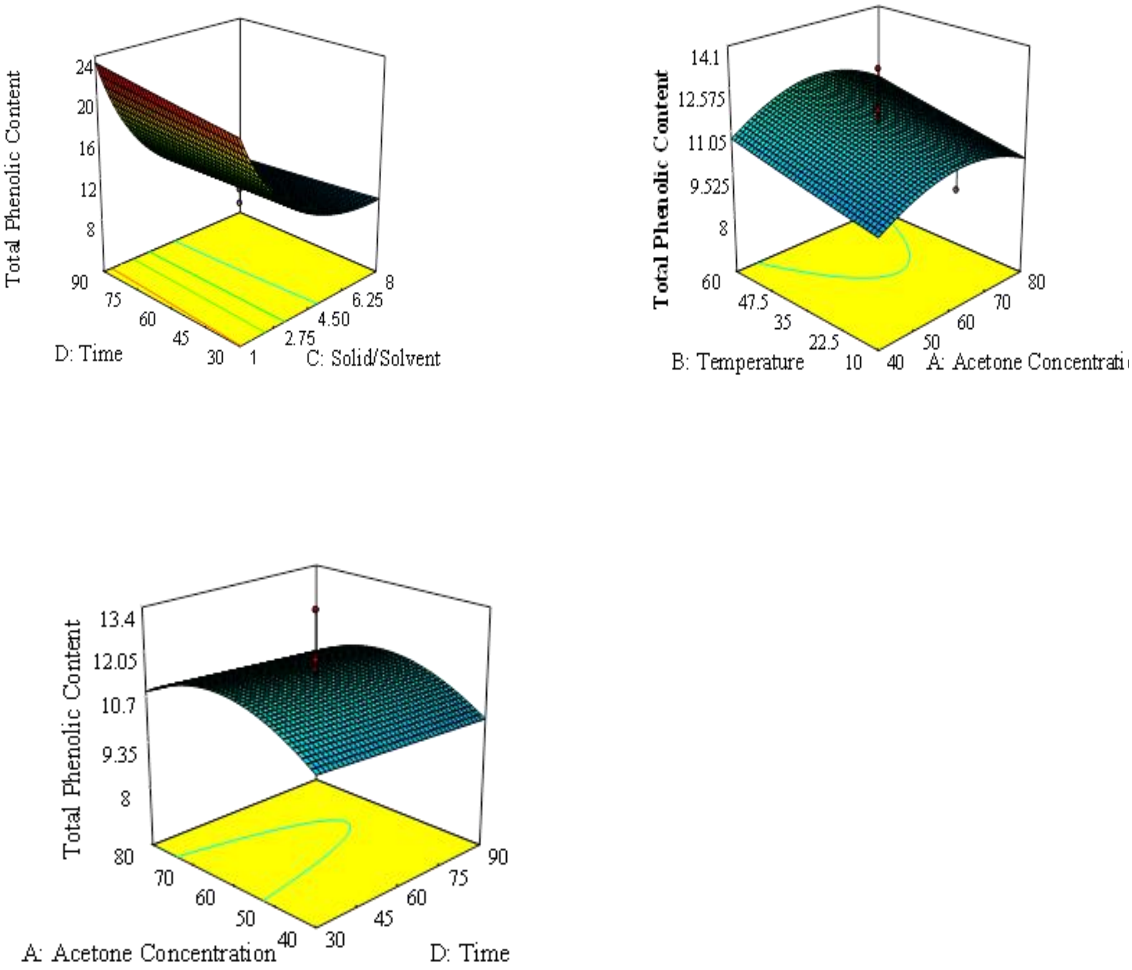


Figure 3-9 Effects of Acetone concentration % (v/v), temperature (°C), solid-to solvent ratio % (w/v), time (minutes) on Total phenolic content (mg/g)GAE DW for varying 2 faactors whilst maintaing other 2 at mean values used in design..

Conditions with highest value of desirability was selected and extractions were carried out in three replicates as in 2.4.3 and total phenolic content determined as in section 2.7.4 and the results shown in Table 3-6.

Table 3-6 Optimal conditions for Total phenolic content under Acetone Extraction

Optimal Conditions				**Total Phenolic Content**	
Acetone Conc. (%)	Extraction Temp °C	Solid/Solvent ratio (%)	Extraction Time (min)	Predicted TPC mg/gGAE DW	Actual TPC mg/g GAE DW
65	60	1	30	21.35	21.70±0.2

The results showed no significant difference between the predicted and experimental values for total phenolic content of the aqueous acetone extracts. Therefore the statistical method could be used to predict phenolic content of apple pomace by response surface methodology within the parameters. Hence total phenolic content of 21.7mg/g GAE DW will serve as the baseline for comparison with subcritical water extraction. Total phenolic content of 21.75mg/g GAE DW and 27.04 mg/g GAE DW have previously been recovered from oven and freeze dried apple pomace respectively using up to 80% (V/V) acetone (Laura et al., 2013). Wijngaard and Bruton reported optimum total phenolic content of 14.15 mg/g GAE DW using 65% aqueous acetone at 25°C for 60minutes. Aqueous acetone, 70% at 20°C was used by Suarez et al., and recorded 6.48 mg/g GAE DW (Suarez et al., 2010).

3.2.6 Model selection for Fraction of Solids Solubilized under Acetone Extraction

The fraction of solids solubilized in all extracts were determined as outlined in 2.2.4 and ranges from 9.7% to 19.4% with a mean value of 16.23%.

Similar rigorous procedures for selecting appropriate model were pursued as in 3.2.5 and the fraction of solids solubilized per 100g followed a reduced quadratic model as in Equation 3-1

The proposed model was significant ($p<0.0001$) and an insignificant lack of fit ($p=0.6319$). In this case $A, C, AC, BC, BD, A^2, D^2$ are significant model terms ($P<0.05$).

Overall regression coefficient was 0.9506 and R^2_{Adj}(0.9163) with undefined R^2_{Pri} and predictive residual sum of squares (PRESS) because the leverage in the analysis was 1.00. Coefficient of variation was 4.65% and all variances of the coefficients were inflated by less than 1.5. The final equation in terms of actual factors is Equation 3-4.

Equation 3-4 Model Equation for Fraction of Solid Solubilised (FSS) under Acetone Extraction

$$\begin{aligned}\mathrm{FSS} = {} & -2.48025 - 0.082150A + 0.033668B + 0.88060C + 0.59345D - 0.010155\mathrm{AC} \\ & + 5.69564 \times 10^{-3}BC - 1.10062 \times 10^{-3}\mathrm{BD} + 1.63244 \times 10^{-3}A^2 \\ & - 4.65261 \times 10^{-3}B^2\end{aligned}$$

The diagnostic case statistics report is showing experimental (actual) values of fraction of solids solubilized per 100g of dried apple pomace and those predicted using the model within the design parameters are shown Table 3-7.

3.2.6.1 Response surface plots for Fraction of Solids Solubilized under Acetone Extraction

Response surface plots for fraction of solids solubilised from the acetone extracts for the various combinations of design parameters are shown in the figures 3-10 to 3-12 below;

Solubility of solids in solvents depends on the activity coefficient which changes with temperature and has the capacity to modify activity coefficient. Fraction of solids solubilised increases linearly with temperature and solid-to solvent ratio (Figure 3-12) and decreases drastically at higher temperatures with extended extraction time (Figure 3-11).

Table 3-7 Experimental and predicted of fraction of solids solubilized g / 100g of starting material under different extraction conditions.

Standard Order	**Experimental condition**	**Actual Value(g/100g)**	**Predicted Value (g/100g)**
1	40-10-1-30	10.00	11.00
2	80-10-1-30	15.40	15.14
4	80-60-1-30	15.40	15.46
5	40-10-8-30	15.50	14.72
6	80-10-8-30	16.00	16.02
7	40-60-8-30	16.40	17.03
8	80-60-8-30	19.00	18.33
9	40-10-1-90	13.30	12.45
11	40-60-1-90	9.70	9.46
12	80-60-1-90	13.50	13.61
13	40-10-8-90	16.10	16.17
14	80-10-8-90	16.70	17.47
15	40-60-8-90	15.00	15.18
16	80-60-8-90	16.50	16.48
17	20-35-4.5-60	18.20	18.20
19	60-10-4.5-60	18.40	18.48
21	60-35-1-60	16.30	16.66
25	60-35-4.5-60	18.50	18.31
26	60-35-4.5-60	19.20	18.31
27	60-35-4.5-60	17.20	18.31
28	60-35-4.5-60	18.50	18.31
29	60-35-4.5-60	17.70	18.31
30	60-35-4.5-60	19.20	18.31

Rows 3, 9,10,12,16, 22 and 27 were ignored for the analysis, Experimental condition 60-35-4.5-60 reads as; 60%(v/v) acetone concentration,at a temperature of 35°C, 4.5%(w/v) solid-to-solvent ratio for 60 minutes extraction time.

Positive coefficient of acetone concentration in the quadratic term describes the curvature of the response plots in (Figure 3-10) whose increment positively impact on faction of solids solubilised.

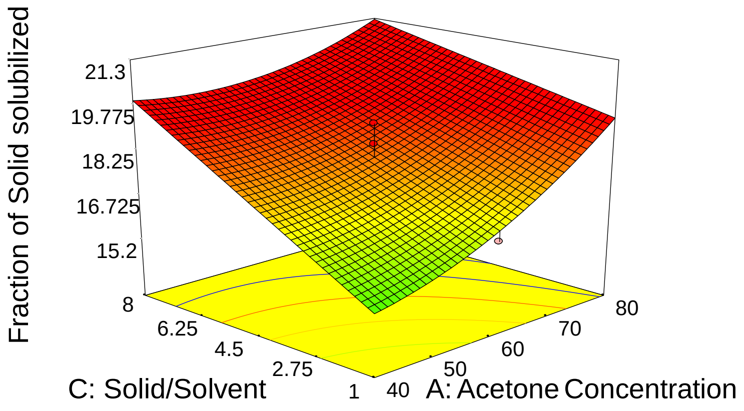


Figure 3-10 Effects of solid-to-solvent ratio (%w/v) and acetone concentration (%v/v)on fraction of solid solubilised (g/100g)during acetone extraction at temperature 35°C for 60 minutes extraction time.

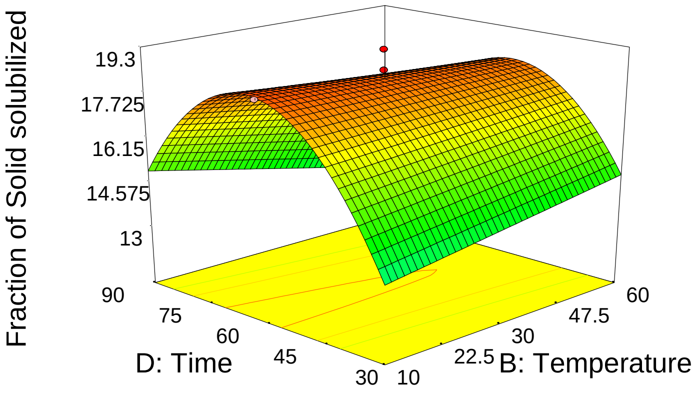


Figure 3-11 Effects of time (minutes)and temperature (°C)on fraction of solid solubilised (g/100g) using a fixed concentration of 60%(v/v) and 4.5% solid-to-solvent ratio.

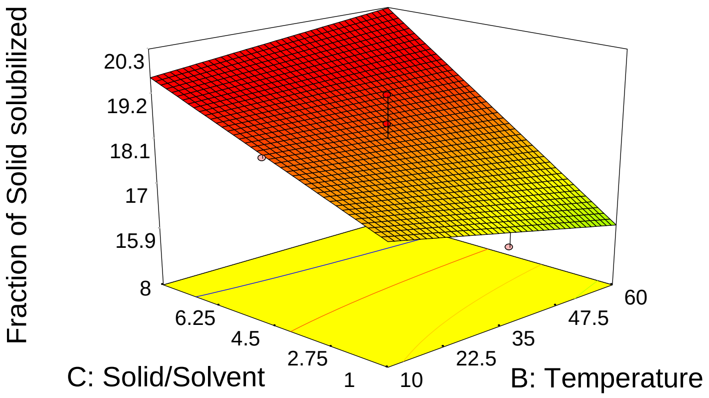


Figure 3-12 solid to solvent ratio (%w/v) and temperature effect (°C) on fraction of solid solubilised (g/100g) using 60%(v/v) acetone concentration for 60 minutes extraction time.

3.2.6.2 Optimization of Process and Verification of Model for fraction of solids solubilized

Conditions for optimization were based on the information from the response surface plots to achieve maximum solubility using numerical optimisation. Optimised conditions were obtained from the many solutions generated by the design expert. Conditions with the highest desirability were selected and extractions were carried out in three replicates as in 2.2.2 and fraction of solids determined as in 2.2.4 and the results shown in Table 3-8.

Table 3-8 Optimal conditions for Fraction of solids solubilised under Acetone Extraction

Optimal Conditions				**Fraction of solids solubilised**	
Acetone Conc. (%)	Extraction Temp (°C)	Solid/solvent ratio (%)	Extraction time (min)	Predicted g/100g	Actual g/100g
78	21	4.7	54	19.92	19.20±0.1

3.2.7 Separation and Identification of Phenolic Compounds in Acetone extracts

Separations of phenolic compounds were done using the protocol described fully in 2.2.9. The phenolic compounds were identified by comparing retention times (t_R) and spectra data of known complimentary phenolic standards at their maximum absorbance. Two main phenolic groups were observed as flavonoids and phenolic acid. Representative phenolic compounds were Chlorogenic acid, Caffeic acid Phloridzin, Procyanidin B2, Epicatechin, Quercetin -3-galactoside and Quercetin-3-glucoside. These compounds were identified in commercial apple pomace and documented in literature (Schieber et al., 2001, Çam and Aaby, 2010, Diñeiro García et al., 2009, Suarez et al., 2010). Typical chromatogram of the phenolic compounds at 320nm is shown in Figure 3-13.

Quercetin glycosides which were thought to be very difficult to separate because they related closely (Tsao and McCallum, 2009), were well resolved by the method. Additionally 3 other quercetin glycosides were resolved but standards were not readily available for their identification. These quercetin glycosides could be quercetin -3-xyloside, quercetin-3-arabinoside and quercetin-3-O-rhamnoside according to elution order (Schieber et al., 2001a, Serena et al., 2007). Phloridzin, Epicatechin and Procyanidin B2 were detected at 280nm, and

Chlorogenic and Caffeic acids at 320nm, and quercetin glycosides at 370nm.The retention times and of the various standards reported as ± standard error is recorded in Table 3-9.

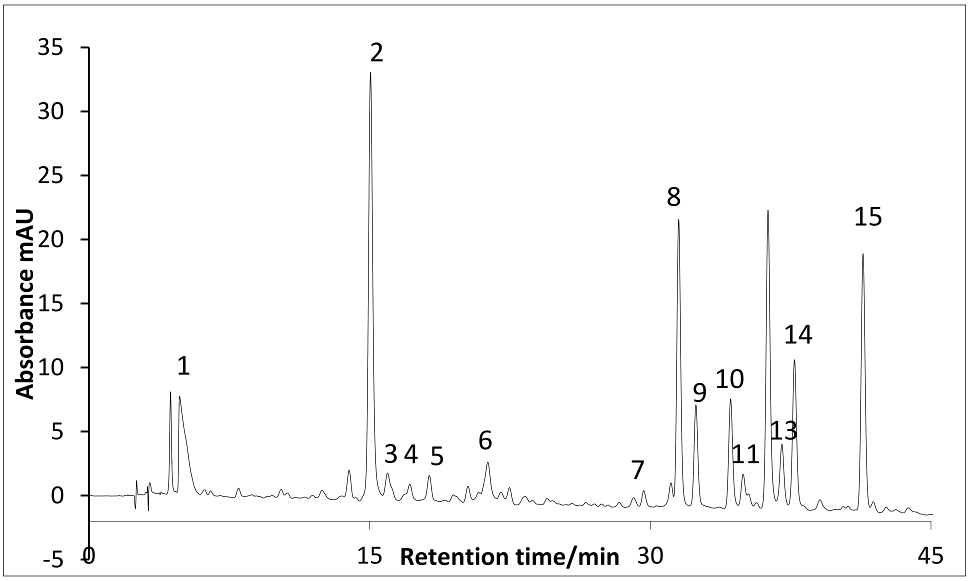


Figure 3-13 Chromatogram (320nm) of phenolic compounds isolated from the apple pomace using aqueous acetone. 1= acetone, 2=chlorogenic acid, 3= procyanidin B2, 4= caffeic acid, 5= epicatechin, 7-Ferulic acid, 8= quercetin-3-galactoside, 9=quercetin-3-glucoside, 15= phloridzin.

3.2.8 Quantification of Phenolic Compounds

Concentration of phenolic compounds in all the extracts were obtained as described in 2.2.10 and the regression equations as well as coefficient (R^2) for the standard calibration graph are reported in Table 3-10.

Table 3-9 Retention time of selected Phenolic standards

Phenolic standard	**Retention time (min)**
5-HMF	7.3±0.05
Furfural	10.4±0.06
Protocatechuic aldehyde	12.4± 0.06
Catechin	13.03 ± 0.01
Chlorogenic acid	15.17±0.05
Procyanidin B2	16.13±0.07
Caffeic acid	17.5± 0.08
Epicatechin	18.6 ±0.07
P-Coumaric acid	25.3 ± 0.08
Ferulic acid	29.3±0.20
Quercetin- 3- galactoside	31.47± 0.11
Quercetin -3-glucoside	32.4±0.01
Phloridzin	41.5 ± 0.23
Phloretin	50.32±0.12
Quercetin	57±0.25

Table 3-10 Equation for calibration of standard phenolic compounds

Phenolic Standard	**Regression equation**	**Correlation Coefficient** (R^2)
Chlorogenic acid	$y = 25667x$	0.9992
Procyanidin B2	$y = 4.9706x$	0.9833
Quercetin-3- galactoside	$y = 26.232x$	0.9998
Quercetin-3- glucoside	$y = 13829x$	1.0000
Phloridzin	$y = 14704x$	0.9999
Epicatechin	$y = 6210x$	0.9998
Catechin	$y = 5901.3x$	1.0000
Protocatechiuc aldehyde	$y = 31800x$	0.9971
5-HMF	$y = 401717x$	0.9998
Furfural	$y = 42138x$	0.9997

Table 3-11 Concentration of Phenolic Compounds (mg/kg)

Std Order	CGA	PHL	Q-3-gal	Q-3-glu	E-CAT	Pr-B2
1	183.17 ± 4.7	686.35±21.5	159.99±2.4	111.14±2.6	ND	ND
15	170.85 ± 4.0	545.01±10.6	128.88±1.9	89.45±1.7	142.50±9.4	216.85±11.8
19	187.78 ± 7.1	677.32±17.1	171.33±0.5	115.22±3.0	130.30±10.8	201.55±19.8
22	160.99 ± 5.7	562.13±18.9	142.92±4.7	98.83±3.7	132.9±9.7	208.33±1.1
16	201.80±8.7	722.53±25.3	175.09±8.2	117.74±4.7	193.6±33.1	165.80±26.0
23	191.88±5.9	634.49±15.9	174.67±0.4	117.84±2.5	141.5±8.0	227.81±4.4
6	177.39±12.2	641.86±37.7	174.10±12.6	116.91±7.6	173.2±37.9	140.35±28.3
26	190.15±6.8	693.36±20.8	177.78±0.3	118.84±3.3	140.7±10.7	451.69±22.4
14	175.36±7.6	636.60±23.2	173.16±8.1	116.47±4.7	167.4±32.8	150.40±23.3
20	157.05±22.0	776.84±52.9	176.32±18.2	134.37±0.0	ND	ND
11	221.58±9.0	785.27±30.8	186.58±6.1	128.20±2.5	ND	ND
21	184.12±10.9	813.70±31.9	187.83±7.1	131.44±6.7	ND	ND
13	146.69±6.2	484.60±20.6	133.68±5.3	92.88±3.8	132.2±9.1	210.25±5.7
9	167.89±8.7	723.64±36.7	162.88±7.8	114.46±5.9	ND	ND
28	191.38±8.4	713.80±5.7	181.05±1.3	121.81±4.4	152.5±20.0	224.06±3.1
7	162.72±10.6	588.53±6.9	135.03±1.4	93.17±1.2	141.7±5.9	216.38±12.8
18	34.49±4.2	273.55±33.1	30.34±6.5	29.22±0.0	ND	ND
5	156.00±3.5	516.75±12.2	136.02±2.7	94.57±2.1	133.1±6.9	218.26±9.0
25	191.19±5.5	727.07±12.3	176.54±3.3	122.26±2.7	132.5±4.0	217.18±5.4
17	140.67±0.9	314.72±4.2	147.77±0.8	103.45±1.0	109.9±1.6	207.83±10.1
24	191.99±2.9	707.97±18.1	178.16±1.9	119.19±2.2	157.82±10.7	222.60±2.1
12	168.67±14.8	894.62±62.4	172.08±15.4	119.92±0.0	ND	ND
8	190.21±11.3	717.27±28.7	173.37±8.1	116.41±4.3	193.27±19.3	167.11±28.0
30	200.41±3.1	705.85±14.4	182.74±1.6	125.56±1.4	148.98±0.2	225.72±6.0
29	193.29±2.5	709.51±13.0	184.98±1.9	120.09±1.1	142.30±1.8	212.80±0.6
20	248.06±4.7	784.18±20.2	180.15±1.7	120.74±2.1	264.15±30.0	339.78±18.7
10	143.60±13.7	845.59±67.5	164.39±14.0	ND	ND	ND
2	124.51±11.6	847.53±64.9	173.26±11.8	ND	ND	ND
3	189.90±0.9	674.83±44.2	149.79±0.9	104.02±1.4	ND	ND
27	192.57±3.0	682.51±15.8	175.66±1.7	121.14±1.6	138.97±5.2	216.97±3.1

Std= standard, CGA- Chlorogenic acid; PHL-Phloridzin; Q-3-gal-Quercetin-3-galatoside; Q-3-glu- Quercetin-3-glucoside; E-CAT- Epicatechin; Pr-B2- Procyanidin B2; ND not detected.

Concentrations of the phenolic compounds of the various combinations of the independent variables were analysed statistically using the Stat-Ease software to study the influence of the design variables on their yield in order to give a better understanding of their behaviour in terms interactions between the independent factors and to optimised the extraction conditions.

Summary of the regression analysis for identified phenolic compounds are shown in Table 3-12 and detailed ANOVA results are in the appendix.

Table 3-12 Summary of significance of Design factors and interaction terms under acetone extraction

Response	**Significance level ($p<0.05$)**											
	A	B	C	D	AC	AD	BC	BD	CD	A^2	B^2	C^2
CGA	✓	✓			✓			✓		✓		✓
PHL	✓	✓	✓	✓			✓	✓	✓	✓		✓
Q-gal	✓		✓		✓	✓		✓			✓	
Q-glu	✓	✓	✓		✓					✓		✓
Pr- B2	✓	✓	✓		✓	✓				✓		✓
E-CAT	✓	✓	✓									✓
TPC-hplc		✓	✓		✓				✓	✓		✓
TPC-FC		✓	✓						✓	✓		✓
FSS	✓		✓		✓		✓	✓		✓		
ORAC		✓	✓						✓	✓		✓

CGA-Chlorogenic acid; PHL-Phloridzin; Q-gal- Quercetin-galactoside; Q-glu- Quercetin glucoside; Pr-B2-Procyanidin B2; E-CAT-epicatechin; TPC-hplc- total phenolic content(HPLC); TPC- FC- total phenolic- Folin-Ciocalteau; FSS- fraction of solid solubilised; ✓-significant. A-acetone concntration; B-Temperature; C-Solid-to-solvent ratio; and D-extraction time

3.2.9 Model Analysis of Individual Phenolic Compounds under Acetone Extraction

All selected models for individual phenolic compounds were significant ($P<0.05$) and insignificant lack of fit ($P>0.05$), except for Chlorogenic acid Quercetin 3-glucoside which had significant lack of fit ($P<0.05$) and may be due to the large blocking effect. Selected models had satisfactory level of adequacies in all with $R^2 > 0.9$ and reasonable agreement between R^2_{Adj} and R^2_{Pri} was observed. Coefficients of variations were < 5% for each of the measurements at the 95% confidence interval. Yields of phenolic compounds were significantly affected by acetone concentration, solid- to- solvent ratio, temperature as well as their interactions.

3.2.9.1 Predictive Model for Extraction of Chlorogenic acid under Acetone Extraction

Concentration of Chlorogenic acid in extracts ranged from 124.5 to 221.58 mg/kg with mean amount of 176.24 mg/kg. The predictive model in terms of actual factors is shown in Equation 3-5.

Equation 3-5 Model Equation for Chlorogenic acid under Acetone Extraction

$$\begin{aligned} Chlorogenic\ Acid \\ &= +100.52918 + 3.21207A + 0.29177B - 6.15519C + 3.24996 \times 10^{-3}D \\ &+ 0.24195\mathrm{AC} - 0.038655BC + 5.36020 \times 10^{-3}\mathrm{BD} - 0.026760\mathrm{CD} \\ &- 0.037565A^2 - 0.56300C^2 \end{aligned}$$

Hydrocinnamic acids are polar among polyphenolic compounds and extracting them from matrices will require a certain reasonable level of polarity of solvent. An increase in temperature from 10 to 60^{o}C for 1% loading at acetone concentration 40%(v/v), caused yield of Chlorogenic acid to increase by 14% but decreases by approximately 20% as concentration of acetone approaches 80%(v/v).Acetone concentration of around 52% at 40^{o}C was reported as good for extracting Chlorogenic acid from apple pomace (Wijngaard and Brunton, 2010) contrary to 46% at 60^{o}C of acetone under the current investigation. The investigation showed that decreasing the concentration of acetone and increasing temperature favours yield of Chlorogenic acid. Optimum concentration of 206.3mg/kg Chlorogenic acid was achieved and was within range 30 – 1766 mg/kg of selected cider apples (Serena et al., 2007). The variation of Chlorogenic acid depicting the behaviour are shown in Figure 3-14 and Figure 3-15.

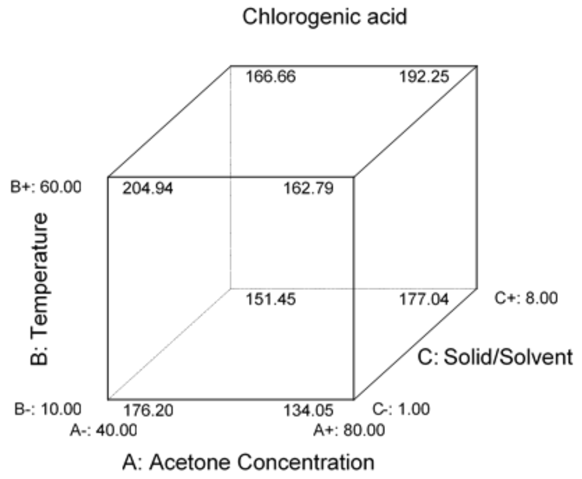


Figure 3-14 Effects of acetone concentration (%v/v), temperature (ºC) and solid -to -solvent ratio (%w/v) of apple pomace on the amount of Chlorogenic acid (mg/kg)for 60 minute extraction time.

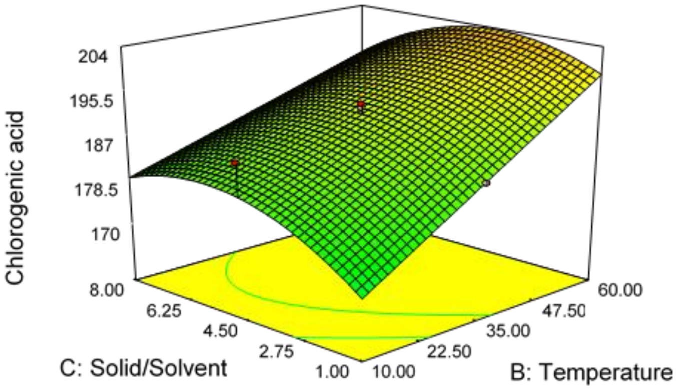


Figure 3-15 Response plot of effects of temperature (°C), and solid to solvent ratio (%w/v) of apple pomace on the amonut of Chlorogenic acid (mg/kg) using 60%(v/v) acetone for 60 minutes extraction time

3.2.9.2 Predictive Model for Extraction of Phloridzin under Acetone Extraction

The concentration of Phloridzin in extracts ranged from 314.7mg/kg to 894.6 mg/kg consistent with 25mg/kg to 1061mg/kg of cider apples (Serena et al., 2007). Equation of model based on the regression analysis in terms of actual factors is shown in Equation 3-6.

Equation 3-6 Model Equation for Phloridzin under Acetone Extraction

$$Phloridzin = +106.89513 + 21.36305A - 1.01061B - 59.51068C + 0.81180D$$
$$+ 0.18934B\mathrm{C} + 0.016002BD - 0.20193\mathrm{CD} - 0.14966A^2 + 4.52601C^2$$

Phloridzin concentration increased by 16% at 1% solid –solvent ratio as the acetone concentration increases to 80% (v/v), and decrease by 24% as loading approaches 8%. Temperature had minimal effect on the recovery of the dihydrochalcone as it only increased by about 1% when temperature increased from 10 to 60°C as shown in Figure 3-16.

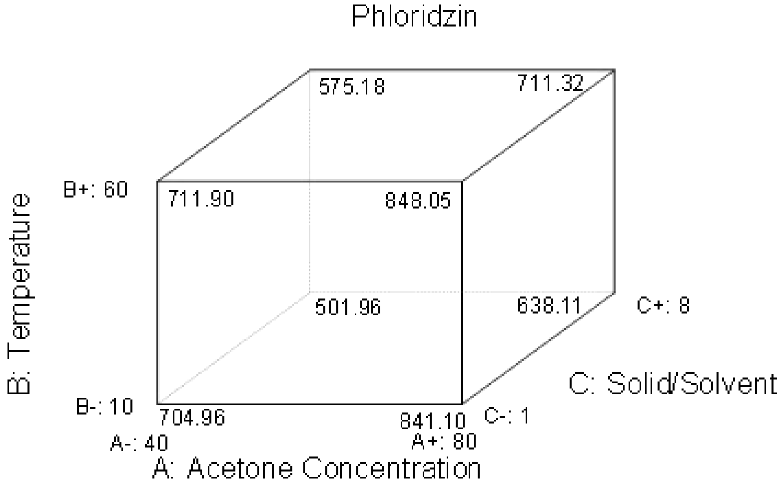


Figure 3-16 Effects of acetone concentration %(v/v), temperature (°C) and solid-to-solvent ratio (%w/v) of apple pomace on the ampunt of Phloridzin (mg/kg) for 60 minutes extraction time.

The 3D plots depicting how Phloridzin responds when two independent variables were changed and the other two fixed at mean level are shown in Figure 3-17and Figure 3-18

Optimum concentration of Phloridzin (858.92mg/kg) was achieved using 73% acetone at 60°C for 60 minutes as against 75% at 40°C for 60 minutes reported earlier (Wijngaard and Brunton, 2010).

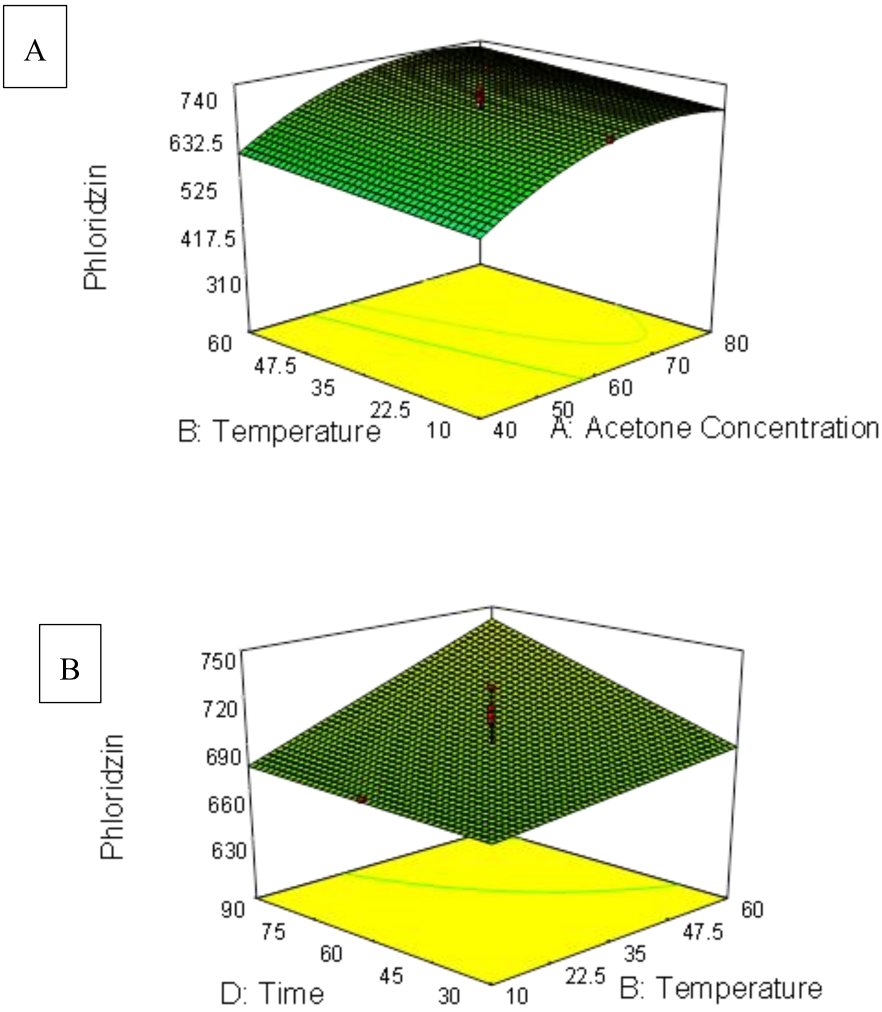


Figure 3-17(A)-Effects acetone concentration %(v/v)-and temperature (°C)using 4.5%(w/v) solid-to-solvent ratio for 60 minutes extraction time on the amount of Phloridzin (mg/kg). (B)- Time (minutes) effect on the amount of Phloridzin (mg/kg) using acetone concentration of 60%(v/v) with 4.5% solid-to-solvent ratio.

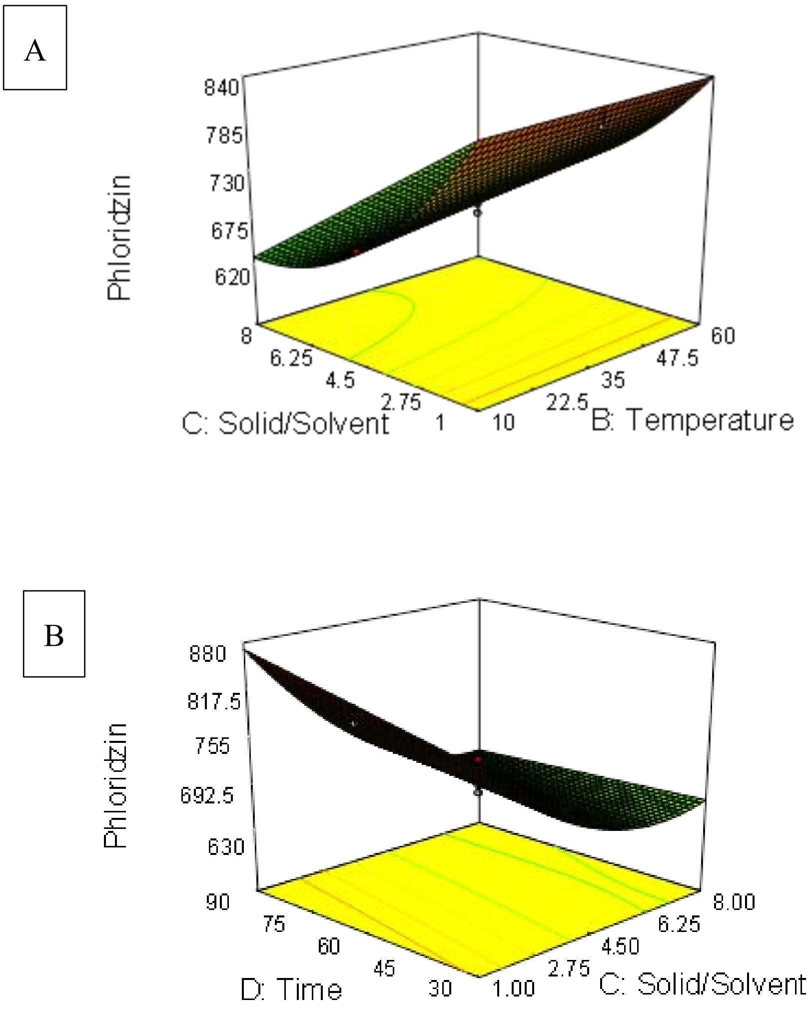


Figure 3-18 (A)-Effects of Solid-to Solvent ratio %(w/v) and Temperature (°C) using 60%(v/v) acetone concentration for extraction time of 60 minutes on the amount of Phloridzin (mg/kg); (B)-Time(minutes) and solid-to-solventeffects on the amount of Phloridzin using 60%(v/v) acetone concectration at temperature 35°C.

3.2.9.3 Predictive Model for Extraction of Quercetin glycosides under acetone extraction

Quercetin-3-galactoside dominates among other quercetin glycosides in apple peels (Tsao and McCallum, 2009) and ranged in the extract from 133.7-187.8 mg/kg dry weight of apple pomace with mean concentration of 168.6 mg/kg. Quercetin-3- glucoside ranged in extracts from 60 -128.2mg/kg. Both results agree with previous reports as 50-520mg/kg for

Quercetin-3-galactoside, and 9-152 mg/kg of quercetin-3-glucoside in cider apple peels (Serena et al., 2007). Transformed quadratic models were suitable and described the behaviour of the Quercetin glycosides as in the Equation 3-7.

Equation 3-7 Transformed Reduced Quadratic Model equations for Quercetin glycosides

$$\frac{1.0}{Sqrt(Q-gal)} = +0.086163 - 1.15229 \times 10^{-4}A - 3.72357 \times 10^{-4}B + 2.1303710^{-3}C - 6.64449 \times 10^{-5}D - 2.7551 \times 10^{-5}A\mathrm{C} + 1.52022 \times 10^{-6}AD - 1.13192 \times 10^{-6}\mathrm{BD} + 6.07617 \times 10^{-6}B^2$$

$$\frac{1.0}{Sqrt(Q-glu)} = +0.099931 - 4.32652 \times 10^{-4}A + 1.64815 \times 10^{-3}C - 1.0606 \times 10^{-4}D - 1.5 \times 4954610^{-4}AC + 1.76547 \times 10^{-6}A\mathrm{D} + 1.06599 \times 10^{-5}A^2 + 6.69141 \times 10^{-4}C^2$$

Quercetin -3-galactoside concentration increases by 5.24% when concentration of acetone was increased to 80% (v/v) and slightly when temperature increases from 10- 60^{o}C with increasing solid-to-solvent ratio as reflected in Figure 3-19. Interaction between acetone concentration and solid-to solvent ratio (AC) was more important than between temperature and time (BD) as revealed by their negative coefficient values which was higher in AC (2.7551×10^{-5}) than BD (1.13192×10^{-6}). The negative coefficient values of temperature and time as well as their interaction suggest that, overtime with increasing temperature, could result in less recovery of the glycoside as shown in Figure 3-19. Decrease in concentration of the glycoside may be due to degradation or hydrolysis of the sugar moiety attached to the quercetin aglycone. Similar fears were reported during the solvent extraction of Quercetin glycosides from ''Idared'' apple peels using ultra- sonication (Vasantha Rupasinghe et al., 2011).

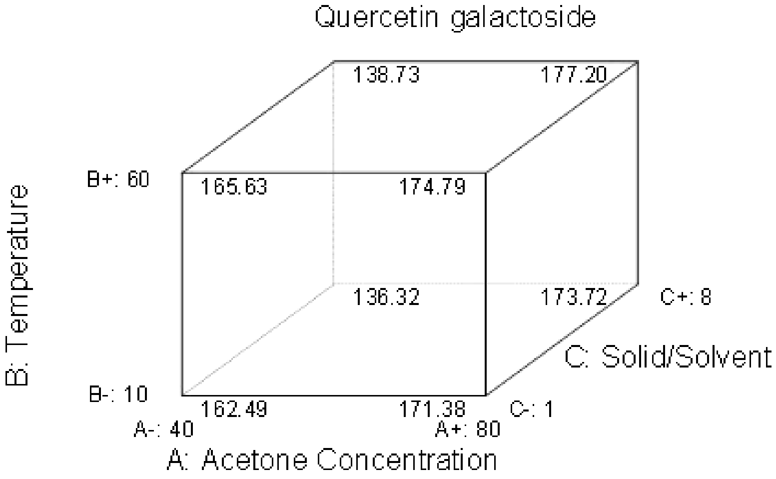


Figure 3-19 The effects of temperature (ºC), acetone concentration %(v/v) and solid-to-solvent ratio %(w/v) on the amount of quercetin-3-galactoside (mg/kg) for 60 minutes extraction time.

The behaviour of quercetin-3-galactoside when independent variables changes are shown in the 3D plots in Figure 3-20 and Figure 3-21.

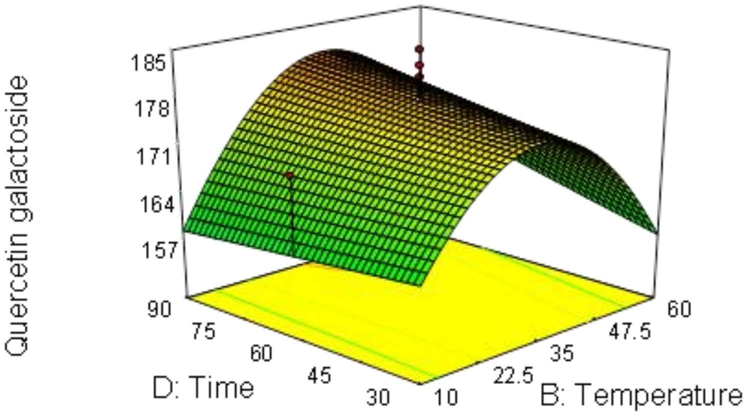


Figure 3-20 The effect of time (minutes)and temperature (°C) on the amount of quercetin-3-galactoside (mg/kg) for 60%(v/v) acetone concentration for 4.5%(w/v) solid-to-solvent ratio.

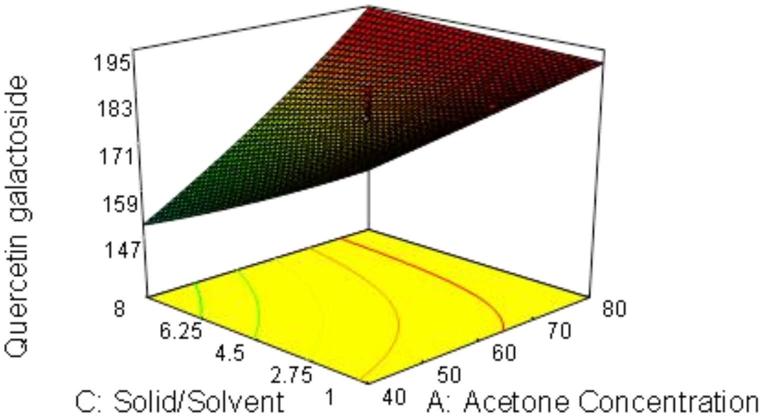


Figure 3-21 Effects of acetone concentration %(v/v) and solid-to solvent ratio %(w/v) on the amount of quercetin-3-galactoside at temperature 35°C for 6o minutes extraction time.

Optimal acetone concentration of 76% (v/v) with 6% solid/solvent ratio was good for extracting quercetin-3-galactoside at 41°C for 58 minutes extraction time. A predicted concentration of 189 mg/kg of quercetin-3-galactoside was suggested at the optimal conditions.

Quercentin-3-glucoside behaves differently from Quercetin -3-galactoside although they fall in the same category of quercetin glycosides. Interactions between experimental factors were different. Solid-to-solvent ratio term influenced positively the yield of quercetin -3-galactoside whereas temperature controlled the elution of quercetin-3-glucoside.

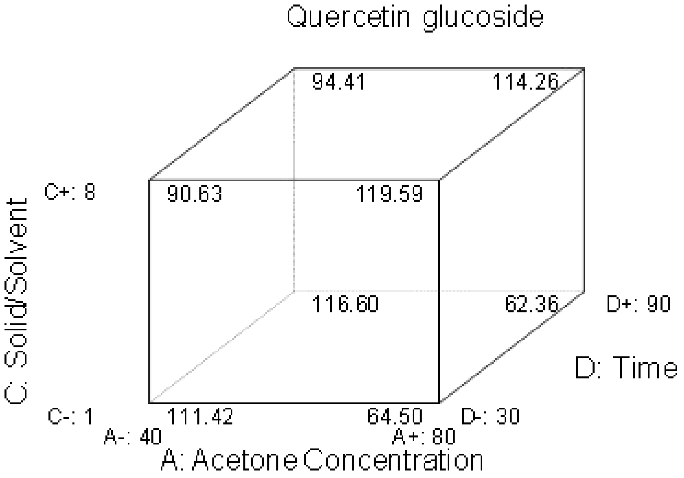


Figure 3-22 Effects of acetone concentration %(v/v), time(minutes) and solid-to solvent ratio %(w/v) on the amount of quercetin-3-glucooside at temperature 35ºC

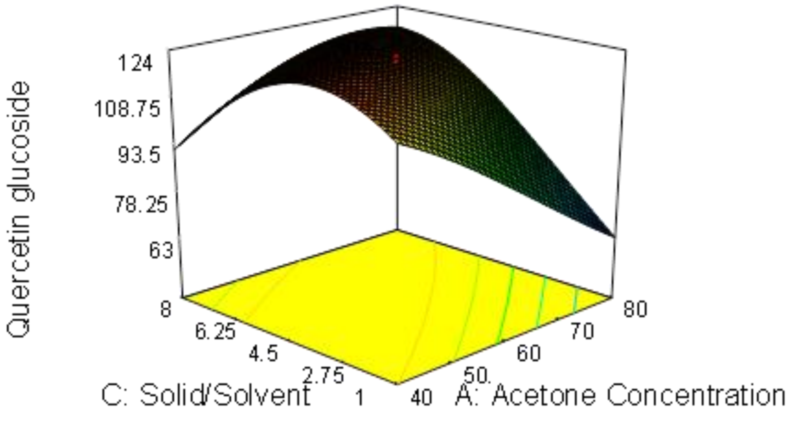


Figure 3-23 Effects of acetone concentration %(v/v) and solid-to solvent ratio %(w/v) on the amount of quercetin-3-glucoside at temperature 35ºC for 6o minutes extraction time.

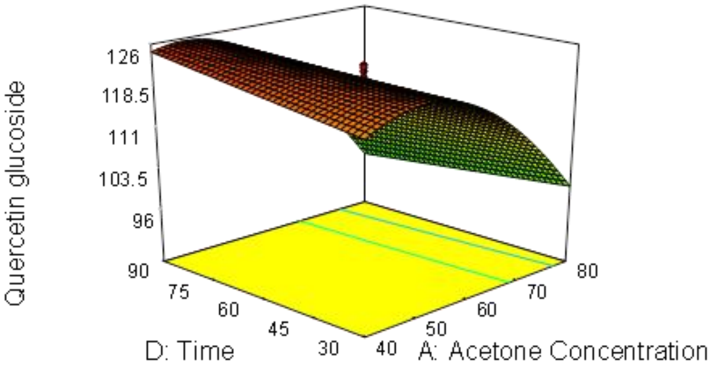


Figure 3-24 The effect of time (minutes)and acetone concentration %(v/v) on the amount of quercetin-3- glucoside (mg/kg) at temperature 35°C 60%(v/v) for 4.5%(w/v) solid-to-solvent ratio.

The optimal conditions for extracting Quercetin-3-glucoside using aqueous acetone from the apple pomace were 40% (v/v) acetone, 3.5% solid-to solvent ratio for 31 minutes at 23°C. Conditions were different to those of quercetin-3- galactoside. It is very important to emphasise that there are no data available in literature to the best of my knowledge as regards good extraction parameters for extracting Quercetin glycosides from cider apple pomace using acetone as an extraction solvent.

3.2.9.4 Predictive Model for Extraction of Epicatechin under acetone extraction

Epicatechin concentration in extract ranges from 0 – 193 mg/kg in extract which was identified as the major flava-3-ol in some selected cider apples with concentrations from 46mg/kg to 2225mg/kg fresh weight (Serena et al., 2007). The predictive model from the regression analysis is as in Equation 3-8.

Equation 3-8 Model Equation for Epicatechin under Acetone Extraction

$$Epicatechin = -53.92179 - 0.12460A - 0.037316B + 56.08802C + 0.16379A\mathrm{C} + 0.051178BC - 5.00284C^2$$

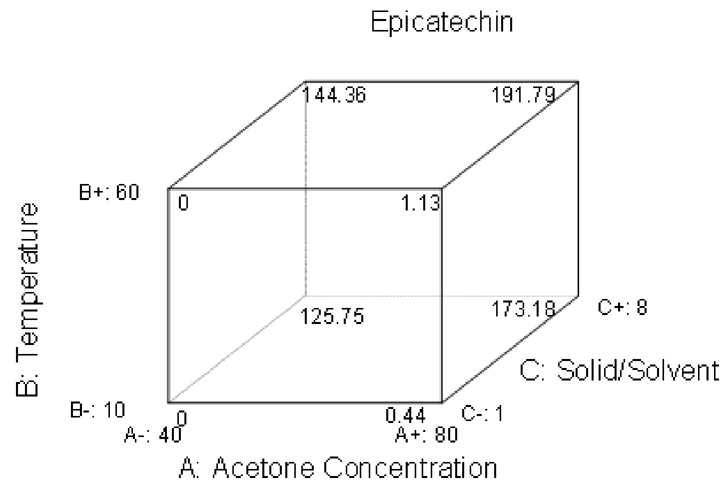


Figure 3-25 Effects of acetone concentration %(v/v), temperature (°C) and solid-to-solvent ratio %(w/v) on the amount of Epicatechin (mg/kg) dry weight apple pomace for 60 minutes extraction time.

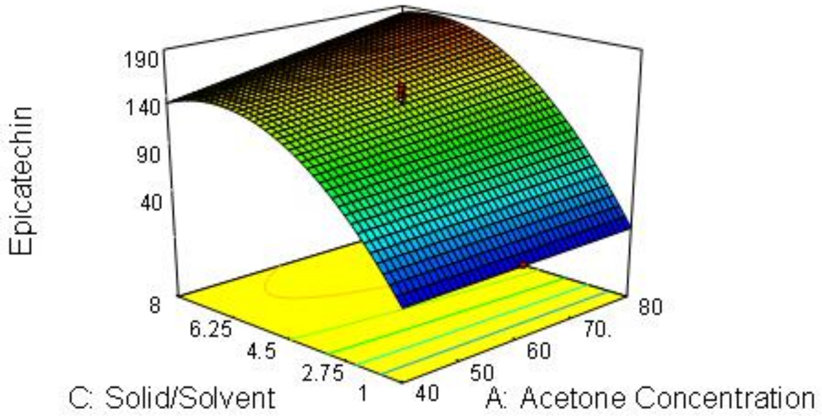


Figure 3-26 Effect of solid-to-solvent ratio %(w/v) and acetone concentration %(v/v) on the amount of Epicatechin (mg/kg) dry weight of apple pomace at temperature 35°C for 60 minutes extraction time.

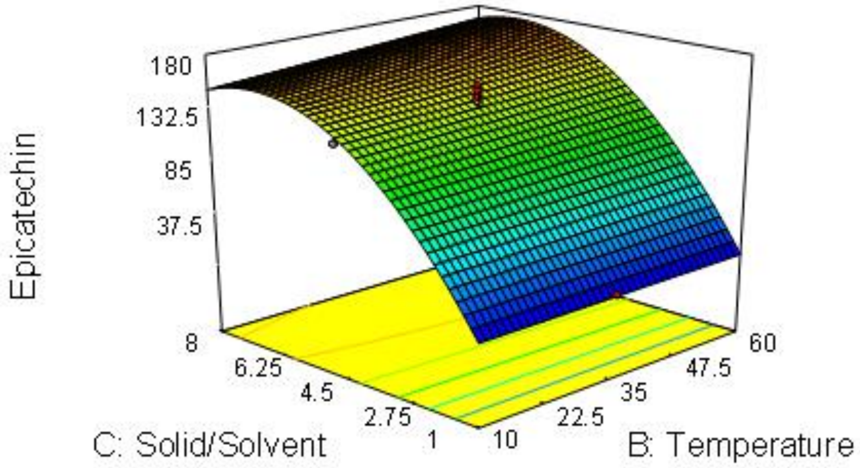


Figure 3-27 Effect of solid-to solvent ratio %(w/v) and temperature (°C) on the amount of Epicatechin (mg/kg) dry weight of apple pomace using 60%(v/v) of actone for 60 minutes extraction time.

3.2.9.5 Predictive Model for Extraction of Procyanidin B2 under acetone

Procyanidin B2, is a major representative of the various groups of the proanthocyanidins in apple peels (Schieber et al., 2001a) and ranged in the extract from 0 (not detectable) to 227.8mg/kg with mean concentration of 137.68mg /kg. Result was consistent with previous reports (56mg/kg to 1362mg/kg) of some selected British cider apples (Serena et al., 2007). Predicted model equation in terms of actual factors of Procyanidin B2 is shown in Equation 3-9.

Equation 3-9 Model Equation for Procyanidin B2 under Acetone Extraction

$$
\begin{aligned}
Procyanidin\ B2 &= -182.02469 + 2.96978A - 0.011758B + 124.61390C - 0.35523D \\
&- 0.21364A\mathrm{C} + 0.034340BC - 0.022831A^2 - 9.59429C^2 + 2.80355 \\
&\times 10^{-3}B^2
\end{aligned}
$$

The variation Procyanidin B2 with experimental factors are shown in Figure 3-28 and the surface response plots as two of the independent variables changes while maintaining the other two at average levels are shown in Figure 3-29 and Figure 3-29.

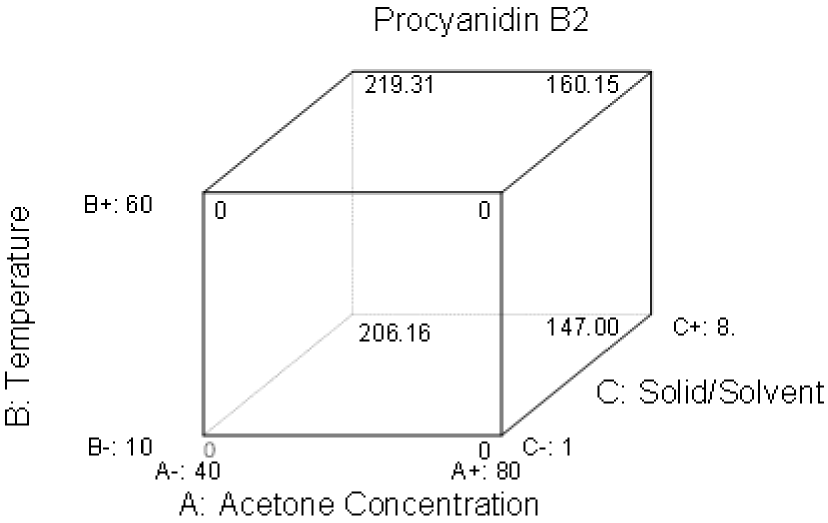


Figure 3-28 Effect of acetone concentration %(v/v), solid- to- solvent ratio %(w/v)and temperature (oC)on the amount of Procyanidin B2 (mg/kg) dry weight for 60 minutes extraction time.of apple pomace.

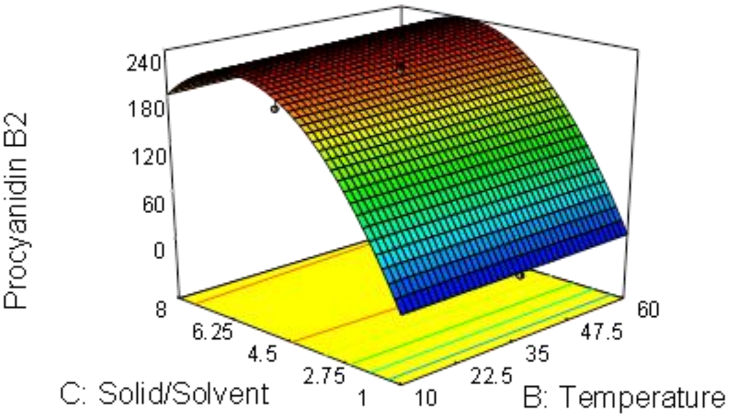


Figure 3-29 Effect of solid- to- solvent ratio %(w/v) and temperature (°C)on the amountof Procyanidin B2 (mg/kg) dry weight for actone concentration of 60%(v/v) for 60 minutes extraction time.

The amount of Procyanidin B2 increases initially as loading (solid/solvent), temperature and acetone concentration increases and decreases significantly for further increase in these parameters.

Optimal solvent concentration and solid-solvent ratio for extracting Procyanidin B2 from the apple pomace at 25°C for 40 minutes were 54% (v/v) and 6% respectively contrary to 70 % (v/v) of acetone reported as good for extracting Procyanidins from the pomace (Hussein (Hussein et al., 1990, Monrad et al., 2010).

3.2.9.6 Overall effect of Design Variables on Total Phenolic Content under HPLC

From the forgoing analysis, it was shown that the most significant factors were acetone concentration and solid-to solvent ratio as well as their interaction. This was reflected in the nature of the response plots of total phenolic content (mg/kg) quantified by HPLC method as in Figure 3-30.

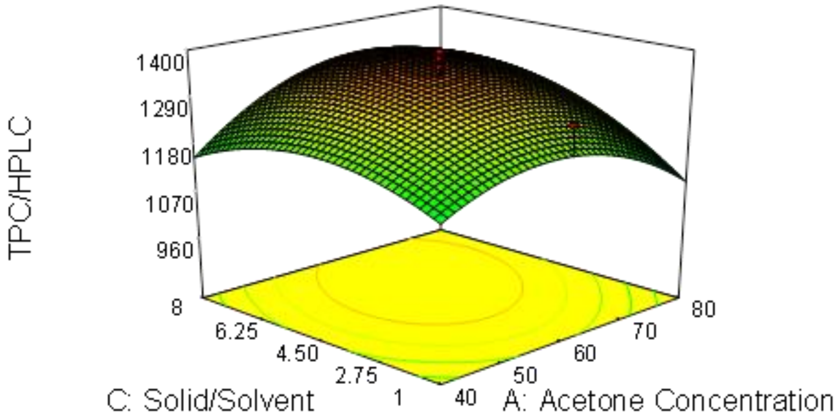


Figure 3-30 Effect of acetone concentration %(v/v) and solid-to-solvent ratio %(w/v) on total phenolic content (TPC, mg/kg) of acetone extracts of the apple pomace by the HPLC determination.

Acetone concentration and solid-to-solvent ratio significantly affected the overall yields of extraction of polyphenolic compounds. Optimised conditions of 65 % (v/v) of acetone, 6% solid-to solvent ratio for 60 min at 60°C were suggested using the predicted model with optimal total phenolic content of 1394.01mg/kg. These conditions were comparable with the results of total phenolic content by the Folin-Ciocalteau method in this investigation except for solid-to solvent ratio. The spectrophotometric method predicted 1% loading to be the best for total phenolic content, however this loading was too dilute to detect, epicatechin and procyanidin B2. Higher amounts of phenolic compounds were mobilised around the optimised conditions. Folin-Ciocalteu method for determination of total phenolics` is not specific and often interfered with sugars and other oxidisable compounds. The chromatographic methods allowed quantification of individual phenolic compounds present in the extracts without any interference. This accounted for the differences in total phenolic content determined by the separate methods. The spectrophotometric assay quantifies total phenolic content in Gallic acid equivalents (GAE). Gallic acid is not found in apples and therefore complicates comparison between the two methods. Chromatographic determination have always been less than the spectrophotometric and have been reported in literature (Escarpa and González, 2001). The HPLC method may not well resolve all phenolic compounds in the extract. For instance oligomeric flavanols which represent about 71-90% of polyphenolic content in apples (Vrhovsek et al., 2004), was not observed in extracts under HPLC used because they might not be retained by the stationary phase.

3.2.10 Determination of Antioxidant activity of solvent extracts by ORAC assay

ORAC assay attempts to mimic antioxidant activity of phenolic compounds by making use of a biological relevant radical source and combines both time and extent of antioxidant activity (Ou et al., 2002). Data from the assay are standardized which permit results across laboratories to be compared (Zulueta et al., 2009). Antioxidant capacities of many food beverages have been determined by ORAC assay and enjoy some level of acceptability in the nutraceutical industry to the extent that ORAC values are coated on the labels of some functional foods (Bank and Schauss, 2004, T. Wright, 2004). The assay requires up to date equipment which are very expensive and also time wasting (Dai and Mumper, 2010) recording wide variations of results across equipment (Zulueta et al., 2009). Furthermore ORAC assay is limited by its capacity to scavenge other reactive oxygen species like, O_2^- HO^-, $ONOO^-$ and singlet oxygen found in biological systems which have different mechanism and therefore cannot represent antioxidant activity (Ou et al., 2002). Nonetheless ORAC assay is still preferred over other antioxidant methods because, the assay is capable of detecting both ''hydrophilic and hydrophobic antioxidants'' by modifying the source of the radical and solvents (Prior et al., 2005). The separation or fractionation of phenolic compounds into individual ones in the extracts will be more expensive and unproductive as a result of the complex nature of the extracts. Therefore, overall determination of the antioxidant capacity of the extracts may be significant as there is report about synergistic nature of phenolic compounds from plant sources (Dai and Mumper, 2010).

Antioxidant capacities of all extracts were determined by ORAC assay. 1ml of extract was pipetted into an Eppendorf tube which was loosely capped and freeze dried for 48hours according to procedure described in 2.2.1.2 and ORAC analysis based on the procedure described by Huang et al., 2002 with slight modification and outlined in section 2.2.11.

ORAC values varied in extracts from 1433µmol TE/g DW to 6260µmol TE/g DW with mean ORAC value of 2732µmol TE/g DW. Data for direct comparison of antioxidant activity by ORAC of selected cider apple peels were not available. However, Budak et al.,2015 reported ORAC value of 9.84 µmol TE/ml in cider (Budak et al., 2015) and 6.2 µmol

TE/ml in apple juice have also been reported (Seeram et al., 2008). ORAC value of 44.07 µmol TE/g fresh weight of ethanolic extract of rare apple peels was published (Giomaro et al., 2014). However it was difficult to compare results of different experimental conditions

and the units in which reported data were expressed. Moreover data expressed in fresh weight are not easy to compare with those of dry weight and also phycoerythrin was used as the protein source whereas this current investigation employed sodium fluorescein as the fluorescent agent.

The effect of independent experimental variables on the antioxidant activities of extracts was evaluated by subjecting the ORAC values to regression analysis (ANOVA) to fit a polynomial equation. Results of the analysis of variance are shown in Table 3-13.

Table 3-13 ANOVA for Response surface Reduced Quadratic Model for ORAC-Solvent Extraction

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p value**
$Model$	3.978×10^{-4}	7	5.683×10^{-5}	55.79	< 0.0001
A	1.262×10^{-6}	1	1.262×10^{-6}	1.24	0.2812
B	9.014×10^{-6}	1	9.014×10^{-6}	8.85	0.0085
C	3.346×10^{-4}	1	3.346×10^{-4}	328.46	< 0.0001
D	1.382×10^{-7}	1	1.054×10^{-4}	0.73	0.7172
CD	5.336×10^{-6}	1	5.336×10^{-6}	5.24	0.0352
A^2	2.594×10^{-5}	1	2.594×10^{-5}	25.46	< 0.0001
C^2	4.530×10^{-5}	1	4.530×10^{-5}	44.47	< 0.0001
$Residual$	1.732×10^{-5}	17	1.019×10^{-6}		
$Lack\ of\ Fit$	8.527×10^{-6}	12	7.106×10^{-7}	0.40	0.9078
$Pure\ Error$	8.791×10^{-6}	5	1.758×10^{-6}		
$Cor\ Total$	4.151×10^{-4}	24			

A= acetone concentration (%v/v), B=Temperature (°C), C= solid-to- solvent ratio, (%w/v) and D= extraction time (minutes)

The proposed model was significant ($p<0.05$) and had an insignificant lack of fit ($p>0.05$). Model terms B, C, CD, A^2, C^2 were significant ($p<0.05$). The Predicted R^2 was in reasonable agreement with adjusted R^2 as shown in Table 3-14.

Table 3-14 Adequacy Level for Response Surface Reduced Quadratic Model for ORAC under Solvent Extraction

Std. Dev.	1.009×10^{-3}	$\boldsymbol{R^2}$	0.9583
Mean	0.021	$\boldsymbol{R^2_{Adj}}$	0.9411
C.V. %	4.89	$\boldsymbol{R^2_{Pri}}$	0.9209
PRESS	3.282×10^{-5}	$\boldsymbol{Precision_{adeq}}$	22.964

The signal to noise ratio (adequate precision) of 22.964 > 4 was good with relatively small measure of dispersion in ORAC values (C.V=4.89%) with acceptable variance of inflation VIF (≤ 1.05).

Final equation in terms of design factors are shown in Equation 3-10

Equation 3-10 Predictive Model Equation for ORAC under solvent extraction

$$\frac{1}{\sqrt{ORAC}} = +0.022036 - 3.11539 \times 10^{-4}A - 2.80293 \times 10^{-5}B + 3.10225 \times 10^{-3}C - 2.39293 \times 10^{-5}D + 5.98469 \times 10^{-6}C\,D + 2.49482 \times 10^{-6}A^2 - 2.26394 \times 10^{-3}C^2$$

The case statistics results which show the actual ORAC values determined under experiments and those predicted by the model equation is shown in Table 3-15 below.

Table 3-15 Actual ORAC values vs predicted ORAC values (µmol TE/g DW)

Standard order	**Sample**	**Actual ORAC (µmol TE/g DW)**	**Predicted ORAC (µmol TE/g DW)**
1	40-10-1-30	4120	3906
2	80-10-1-30	4216	4444
3	40-60-1-30	4944	5102
5	40-10-8-30	1725	1736
6	80-10-8-30	1724	1736
7	40-60-8-30	1925	1890
8	80-60-1-90	1919	2066
9	40-10-1-90	5856	4444
11	40-60-1-90	4809	5917
12	80-60-1-90	16260	5917
14	80-10-8-90	1694	1479
15	40-60-8-90	1614	1600
16	80-60-8-90	1814	1736
17	20-35-4.5-60	1586	1736
18	100-35-4.5-60	1643	1600
19	60-10-4.5-60	1974	1736
20	60-85-4.5-60	2975	2066
21	60-35-1-60	2561	2770
22	60-35-11.5-60	1959	2268
23	60-35-4.5-5	2298	2268
24	60-35-4.5-120	21.59	2268
25	60-35-4.5-60	2766	2268
26	60-35-4.5-60	2394	2268
27	60-35-4.5-60	2189	2268
28	60-35-4.5-60	2307	2267
29	60-35-4.5-60	1881	3906
30	60-35-4.5-60	2377	4444

3.2.10.1 Analysis of Response Surface Plots for ORAC under Solvent extraction

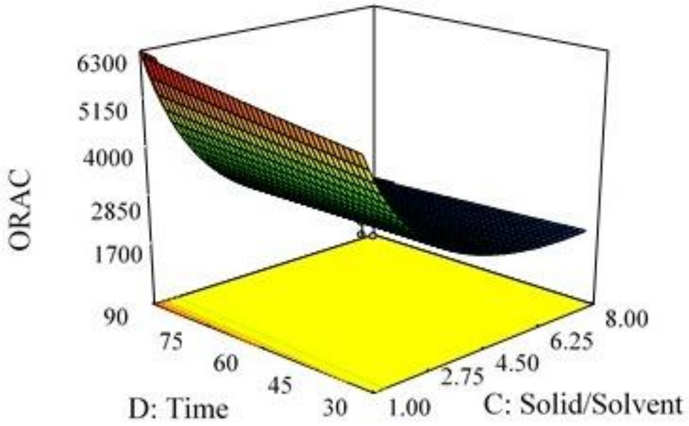


Figure 3-31 Response surface plot for effects of time (minutes) and solid-to-solvent ratio %(w/v) on ORAC values (µmol TE/g DW)with 60%(v/v) acetone at temperature 35°C.

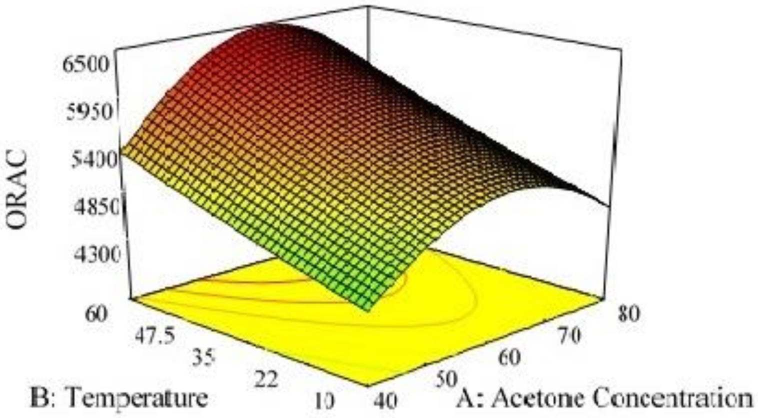


Figure 3-32 Response surface plot for effects of temperature (°C)and acetone concentration %(v/v)on ORAC values (µmol TE/g DW) with 1% solid-to-solvent ratiofor 60 minutes extraction time.

Similar interaction of experimental parameters (solid/solvent ratio and time) were observed between total phenolic content and ORAC activity (see Figure 3-9) suggesting positive relationship between phenolic content and antioxidant activity. Significant correlations between total phenolic content and antioxidant activity methods have previously been reported (Javanmardi et al., 2003, Kaur and Kapoor, 2002, Kratchanova et al., 2010, Velioglu et al., 1998). Also a weak correlation between antioxidant activity with phenolic concentration have also been reported (Suarez et al., 2010).

Lower acetone concentration (58 % (v/v), at 60^{o}C, 60min) selectively favoured extraction of antioxidant compounds as against (65 % (v/v), at 60^{o}C, 30min) for total phenolic content.

3.3 Conclusions

Results have shown that statistics can be used to design an experiment to study the interrelationship between experimental factors and to predict the region of optimum values. . The potential interaction of operational parameters effects of experimental factors such; nature of apple pomace, solid-to-solvent ratio, temperature, solvent concentration and residence time on yields of solubilisation of the apple pomace, polyphenolic content and antioxidant activity of extracts were studied using response surface methodology. Designed conditions demonstrated selectivity towards significant recovery of all responses studied Significant amounts of phenolic and flavonoids compounds like Chlorogenic acid, Phloridzin, Quercetin glycosides, Epicatechin and Procyanidin B2 were extracted using aqueous acetone as a solvent from dried apple pomace. Individual interaction of the polyphenolic compounds with design parameters differed, particularly with Quercetin-3- galactoside and Quercetin-3- galactoside exhibiting separate relationship with experimental conditions although both classified as Quercetin glycosides. Addition of water to improve polarity of the solvent demonstrated to be good for extracting Chlorogenic acid and Procyanidins from the pomace residue. Extract from the by-product and apple juice and cider production, exhibited high antioxidant activity by the oxygen radical absorbance capacity (ORAC) which can be alternative source of natural antioxidant compounds to be used in the cosmetic, nutraceutical and pharmaceutical industries

.

Chapter 4

Optimisation of Subcritical water Mediated Extraction of polyphenolic compounds from apple pomace using Response Surface Methodologies

4.1 Introduction

Application of critical fluids in the extraction of bioactive ingredients have received considerable attention in recent times due to their environmentally friendliness. Subcritical water extraction (temperature between 100-374°C), a relatively new technique has high potential to replace organic solvents because water is cheap, nontoxic, and readily available (Liang and Fan, 2013). Water under subcritical conditions has dielectric constant similar to acetone and ethanol with decreased viscosity and high diffusivity at the high temperature (Akiya and Savage, 2002).Subcritical water has been shown to demonstrate selectivity towards separate groups of compounds at selected temperatures depending on their polarities. Polar compounds are extracted at lower subcritical temperatures whereas less polar ones are recovered at higher temperatures (Ibañez et al., 2003). Water under subcritical conditions has high concentrations of H^+ and OH^- ions which can catalyse acid-base reactions (Arai et al., 2013). Therefore other water mediated reactions such as hydrolysis and dehydration can occur at the high temperature (Brunner, 2009). For example hemicellulose, proteins and lignin from plant biomass can hydrolyse at the high subcritical water conditions to produce oligomer sugars and amino acids (Brunner, 2009). The modified physico-chemical properties at the elevated temperature contribute to the disruption of solute-matrix interactions thereby improving solubility and mass transfer effects leading to elution of compounds that are strongly bound to cell wall. The released compounds can undergo transformation at the high temperatures to generate other compounds of different chemical properties and structures(Plaza et al., 2010). Utilisation of subcritical water towards recovery of bioactives from plant materials has been investigated (Herrero et al., 2012, Plaza et al., 2010).

Recovery of phenolic and antioxidant compounds from pressurised hot water extraction from apple by-products has been reported (Çam and Aaby, 2010, Plaza et al., 2013). Subcritical water with ethanol as a modifier was employed to recover antioxidant compounds from lyophilised apple pomace (Wijngaard and Brunton, 2009).

The current research assessed the utility of subcritical water in the recovery of polyphenolic compounds from wet apple pomace. No organic co-solvents or modifiers were applied or addition of acid/base to the reaction mixture to transform the pH under the subcritical water conditions. Different levels of loading of industrial apple pomace under wet and dried conditions were employed to assess efficient solubilisation of the apple pomace residue for recovery of polyphenolics under subcritical water conditions. Recovery of polyphenolic antioxidant compounds was achieved through optimisation of subcritical water mediated operation parameters such as temperature, residence time and solid-to-solvent ratio in a batch reactor. Response surface methodology procedure similar to chapter 3 was employed to study the relationship between experimental factors to maximise extraction yields of responses. The composition and structure of the polyphenolics in the various extracts as well the antioxidant capacities were analysed.

4.2 Results and Discussion

4.2.1 Homogenization of apple pomace

The homogenized apple pomace although still heterogeneous was a better sample to use than the raw apple pomace. Result of the mean dry matter content of the homogenised apple pomace was 26.2 ± 0.1g/100g fresh weight and the amount of water removed during freeze drying of the homogenised pomace was 71.7 ± 0.4g/100g fresh weight.

4.2.2 Screening Experiment under subcritical water extraction

4.2.2.1 Nature of sample

Wet and dried homogenised apple pomace were compared for recovery of polyphenolic compounds for consideration under subcritical water extraction at 150^{o}C for 20 minutes Variation of total phenolic content in terms of dry weight basis for wet and dried apple pomace are shown in Figure 4-1.

Analysis of variance (ANOVA) was conducted using the statistical package XLSTAT-2014.02 version with post hoc Tukey test for total phenolic content (TPC) of the wet and dried apples pomace. in terms mg/g GAE DW. The results shows no significant difference in total phenolic content between corresponding wet and dried samples for 1%, 2% and 4% ($p>0.05$). Results implies, the amount of phenolic compounds that can be recovered using 1% wet and dried apple pomace will be similar and so for 2% and 4% loadings.

However recoveries for total phenolic content differ between 6% and 8% loadings with slightly higher recoveries achieved with the wet samples compared to corresponding dried ones. Drying of the apple pomace may cause some changes or transformation of some of its structural properties and the physical characteristics that were visible were shrinking of the size of their particles and changes in porosity.

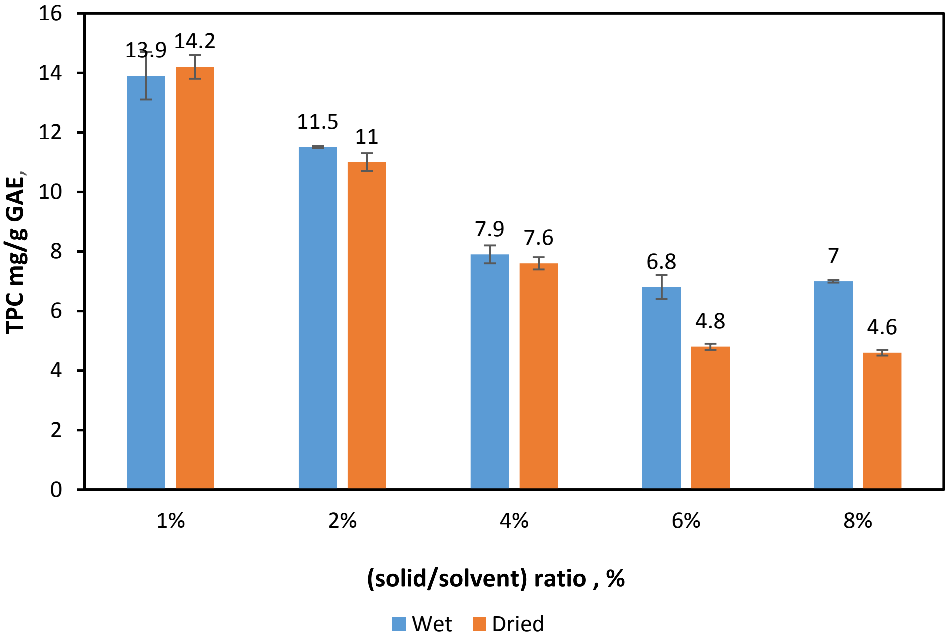


Figure 4-1 Comparison of Total phenolic content (TPC) of wet and dried homogenized apple pomace during subcritical water mediated hydrolysis at 150°C and 20 minutes extraction time.

Alterations in the properties of the apple pomace as a result of drying had the potential to affect it uptake of water during the rehydration process. The absorptive ability in other words porosity changes limiting their rehydrating capacities may result in the lower amount of phenolic content in the 6 % and 8% of the dried samples consistent with similar observation previously reported for dried plant materials (Krokida and Philippopoulos, 2005, M.K and C., 2005, Witrowa-Rajchert and Lewicki, 2006). Generally there are three processes that occur during rehydration of dried biomass which are taking place at the same time. 1) Absorption of water by the dried biomass; 2) Swelling or expanding of biomass as it takes up water; 3) Elution of the soluble ingredients into the extraction fluid (Lewicki, 1998, McMinn and Magee, 1997). In view of the fact that, more solids are in the 6% and 8% of dried pomace, which may delay leaching process hence lower amounts of phenolic content in extracts. For higher loading the dried apple pomace will always lag behind by a step in the general mechanism involved in extraction of biomass under subcritical water extraction. The mechanism involved the diffusion of the solute from the core material to the surface to be transferred into the extraction fluid which would be finally be removed from the extraction cell (Ong et al., 2006). Depending on the amount of water available, the dried samples will always need to be rehydrated first before the actives are diffused from the core to the surface. Hence may explain why the higher loading for dried material recorded lower total phenolic values compared to corresponding wet samples. High biomass loading lead to incomplete extraction due to insufficient volume of water as solvent (Teo et al., 2010). Results in lower loadings (1-4%) confirms freeze drying operation did not do damage to phenolic compounds consistent with earlier reports, that operation was a mild one as excessive heat treatment may cause degradation of the phenolic compounds (Luthria, 2006, Peschel et al., 2006, Salimi Hizaji et al., 2011). Therefore the wet homogenized apple pomace was used for further investigations under the subcritical water mediated hydrolysis of the apple pomace to cut energy cost of drying, and to benefit the advantage of subcritical water extraction which can be used for wet samples directly without first drying them.

4.2.2.2 Effect of temperature

The impact of temperature on the recovery of polyphenolic compounds from the wet homogenised apple pomace was investigated under the single factor experiment. The solid – to- solvent ratio used was 1% with a residence time of 20 minutes for subcritical water temperatures 100°C, 160°C and 200°C. Total phenolic content increases from 6.93 mg/g GAE DW to 46.25mg/g GAE DW as temperature was increased from 100°C to 200°C as shown in Figure 4-2.

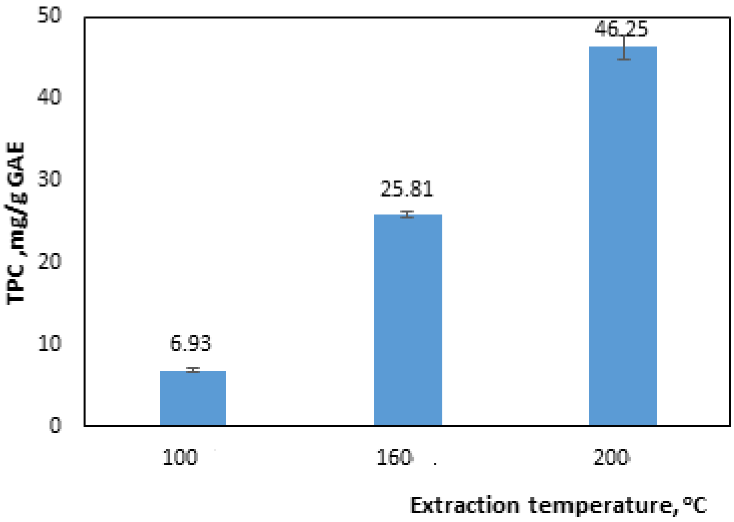


Figure 4-2 Effects of temperature on total phenolic content (TPC, mg/g GAE)under subcritical water extraction using 1% solid-to-solvent ratio of wet homogenised apple pomace for resedience time of 20 minutes..

The high temperatures coupled with high pressure applied were able to break the cohesive forces holding the apple pomace particles together in the matrix. Associated forces may include van der Waals forces, dipole-dipole interaction (forces) and hydrogen bonding. Both adhesive and cohesive forces existing within the solute and the apple pomace matrix were disorganized and the solute was pushed into the subcritical water by simple convective mass transfer (Smith, 2002). However increasing temperature beyond certain limits may lead to degradation of phenolic compounds or even generate unwanted substances. Browning of extracts was observed beyond 150^{o}C due to possible formation of melanoidins which have brown colour (Wijngaard and Brunton, 2009). At 200^{o}C, the product was darker and had a different smell which could be attributed to non-enzymatic browning between proteins and carbohydrate, in addition to caramelisation of sugars in the extract (Rodríguez-Meizosoa et al., 2010). Therefore temperature 200°C was selected for the upper limit since it recorded higher total phenolic content of 46.25 mg/g GAE and 100°C was the lower limit.

4.2.2.3 Effect of residence time

The investigation of the impact of residence time was done using 1% (solid/solvent) loading at 200°C for extraction time of 0, 20 and 30 and 60 minute's residence time. The results showed that total phenolic content increased by about 36% for extraction time from 0 to 20 minutes and remained about the same beyond 30 minutes as shown in Figure 4-3.

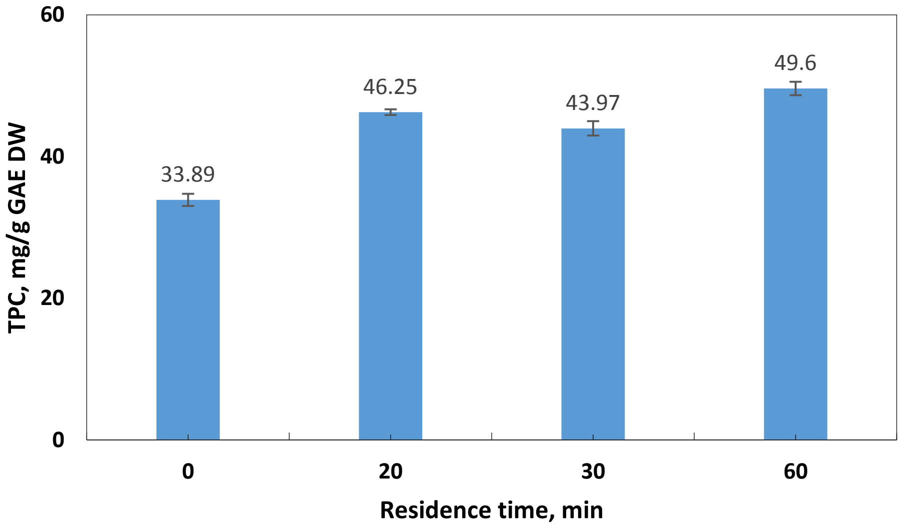


Figure 4-3 Effect of Residence time on total phenolic content(TPC mg/g GAE DW) under Subcritical water extraction using 1% solid-to-solvent ratio of wet homogenised apple pomace at temperature 200°C.

Results did not show a highly significant difference between total phenolic content at 20 and 30 and 60 minutes. Total phenolic content increase by 7.24% moving from 20 to 60 minutes and this difference could be attributed to non-homogeneous nature of the apple pomace used in the extraction. Similar observations under subcritical water extraction of phenolic compounds from pomegranate seed residue where made (He et al., 2012) The results revealed that, total phenolic content at 30 minutes extraction was not different from that of 120 minutes and concluded that, there was no advantage in extracting the phenolic compounds beyond 30 minutes. Within this period, the solvent would have been able to saturate the apple pomace matrix with sufficient energy supplied thereby eluting the phenolic compounds earlier. Hence extending the time at this high temperature may lead to decomposition of the phenolic compounds that were already assembled (Luque-Rodríguez et al., 2007). Maximum residence time of 30 minutes where achieved during extraction of antioxidant compounds from grape pomace using pressurized water (Joana Gil-Chávez et al., 2013). Therefore residence time of 10 and 30 minutes were selected and used as the lower and upper limits respectively.

4.2.3 Experimental design by response surface methodology under Subcritical water extraction

Total phenolic content under the subcritical water mediated hydrolysis was influenced by loading (solid/solvent) ratio, extraction temperature and residence time from the screening experiments. The lower and upper limits identified during the exercise were used to design an experiment with the help of the design expert Stat- Ease 7.0.0 (Inc Minneapolis, USA), for optimization of phenolic compounds from the apple pomace by response surface methodology using the central composite rotatable design. In all 20 experimental points were realised, consisting of 14 trials and 6 replicates runs around the centre points. The actual design points are shown in Table 2-2 and design summary in Table 4-1.

Table 4-1 Design summary under Subcritical Water Extraction (SWE)

Factor	**Name**	**Units**	**Low Actual**	**High Actual**	**Mean**	**Std. Dev.**
A	Solid/Solvent	%	1	8	4.55	2.64
B	Temperature	°C	100	200	150	41.31
C	Residence Time	Min	10	30	20	8.30

Subcritical water extractions were performed for each of the experimental point using the wet homogenized apple pomace with dry matter content measured as 27% according to the method described in 2.2.3 and total phenolic content (TPC) measured as in 2.2.5. Also fractions of solids solubilised were determined based on the method described in 2.2.4 and recorded in Table 4-2.

4.2.4 Selection of appropriate model for optimisation of total phenolic content under subcritical water extraction

The procedure for selecting the appropriate model for total phenolic content is similar to one described in 3.2.5 under aqueous acetone extraction. The guiding principles have been that, selected model should be significant and lack of fit insignificant in addition to satisfactory levels of adequacy. A transformed reduced quadratic model was proposed to follow a generalised second order polynomial as in Equation 4-1.**Error! Reference source not found.**

Equation 4-1 Generalised transformed second order Polynomial

$$\frac{1}{\sqrt{y}} = \beta_0 + \sum_{i=1}^{4} \beta_i \, x_i + \sum_{i=1}^{4} \beta_{ii} \, x_i^2 + \sum \sum_{i<j=1}^{4} \beta_{ij} \, x_i x_j$$

Where β0, βi βii and βij are the respective coefficients of regression for intercept, linear, quadratic and interaction terms and x_i, x_j are the coded design variables.

Table 4-2 Mean total phenolic content and fraction of solid solubilised under the subcritical water extraction

Standard order	**Sample**	**Response**	
		TPC (mg/g)	**FSS(g/100g)**
6	8-100-30	2.3 ± 0.20	14.7 ± 0.22
11	4.5-66-20	3.5± 0.06	16.5± 0.17
9	0.5-150-20	54.3 ± 0.50	27.4 ± 1.58
18	4.5-150-20	8.7 ± 0.04	23.8 ± 0.43
2	8-100-10	2.2 ± 0.01	14.2 ± 0.17
3	1-200-10	51.1 ± 1.60	25.8 ± 0.29
4	8-200-10	12.9 ± 0.04	12.9 ± 0.00
12	4.5-234-20	31.1 ± 0.05	14.3 ± 2.14
15	4.5-150-20	7.8 ± 0.06	22.9 ± 0.19
20	4.5-150-20	7.8 ± 0.05	22.2 ± 0.21
14	4.5-150-37	9.3 ± 0.11	24.8 ± 0.34
13	4.5-150-3	5.7 ± 0.20	21.9 ± 0.14
1	1-100-10	28.2 ± 1.9	21.2 ± 0.49
7	1-200-30	60.3 ± 0.13	18.7 ± 0.58
17	4.5-150-20	7.0 ± 0.01	23.4 ± 0.07
8	8-200-30	13.3 ± 0.17	11.6 ± 0.06
16	4.5-150-20	8.6 ± 0.13	26.3 ± 0.56
5	1-100-30	33.6 ± 0.88	24.4 ± 0.29
10	9-150-20	3.1 ± 0.08	14.2 ± 0.3
19	4.5-150-20	7.5 ± 0.17	25.2± 0.19

Sample 8-100-30 denotes 8% solid/solvent @100°C for 30 minutes.

The stepwise regression analysis for total phenolic content using the stat-Ease design software shows that the model was highly significant ($p<0.0001$) and the lack of fit not significant ($p>0.05$). Results of the statistical analysis of variance ANOVA is shown in

Table 4-3.

Table 4-3 ANOVA for Response Surface Reduced Quadratic Model for Total Phenolic Content under SWE

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p value**	
Model	0.44	6	0.073	285.70	< 0.0001	*significant*
A	0.24	1	0.24	932.80	< 0.0001	
B	0.10	1	0.10	398.90	< 0.0001	
C	2.373×10^{-5}	1	2.373×10^{-5}	0.093	0.7671	
AB	0.049	1	0.049	189.79	< 0.0001	
A^2	2.449×10^{-3}	1	2.449×10^{-3}	9.56	0.0114	
C^2	1.413×10^{-3}	1	1.413×10^{-3}	5.52	0.0407	
Residual	2.561×10^{-3}	10	2.561×10^{-4}			
Lack of Fit	1.475×10^{-3}	5	2.951×10^{-4}	1.36	0.3724	*not significant*
Pure Error	1.086×10^{-3}	5	2.172×10^{-4}			
Cor Total	0.44	16				

A= solid-to-solvent ratio, B= Temperature and C= Residence Time
Row 10,1,12 were not used for analysis

All design factors were significant except time ($p<0.05$) except residence time ($p>0.05$) for their linear terms, in addition to the interaction between (solid/solvent) and temperature ($p<0.05$), and only solid/solvent ratio and time was significant in the quadratic term.

Table 4-4 Adequacy Level for Response Surface Reduced quadratic Model for TPC under SWE

Std. Dev.	0.016	$\boldsymbol{R^2}$	0.9942
Mean	0.36	$\boldsymbol{R^2_{Adj}}$	0.9907
C.V. %	4.47	$\boldsymbol{R^2_{Pri}}$	0.9742
PRESS	0.011	$\boldsymbol{Precision_{adeq}}$	52.475

The R^2_{Pri} value of 0.9742 was in reasonable agreement with the R^2_{Adj} of 0.9907 and "Adeq Precision" which measures the signal to noise ratio of 52.475 was good. A ratio greater than 4 was desirable and the model can be used to navigate the design space.

Coefficient estimates for intercept, linear, interaction and quadratic in terms of design factors are represented in Table 4-5

Table 4-5 Coefficient Estimates for factors of Reduced quadratic Model for TPC under SWE

Factor	**Coefficient Estimate**	**df**	**Standard Error**	**95% CI Low**	**95% CI High**	**VIF**
$Intercept$	0.35	1	6.146×10^{-3}	0.34	0.37	
A-	0.16	1	5.291×10^{-3}	0.15	0.17	1.12
B	-0.11	1	5.400×10^{-3}	-0.12	-0.096	1.07
C-	-1.769×10^{-3}	1	5.812×10^{-3}	-0.015	0.011	1.29
AB	-0.087	1	6.318×10^{-3}	-0.01	-0.073	1.08
A^2	-0.021	1	6.703×10^{-3}	-0.036	-5.793×10^{-3}	1.15
C^2	-0.014	1	5.764×10^{-3}	-0.026	-6.944×10^{-4}	1.21

A= solid-to-solvent ratio, B=Temperature and C=residence time

The final equation in terms of actual factors is shown in .

Equation 4-2.

Equation 4-2 Transformed Quadratic Model Equation for TPC under SWE

$$\frac{1}{\sqrt{TPC}} = +0.049943 + 0.13601A + 8.12397 \times 10^{-5}B + 5.23829 \times 10^{-3}C - 4.97393 \times 10^{-4}AB - 1.69217 \times 10^{-3}A^2 - 1.35380 \times 10^{-4}C^2$$

Where $A, B, C and\ TPC$ are solid-to–solvent ratio, temperature, residence time, and total phenolic content respectively.

4.2.4.1 Response surface plots for Total phenolic content under subcritical water extraction

Results of total phenolic content of the subcritical water mediated hydrolysis showing the effects of solid-to solvent ratio between 1 - 8%, temperature 100-200°C and time from 10 - 30 minutes are presented in Figure 4-4 and the response surface plots of solid-to solvent ratio and temperature interaction at a fixed time of 20 minutes is shown in Figure 4-5.

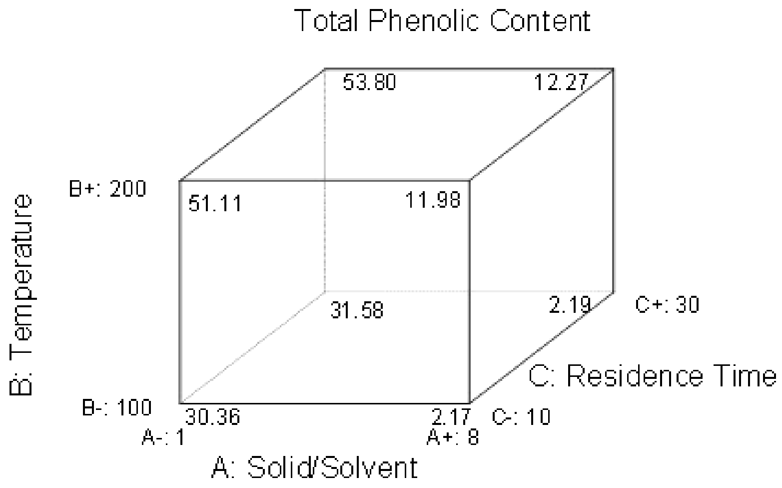


Figure 4-4 Effects of Solid- to –solvent ratio %(w/v), temperature $(^{o}C)$and residence time (minutes) on total phenolic content (mg/g GAE DW) under subcritical water extraction.of apple pomace.

Increase in temperature from 100°C to 200°C at 1% increases total phenolic content by approximately 41%, and however slightly affected (5% increase) when time is extended to 30 minutes as seen Figure 4-4. The negative intercept of solid-to solvent ratio in the quadratic term (-1.69217×10^{-3}) explains the curvature of the response plot and there was no advantage to recovery of phenolic compounds by increasing both temperature and amount of solids at the same time.

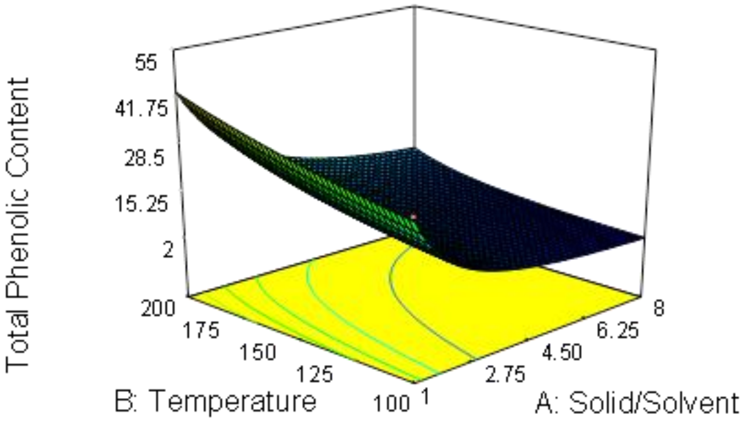


Figure 4-5 Effects of Solid-to-solvent ratio %(w/v) and, temperature (°C) on total phenolic content (mg/g GAE DW) during subcritical water extraction of apple pomace at a fixed time of 20minutes.

The case statistics report showing the experimental (actual) values of Total phenolic content and the predicted are shown in

Table 4-6. Experimental values agreed with the predicted values to a large extent according to the proposed model. Therefore statistical technique could be used to predict total phenolic content within the experimental conditions.

Table 4-6 Case Statistics of Report for Actual and Predicted TPC under SWE

Standard Order	Sample	Actual TPC Value (mg/g GAE)	Predicted TPC Value (mg/g GAE)
1	1-100-10	28.2	30.87
2	8-100-10	2.2	2.16
3	1-200-10	51.1	51.02
4	8-200-10	12.9	11.89
5	1-100-30	33.6	30.87
6	8-100-30	2.3	2.16
8	8-200-30	13.3	11.89
9	0.5-150-20	54.3	51.02
10	9.5-150-20	3.1	3.43
11	4.5-150-20	3.5	3.43
14	4.5-150-37	9.3	10.41
15	4.5-150-20	7.8	8.16
16	4.5-150-20	8.6	8.16
17	4.5-150-20	7.0	8.16
18	4.5-150-20	8.7	8.16
19	4.5-150-20	7.5	8.16
20	4.5-150-20	7.8	8.16

4.2.4.2 Optimisation of Process and Verification of Model for total phenolic content under SWE

Procedure for optimization of the process using the selected model did not vary from what has been described previously in Chapter 2. The best solution with maximum desirability from the numerical optimization was selected as shown in Table 4-7 and extractions were done in triplicate and the phenolic content determined. Because there was no maximum as shown in the surface response, the maximum total phenolic content within the limits of design variables was at 200^{o}C.

Table 4-7 Optimal conditions for extracting phenolic compounds with TPC content of predicted and experimental values

Solid/solvent (%)	**Extraction Temp (°C)**	**Extraction time (min)**	**Predicted TPC (mg/g) GAE DW**	**Actual TPC (m/g) GAE DW**
1	200	30	53.76	49.86

4.2.5 Model selection for fraction of solids solubilized under subcritical mediated hydrolysis

Solubilisation of solids in the liquid extracts during the subcritical water mediated hydrolysis of the apple pomace was determined according to the procedure described in section 2.2.4.The fraction of solids solubilised (g100g) in extracts ranged from 11.6 to 27.4g/100g with a mean value of 20.3g/100g.Subjecting the results of fraction of the solids solubilised to regression analysis revealed a reduced form of the general quadratic model as in Equation 3-1. Analysis of variance showed that the reduced quadratic model was significant ($p<0.05$) and lack of fit insignificant ($p>0.05$) as shown in Table 4-8.

Table 4-8 ANOVA for Response Surface Reduced Quadratic Model for fraction of solid solubilised under SWE

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p value**	
$Model$	441.14	6	73.52	27.42	< 0.0001	$significant$
A	204.12	1	204.12	76.16	< 0.0001	
$B.$	6.20	1	6.20	2.31	0.1524	
C	2.999×10^{-3}	1	2.999×10^{-3}	8.576×10^{-4}	0.9771	
BC	18.30	1	18.30	6.83	0.0215	
A^2	41.53	1	41.53	15.49	0.0215	
B^2	141.72	1	141.72	52.86	< 0.0001	
$Residual$	34.85	13	2.68			
$Lack\ of\ Fit$	23.28	8	2.91	1.26	0.4179	$not\ significant$
$Pure\ Error$	11.57	5	2.31			
$Cor\ Total$	476.00	19				

A= Solid-to-solvent ratio, B=Temperature and C=Residence time

$A, BC, A^2\ and\ B^2$ were the significant model terms (p<0.05). The model had satisfactory level of adequacy where the Predicted R^2 value of 0.8137 reasonable agrees to the adjusted R^2 value of 0.8930 as shown Table 4-9.

Table 4-9 Adequacy Level for Response Surface Reduced Quadratic Model for FSS under Subcritical water Extraction

$\boldsymbol{Std.Dev.}$	1.64	$\boldsymbol{R^2}$	0.9268
$\boldsymbol{Mean}$	20.21	$\boldsymbol{R^2_{Adj}}$	0.8931
$\boldsymbol{C.V.\%}$	8.10	$\boldsymbol{R^2_{Pri}}$	0.8099
$\boldsymbol{PRESS}$	88.68	$\boldsymbol{Precision_{adeq}}$	14.174

Adequate precision value of 14.174 > 4 was good and the model can be used to navigate the design space.

The regression analyses showing estimates for coefficients of the intercept, linear, interaction and quadratic terms at 95% confidence interval are shown in Table 4-10.

Table 4-10 Coefficients Estimates terms for Reduced Quadratic Model for FSS under subcritical water extraction

Factor	**Coefficient Estimate**	**df**	**Standard Error**	**95% CI Low**	**95% CI High**	**VIF**
$Intercept$	23.75	1	0.58	22.50	24.99	
A	-4.28	1	0.49	-5.34	-3.22	1.02
B	-0.67	1	0.44	-1.63	0.28	1.00
C	0.013	1	0.44	-0.94	0.97	1.00
BC	-1.51	1	0.58	-2.76	-0.26	1.00
A^2	-2.39	1	0.61	-3.70	-1.08	1.02
B^2	-3.11	1	0.43	-4.03	-2.19	1.00

The final equation of the model in terms of actual factors is shown Equation 4-3

Equation 4-3 Quadratic Model Equation for FSS under Subcritical water extraction

$$y = -9.75204 + 0.53008A + 0.42011B + 0.45505C - 3.025 \times 10^{-3}BC - 0.19488A^2 - 1.24362 \times 10^{-3}B^2$$

Where A, B, C , and y are respectively, loading (solid/solvent), temperature, time and fraction of solids solubilised in g/100g.

The interaction between extraction temperature and time under subcritical water extraction played an important factor in the solubilisation similar to aqueous acetone solubilisation. This strong relationship between temperature and extraction time have been confirmed under pressurised water in terms recovery of solids (Smith, 2002). Solid-to-solvent ratio demonstrated similar effects for linear and the quadratic terms for both procedures. Temperature exhibited significance ($p<0.05$) for quadratic term under subcritical water whereas time was significant ($p<0.05$) under aqueous acetone extract in addition to loading.

4.2.5.1 Response surface plots for fraction of solids solubilized under subcritical water extraction

The effects of solid-to-solvent ratio, temperature and residence time on fraction of solids solubilised are shown in Figure 4-6. Solubilisation increases with increased in temperature up to 150^{o}C and decreases thereafter as shown in Figure 4-7.Variation of solid-to-solvent ratio with residence time is represented in Figure 4-9.

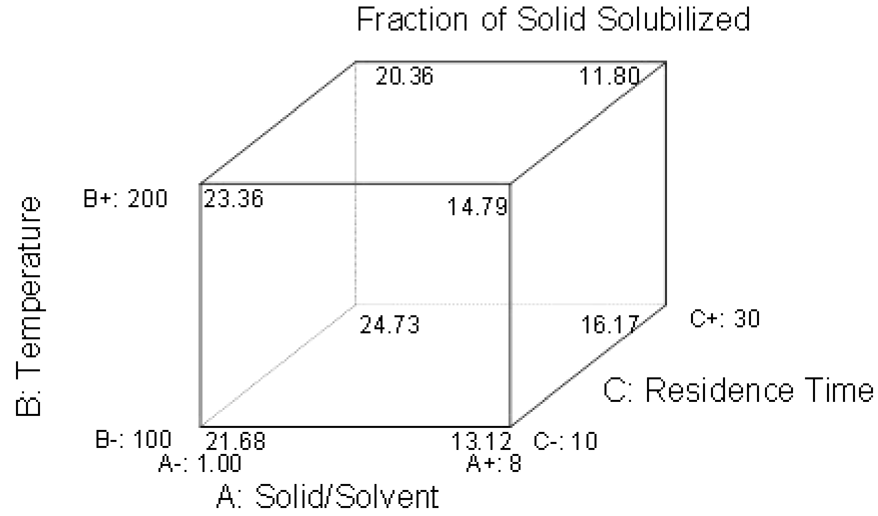


Figure 4-6 Effects of temperature (^{o}C), solid-to-solvent ratio %(w/v)and residence time on Fraction of solids solubilised (g/100g) during subcritical water extraction of apple pomace.

Increasing temperature may cause degradation of other heat sensitive ingredients from the solid matrix. It was expected that more solids would have been eluting at the higher temperature which was not observed. Apple pomace composed of considerable amounts of carbohydrates and proteins which decomposed to their simpler monomers at high temperatures. For instance carbohydrates under subcritical water undergoes hydrolysis to form simple sugars and other disaccharides (Toor et al., 2011). Heteropolymer, hemicellulose solubilises at temperatures above 180°C under both acid- base catalysis to produce several monosaccharides like xylose, mannose, glucose and galactose (Bobleter, 1994). Proteins which comprise several peptide bonds can undergo decarboxylation and deamination to form amino acids and sugars through the cleavage of the peptide- chains at the high temperature (Toor et al., 2011). All these reactions occurring are expected to increase amount of solids in the extract which was not observed. The possible explanation could be that, under the high pressure –temperature conditions these molecules may combine to form macromolecules which can precipitate and fall back to the residue during processing of products from the batch reactor and are not accounted for in the estimation of yield of solid solubilised. Auto polymerization of 5-HMF to produce less soluble humins in water have previously been reported (Gaset et al., 1985).

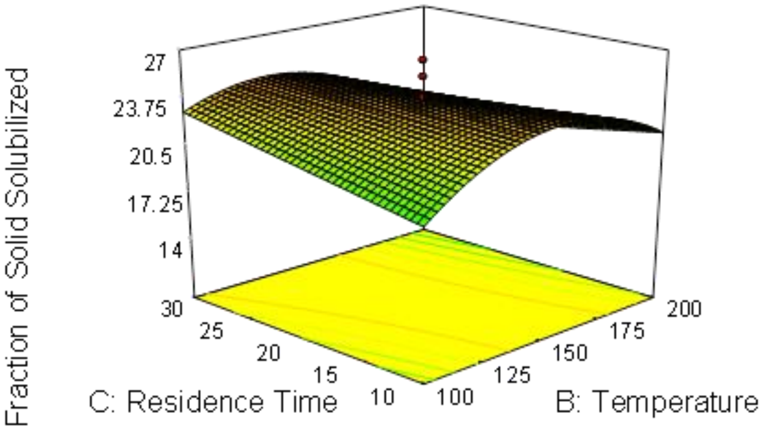


Figure 4-7 Response surface plot of temperature (°C) and residence time (minutes) on Fraction of solids solubilised (g/100g) under subcritical water extraction of apple pomace.

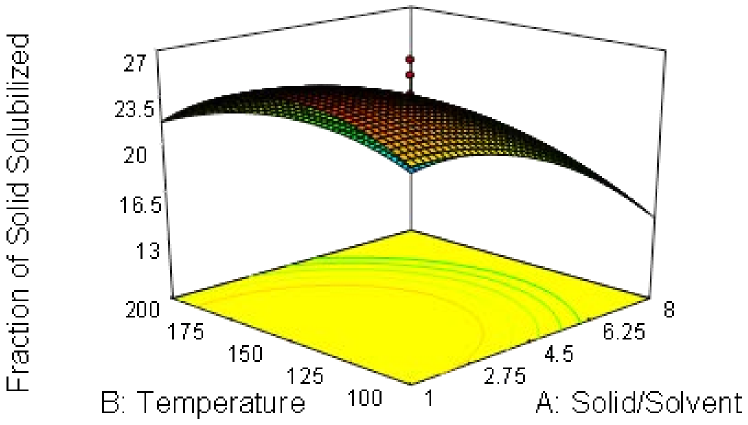


Figure 4-8 Response surface plot of temperature (°C) and Solid- to- solvent ratio %(w/v) effects on Fraction of solids solubilised under subcritical water extraction of apple pomace at a residence time of 20 minutes.

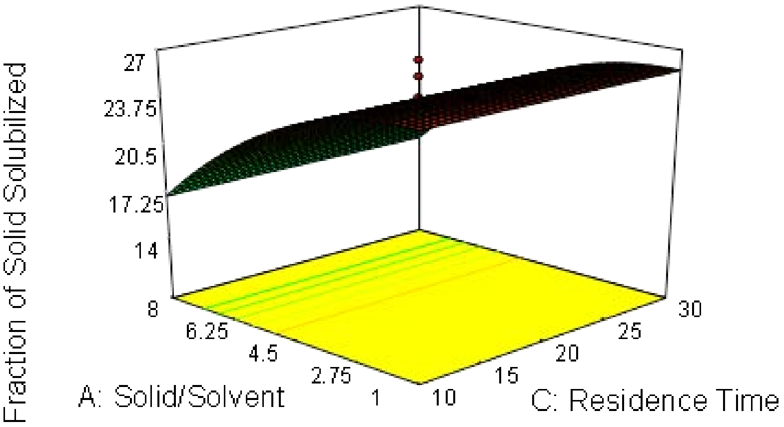


Figure 4-9 Response surface plot of solid-to-solvent ratio %(w/v) and residence time (minutes) relationship on Fraction of solids solubilised (g/100g) under subcritical water extraction of apple pomace at temperature 150°C.

The case statistics report showing the experimental (actual) values of fraction of solids solubilized per 100g of starting material and the predicted are shown in

Table 4-11.

Table 4-11 Experimental and predicted of fraction of solids solubilized g /100g of apple pomace under subcritical water extraction

Standard Order	**Sample**	**Actual Value** (g/**100g**)	**Predicted Value** (g/**100g**)
1	1-100-10	21.20	21.68
2	8-100-10	14.20	13.12
3	1-200-10	25.80	23.36
4	8-20010	12.90	14.79
5	1-100-30	24.40	24.73
6	8-100-30	14.70	16.17
7	1-200-30	18.70	20.36
8	8-200-30	11.60	11.80
9	0.5-150-20	27.40	25.53
10	9.5-150-20	14.20	12.76
11	4.5-66-20	16.50	16.09
12	4.5-234-20	14.30	13.38
13	4.5-150-3	21.90	23.76
14	4.5-150-376	24.80	23.82
15	4.5-150-20	22.90	23.79
16	4.5-150-20	26.30	23.77
17	4.5-150-20	23.40	23.75
18	4.5-150-20	23.80	23.75
19	4.5-150-20	25.20	23.75
20	4.5-150-20	22.20	23.75

Experimental and predicted values agree to a large extent.

4.2.5.2 Optimisation of Process and Verification of Model for fraction of solids solubilised under SWE

The solution with maximum desirability of the numerical optimisation was selected and the fraction of solids solubilised, was determined and compared with the predicted value as shown in Table 4-12.

Table 4-12 Optimal conditions for Fraction of solids solubilised/100g of apple pomace of predicted and experimental values

solid/solvent (%)	**Extraction Temp (°C)**	**Extraction time (min)**	**Predicted FSS (%)**	**Actual FSS (%)**
1	153	30	26.1	28.2

The observed value agrees with the predicted value as variations can be attributed to the non-homogeneous nature of the apple pomace. Subcritical water was able to solubilise more solids compared with the solvent extraction using acetone because subcritical water was not just extracting but hydrolysing as well. 28.2% of solids solubilised under subcritical water against 19.2% under aqueous acetone. Subcritical water was more efficient than acetone in the recovery of solutes from roots of rhizomes of piper widely used as medicinal soft drink in Europe (Kubátová et al., 2001). The high H^+ ions coupled with high temperature and pressure under the subcritical water conditions promoted cleavage of the bonds and disrupted cell walls within the solid matrix thereby pushing more solids into solution (Brunner, 2009).

4.2.6 Identification of polyphenolic compounds under Subcritical water extracts

The extracts from the various design parameters combinations from the subcritical water extraction were analysed by the HPLC-DAD method described previously in 2.2.9.

The HPLC –DAD characterisation of the polyphenolic compounds of subcritical conditions at 100°C reveals among others similar compounds under the aqueous acetone extraction. The main phenolic compounds identified were, Chrologenic acid, Phloridzin, Quercetin-3-galactoside, Quercetin-3-glucoside, Procyanidins B2., and Phloretin glycosides. Monomeric procyanidins like Catechin and Epicatechin were barely detected and the reason could that the UV-DAD was not very good enough for their detection. Protocatechuic aldehyde was detected at 150°C and beyond. These polyphenolic compounds with exception of Protocatechuic aldehyde were the main polyphenolic compounds recovered from apple pomace using pressurised ethanol/water as solvents, with other phenolic compounds like Caffeic acid, P- Coumaric acid, Catechins and Procyanidins, detected only at smaller concentrations (Wijngaard and Brunton, 2009). Phenolic compounds like anthocyanidins were not detected although the apple pomace sample used was a blend of varieties of cider apples including the red ones which were thought to contain high levels of the flavonoid. Anthocyanidins are highly unstable and may even oxidize before the start of the analysis (Delgado-Vargas et al., 2000). However there were other peaks typically of phenolic nature by their UV- spectra but standards were not available to identify them. Other compounds like 5-hydroxymethylfurral (5-HMF), Furfural and other hydrolysis products were observed as temperature increases from 100°C. Typical chromatograms of the subcritical water mediated hydrolysis at 280nm and 320nm are shown in Figs 4-10-12.

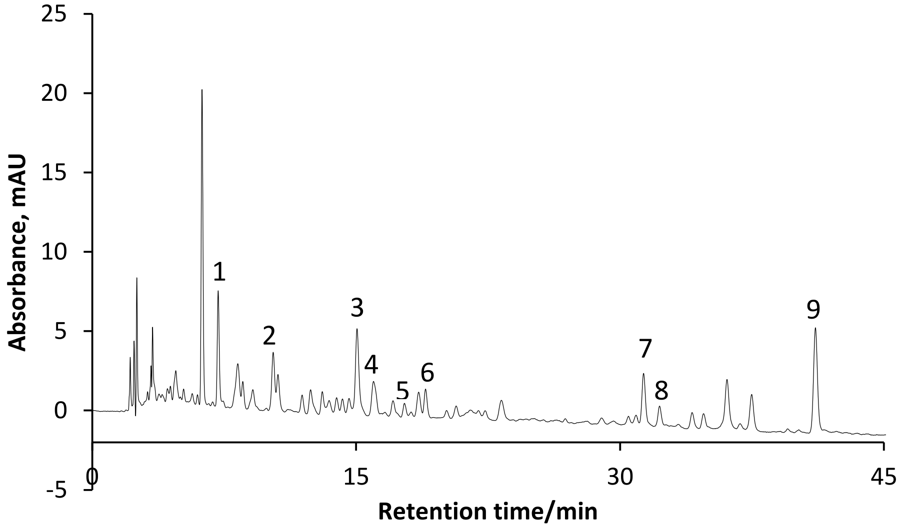


Figure 4-10 Chromatogram of subcritical water extract for 8% solid-to-solvent ratio at 100°C for 30 minutes; 1-5HMF; 2-furfural, 3- Chlorogenic acid; 4- Procyanidin b2; 5-Caffeic acid); 6-Epicatechin; 7-Quercetin-3-galactosid; 8-Quercetin-3-glucoside, 9-Phlorodzin

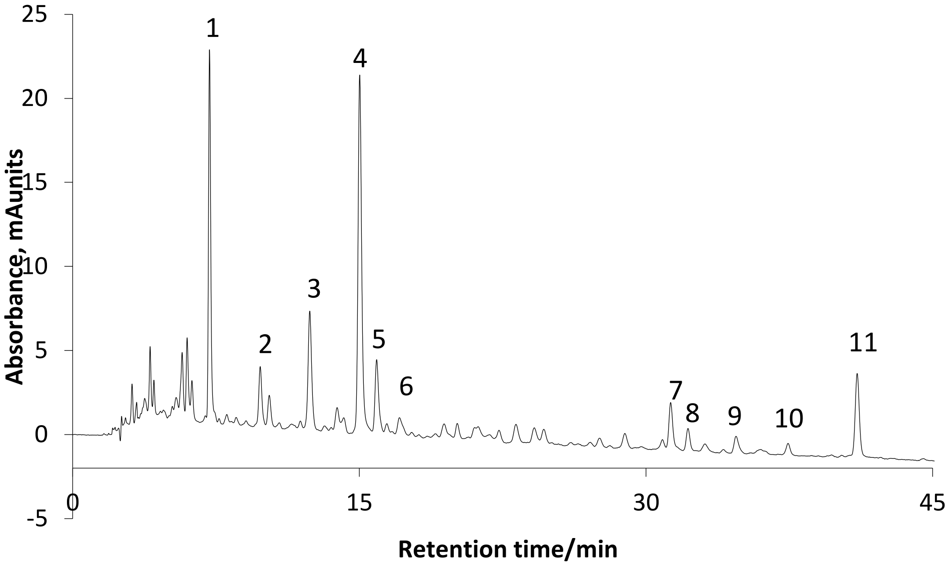


Figure 4-11 Chromatogram of subcritical water extract for 4.5% solid-to-solvent ratio at 150°C for 30minutes; 1-5HMF; 2-furfural, 3- Protocatechuic aldehyde; 4- Chlorogenic acid; 5-(isomer of Chlorogenic acid); 6-caffeic acid; 7-Quercetin-3-galactosid; 8-Quercetin-3-glucoside, 9 and 10-are unidenfied polyphenolic compounds and 11-Phlorodzin.

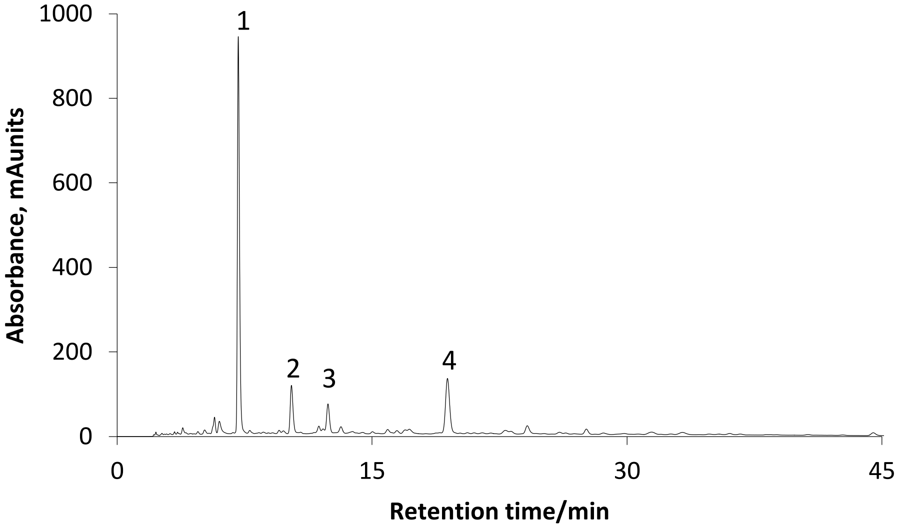


Figure 4-12 Chromatogram of subcritical water extract of apple pomace for 8% solid-to solvent ratio at 200°C for 30minutes; 1-5HMF; 2-furfural, 3- Protocatechuic aldehyde; 4- unidentified compound.

4.2.7 Quantification of the phenolic compounds in the subcritical water extract

Phenolic compounds identified were quantified based on the procedure outlined in section 2.2.10 and were recorded as mean values ± standard error and expressed in mg/kg of dry weight of apple pomace as shown in Table 4-13 and Table 4-14.

4.2.8 Model Analysis of individual phenolic compounds under Subcritical water extraction

Polyphenolic compounds (Chlorogenic acid, Phloridzin, Quercetin-3-galactoside, Quercetin-3-glucoside, Procyanidin B2), degradation compounds (Protocatechuic aldehyde, 5-HMF and Furfural) were evaluated for optimization using the concentrations in Table 4-13 and Table 4-14 .Detailed ANOVA results at 95% confidence interval are shown in the appendix section and summary on the level of significance of design factors and their interaction are shown in Table 4-15.

Table 4-13 Mean concentration of Phenolic compounds (mg/kg) dry weight under subcritical water extraction of apple pomace

Std Order	**Sample**	**CGA**	**PHL**	**Q-3-gal**	**Q-3-glu**
6	8-100-30	35.05 ± 6.3	38.74 ± 3.6	16.54 ± 2.5	10.84 ± 1.9
11	4.5-66-20	6.91±0.0	50.24±5.0	22.36 ± 6.9	ND
9	0.5-150-20	ND	ND	ND	ND
18	4.5-150-20	60.37±1.8	57.61 ± 2.4	12.712 ± 0.7	21.43 ± 0.2
2	8-100-10	24.67 ± 1.3	31.43 ± 1.32	16.66 ± 0.5	12.91± 0.4
3	1-200-10	ND	ND	ND	ND
4	8-200-10	ND	ND	ND	ND
12	4.5-234-20	ND	ND	ND	ND
15	4.5-150-20	60.14±0.4	48.30 ± 7.0	14.45 ± 0.5	18.68 ±
20	4.5-150-20	59.18 ± 2.8	43.48 ± 1.7	11.29 ± 0.2	19.46 ±
14	4.5-150-37	54.90 ± 1.7	44.80 ± 2.3	10.59 ± 2.4	20.88 ± 2.6
13	4.5-150-3	59.4 ± 2.4	50.49 ± 2.8	25.07 ± 1.1	22.97 ±1.7
1	1-100-10	58.39±0.6	60.73 ±	76.16 ± 0.7	ND
7	1-200-30	ND	ND	ND	ND
17	4.5-150-20	60.56 ± 1.5	45.29 ± 2.6	15.30 ± 0.9	18.32 ± 4.9
8	8-200-30	ND	ND	ND	ND
16	4.5-150-20	34.49±1.2	22.37±4.3	25.33 ± 1.67	28.24 ± 4.7
5	1-100-30	122.21±1.9	250.20 ± 22.2	97.00± 0.7	ND
10	9.5-150-20	40.21±0.4	31.53 ± 1.2	5.83 ± 0.7	8.87 ± 2.9
19	4.5-150-20	78.33±2.7	89.21 ± 1.02	19.24 ± 1.4	23.59 ± 4.9

Sample 8-100-30 reads; 8% solid/solvent @ 100°C for 30 minutes;
CGA- Chlorogenic acid; PHL- Phloridzin; Q-3-gal- Quercetin-3-galactoside; Q-3-glu- Quercetin-3-glucoside, ND –not detected.

Table 4-14 Mean concentration of Procyanidin B2, Protocatechuic aldehyde, 5-HMF and Furfural all (mg/kg) dry weight under subcritical water extract of the apple pomace.

Std order	Sample	Pr-B2	PCA	Furfural	5-HMF
6	8-100-30	57.67 ± 5	ND	16.09 ± 1.7	4.19 ± 0
11	4.5-66-20	ND	ND	ND	ND
9	0.5-150-20	ND	ND	ND	150.89 ± 8.1
18	4.5-150-20	ND	39.6 ± 0.3	628.75±71	194.05 ± 33.0
2	8-100-10	48.55 ± 0.1	ND	4.40 ± 0.23	2.02 ± 1.4
3	1-200-10	ND	674.2 ± 0.1	17980.39± 406	3754.93 ± 67.7
4	8-200-10	ND	337.8 ± 0.5	6653.63± 103	633.27 ± 2.7
12	4.5-234-20	ND	1133.2 ± 0.6	15787.68± 265	1543.86 ± 13.5
15	4.5-150-20	ND	40.5 ± 0.2	590.89 ± 42.5	168.32 ± 6.1
20	4.5-150-20	ND	38.6 ± 1.4	631.12 ± 14.5	175.12 ± 6.0
14	4.5-150-37	ND	62.2 ± 0.0	1042.49± 74.1	342.83 ± 1.8
13	4.5-150-3	ND	18.4 ± 0.1	177.61 ± 20.3	33.36 ± 1.8
1	1-100-10	ND	ND	46.27±0.4	6.48 ± 0.2
7	1-200-30	ND	966.4 ± 0.1	40330.39± 786	5767.29±170.5
17	4.5-150-20	ND	39.7 ± 0.1	588.45 ± 1.57	162.24 ± 3.1
8	8-200-30	ND	357.4 ± 23.9	9038.32 ± 158.6	795.93 ± 1.8
16	4.5-150-20	**ND**	41.8 ± 0.1	753.07 ± 148	201.94 ±46.3
5	1-100-30	ND	ND	97.78 ± 9.3	4.34 ± 0.5
10	9-150-20	ND	44.5 ± 1.1	652.88 ± 1.03	212.95 ± 5.0
19	4.5-150-20	ND	41.6 ± 0.0	594.97± 11.9	185.97 ± 7.0

ND= not detected; 1-100-30 reads 1% solid-to-solvent ratio at 100°C for 30 minutes residence time.

Solid-to-solvent ratio, temperature and their interaction had significant effects on the yield of all responses except for FSS which has been previously discussed. Effect of time was not significant on extraction of phenolic compounds, consistent with earlier reports (Plaza et al., 2013). Residence time exerted its influence on the yield of Protocatechuic aldehyde, 5-HMF and furfural as well it's interaction with temperature.

Table 4-15 Summary of significance level of design factors and interaction terms under subcritical water extraction

Response	**Significant level ($p<0.05$)**								
	A	B	C	AB	AC	BC	A^2	B^2	C^2
CGA	✓	✓		✓				✓	
PHL	✓	✓		✓			✓	✓	
Q-gal.	✓	✓		✓			✓		
Q-glu.	✓	✓		✓			✓		
Pr-B2	✓	✓		✓			✓		
PCA	✓	✓	✓		✓	✓	✓		
5-HMF	✓	✓	✓	✓		✓	✓		
Furfural	✓	✓	✓	✓		✓	✓		
ORAC	✓	✓		✓			✓	✓	
TPC	✓	✓		✓			✓		
FSS	✓					✓	✓	✓	✓

CGA-chlorogenic acid; PHL-phloridzin; Q-gal- quercetin-galactoside; Q-glu- quercetin glucoside; Pr-B2-procyanidin B2; TPC-total phenolic content, FSS fraction of solid solubilised. A-solid-to-solvent ratio; B-temperature, and C- residence time in reactor

High temperatures and extended time favour the yield of the decomposition products. All proposed models were significant ($p<0.05$) with satisfactory level of adequacies ($R^2 >0.9$) and predicted R^2 largely agreed with adjusted $R^{2.}$ Coefficient of variations and variance inflation factors were at acceptable levels.

4.2.8.1 Predictive Model for Extracting Chlorogenic acid under Subcritical water

Maximum Chlorogenic acid determined from the subcritical water extract was 122.2 mg/kg which represents 55% of that recovered using aqueous acetone (221.58mg/kg).

Final equation in terms of actual factors is shown in Equation 4-4.

Equation 4-4 Predictive Model Equation for Chlorogenic acid under Subcritical water extraction

$$\sqrt{Chlorogenic\ acid} = -14.66360 - 1.11557A + 0.38727B + 0.19207C + 5.57786 \times 10^{-3}AB - 1.09174 \times 10^{-3}BC - 1.55493 \times 10^{-3}B^2$$

Yield of Chlorogenic acid was significantly affected by loading ratio (solid/solvent) and temperature as well as their interaction ($P<0.05$). Only temperature shows significant effect in the quadratic term ($p<0.05$). Residence time had no significant influence on the recovery of Chlorogenic acid ($p > 0.05$). Maximum concentration of Chlorogenic acid was extracted around 100^{o}C.Chlorogenic acid had been reported to degrade at temperature 112^{o}C during pressurised liquid extraction of antioxidants from apple pomace using ethanol and water as solvent (Wijngaard and Brunton, 2009). A phenolic compound was observed at temperature 150^{o}C with retention time 16.06 minutes which share similar spectra characteristics with Chlorogenic acid but with different retention times. This compound was observed to evolve from standard Chlorogenic acid investigation under temperature 60^{o}C over time in a control experiment and could be an isomer or decomposition product of

Chlorogenic acid (see peak number 4 in Figure 4-13). Chlorogenic acid which is an ester of Caffeic acid and Quinic acid has several isomers and classified based on the number and position of the acyl groups attached to them. The isomers include 5-O- caffeeoylquinic acid (common one), 4-O- caffeeoylquinic acid and others with two Caffeic acid molecules and referred to as iso Chlorogenic acid comprising 3,4, dicaffeeoyliquinic acid and 3,5, dicaffeeoyliquinic acid (Clifford, 2000). Compound (peak 4) was not observed in solvent extracts of the dried apple pomace. On the other hand Winjgaard and Brunton reported, an increased in Chlorogenic acid concentration at temperatures higher than 160^{o}C and attributed the release from a possible noncovalent relationship of Chlorogenic acid with melanoidins. Similarly Plaza and co-workers reported maximum concentration of 5-caffeoquinic acid (Chlorogenic acid) from apple pomace could be obtained between 175-200^{o}C,which was not verifiefd (Plaza et al., 2013). Chlorogenic acid or any of its isomers were not detected at temperature 200^{o}C in this investigation. The transformed polynomial model for Chlorogenic acid was used to navigate the design space and no prediction of any higher concentrations at higher temperatures was observed. . Predictive capacity of the model was tested which can be seen in the case statistics reports of Chlorogenic acid under the appendix section.

Response surface plots for recovery of Chlorogenic acid showing the effects of solid/solvent ratio, temperature at a fixed residence time of 20 minutes residence time is shown in Figure 4-14.

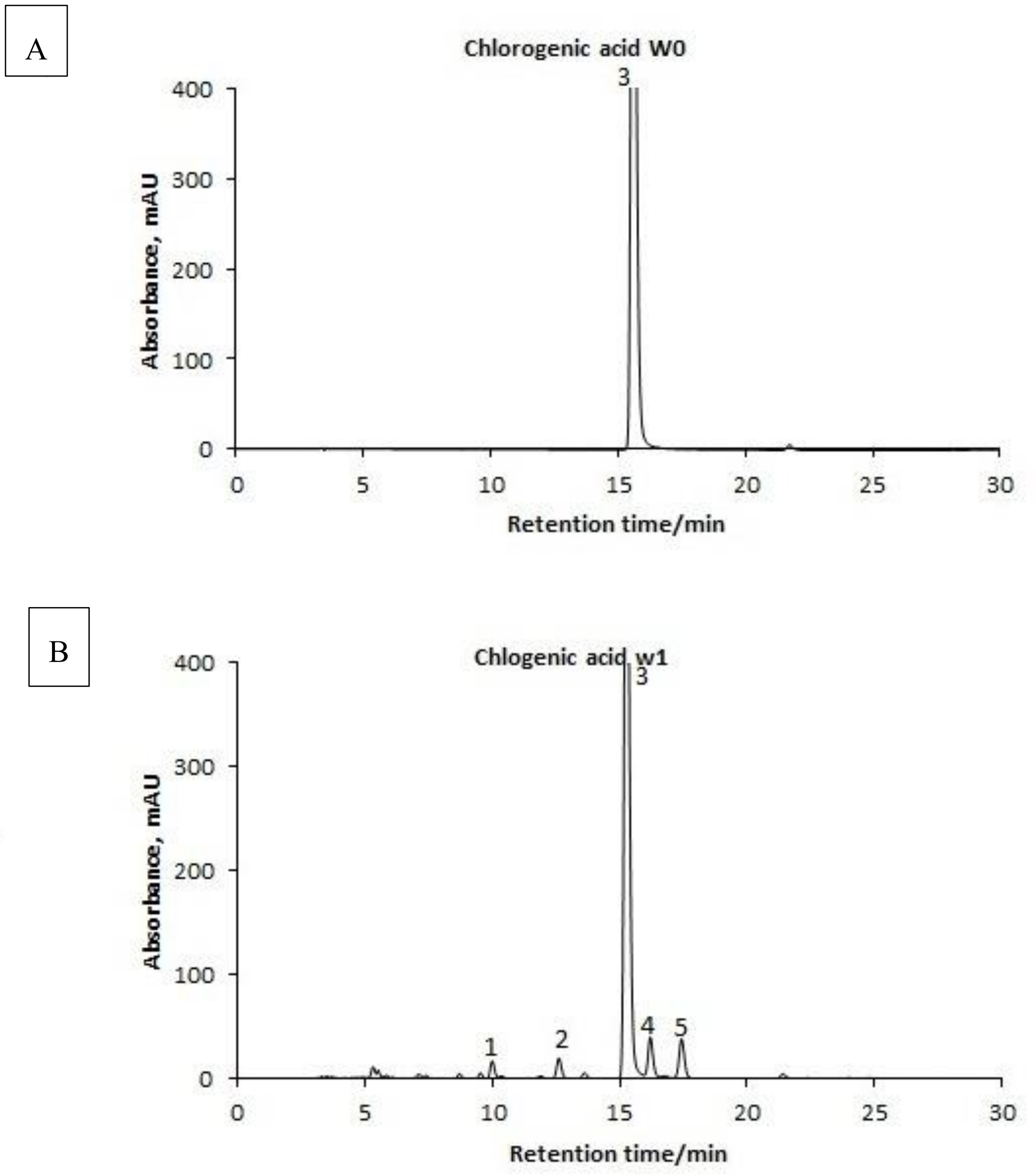


Figure 4-13 . A= Chromatogram of standard Chrologenic acid solution. B= Chromatogram of the standard Chlorogenic acid solution after 7 days period Decomposition Products of stanadard Chlorogenic acid at 65°C observed are.1= unidentified Phenolic acid; 2= Protocatechuic aldehyde; 3= Chlorogenic acid; 4= unidentified Phenolic acid; 5= Cafeic acid.

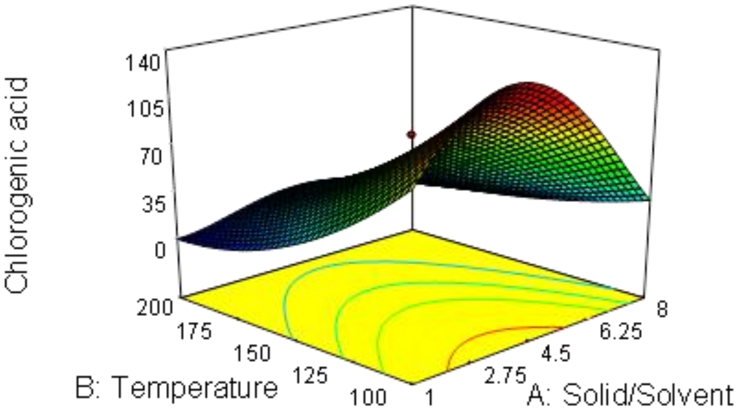


Figure 4-14 Response surface plot for the effects of temperature (°C)and solid/solvent ratio %(w/v) on the amount of Chlorogenic acid (mg/kg) der weight under subcritical water extraction of apple pomace for a residence time 20 minutes

4.2.8.2 Predictive Model for Extracting Phloridzin under Subcritical water

Maximum concentration of the dihydrochalcone glycosides Phloridzin in extracts was 250.2mg/kg at 100°C which is consistent with similar extractions using ethanol/water as solvents for extraction (Wijngaard and Brunton, 2009). However it began to degrade at around 120°C. Phloridzin was not detected at temperature 200°C.

The predictive model equation in terms of actual factors for extracting Phloridzin is shown below Equation 4-5.

Equation 4-5 Model Equation for Phloridzin under Subcritical water extraction

$$\sqrt{Phloridzin} = +8.95591 - 3.88579A + 0.22440B + 0.014146AB + 0.11739A^2 - 1.32240 \times 10^{-3}B^2$$

The response surface plots shows highest amount of Phloridzin at 100°C and stay relatively constant until around 120°C and gradually decreases to zero at 200°C. The model could be used to navigate the design space. Similar to Chlorogenic acid, Plaza et al., 2013 reported a deviation of response surface plots for Phloridzin under subcritical water extraction of apple peels. Response surface plots showing the effects of temperature, solid- to- solvent ratio at fixed residence time of 20 minutes on the yield of Phloridzin is shown in Figure 4-15.

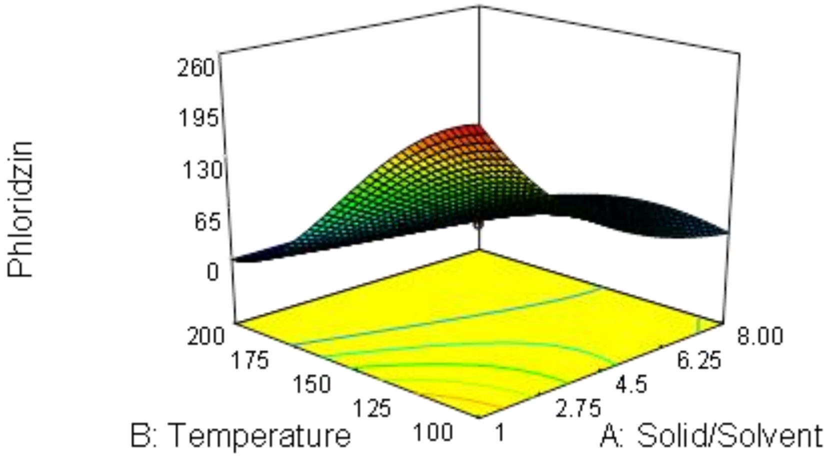


Figure 4-15 Response surface plot for the effects of temperature (°C) and solid/solvent ratio %(w/v) on the amount of Phloridzin (mg/kg) dry weight under subcritical water extraction of apple pomace for residence time of 20 minutes.

The predictive capacity of the transformed quadratic model for Phloridzin was assessed in the case statistics report which showed to a large extent the agreement between experimental results against those predicted by the model within the experimental parameter limits (appendix).

4.2.8.3 Predictive Model for Extracting Procyanidin B2 under Subcritical water

Structural and molecular weight variation within Proanthocyanidins makes their evaluation very challenging. Moreover their complexation with other non-soluble polymers under estimate their quantification due to incomplete extraction (Pérez-Jiménez et al., 2009). About 50-93% of apple Procyanidins may be retained within cell wall material during processing of apple juice (Le Bourvellec and Renard, 2012).

Concentration of Procyanidin B2 under subcritical water extraction varied from 0.00 – 57.67± 5.30mg/kg DW and the yield of Procyanidin B2 was both solid-to solvent ratio and temperature dependent as well as their interaction. Residence time had no influence on overall amount of Procyanidin B2 under the subcritical water extraction within selected range (see Table 4-15). Procyanidin B2 was not detected at 150^{o}C and beyond suggesting that the amount originally accumulated at 100^{o}C would have been degrading. Therefore it was not advisable to pursue predictive modelling based on the selected range although the design expert suggested predictive model and this could not be relied upon due to limited data because contour plots may be misleading. Optimal temperature conditions of 80-140^{o}C were reported for extracting Procyanidins from grape pomace using ethanol /water mixture as solvents (Monrad et al., 2010).

4.2.8.4 Predictive Model for Extracting Quercetin glycosides under Subcritical water

The two quercetin glycosides have again differed in the way they responded to changes in temperature and solid- to- solvent ratio. From both the aqueous acetone and subcritical water extraction methods, quercetin-3-galactoside was influenced overall slightly by temperature whereas quercetin-3-glucoside is been controlled by solid-to-solvent ratio both in the quadratic terms. The interaction between solid-to solvent ratio and temperature was positive for Quercetin-3-galactoside and negative Quercetin-3-gluctoside as shown in the. predictive equations. See Equation 4-6.

Equation 4-6 Predictive model equations for Quercetin glycosides

$$Quercetin-3-galactoside = +95.97583 - 31.76127A + 0.45032B + 0.085086AB + 1.63824A^2 - 4.32336 \times 10^{-3}\,B^2$$

$$quercetin-3-glucoside = -15.02656 + 16.48077A + 0.016964B - 0.016964AB - 1.45421A^2$$

The difference in the behaviour could be attributed to the structural configuration of galactose and glucose been sugar moieties attached to the quercetin aglycone. Although glucose and galactose can both occur in either open or cyclic structures, the five (5) hydroxyl groups in glucose can be arranged in a specific way on the six- carbon backbone whereas galactose has a carbonyl towards end of chain. These configurations contribute to the different boiling points of the sugars despite identical molecular weights as they are isomers (180.156g/mol for glucose and 180.16g/mol for galactose). Boiling points of glucose and galactose are 146- 150^{o}C and 167^{o}C respectively.

Boiling points are reflection of the strength of the forces between molecules and indication of ease of separation of components which are bound together. Both quercetin glycosides have positive linear temperature coefficients which suggested their concentrations increase in extracts initially when temperature was raised up to 100^{o}C. However further increase in temperature negatively affects the yields of the glycosides. The coefficients values of interaction between solid-to solvent and temperature for both glycosides indicates that it was not advantageous to the yield of quercetin-3-galactoside when both factors are increased simultaneously whereas it positively influence the amount of the quercetin-3-glucoside as shown in the response surface plots. Optimal extraction conditions of 1% solid-to solvent ratio, at 100°C for 29 minutes for extracting quercetin -3-galactoside against 4.75% solid-to solvent ratio, at 104°C for 27 minutes for quercetin-3-glucoside.

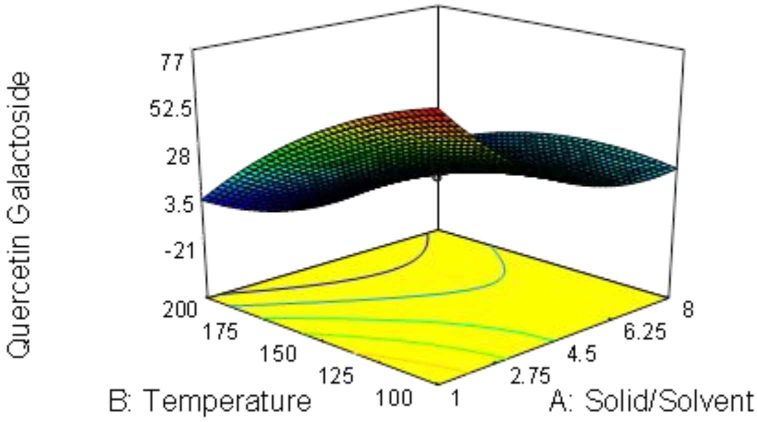


Figure 4-16 Response surface plot for the effects of temperature (°C) and solid/solvent ratio %(w/v) on the amount of Quercetin-3- galactoside (mg/kg) dry weight for 20 minutes residence time under subcritical water extraction of apple pomace .

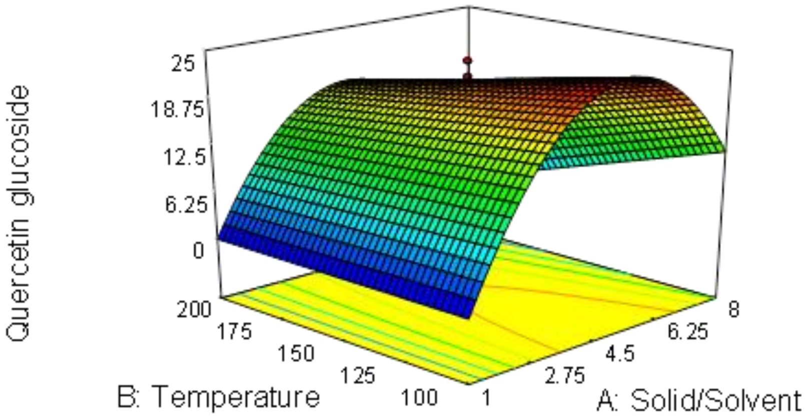


Figure 4-17 Response surface plot for the effects of temperature (°C)and solid/solvent ratio %(w/v)on the amount of Quercetin-3- glucoside (mg/kg) dry weight for 20 minutes residence time under subcritical water extraction of apple pomace .

4.2.8.5 Predictive Model for Extracting Protocatechuic aldehyde under Subcritical water

3, 4 – Dihydroxybenzaldehyde otherwise known as Protocatechuic aldehyde is not found freely in apples and no mention of it so far during subcritical water extraction of apple pomace phenolics to the best of my knowledge. Small amount (4μg/ml) of Protocatechuic aldehyde had evolved after 7days when standard Chlorogenic acid (1mg/ml) was investigated for degradation at 65℃ (see Figure 4-13). The phenolic aldehyde has been reported to be a stable product of thermal degradation of Chlorogenic acid (Moon and Shibamoto, 2010). Protocatechuic aldehyde was also the main stable compound from Caffeic acid degradation under subcritical water conditions within 160-240℃ (Khuwijitjaru et al., 2014). Either of these reports was acceptable because Chlorogenic acid is an ester of Quinic and Caffeic acids and Protocatechuic aldehyde could be released from both hydrocinnamic acids undergoing degradation.

The UV spectra of Protocatechuic aldehyde standard (red marking) and those in samples (blue marking) are shown in Figure 4-18.

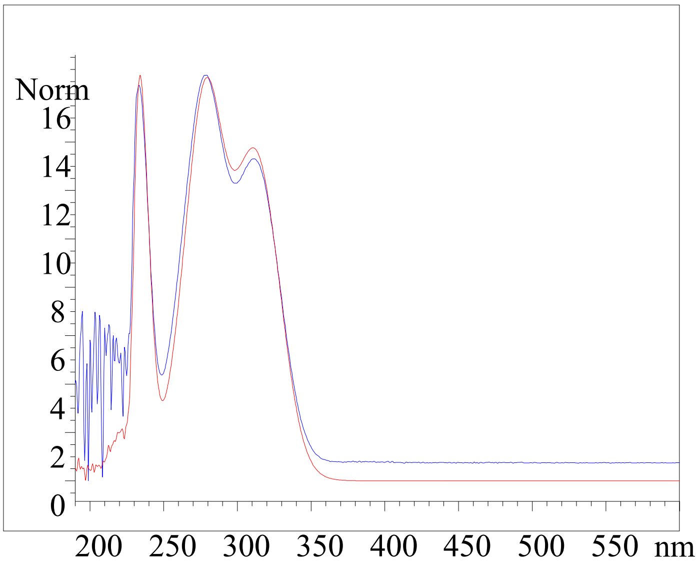


Figure 4-18 UV- Spectra of Protocatechuic aldehyde standard and in extract from the subcrtical water extraction of the apple pomace at 280nm.

Water soluble Protocatechuic aldehyde in the extracts ranged from 0 – 966.4 mg/kg within the limits of design parameters. Protocatechuic aldehyde was not detected at 100°C but only noticeable at 150°C and was stable in the extract even up to 234°C.

The transformed quadratic model was significant ($p<0.05$) with unfortunately a significant lack of fit ($p>0.05$) which was bad but yet the model should fit. All model terms were significant as well as their interactions. Only solid to solvent ratio was significant in the quadratic term as shown in Equation 4-7.

Equation 4-7 Predictive model equation for Protocatechuic aldehyde under subcritical water extraction

$$\sqrt{PCA} = -25.29499 - 2.30741A + 0.27115B - 0.052261C - 0.014120AB - 0.016414AC + 1.41183 \times 10^{-3}BC + 0.44974 \times 10^{-3}A^2$$

Yield of Protocatechuic aldehyde increases from 150°C to 200°C with increasing residence time up to 30 minutes. However it decreases as solid to solvent ratio approaches 8% meaning decreasing the loading and increasing both temperature and residence time improves the yield of Protocatechuic aldehyde. Similar effect of experimental parameters on total phenolic content and antioxidant activity by ORAC were observed. Response surface plots depicting this behaviour of temperature, solid to solvent and residence time are shown Fig 4-19-21 below;

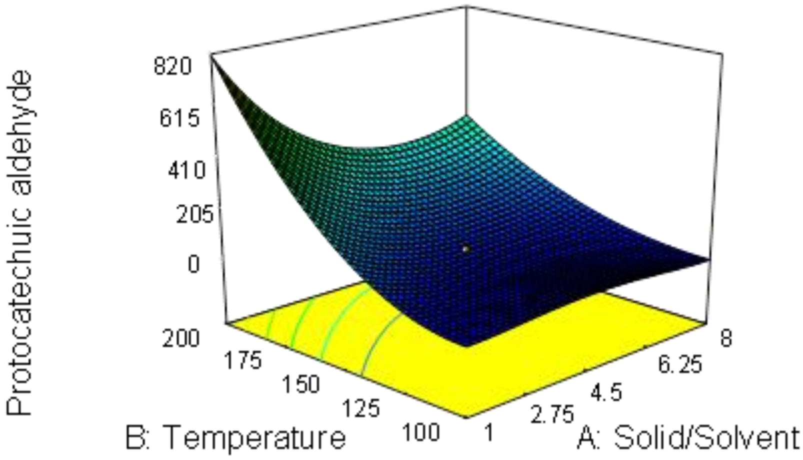


Figure 4-19 Response surface plot for the effects of temperature (°C) solid/solvent ratio %(w/v) on theamount of Protocatechuic aldehyde (mg/kg) dry weight for 20 minutes residence time under subcritical water extraction of apple pomace .

The predictive power of the model was assessed by the case statistics report shown in appendix section, where experimental values agree with the predicted ones. Model could be used to describe the behaviour of Protocatechuic aldehyde within the experimental variable limits. Numerical optimisation of extraction conditions for Protocatechuic aldehyde according to the maximum desirability value of 0.986 were 1% solid-to-solvent, 200°C for 30 minutes residence time.

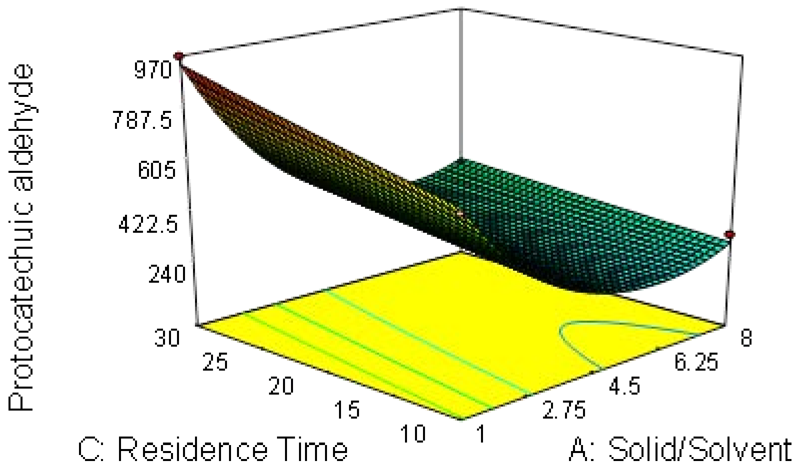


Figure 4-20 Response surface plot for the effects of solid/solvent ratio %(w/v) and residence time (minutes) on the amount Protocatechuic aldehyde (mg/kg) dry weight under subcritical water extraction of apple pomace temperature of 200°C.

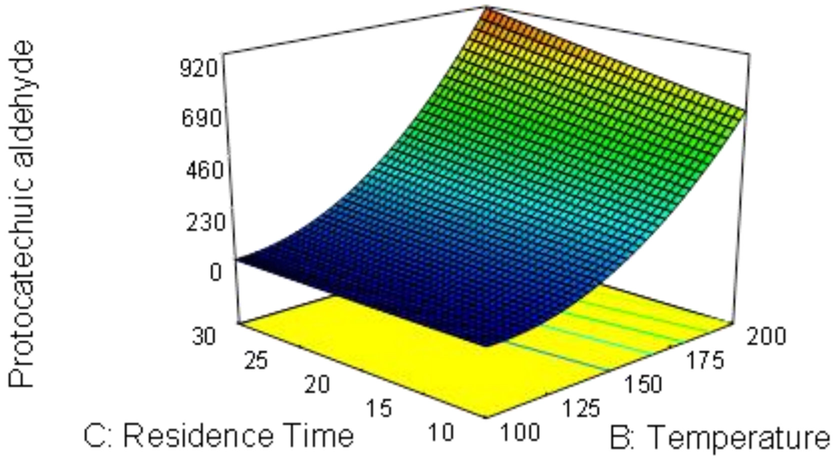


Figure 4-21 Response surface plot for the effects of temperature (°C)and residence time (minutes) on the amount of Protocatechuic aldehyde (mg/kg) dry weight with 1% solid-to-solvent ratio under subcritical water extraction of apple pomace .

Protocatechuic aldehyde demonstrates a strong effect by suppressing replication of hepatitis B-virus both in vitro and in vivo studies (Zhou et al., 2007).

4.2.8.6 Predictive Model for 5-HMF and Furfural under Subcritical water extraction

HMF and furfural constitutes furan derivatives and are by-products of hexose and xylose degradation respectively (Ulbricht et al., 1984). The subcritical water extracts of the apple pomace contained varying concentration of monomeric sugars which degraded at higher temperatures and extended periods under the batch operations to produce the two Maillard agents. Statistical analysis of variance results described a transformed square root models for both 5-HMF and furfural shown Equation 4-8.

Equation 4-8 Predictive models for 5-HMF and Furfural under the subcritical water extraction

$$\sqrt{5HMF} = -57.06203 - 1.78796A + 0.63427B - 0.26542C - 0.059059AB - 0.037606AC + 4.38730 \times 10^{-3}BC + 0.92923A^2$$

$$\sqrt{Furfural} = -77.74299 - 22.99902A + 1.22185B - 2.21599C - 0.098922AB + 0.021109BC + 3.49756A^2$$

5-HMF and furfural varied significantly with temperature and solid to solvent ratio. High temperature favours the formation of furanic compounds which are also influenced by pH (Purlis, 2010). 5- HMF and furfural were not detected at temperatures 66°C which is consistent with results reported earlier (Çam and Aaby, 2010, Schieber et al., 2001a). Lower concentrations of 2.02 mg/kg and 4.4 mg/kg of 5-HMF and furfural were recorded at 100°C respectively. Concentrations of 5767.3 mg/kg and 40330.4 mg/kg of HMF and furfural respectively were achieved at 200°C with corresponding increase in browning of extract suggesting caramerisation reactions taking place.

4.2.8.6.1 Response Surface Plots for 5-HMF

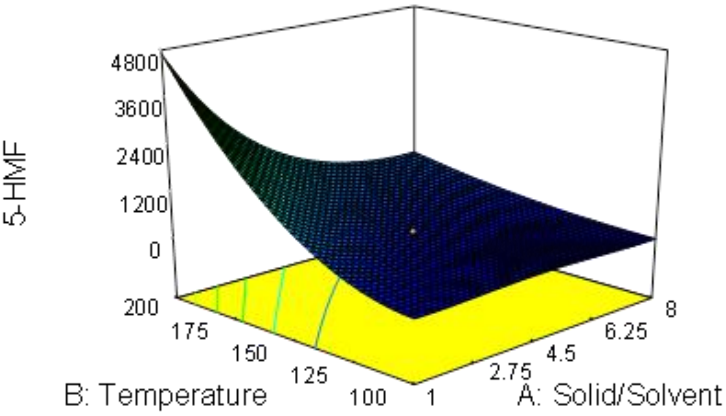


Figure 4-22 Response surface plot of effects of temperature (°C) and solid-to solvent ratio %(w/v) on 5-HMF (mg/kg)dry weight for 20 minutes residence time under the subcritical water extraction of apple pomace .

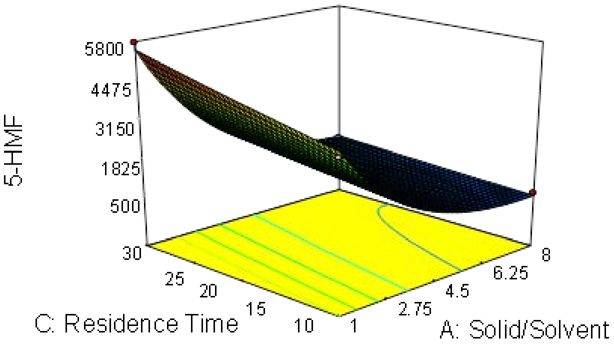


Figure 4-23 Response surface plot of effects of residence time (minutes) and solid-to solvent ratio %(w/v) on 5-HMF (mg/kg)dry weight at 200°C under the subcritical water extraction of apple pomace.

Increase in temperature under subcritical conditions decreased the pH of water which enhances the dehydration reaction to 5-HMF (Aida et al., 2007).

4.2.8.6.2 Response Surface Plots for Furfural

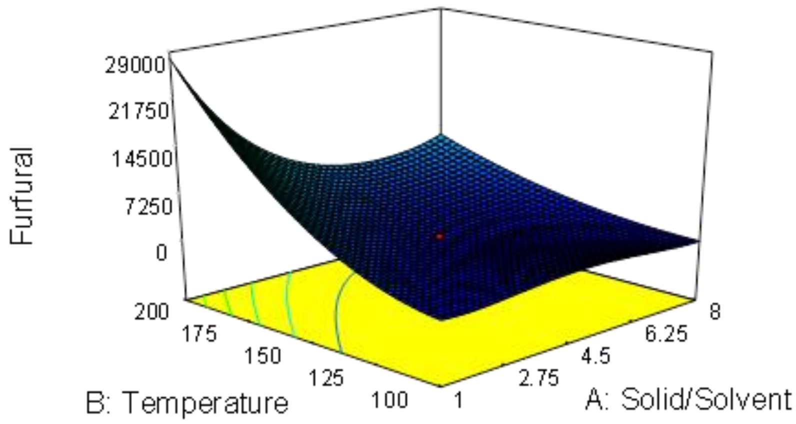


Figure 4-24 Response surface plot of effects of temperature (°C)and solid-to solvent ratio %(w/v) on furfural (mg/kg) dry weight for 20 minutes extraction time under the subcritical water extraction of apple pomace.

The diagnostic case statistics reports for 5-HMF and Furfural are shown in the appendix section. It is very important to note that higher concentrations recorded for 5-HMF and furfural may not exactly be correct, but an indication of how both concentrations would be present in extracts particularly at higher temperatures. Maximum concentrations used for the calibration curve of the furanic compounds were 1mg/l which by far below those recorded. Concentration higher than the maximum may deviate from the Beer Lamberts Law and this phenomenon could account for the higher residuals recorded for both 5-HMF and furfural in the case statistics reports.

Conditions for limiting extraction of 5-HMF while maximising extraction of polyphenolic compounds was 6.61%, Solid-to-Solvent, 101.08℃ for 25 minutes residence time, and those of furfural were 3.71%, 105.59℃ for 25 minutes residence time.

4.2.9 Comparison of polyphenolic compounds under aqueous acetone and subcritical extracts

Extracts of subcritical water were compared with those of the aqueous acetone to evaluate the efficacy of the subcritical water in overall recovery of polyphenolic compounds from the apple pomace. Concentrations of components in both extracts significantly varied although polyphenolic profiles were largely similar particularly for extracts for subcritical water at 100^{o}C.

Total phenolic content (antioxidant activity in terms of Folin-Ciocalteu method) of subcritical water extract was 2.4 times higher than those recovered under the acetone extracts. Higher temperatures under the subcritical conditions caused generation of new compounds like Protocatechuic aldehyde which can be oxidized by the Folin reagent to result in increase in antioxidant activity. The higher total phenolic value under subcritical water mediated hydrolysis could also be attributed to phenolic compounds that were not extracted under the aqueous acetone extraction. However individual polyphenolic concentrations of subcritical water extracts were lower compared to those of the aqueous acetone. Summary of maximum concentrations are recorded in Table 4-16.

Table 4-16 Optimised concentrations of responses under aqueous acetone and subcritical water

Response	**Maximum Amounts Aqueous Acetone extraction**	**Maximum Amounts Subcritical water extraction**
Total phenolic content	22.55 (mg/g GAE DW)	54.30 (mg/g GAE DW)
Fraction of solids solubilised	19.20/100g	28.2/100g
Chlorogenic acid	221.58 mg/kg	122.2 mg/kg
Phloridzin	894.62 mg/kg	250.20mg/kg
Procyanidin B2	227.810mg/kg	57.67 mg/kg
Epicatechin	193.50 mg/kg	N/A
Quercetin-3-galactoside	187.83 mg/kg	76.12 mg/kg
Quercetin-3-glucoside	128.20	23.59 mg/kg
Protocatechuic aldehyde	ND	966.40 mg/kg
5-HMF	ND	5767.29 mg/kg
Furfural	ND	40330.39 mg/kg

ND- Not detected

Results of optimisation of polyphenolic compounds during organic solvent extraction using acetone suggested water may be a good extraction solvent for Chlorogenic acid and Procyanidin B2. However the results under subcritical water reveals only about 55% of Chlorogenic acid of the acetone extracts value were recovered at 100°C under subcritical water. Yields of Chlorogenic acid from the apple pomace under subcritical water extraction could be higher but due to degradation as a result of higher temperature may form other new and compounds Concentration of Procyanidin B2 was even less as a quarter of its equivalent amounts were recovered under subcritical water extraction compared to the aqueous acetone. Phloridzin concentrations dominated among all polyphenolic compounds and 72% of the amount extracted using acetone was recorded during subcritical water extraction. The situations were not different for Quercetin glycosides as shown in the table 4.16 above. Higher temperatures under the subcritical water conditions caused degradation of phenolic compounds and targeting phenolic compounds at lower temperatures (~100^{o}C) will results in dilute extracts with lower antioxidant activity. The combination of organic solvent and water under the subcritical conditions may improve the yield.

Subcritical water was rather superior in terms of solubilisation of components from the apple pomace than organic solvent acetone. Approximately 37% more solids were solubilised under optimised subcritical conditions compared to those of aqueous acetone. Hence subcritical water was more efficient in terms of solubilisation and generation of higher antioxidant compounds.

4.2.10 Determination of Antioxidant Capacity of Subcritical Water Extracts

An in vitro assessment of antioxidant activity of subcritical water by ORAC assay was conducted according to the procedure previously outlined in section 2.2.11. However it is very important to note these results cannot represent what happens in vivo. Results of ORAC analysis are recorded in Table 4-17.

ORAC values ranges from 418 to 99285µmol/g DW of apple pomace with mean value of 11341µmol/g DW.

4.2.11 Model Selection for Antioxidant Activity by ORAC

Regression analysis was performed using the mean ORAC values measured to study the relationship between design variables and the antioxidant capacity. Summary of the ANOVA results are shown Table 4-15.

Solid/solvent ratio, and extraction temperature had significance on the ORAC value as well as their interaction ($p<0.0001$). Residence time demonstrated least significant consistent with other findings (Plaza et al, 2013). Temperature was the most controlling factor in the quadratic term. A transformed quadratic model similar to that of total phenolic came out to be the most significant ($P<0.0001$) and has an insignificant lack of fit ($P>0.05$).

Table 4-17 Mean ORAC values of subcritical water extract (μmol/g DW) of apple pomace

Run	Sample	Mean ORAC value	(± standard Error)
1	8-100-30	444	18
2	4.5-66-20	1523	112
3	0.5-150	92972	0
4	4.5-150-20	2000	16
5	8-100-10	418	14
6	1-200-10	31760	3701
7	8-200-10	1641	180
8	4.5-234-20	3776	301
9	4.5-150-20	1543	59
10	4.5-150-20	1672	86
11	4.5-150-36	1356	161
12	4.5-150-3	1192	46
13	1-100-10	7415	787
14	1-200-30	99285	7201
15	4.5-150-20	1580	170
16	8-200-30	3741	105
17	4.5-150-20	2690	228
18	1-100-30	13973	1145
19	9.5-150-20	437	16
20	4.5-150-20	3627	166

8-100-30 means 8% solid/solvent at 100°C for 30 minutes

The coefficient of variation was 9.65% with R^2_{Pri} (0.96) in agreement with R^2_{PriAdj} (0.98). Hence selected model could be used to navigate the design space. The predictive model in terms of actual factors is shown in Equation 4-9.

Equation 4-9 Predictive Model Equation for ORAC under subcritical water extraction

$$\frac{1}{\sqrt{ORAC}} = -0.22457 + 7.11598 \times 10^{-3}A + 4.99189 \times 10^{-4}B - 1.92259 \times 10^{-4}C - 3.14617 \times 10^{-5}AB + 1.68692 \times 10^{-4}A^2 - 1.75480 \times 10^{-6}B^2$$

The predictive capacity of the model was reviewed in the case statistics report showing agreements to a large extent the actual values measured and those predicted using the model.

4.2.11.1 Analysis of Response Surface Plots for ORAC

ORAC values increased with temperature from 100°C to 200°C, but decreasing exponentially as the solid/solvent ratio increases. The negative interaction coefficient of solid/solvent ratio and temperature suggest that, is unpractical to achieve higher amount of antioxidant activity by increasing both parameters simultaneously as seen Figure 4-25.

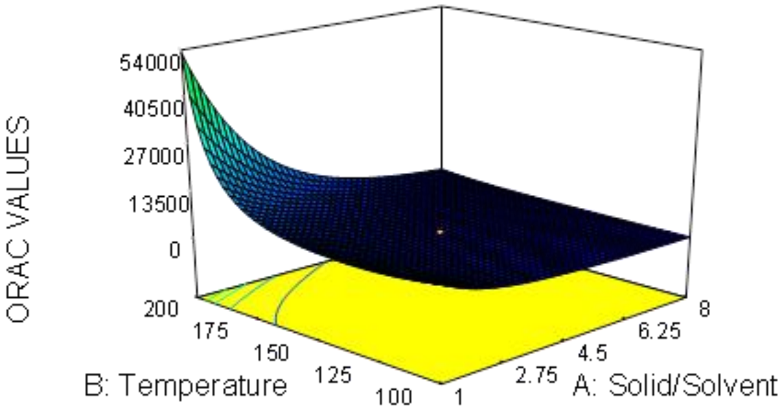


Figure 4-25 Response surface plot for effects of temperature (°C) and solid/solvent ratio %(w/v) on ORAC values (μmol TE/g DW) under the subcritical water extraction of apple pomace for 20 minutes residence time.

Antioxidant capacity by the ORAC at 200°C was about 5 times that at 100°C. An average increase of five times of antioxidant activity was reported using TEAC assay from 100 to 200°C, under subcritical water extraction of phytochemicals from plant sources (Rodríguez-Meizosoa et al., 2010). ORAC values correlates positively with total phenolic content of the extracts because similar trends were observed and can be seen clearly by their surface response behaviour. Both ORAC and total phenolic are antioxidant capacities assays with different mechanisms as previously discussed in Chapter 1. Similar observations were previously reported of strong relationship between total phenolic content and antioxidant activities (Karacabey and Mazza, 2010, Plaza et al., 2013, Yu et al., 2005). However increasing temperature increases both Total phenolic content and antioxidant activity with diminishing concentration of ordinary phenolic compounds and amount of soluble solids in extracts. Furthermore, it is very important to note that, higher antioxidant activity at higher temperatures cannot be attributed to ordinary phenolic compounds alone. Other new-phenolic compounds with antioxidant properties may be formed which could not be resolved by the chromatographic method so chosen. Several researchers have attributed the increased antioxidant activity at higher temperatures due to formation of new antioxidant compounds (Herrero et al., 2012, Plaza et al., 2010). These new compounds may have different chemical structures from the parent polyphenolic compounds because antioxidant capacities of polyphenolic compounds are affected by their chemical structure (Cao et al., 1997, Fukumoto and Mazza, 2000, Lien et al., 1999).

Water soluble Protocatechuic aldehyde identified as a degradation product of some hydrocinnamic acids was stable in the extract up to 230°C and may contribute to antioxidant activity at the high temperatures. Antioxidant activity and anti-inflammatory effects of Protocatechuic aldehyde on human umbilical vascular endothelial cells (HUVECS) has been reported (Zhou et al., 2005). Although antioxidant capacities of aldehydes are less than corresponding acids, the ORAC value of Protocateuchuic aldehyde was comparable to those of Caffeic acid and slightly higher than Chlorogenic acid (Dávalos et al., 2004). The surface response graph shows the possibility to extract antioxidant compounds at higher temperature but nonetheless this condition was not suitable as higher amounts of 5-HMF and furfural were observed. These two compounds which constitute possible agents of Maillard reaction have toxicological issues in food samples (Morales and Jimenez, 2009). Similar fears were reported previously (Çam and Aaby, 2010, Plaza et al., 2013, Wijngaard and Brunton, 2009). Hydroxymethylfurfural was shown to be initiator and promoter of colon cancer (X.M. Zhang et al., 1993), neurotoxicity (Bakhiya et al., 2009). They can undergo metabolism to 5-sulphooxymethylfurfural which to some extent may be harmful (Arribas-Lorenzo and Morales, 2010). Higher concentrations of HMF were reported to be cytotoxic which causes irritation to eyes, upper respiratory tract, skin, and membranes of the mucous and oral dose of LD50 of 3.1g/kg body weight has been reported in rats(Ulbricht et al., 1984). Unfortunately data on levels of toxicity of 5-HMF in food had not been systematically documented (Abraham et al., 2011) and therefore giving confusing estimates for recommended intake with no conclusive standards about the dangers to human health due to exposure (Janzowski et al., 2000). 40mg/kg and 50mg/kg of 5-HMF were recommended as limits in honey and apple juice respectively (EU, 2001) Other standard bodies like international Federation of fruit juice Processors and Turkish standards gave a maximum of 10mg/kg and 60mg/kg 5-HMF in fruit juices respectively (Gaspar and Lucena, 2009, TSE, 2003).

However, research on toxicological and genotoxicity effects of furfural and its derivatives are still ongoing (EFSA, 2005, NTP, 2008). Optimized conditions for ORAC values of extract with maximising majority of phenolic compounds, and minimising evolution of both 5-HMF and Furfural are 1.4% solid-to-solvent ratio at 100℃ for a residence of 30 minutes. This results is consistent with previous findings under pressurised liquid extraction of apple pomace with 60% ethanol in water where optimal antioxidant activity using DPPH assay recorded at 102℃ with minimisation of 5-HMF (Wijngaard and Brunton, 2009).Minimum concentrations for 5-HMF and Furfural predicted for optimised conditions for ORAC under the subcritical water extraction were 1.12mg/kg and 4.80mg/kg respectively which are within the acceptable limits.

4.3 Conclusions

Subcritical water mediated extraction of bioactive compounds was shown to be more efficient than aqueous acetone and was thought to be due to the simultaneous hydrolysis and solubilisation of the apple pomace thereby overcoming the potential stearic hindrance of the cell walls. Temperature played an important role in the extraction of antioxidant compounds from the apple pomace. Polyphenolic compounds were successfully extracted between 100 and 150^{o}C, and individual polyphenolic species differed in their response to temperature.

However total phenolic content and antioxidant activity as determined by ORAC assay continued to increase with increasing temperature up to 200^{o}C. Analysis of the extracts at 200^{o}C revealed the dominant polyphenolics (Chlorogenic acid, Procyanidin B2, Phloridzin and Querctin glycosides) observed up to 150^{o}C were no longer detected. Subcritical water extracts at 150^{o}C and beyond exhibited high antioxidant activities and this was apportioned to the synthesis of novel compounds which may possess potentially high antioxidant capacities. Therefore phenolic acids and flavonoids observed in extracts below 150^{o}C would have little contribution to antioxidant activities at the high temperatures. Protocatechuic aldehyde was identified for the first time only in the subcritical water extract and to date has not been identified in solvent extracts of cider apple pomace extracts. The water soluble phenolic aldehyde was stable in extract even at 234^{o}C and possibly could contribute to the high antioxidant activity at the high temperature. Therefore the stability of Protocatechuic aldehyde at this high temperature contradicts the commonly held school of thought that phenolic compounds are thermoliable and subcritical water at high temperature could be employed to recover high antioxidant compounds from the add pomace to add value to the residue.

Chapter 5

Encapsulation of Polyphenolic fraction of subcritical water extract of apple pomace with naturally occurring polymers to improve oxidative stability

5.1 Introduction

Polyphenolic compounds can undergo oxidation and therefore molecular transformation when exposed to extreme environmental conditions. The subcritical water extract derived from apple pomace was shown to contain polyphenolic compounds with relatively high antioxidant activity. Stabilisation of the high antioxidant activity is therefore very important to ensure efficacy before, and during cosmetic and pharmaceutical applications. Encapsulation has been widely studied as a route to stabilise and protect bioactive compounds against oxidation within the food, cosmetic and pharmaceutical industries (Desai and Park, 2005).

Encapsulation involves the creation of a barrier around an active ingredient thereby, modulating the interaction with the environment (Gibbs et al 1999, Mozafari 2006). The barrier material often referred to as the carrier can be derived from natural or modified polymeric material such as hemicellulose, lipids, starch, gum or synthetic polymers. Several microencapsulation techniques have been applied for food ingredients and have been discussed under section 1.7.1. Freeze and spray drying technologies have been applied extensively in recent times in the cosmetic and pharmaceutical industries to stabilise ingredients against oxidation, improve shelf-life, enhances solubility and bioavailability during applications (Joana Gil-Chávez et al., 2013, Mourtzinos et al., 2007, Szente and Szejtli, 2004). During the spray drying process, as the solvent usually water rapidly dries as result of contact with hot processed gas and causes simultaneous entrapment of the active ingredient (usually smaller) into the larger molecules (Gharsalloaui et al 2007).

The objective of this chapter was to assess the suitability of coupling extraction with encapsulation simultaneously by making use of polymeric substances co-extracted with the polyphenolic compounds as a carrier to improve stability against oxidation and to reduce the transfer rate of the polyphenolic antioxidant compounds to the external environment.

Previous experiments in the laboratory have shown hemicellulose, proteins and lignin were co-extracted with polyphenolics under subcritical water condition. Interactions between polyphenols and carbohydrates in plant materials have been established (Le Bourvellec and Renard, 2012). Chlorogenic acid, cafeic, gallic acid, kaemferol, quercetin, myricetin, and green tea flavonoids have been encapsulated with soy protein (Rawel et al., 2002). Lipid nano-capsules were used as a carrier for quercetin delivery (Barras et al., 2009). Matsunaga et al., 2014 demonstrated direct formation of microparticulate polysaccharide powders from *Ganoderma Lucidum,* a mushroom with claimed health promoting properties after hydrolysing with subcritical water between 100°C-190°C (Matsunaga et al., 2014). On-line micronisation of quercetin and its derivatives extracted from onion waste with pressurised hot water and supercritical antisolvent processes have also been reported (Andersson et al., 2012).

The current study employed spray drying technique to form an encapsulated product made up of polyphenolics and naturally occurring carbohydrate polymers co-extracted with the polyphenolics during subcritical water extraction of apple pomace. In addition, a comparative study was conducted using the subcritical water extract to form an inclusion complex upon mixing with β-Cyclodextrin, a molecule widely used for encapsulation.

Cyclodextrins are oligomers of glucose capable of forming inclusion complexes (Paramera et al., 2011). The micronised particles produced from the drying process of subcritical water extract of the apple pomace and the extract mixed with β-Cyclodextrins to form inclusion complex ,were investigated for improved storage and stability in terms of antioxidant activity. Physical characteristics such morphologies of encapsulated products and FT-IR spectra of the powders were explored to elucidate evidence of encapsulation.

5.2 Results and Discussions

5.2.1 Powder production

Freeze dried powders of both subcritical water extract and those of the inclusion complex were obtained by freeze drying portions of the samples by the procedure similar to that described in section 2.2.1.2. Corresponding spray dried powders obtained as described in 2.2.13.

Colour of the directly encapsulated polyphenolic fraction (SWE) of the apple pomace with natural occurring polymers co-extracted under the subcritical water mediated was yellowish brown. Those of the HP-β-Cyclodextrins (SWE+ HP-β-CD) encapsulated reflected the colour of the directly encapsulated product in extract and were lightly brownish.

Different conditions of inlet and outlet temperatures spray drying operations were selected by trial and error to favour the production of dried powders of the subcritical water extract. Direct encapsulated subcritical water extract (SWE) were obtained at inlet temperatures from 140 to 170^{o}C. It was difficult to obtain fine and dried powders below 140^{o}C and beyond 180^{o}C for the subcritical water extract alone whose concentration was only 2.75% (w/v) solids. Wet products were observed below 140^{o}C due to insufficient drying and sticky brown products at 180°C due to caramelisation reaction of the monomeric sugars in the extract at the high temperatures.

However spray drying of HPβ -Cyclodextrins encapsulated with the subcritical water extract (SWE+ HP-β-CD) was smooth without any challenges. The incorporation of HPβ – Cyclodextrins reduced the stickiness during spray drying operation and whose inclusion raised the glass transition temperature of the subcritical water extracts thereby reducing the stickiness (Caparino et al., 2012). Previous reported research suggested, many challenges during spray drying of substances containing higher sugar levels (glucose, fructose, sucrose) without a wall material. However the current research has demonstrated successfully, production of micronized powders from subcritical water extracts from apple pomace.

5.2.2 Characterisation of Powders

5.2.2.1 Moisture Content of powder

Residual water or moisture content associated with solid raw materials for pharmaceutical and cosmetic applications were known to affect significantly their physico-chemical properties. Properties such as rate of dissolution, flow and compactibility, deterioration or degradation are affected by residual water as a result of prolonged exposure to the surroundings (Ahlneck and Zografi, 1990).

Average moisture content (g water/100g) of the powders are shown in Table 5-1.

Table 5-1 Some physico-chemical properties of spray dried powders of subcritical water extract and extract encapsulated in HPβ-Cyclodextrin

Powder Sample	**Moisture %**	**Hygroscopicity g/100g**	**Density g/cm^3**	**Particle size µm**
SWE+HPβ-CD	5.59 ± 0.4	5.08 ± 0.01	1.5030 ± 0.003	3.46 ±0.04
SWE	7.22 ± 0.01	9.30 ± 0.11	1.5602 ± 0.001	3.41 ± 0.15

Moisture content of directly encapsulated subcritical water extract powders were significantly different from those of the inclusion complex by HPβ-cyclodextrin which suggested that increasing the solid content by mixing with HPβ-Cyclodextrin decreases the amount of moisture content of powders (approximately 22.6% reduction). Moisture content of microcapsules depended on type and concentration of the wall material (Şahin Nadeem et al., 2011). Similar results have been documented during spray drying of phenolic compounds using maltodextrin (Pang et al., 2014). Higher moisture content of powders by subcritical water extracts alone suggested powder may be taking up water from the surroundings.

5.2.2.2 Hygroscopicity of Powders

Hygroscopicity is defined as the estimation of the ability of a substance to absorb moisture from a relatively high humid environment. The property is an important one to consider during storage of the powders (Haugaard et al., 1978, Rodríguez-Hernández et al., 2005, Slade et al., 1991). Hygroscopicity of the spray dried powders were determined according to the procedure described in section 2.2.16 and results are listed in Table 5-1

Spray dried powders of the subcritical water extract (SWE) exhibited high hygroscopicity compared with HPβ- cyclodextrin encapsulated subcritical water extract. The absorptive capacity of micronised subcritical water extract in moist environment was approximately twice that of HPβ -cyclodextrins encapsulated spray dried powder. Addition of HPβ -cyclodextrin reduces the hygroscopic nature of the extract. Data on hygroscopicity of spray dried powders of subcritical water extract of apple pomace were not readily available for comparison. However, mean hygroscopicity of 23g/100g was reported for apple pomace during vacuum drying at temperatures between 80 and 110°C with hygroscopicity decreasing at higher temperatures (Yan and Kerr, 2013). Hygroscopicity of instant coffee ranges from

9.09 - 10.32% and that of tomato soup powder 4.20 - 6.64% (Jaya and Das, 2004). A cut-off value for hygroscopicity of mango power was reported to be 5.13% to 9.38% (Caparino et al., 2012). Many researchers have reported reduction of hygroscopicity of extracts when carriers were added. For instance hygroscopicity of acai extract reduced significantly when significant maltodextrin concentration was used (Tonon et al., 2008). Least hygroscopicity of powder was obtained using 23% of maltodextrin with Cactus pear juice (Rodríguez-Hernández et al., 2005). Reduction of hygroscopicity of mango powder with maltodextrin has also been reported. Sugars are generally hygroscopic and readily cause stickiness and lowering glass transition temperatures of high sugar containing powders (Jaya and Das, 2004). Glucose and fructose are accountable for the strong relationship with water molecules due to the polar nature of their terminal structures and leaves many products caking over storage (B. R. Bhandari et al., 1997, Haugaard et al., 1978, Slade et al., 1991). Powder stickiness can be reduced by addition of carriers like cyclodextrin or maltodextrin to increase glass transition temperature thereby eliminating the problem during storage (Jaya and Das, 2004, Tonon et al., 2008). The physical state of the drying powders under the spray dry operation were already changing rapidly with stickiness during collection consistent with previously reported investigations for sugar containing samples (B. R. Bhandari et al., 1997). Stickiness of powders was lesser in the SWE+HPβ-CD compared to the auto-encapsulated subcritical water extract (SWE).

5.2.2.3 Density and Particle size of Powders

Many methods for determining particle density of solids have been applied and reported in literature (DeCarlo et al., 2004, Ehara and Shin, 1998, Le Bronec et al., 1999, McMurry et al., 2002, Morawska et al., 1999, Pitz et al., 2003, Schreiner et al., 2002).

However density of particles that made up the powder under this investigation were determined by gas displacement technique and described fully in section 2.2.14. Gas displacement methods are thought to be one of the most reliable to obtaining ‘’true, absolute and apparent volume and density’’ (Micrometrics, 2015). Average density of the powders recorded for 5 cycles at different times is listed in table 5-1 and graphically shown in Figure 5-1.

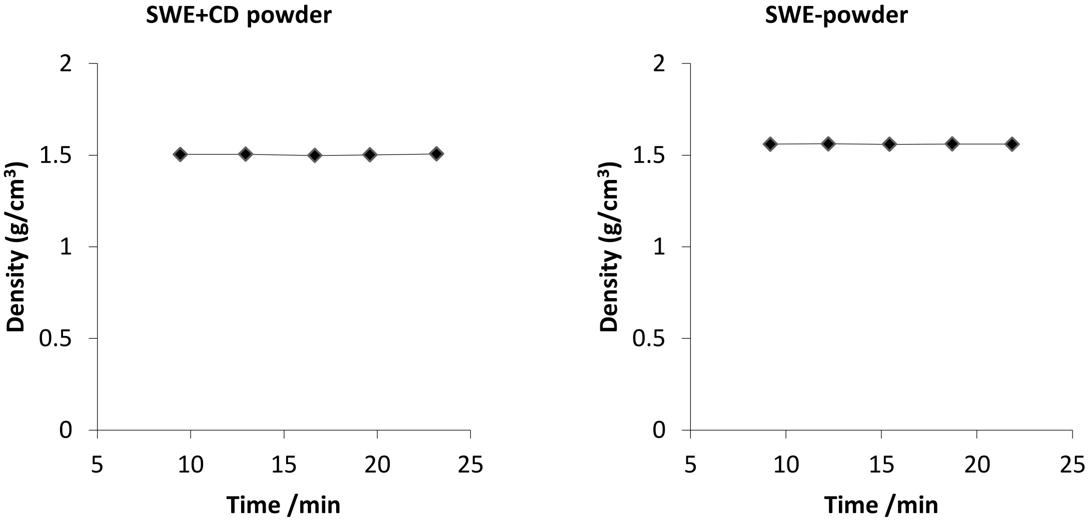


Figure 5-1 Density of powder as a function of time under 5 cycles with purge fill pressure of 19.50psig and equilibrating rate at 0.020psig per minute.

The SWE Powder was slightly denser than powders obtained after mixing with HPβ-Cyclodextrins. The marginal increase in the volume of the HPβ-Cyclodextrins can be attributed to the less dense nature of the Cyclodextrins and may have higher volume compared to the compact co-extracted polymers with polyphenolics under subcritical water extract.

Particle size estimation of powders was done using the HELOS/RODOS laser diffractometer described in section 2.2.15 at a pressure of 4-bar and recorded in table 6-1. The cumulative distribution curves for both powder samples are presented in Figure 5-2.

Mean particle size of (SWE+HPβ-CD) and SWE were 3.42µm and 3.35µm respectively. Differences in the average particle size were not significant ($p<0.05$). The closeness in the value of particle size for both could be because the feeding rate during spray dry operations were similar. Different particle sizes were reported to have been produced due to different pumping rates in spray dry operations (Nath and Satpathy, 1998).the marginal increase could also be due to higher solid content of the SWE+HPβ-CD due to the inclusion of the Cyclodextrins.

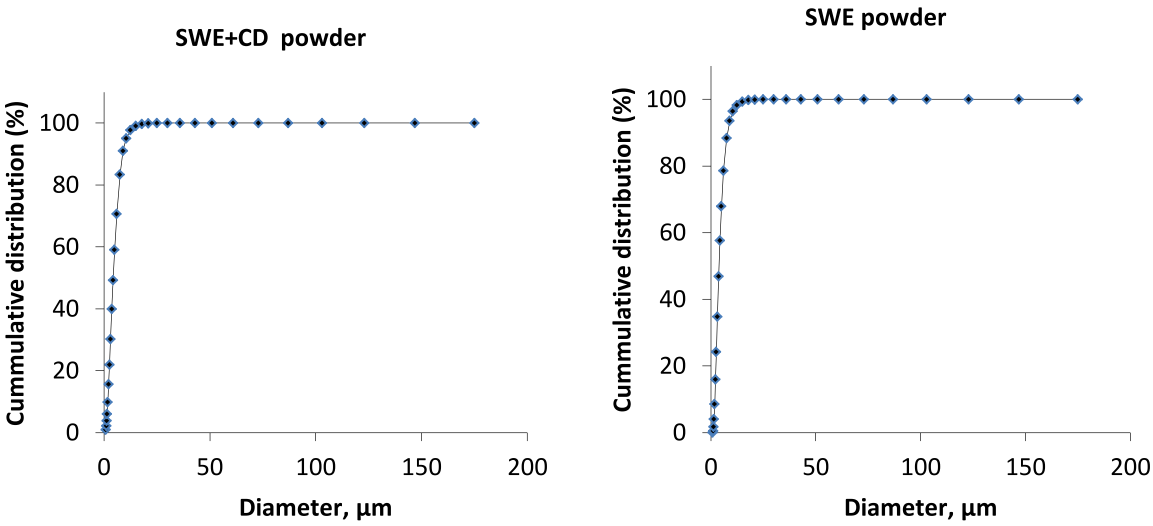


Figure 5-2 Cumulative distribution of particle size percent vs the upper limit of each size class from the HELOS and RODOS particle size analyzer using density values of 1.5g/cm^3 (SWE+CD powder) and 1.56g/cm^3(SWE powder)

The ESEM morphology analysis reaffirms, particle size were in micrometres. Micronized powders are suitable for cosmetic and pharmaceutical formulations and can help determine how much of the particles in nano size can be present in the formulation. Particle size of raw material for cosmetics influences the physical appearance, feel, stability of the end products. Particle size analysis is an indicator of quality and performance as size impacts flow and compaction properties. Larger particles will usually flow better than smaller particles (HORIBA, 2012). Particle sizes below 0.1µm are not good for cosmetic formulations and the issue had been addressed by FDA-United states and the cosmetic industry (FDA, 2007, Miller et al., 2006). Based on the characterization so far the HPβ-CD encapsulated powder would be preferred to the powder generated from the subcritical extract only for pharmaceutical and cosmetic formulations.

5.2.2.4 Morphology of Powders

Particle morphology of Pure HPβ-Cyclodextrins, freeze and spray dried encapsulated products were observed under Environmental Scanning Electron Microscopy (ESEM) as described in section 2.2.17 Nature of the crystals from the images captured helped to draw meaningful conclusions about the micronisation process. Particle morphologies of freeze dried samples were significantly different from spray dried samples. Morphology of the freeze dried subcritical water extract showed smooth flake-like and aggregates of particles with varying sizes at one location and amorphous, porous at another (Figure 5-4). Similar micrographs were observed and reported typically for freeze dried powders (Caparino et al., 2012, Che Man et al., 1999, Desobry et al., 1997, Laine et al., 2008). The SEM images with different resolutions are shown in Figure 5-4.

SEM images of the freeze dried encapsulated subcritical water extracts with HP β-cyclodextrins consisted of smooth spherical surface with less shrunken. Absence of cracks or pores in the freeze dried microencapsulated with HP-β-CD could be an indicator of preservation of the active ingredient. Similar micrographs for freeze dried quercetin encapsulated in β-CD were observed and reported (Pralhad and Rajendrakumar, 2004). The ice in the material during the freeze drying process prevented the collapse and shrinkage of the particles (Ratti, 2001) see Figure 5-5.

However shrinkage or formation of dent particles in the spray dried powder was pronounced. The combined effects of drying rate and the mechanism of atomization occurring at the initial drying stages were responsible for this behaviour (Rosenberg et al., 1985, Sheu and Rosenberg, 1998). Viscoelasticity of the wall material prior to expansion of droplets could possible contribute to the deflated morphology and rapid solidification due to high rate of drying leading to formation of crust on particle surface which hinders the inflation of the microcapsules hence shrunken surface (Anandharamakrishnan et al., 2007, Rajam et al., 2012).

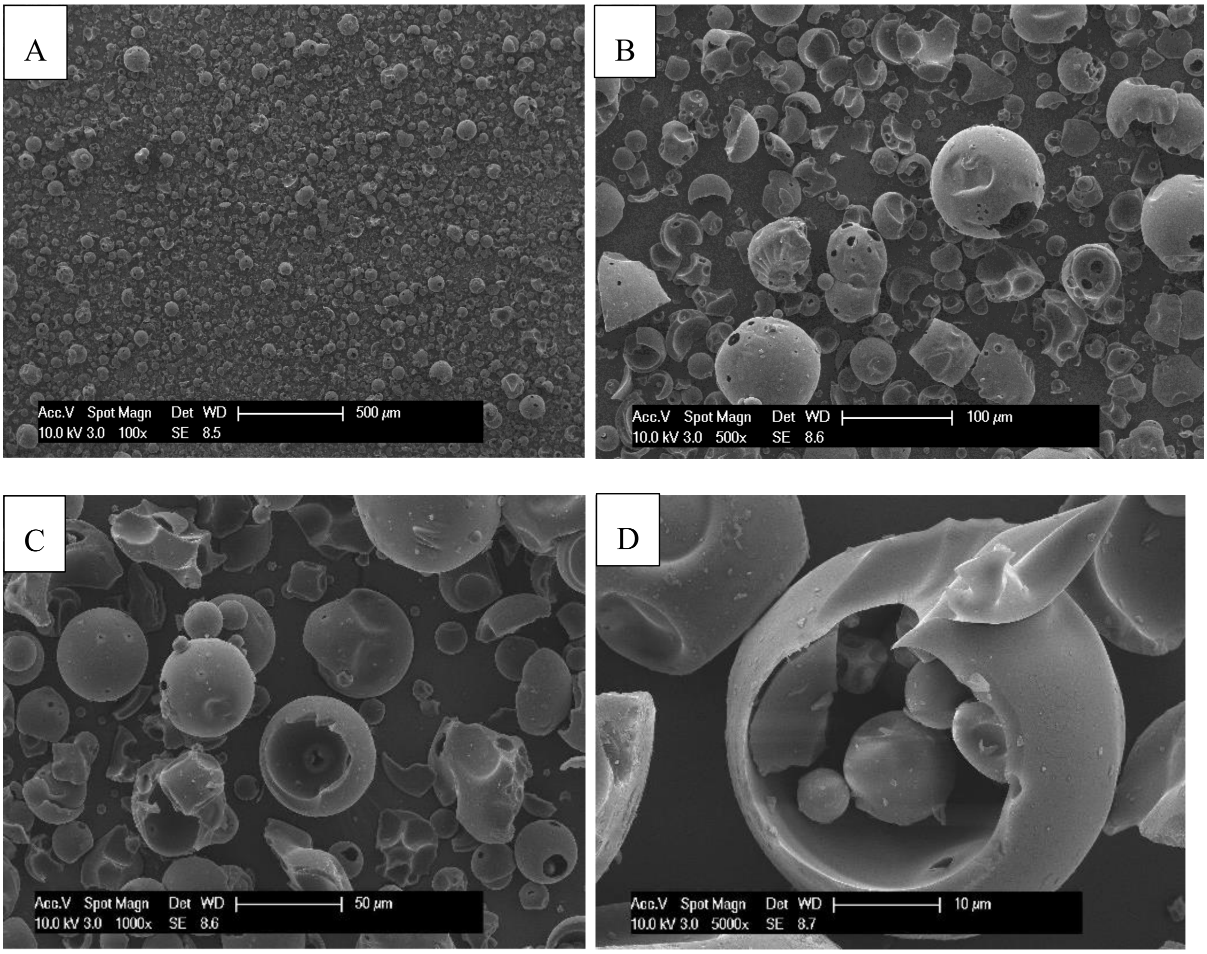


Figure 5-3 SEM images of pure HPβ-Cyclodextrin s Powder ; with Magnifications; A=100X; B=500X; C=1000X and D=5000X

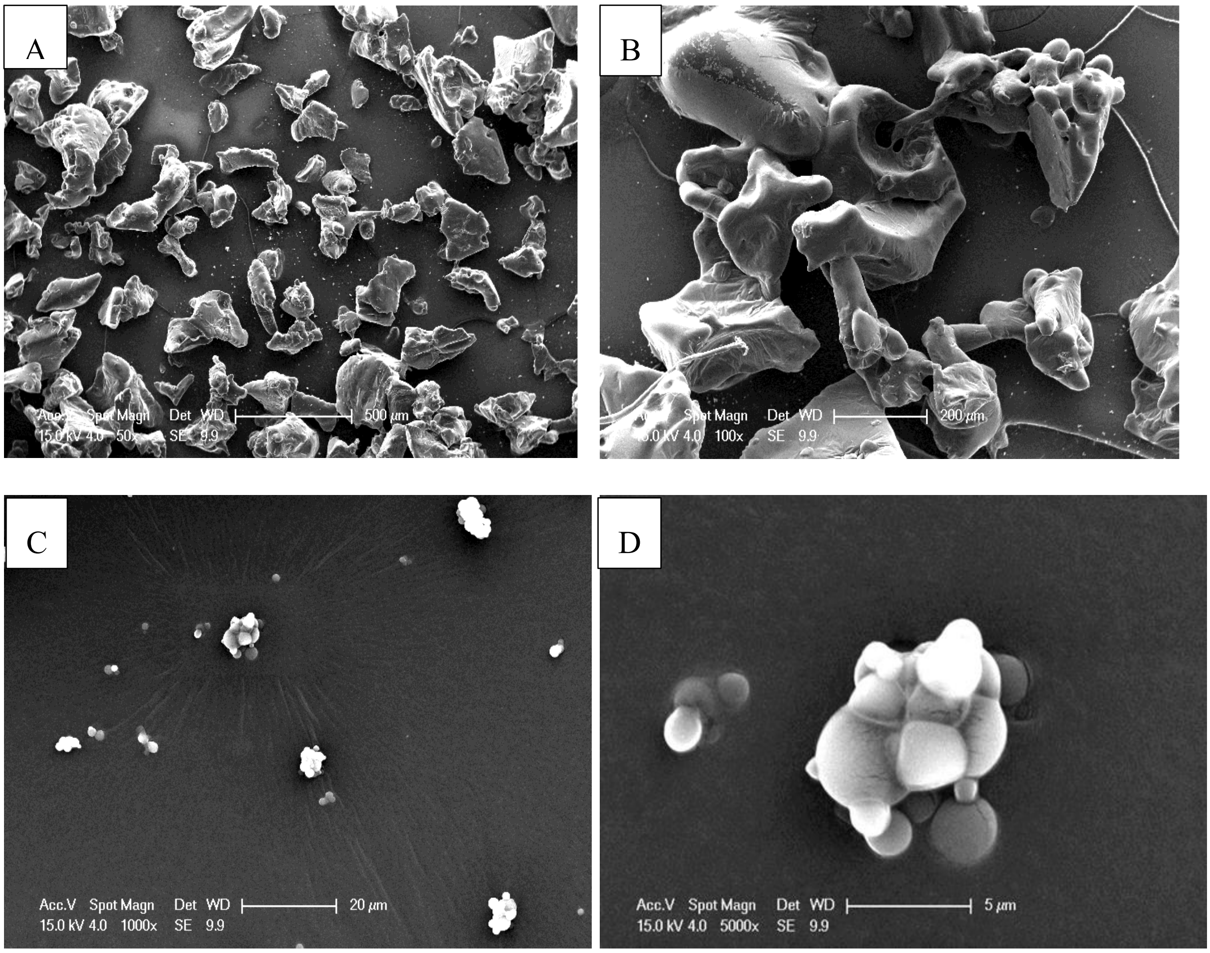


Figure 5-4 SEM images of freez dried encapsulated phenolic fraction with natural polysaccharides co-extracted under subcritical water extraction of apple pomace(SWE); with Magnifications; A=50X; B=100X; C=1000X and D=5000X

Crust formation on surfaces of powder suggests incomplete encapsulation that yielded the dented morphology (Figure 5-6) (Poomkokrak et al., 2015). Structural collapse and blow-holes on the surface of spray dried microencapsulated β-Cyclodexrin has also been reported (Al-Hakim and Stapley, 2004, Hundre et al., 2015, Rajam et al., 2012). Presence of holes on the cylindrical/spherical surfaces within microencapsulated structure could also indicate incomplete encapsulation because similar structures were seen in β- Cyclodextrins SEM images alone. The solid content of the HPβ-Cyclodextrins was in excess compared to subcritical water extract (4:1) and the possibility of incomplete encapsulation cannot be discounted. The subcritical water extract which is a complex mixture of molecules with varying sizes could have an influence in the morphology of encapsulated HPβ - Cyclodextrins. The polyphenolic fraction with natural carbohydrates co-extracted could be bigger in terms of structure for HPβ - Cyclodextrins to host The shape of HPβ - Cyclodextrin is spherical with a hollow hole and changed as a result of the incorporation consistent with results of Cyclodextrin encapsulation (Scalia et al., 1999).

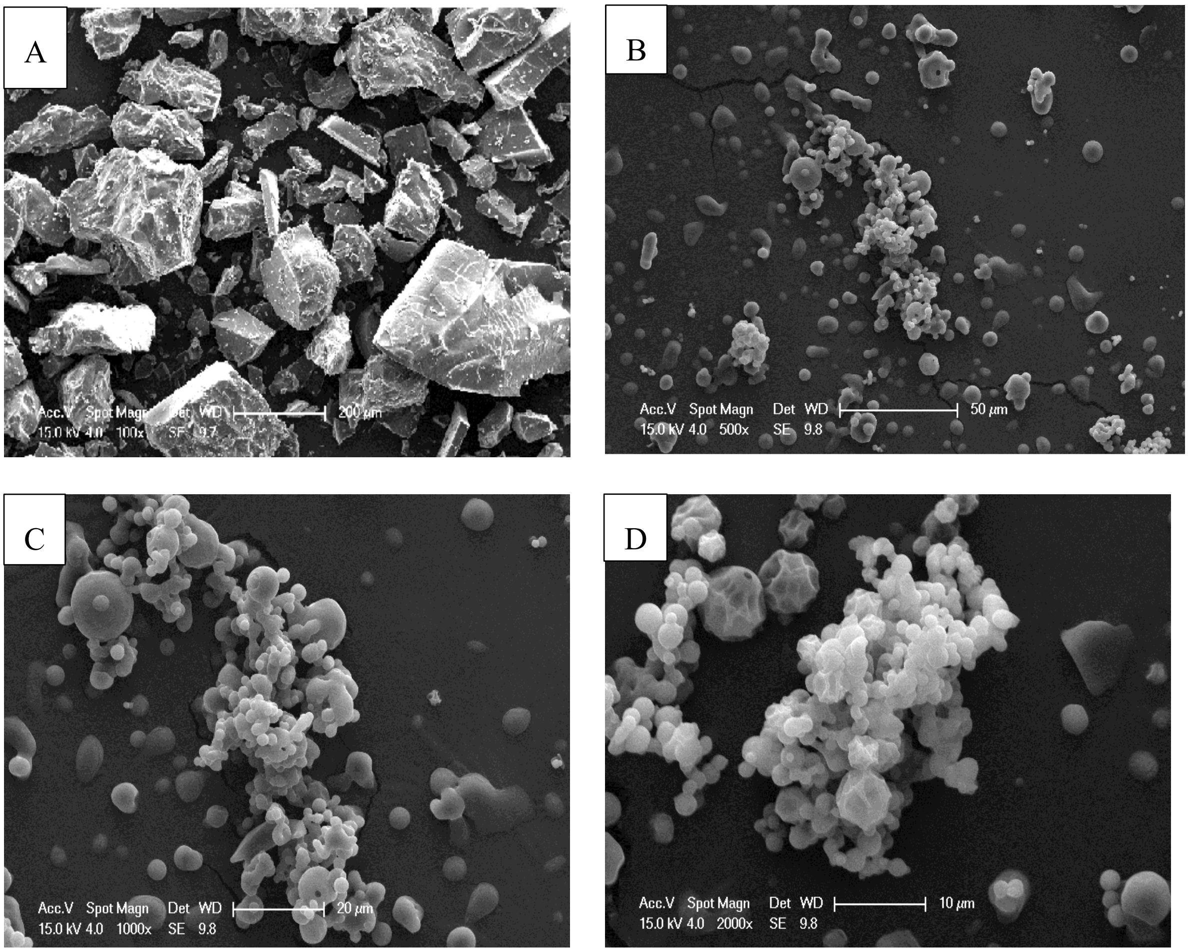


Figure 5-5 SEM images of freez dried encapsulated subcritical water extract of apple pomace with HP-β-Cyclodextrins (SWE+ HP-β-CD);with Magnifications; A=100X; B=500X; C=1000X and D=2000X

Morphologies of spray dried subcritical water extract was an agglomerated one comprising of smaller and bigger molecules crosslinked together (Figure 5-7).

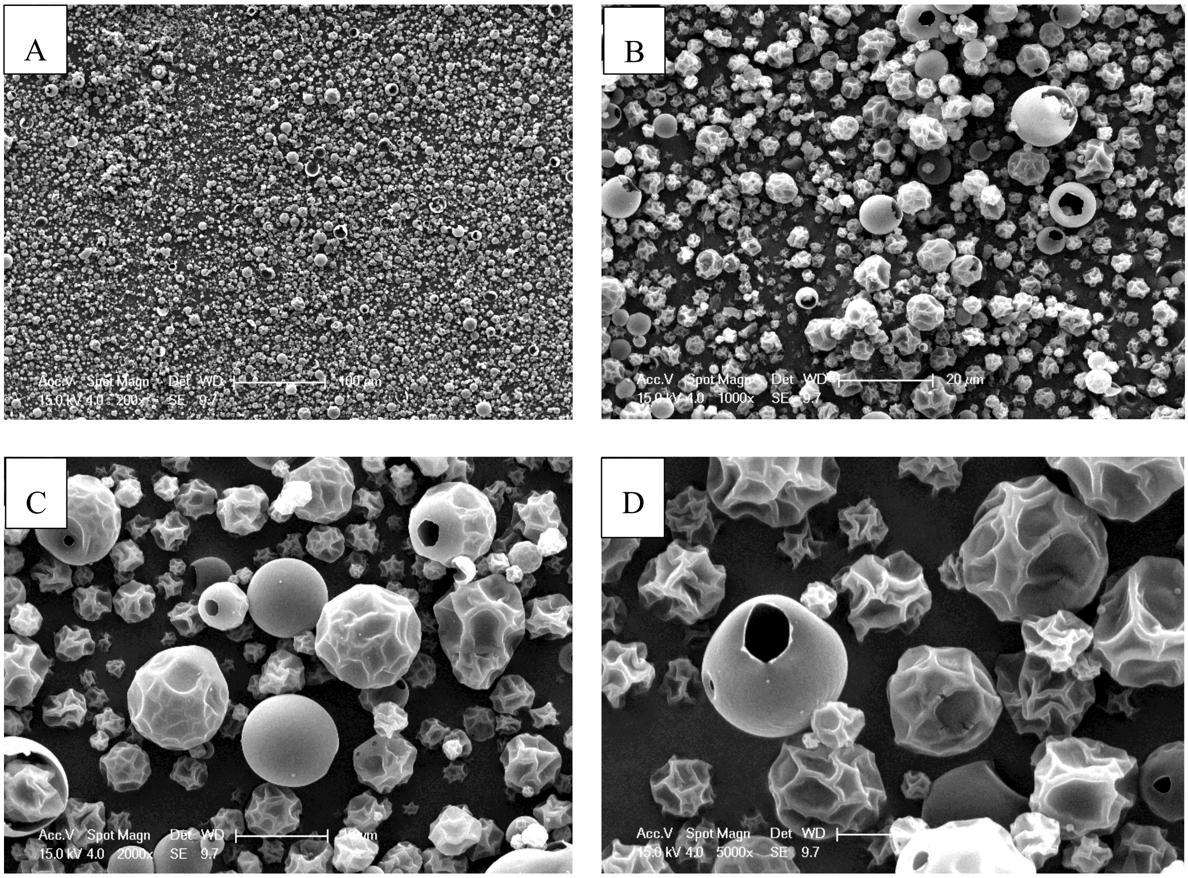


Figure 5-6 SEM images of spray dried encapsulated subcritical water extract of apple pomace with HP-β-Cyclodextrins (SWE+ HP-β-CD) with Magnifications; A=200X; B=1000X; C=2000X and D=5000X

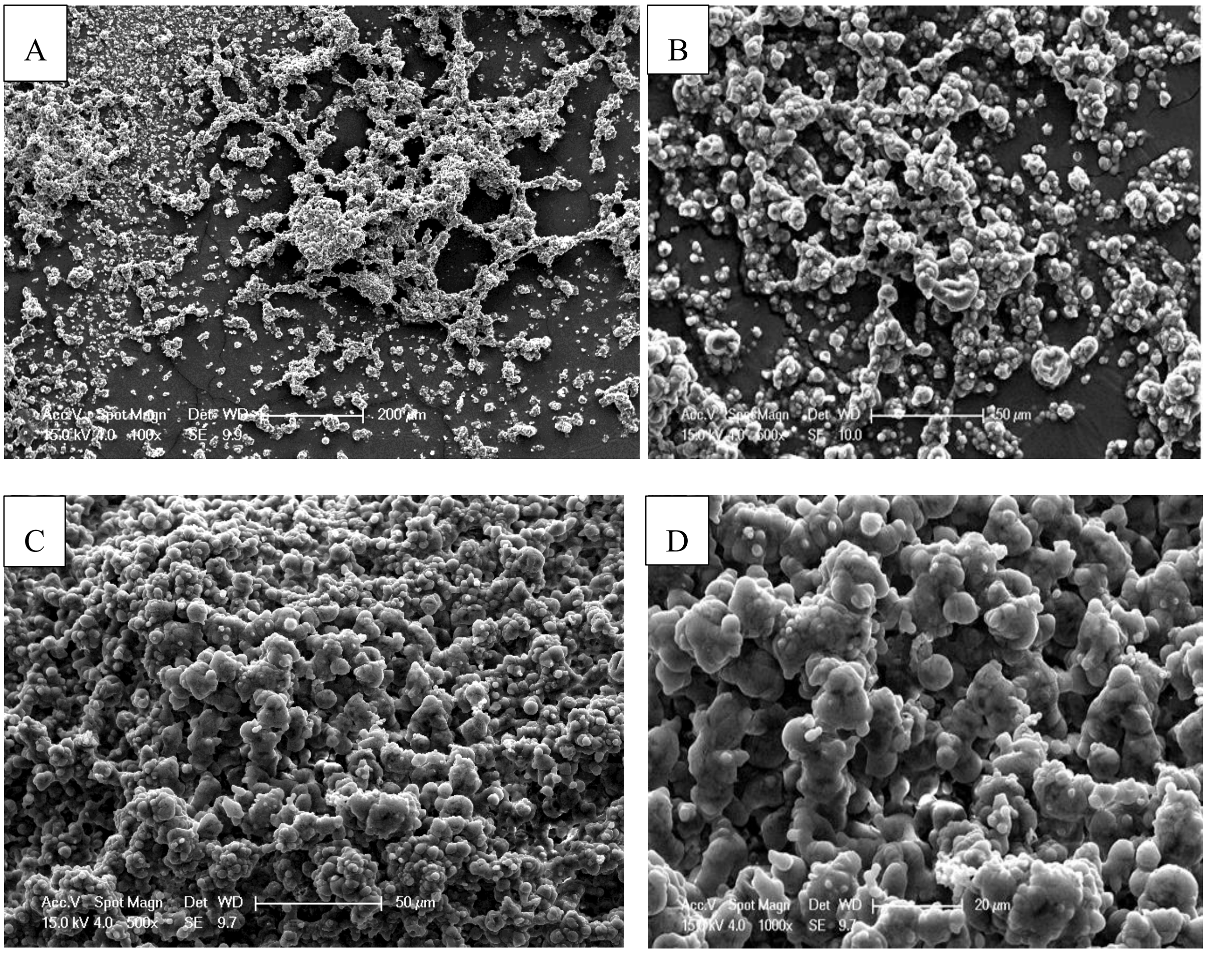
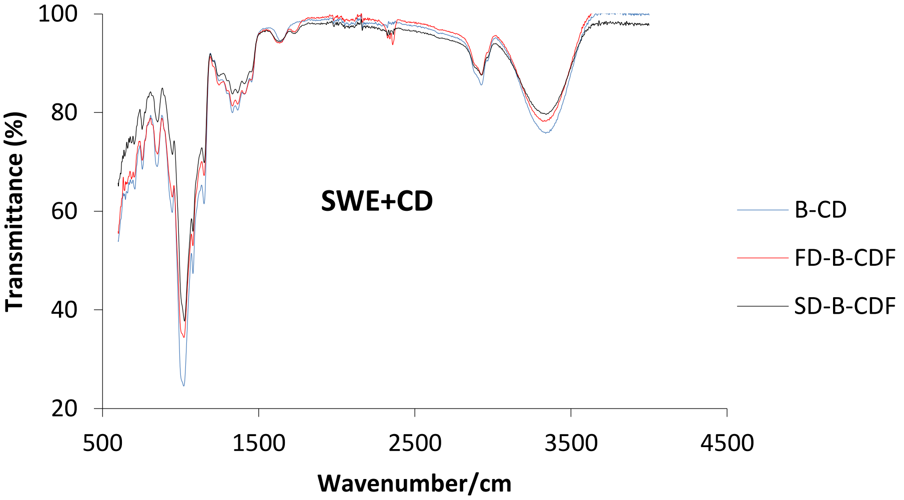


Figure 5-7 SEM images of spray dried encapsulated phenolic fraction with natural polysaccharides co-extracted under subcritical water extraction of apple pomace(SWE) with Magnifications; A=100X; B=200X; C=500X and D=1000X

5.2.3 Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR spectroscopy was an additional technique used to study the interaction between HP-β-CD and the directly encapsulated subcritical water extract to support evidence of encapsulation. FT-IR application was based on the theory that, bonds or group of bonds vibrates or oscillate at defined frequencies. The vibrational spectra are unique physical characteristics or properties of molecules and therefore can be employed as a fingerprint for identifying an unknown by simply comparing the spectrum with that of a standard spectrum previously determined (Coates, 2000).



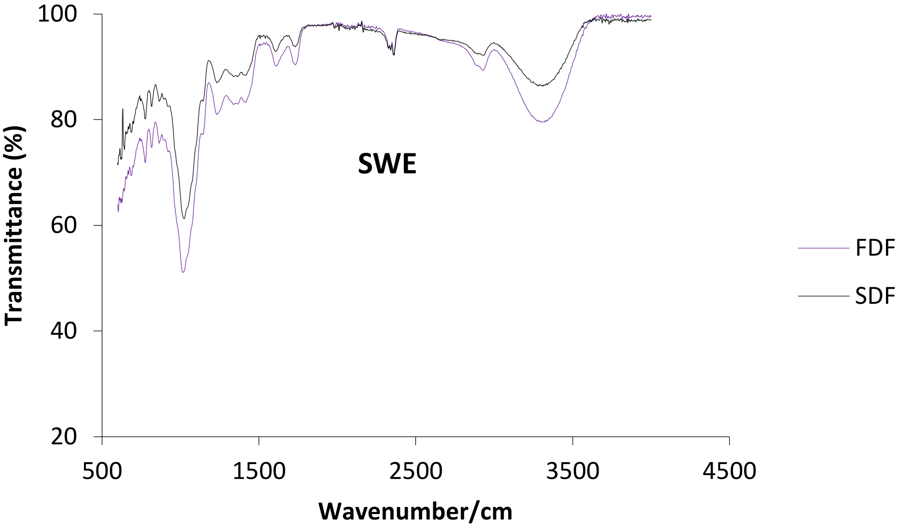


Figure 5-8 FTIR-spectra of HPβ-cyclodextrin(B-CD), spray dried HPβ-cyclodextrin with SWE (B-CD-SDF), Freez dried HPβ-cyclodextrin with SWE (FD-B-CDF), spray dried SWE (SDF) and Freeze dried SWE (FDF).

Molecular structure of the powders were characterised by exploring representative functional groupings within the compounds. Scanning was done within 4000cm^{-1} – 600cm^{-1} wavenumbers according to the procedure described in section 3.2.13. Infrared spectra of HPβ-

Cyclodextrin spray and freeze dried HPβ-Cyclodextrin with SWE, spray and freeze dried SWE, powders are shown in Figure 5-8.

There was no significant difference in the FTIR spectra between spray and freeze dried encapsulate products, only slight variations in the intensities which can be attributed to mode of dehydration. The spectra were monitored for the presence and absence of important peaks/bands (frequency) within a given spectrum. Functional groupings that were considered for the monitoring are shown in Table 5-2.

Table 5-2 Infrared Absortption characteristics of selected functional groups (Koji Nakanishi, 1963)

Functional group	**Bond**	**Wavenumber(frequency) cm^{-1}**
Alcohols and phenols	O-H stretching	3500-3200
Alkanes	C-H stretching	3000-2850
General carbonyls	C=O stretching	1760-1665
Carboxylic acids	C=O stretching	1760-1769
Esters and others	C-OH stretching	1320-1000

The FTIR spectrum of pure HPβ-Cyclodextrin contains all the functional groups listed in Table 5-2 , with the exception of carboxylic acid group (C=O). However the C=O was present in the direct encapsulated product from subcritical water extract of the apple pomace and was observed in the encapsulated HPβ-Cyclodextrin with the subcritical water extract as shown in Figure 5-8.The different chemical structure showed in the FTIR spectra of HPβ- and encapsulated Cyclodextrin was as a result of the interaction between the polyphenolic fraction and the co-extracted carbohydrate polymers in extract with HPβ-Cyclodextrin. The spectrum of the incorporated HPβ-Cyclodextrin showed a shift in the frequencies, and increased intensity due to the involvement of hydrogen bonding and generally shifted bands to higher frequencies as shown in Table 5-3 and Table 5-4.

Table 5-3 Comparison between frequencies of pure HPβ-CD and corresponding inclusion complex for spray dried powder

Bond	**Frequency**		**Change Δδ**
	HPβ-CD	**HPβ-CD-Inclusion Complex**	
O-H	3338.18	3341.07	+2.89
C-H	2925.48	2926.45	+0.97
C=O(general carbonyl)	1643.05	1627.63	-15.42
C=O carboxyl	-	1731.76	+1731.76
C-OH	1020.16	1024.98	+4.82

Table 5-4 Comparison between frequencies of pure HPβ-CD and corresponding inclusion complex for freeze dried powder

Bond	**Frequency (cm^{-1})**		Change Δδ
	HPβ-CD	HPβ-CD-Inclusion Complex	
O-H	3338.18	3317.93	-20.25
C-H	2925.48	2928.38	+2.90
C=O(general carbonyl)	1643.05	1634.38	-8.70
C=O carboxyl	-	1726.94	+1726.94
C-OH	1020.16	1021.12	+0.96

Carboxylic acid group (C=O) is peculiar to polyphenolic compounds and a strong band was reported to have appeared around 1740cm^{-1} when quercetin was encapsulated in a surfactant Poloxamers (Fraile et al., 2014). A shift of the ester (C-OH) band from 1183- 1206 cm^{-1} (Δδ= +23) for dimethyl-β-Cyclodextrin (Chun and Yun, 1993) and 1180 to 1154cm-1 (Δδ= +26) for Proxicam complexed with β-Cyclodextrin (Otero-Espinar et al., 1992).

Results so far confirmed interaction between HPβ-CD and the phenolic extract but not very indicative enough to support evidence of encapsulation because FTIR spectroscopic method is less clarifying technique compared to other methods suitable for detecting inclusion complexes (Baboota et al., 2005, Erden and Çelebi, 1988, Lee et al., 2006).

5.2.4 Stability Test

Prior to the stability test, the antioxidant activity of the liquid subcritical water extract of the apple pomace and corresponding spray dried powders were determined by both ORAC assay described in section 2.2.11 and the total phenolic content. The results are shown in Table 5-5 and Table 5-6.

Table 5-5 Antioxidant activity changes of subcritical water extract before and after spray drying

Antioxidant activity	**Subcritical water extract**		**Percentage loss in antioxidant activity (%)**
	Before spray drying	**After spray drying**	
TPC (mg/l) GAE	574.1 ± 13.9	318.8 ± 11.2	44.6
ORAC(μmolTE/g)DW	1517.6 ± 93	811.7± 20	46.5

Table 5-6 Antioxidant activity changes of subcritical water extract with HPβ-CD before and after spray drying

Antioxidant activity	**Subcritical water extract with HPβ-CD**		**Percentage loss in antioxidant activity (%)**
	Before spray drying	**After spray drying**	
TPC (mg/l) GAE	530.0 ± 4.4	513.5 ± 16	3.2

Clearly from the results it shows that spray drying had significant impact on antioxidant activity. Average loss in antioxidant activity of the directly encapsulated product derived from the spray drying process was 46.5% and that of the inclusion complex 3.2%. The loss could be due to degradation of thermo sensitive antioxidant compounds in extracts. Decrease in polyphenolic content by 25% had been reported after spray drying of elderberry juice and the polyphenolic content decreases further with increasing inlet temperature (Murugesan and Orsat, 2011). In related findings, decreases of 28 to 50% in antioxidant activity were observed during spray drying of extracts from grape pomace (Larrauri et al., 1997). Also approximately 42% loss in antioxidant activity reported during spray drying of *Momordica cochinchinensis,* fruit aril powder (Kha et al., 2010).

Higher temperatures under subcritical water extraction already demonstrated the diminishing concentration of dominant polyphenolic compounds at lower subcritical water temperatures as seen in Chapter 4 .So the higher inlet temperatures under spray drying process significantly affected antioxidant activity and of total polyphenolic content of the subcritical water extract. The subcritical water extracts were obtained at 150^{o}C and then spray dried at 170^{o}C, and the increased in temperature had negative effect on both the antioxidant activity and Polyphenolic content. Degradation of active compounds with increasing temperature had already been reported (Desobry et al., 1997, Kosaraju et al., 2006). However the HPβ-CD demonstrated significant protection of the polyphenols as shown in the results above.

5.2.4.1 Storage stability Test

The relatively high hygroscopic and caking nature of the directly encapsulated powder (SWE) from subcritical water extract was a potential challenge to the stability studies to be conducted in the solid form. However aqueous forms were used for analysis because molecular encapsulation can occur both in the solid or in a solution (Singh et al., 2010). Both solid and liquid state inclusion complexes of β-Cyclodextrin with the glycosides, hesperidin, hesperetin, narigeninn and naringin have been reported using NMR, FT-IR, DSC and X-ray techniques to support evidence of encapsulation (Ficarra et al., 2002, Fronza et al., 2002).

Loss of antioxidant activity of SWE and SWE+ HPβ-CD were monitored at facilitated conditions of 65^{o}C to study the effect of time on the retention of polyphenolic antioxidant compounds. The procedure had been described in section 2.2.19. A Control experiment using Chlorogenic acid was set up similarly to aid in the understanding of stability of phenolic compounds against time at the facilitated conditions. Folin Ciocalteu assay proposed among standardised methods for quality control and antioxidant activity determination was adopted and assay was very straightforward and reproducible. Assay principle and procedure had been outlined in section 2.2.5.

The variations in antioxidant activity during the storage are presented in Figure 5-9. As shown in the Figure 5-9 , antioxidant activity decreases with time at the constant temperature 65°C. Decrease of 44% of antioxidant activity of the subcritical extract alone compared 25% loss of the subcritical water encapsulated in HPβ-CD over the 35days period.

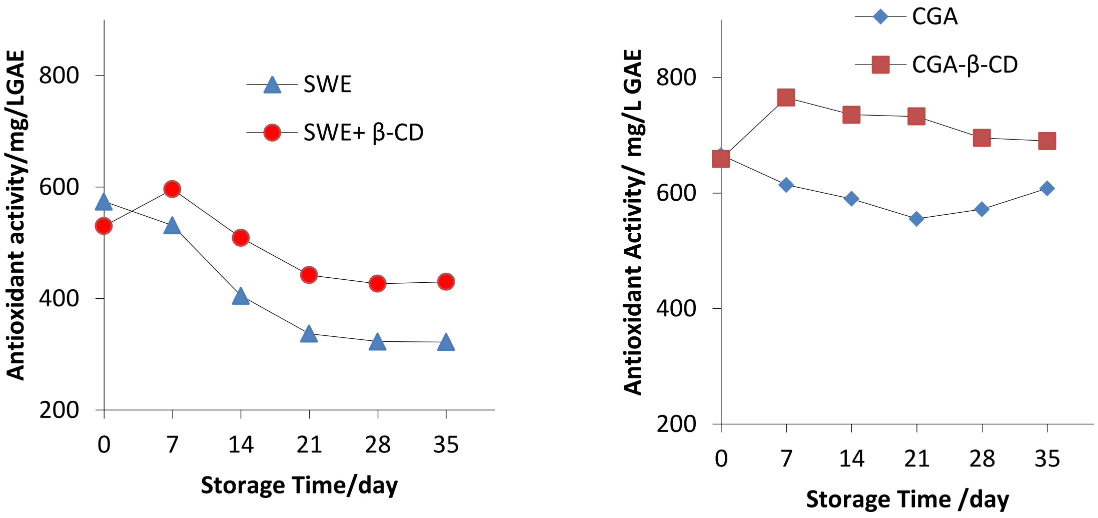


Figure 5-9 Antioxidant activity for subcritical water extract (SWE), extract with cyclodextrin (SWE+β-CD), Chlorogenic acid (CGA) and Chlorogenic acid with cyclodextrin (CGA-β-CD) following storage at 65°C for 35 days.

Initial antioxidant activity of the HPβ- Cyclodextrins included samples of both subcritical water extract and standard Chlorogenic acid were less because HPβ- Cyclodextrins displaced some volume of sample prior to analysis. However antioxidant activity sharply increases within the 7 days period and began to decrease thereof. One –way ANOVA of antioxidant activity tested against time for each treatment with post hoc Tukey comparisons at 95% confidence interval were conducted. The null hypothesis was that all means (antioxidant activity) were equal against the alternative hypotheses that, at least one mean (antioxidant activity) was different. The results are shown in Table 5-7.

The Tukey pairwise comparisons using the Tukey method at 95% confidence interval for all treatments are shown in Table 5-8. Means of antioxidant activities that do not share same letter for a particular set of treatment are significantly different ($p<0.05$).

There was no significant change in antioxidant activity for subcritical water extracts within the first 7days ($p>0.05$). However antioxidant activity changed significantly after the 7days ($p<0.05$) and no significant differences were observed from 14- 35days ($p<0.05$). In the control experiment using Chlorogenic acid, antioxidant activity significantly changed after 7days ($p>0.05$) and no significant difference in antioxidant activity measured for 14-28 days were observed ($p<0.05$). Antioxidant activity of the 35th day of Chlorogenic acid was significantly different and this may be as a result of high antioxidant activity of degradation products of Chlorogenic acid or may simply be an experimental error. For the HPβ-CD complexes, antioxidant activities at day 7 were significantly higher compared to day 0 (initial) antioxidant activities ($p<0.05$).No significant difference in antioxidant activities for days, 21,28 and 35 for HPβ-CD encapsulated subcritical water extract were observed ($p>0.05$).

Similarly no signficant difference in antioxidant activities for days 0, 28, 35 for HPβ-CD encapsulated Chlorogenic acid ($p>0.05$).

ANOVA was performed with post hoc Tukey analysis at 95% confidence level to establish the protective effect of HPβ-CD on the polyphenolic antioxidant activity over the storage period of 35 days and the results are shown in Table 5-9.

Table 5-7 One-way ANOVA of Antioxidant activity versus time for Subcritical water extract (SWE), Subcritical water extract + HPβ-Cyclodextrin (SWE+ HPβ-CD), Chlorogenic acid (CGA) and Chlorogenic acid + HPβ-Cyclodextrin (CGA+ HPβ-CD)

Subcritical water extract (SWE)

Source	DF	Adj SS	Adj MS	F-value	P-Value
Time(days)	5	169478	33895.5	37.55	0.0
Error	11	9930	902.7		
Total	16	179407			

Subcritical water extract + HPβ-Cyclodextrin (SWE+ HPβ-CD)

Source	DF	Adj SS	Adj MS	F-value	P-Value
Time(days)	5	69182	13836.5	90.54	0.0
Error	11	1681	152.8		
Total	16	70863			

Chlorogenic acid (CGA)

Source	DF	Adj SS	Adj MS	F-value	P-Value
Time(days)	5	16821	3364.2	13.66	0.0
Error	11	2710	246.3		
Total	16	19530			

Chlorogenic acid + HPβ-Cyclodextrin (CGA+ HPβ-CD)

Source	DF	Adj SS	Adj MS	F-value	P-Value
Time(days)	5	18769	3753.9	26.28	0.0
Error	10	1428	142.8		
Total	15	201980			

Clearly from Table 5-9, only weeks 0 and 1 antioxidant activities were comparable for both encapsulated and non-encapsulated subcritical water extracts samples (Group A).

It implies that there was no significant difference between antioxidant activities for both treatments within the first 7days ($p<0.05$).

However antioxidant activity for weeks (2, 3, 4 and5) of HPβ-CD encapsulated subcritical water extracts were significantly higher than corresponding non-encapsulated samples ($p>0.05$). Coincidentally, only week 0 of control experiment using Chlorogenic acid was comparable week 1 of Chlorogenic acid + HPβ-Cyclodextrin (Group D, $p<0.05$). Encapsulation of Chlorogenic acid with HPβ-Cyclodextrin caused significant variation of the antioxidant activity after 7 days ($p<0.05$). Weeks 1,2,3,4 and 5 of Chlorogenic acid samples were significantly lower ($p<0.05$) in antioxidant activity than week 1,2,3,4, and 5 of HPβ-CD complex of Chlorogenic acid.

Cyclodextrin offered good retention of the polyphenolic antioxidant compounds against degradation, and was confirmed in the control experiment using Chlorogenic acid standard. Nearly similar trends were observed in both experiments as shown in Figure 5-9. The slightest difference can be due the different environments of both experimental setups. Subcritical water extract was a complex mixture polyphenolic, other bioactive compounds and carbohydrate polymers, were as the control experiment contained largely Chlorogenic acid.

Cyclodextrin is considered as secondary antioxidant and was reported to have protective effect on ascorbic acid and phenolic compound 2, 2, 5, 7, 8-pentamethylchroman-6-ol (PMC) (Núñez-Delicado et al., 1997). Many other reports of protective effect of Cyclodextrin on antioxidant activity of bioactive compounds are already available (Antas, 2015, Kalogeropoulos et al., 2010, Navarro et al., 2011).

Protective effects of the Cyclodextrin against oxidation and degradation were apparently due to the complexation of the SWE and the Chlorogenic acid into its hydrophobic cavity. Therefore the HPβ-Cyclodextrin can be employed as a carrier to prolong shelf-life of the phenolic antioxidant compounds and to mask any undesired taste and colour for applications in nutraceutical, pharmaceutical industries.

Table 5-8 Tukey Pairwise Comparisons of antioxidant activity versus time for Subcritical water extract (SWE), Subcritical water extract + HPβ-Cyclodextrin (SWE+ HPβ-CD), Chlorogenic acid (CGA) and Chlorogenic acid + HPβ-Cyclodextrin (CGA+ HPβ-CD).

Subcritical water extract (SWE)

Storage Time(days)	N	Mean	Grouping	
0	3	574.1	A	
7	2	530.0	A	
14	3	380.0		B
21	3	336.8		B
35	3	329.4		B
28	3	324.4		B

Subcritical water extract + HPβ-Cyclodextrin (SWE+ HPβ-CD)

Storage Time(days)	N	Mean	Grouping		
7	3	595.6	A		
0	2	530.0		B	
14	3	509.4		B	
35	3	429.8			C
21	3	429.8			C
28	3	426.5			C

Chlorogenic acid (CGA)

Storage Time(days)	N	Mean	Grouping		
0	2	665.6	A		
7	3	613.9		B	
35	3	608.5		B	
14	3	590.0		B	C
28	3	580.4			C
21	3	555.2			C

Chlorogenic acid + HPβ-Cyclodextrin (CGA+ HPβ-CD)

Storage Time(days)	N	Mean	Grouping		
7	3	764.7	A		
21	3	732.5	A	B	
14	3	728.1		B	
28	2	695.3		B	C
35	3	687.2			C
0	3	656.5			C

Table 5-9 Tukey Pairwise Comparisons of antioxidant activity SWE and SWE+HPβ-CD against time and CGA and CGA+HPβ-CD against time.

SWE and SWE+HPβ-CD against time								
StorageTime (weeks)	**N**	**Mean**	**Grouping**					
W1 β-CD	3	595.6	A					
W0	3	574.1	A					
W1	2	530.0	A	B				
W0 β-CD	3	530.0	A	B				
W2 β-CD	3	478.0		B	C			
W3 β-CD	3	437.7			C	D		
W5 β-CD	3	429.8			C	D		
W4 β-CD	2	426.5			C	D	E	
W2	3	380.0				D	E	F
W3	3	336.4					E	F
W5	3	329.4						F
W4	3	324.4						F

CGA and CGA+HPβ-CD against time										
Storage Time (weeks)	**N**	**Mean**	**Grouping**							
W1-β-CD	3	764.7	A							
W3 β-CD1	3	732.5	A	B						
W2 β-CD	3	728.1	A	B	C					
W4 β-CD	2	695.3		B	C	D				
W5 β-CD	3	687.2			C	D				
W0	3	665.6				D				
W0 β-CD	3	656.5				D	E			
W1	2	613.9					E	F		
W5	3	608.5						F		
W2	3	590.0						F	G	
W4	3	580.4						F	G	
W3	3	555.2							G	

W0 =week 0 for non encapsulated samples, W0 β-CD= week 0 for encapsulated samples with Cyclodextrins.

5.3 Conclusions

Micronisation of subcritical water extract was successfully demonstrated by spray drying method with and without the carrier HPβ-Cyclodextrin. Particle sizes of powders suitable for cosmetic formulations were achieved. However spray drying methods negatively affected antioxidant activity of the micronized samples. Directly encapsulated total antioxidant activity was approximately 50% less than the liquid subcritical water extract. Powders generated were found to be hygroscopic and can negatively affect their applications in formulation and also increase cost of storage. Hydroxyl propyl-β-cyclodextrin as a carrier was able to reduce the hygroscopicity of the subcritical water extract, mask the brown colour, and in addition, demonstrated protective effect against oxidation and degradation and thereby prolonging the shelf life of the antioxidants compounds. Electron scanning microscopy (SEM) and Fourier transform infra-red spectroscopy (FTIR) were selected to characterised powders to support evidence of encapsulation of the subcritical water extract into the hydrophobic cavity of the Cyclodextrins. Both techniques have revealed some level of interaction between the host HPβ-CD and subcritical water extract. However the results could not be conclusive enough to endorse complete evidence of encapsulation.

Chapter 6

Conclusion and Future Work

6.1 Conclusions

The current investigations have shown that apple pomace, a by-product of apple juice and cider productions contain high levels of antioxidant compounds that can be used in nutraceutical, pharmaceutical and cosmetic industries. Recoveries of these polyphenolic antioxidant compounds from the apple pomace were done by employing two separate methods. Aqueous acetone for organic solvent extraction and novel subcritical water mediated hydrolysis. Extracts were analysed for total phenolic content, solubilisation and antioxidant activity. Phenolic compounds like Chlorogenic acid, Phloridzin, Procyanidins were the main representative phenolic acid and flavonoids identified in extracts from both techniques. Water soluble Protocatechuic aldehyde was identified only in extract of subcritical water mediated hydrolysis technique for the first time under subcritical water mediated hydrolysis of cider apple pomace. The phenolic aldehyde was not identified in the acetone extraction. Multivariate statistics methods were used to design the experiments by response surface methodology (RSM) to fit polynomial equations to observed data and then applied to predict the behaviour of all responses based on the set of experimental variables. Both extraction techniques have shown selectivity of solvent concentration, solid-to-solvent ratio, and temperature and residence time on polyphenolic compounds based on structure and composition. Solvent selectivity based on the models could serve a crucial role in recovery of polyphenols from the apple pomace. Overall subcritical water extraction technique was shorter in extraction time and more efficient for solubilisation, higher total phenolic content and antioxidant activity by ORAC assay. Higher yields of solubilisation were achieved under the subcritical extraction due to better mass transfer rates where penetrability of the pressurised water within the sample matrix increased. Total phenolic content and antioxidant activity increased with increasing temperature with corresponding high levels of Maillard products and the water soluble Protocatechuic aldehyde in extracts. Therefore subcritical water extraction could be applied to recover high polyphenolic antioxidant compounds from fresh wet apple pomace to replace synthetic antioxidant in food and cosmetic application. Concerns regarding safety of artificial antioxidant by consumers would therefore be addressed.

Auto-encapsulation of the subcritical water extract was achieved by utilisation of natural occurring polysaccharides co-extracted with polyphenolic compounds by spray drying process. At the same time the extract were encapsulated with addition HPβ-CD. Relatively sticky and hygroscopic encapsulated products were generated, with the level of hygroscopicity decreased when HPβ-CD was added during the encapsulation process. Cyclodextrins offered protection against degradation of antioxidant compounds during the spray drying operation. Additionally it slowed down polyphenolic antioxidant degradation during storage by increasing the shelf-life of the antioxidant and can be effectively applied in formulation of cosmetic and nutraceutical products.

6.2 Recommendations for Future Work

Some areas in the research have been identified and require further investigations to give a better understanding of the utility of subcritical water in terms of solubilisation of the apple pomace and the release of high antioxidant compounds at the higher temperatures. For instance the batch reactor configuration used in the research should be replaced with a sem-continuous reactor system where products are withdrawn at subcritical water conditions of interest. The withdrawn products shouldn’t be centrifuged but rather analysed directly for solubilisation, phenolic content and antioxidant activities. In this way there will be a better understanding of solubilisation of the bioactives from the apple pomace and high quality of products can be guaranteed under the continuous reactor configuration.

A new liquid chromatographic method either normal or reverse phase mode should be developed coupled with mass spectrometry (LC-MS) for resolution and identification of suspected high molecular weight compounds whose standards are not commercially available. Additionally analytical method should be explored for fractionation of the extract at various temperature conditions to identify compounds and analyse their contribution to overall antioxidant activity. Monomeric sugar analysis of extract should also be explored to correlate data between levels of total sugars and stickiness of products generated from the micronisation by spray drying operation.

References

Abraham, K., Gürtler, R., Berg, K., et al. (2011) Toxicology and risk assessment of 5-Hydroxymethylfurfural in food. **Molecular nutrition & food research**, 55: (5): 667-678.

Adom, K.K. and Liu, R.H. (2002) Antioxidant activity of grains. . **Journal of Agricultural and Food Chemistry**, 50: 6182-6187.

Afaq, F., Adhami, V.M. and Ahmad, N. (2003) Prevention of short-term ultraviolet B radiation-mediated damages by resveratrol in SKH-1 hairless mice. **Toxicology and applied pharmacology**, 186: (1): 28-37.

Agrahari, P.R. and Khurdiya, D.S. (2003) Studies on preparation and storage of RTS beverage from pulp of culled apple pomace. **Indian Food Packer**, 57: (2): 56-61.

Aherne, S.A. and O'Brien, N.M. (2002) Dietary flavonols: chemistry, food content, and metabolism. **Nutrition**, 18: (1): 75-81.

Ahlneck, C. and Zografi, G. (1990) The molecular basis of moisture effects on the physical and chemical stability of drugs in the solid state. **International journal of pharmaceutics**, 62: (2–3): 87-95.

Aida, T.M., Sato, Y., Watanabe, M., et al. (2007) Dehydration of -glucose in high temperature water at pressures up to 80MPa. **The Journal of Supercritical Fluids**, 381–388.

Akiya, N. and Savage, P.E. (2002) Roles of water for chemical reactions in high-temperature water. **Chemical Reviews**, 102: (8): 2725-2750.

Alexis (2008) " Flavonoids-Stilbenoids-Phenolic acids". **Polyphenols**. Alexis Biochemicals.

Allison, P. (2012) **When Can You Safely Ignore Multicollinearity?** [online]. http://statisticalhorizons.com/ Statistical Horizon [Accessed

Ames, B.N., Shigenaga, M.K. and Hagen, T.M. (1993) Oxidants, antioxidants, and the degenerative diseases of aging. **Proceedings of the National Academy of Sciences**, 90: 7915-7922.

Anandharamakrishnan, C., Rielly, C.D. and Stapley, A.G.F. (2007) Effects of Process Variables on the Denaturation of Whey Proteins during Spray Drying. **Drying Technology**, 25: (5): 799-807.

Andersson, J.M., Lindahl, S., Turner, C., et al. (2012) Pressurised hot water extraction with on-line particle formation by supercritical fluid technology. **Food Chemistry**, 134: (4): 1724-1731.

Anselmi, C., Centini, M., Maggiore, M., et al. (2008) Non-covalent inclusion of ferulic acid with a-cyclodextrin improves photo-stability and delivery: NMR and modeling studies. **Journal of Pharmaceutical and Biomedical Analysis**, 46: 645-652.

Antas, C. (2015) **AN ASSESSMENT OF THE UTILITY OF SUBCRITICAL WATER TO RECOVER BIOACTIVE COMPOUNDS FROM CIDER LEES**. PhD, University of Birmingham.

Arai, Y., Sako, T. and Takebayashi, Y. (2013) **Supercritical fluids: molecular interactions, physical properties and new applications**. Springer Science & Business Media.

Aron, P.M. and Kennedy, J.A. (2008) Flavan-3-ols: nature, occurrence and biological activity. **Molecular nutrition & food research**, 52: 79-104.

Arranz, S., Silván, J.M. and Saura-Calixto, F. (2010) Nonextractable polyphenols, usually ignored, are the major part of dietary polyphenols: a study on the Spanish diet. **Molecular nutrition & food research**, 54: (11): 1646-1658.

Arribas-Lorenzo, G. and Morales, F.J. (2010) Estimation of dietary intake of 5-hydroxymethylfurfural and related substances from coffee to Spanish population. **Food and Chemical Toxicology**, 48: 644–649.

Arts, I.C., van de Putte, B. and Hollman, P.C. (2000a) Catechin contents of foods commonly consumed in The Netherlands. 1. Fruits, vegetables, staple foods, and processed foods. **Journal of Agricultural and Food Chemistry**, 48: (5): 1746-1751.

Arts, I.C., van de Putte, B. and Hollman, P.C. (2000b) Catechin contents of foods commonly consumed in The Netherlands. 2. Tea, wine, fruit juices, and chocolate milk. **Journal of Agricultural and Food Chemistry**, 48: (5): 1752-1757.

Awika, J.M., Rooney, L.W., Wu, X., et al. (2003) Screening methods to measure antioxidant activity of sorghum (Sorghum bicolor) and sorghum products. **Journal of Agricultural and Food Chemistry**, 51: (23): 6657-6662.

Baboota, S., Dhaliwal, M., Kohli, K., et al. (2005) Inclusion complexation of Rofecoxib with dimethyl β-cyclodextrin. **Indian journal of pharmaceutical sciences**, 67: (2): 226-229.

Badarinath, A.V., RAo, K.M., Madhu SudhanaChetty, C., et al. (2010) A Review on In-Vitro Antioxidant Methods: Comparisons, Correlations and Considerations. **International Journal of Pharm Tech Research**, 2: (2): 1276-1285.

Baei, M.S., Mahmoudi, M. and Yunesi, H. (2008) A kinetic model for citric acid production from apple pomac by Aspergillus niger. **African Journal of Biotechnology**, 7: (19): 3487-3489.

Bagchi, D., Bagchi, M., Stohs, S.J., et al. (2000) Free radicals and grape seed proanthocyanidin extract: importance in human health and disease prevention. **Toxicology**, 148: (2): 187-197.

Bai, X.-L., Yue, T.-L., Yuan, Y.-H., et al. (2010) Optimization of microwave-assisted extraction of polyphenols from apple pomace using response surface methodology and HPLC analysis. **Journal of Separation Science**, 33: 3751-3758.

Bakhiya, N., Monien, B., Frank, H., et al. (2009) Renal organic anion transporters OAT1 and OAT3 mediate the cellular accumulation of 5-sulfooxymethylfurfural, a reactive nephrotoxic metabolite of the Maillard product 5-hydroxymethylfurfural. **Biochemical Pharmacology**, 78: 414-419.

Baldisserotto, A., Malisard, i.G., Scalambra, E., et al. (2012) Synthesis, Antioxidant and Antimicrobial Activity of a New Phloridzin Derivative for Dermo-Cosmetic Applications. **Molecules**, 17: 13275-13289.

Bank, G. and Schauss, A. (2004) **Antioxidant testing: an ORAC update.**
[online]. Nutraceuticals World 2004, http://www.nutraceuticalsworld.com/March042.htm.
[Accessed

Barbero, G., Liazid, A., Palma, M., et al. (2008) Ultrasound-assisted extraction of capsaicinoids from peppers. **Talanta**, 75: (5): 1332-1337.

Barras, A., Mezzetti, A., Richard, A., et al. (2009) Formulation and characterization of polyphenol-loaded lipid nanocapsules. **International journal of pharmaceutics**, 379: (2): 270-277.

Bartosz, G., Janaszewska, A., Ertel, D., et al. (1998) Simple determination of peroxyl radical-trapping capacity. **Biochemistry and molecular biology international**, 46: 519-529.

Bhandari, B. (2004) Spray drying: an encapsulation technique for food flavors.

Bhandari, B.R., D'Arcy, B.D. and Padukka, I. (1999) Encapsulation of lemon oil by paste method using b-cyclodextrin: encapsulation efficiency and profile of oil volatiles. **Journal of Agricultural and Food Chemistry**, 47: 5194-5197.

Bhandari, B.R., Datta, N. and Howes, T. (1997) Problems Associated With Spray Drying Of Sugar-Rich Foods. **Drying Technology**, 15: (2): 671-684.

Bhushan, S., Kalia, K., Sharma, M., et al. (2008) Processing of apple pomace for bioactive molecules. **Critical Reviews in Biotechnology**, 28: 285-296.

Biedrzycka, E. and Amarowicz, R. (2008) Diet and health: apple polyphenols as antioxidants. **Food Reviews International**, 24: (2): 235-251.

Bobleter, O. (1994) Hydrothermal degradation of polymers derived from plants. **Polymer Science**, 19: 797-841.

Boccia, M., Kopf, S. and Baratti, C. (1999) Phlorizin, a competitive inhibitor of glucose transport, facilitates memory storage in mice. **Neurobiology of learning and memory**, 71: (1): 104-112.

Box, G.E. and Draper, N.R. (2007) **Response surfaces, mixtures, and ridge analyses**. John Wiley & Sons.

Brendan, L. (2010) "Apples". Washington DC, United State International Trade Commission.

Brunner, G. (2009) Near critical and supercritical water. Part I. Hydrolytic and hydrothermal processes. **Journal of Supercritical Fluids**, 47: 373-381.

Budak, N.H., Ozçelik, F. and Güzel-Seydim, Z.B. (2015) Antioxidant Activity and Phenolic Content of Apple Cider. **Turkish Journal of Agriculture-Food Science and Technology**, 3: (6).

Buskirk, D. and Rust, S. (2008) Feeding Apples or Apple pomace in Cattle Diets. **Cattle Call** 13: (4).

Cabelli, D.E. and Bielski, B.H. (1983) Kinetics and mechanism for the oxidation of ascorbic acid/ascorbate by HO2/O2-(hydroperoxyl/superoxide) radicals. A pulse radiolysis and stopped-flow photolysis study. **The Journal of Physical Chemistry**, 87: (10): 1809-1812.

Caldeira, I., Pereira, R., Clímaco, M.C., et al. (2004) Improved method for extraction of aroma compounds in aged brandies and aqueous alcoholic wood extracts using ultrasound. **Analytica Chimica Acta**, 513: (1): 125-134.

Çam, M. and Aaby, K. (2010) Optimization of Extraction of Apple Pomace Phenolics with Water by Response Surface Methodology. **Journal of Agricultural and Food Chemistry**, 58: (16): 9103-9111.

Cao, G. and Prior, R.L. (1999) Measurement of oxygen radical absorbance capacity in biological samples. **Methods Enzymol**, 299: 50-62.

Cao, G., Sofic, E. and Prior, R.L. (1997) Antioxidant and prooxidant behavior of flavonoids: Structure-activity relationship. **Free Radical Biology and Medicine**, 22: 749-670.

Cao, G., Verdon, C.P., Wu, A., et al. (1995) Automated assay of oxygen radical absorbance capacity with the COBAS FARA II. **Clinical Chemistry**, 41: (12): 1738-1744.

Caparino, O.A., Tang, J., Nindo, C.I., et al. (2012) Effect of drying methods on the physical properties and microstructures of mango (Philippine 'Carabao' var.) powder. **Journal of Food Engineering**, 111: (1): 135-148.

Cardoso, A., Lemos, L., Frighetto, M., et al. (2011) Total phenolics extracted from the skin of fuji apple and incorporated by liposome in galenic bases: an alternative to use by-products of food industry. **Romanian Biotechnological Letters**, 16: (6): 41.

Carpita, N.C. and Gibeaut, D.M. (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. **Plant Journal**, 3: (1): 1-30.

Carson, K.J., Collins, J.L. and Penfield, M.P. (1994) Unrefined, dried apple pomace as a potential food ingredient **Journal of Food Science**, 59: 1213-1215.

Cetkovic, G., J., C.a.-B., Djilas, S., et al. (2008) Assessment of polyphenolic content and in vitro antiradical characteristics of apple pomace. **Food Chemistry**, 109: (2): 340-347.

Chandrasekara, A. and Shahidi, F. (2010) Content of insoluble bound phenolics in millets and their contribution to antioxidant capacity. **Journal of Agricultural and Food Chemistry**, 58: 6706-6714.

Chang, A. (2006) **Study of particle formation using supercritical CO2 as an antisolvent**. PhD, North Carolina State University.

Chaudhary, K., Ethiraj, S., Lakshminarayana, K., et al. (1978) Citric acid production from Indian cane molasses by Aspergillus niger under solid state fermentation conditions. **Journal of fermentation technology**, 56: (5): 554-557.

Che Man, Y.B., Irwandi, J. and Abdullah, W.J.W. (1999) Effect of different types of maltodextrin and drying methods on physico-chemical and sensory properties of encapsulated durian flavour. **Journal of the Science of Food and Agriculture**, 79: (8): 1075-1080.

Chen, F., Sun, Y., Zhao, G., et al. (2007) Optimization of ultrasound-assisted extraction of anthocyanins in red raspberries and identification of anthocyanins in extract using high-performance liquid chromatography–mass spectrometry. **Ultrasonics Sonochemistry**, 14: (6): 767-778.

Chun, I.K. and Yun, D.S. (1993) Inclusion complexation of hydrocortisone butyrate with cyclodextrins and dimethyl-β-cyclodextrin in aqueous solution and in solid state. **International journal of pharmaceutics**, 96: (1): 91-103.

Clifford, M.N. (2000) Chlorogenic acids and other cinnamates–nature, occurrence, dietary burden, absorption and metabolism. **Journal of the Science of Food and Agriculture**, 80: (7): 1033-1043.

Coates, J. (2000) Interpretation of infrared spectra, a practical approach. **Encyclopedia of analytical chemistry**.

Cocero, M.J. and Martin, A. (2008) Precipitation processes with supercritical fluids: patents review. **Recent Patents on Engineering**, 2: (1): 9-20.

Cocero, M.J., Martín, Á., Mattea, F., et al. (2009) Encapsulation and co-precipitation processes with supercritical fluids: fundamentals and applications. **The Journal of Supercritical Fluids**, 47: (3): 546-555.

COELHO, M.A.Z., LEITE, S.G.F., Rosa, M.d.F., et al. (2001) Aproveitamento de resíduos agroindustriais: produção de enzimas a partir da casca de coco verde. **Boletim CEPPA**, 19: (01): 33-42.

Cortell, J. and Kennedy, J. (2006) Effect of shading on accumulation of flavonoid compounds in (Vitis vinifera L.) pinot noir fruit and extraction in a model system. . **Journal of Agricultural and Food Chemistry**, 54: 8510-8520.

Crozier, A., Jaganath, I.B. and Clifford, M.N. (eds.) (2006) **"Phenols, Polyphenols and Tannins: An Overview"**, Oxford, UK,: Blackwell Publishing Ltd.

Cruz, A.G., Scullin, C., Mu, C., et al. (2013) Impact of high biomass loading on ionic liquid pretreatment. **Biotechnology for biofuels**, 6: (1): 1-10.

D'Archivio, M., Filesi, C., Di Benedetto , R., et al. (2007) Polyphenols, dietary sources and bioavailability. **Annali dell'Istituto Superiore Di Sanita**, 43: 348-361.

Dai , J. and Mumper, R.J. (2010) Plant Phenolics: Extraction, Analysis and their Antioxidant and Anticancer Properties. **Molecules**, 15: 7313-7352.

Darr, D., DUNSTON, S., FAUST, H., et al. (1996) Effectiveness of Antioxidants (Vitamin C and E) With and. **Acta Derm Vcnereol (Stockh)**, 76: 264-268.

Dávalos, A., Gómez-Cordovés, C. and Bartolomé, B. (2004) Extending Applicability of the Oxygen Radical Absorbance Capacity (ORAC−Fluorescein) Assay. **Journal of Agricultural and Food Chemistry**, 52: (1): 48-54.

Debenedetti, P.G., Tom, J.W., Kwauk, X., et al. (1993) Rapid expansion of supercritical solutions (RESS): fundamentals and applications. **Fluid Phase Equilibria**, 82: 311-321.

DeCarlo, P.F., Slowik, J.G., Worsnop, D.R., et al. (2004) Particle Morphology and Density Characterization by Combined Mobility and Aerodynamic Diameter Measurements. Part 1: Theory. **Aerosol Science and Technology**, 38: (12): 1185-1205.

Del Valle, J.M. and Aguilera, J.M. (1999) Review: high pressure CO2 extraction. Fundamentals and applications in the food industry. **Food Science & Technology International**, 5: 1-24.

Delgado-Vargas, F., Jimenez, A.R. and Pardes-Lopez, O. (2000) Natural pigments: carotenoids, anthocyanins and betalainsecharacteristics, biosynthesis, processing and stability. **Critical Reviews in Food Science and Nutrition**, 40: 173-289.

Desai, K.G.H. and Park, H.J. (2005) Recent developments in microencapsulation of food ingredients. **Drying Technology**, 23: 1361-1394.

Desobry, S.A., Netto, F.M. and Labuza, T.P. (1997) Comparison of Spray-drying, Drum-drying and Freeze-drying for β-Carotene Encapsulation and Preservation. **Journal of Food Science**, 62: (6): 1158-1162.

Devaravjan, A. (1997) **Solid state fermentation of apple pomace: methods of ethanol recovery, physic- chemical evaluation and acceptability of animal feed produced, MSc thesis**. Dr YS Parmar University of Horticulture and Forestry Nauni Solan HP.

Dhillon, G.S., Brar, S.K., Verma, M., et al. (2011) Enhance solid state Citric acid bio-production using apple pomace waste through surface response methodology. **Journal of Appllied Microbiology***,* 10: 1365-2672.

Dimitrios, B. (2006) Sources of natural phenolics antioxidants. **Trends in Food and Technolgy** 17: 505-512.

Diñeiro García, Y., Valles, B.S. and Picinelli Lobo, A. (2009) Phenolic and antioxidant composition of by-products from the cider industry: Apple pomace. **Food Chemistry***,* 117: (4): 731-738.

Douglas, R. (1913) "Food product". Google Patents.

DuPont, M.S., Bennett, R.N., Mellon, F.A., et al. (2002) Polyphenols from alcoholic apple cider are absorbed, metabolized and excreted by humans. **The Journal of nutrition***,* 132: (2): 172-175.

Dziezak, J.D. (1998) Microencapsulation and encapsulated food ingredients. **Food Technology***,* 42: 136-151.

EFSA (2005) Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food (AFC) on request from the commission related to flavouring group evaluation 13: furfuryl and furan derivatives with and without additional side-chain substitutes and heteroatomes from chemical group 14 (commission regulation (EC) No. 1565/2000 of 18 July 2000). . **EFSA J.***,* 215: 1-73.

Ehara, K. and Shin, S. (1998) Measurement of density distribution of aerosol particles by successive classification of particles according to their mass and diameter. **Journal of aerosol science***,* 29: S19-S20.

Endreß, H.-U. (2000) High quality resulting from product integrated environment protection-PIUS. **Fruit Processing***,* 10: 273-276.

Erden, N. and Çelebi, N. (1988) A study of the inclusion complex of naproxen with β-cyclodextrin. **International journal of pharmaceutics***,* 48: (1): 83-89.

Ersus, S. and Yurdagel, U. (2007) Microencapsulation of anthocyanin pigments of black carrot (Daucus carota L.) by spray drier. **Journal of Food Engineering***,* 80: (3): 805-812.

Escarpa, A. and Gonzalez, M. (1998) High-performance liquid chromatography with diode-array detection for the performance of phenolic compounds in peel and pulp from different apple varieties. **Journal of Chromatography** A*,* 823: 331-337.

Escarpa, A. and González, M.C. (2001) Approach to the content of total extractable phenolic compounds from different food samples by comparison of chromatographic and spectrophotometric methods. **Analytica Chimica Acta***,* 427: (1): 119-127.

EU (2001) "Council directive 2001\1100\EC of December 2001 relating to honey". In Union, E. (Ed.) **2001\1100\EC**. **Journal of European Communities**.

Fages, J., Hubert, L., jean- Jacques, L., et al. (2004) Particle generation for pharmaceutical applications using supercritical fluid technology. **Powder Technology**, 141: 219-226.

Fang, Z. and Bhandari, B.R. (2010) Encapsulation of polyphenols - a review. **Trends in Food Science and Technology**, 21: 510-523.

Fargione, J., Hill, J., Tilman, D., et al. (2008) Land clearing and the biofuel carbon debt. **Science**, 319: (5867): 1235-1238.

FDA (2007) "A Report of the U.S. Food and Drug Administration Nanotechnology Task Force.". Nanotechnology, U.S. Food and Drug Administration.

Ficarra, R., Tommasini, S., Raneri, D., et al. (2002) Study of flavonoids/β-cyclodextrins inclusion complexes by NMR, FT-IR, DSC, X-ray investigation. **Journal of Pharmaceutical and Biomedical Analysis**, 29: (6): 1005-1014.

Fischer, R. (1984) Apple pomace as a source of energy – does it pay off? . **Flussiges Obst**, 51: 534-539.

FOA (2011) **Food and Agricultural Organization of the United Nations** [online]. http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567 FOASTAT [Accessed 2011].

Foo, L.Y. and Lu, Y. (1999) Isolation and identification of procyanidins in apple pomace. **Food Chemistry**, 64: (4): 511-518.

Fox, G.F., Asmussen, R., Fischer, K., et al. (1991) Aufwand und Nutzen der Apfeltresterverwertung. **Flu¨ ssiges Obstet.**, 58: 492-499.

Fraile, M., Buratto, R., Gómez, B., et al. (2014) Enhanced Delivery of Quercetin by Encapsulation in Poloxamers by Supercritical Antisolvent Process. **Industrial & Engineering Chemistry Research**, 53: (11): 4318-4327.

Fronza, G., Fuganti, C., Genesio, E., et al. (2002) Structural Features of the β-CD Complexes with Naringin and its Dihydrochalcone and Aglycon Derivatives by 1H NMR. **Journal of inclusion phenomena and macrocyclic chemistry**, 44: (1-4): 225-228.

Fuchs, J., Huflejt, M.E., Rothfuss, L.M., et al. (1989) Impairment of enzymic and nonenzymic antioxidants in skin by UVB irradiation. **Journal of Investigative Dermatology**, 93: (6): 769-773.

Fukumoto, L.R. and Mazza, G. (2000) Assessing antioxidant and prooxidant activity of phenolic compounds. **Journal of Agricultural and Food Chemistry**, 48: 3597–3604.

Gallagher, P., Coffey, M., Krukonis, V., et al. (1989) "Gas antisolvent recrystallization: new process to recrystallize compounds insoluble in supercritical fluids". **Supercritical fluids Science and Technology,** 406: 334-354.

Gaset, A., Rigal, L., Paillassa, G., et al. (1985) "FR 2551754 A1 19850315.".

Gaspar, E. and Lucena, A.F.F. (2009) Improved HPLC methodology for food control - furfurals and patulin as markers of quality. **Food Chemistry.**, 114: (4): 1576–1582.

Gaudout, D., Megard, D., Inisan, C., et al. (2006) "Phloridzin-rich phenolic fraction and use thereof as a cosmetic, dietary or nutraceutical agent". Google Patents.

Georgetti, S.R., Casagrande, R., Souza, C.R.F., et al. (2008) Spray drying of the soybean extract: Effects on chemical properties and antioxidant activity. **LWT-Food Science and Technology**, 41: (8): 1521-1527.

Ghafoor, K., Choi, Y., Jeon, J., et al. (2009) Optimization of ultrasound-assisted extraction of phenolic compounds, antioxidants, and anthocyanins from grape (Vitis vinifera) seeds. . **Journal of. Agricutural and Food Chemistry**, 57: 4988-4994.

Ghiselli, A., Serafini, M., Maiani, G., et al. (1995) A fluorescence-based method for measuring total plasma antioxidant capability. **Free Radical Biology and Medicine**, 18: (1): 29-36.

Gibbs, F., Selim Kermasha, Inteaz Alli, et al. (1999) Encapsulation in the food industry: a review. **International Journal of Food Sciences and Nutrition**, 50: (3): 213-224.

Gilmour, S.G. (2006) Response surface designs for experiments in bioprocessing. **Biometrics**, 62: (2): 323-331.

Giomaro, G., Karioti, A., Bilia, A.R., et al. (2014) Polyphenols profile and antioxidant activity of skin and pulp of a rare apple from Marche region (Italy). **Chemistry Central Journal**, 8: 45-45.

Giunchedi, P. and Conte, U. (1995) Spray-drying as a preparation method of microparticulate drug delivery systems: an overview. **STP Pharma Sciences**, 5: (4): 276-290.

Gradinaru, G., Biliaderis, C.G., Kallithraka, S., et al. (2003) Thermal stability of Hibiscus sabdariffa L. anthocyanins in solution and in solid state: effects of copigmentation and glass transition. **Food Chemistry**, 83: 423-436.

Graham, H.N. (1992) Green tea composition, consumption, and polyphenol chemistry. **Preventive medicine**, 21: (3): 334-350.

Gunst, R.F. (1996) Response Surface Methodology: Process and Product Optimization Using Designed Experiments. **Technometrics**, 38: (3): 284-286.

Guyot, S., Marnet, N., Sanoner, P., et al. (2003) Variability of the Polyphenolic Composition of Cider Apple (Malus domestica) Fruits and Juices. **Journal of Agricultural and Food Chemistry**, 51: (21): 6240-6247.

Hair, J.F.J., Anderson, R.E., Tatham, R.L., et al. (1995) **Multivariate Data Analysis (3rd ed)**. New York: Macmillan.

Haiyun, D., Jianbin, C., Guomei, Z., et al. (2003) Preparation and spectral investigation on inclusion complex of β-cyclodextrin with rutin. **Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy**, 59: (14): 3421-3429.

Hamburger, M., Baumann, D. and Adler, S. (2004) Supercritical carbon dioxide extraction of selected medicinal plants—Effects of high pressure and added ethanol on yield of extracted substances. **Phytochemical. Analysis**, 15: 46-54.

Hang, Y.D., Lee, C.Y. and Woodams, E.E. (1982) A Solid state fermentation system for production of ethanol from apple pomace. **Journal of Food Science**, 47: 1851-1852.

Hang, Y.D. and Woodams, E.E. (1986) A solid state fermentation of apple pomace for citric acid production using Aspergillus. **MIRCEN Journal of Applied Microbiology and Biotechnology**, 2: (2): 283-287.

Harborne, J. (1993) **The flavonoids: Advances in research since 1986**. London: Chapman and Hall.

Harborne, J. and Williams, C. (2000) Advances in flavonoid research since **Phytochemistry**, 55: 481-504.

Harholt, J., Suttangkakul, A. and Scheller, H.V. (2010) Biosynthesis of pectin. **Plant physiology**, 153: (2): 384-395.

Harman, D. (2002) Aging: a theory based on free radical and radiation chemistry. **Science's SAGE KE**, 2002: (37): 14.

Haugaard, I.S., Krag, J., Pisecky, J., et al. (1978) **Analytical methods for dry milk powders. Denmark: Niro Atomizer** . San Diego: Academic Press.

Hawthorne, S.B., Yang, M. and Miller, N.J. (1994) Extraction of organic pollutants from environmental solids with sub- and supercritical water. **Analytical Chemistry.**, 66: 2912.

He, L., Zhang, X., Xu, H., et al. (2012) Subcritical water extraction of phenolic compounds from pomegranate (Punica granatum L.) seed residues and investigation into their antioxidant activities with HPLC–ABTS+ assay. **Food and Bioproducts Processing**, 90: (2): 215-223.

Heinonen, I.M., Meyer, A.S. and Frankel, E.N. (1998) Antioxidant activity of berry phenolics on human low-density lipoprotein and liposome oxidation.**Journal of Agricultural and Food Chemistry**, 46: (10): 4107-4112.

Helene, M., Giovanetti, C. , Nogueira, A., de Oliveira, P., C.L, et al. (2012) **Characterization of Apple Pectin- A Chromatographic Approach Chapter 14 325-342**. **INTECH**.

Hellström, J.K. and Mattila, P.H. (2008) HPLC Determination of Extractable and Unextractable Proanthocyanidins in Plant Materials. **Journal of Agricultural and Food Chemistry***,* 56: (17): 7617-7624.

Herrero, M., Castro-Puyana, M., Rocamora, L., et al. (2012) Formation and relevance of 5-hydroxymethylfurfural in bioactive subcritical water extracts from olive leaves. **Food Reviews International***,* 47: (1): 31-37.

Herrero, M., Cifuentes, A. and Ibañez, E. (2006) Sub- and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae: A review. **Food Chemistry***,* 98: (1): 136-148.

Herrmann, G., Wlaschek, M., Lange, T.S., et al. (1993) UVA irradiation stimulates the synthesis of various matrix-metalloproteinases (MMPs) in cultured human fibroblasts. **Experimental dermatology***,* 2: (2): 92-97.

Hismath, I., Wan Aida, W. and Ho, C. (2011) Optimization of extraction conditions for phenolic compounds from neem (Azadirachta indica) leaves. **International Food Research Journal***,* 18: (3).

Hollman, P.C., Cassidy, A., Comte, B., et al. (2011) The biological relevance of direct antioxidant effects of polyphenols for cardiovascular health in humans is not established. **The Journal of Nutrition***,* 141: (5): 989S-1009S.

Hollman, P.C. and Katan, M. (1999) Dietary flavonoids: intake, health effects and bioavailability. **Chemical Toxicology.** *,* 37: 937-942.

Hollman, P.C., van Trijp, J., Buysman, M., et al. (1997) Relative bioavailability of the various antioxidant flavonoid quercetin from various foods in man. **FEBS Let 1997***,* 418: (1-2): 152-156.

HORIBA (2012) **A Guidebook to particle size analyis.** USA: HORIBA Scuientific.

Hoseyini, M., Asefi, N. and Mozaffari, M. (2011) Production of Citric Acid from Apple Pomace by Using Surface Culture Method. **Agricultural Journal***,* 6: (5): 226-230.

Huang, D., Ou, B., Hampsch-Woodill, M., et al. (2002) High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. **Journal of Agricultural and Food Chemistry***,* 50: (16): 4437-4444.

Huang, D., Ou, B. and Prior, R.L. (2005) The Chemistry behind Antioxidant Capacity Assays. **Journal of Agricultural and Food Chemistry***,* 53: (6): 1841-1856.

Huang, W.-y., Zhang, H.-c., Liu, W.-x., et al. (2012) Survey of antioxidant capacity and phenolic composition of blueberry, blackberry, and strawberry in Nanjing. **Journal of Zhejiang University of Science B**, 13: (2): 94-102.

Hundre, S.Y., Karthik, P. and Anandharamakrishnan, C. (2015) Effect of whey protein isolate and β-cyclodextrin wall systems on stability of microencapsulated vanillin by spray–freeze drying method. **Food Chemistry**, 174: (0): 16-24.

Hunter, M.D. and Hull, L.A. (1993) Variation in concentrations of phloridzin and phloretin in apple foliage. **Phytochemistry**, 34: 1251-1254.

Hussein, L., Fattah, M.A. and Salem, E. (1990) Characterization of pure proanthocyanidins isolated from the hulls of faba beans. **Agric. Food Chem**, 38: 95.

Ibañez, E., Kubátová, A., Señoráns, F.J., et al. (2003) Subcritical Water Extraction of Antioxidant Compounds from Rosemary Plants. **Journal of Agricultural and Food Chemistry**, 51: (2): 375-382.

Ibáñez, E., Mendiola, J.A., Rodríguez-Meizoso, I., et al. (2008) Antioxidants in plant foods and microalgae extracted using compressed fluids. **EJEAFChe**, 7: (8): 3301-3309.

Ignat, I., Volf, I. and Popa, V.I. (2011) A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. **Food Chemistry**, 126: 1821-1835.

Ishige, K., Schubert, D. and Sagara, Y. (2001) Flavonoids protect neuronal cells from oxidant stress by three distinct mechanisms. **Free Radical Biology and Medicine**, 30: 433-446.

Issenhuth, F. (2008) The new generation of Panzym mash. **Fruit Processing**, 18: (5): 254-255.

Jane, C. (ed.) (2007) **Drought feeding and management of sheep: A guide to farmers and land management**, Victoria, Australia: Victorian Government, Department of Primary Indusries.

Janzowski, C., Glaab, V., Samim, E., et al. (2000) 5-Hydroxymethylfurfural: assessment of mutagenicity, DNA-damaging potential and reactivity towards cellular glutathione. **Food and Chemical Toxicology**, 38: 801-809.

Javanmardi, J., Stushnoff, C., Locke, E., et al. (2003) Antioxidant activity and total phenolic content of Iranian Ocimum accessions. **Food Chemistry**, 83: (4): 547-550.

Jaya, S. and Das, H. (2004) Effect of maltodextrin, glycerol monostearate and tricalcium phosphate on vacuum dried mango powder properties. **Journal of Food Engineering**, 63: (2): 125-134.

Joana Gil-Chávez, G., Villa, J.A., Fernando Ayala-Zavala, J., et al. (2013) Technologies for Extraction and Production of Bioactive Compounds to be Used as Nutraceuticals and Food

Ingredients: An Overview. **Comprehensive Reviews in Food Science and Food Safety**, 12: (1): 5-23.

Johansen, K. (2000) Cider Production in England and France–and Denmark? **Brygmesteren (Denmark)**.

Joshi, V.K. (1997) **Fruit wines, 2nd edition** Dr. YS Parmar University of Horticulture and Forestry, Solan, India.

Joshi, V.K. (1998) **Apple pomace utilization – Present status and future strategies:** . New Delhi: Education Publishers and Distributors.

Joshi, V.K. and Attri, B.L. (1991) Importance and scope of fruit based fermented beverages in India. **Beverage and Food World**, 17: (4): 9-10.

Joshi, V.K. and Attri, D. (2006) Solid state fermentation of apple pomace for the production of value added products. **Natural Product Radiance**, 5: (4): 289-296.

Joshi, V.K., Kaushal, N.K. and Thakur, N.S. (1996) Apple pomace sauce development, quality of fresh and stored products. **Journal of. Food Science and. Technology.**, 33: 414-419.

Joshi, V.K. and Pandey, A. (1999) **Biotechnology: food fermentation.** New Delhi: Educational Publish Distributors.

Joshi, V.K. and Sandhu, D.K. (1996) Preparation and evaluation of animal feed using solid state fermentation of apple. **Bioresource Technology**, 56: 251-255.

Jules, J., James, N.C., Susan, K.B., et al. (1996) **Fruit Breed**. John Wiley & Sons.

Jumbu, N. (2014) **An investigation into the antioxidant activity of a cider yeast extract with the aim of process optimisation**. University of Birmingham.

Jung, J. and Perrut, M. (2001) Particle design using supercritical fluids: literature and patent survey. **The Journal of Supercritical Fluids**, 20: (3): 179-219.

Kahkonen, M.P., Hopia, A.I. and Heinonen, M. (2001) Berry phenolics and their antioxidant activity **Journal of Agricultural and Food Chemistry**, 49: (8): 4076-4082.

Kalogeropoulos, N., Yannakopoulou, K., Gioxari, A., et al. (2010) Polyphenol characterization and encapsulation in β-cyclodextrin of a flavonoid-rich Hypericum perforatum (St John's wort) extract. **LWT - Food Science and Technology**, 43: (6): 882-889.

Kapoor, K., Chaudhari, K. and Tauro, P. (1982) **Prescott & Dunn's Industrial Microbiology. 4th edition**. West Port CT: AVI Publishing Co Inc.

Karacabey, E. and Mazza, G. (2010) Optimization of antioxidant activity of grape cane using response surface methodology. **Food Chemistry**, 119: 343-348.

Katiyar, S.K., Ahmad, N. and Mukhtar, H. (2000) Green tea and skin. **Archives of Dermatology**, 136: (8): 989-994.

Kaur, C. and Kapoor, H.C. (2002) Anti-oxidant activity and total phenolic content of some Asian vegetables. **International Journal of Food Science & Technology**, 37: (2): 153-161.

Kaushal, N. and Joshi, V. (1995) Preparation and evaluation of apple pomace based cookies. **Indian Food Packer**, 49: (5): 17-24.

Kaushal, N., Joshi, V. and Sharma, R. (2002) Effect of stage of apple pomace collection and the treatment on the physical-chemical and sensory qualities of pomace papad (fruit cloth). **Journal of Food Science and Technology**, 39: 388-393.

Kennedy, M., List, D., Lu, Y., et al. (1999) **Apple pomace and products derived from apple pomace: Uses, composition and analysis.** Berlin: Heidelberg: Springer.

Kertesz, Z. (1951) **The pectic substances. Interscience Publishers,** New York.

Kha, T.C., Nguyen, M.H. and Roach, P.D. (2010) Effects of spray drying conditions on the physicochemical and antioxidant properties of the Gac (Momordica cochinchinensis) fruit aril powder. **Journal of Food Engineering**, 98: 385–392.

Khan, M., K, Albert-Vian, M., Fabiano-Tixier, A.S., et al. (2010) Ultrasound-assisted extraction of polyphenols (flavanone glycosides) from orange (*citrus sininsis* L.) peel. **Food Chemistry,** 119(2):851-858.

Khosravi, K. and Shojaosadati, S.A. (2003) A solid state fermentation system for production of ethanol from apple pomace. **Fanni va Muhandisi-i Mudarris**, 10: 55-60.

Khuwijitjaru, P., Suaylam, B. and Adachi, S. (2014) Degradation of Caffeic Acid in Subcritical Water and Online HPLC-DPPH Assay of Degradation Products. **Journal of Agricultural and Food Chemistry**, 62: (8): 1945-1949.

Kim, J.H., Paxton, T.E. and Tomasko, D.L. (1996) Microencapsulation of naproxen using rapid expansion of supercritical solutions. **Biotechnology progress**, 12: (5): 650-661.

Kim, K.-H., Tsao, R., Yang, R., et al. (2006) Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effect of hydrolysis conditions. **Food Chemistry**, 95: 466-473.

King, J.W. (2000) Advances in critical fluid technology for food processing. **Food Science & Technology Today**, 14: 186-191.

Klein-Marcuschamer, D., Simmons, B.A. and Blanch, H.W. (2011) Techno-economic analysis of a lignocellulosic ethanol biorefinery with ionic liquid pre-treatment. **Biofuels, Bioproducts and Biorefining**, 5: (5): 562-569.

Knekt, P., Jarvinen, R., Seppanen, R., et al. (1997) Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. **American Journal of Epidemiology**, 146: 223-230.

Knez, Z. and Weidner, E. (2003) particle formation and particle design using supercritical fluid. **Current Opinion in Solid State and Materials Science**, 7: 353-361.

Kong, A.-N.T., Yu, R., Chen, C., et al. (2000) Signal transduction events elicited by natural products: role of MAPK and caspase pathways in homeostatic response and induction of apoptosis. **Archives of pharmaceutical research**, 23: (1): 1-16.

Kosaraju, S.L., D'ath, L. and Lawrence, A. (2006) Preparation and characterisation of chitosan microspheres for antioxidant delivery. **Carbohydrate polymers**, 64: (2): 163-167.

Kosaraju, S.L., Labbett, D., Emin, M., et al. (2008) Delivering polyphenols for healthy ageing. **Nutrition & Dietetics**, 65: (s3): S48-S52.

Kratchanova, M., Denev, P., Ciz, M., et al. (2010) Evaluation of antioxidant activity of medicinal plants containing polyphenol compounds. Comparison of two extraction systems. **Acta Biochim Pol**, 57: (2): 229-234.

Krishnaiah, D., Sarbatly, R., Hafiz, A., et al. (2009a) Study on retention of bioactive components of Morinda citrifolia L. using spray-drying. **Journal of Applied Sciences**, 9: (17): 3092-3097.

Krishnaiah, D., Sarbatly, R., Rao, Z.R.M., et al. (2009b) Optimal operating conditions of spray dried noni fruit extract using K-carrageenan as adjuvant. **Journal of Applied Sciences**, 9: (17): 3062-3067.

Krishnakumar, V. and Gordon, I. (1996) Antioxidants- trends and developments. **International food ingredients**, 12: 41-44.

Krokida, M. and Philippopoulos, C. (2005) Rehydration of dehydrated foods. **Drying Technology**, 23: (4): 799-830.

Kroon, P.A., Clifford, M.N., Crozier, A., et al. (2004) How should we assess the effects of exposure to dietary polyphenols in vitro? **The American journal of clinical nutrition**, 80: (1): 15-21.

Kubátová, A., Miller, D.J. and Hawthorne, S.B. (2001) Comparison of subcritical water and organic solvents for extracting kava lactones from kava root. **Journal of Chromatography A**, 923: (1–2): 187-194.

Laine, P., Kylli, P., Heinonen, M., et al. (2008) Storage stability of microencapsulated cloudberry (Rubus chamaemorus) phenolics. **Journal of Agricultural and Food Chemistry**, 56: 11251-11261.

Lang, Q. and Wai, C.M. (2001) Supercritical fluid extraction in herbal and natural product studies—A practical review. **Talanta**, 53: 771-782.

Larrauri, J.A., Rupérez, P. and Saura-Calixto, F. (1997) Effect of Drying Temperature on the Stability of Polyphenols and Antioxidant Activity of Red Grape Pomace Peels. **Journal of Agricultural and Food Chemistry**, 45: (4): 1390-1393.

Laura, M., Daniel, R., Ana, B.M.D., et al. (2013) Valorisation of Apple Peels. **European Journal of Food Research & Review**, 3: (1): 1-15.

Le Bourvellec, C. and Renard, C. (2012) Interactions between polyphenols and macromolecules: quantification methods and mechanisms. **Critical Reviews in Food Science and Nutrition**, 52: (3): 213-248.

Le Bronec, E., Renoux, A., Boulaud, D., et al. (1999) Effect of gravity in differential mobility analysers. A new method to determine the density and mass of aerosol particles. **Journal of aerosol science**, 30: (1): 89-103.

Lea, A. (1990) **Bitterness and Astringency: The Procyanidins of Fermented Apple Ciders,**. Amsterdam: Elsevier.

Lee, P.S., Han, J.-Y., Song, T.W., et al. (2006) Physicochemical characteristics and bioavailability of a novel intestinal metabolite of ginseng saponin (IH901) complexed with β-cyclodextrin. **International journal of pharmaceutics**, 316: (1): 29-36.

Lemanska, K., Szymusiak, H., Tyrakowska, B., et al. (2001) The influence of pH on the antioxidant properties and the mechanisms of antioxidant action of hydroxyflavones. **Free Radical Biology and Medicine**, 31: 869-881.

Lewicki, P.P. (1998) Effect of pre-drying treatment, drying and rehydration on plant tissue properties: a review. **International Journal of Food Properties**, 1: 1-22.

Liang, X. and Fan, Q. (2013) Application of Sub-Critical Water Extraction in Pharmaceutical Industry. **Journal of Materials Science and Chemical Engineering**, 1(5): 6.

Lien, E.J., Ren, S., Bui, H., et al. (1999) Quantitative structure-activity relationship analysis of phenolic antioxidants. **Free Radical Biology and Medicine**, 26: 285–294.

Liu, R.H. (2003) Health benefits of fruits and vegetables are from additive and synergistic combination of phytochemicals. **The American journal of clinical nutrition**, 78: (3): 517-520.

Liyana-Pathirana, C. and Shahidi, F. (2005) Optimization of extraction of phenolic compounds from wheat using response surface methodology. **Food Chemistry**, 93: (1): 47-56.

López-García, M.Á., López, Ó., Maya, I., et al. (2010) Complexation of hydroxytyrosol with β-cyclodextrins. An efficient photoprotection. **Tetrahedron**, 66: (40): 8006-8011.

Lotito, S.B. and Frei, B. (2004) Relevance of Apple polyphenols as antioxidants in Human plasma: Contrasting in vitro and in vivo effects. **Free Radical Biology and Medicine**, 36: (2): 201-211.

Lu, Y. and Foo, L.Y. (1997) Identification and quantification of major polyphenols in apple pomace. **Food Chemistry**, 59: (2): 187-194.

Lu, Y. and Foo, L.Y. (2000) Antioxidant and radical scavenging activities of polyphenols from apple pomace and vegetables. **Food Chemistry**, 68: (1): 81-85.

Lucas-Abella´n, C., Fortea, M.I., Gabaldo´n, J.A., et al. (2008) Encapsulation of quercetin and myricetin in cyclodextrins at acidic pH. **Journal of Agricultural and Food Chemistry**, 56: 255-259.

Lucas-Abellán, C., Fortea, I., López-Nicolás, J.M., et al. (2007) Cyclodextrins as resveratrol carrier system. **Food Chemistry**, 104: (1): 39-44.

Lundt, I., Rosenbohm, C. and Christensen, T.M.I.E. (2003) **Carbohydrate Resources**, 338: 637.

Luque-Rodríguez, J., de Castro, M.L. and Pérez-Juan, P. (2007) Dynamic superheated liquid extraction of anthocyanins and other phenolics from red grape skins of winemaking residues. **Bioresource Technology**, 98: (14): 2705-2713.

Luque de Castro, M.D. and Garcia-Ayuso, L.E. (1998) Soxhlet extraction of solid materials: An outdated technique with a promising innovative future. **Analytica Chimica Acta,**, 369: 1-10.

Luque de Castro, M.D., Jiménez-Carmona, M.M. and Fernández-Pérez, V. (1999) Towards more rational techniques for the isolation of valuable essential oils from plants. **TrAC Trends in Analytical Chemistry**, 18: (11): 708-716.

Luque de Castro, M.D., ValcaÂrcel, M. and Tena, M.T. (1994) **Analytical Supercritical Fluid Extraction** New York. : Springer-Verlag.

Lussignoli, S., Fraccaroli, M., Andrioli, G., et al. (1999) A microplate-based colorimetric assay of the total peroxyl radical trapping capability of human plasma. **Analytical. Biochemistry**, 269: 38-44.

Luthria, D.L. (2006) Significance of sample preparation in developing analytical methodologies for accurate estimation of bioactive compounds in functional foods. **Journal of the Science of Food and Agriculture**, 86: 2266-2272.

M.K, K. and C., P. (2005) Rehydration of dehydrated foods. **Journal of. Drying Technology**, 23: 799-830.

Ma, Y.-Q., Chen, J.-C., Liu, D.-H., et al. (2009) Simultaneous extraction of phenolic compounds of citrus peel extracts: Effect of ultrasound. **Ultrasonics Sonochemistry**, 16: (1): 57-62.

Madene, A., Jacquot, M., Scher, J., et al. (2006) Flavour encapsulation and controlled release– a review. **International Journal of Food Science & Technology**, 41: (1): 1-21.

Mahawar, M., Singh, A. and Jalgaonkar, K. (2012) Utility of apple pomace as a substrate for various products: A review. **Food and Bioproducts Processing**, 90: 597-605.

Manach, C., Scalbert, A., Morand, C., et al. (2004) Polyphenols: food sources and bioavailability. **American Journal of Clinical Nutrition**, 79: 727-747.

Marcon, M.V., Carneiro, P.I.B., Wosiacki G., et al. (2005) Pectins from Apple Pomace – Characterization by 13C and 1H NMR Spectroscopy. **Ann. Magn. Reson**, 4: (3): 56-63.

Mariethoz, E., Richard, M.-J., Polla, L.L., et al. (1998) Oxidant/antioxidant imbalance in skin aging: environmental and adaptative factors. **Reviews on environmental health**, 13 (3): 147-168.

Matson, D.W., Fulton, J.L., Petersen, R.C., et al. (1987) Rapid expansion of supercritical fluid solutions: solute formation of powders, thin films, and fibers. **Industrial & Engineering Chemistry Research**, 26: (11): 2298-2306.

Matsunaga, Y., Machmudah, S., Kanda, W., et al. (2014) Subcritical water extraction and direct formation of microparticulate polysaccharides powders from Ganoderma lucidum. **International Journal of Technology**, 5: 40-50.

May, C.D. (1990) Industrial pectins: Sources, production and applications.**Carbohydrate polymers**, 12: 79-84.

Mazza, G., Cacace, J. and Kay, C. (2004) Methods of analysis for anthocyanins in plants and biological fluids. **J AOAC International**, 87: 129-145.

Mazza, G. and Miniati, E. (1991) **Anthocyanins in fruits, vegetables, and grains**. Boca Raton: CRC Press.

McHugh, M. and Krukonis, V. (2013) **Supercritical fluid extraction: principles and practice**. Elsevier.

McMinn, W.A.M. and Magee, T.R.A. (1997) Quality and physical structure of dehydrated starch based system. **Drying Technology**, 15: 49-55.

McMurry, P.H., Wang, X., Park, K., et al. (2002) The relationship between mass and mobility for atmospheric particles: A new technique for measuring particle density. **Aerosol Science & Technology**, 36: (2): 227-238.

Mercader-Ros, M., Lucas-Abellán, C., Fortea, M., et al. (2010) Effect of HP-β-cyclodextrins complexation on the antioxidant activity of flavonols. **Food Chemistry**, 118: (3): 769-773.

Meterc, D., Petermann, M. and Weidner, E. (2008) Drying of aqueous green tea extracts using a supercritical fluid spray process. **The Journal of Supercritical Fluids**, 45: (2): 253-259.

Meyer, C.A., Jr, McClintock, R.B., Silvestri, G.J., et al. (1999) **ASME Steam Tables: Thermodynamics and Transport Properties of steam 6th Edition**.

Micrometrics (2015) http://www.micromeritics.com/Product-Showcase/AccuPyc-II-1340.aspx [online]. [Accessed

Miguel, F., Martin, A., Gamse, T., et al. (2006) Supercritical anti solvent precipitation of lycopene: Effect of the operating parameters. **The Journal of Supercritical Fluids**, 36: (3): 225-235.

Miller, G., Archer, L., Pica, E., et al. (2006) Nanomaterials, sunscreens and cosmetics: small ingredients, big risks: Friends of the Earth Australia & Friends of the Earth United States.

Mishima, K., Matsuyama, K., Tanabe, D., et al. (2000) Microencapsulation of proteins by rapid expansion of supercritical solution with a nonsolvent. **AIChE Journal**, 46: (4): 857-865.

Mittal, A., Elmets, C. and Katiyar, S. (2003) Dietary feeding of proanthocyanidins from grape seeds prevents photocarcinogenesis in SKH-1 hairless mice: relationship to decreased fat and lipid peroxidation. **Carcinogenesis**, 24: (8): 1379-1388.

Mohamed, R.S., Debenedetti, P.G. and Prud'homme, R.K. (1989) Effects of process conditions on crystals obtained from supercritical mixtures. **AIChE Journal**, 35: (2): 325-328.

Mohsen-Nia, M., Amiri, H. and Jazi, B. (2010) Dielectric Constants of Water, Methanol, Ethanol, Butanol and Acetone: Measurement and Computational Study. **Journal of Solution Chemistry**, 39: (5): 701-708.

Monrad, J.K., Howard, L.R., King, J.W., et al. (2010) Subcritical Solvent Extraction of Procyanidins from Dried Red Grape Pomace. **Journal of Agricultural and Food Chemistry**, 58: (7): 4014-4021.

Moon, J.K. and Shibamoto, T. (2010) Formation of volatile chemicals from thermal degradation of less volatile coffee **Journal of Agricultural and Food Chemistry**, 58: 5465.

Morales, F.J. and Jimenez, -.P., S. (2009) Free radical scavenging capacity of Maillard reaction products as related to color and fluorescence. **Food Chemistry**, 72: 119-196.

Morawska, L., Johnson, G., Ristovski, Z., et al. (1999) Relation between particle mass and number for submicrometer airborne particles. **Atmospheric Environment**, 33: (13): 1983-1990.

Morgan, J. and Richards, A. (1993) **The Book of Apples**. London: Ebury Press.

Morimoto, S., Nonaka, G.I. and Nishioka, I. (1985) Tannins and Related Compounds XXXV. Proanthocyanidins with a Doubly Linked Unit from the Root Bark of Cinnamomum sieboldii MEISNER. **Chemical and pharmaceutical bulletin**, 33: 4338-4345.

Mourtzinos, I., Salta, F., Yannakopoulou, K., et al. (2007) Encapsulation of olive leaf extract in β-cyclodextrin. **Journal. of Agricultural and Food Chemistry**, 55: (20): 8088-8094.

Mozafari, M.R. (2006) "Bioactive entrapment and targeting using nanocarrier technologies: an introduction". **Nanocarrier technologies**. Springer 1-16.

Munin, A. and Florence, E.-L. (2011) Encapsulation of Natural Polyphenolic Compounds; a Review **Pharmaceutics**, 3: 793-829.

Murugesan, R. and Orsat, V. (2011) Spray drying of elderberry (Sambucus nigra L.) juice to maintain its phenolic content. **Drying Technology**, 29: (14): 1729-1740.

Myers, R.H., Montgomery, D.C. and Anderson-Cook, C.M. (2009) **Response surface methodology: process and product optimization using designed experiments**. John Wiley & Sons.

Nath, S. and Satpathy, G.R. (1998) A systematic approach for investigation of spray drying processes. **Drying Technology**, 16: (6): 1173-1193.

Navarro, P., Nicolas, T.S., Gabaldon, J.A., et al. (2011) Effects of cyclodextrin type on vitamin C, antioxidant activity, and sensory attributes of a mandarin juice enriched with pomegranate and goji berries. **Journal of Food Science**, 76: (5): S319-S324.

Nelson, J. and Falk, R. (1992) The efficacy of phloridzin and phloretin on tumor cell growth. **Anticancer research**, 13: (6A): 2287-2292.

Nicholas, L. (2012) " Anaerobic Digestion plant at court farm, Herefordshire In: Transport statement in support of retrospective planning application.".

Nieto, A., Borrull, F., Pocurull, E., et al. (2010) Pressurized liquid extraction: a useful technique to extract pharmaceuticals and personal-care products from sewage sludge. **TrAC Trends in Analytical Chemistry**, 29: (7): 752-764.

Niki, E. (2002) Antioxidant activity: are we measuring it correctly? **Nutrition**, 18: 524-525.

Nogueira, A. and Wosiacki, G. (2012) **Apple cider fermentation**. CRC Press.

NTP (2008) "Toxicology and carcinogenesis studies of 5-(hydroxymethyl)-2-furfural (CAS No. 67-47-0) in F344/N rats and B6C3F, mice (gavage studies)". **National Toxicology. Program. Technical. Report Series**. <http://ntp.niehs.nih.gov/>.

Núñez-Delicado, E., Sánchez-Ferrer, A. and García-Carmona, F. (1997) Cyclodextrins as Secondary Antioxidants: Synergism with Ascorbic Acid. **Journal of Agricultural and Food Chemistry**, 45: (8): 2830-2835.

O'Rourke, D., Janic, J. and Sansavini, S. (2003) World apple cultivar dynamics, horticultural science news. Plant Species Project. **Chronica Horticulturae**, 43: (3): 10-14.

Oetjen, G.W. (2004) **Freeze-Drying**. Wiley Online Library.

Olsson, C. and Westman, G. (2013) Direct dissolution of cellulose: background, means and applications. **Cellulose–fundamental aspects, InTech, Chapter**, 6: 144-178.

Ong, E.S., Cheong, J.S.H. and Goh, D. (2006) Pressurized hot water extraction of bioactive or marker compounds in botanicals and medicinal plant materials: Review. **Chromatography. A** 1112: 92-102.

Otero-Espinar, F., Anguiano-Igea, S., Garcia-Gonzalez, N., et al. (1992) Interaction of naproxen with β-cyclodextrin in solution and in the solid state. **International journal of pharmaceutics**, 79: (1): 149-157.

Ou, B., Hampsch-Woodill, M. and Prior, R.L. (2001) Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. **Journal of Agricultural and. Food Chemistry**, 49: 4619-4926.

Ou, B., Huang, D., Hampsch-Woodill, M., et al. (2002) Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: a comparative study. **Journal of Agricultural and. Food Chemistry**, 50: 3122-3128.

Pacheco-Palencia, L.A., Duncan, C.E. and Talcott, S.T. (2009) Phytochemical composition and thermal stability of two commercial açai species, Euterpe oleracea and Euterpe precatoria. **Food Chemistry**, 115: (4): 1199-1205.

Pagington, J. (1986) Beta-cyclodextrin and its uses in the flavour industry. **Developments in food flavours/edited by GG Birch and MG Lindley**.

Pang, S.F., Yusoff, M.M. and Gimbun, J. (2014) Assessment of phenolic compounds stability and retention during spray drying of Orthosiphon stamineus extracts. **Food Hydrocolloids**, 37: (0): 159-165.

Parma, M. (2003) **Utilization of apple pomace for production and evaluation of pectinase(s) MSc thesis**. Dr YS Parma University of Horticulture and Forestry,Nauni.

Patel, R., Patel, M. and Suthar, A. (2009) Spray drying technology: an overview. **Indian Journal of Science and Technology**, 2: (10): 44-47.

Pérez-Jiménez, J., Arranz, S. and Saura-Calixto, F. (2009) Proanthocyanidin content in foods is largely underestimated in the literature data: An approach to quantification of the missing proanthocyanidins. **Food Research International**, 42: (10): 1381-1388.

Peschel, W., Sanchez-Rabaneda, F., Diekmann, W., et al. (2006) An industrial approach in the search of natural antioxidants from vegetable and fruit wastes. **Food Chemistry**, 97: 137-150.

Pestov, D., Levit, N., Guney-Altav, O., et al. (2003) "Development of encapsulated microspheres from a multiple-component, rapidly expanding supercritical solution from sensing applications". In IN G. Brunner, I.K., M. Perrut (Eds.) (Ed.) **Proceedings of the Sixth International Symposium on Supercritical Fluids**. Versalles, France.

Petersen, R.C. (1835) Analyse des phloridzin. **Annals of the New York Academy of Sciences Fr**, 15: (178).

Petersen, R.C., Matson, D.W. and Smith, R.D. (1986) Rapid precipitation of low vapor pressure solids from supercritical fluid solutions: the formation of thin films and powders. **Journal of the American Chemical Society**, 108: (8): 2100-2102.

Pinelo, M., Ruiz-Rodriguez, A., Sineiro, J., et al. (2007) "Supercritical fluid and solid--liquid extraction of phenolic antioxidants from grape pomace: a comparative study.(Report)". **European Food Research and Technology**. springer.

Pingret, D., Fabiano-Tixier, A.-S., Le Bourvellec, C., et al. (2012) Lab and pilot-sacle ultrasound water extraction of polyphenols from apple pomace. **Journal of Food Engineering**, 111: 73-81.

Pitz, M., Cyrys, J., Karg, E., et al. (2003) Variability of apparent particle density of an urban aerosol. **Environmental Science & Technology**, 37: (19): 4336-4342.

Plaza, M., Abrahamsson, V. and Turner, C. (2013) Extraction and Neoformation of Antioxidant Compounds by Pressurized Hot Water Extraction from Apple Byproducts. **Journal of Agricultural and Food Chemistry**, 61: 5500-5510.

Plaza, M., Amigo-Benavent, M., Del Castillo, M.D., et al. (2010) Facts about the formation of new antioxidants in natural samples after subcritical water extraction. **Food Research International**, 43: (10): 2341-2348.

Poomkokrak, J., Niamnuy, C., Choicharoen, K., et al. (2015) Encapsulation of soybean extract using spray drying. **Journal of Food Science and Agricultural Technology**, 1: (1): 105-110.

Porter, L.J. (ed.) (1988) **Flavans and Proanthocyanidins.**, New York: Chapman and Hall.

Porter, L.J. (ed.) (1989) **Tannins.**, San Diego, CA: Academic Press.

Pouillot, A., Luigi, L.P., Philippe, T., et al. (2011) **Natural Antioxidant and their effects on the skin**. John Wiley & Sons,Inc.

Pralhad, T. and Rajendrakumar, K. (2004) Study of freeze-dried quercetin–cyclodextrin binary systems by DSC, FT-IR, X-ray diffraction and SEM analysis. **Journal of Pharmaceutical and Biomedical Analysis**, 34: (2): 333-339.

Prior, R.L. and Gu, L. (2005) Occurrence and biological significance of proanthocyanidins in the American diet: Review. **Phytochemistry**, 66: 2264-2280.

Prior, R.L., Hoang, H., Gu, L., et al. (2003) Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORACFL)) of plasma and other biological and food samples. **Journal of Agricultural and Food Chemistry**, 51: 3273-3279.

Prior, R.L., Wu, X. and Schaich, K. (2005) Standardized Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements. **Journal of Agricultural and Food Chemistry**, 53: (10): 4290-4302.

Puel, C., Quintin, A., Mathey, J., et al. (2005) Prevention of bone loss by phloridzin, an apple polyphenol, in ovariectomized rats under inflammation conditions. **Calcified tissue international**, 77: (5): 311-318.

Pulido, R., Bravo, L. and Saura-Calixto, F. (2000) Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. **Journal of Agricultural and Food Chemistry**, 48: (8): 3396-3402.

Purlis, E. (2010) Browning development in bakery products – A review. **Journal of Food Engineering**, 99: (3): 239-249.

Rajam, R., Karthik, P., Parthasarathi, S., et al. (2012) Effect of whey protein – alginate wall systems on survival of microencapsulated Lactobacillus plantarum in simulated gastrointestinal conditions. **Journal of Functional Foods**, 4: (4): 891-898.

Rasmussen, S., Frederiksen, H., Struntze, K.K., et al. (2005) Dietary proanthocyanidins: occurrence, dietary intake, bioavailability, and protection against cardiovascular disease. **Molecular nutrition & food research**, 49: 159-174.

Ratnam, D.V., Ankola, D., Bhardwaj, V., et al. (2006) Role of antioxidants in prophylaxis and therapy: A pharmaceutical perspective. **Journal of Controlled Release**, 113: (3): 189-207.

Ratti, C. (2001) Hot air and freeze-drying of high-value foods: a review. **Food Engineering**, 49: 311-319.

Raventós , M., Duarte, S. and Alarcroŕ, R. (2002) Application and possibilities of supercritical CO2 extraction in food processing industry: an overview. **Food Scencei and Technol international**, 8: 269-284.

Rawel, H.M., Czajka, D., Rohn, S., et al. (2002) Interactions of different phenolic acids and flavonoids with soy proteins. **International journal of biological macromolecules**, 30: (3): 137-150.

Re, R., Pellegrini, N., Proteggente, A., et al. (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. **Free Radical Biology and Medicine**, 26: (9): 1231-1237.

Reis, S.F., Rai, D.K. and Abu-Ghannam, N. (2012) Water at room temperature as a solvent for the extraction of apple pomace phenolic compounds. **Food Chemistry**, 135: 1991-1998.

Reverchon, E. (2002) Supercritical-assisted atomization to produce micro-and/or nanoparticles of controlled size and distribution. **Industrial & Engineering Chemistry Research**, 41: (10): 2405-2411.

Rezk, B.M., Haenen, G.R., van der Vijgh, W.J., et al. (2002) The antioxidant activity of phloretin: the disclosure of a new antioxidant pharmacophore in flavonoids. **Biochemical and biophysical research communications**, 295: (1): 9-13.

Rice-Evans, Miller, N. and Paganga, G. (1997) Antioxidant properties of phenolic compounds. **Trends Plant Sci**, 2: 152-159.

Rice-Evans, Miller, N.J., Bolwell, P.G., et al. (1995) The relative antioxidant activities of plant-derived polyphenolic flavonoids. **Free Radical Research**, 22: 375-383.

Robert, P., Gorena, T., Romero, N., et al. (2010) Encapsulation of polyphenols and anthocyanins from pomegranate (Punica granatum) by spray drying. **International Journal of Food Science & Technology**, 45: (7): 1386-1394.

Rocha, S., Generalov, R., Pereira, M.d.C., et al. (2011) Epigallocatechin gallate-loaded polysaccharide nanoparticles for prostate cancer chemoprevention. **Nanomedicine**, 6: (1): 79-87.

Rodrigues, S. and Pinto, G.A.S. (2007) Ultrasound extraction of phenolic compounds from coconut (Cocos nucifera) shell powder. **Journal of Food Engineering**, 80: (3): 869-872.

Rodríguez-Hernández, G.R., González-García, R., Grajales-Lagunes, A., et al. (2005) Spray-Drying of Cactus Pear Juice (Opuntia streptacantha): Effect on the Physicochemical Properties of Powder and Reconstituted Product. **Drying Technology**, 23: (4): 955-973.

Rodríguez-Meizosoa, I., Jaimeb, L., Santoyob, S., et al. (2010) Subcritical water extraction and characterization of bioactive compounds from Haematococcus pluvialis microalga. **Journal of Pharmaceutical and Biomedical Analysis**, 51: 456-463.

Rogerson, P. (2001) **Statistical methods for geography**. Sage.

Rolin, C. and De Vries, J. (1990) **Pectin.** London: Elsevier.

Rosenberg, M., Kopelman, I.J. and Talmon, Y. (1985) A Scanning Electron Microscopy Study of Microencapsulation. **Journal of Food Science**, 50: (1): 139-144.

Roukas, T. (1991) Production of citric acid from beet molasses by immobilized cells of Aspergillus niger. **Journal of Food Science**, 56: (3): 878-880.

Roupas, P. and Noakes, M. (2010) Apples, their antioxidants and benefits to human health. **Australia: CSIRO**, 1-56.

Rust, S.R. (1991) "Feeding food industry by-product to ruminants". **52nd Annual Minnesota nutrition conference**.

Sablani, S.S., Shrestha, A.K. and Bhandari, B.R. (2008) A new method of producing date powder granules: Physicochemical characteristics of powder. **Journal of Food Engineering**, 87: (3): 416-421.

Şahin Nadeem, H., Torun, M. and Özdemir, F. (2011) Spray drying of the mountain tea (Sideritis stricta) water extract by using different hydrocolloid carriers. **LWT - Food Science and Technology**, 44: (7): 1626-1635.

Salimi Hizaji, A., Maghsoudlou, Y. and Jafari, S. (2011) Effect of water temperature, variety and shelf life on rehydration kinetics of microwave dried potato cubes. **Latin American applied research**, 41: (3): 249-254.

Salles, B., Provot, C., Calsou, P., et al. (1995) A chemiluminescent microplate assay to detect DNA damage induced by genotoxic treatments. **Analytical Biochemistry**, 232: (1): 37-42.

Sanchez-Moreno, C. (2002) Review: Methods used to evaluate the free radical scavenging activity in foods and biological systems. **Food Science and Technology International**, 8: 121-137.

Sanroman, A., Pintado, J. and Lema, J. (1994) A comparison of two techniques (adsorption and entrapment) for the immobilization of Aspergillus niger in polyurethane foam. **Biotechnology Techniques**, 8: (6): 389-394.

Sartor, V., Henderson, P.T. and Schuster, G.B. (1999) Radical cation transport and reaction in RNA/DNA hybrid duplexes: effect of global structure on reactivity. **Journal of the American Chemical Society**, 121: (48): 11027-11033.

Sato, M.F., Vieira, R.G., Zardo, D.M., et al. (2010) Apple pomace from eleven cultivars: an approach to identify sources of bioactive compounds. **Acta Scientiarum. Agronomy**, 32: 29-35.

Saura-Calixto, F. (1998) Antioxidant Dietary Fiber Product: A New Concept and a Potential Food Ingredient. **Journal of Agricultural and Food Chemistry**, 46: (10): 4303-4306.

Saura-Calixto, F., Serrano, J. and Goñi, I. (2007) Intake and bioaccessibility of total polyphenols in a whole diet. **Food Chemistry**, 101: 492-501.

Savary, B.J., Hotchkiss, A.T., Fishman, M.L., et al. (2003) **Development of a Valencia orange pectin methyl esterase for generating novel pectin products.** The Netherlands: Kluwer Academic Publishers.

Scalbert, A., Johnson, I.T. and Saltmarsh, M. (2005a) Polyphenols: antioxidants and beyond. **The American Journal of Clinical Nutrition**, 81: (1): 215S-217S.

Scalbert, A., Manach, C., Morand, C., et al. (2005b) Dietary polyphenols and the prevention of diseases. **Critical Reviews in Food Science and Nutrition**, 45: (4): 287-306.

Scalbert, A. and Williamson, G. (2000) Dietary intake and bioavailability of polyphenols. **The Journal of Nutrition**, 130: (8): 2073S-2085S.

Scalia, S., Villani, S. and Casolari, A. (1999) Inclusion complexation of the sunscreen agent 2-ethylhexyl-p-dimethylaminobenzoate with hydroxypropyl- beta-cyclodextrin: effect on photostability. **Journal of pharmacy and pharmacology**, 51: 1367-1374.

Schieber, A., Keller, P. and Carle, R. (2001a) Determination of phenolic acids and flavonoids of apple and pear by high- performance liquid chromatography. **Chromatography. A**, 910: 265-273.

Schieber, A., Keller, P. and Carle, R. (2001b) Determination of phenolic acids and flavonoids of apple and pear by high-performance liquid chromatography. **Journal of Chromatography A**, 910: 265-273.

Schreiner, J., Voigt, C., Zink, P., et al. (2002) A mass spectrometer system for analysis of polar stratospheric aerosols. **Review of scientific instruments**, 73: (2): 446-452.

Seeram, N.P., Aviram, M., Zhang, Y., et al. (2008) Comparison of antioxidant potency of commonly consumed polyphenol-rich beverages in the United States. **Journal of Agricultural and Food Chemistry**, 56: (4): 1415-1422.

Serena, C.M., William, M. and Crozier, A. (2007) Flavonoids and Chlorogenic acid profiles of English cider apples. **Journal of the Science of Food and Agriculture**, 87: 719-728.

Shalini, R. and Gubta, D.K. (2010) Utilization of pomace from apple processing industries: a review. **Food Science Technol**, 47: (4): 365-371.

Shankaranand, V. and Lonsane, B. (1994) Coffee husk: an inexpensive substrate for production of citric acid by Aspergillus niger in a solid-state fermentation system. **World Journal of Microbiology and Biotechnology**, 10: (2): 165-168.

Shari, K. (2013) "How to get Started with Design- Expert Software". In Stat-Ease (Ed.) www.statease.com/webinar.html.

Shariati, A. and Peters, C.J. (2003) Recent developments in particle design using supercritical fluids. **Current Opinion in Solid State and Materials Science**, 7: (4): 371-383.

Sharma, T.R., Lal, B.B., Kumar, S., et al. (1985) Pectin from different varieties of Himachal Pradesh apples. **Indian Food Packer**, 39: (4): 53-57.

Sheu, T.Y. and Rosenberg, M. (1998) Microstructure of Microcapsules Consisting of Whey Proteins and Carbohydrates. **Journal of Food Science**, 63: (3): 491-494.

Shindo, Y., Witt, E., Han, D., et al. (1994) Enzymic and non-enzymic antioxidants in epidermis and dermis of human skin. **Journal of Investigative Dermatology**, 102: (1): 122-124.

Shojaosadati, S.A. and Babaeipour, V. (2002) Citric Acid Production from Apple Pomace in Multi-layer Packed Bed Solid State Bioreactor. **Process Biochemistry**, 37: 909-914.

Shuang, L., Hou, W., Yao, P., et al. (2010) Quercetin protects against ethanol-induced oxidative damage in rat primary hepatocytes. **Toxicology in Vitro**, 24: (2): 516-522.

Sievers, R., Huang, E., Villa, J., et al. (2003) Micronization of water-soluble or alcohol-soluble pharmaceuticals and model compounds with a low-temperature Bubble Dryer®. **The Journal of Supercritical Fluids**, 26: (1): 9-16.

Sihvonen, M., Jarvenpaa, E., Hietaniemi, V., et al. (1999) Advances in supercritical carbon dioxide technologies. **Trends in Food and Technolgy**, 10: 217-222.

Silva, E., Rogez, H. and Larondelle, Y. (2007) Optimization of extraction of phenolics from Inga edulis leaves using response surface methodology. **Separation and Purification Technology**, 55: (3): 381-387.

Singh, R., Bharti, N., Madan, J., et al. (2010) Characterization of cyclodextrin inclusion complexes—a review. **Journal of. Pharmaceutical. Science.and Technology**, 2: (3): 171-183.

Singleton, V.L., Orthofer, R. and Lamuela-Raventos, R.M. (1999) Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. **Methods in Enzymology**, 299: 152-178.

Singleton, V.L. and Rossi, J.A. (1965a) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. **American journal of Enology and Viticulture**, 16: (3): 144-158.

Singleton, V.L. and Rossi, J.A., Jr (1965b) Colorimetry of total phenolics with phosphomolybdicphosphotungstic acid reagents. **American Journal of Enology.and Viticuture**, 16: 144-158.

Sivaraman, T., Kumar, T.K.S., Jayaraman, G., et al. (1997) The Mechanism of 2,2,2-Trichloroacetic Acid-Induced Protein Precipitation. **Journal of Protein Chemistry**, 16: (4): 291-297.

Slade, L., Levine, H. and Reid, D.S. (1991) Beyond water activity: Recent advances based on an alternative approach to the assessment of food quality and safety. **Critical Reviews in Food Science and Nutrition**, 30: (2-3): 115-360.

Smith, R.M. (2002) Extractions with superheated water. **Journal of Chromatography A**, 975: (1): 31-46.

Song, J., Huang, J., Xu, K.-Z., et al. (2003) Production of Citric Acid from Apple Pomace Enzymolyzed by Cellulase. **The Chinese Journal of Process Engineering**, 3: (5).

Spencer, J.P., Rice-Evans, C. and Williams, R.J. (2003) Modulation of prosurvival Akt/protein kinase B and ER K1/2 signaling cascades by quercetin and its in vivo metabolites underlie their action on neuronal viability.**Journal of Biological Chemistry**, 278: 34783-34793.

Srinivasan, V.S. (2001) Bioavailability of nutrients: a practical approach to in vitro demonstration of the availability of nutrients in multivitamin-mineral combination products. **The Journal of nutrition**, 131: (4): 1349S-1350S.

Streilen, J. and Gilchrist, B. (1995) UVB susceptibility: possible relationship to photoaging and photocarcinogenesis. **Photodamage. Philadelphia, Pa: Lippincott–Williams & Wilkins**, 68-80.

Suarez, B., Ángel L. Álvarez, Yolanda Diñeiro García , et al. (2010) Phenolic profiles, antioxidant activity and in vitro antiviral properties of apple pomace. **Food Chemistry**, 120: 339-342.

Sun, J., Chu, Y.-F., Wu, X., et al. (2002) Antioxidant and antiproliferative activities of common fruits. **Journal of Agricultural and Food Chemistry**, 50: (25): 7449-7454.

Taasoli, G. and Kafilzadeh, F. (2008) Effects of Dried and Ensiled Apple Pomace from Puree Making on Performance of Finishing Lambs. **Pakistan Journal of Biological Sciences**, 11: 294-297.

Tang, B., Ma, L., Wang, H.-y., et al. (2002) Study on the supramolecular interaction of curcumin and β-cyclodextrin by spectrophotometry and its analytical application. **Journal of Agricultural and Food Chemistry**, 50: (6): 1355-1361.

Teo, C.C., Swee, N.T., Jean, W.H.Y., et al. (2010) Pressurized hot water extraction (PHWE). **Chromatography. A**, 1217: 2484-2494.

Tom, J.W. and Debenedetti, P.G. (1991) Formation of bioerodible polymeric microspheres and microparticles by rapid expansion of supercritical solutions. **Biotechnology progress**, 7: (5): 403-411.

Tommasini, S., Calabrò, M., Stancanelli, R., et al. (2005) The inclusion complexes of hesperetin and its 7-rhamnoglucoside with (2-hydroxypropyl)-β-cyclodextrin. **Journal of Pharmaceutical and Biomedical Analysis**, 39: (3): 572-580.

Tomren, M., Masson, M., Loftsson, T., et al. (2007) Studies on curcumin and curcuminoids: XXXI. Symmetric and asymmetric curcuminoids: stability, activity and complexation with cyclodextrin. **International journal of pharmaceutics**, 338: (1): 27-34.

Tonon, R.V., Brabet, C. and Hubinger, M.D. (2008) Influence of process conditions on the physicochemical properties of açai (Euterpe oleraceae Mart.) powder produced by spray drying. **Journal of Food Engineering**, 88: (3): 411-418.

Toor, S.S., Rosendahl, L. and Rudolf, A. (2011) Hydrothermal liquefaction of biomass: A review of subcritical water technologies. **Energy** 36: 2328-2342.

Tran, C.T. and Mitchell, D.A. (1995) Pineapple Waste a Novel Substrate for Citric Acid Production by Solid-state Fermentation. **Biotechnology. Letters.**, 17: (10): 1107-1110.

Tsao, R. and McCallum, J. (2009) **Chemistry of Flavonoids. In ; Eds.; Blackwell Publishing: USA, 2009; Chapter 5, pp.** . USA: Ames, IA.

TSE (2003) " Standard of Apple Juice Concentrate TS 3686". **Institute of Turkish Standards**. Ankara, Turkey.

Ulbricht, R., J., Northup, S., J. and Thomas, J., A. (1984) A Review of 5-Hydroxymethylfurfural (HMF) in Parenteral Solutions. **Toxicological Sciences**, 4: (5): 843-853.

Valcárcel, M. and Tena, M.T. (1997) Applications of supercritical fluid extraction in food analysis. **Fresenius Journal of Analytical Chemistry**, 358: 561-573.

Vasantha Rupasinghe, H.P., Kathirvel, P. and Huber, G.M. (2011) Ultrasonication-Assisted Solvent Extraction of Quercetin Glycosides from 'Idared' Apple Peels. **Molecules**, 16: (12): 9783.

Vasil'ev, Y.I., Morozov, A.N., Zaiko, G.M., et al. (1976) The utilization of waste from vegetable and fruit canning plants. **Konservnaya-i-Ovoshchesushil naya-Promyshlennost**, 3: 32-34.

Veeriah, S., Kautenburger, T., Habermann, N., et al. (2006) Apple flavonoids inhibit growth of HT29 human colon cancer cells and modulate expression of genes involved in the biotransformation of xenobiotics. **Molecular carcinogenesis**, 45: (3): 164-174.

Vehring, R. (2008) Pharmaceutical particle engineering via spray drying. **Pharmaceutical Research**, 25: (5): 999-1022.

Velioglu, Y.S., Mazza, G., Gao, L., et al. (1998) Antioxidant Activity and Total Phenolics in Selected Fruits, Vegetables, and Grain Products. **Journal of Agricultural and Food Chemistry**, 46: (10): 4113-4117.

Vendruscolo, F., Albuquerque, P.M., Streit, F., et al. (2008) Apple Pomace: A Versatile Substrate for Biotechnological Applications. **Critical. Reviews.in Biotechnology**, 28: (1): 1-12.

Véronique, C. (2005) Polyphenols in foods are more complex than often thought. **Am J Clin Nutr**, 81: (1): 2235-2295.

Vertuani, S., Beghelli, E., Scalambra, E., et al. (2011) Activity and stability studies of verbascoside, a novel antioxidant, in dermo-cosmetic and pharmaceutical topical formulations. **Molecules**, 16: 7068-7080.

Via, J., Taylor, L.T. and Schweighardt, F.K. (1994) Experimental determination of changes in methanol modifier concentration in premixed carbon dioxide cylinders. **Analytical. Chemistry.**, 66: 1459-1461.

Vinatoru, M. (2001) An overview of the ultrasonically assisted extraction of bioactive principles from herbs. **Ultrasonics Sonochemistry**, 8: 303-313.

Vinson, J.A., Dabbagh, Y.A., Serry, M.M., et al. (1995) Plant flavonoids, especially tea flavonols, are powerful antioxidants using an in vitro oxidation model for heart disease. **Journal of Agricultural and Food Chemistry**, 43: (11): 2800-2802.

Vinson, J.A., Su, X., Zubik, L., et al. (2001) Phenol antioxidant quantity and quality in foods: fruits. **Journal of Agricultural and Food Chemistry**, 49: 5315-5321.

Virot, M., Tomao, V., Le Bourvellec, C., et al. (2010) Towards the industrial production of antioxidants from food processing by-products with ultrasound-assisted extraction. **Ultrasonics Sonochemistry**, 17: (6): 1066-1074.

Visioli, F., Bernaert, H., Corti, R., et al. (2009) Chocolate, lifestyle, and health. **Critical Reviews in Food Science and Nutrition**, 49: (4): 299-312.

Voragen, A.G.J., Pilnik, W. and Thibault, J.F. (1995) **Pectins.** New York: Marcel Dekker.

Vrhovsek, U., Rigo, A., Tonon, D., et al. (2004) Quantitation of Polyphenols in Different Apple Varieties. **Journal of Agricultural and Food Chemistry**, 52: (21): 6532-6538.

Walter, R.H. and Sherman, R.M. (1976) Fuel value of grape and apple processing wastes. **Journal of Agricultural and Food Chemistry**, 24: (6): 1244-1245.

Wang, J., Sun, B., Cao, Y., et al. (2008) Optimisation of ultrasound-assisted extraction of phenolic compounds from wheat bran. **Food Chemistry**, 106: (2): 804-810.

Wang, L. and Weller, C.L. (2006) Recent advances in extraction of nutraceuticals from plants. **Trends in Food Science & Technology**, 17: 300-312.

Wang, Y., Dave, R.N. and Pfeffer, R. (2004) Polymer coating/encapsulation of nanoparticles using a supercritical anti-solvent process. **The Journal of Supercritical Fluids**, 28: (1): 85-99.

Waterhouse, A.L. (2001) Determination of Total Phenolics. **Current Protocols in Food Analytical Chemistry**, I1.1.1-I1.1.8.

Way, R.D., Aldwinckle, H.S., Lamb, R.C., et al. (1990) **Apples (Malus)** Hort. Sci., Wageningen (Acta Hort. 290): Int. Soc.

Weidner, E. (1996) **Herstellung von Pulvern durch Versprühen gashaltiger Lösungen**.

Weidner, E. (2009) High pressure micronization for food applications. **Supercritical Fluids** 47: 556-565.

Weidner, E., Kilzer, A., Petermann, M.M., et al. (2000) "Verfahren zur Erzeugung von Polyurethanpartikeln". In Patent, D. (Ed.).

Weidner, E., Knez, Z. and Novak, Z. (1994a) **3rd International Symposium on Supercritical Fluids** Strasbourg Proc. Tome 3

Weidner, E., Knez, Z. and Novak, Z. (1994b) A process and equipment for production and fractionation of fine particles from gas saturated solutions. **World Patent WO**, 95: 21688.

Whitaker, M.J., Hao, J., Davies, O.R., et al. (2005) The production of protein-loaded microparticles by supercritical fluid enhanced mixing and spraying. **Journal of Controlled Release**, 101: (1): 85-92.

Whiting, G.C. and Coggins, R.A. (1975) Estimation of the monomeric phenolics of ciders. **Journal of the Science of Food and Agriculture**, 26: (12): 1833-1838.

Wijngaard, H.H. and Brunton, N. (2009) The optimization of extraction of antioxidants from apple pomace by pressurized liquids. **Journal of Agricultural and Food Chemistry**, 57: (22): 10625-10631.

Wijngaard, H.H. and Brunton, N. (2010) The optimisation of solid–liquid extraction of antioxidants from apple pomace by response surface methodology. **Journal of Food Engineering**, 96: (1): 134-140.

Wiseman, S., Mulder, T. and Rietveld, A. (2001) Tea flavonoids: bioavailability in vivo and effects on cell signaling pathways in vitro. **Antioxidants and Redox Signaling**, 3: (6): 1009-1021.

Witrowa-Rajchert, D. and Lewicki, P.P. (2006) Rehydration properties of dried plant tissues. **International Journal of Food Science & Technology**, 41: (9): 1040-1046.

Wright, J.S., Johnson, E.R. and DiLabio, G.A. (2001) Predicting the activity of phenolic antioxidants: Theoretical method, analysis of substituent effects, and application to major families of antioxidants. **Journal of the American Chemical Society**, 123: 1173-1183.

Wright, T. (2004) **Antioxidants update.** [online]. Nutraceuticals World 2004, http://www.nutraceuticalsworld.com/March041.htm. [Accessed]

Xia, T., Shi, S. and Wan, X. (2006) Impact of ultrasonic-assisted extraction on the chemical and sensory quality of tea infusion. **Journal of Food Engineering**, 74: (4): 557-560.

Yan, H. and Kerr, W.L. (2013) Total phenolics content, anthocyanins, and dietary fiber content of apple pomace powders produced by vacuum-belt drying. **Journal of the Science of Food and Agriculture**, 93: (6): 1499-1504.

Yang, Y., Gharaibeh, A., Hawthorne, S.B., et al. (1995) Combined temperature/modifier effects on supercritical CO_2 extraction efficiencies of polycyclic aromatic hydrocarbons from environmental samples**Analytical. Chemistry 67:641-656.**

Yeo, S.-D. and Kiran, E. (2005) Formation of polymer particles with supercritical fluids: a review. **The Journal of Supercritical Fluids**, 34: (3): 287-308.

Yildiz, F. (ed.) (2010) **Advances in food Biochemistry**, United State of America: CRC.
Yu, J., Ahmedna, M. and Goktepe, I. (2005) Effects of processing methods and extraction solvents on concentration and antioxidant activity of peanut skin phenolics. **Food Chemistry**, 90: 199–206.

Yue, T., Bai, X., Zhang, H., et al. (2012) Fractionation and anti-inflammatory effects of polyphenol –enriched extracts from apple pomace,. **Bangladesh Journal of Pharmacology**, 7: 28-32.

Zhang, L., Mou, D. and Du, Y. (2007) Procyanidins: extraction and micro-encapsulation. **Journal of the Science of Food and Agriculture**, 87: (12): 2192-2197.

Zhang, X.M., Chan, C.C., Stamp, D., et al. (1993) Initiation and promotion of colonic aberrant crypt foci in rats by 5-hydroxymethyl-2-furaldehyde in thermolyzed sucrose **Carcinogenesis**, 14: 773-775.

Zhou, Z., Liu, Y., Miao, A.-D., et al. (2005) Protocatechuic aldehyde suppresses TNF-α-induced ICAM-1 and VCAM-1 expression in human umbilical vein endothelial cells. **European Journal of Pharmacology**, 513: (1–2): 1-8.

Zhou, Z., Zhang, Y., Ding, X.-R., et al. (2007) Protocatechuic aldehyde inhibits hepatitis B virus replication both in vitro and in vivo. **Antiviral Research**, 74: (1): 59-64.

Zsigmondy, R. and Bachmann, W. (1918) Ueber neue filter. **Zeitschrift für anorganische und allgemeine Chemie**, 103: (1): 119-128.

Zulueta, A., Esteve, M.J. and Frígola, A. (2009) ORAC and TEAC assays comparison to measure the antioxidant capacity of food products. **Food Chemistry**, 114: 310-316.

Appendices

APPENDIX A-

RESULTS OF ANALYSIS OF VARIANCE FOR POLYPHENOLIC COMPOUNDS UNDER ACETONE EXTRACTION.

These are the results from the stat-Ease design expert.

Appendix A1- Chlorogenic acid

ANOVA for Response Surface Reduced Quadratic Model for Chlorogenic acid

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	10841.32	10	1084.13	28.92	< 0.0001	significant
A-Acetone Concentration	244.98	1	244.98	6.53	0.0228	
B-Temperature	1804.86	1	1804.86	48.14	< 0.0001	
C-Solid/Solvent	20.56	1	20.56	0.55	0.4712	
D-Time	95.46	1	95.46	2.55	0.1329	
AC	4041.81	1	4041.81	107.80	< 0.0001	
BC	161.20	1	161.20	4.30	0.0571	
BD	227.73	1	227.73	6.07	0.0273	
CD	111.25	1	111.25	2.97	0.1070	
A^2	2769.28	1	2769.28	73.86	< 0.0001	
C^2	235.45	1	235.45	6.28	0.0252	
Residual	524.89	14	37.49			
Lack of Fit	518.84	10	51.88	34.31	0.0019	significant
Pure Error	6.05	4	1.51			
Cor Total	11366.21	24				

These Rows Were Ignored for this Analysis. 5, 16, 12, 9, 18

Std. Dev.	6.12	R-Squared	0.9538
Mean	175.62	Adj R-Squared	0.9208
C.V. %	3.49	Pred R-Squared	0.8029
PRESS	2240.45	Adeq Precision	19.877

Factor	Coefficient Estimate	df	Standard Error	95% CI		VIF
				Low	High	
Intercept	192.60	1	2.17	187.95	197.24	
A-Acetone Concentration	-4.14	1	1.62	-7.61	-0.67	1.30
B-Temperature	10.99	1	1.58	7.59	14.38	1.06
C-Solid/Solvent	1.18	1	1.59	-2.23	4.58	1.07
D-Time	2.11	1	1.32	-0.73	4.95	1.04
AC	16.94	1	1.63	13.44	20.43	1.06
BC	-3.38	1	1.63	-6.88	0.12	1.06
BD	4.02	1	1.63	0.52	7.52	1.06
CD	-2.81	1	1.63	-6.31	0.69	1.06
A^2	-15.03	1	1.75	-18.78	-11.28	1.35
C^2	-6.90	1	2.75	-12.80	-0.99	1.16

Case statistics report for chlorogenic acid under acetone extraction

Standard Order	Actual Value	Predicted Value	Residual	Leverage
1	183.17	175.30	7.87	0.537
2	124.51	133.15	-8.64	0.549
3	189.90	196.00	-6.10	0.523
4	157.05	153.84	3.21	0.536
5	156.00	156.17	-0.17	0.597
6	177.39	181.76	-4.37	0.554
7	162.72	163.33	-0.61	0.543
8	190.21	188.93	1.28	0.629
9	167.89	177.11	-9.22	0.532
10	143.60	134.95	8.65	0.545
11	221.58	213.88	7.70	0.523
12	168.67	171.73	-3.06	0.536
13	146.69	146.73	-0.042	0.552
14	175.36	172.33	3.03	0.602
15	170.85	169.98	0.87	0.721
17	140.67	140.77	-0.10	0.95 #
19	187.78	181.61	6.17	0.176
21	184.12	184.52	-0.40	0.176
23	191.88	188.72	3.16	0.283
24	191.99	196.82	-4.83	0.311
25	191.19	192.60	-1.41	0.125
26	190.15	192.60	-2.45	0.125
27	192.57	192.60	-0.025	0.125
28	191.38	192.60	-1.22	0.125

29	193.29	192.60	0.69	0.125

Appendix A2- Phloridzin

ANOVA for Response Surface Reduced Quadratic Model for Phloridzin

	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	3.720E+005	9	41332.68	80.24	< 0.0001	significant
A-Acetone Concentration	75116.17	1	75116.17	145.83	< 0.0001	
B-Temperature	6781.78	1	6781.78	13.17	0.0021	
C-Solid/Solvent	1.217E+005	1	1.217E+005	236.24	< 0.0001	
D-Time	4509.95	1	4509.95	8.76	0.0088	
BC	4391.71	1	4391.71	8.53	0.0096	
BD	2304.48	1	2304.48	4.47	0.0495	
CD	7192.74	1	7192.74	13.96	0.0016	
A^2	45289.78	1	45289.78	87.92	< 0.0001	
C^2	16034.32	1	16034.32	31.13	< 0.0001	
Residual	8756.80	17	515.11			
Lack of Fit	7530.66	12	627.55	2.56	0.1542	not significant
Pure Error	1226.14	5	245.23			
Cor Total	3.808E+005	26				

These Rows Were Ignored for this Analysis. 16, 12, 9

Std. Dev.	22.70	R-Squared	0.9770
Mean	684.00	Adj R-Squared	0.9648
C.V. %	3.32	Pred R-Squared	0.9071
PRESS	35365.99	Adeq Precision	41.612

Coefficient	Standard	95% CI	95% CI			
Factor	Estimate	df	Error	Low	High	VIF
Intercept	695.99	1	7.57	680.02	711.96	
A-Acetone Concentration	68.07	1	5.64	56.18	79.97	1.22
B-Temperature	20.04	1	5.52	8.39	31.69	1.00
C-Solid/Solvent	-84.93	1	5.53	-96.59	-73.27	1.01
D-Time	13.90	1	4.70	3.99	23.80	1.00
BC	16.57	1	5.67	4.60	28.54	1.00
BD	12.00	1	5.67	0.030	23.97	1.00

CD	-21.20	1	5.67	-33.17	-9.23	1.00
A^2	-59.86	1	6.38	-73.33	-46.39	1.36
C^2	55.44	1	9.94	34.48	76.41	1.21

Case statistics reports for Phloridzin under acetone extraction

					Internally	Externally	Influence on		
Standard	Actual	Predicted			Studentized	Studentized	Fitted Value	Cook's	Run
Order	Value	Value	Residual	Leverage	Residual	Residual	DFFITS	Distance	Order
1	686.35	681.86	4.49	0.457	0.269	0.261	0.240	0.006	26
2	847.53	818.01	29.52	0.470	1.787	1.923	1.811	0.283	11
3	674.83	664.80	10.03	0.456	0.600	0.588	0.539	0.030	3
4	776.84	800.95	-24.11	0.470	-1.458	-1.513	-1.423	0.188	28
5	516.75	521.27	-4.52	0.476	-0.275	-0.268	-0.255	0.007	19
6	641.86	657.42	-15.56	0.476	-0.947	-0.944	-0.900	0.082	4
7	588.53	570.48	18.05	0.476	1.099	1.106	1.055	0.110	23
8	717.27	706.63	10.64	0.476	0.648	0.636	0.607	0.038	1
9	723.64	728.05	-4.41	0.457	-0.264	-0.257	-0.236	0.006	25
10	845.59	864.20	-18.61	0.470	-1.127	-1.136	-1.070	0.113	27
11	785.27	759.00	26.27	0.456	1.570	1.647	1.509	0.207	15
12	894.62	895.15	-0.53	0.469	-0.032	-0.031	-0.029	0.000	30
13	484.60	482.65	1.95	0.476	0.118	0.115	0.110	0.001	7
14	636.60	618.80	17.80	0.476	1.083	1.089	1.039	0.107	13
15	545.01	579.87	-34.86	0.476	-2.122	-2.401	* -2.29	0.409	21
16	722.53	716.02	6.51	0.476	0.396	0.386	0.368	0.014	5
17	314.72	320.39	-5.67	0.95 #	-1.099	-1.106	* -4.74	* 2.22	2
19	677.32	675.95	1.37	0.157	0.066	0.064	0.027	0.000	24
21	813.7	836.37	-22.67	0.174	-1.099	-1.106	-0.507	0.025	20

	0								
23	634.49	670.52	-36.03	0.258	-1.843	-1.999	-1.179	0.118	22
24	707.97	723.78	-15.81	0.279	-0.821	-0.812	-0.506	0.026	10
25	727.07	695.99	31.08	0.111	1.452	1.506	0.533	0.026	6
26	693.36	695.99	-2.63	0.111	-0.123	-0.119	-0.042	0.000	17
27	682.51	695.99	-13.48	0.111	-0.630	-0.619	-0.219	0.005	29
28	713.80	695.99	17.81	0.111	0.832	0.824	0.292	0.009	8
29	709.51	695.99	13.52	0.111	0.632	0.620	0.219	0.005	14
30	705.85	695.99	9.86	0.111	0.461	0.450	0.159	0.003	18

Appendix A3 Quercetin galactoside

ANOVA for Response Surface Reduced Quadratic Model for quercetin galactoside

	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	3.569E-004	8	4.461E-005	23.94	< 0.0001	significant
A-Acetone Concentration	1.570E-004	1	1.570E-004	84.29	< 0.0001	
B-Temperature	2.104E-006	1	2.104E-006	1.13	0.3028	
C-Solid/Solvent	4.256E-005	1	4.256E-005	22.84	0.0002	
D-Time	4.292E-006	1	4.292E-006	2.30	0.1475	
AC	5.329E-005	1	5.329E-005	28.60	< 0.0001	
AD	1.192E-005	1	1.192E-005	6.40	0.0216	
BD	1.033E-005	1	1.033E-005	5.54	0.0308	
B^2	8.474E-005	1	8.474E-005	45.48	< 0.0001	
Residual	3.167E-005	17	1.863E-006			
Lack of Fit	2.873E-005	12	2.394E-006	4.06	0.0661	not significant
Pure Error	2.946E-006	5	5.892E-007			
Cor Total	3.886E-004	25				

Transform: Inverse sqrt Constant: 0 These Rows Were Ignored for this Analysis. 21, 16, 12, 9

Std. Dev.	1.365E-003	R-Squared	0.9185
Mean	0.077	Adj R-Squared	0.8801

C.V. %	1.77	Pred R-Squared	0.7554
PRESS	9.505E-005	Adeq Precision	17.259

Coefficient	Standard	95% CI	95% CI			
Factor	Estimate	df	Error	Low	High	VIF
Intercept	0.075	1	4.375E-004	0.074	0.076	
A-Acetone Concentration	-2.960E-003	1	3.224E-004	-3.640E-003	-2.280E-003	1.06
B-Temperature	-3.735E-004	1	3.515E-004	-1.115E-003	3.681E-004	1.05
C-Solid/Solvent	1.671E-003	1	3.495E-004	9.331E-004	2.408E-003	1.04
D-Time	-4.455E-004	1	2.935E-004	-1.065E-003	1.738E-004	1.03
AC	-1.929E-003	1	3.606E-004	-2.689E-003	-1.168E-003	1.04
AD	9.121E-004	1	3.606E-004	1.513E-004	1.673E-003	1.04
BD	-8.489E-004	1	3.606E-004	-1.610E-003	-8.815E-005	1.04
B^2	3.798E-003	1	5.631E-004	2.610E-003	4.986E-003	1.05

Diagnostics Case statistics report for quercetin galactoside under acetone extraction

					Internally	Externally	Influence on		
Standard	Actual	Predicted			Studentized	Studentized	Fitted Value	Cook's	Run
Order	Value	Value	Residual	Leverage	Residual	Residual	DFFITS	Distance	Order
1	0.079	0.079	1.019E-004	0.477	0.103	0.100	0.096	0.001	26
2	0.076	0.075	9.010E-004	0.453	0.892	0.887	0.807	0.073	11
3	0.082	0.080	1.799E-003	0.490	1.846	2.003	1.965	0.364	3
4	0.075	0.076	-7.120E-004	0.467	-0.714	-0.704	-0.658	0.050	28
5	0.086	0.086	-4.128E-004	0.481	-0.420	-0.410	-0.394	0.018	19
6	0.076	0.075	1.233E-003	0.455	1.223	1.243	1.135	0.139	4
7	0.086	0.087	-1.050E-003	0.495	-1.083	-1.088	-1.078	0.128	23
8	0.07	0.076	4.420E	0.468	0.444	0.433	0.407	0.019	1

	6		-004						
9	0.078	0.078	4.147E-004	0.455	0.411	0.401	0.366	0.016	25
10	0.078	0.078	2.924E-004	0.481	0.297	0.289	0.279	0.009	27
11	0.073	0.075	-2.286E-003	0.559	-2.522	-3.093	* -3.48	0.897	15
12	0.076	0.075	9.747E-004	0.492	1.002	1.002	0.985	0.108	30
13	0.086	0.085	1.352E-003	0.536	1.454	1.508	1.621	0.272	7
14	0.076	0.077	-1.192E-003	0.487	-1.220	-1.239	-1.209	0.157	13
16	0.076	0.075	8.326E-004	0.490	0.855	0.847	0.831	0.078	5
17	0.082	0.081	1.427E-003	0.283	1.234	1.255	0.788	0.067	2
19	0.076	0.079	-2.690E-003	0.111	-2.090	-2.352	-0.832	0.061	24
21	0.073	0.073	-2.809E-004	0.157	-0.224	-0.218	-0.094	0.001	20
23	0.076	0.076	-6.939E-005	0.262	-0.059	-0.057	-0.034	0.000	22
24	0.075	0.074	8.935E-004	0.283	0.773	0.764	0.480	0.026	10
25	0.075	0.075	3.455E-004	0.103	0.267	0.260	0.088	0.001	6
26	0.075	0.075	8.255E-005	0.103	0.064	0.062	0.021	0.000	17
27	0.075	0.075	5.338E-004	0.103	0.413	0.403	0.136	0.002	29
28	0.074	0.075	-5.978E-004	0.103	-0.462	-0.451	-0.153	0.003	8
29	0.074	0.075	-1.392E-003	0.103	-1.076	-1.082	-0.366	0.015	14
30	0.074	0.075	-9.423E-004	0.103	-0.729	-0.718	-0.243	0.007	18

Appendix A5 Quercetin glucoside

ANOVA for Response Surface Reduced Quadratic Model

	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	2.210E-003	7	3.157E-004	68.30	< 0.0001	significant
A-Acetone Concentration	3.335E-004	1	3.335E-004	72.13	< 0.0001	
C-Solid/Solvent	4.147E-004	1	4.147E-004	89.71	< 0.0001	
D-Time	3.310E-010	1	3.310E-010	7.159E-005	0.9934	
AC	1.506E-003	1	1.506E-003	325.70	< 0.0001	
AD	1.550E-005	1	1.550E-005	3.35	0.0870	
A^2	2.024E-004	1	2.024E-004	43.77	< 0.0001	
C^2	3.127E-004	1	3.127E-004	67.64	< 0.0001	
Residual	6.935E-005	15	4.623E-006			
Lack of Fit	6.826E-005	11	6.206E-006	22.85	0.0042	significant
Pure Error	1.086E-006	4	2.716E-007			
Cor Total	2.280E-003	22				

Transform: Inverse sqrt Constant: 0

These Rows Were Ignored for this Analysis. 11, 27, 16, 12, 20, 9, 18

Std. Dev.	2.150E-003	R-Squared	0.9696
Mean	0.097	Adj R-Squared	0.9554
C.V. %	2.21	Pred R-Squared	N/A
PRESS	N/A	Adeq Precision	27.748

Diagnostic Case statistics Reports of quercetin glucoside under acetone extraction

					Internally	Externally	Influence on		
Standard	Actual	Predicted			Studentized	Studentized	Fitted Value	Cook's	Run
Order	Value	Value	Residual	Leverage	Residual	Residual	DFFITS	Distance	Order
1	0.095	0.095	1.192E-004	0.356	0.069	0.067	0.050	0.000	26
3	0.098	0.095	3.312E-003	0.356	1.920	2.136	1.589	0.255	3
4	0.12	0.12	-2.513E	0.633	-1.930	-2.151	* -2.83	0.805	28

			-003						
5	0.10	0.11	-2.214E-003	0.356	-1.283	-1.314	-0.978	0.114	19
6	0.092	0.091	1.044E-003	0.383	0.618	0.605	0.477	0.030	4
7	0.10	0.11	-1.444E-003	0.356	-0.837	-0.828	-0.616	0.048	23
8	0.093	0.091	1.242E-003	0.383	0.736	0.724	0.571	0.042	1
9	0.093	0.093	8.599E-004	0.356	0.498	0.486	0.361	0.017	25
11	0.088	0.093	-4.291E-003	0.356	-2.487	-3.135	* -2.33	0.428	15
12	0.13	0.13	2.513E-003	0.633	1.930	2.151	* 2.83	0.805	30
13	0.10	0.10	8.438E-004	0.356	0.489	0.476	0.354	0.017	7
14	0.093	0.094	-8.924E-004	0.383	-0.529	-0.515	-0.406	0.022	13
15	0.11	0.10	2.814E-003	0.356	1.631	1.738	1.293	0.184	21
16	0.092	0.094	-1.393E-003	0.383	-0.825	-0.816	-0.644	0.053	5
17	0.098	0.098	0.000	1.00 *	2				
19	0.093	0.091	1.691E-003	0.125	0.841	0.832	0.314	0.013	24
23	0.092	0.091	6.420E-004	0.288	0.354	0.343	0.218	0.006	22
24	0.092	0.091	1.340E-004	0.311	0.075	0.073	0.049	0.000	10
25	0.090	0.091	-1.031E-003	0.125	-0.513	-0.500	-0.189	0.005	6
26	0.092	0.091	2.609E-004	0.125	0.130	0.125	0.047	0.000	17
27	0.091	0.091	-6.141E-004	0.125	-0.305	-0.296	-0.112	0.002	29
28	0.091	0.091	-8.644E-004	0.125	-0.430	-0.418	-0.158	0.003	8
29	0.09	0.091	-	0.125	-0.108	-0.105	-0.040	0.000	14

	1		2.178E-004						

Appendix A6 Procyanidin B2

ANOVA for Response Surface Reduced Quadratic Model for Procyanidin B2

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	2.349E+005	9	26097.91	641.83	< 0.0001	significant
A-Acetone Concentration	3174.65	1	3174.65	78.07	< 0.0001	
B-Temperature	195.68	1	195.68	4.81	0.0444	
C-Solid/Solvent	1.352E+005	1	1.352E+005	3324.56	< 0.0001	
D-Time	6.91	1	6.91	0.17	0.6861	
AC	3243.78	1	3243.78	79.77	< 0.0001	
BC	130.95	1	130.95	3.22	0.0929	
A^2	1021.96	1	1021.96	25.13	0.0002	
C^2	65574.56	1	65574.56	1612.68	< 0.0001	
D^2	137.53	1	137.53	3.38	0.0858	
Residual	609.93	15	40.66			
Lack of Fit	493.89	11	44.90	1.55	0.3589	not significant
Pure Error	116.04	4	29.01			
Cor Total	2.355E+005	24				

These Rows Were Ignored for this Analysis. 15, 16, 12, 9, 17

Std. Dev.	6.38	R-Squared	0.9974
Mean	137.68	Adj R-Squared	0.9959
C.V. %	4.63	Pred R-Squared	0.9900
PRESS	2363.38	Adeq Precision	56.629

Coefficient	Standard	95% CI	95% CI			
Factor	Estimate	df	Error	Low	High	VIF
Intercept	216.54	1	2.57	211.07	222.01	
A-Acetone Concentration	-14.63	1	1.66	-18.15	-11.10	1.28
B-Temperature	3.57	1	1.63	0.10	7.04	1.03
C-Solid/Solvent	93.27	1	1.62	89.82	96.72	1.03
D-Time	-0.56	1	1.37	-3.48	2.35	1.03
AC	-14.95	1	1.67	-18.52	-11.39	1.03
BC	3.00	1	1.67	-0.56	6.57	1.03
A^2	-9.13	1	1.82	-13.01	-5.25	1.35

C^2	-117.53	1	2.93	-123.77	-111.29	1.21
D^2	2.52	1	1.37	-0.40	5.45	1.03

Diagnostic Case statistics reports for Procyanidin B2 under acetone extraction

					Internally	Externally	Influence on		
Standard	Actual	Predicted			Studentized	Studentized	Fitted Value	Cook's	Run
Order	Value	Value	Residual	Leverage	Residual	Residual	DFFITS	Distance	Order
1	0.000	-1.20	1.20	0.406	0.243	0.236	0.195	0.004	26
2	0.000	-0.54	0.54	0.461	0.115	0.111	0.103	0.001	11
3	0.000	-0.068	0.068	0.553	0.016	0.015	0.017	0.000	3
4	0.000	0.59	-0.59	0.415	-0.121	-0.117	-0.099	0.001	28
5	218.26	209.25	9.01	0.418	1.853	2.039	1.727	0.247	19
6	140.35	150.08	-9.73	0.416	-1.998	-2.253	-1.901	0.284	4
7	216.38	222.39	-6.01	0.418	-1.236	-1.260	-1.068	0.110	23
8	167.11	163.23	3.88	0.417	0.796	0.786	0.665	0.045	1
9	0.000	-2.32	2.32	0.434	0.484	0.472	0.413	0.018	25
10	0.000	-1.67	1.67	0.422	0.344	0.333	0.285	0.009	27
12	0.000	-0.54	0.54	0.445	0.113	0.109	0.098	0.001	30
13	210.25	208.12	2.13	0.417	0.438	0.426	0.360	0.014	7
14	150.40	148.96	1.44	0.418	0.297	0.288	0.244	0.006	13
15	216.85	221.27	-4.42	0.416	-0.906	-0.900	-0.760	0.058	21
16	165.80	162.10	3.70	0.418	0.760	0.748	0.634	0.041	5
17	207.83	209.27	-1.44	0.95 #	-1.039	-1.042	* -4.70	* 2.19	2
19	201.55	212.97	-11.42	0.208	-2.013	-2.276	-1.166	0.106	24
21	0.000	5.74	-5.74	0.249	-1.039	-1.042	-0.601	0.036	20
23	227.81	226.06	1.75	0.594	0.431	0.419	0.507	0.027	22
24	222.60	225.51	-2.91	0.713	-0.851	-0.843	-1.329	0.180	10
25	217.18	216.54	0.64	0.162	0.109	0.105	0.046	0.000	6
27	216.97	216.54	0.43	0.162	0.073	0.071	0.031	0.000	29
28	224.0	216.54	7.52	0.162	1.288	1.319	0.580	0.032	8

	6								
29	212.80	216.54	-3.74	0.162	-0.641	-0.628	-0.276	0.008	14
30	225.72	216.54	9.18	0.162	1.572	1.662	0.731	0.048	18

Appendix A7 Epicatechin

ANOVA for Response Surface Reduced Quadratic Model

	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	1.607E+005	7	22955.55	213.51	< 0.0001	significant
A-Acetone Concentration	2756.10	1	2756.10	25.63	< 0.0001	
B-Temperature	1022.67	1	1022.67	9.51	0.0064	
C-Solid/Solvent	92991.61	1	92991.61	864.93	< 0.0001	
AC	2004.45	1	2004.45	18.64	0.0004	
BC	327.21	1	327.21	3.04	0.0981	
B^2	7728.21	1	7728.21	71.88	< 0.0001	
C^2	38119.78	1	38119.78	354.56	< 0.0001	
Residual	1935.25	18	107.51			
Lack of Fit	1689.47	13	129.96	2.64	0.1452	not significant
Pure Error	245.78	5	49.16			
Cor Total	1.626E+005	25				

APPENDIX B-

ANALYSIS OF VARIANCE RESULTS FOR POLYPHENOLIC COMPOUNDS UNDER SUBCRITICAL WATER EXTRACTION

Appendix B1- Chlorogenic acid

ANOVA for Response Surface Reduced Quadratic Model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	196.51	6	32.75	62.93	< 0.0001	significant
A-Solid/Solvent	7.62	1	7.62	14.65	0.0040	
B-Temperature	131.81	1	131.81	253.25	< 0.0001	
C-Residence Time	1.09	1	1.09	2.10	0.1809	
AB	7.62	1	7.62	14.65	0.0040	
BC	2.38	1	2.38	4.58	0.0610	
B^2	112.46	1	112.46	216.08	< 0.0001	
Residual	4.68	9	0.52			
Lack of Fit	3.76	5	0.75	3.25	0.1382	not significant
Pure Error	0.93	4	0.23			
Cor Total	201.20	15				

Std. Dev.	0.72	R-Squared	0.9767
Mean	5.52	Adj R-Squared	0.9612
C.V. %	13.07	Pred R-Squared	0.8984
PRESS	20.44	Adeq Precision	22.294

Coefficient Factor	Standard Estimate	95% CI df	95% CI Error	Low	High	VIF
Intercept	7.75	1	0.25	7.19	8.31	
A-Solid/Solvent	-0.98	1	0.26	-1.55	-0.40	1.00
B-Temperature	-3.80	1	0.24	-4.34	-3.26	1.17
C-Residence Time	0.28	1	0.20	-0.16	0.72	1.00
AB	0.98	1	0.26	0.40	1.55	1.00
BC	-0.55	1	0.26	-1.12	0.031	1.00
B^2	-3.89	1	0.26	-4.49	-3.29	1.17

Diagnostics Case statistics reports for Chlorogenic acid under subcritical water extraction

Standard	Actual	Predicted		
Order	Value	Value	Residual	Leverage
1	7.64	8.79	-1.14	0.586
2	4.97	4.88	0.086	0.586
3	0.000	0.33	-0.33	0.691
4	0.000	0.33	-0.33	0.691
5	11.05	10.44	0.61	0.586
6	5.92	6.54	-0.62	0.586
7	0.000	-0.20	0.20	0.691
8	0.000	-0.20	0.20	0.691
11	3.62	3.14	0.47	0.647
13	7.71	7.28	0.43	0.326
14	7.41	8.23	-0.82	0.326
15	7.92	7.75	0.16	0.119
17	7.78	7.75	0.030	0.119
18	7.77	7.75	0.018	0.119
19	8.85	7.75	1.10	0.119
20	7.69	7.75	-0.059	0.119

Appendix B2- Phloridzin

ANOVA for Response Surface Reduced Quadratic Mode

	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	279.36	4	69.84	13.00	0.0004	significant
A-Solid/Solvent	37.65	1	37.65	7.01	0.0227	
B-Temperature	224.74	1	224.74	41.84	< 0.0001	
AB	37.65	1	37.65	7.01	0.0227	
B^2	86.07	1	86.07	16.03	0.0021	
Residual	59.08	11	5.37			
Lack of Fit	6.77	6	1.13	0.11	0.9914	not significant
Pure Error	52.30	5	10.46			
Cor Total	338.44	15				

Std. Dev.	2.32	R-Squared	0.8254
Mean	6.30	Adj R-Squared	0.7620
C.V. %	36.79	Pred R-Squared	0.7528
PRESS	83.67	Adeq Precision	11.693

Coefficient	Standard	95% CI	95% CI			
Factor	Estimate	df	Error	Low	High	VIF
Intercept	8.41	1	0.77	6.72	10.10	
A-Solid/Solvent	-2.38	1	0.90	-4.36	-0.40	1.04
B-Temperature	-5.19	1	0.80	-6.96	-3.43	1.18
AB	2.38	1	0.90	0.40	4.36	1.04
B^2	-3.32	1	0.83	-5.14	-1.49	1.15

Diagnostics Case statistics reports for Phloridzin under subcritical water extraction

Standard	Actual	Predicted		
Order	Value	Value	Residual	Leverage
2	5.61	5.53	0.075	0.407
3	0.000	-0.098	0.098	0.494
4	0.000	-0.098	0.098	0.494
5	15.82	15.05	0.77	0.63 #
6	6.22	5.53	0.69	0.407
7	0.000	-0.098	0.098	0.494
8	0.000	-0.098	0.098	0.494
11	7.09	7.77	-0.68	0.71 #
13	7.11	8.41	-1.31	0.110
14	6.69	8.41	-1.72	0.110
15	6.95	8.41	-1.46	0.110
16	14.96	8.41	6.54	0.110
17	6.73	8.41	-1.68	0.110
18	7.59	8.41	-0.82	0.110
19	9.45	8.41	1.03	0.110
20	6.59	8.41	-1.82	0.110

Appendix B4 Protocatechuic aldehyde

ANOVA for Response Surface Reduced Quadratic Model

	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	1348.18	7	192.60	477.44	< 0.0001	significant
A-Solid/Solvent	48.85	1	48.85	121.09	< 0.0001	
B-Temperature	1112.43	1	1112.43	2757.67	< 0.0001	
C-Residence Time	10.02	1	10.02	24.84	0.0011	
AB	48.85	1	48.85	121.09	< 0.0001	
AC	2.64	1	2.64	6.55	0.0337	
BC	3.99	1	3.99	9.88	0.0137	

A^2	121.41	1	121.41	300.98	< 0.0001	
Residual	3.23	8	0.40			
Lack of Fit	3.18	3	1.06	110.62	< 0.0001	significant
Pure Error	0.048	5	9.580E-003			

Std. Dev.	0.64	R-Squared	0.9976
Mean	9.04	Adj R-Squared	0.9955
C.V. %	7.03	Pred R-Squared	0.9326
PRESS	91.14	Adeq Precision	69.890

Coefficient	Standard	95% CI	95% CI			
Factor	Estimate	df	Error	Low	High	VIF
Intercept	6.28	1	0.22	5.76	6.80	
A-Solid/Solvent	-2.47	1	0.22	-2.99	-1.95	1.00
B-Temperature	11.79	1	0.22	11.27	12.31	1.00
C-Residence Time	0.86	1	0.17	0.46	1.25	1.00
AB	-2.47	1	0.22	-2.99	-1.95	1.00
AC	-0.57	1	0.22	-1.09	-0.057	1.00
BC	0.71	1	0.22	0.19	1.22	1.00
A^2	5.51	1	0.32	4.78	6.24	1.00

Diagnostics Case statistics report of Protocatechuic aldehyde under subcritical water extraction

Standard	Actual	Predicted		
Order	Value	Value	Residual	Leverage
1	0.000	-0.73	0.73	0.823
2	0.000	0.42	-0.42	0.823
3	25.97	26.39	-0.42	0.823
4	18.38	17.65	0.73	0.823
5	0.000	0.73	-0.73	0.823
6	0.000	-0.42	0.42	0.823
7	31.09	30.66	0.42	0.823
8	18.91	19.63	-0.73	0.823
13	4.29	4.84	-0.55	0.332
14	7.89	7.72	0.16	0.332
15	6.36	6.28	0.081	0.125
16	6.47	6.28	0.18	0.125
17	6.30	6.28	0.018	0.125
18	6.29	6.28	0.010	0.125
19	6.45	6.28	0.17	0.125
20	6.21	6.28	-0.070	0.125

Appendix B6 Quercetin glucoside

ANOVA for Response Surface Reduced Quadratic Model

	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	1617.21	4	404.30	58.25	< 0.0001	significant
A-Solid/Solvent	70.51	1	70.51	10.16	0.0086	
B-Temperature	70.51	1	70.51	10.16	0.0086	
AB	70.51	1	70.51	10.16	0.0086	
A^2	1405.69	1	1405.69	202.53	< 0.0001	
Residual	76.35	11	6.94			
Lack of Fit	4.44	6	0.74	0.052	0.9988	not significant
Pure Error	71.90	5	14.38			
Cor Total	1693.56	15				

Std. Dev.	2.63	R-Squared	0.9549
Mean	12.34	Adj R-Squared	0.9385
C.V. %	21.35	Pred R-Squared	0.9377
PRESS	105.49	Adeq Precision	14.745

Coefficient	Standard	95% CI	95% CI			
Factor	Estimate	df	Error	Low	High	VIF
Intercept	21.72	1	0.93	19.66	23.77	
A-Solid/Solvent	2.97	1	0.93	0.92	5.02	1.00
B-Temperature	-2.97	1	0.93	-5.02	-0.92	1.00
AB	-2.97	1	0.93	-5.02	-0.92	1.00
A^2	-18.75	1	1.32	-21.65	-15.85	1.00

Diagnostics Case Statistics for quercetin glucoside under subcritical water extraction

Standard	Actual	Predicted		
Order	Value	Value	Residual	Leverage
1	0.000	3.553E-015	0.000	0.500
2	12.91	11.88	1.03	0.500
3	0.000	3.553E-015	0.000	0.500
4	0.000	3.553E-015	0.000	0.500
5	0.000	3.553E-015	0.000	0.500
6	10.84	11.88	-1.04	0.500

7	0.000	3.553E-015	0.000	0.500
8	0.000	3.553E-015	0.000	0.500
13	22.97	21.72	1.25	0.125
14	20.88	21.72	-0.84	0.125
15	18.68	21.72	-3.04	0.125
16	28.24	21.72	6.52	0.125
17	18.32	21.72	-3.40	0.125
18	21.58	21.72	-0.14	0.125
19	23.59	21.72	1.87	0.125
20	19.46	21.72	-2.26	0.125

Appendix B7 5-HMF

ANOVA for Response Surface Reduced Quadratic Model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	6457.60	7	922.51	253.84	< 0.0001	significant
A-Solid/Solvent	903.20	1	903.20	248.53	< 0.0001	
B-Temperature	4163.29	1	4163.29	1145.58	< 0.0001	
C-Residence Time	39.94	1	39.94	10.99	0.0161	
AB	854.55	1	854.55	235.14	< 0.0001	
AC	13.86	1	13.86	3.81	0.0987	
BC	38.50	1	38.50	10.59	0.0174	
A^2	444.26	1	444.26	122.24	< 0.0001	
Residual	21.81	6	3.63			
Lack of Fit	20.18	1	20.18	62.07	0.0005	significant
Pure Error	1.63	5	0.33			
Cor Total	6479.40	13				

Std. Dev.	1.91	R-Squared	0.9966
Mean	19.96	Adj R-Squared	0.9927
C.V. %	9.55	Pred R-Squared	0.8003
PRESS	1293.84	Adeq Precision	51.278

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	13.45	1	0.78	11.55	15.36	
A-Solid/Solvent	-10.63	1	0.67	-12.27	-8.98	1.00

B-Temperature	22.81	1	0.67	21.16	24.46	1.00
C-Residence Time	2.23	1	0.67	0.59	3.88	1.00
AB	-10.34	1	0.67	-11.98	-8.69	1.00
AC	-1.32	1	0.67	-2.97	0.33	1.00
BC	2.19	1	0.67	0.54	3.84	1.00
A^2	11.38	1	1.03	8.86	13.90	1.00

Diagnostics Case statistics report of 5-HMF Transform Square root

Standard Order	**Actual Value**	**Predicted Value**	**Residual**	**Leverage**	**Internally Studentized Residual**	**Externally Studentized Residual**	**Influence on Fitted Value DFFITS**	**Cook's Distance**
1	2.55	0.96	1.59	0.875	2.356	** 7.88	* 20.84	* 4.86
2	1.42	3.01	-1.59	0.875	-2.356	** -7.88	* -20.84	* 4.86
3	61.28	62.87	-1.59	0.875	-2.356	** -7.88	* -20.84	* 4.86
4	25.16	23.58	1.59	0.875	2.356	** 7.88	* 20.84	* 4.86
5	2.08	3.67	-1.59	0.875	-2.356	** -7.88	* -20.84	* 4.86
6	2.05	0.46	1.59	0.875	2.356	** 7.88	* 20.84	* 4.86
7	75.94	74.35	1.59	0.875	2.356	** 7.88	* 20.84	* 4.86
8	28.21	29.80	-1.59	0.875	-2.356	** -7.88	* -20.84	* 4.86
15	12.97	13.45	-0.48	0.167	-0.276	-0.253	-0.113	0.002
16	14.21	13.45	0.76	0.167	0.435	0.403	0.180	0.005
17	12.74	13.45	-0.72	0.167	-0.412	-0.381	-0.170	0.004
18	13.93	13.45	0.48	0.167	0.274	0.252	0.112	0.002
19	13.64	13.45	0.18	0.167	0.105	0.096	0.043	0.000
20	13.23	13.45	-0.22	0.167	-0.127	-0.116	-0.052	0.000

Appendix B8 Furfural

ANOVA for Response Surface Reduced Quadratic Model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	43014.62	6	7169.10	68.57	< 0.0001	significant
A-Solid/Solvent	3963.17	1	3963.17	37.91	0.0005	
B-Temperature	28746.37	1	28746.37	274.94	< 0.0001	
C-Residence Time	722.58	1	722.58	6.91	0.0340	

AB	2397.46	1	2397.46	22.93	0.0020	
BC	891.19	1	891.19	8.52	0.0224	
A^2	6293.84	1	6293.84	60.20	0.0001	
Residual	731.88	7	104.55			
Lack of Fit	724.56	2	362.28	247.33	< 0.0001	significant
Pure Error	7.32	5	1.46			
Cor Total	43746.50	13				

Std. Dev.	10.23	R-Squared	0.9833
Mean	49.58	Adj R-Squared	0.9689
C.V. %	20.62	Pred R-Squared	0.7348
PRESS	11603.50	Adeq Precision	25.658

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	25.10	1	4.17	15.23	34.97	
A-Solid/Solvent	-22.26	1	3.62	-30.81	-13.71	1.00
B-Temperature	59.94	1	3.62	51.40	68.49	1.00
C-Residence Time	9.50	1	3.62	0.96	18.05	1.00
AB	-17.31	1	3.62	-25.86	-8.76	1.00
BC	10.55	1	3.62	2.01	19.10	1.00
A^2	42.85	1	5.52	29.79	55.90	1.00

Diagnostics Case statistics report of Furfural Transform Square root

Standard Order	**Actual Value**	**Predicted Value**	**Residual**	**Leverage**	**Internally Studentized Residual**	**Externally Studentized Residual**	**Influence on Fitted Value DFFITS**	**Cook's Distance**
1	16.00	14.00	2.01	0.750	0.393	0.368	0.637	0.066
2	2.10	4.11	-2.01	0.750	-0.393	-0.368	-0.637	0.066
3	134.09	147.40	-13.31	0.750	-2.603	** -13.46	* -23.32	* 2.90
4	81.57	68.26	13.31	0.750	2.603	** 13.46	* 23.32	* 2.90
5	9.89	11.90	-2.01	0.750	-0.393	-0.368	-0.637	0.066
6	4.01	2.00	2.01	0.750	0.393	0.368	0.637	0.066
7	200.82	187.52	13.31	0.750	2.603	** 13.46	* 23.32	* 2.90
8	95.07	108.38	-13.31	0.750	-2.603	** -13.46	* -23.32	* 2.90

15	24.31	25.10	-0.79	0.167	-0.085	-0.079	-0.035	0.000
16	27.44	25.10	2.34	0.167	0.251	0.233	0.104	0.002
17	24.26	25.10	-0.84	0.167	-0.090	-0.084	-0.037	0.000
18	25.07	25.10	-0.025	0.167	-0.003	-0.002	-0.001	0.000
19	24.39	25.10	-0.71	0.167	-0.076	-0.070	-0.031	0.000
20	25.12	25.10	0.023	0.167	0.002	0.002	0.001	0.000