Extraction of Anthocyanins from Dried Blackcurrant (*Ribes nigrum* L.) Skins and evaluation of their potential as Natural Colourants

A Thesis Submitted for the Degree of Doctor of Philosophy

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DEDICATION

Special Dedicated to my Beloved Husband Mohd Firdauz, daughter Jannatul Adni and son Muhammad Naim for their Support and Unconditional Love

ABSTRACT

Blackcurrant skins is a by-product from the blackcurrant pressing process during the production of the juice, jams, jellies and nutraceutical ingredients. Blackcurrant skins are rich in polyphenolic compounds, primarily anthocyanins, which can be potentially utilised by the food industry as alternatives to synthetic colourants and as natural ingredients that can exert health promoting properties. Overall, the aim of the work presented in this thesis was to address key parts of a potential valorisation strategy for blackcurrant skins, focusing from the efficient drying process to the extraction of anthocyanins, and finally the application of anthocyanins as natural colourants. More specifically, the work aimed to address a number of objectives including comparing different approaches for drying the skins at laboratory and commercial scale, understanding the key factors influencing anthocyanin extraction from dried blackcurrant skins (DBS) (i.e. solvent, temperature, time), and evaluating different copigmentation methods for the stabilisation of the extracted anthocyanins and their colour.

To address the first objective, different drying processes of blackcurrant pressed residues including industrial rotary drum drying, laboratory oven drying and freeze drying were investigated. Furthermore, in the case of industrial rotary drum drying the effect of different drying parameters such as temperatures, residence time, the ratio of air speed to drum rotor speed and particle sizes were also evaluated. The results demonstrated that industrial rotary drying showed significantly ($p \leq 0.05$) higher total free anthocyanins (17.9 ± 0.4 mg/g) compared to freeze and oven drying, with the key factors influencing these identified as the hot air speed with gradient temperatures. This work indicated that industrial drum drying under optimised conditions prevents overheating of the blackcurrant skins which may lead to degradation of anthocyanins especially in the case of smaller particle sizes.

To address the second objective, a representative DBS sample from industrial rotary drum drying was extracted using alternative extraction methods to the conventional solvent-based extraction methods (i.e. with ethanol, methanol and methanol/water mixtures), including using food grade acetic acid and hot water at different temperatures and extraction times in order to identify the conditions that provided high yields of anthocyanins and other phenolic compounds such as hydroxycinnamic acids and flavonols. The composition, antioxidant activities and colour profiles of the obtained anthocyanins-containing extracts were analysed with the aim primarily to understand the influence of pH, temperature and solvent on colour formation and anthocyanin stability. Acetic acid/water mixtures (pH 1.5) resulted in extracts with the highest free anthocyanins (consisting primarily of delphinidin-3-O-glucoside, delphinidin-3-O-rutinoside, cyanidin-3-O-rutinoside and cyanidin-3-O-glucoside) and total phenolic content [16.6 mg/g and 37.0 mg gallic acid equivalent (GAE)/g], respectively amongst all solvents after 2 h extraction, which also demonstrated high antioxidant activities and high colour intensity and stability.

To address the third and fourth objectives, the anthocyanin stability and colour stability of the extracts were enhanced through an intermolecular copigmentation approach involving non-covalent interactions between anthocyanins and various phenolic acids. This phenomenon is known as charge-transfer complex formation, or π–π interactions. The phenolic acids used as copigments were ferulic, caffeic, chlorogenic, tannic and rosmarinic acids, at pH 3.0 and 6.0 in buffers and solvent/buffer solutions. The results indicated that the anthocyanins/ferulic acid complexes at pH 3.0 exhibited the strongest interaction and resulted in the highest colour and anthocyanin stability during storage at 4 °C for 140 days, most likely because ferulic acid contains methoxylated derivatives that induce a stronger copigmentation effect than phenolic acids with hydroxyl substituents such as caffeic, chlorogenic, tannic and rosmarinic acids. Furthermore, the effect of copigmentation between anthocyanins and copigment through non-covalent bond in the intermolecular

copigmentation and covalent bond in the enzymatic acylation were compared. In terms of the enzymatic acylation approach using lipase as the biocatalyst, acylated anthocyanins were successfully synthesised using cinnamic acid methyl ester as the acyl donor, with the highest conversion yields obtained for cyanidin 3-(6″- cinnamoyl)-glucoside (~15.1%) and delphinidin 3-(6″- cinnamoyl)-glucoside (~10.1%). Interestingly, the acylated anthocyanins were more stable and demonstrated better stability and better colour stability during storage compared to the non-acylated anthocyanins, indicating that the acyl group which is covalently bound to the basic anthocyanin structure protects the anthocyanin molecules from hydration.

Overall, the work presented in this thesis demonstrated that a valorisation process for DBS targeting the extraction of natural anthocyanins is potentially feasible without the need for large amounts of solvents. Moreover, the stability of the extracted anthocyanins can be enhanced through a number of copigmentation approaches, rendering them suitable alternatives to synthetic colourants for food applications.

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AUTHOR'S DECLARATION

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

(Ezzat Binti Mohamad Azman)

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Ezzat Binti Mohamad Azman

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CHAPTER 1 – GENERAL INTRODUCTION

The blackcurrant (*Ribes nigrum* L.) is widely cultivated in Europe, Northern America and New Zealand. Besides being consumed fresh, it is grown commercially to be processed into juice, jams, jellies and nutraceutical ingredients. Blackcurrants have been labelled as 'superfruits' due to their health promoting properties, including immunomodulatory, anti-microbial and anti-inflammatory properties, and their potential to reduce the risk of cardiovascular diseases and cancer (Bishayee et al., 2011; Gopalan et al., 2012).

In the United Kingdom, 75% of the total fresh blackcurrant production is processed into juice; ~19% remains as a by-product after processing, which consists mainly of skins, seeds and stems (Pap et al., 2005). In terms of waste treatment, blackcurrant pressed residues are directly discarded into soils. This however causes serious environmental problems due to their low pH as well as their high moisture content, which can potentially have a negative effect on the soil microbiome. The low pH and their often rancid taste limit the application of blackcurrant by-products as animal feed (Basegmez et al., 2017). As a result, alternative waste management approaches are being investigated, which primarily focus on the extraction of medium to high added value ingredients from such residues, such as anthocyanins, which can potentially have applications as food ingredients, or as active/functional ingredients for cosmetic and nutraceutical applications.

In order to utilise the pressed blackcurrant residues as sources of added value components, a drying step is usually required to increase the stability of the targeted phytochemicals present in the by-products, inhibit growth of spoilage microorganisms, and minimise the occurrence of enzymatic and non-enzymatic browning reactions taking place (Yang & Zhao, 2013). Rotary drum dryers are commonly used in the industry to dry food materials; in this case, a hot gas flowing through the dryer provides the heat required for the

vaporisation of water (Lisboa et al., 2007). Besides the temperature of the dryer, key parameters likely to affect the drying process include the residence time, the ratio of air speed to drum rotor speed and the particle size of the material (Gómez-de la Cruz et al., 2018).

Blackcurrant by-products are rich in anthocyanins (250 mg/100 g of fresh fruit) and other phenolic compounds, such as hydroxycinnamic acid (caffeic, ferulic and *p*-coumaric acids) and flavonols (kaempferol, myricetin and quercetin). The blackcurrant contains four major anthocyanins, namely delphinidin-3-O-glucoside, delphinidin-3-O-rutinoside, cyanidin-3-O-rutinoside and cyanidin-3-O-glucoside; these are responsible for up to 97% of the total anthocyanin content. Anthocyanins exist almost exclusively in the skins, while lesser amounts are usually found in the flesh and seeds of blackcurrant berries. Moreover, anthocyanins have been reported to possess significant health-promoting properties, which are linked to their antioxidant, anti-cancer, anti-inflammatory and anti-diabetic activities (Slimestad & Solheim, 2002; Mazza, 2018; Farzaei et al., 2018).

The most common steps used to extract phenolic compounds, including anthocyanins, from plant sources such as blackcurrants, grapes, blueberries and cherries, include grinding and drying of the plant samples followed by extraction with a suitable organic solvent (Merken & Beecher, 2000). The main advantage of using a conventional solid-liquid extraction method is that it is a simple and well established method that generally gives good extraction yield and is potentially, depending on the raw material and target phenolic product, economically viable and scalable. The main disadvantages include the requirement to use large volumes of organic solvents such as methanol and acetone, which are potential environmental pollutants and health hazards. Moreover, solvent extraction often requires long extraction times and might cause degradation of targeted phenolic components due to hydrolysis and oxidation during extraction (Altuner et al., 2012; Mahugo Santana et al., 2009). The key factors influencing the extraction yield in the case of

anthocyanins include the solvent type, temperature, particle size, solvent-to-solid ratio and the extraction time (Pinelo et al., 2004; Hayouni et al., 2007).

Among the various environmental factors influencing the extraction yield of anthocyanins from natural plant sources, as well as their stability, is the pH, as different pH values lead to changes in the structural conformation and colour of anthocyanins. Structurally, anthocyanins exist in four major chemical forms: the red flavylium cation (pH 1.0 – 3.0), the blue quinonoidal base (pH 6.0 – 7.0), the colourless carbinol pseudobase (pH 4.5) and the colourless chalcone (pH 4.5) (Brouillard & Delaporte, 1977). In the food industry, there is a high demand to replace synthetic food colourants with natural ones, especially those originating from vegetable and fruit sources. Thus, anthocyanins being natural products are regarded as potential strong candidates for the replacement of synthetic dyes due to their bright and attractive colours (orange, red, purple), high water solubility and possible health benefits (Cavalcanti et al., 2011; Castaneda-Ovando et al., 2009). However, the main challenges that need to be tackled in order for anthocyanins to be incorporated into food matrices include their poor stability during processing and storage. Moreover, anthocyanins exhibit rapid colour fading when exposed to light, oxygen, high temperature (> 70°C) and pH (pH 4.0 – 6.0), which can be exposed during different food processing operations. Due to this, anthocyanins can be applied as natural colourants in acidic foods such as jellies and jams, but are very unstable at neutral or slightly alkaline foods, such as dairy products (ice cream and milk) (Jing & Giusti, 2005).

Acylated anthocyanins are more colour stable during processing and storage than non-acylated anthocyanins, however blackcurrant contains only the latter (Rose et al., 2018). A method that can be used to maintain anthocyanin and consequently colour stability is copigmentation, which aims to protect the anthocyanin chromophore (flavylium cation form) against nucleophilic attack by water molecules, leading to colour loss. Copigmentation

involves a number of interactions including intermolecular copigmentation, intramolecular copigmentation and self-association. The level of these interactions can affect considerably the stability, shelf life and colour of food products, and influence consumer acceptance. However, to our knowledge, there have been no works investigating the effects of intermolecular and intramolecular copigmentation of anthocyanins extracted from dried blackcurrant skins (DBS) on colour stability in different environments, including acidic, neutral and slightly alkaline environments. Overall, the aims of this research were to investigate a potential valorisation strategy for blackcurrant skins, starting from the efficient drying process in protecting anthocyanins and develop a potentially scalable process for the efficient extraction of anthocyanins from DBS that exhibit high antioxidant activity and colour intensity. Then, a deep understanding of the potential copigmentation methodologies will be developed that can be used to enhance anthocyanin and colour stability in various conditions of relevance to food operations and storage.

Hence, the specific objectives that were set out in order to accomplish the research aims were:

1. To investigate the key processing parameters (i.e. temperature, residence time, the ratio of air speed to drum rotor speed, sample particle size) affecting the efficiency of the drying process of blackcurrant skins and the extraction of anthocyanins, hydroxycinnamic acids and flavonols **(CHAPTER 3)**.

2. To evaluate the effect of solvent type, temperature and time on the yield of total anthocyanins extracted from dried blackcurrant skins, identify and quantify the individual anthocyanins, hydroxycinnamic acids and flavonols, and analyse the colour profiles and antioxidant activities of the extracts **(CHAPTER 4)**.

3. To investigate methods to increase colour stability through the conjugation of anthocyanins with phenolic compounds, such as ferulic, caffeic, chlorogenic, tannic and rosmarinic acid, via intermolecular copigmentation and to study the effect of pH and temperature on anthocyanin colour and stability during storage in order to evaluate their commercial potential as natural colourants **(CHAPTER 5)**.

4. To investigate the effects of different acyl donors, such as methyl cinnamate and methyl ferulate, in producing acylated anthocyanins through an enzymatic acylation process and measure the stability of acylated anthocyanins at different pH and temperature during storage, targeting commercial applications as natural colourants **(CHAPTER 6)**.

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CHAPTER 2 - LITERATURE REVIEW

2.1 Blackcurrant

Blackcurrant (*Ribes nigrum* L.) is recognised as an economically important soft fruit crop in Europe, Russia, northern Asia, New Zealand and North America (Battino et al., 2009). Blackcurrant has been produced in the United Kingdom for more than 500 years (Keep, 1995), and data from 2017 reported that there were approximately 50 growers that occupy 2,400 hectares in British Isles producing around 11,000 tonnes of blackcurrant per year (IBA, 2018). Blackcurrant is generally cultivated for food industry related applications, such as natural colourants and preservatives for foods, as well as a source of ascorbic acid (180 mg/100g) and polyphenols (500-1342 mg/100 g), and have been associated with health promoting effects in the human body (Moyer et al., 2002; Markowski & Pluta, 2008; Brennan & Graham, 2009).

The biological activities of phenolic compounds such as anthocyanins, procyanidins, flavanols and phenolic acids is mainly associated with their high antioxidant activity (Slimestad & Solheim, 2002; Tabart et al., 2007; Szajdek & Borowska, 2008). Moreover, the fresh blackcurrant fruit contains many functional and bioactive compounds such as organic acids (major: citric acid; minor: succinic, malic, oxalic, and quinic acids), a diverse range of vitamins (C, A, E and B_6), and multi-amino acids which may protect the human body against chronic diseases. Blackcurrant contains also a diverse range of minerals, such as phosphorus, calcium, magnesium, iron, zinc, and unsaturated fatty acids, primarily linolenic acid (USDA, 2011).

2.1.1 Cultivation

Blackcurrant is a fairly long-term crop, costly to establish, and with no sufficient return on capital costs until the third or fourth year of its cultivation. Blackcurrant prefers damp fertile soils and planting is usually done in the autumn or winter to allow the plants to become established before growth starts in the spring. Generally, the blackcurrant crop forms woody bushes occupying about 1.5 metres in height and width (**Figure 2-1**). During March and April, the buds unfold and produce leaves; the flowers unfold and to some extent are protected by the immediate truss leaves. If the flowers are successfully pollinated the berries start yielding and ripen fruits are harvested during July and August (MAAF, 1981), with the berry at the top of bush ripening first. Therefore, berries should be hand-picked individually. Fresh blackcurrant has a very short seasonal availability as well as a sour taste. Other than being consumed fresh within few days after harvesting, berries can be also processed directly into jam, jelly or smoothies.

Figure 2-1. Blackcurrant bush (left) and fruits (right) (Vagiri, 2014).

In the United Kingdom, 'Baldwin' is the main commercial cultivar since the late nineteenth century, and is of unknown origin. New varieties of cultivars have derived from 'Baldwin' such as Ben Connan, Ben Hope, Ben Lomond, Ben Sarek, Ben Tirran, Big Ben, Ebony, Foxendown and Titania. **Table 2-1** shows that each variety ripens at different seasonal periods and some of them are frost tolerant cultivars (Vagiri et al., 2012). Additionally, their chemical compositions are considerably influenced by seasonal variation,

geographical location, climate, treatment during plant growth and physical conditions (Bakowska-Barczak et al., 2009; Khoo et al., 2012).

Table 2-1. Blackcurrant varieties cultivars at different seasonal periods (McSeveney, 2012)

Early Cultivars	Mid-Season Cultivars	Late-Season Cultivars
Ebony	Ben Hope	Ben Tirran
Big Ben	Ben Loyal	Ben Maia
Ben Gairn	Ben Lomond	
	Tiben	
	Ben Connan	
	Ben Finlay	
	Ben Sarek	

As shown in **Table 2-2**, in year 2017, the blackcurrant production worldwide was 188,582 tonnes with 153,932 tonnes being produced in Europe. This worldwide production decreased from 250,037 tonnes in 2015 to 186,682 tonnes in 2016 and slightly increased to 188,582 tonnes in 2017. By far the largest European producer was Poland with an annual production of more than 100,000 tonnes (IBA, 2018), with Ukraine being second. The United Kingdom usually appears as the third largest producer within Europe, with its production figures fluctuating the past few years, between 13,400 tonnes (in 2015) and 11,000 tonnes (in 2017). Non-European blackcurrant producing countries include Australia, Canada, China, New Zealand and U.S.A. Among these non-European countries, China is the main producer, followed by New Zealand and Australia (IBA, 2018).

Table 2-2. Worldwide production of blackcurrant in 2015, 2016 and 2017 (adapted from www.internationalblackcurrantassociation.com)

Country	2015			2016			2017		
	Hectares	**Total Yield tonnes**	**Actual Crop Harvest**	**Hectares**	**Total Yield tonnes**	**Actual Crop Harvest**	**Hectares**	**Total Yield tonnes**	**Actual Crop Harvest**
AUSTRIA	70	1,330	**155**	121	600	**20**	175	800	**162**
DENMARK	1,100	7,070	**5,000**	700	2,070	**2,500**	580	1,680	**1,960**
ESTONIA	318	200	**150**	-	170	**-**	198	190	**120**
FINLAND	1,400	810	**1,200**	1,387	950	**1,000**	1,870	1,400	**1,560**
FRANCE	2,000	9,400	**8,000**	2,000	6,720	**3,500**	2,000	8,160	**3,500**
GERMANY	1,600	7,180	**7,000**	1,600	6,810	**-**	1,440	5,620	**7,000**
HUNGARY	280	1,060	**550**	280	1,730	**-**	-	-	**-**
LATVIA	542	500	**-**	-	400	**-**	1,028	300	**1,500**
LITHUANIA	3,000	3,340	**3,500**	2,030	2,910	**2,500**	2,850	3,310	**4,230**
NETHERLANS	350	2,820	**2,300**	280	2,200	**1,320**	230	1,900	**840**
NORWAY	190	860	**742**	190	800	**742**	190	-	**560**
POLAND	32,000	120,800	**140,000**	36,000	130,820	**131,000**	36,500	140,500	**101,500**
RUSSIA	*No statistics available for Russian production as growers not required to keep specific fruit-type statistics; including blackcurrant.*								
SWEDEN	-	250		-	240	**-**	-	200	-
UNITED KINGDOM	2,400	15,200	**13,400**	2,400	11,400	**13,000**	2,400	13,900	**11,000**
UKRAINE	6,200	36,000	**25,540**	6,300	-	**24,500**	6,200	28,150	**20,000**
EUROPE TOTAL	**51,450**	**206,820**	**207,537**	**53,288**	**167,820**	**180,082**	**55,661**	**206,110**	**153,932**
NON-EU TOTAL	**10,730**	**53,422**	**42,500**	**7,600**	**59,865**	**6,600**	**6,200**	**-**	**34,650**
WORLD TOTAL	**62,180**	**260,242**	**250,037**	**60,888**	**227,685**	**186,682**	**61,861**	**-**	**188,582**

2.1.2 Characteristics

Besides being consumed fresh, blackcurrants are mainly cultivated to be used in juice and beverage production and are also processed to produce a range of functional ingredients that can be incorporated into jams, jellies, purées and teas (Varming et al., 2004). In fact, in the United Kingdom only, 75% of the total fresh blackcurrant production is processed into juice under a world famous commercial brand named 'RibenaTM' (Vagiri, 2014). **Table 2-3** shows that blackcurrant has a high concentration of bioactive compounds such as anthocyanins, flavonols, and total phenolics (Kähkönen et al., 2001). Apart from anthocyanins, other components such as flavonoids, hydroxycynnamic acids, *p*-coumaric acid, myrcetin, quercetin, kaempferol glycosides and isorhamnetin shape the polyphenol profile of the blackcurrant fruit (Landbo & Meyer, 2004; Oszmian´ski & Wojdylo, 2009; Sójka & Król, 2009).

Table 2-3. Nutritional values and polyphenol compounds of blackcurrants (Kähkönen et al., 2001; USDA, 2011)

Nutrients/100 g	**Blackcurrant, Raw**
Calories (kcal)	63.0
Total fat (g)	0.41
Saturated fat (g)	0.034
Poly-unsaturated fat (g)	0.179
Mono-unsaturated fat (g)	0.058
Cholesterol (mg)	0.00
Sodium (mg)	2.0
Potassium (mg)	322.0
Carbohydrate (g)	15.38
Total fibre (g)	NA
Sugars (g)	NA
Protein (g)	1.40
Calcium (mg)	55.0
Vitamin C (mg)	181.0
Vitamin A (IU)	230.0
Water (g)	81.96
Anthocyanins (mg) (expressed as cyanidin-3-glucoside)	756 - 1297
Flavonol (mg) (expressed as quercetin-3-rutinoside)	72 - 87
Total Phenolics (mg) (expressed as gallic acid)	2230 - 2790

In terms of their health-promoting activities, these have been associated with the blackcurrant anthocyanins and primarily delphinidin-3-O-rutinoside, which has a relaxation mechanism in the bovine ciliary smooth muscles and can also improve the visual function (Matsumoto et al., 2005a). Studies using *in vitro* models have shown that the above mentioned bioactive compounds exhibit potential anti-inflammatory, anti-carcinogenic, vascomodulatory and anti-haemostatic activities (Hollands et al., 2008; Brennan & Graham, 2009; Karjalainen et al., 2009). Moreover, studies have also shown that blackcurrant juice is able to increase blood flow, induce peripheral circulation as well as reduce muscle fatigue in humans (Matsumoto et al., 2005b).

2.2 Current waste management strategies

Waste management is one of the important factors in food processing, whereas the majority of plant-derived by-products (often referred to as biomass) is usually marketed as animal feed (Adebowale, 1985). Without an efficient utilisation of these by-products, their surplus is unavoidably expected to generate high quantities of waste and pollutants, with adverse effects on the environment (Szenes, 1995). Recently, research has been focused on the investigation of new alternatives for these by-products as potential sources of value added ingredients (O'Shea et al., 2012). For example, in the apple processing industry including the production of apple juice or canned apple products, the residue of apple pomace can be used as a raw material for pectin production (Hui, 2006).

2.2.1 Blackcurrant juice processing

The production of blackcurrant juice includes various processing stages such as milling, pressing, pasteurization, filtration, clarification and concentration as shown in **Figure 2-2**. In addition, the blackcurrant mash and juice are depectinised with pectinase

enzymes to facilitate the pressing process, as well as the clarification of blackcurrant juice (Raju & Bawa, 2006). Generally, in the juice clarification process, pectinases are often combined with amylases and cellulases in order to remove the negative charge of the pectin deposits, thus decrease the turbidity/cloudiness of fresh fruit juices (Yamasaki et al., 1967).

In soft fruit juice processing such as in the case of blackcurrant, the enzymatic treatment not only facilitates the pressing process, but may also increase the yields of anthocyanin pigment and the colour density of the final product. Pectinases also hydrolyse anthocyanins to aglycones and endogenous sugars, which in turn form readily pectin-based gels at low pH levels (Buchert et al., 2005; Wang et al., 2009). Moreover, the degradation of the pectin in the juice results in the increase of its astringency at the same time (Bajec & Pickering, 2008).

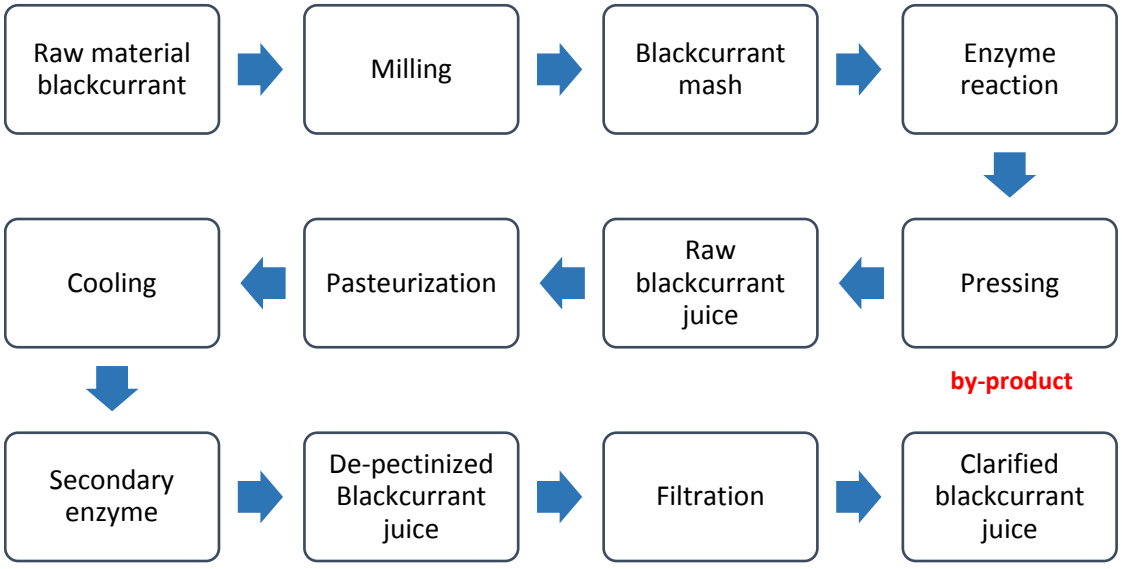

Figure 2-2. Blackcurrant juice processing flow (Woodward et al., 2011)

The by-products of the blackcurrant juice process (skins, seeds and stems as shown in **Figure 2-3**) are collected during the pressing stage. In 2010 in Europe, only minimum amounts of these by-products were upgraded or recycled. The advantage would be significant if a process is employed for the extraction of polyphenols from these by-products

before further utilisation or treatment of the by-products, i.e. use as animal feed, composting or discharge (Arvanitoyannis, 2010). Many recent studies have highlighted the health promoting effects of polyphenols extracted from blackcurrant by-products and their potential for further applications as functional food ingredients (Archaina et al., 2008; Miladinovic et al., 2018).

Figure 2-3. Dried blackcurrant by-products consisting of skins, seeds, and stems.

2.2.2 Blackcurrant waste

Apart from the pomace, the skins that are left behind as residue during juice processing, shown in **Figure 2-4,** are a rich source of polyphenols and especially of anthocyanins (250 mg/100 g of berries) (Vagiri, 2012). Anthocyanins exist almost exclusively in the skins, while lesser amounts are usually found in the flesh and seeds of blackcurrant berries. However, their individual anthocyanin content can vary considerably according to genotype, light, temperature and environment factors (Horbowicz et al., 2008; Tiwari & Cummins, 2013). Other than anthocyanins, blackcurrant solid residues are also a rich source of antioxidant compounds, which have potential uses in the manufacture of dietary supplements or food ingredients that may provide nutritional benefits (Lapornik et al., 2005; Kapasakalidis et al., 2009). The oil from blackcurrant seeds contains various fatty acids which show significant levels of antioxidant activities, primarily γ-linolenic and other fatty acids of minor nutritional importance such as α-linolenic and stearidonic acids.

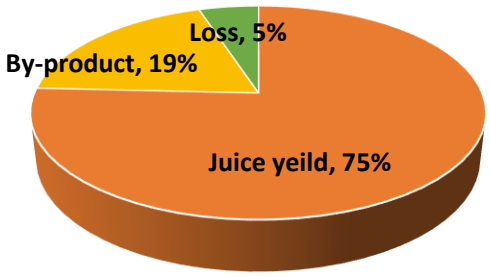

Figure 2-4. Production yields during blackcurrant juice processing (adapted from Pap et al., 2005).

2.2.3 Drying stage of blackcurrant by-products

Rotary dryers are commonly used by the food industry to dry particulate solids fruits or vegetables including their by-products (Keey, 2013). Commonly, the size of these dryers varies from 0.3 to 5 m in diameter by 2 to 90 m in length. During the drying process, solids with high moisture content (above 50%, w/w) enter one end of the rotary drum, go through a long cylindrical shell drum that is rotating, and exit at the end when suitably dried, as shown in **Figure 2-5**. The drying medium is usually hot air or combustion gases, which flow directly throughout the drum length and provide the sensible and latent heat required for water evaporation. Rotary drum dryers contain flight lifters that lift the cascading materials from the bottom of the dryer and drop them through the hot air to allow the effective contact between solids and hot air (**Figure 2-6**) (Fakouri, 2013; Chakraverty et al., 2003).

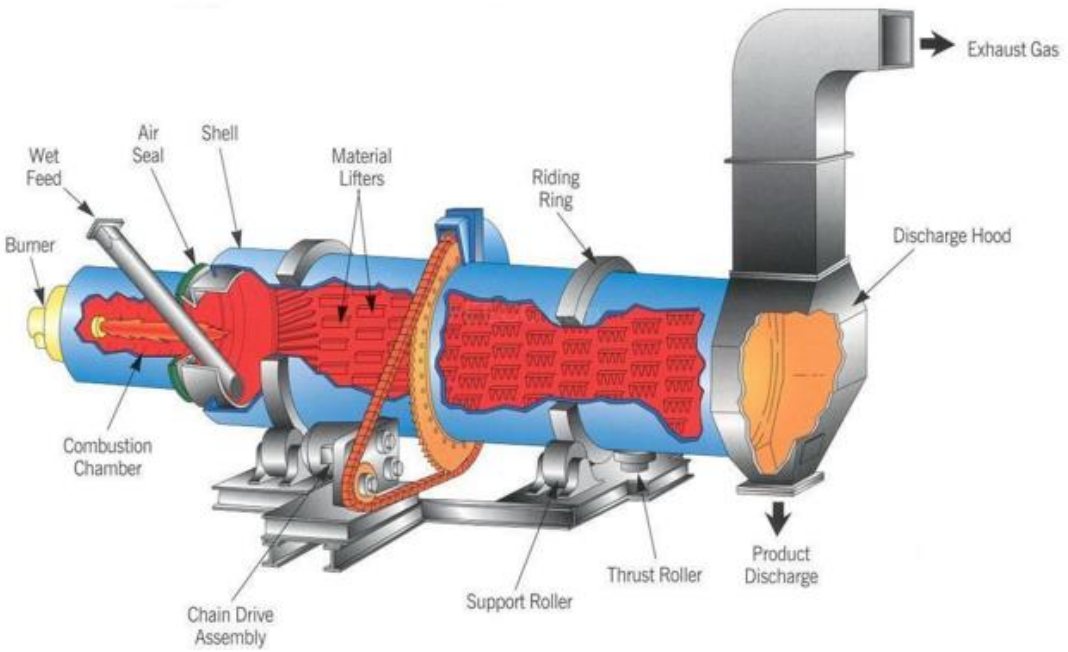

Figure 2-5. Mechanical construction of a direct heat rotary dryer (KBW Machinery, 2017)

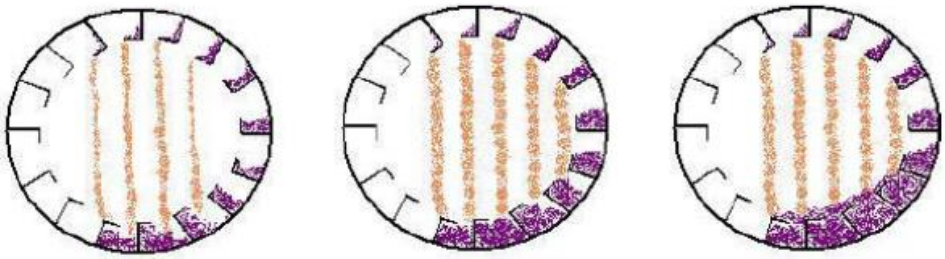

Figure 2-6. Illustration of the contact between the cascading particles and hot air in the rotary drum dryer (Fakouri et al., 2013)

The temperatures during the drying process using rotary dryers fluctuate between 25 °C at the beginning of the drying stage to a maximum temperature of 1200 °C in the combustion chamber, before eventually decreasing to 200 °C at the starting of the rotary drum dryer to 95 °C at the end of the drying process. **Figure 2-7** shows that the moisture content decreases rapidly (~52%) in the first two metres of the rotary drum dryer, and decreases slowly in the last segments. This is due to an increase in temperature at the beginning of the drying process, and after a while the heat transfer decreases; this causes the product to stop heating and decreases its temperature.

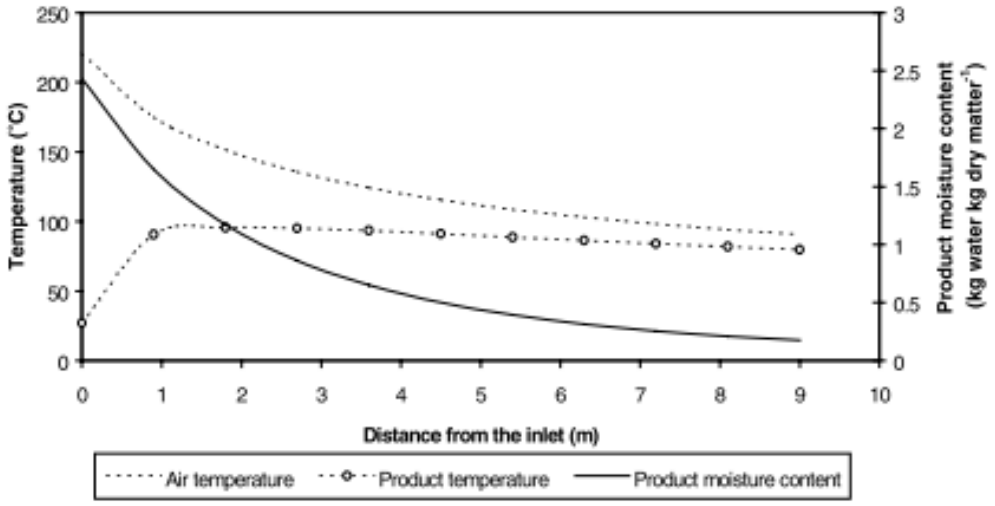

Figure 2-7. Changes of air and product temperature and moisture content of the product along the rotary drum dryer during the drying process (Iguaz et al, 2003).

2.2.4 Oven drying

Ovens generally heat the material via thermal convection. They provide uniform temperatures throughout the drying process and are widely used in drying, sterilization, thermostatic storage, heat treatment and other industrial laboratory functions. However, they are not suitable to use for volatile, flammable and explosive items. The typical sizes of the internal dimensions for laboratory ovens are 340 × 370 × 330 mm and 950 × 950 × 1200 mm for the industrial ovens, with the temperature being able to be controlled up to 300 °C (Riley Surface World, 2018). In addition, a cooling system, shelves, air filtration, timers and alarms are among the features provided in industrial drying ovens (Engineering360, 2018).

(a)

(b)

Figure 2-8. Images of the typical (a) industrial drying oven and (b) laboratory drying oven (Riley Surface World, 2018).

As shown in **Figure 2-8**, the heating element rack is located separately in the external envelope in order to prevent radiant heat from affecting the sample. However, this is not enough to heat and dry a sample. Therefore, a centrifugal flow type fan is used to speed up the drying process by recirculating the temperature evenly within the oven. The oven is functioning when the air been drawn into the fan from the centre of the oven is passed over the heating element. Then, the heated air is distributed by air flow baffles and then recirculated by the fan (Engineering360, 2018).

2.2.5 Freeze drying

Freeze drying, also known as lyophilisation or cryodessication, is a water removal method at low temperature instead of using heat. It involves a number of processing steps, including freezing the product, lowering the pressure and then removing the ice from the solid state to vapour phase by sublimation (Liu et al., 2008). The low temperature used during the process is a significant advantage of freeze drying over other drying methods, particularly for heat sensitive products. Moreover, the original shape of the product is maintained and the quality of the dried product is well preserved (Ratti, 2001). Generally,

freeze drying is used in a number of industrial applications including biological (e.g. bacteria and yeasts), biomedical (e.g. surgical transplants) and food processing (e.g. coffee and fruits) (Ratti, 2008). However, despite of its many advantages, freeze drying has always been recognised as the most expensive process for manufacturing of a dehydrated product. An industrial type freeze drier is depicted in **Figure 2-9 (a)**.

There are three stages in the freeze drying process including: 1) Freezing; 2) Primary drying and 3) Secondary drying. During freezing, the material is frozen below its triple point [**Figure 2-9 (b)**] to ensure that sublimation occurs rather than melting. Solid, liquid and gas are among the phases of the material that coexist at this point. Freezing is the most important phase since it influences the speed of reconstitution, the duration of the freeze-drying cycle, product stability and appropriate crystallization (Rey & May, 2004). Also at this phase, the pressure in the freeze dryer is lowered to the range of a few millibars to initiate primary drying. This results in a constant and low temperature in the freeze dryer, thus speeds up the sublimation as ~95% of water is progressively removed from the frozen mass (Franks, 1998). Subsequently in the secondary drying phase, the shelf temperature is raised higher than the primary drying phase so that unfrozen water molecules can be removed (Zhai et al., 2003).

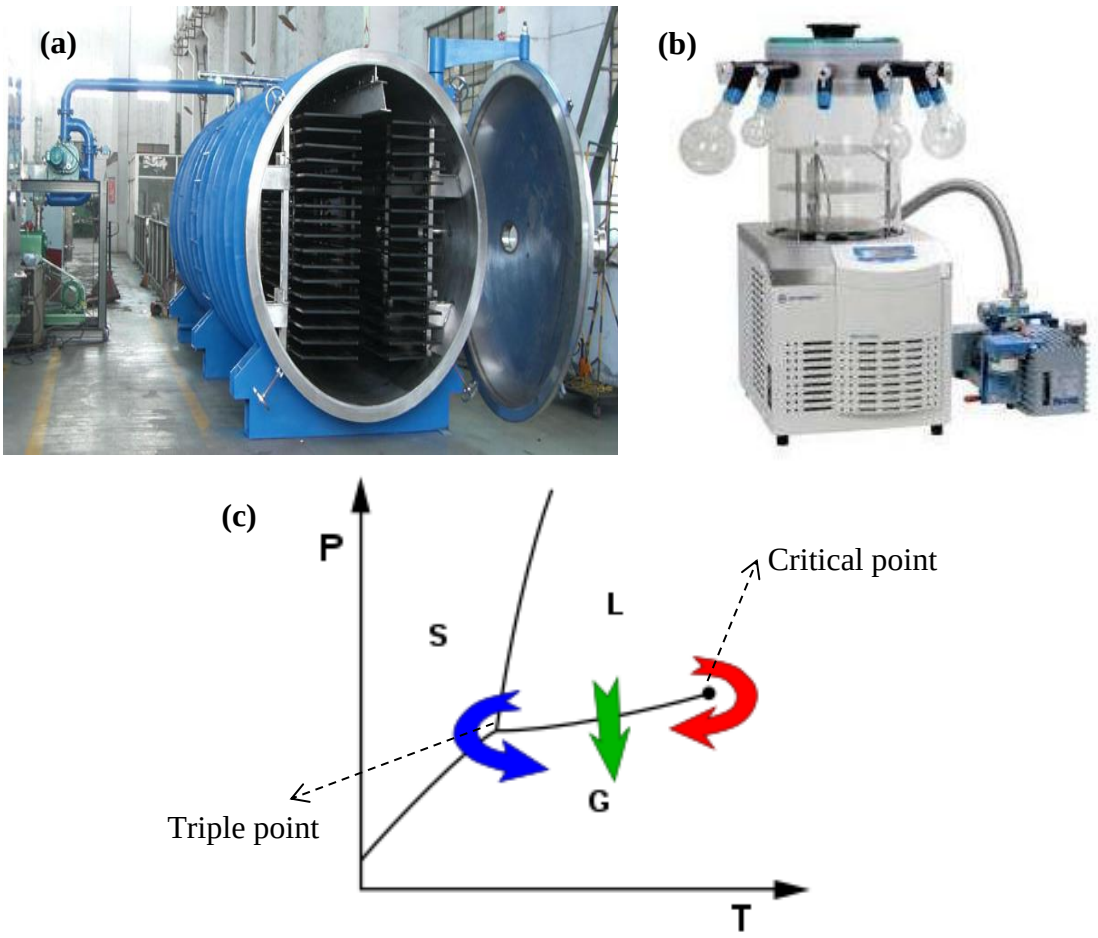

Figure 2-9. Images of the typical (a) industrial freeze dryer and (b) laboratory freeze dryer. (c) The typical phase diagram of freeze drying. Freeze drying brings the system around the triple point, avoiding the direct liquid (L) to gas (G) transition (green arrow).

2.3 Polyphenols

Polyphenols are a structural class of natural chemical compounds containing numerous phenol groups. They are widely distributed in the plant kingdom and are often found in fruits, vegetables and flowers (Ross & Kasum, 2002; Wilig, 2009). These compounds feature several hydroxyl groups on an aromatic ring and their structures are different from a simple phenolic molecule as they often form a complex high-molecular mass polymer (Balasundram et al., 2006). Biochemically, polyphenols are derived from the stimulation of two main synthetic pathways; the shikimate pathway and the acetate pathway (Bravo, 1998). The main groups of polyphenols are known as tannins, lignins and flavonoids. To date, more than 9,000 polyphenolic structures have been identified, with the flavonoid family being the largest (Whiting et al., 2001).

In nature, these compounds are secondary metabolites of plants and are physiologically and morphologically important for plant growth and reproduction. They have diverse biological functions such as providing efficient resistance against pathogens and predators and protect crops from disease and pre-harvest seed germination (Popa et al., 2002; Ross & Kasum, 2002). As a large group of bioactive chemicals, phenolics may act as phytoalexins (Popa et al., 2008), anti-feedants, attractants for pollinators, contributors to plant pigmentation, UV light protectors and antioxidants (Naczk & Shahidi, 2006). Moreover, these compounds also contribute to the colour and sensory characteristics of fruits and vegetables (Alasalvar et al., 2001).

2.3.1 Flavonoids

Flavonoids are the largest class of polyphenols, with a basic skeletal structure of diphenylpropanes (C_6-C_3-C_6), consisting of two aromatic rings (A and B) that are linked by three carbons and form an oxygenated heterocycle ring (C) (**Figure 2-10**). Naturally, the A and C rings usually arise from the acetate pathway biosynthesis and have a characteristic hydroxylation pattern at the 5 and 7 position. On the other hand, the B ring is derived from the shikimate pathway with the hydroxylation pattern taking place at the 4', 3'4', or the 3'4'5' positions (Croft, 1998).

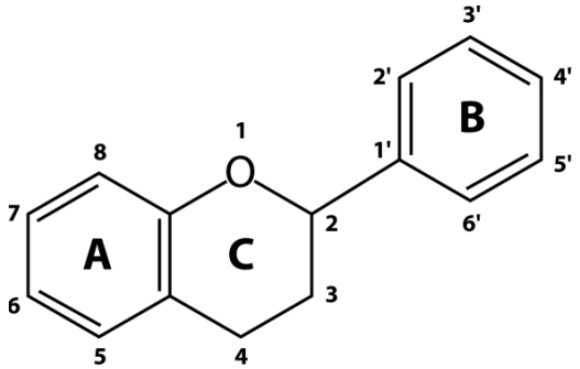

Figure 2-10. General structure and numbering pattern for common food flavonoids (Wilig, 2009).

According to the variations in the heterocyclic C ring, flavonoids can be divided into six major subclasses according to the degree of oxidation of the oxygen heterocycle, namely flavones, flavonols, flavanones, catechins, anthocyanidins, and isoflavones (**Figure 2-11**) (He & Giusti, 2010). Flavonoids have been found to be the most abundant polyphenols in the human diet. There are several factors that influence the formation of flavonoids in plants including the plant variety, plant genetics, environmental conditions, ultraviolet exposure, germination, ripeness stage and food processing and storage (Bravo, 1998). The biosynthesis of flavonoids is stimulated by sunlight (ultraviolet radiation), therefore higher concentrations of flavonoids can typically be found in the outer layers of fruits and vegetables, i.e. the skins (Manach et al., 2004; Vanderauwera et al., 2005).

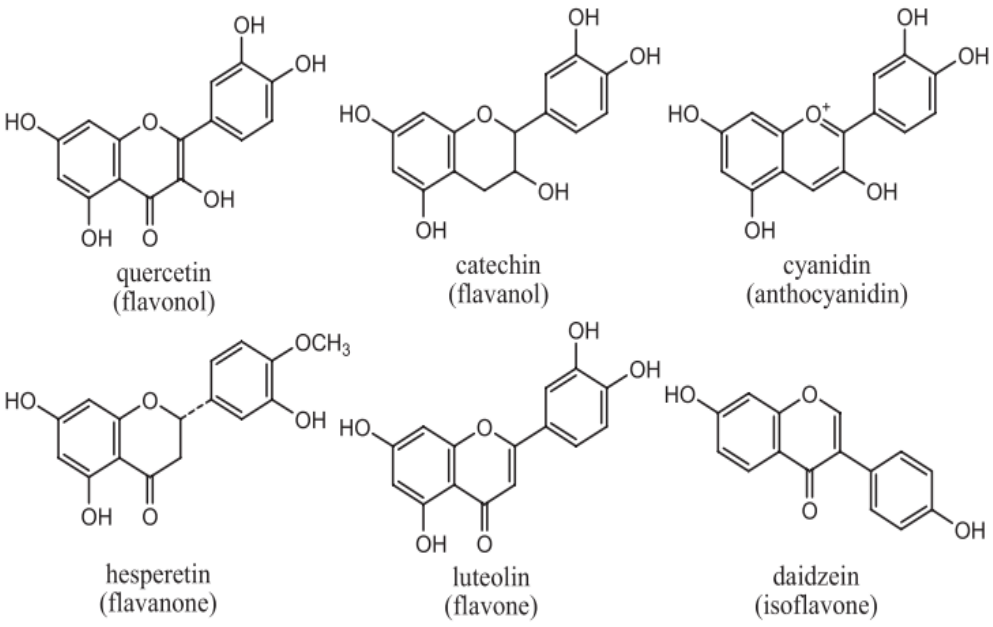

Figure 2-11. Chemical structure of common flavonoid sub-classes (He & Giusti, 2010).

2.4 Anthocyanins

2.4.1 Properties

The terminology of anthocyanins originates from two Greek words, anthos (flower) and kyanos (dark blue) (Delgado-Vargas & Paredes-Lopez, 2002). To date, there are over 635 anthocyanins identified in nature (Andersen & Jordheim, 2008) and have been widely

studied in recent decades. Anthocyanins play several important roles for plants including as colour attractant for animals, thus helping plants in both seed dispersal and the pollination processes. Moreover, due to their strong absorption of light, anthocyanins are believed to be important in protecting plants from UV-induced DNA damage (Mazza & Miniati, 1993). Other than acting as secondary metabolites in plants, anthocyanins have also gained considerable interest as food-colourants and as potential antioxidants, phytoalexins and antibacterial agents (Kang et al., 2003). As shown in **Table 2-4**, a higher content of anthocyanins is found in fruits compared to vegetables, especially in various berries and blackcurrants. However, amongst vegetables, red cabbage is shown to contain high amounts of anthocyanins as well, closer to that of blueberries (Wu et al., 2006).

Table 2-4. Concentration of anthocyanins in most common fruits and vegetables (Wu et al., 2006).

Fruit or vegetable	**Total anthocyanin content (mg/100 g of fresh weight)**
Apple	0.6 – 14.2
Blackberry	177 – 300.5
Blueberry	308.9 – 486.5
Cherry, sweet	100.7 – 143.3
Chokeberry	1480
Blackcurrant	361 - 591
Redcurrant	12.8
Elderberry	1375
Gooseberry	0.7 – 10.5
Grape	37.6 – 120.1
Nectarine	5.3 – 8.3
Peach	3.6 – 6.0
Plum	14.4 – 146.1
Raspberry	72.4 - 687
Strawberry	17.9 – 41.7
Black bean	44.5
Eggplant	85.7
Red cabbage	281.2 – 362.8
Red leaf lettuce	0.7 – 3.7
Red onion	48.5
Red radish	70.1 – 130.1
Small red bean	6.7

The basic structure of aglycones (anthocyanidins) share a C-6 (A-ring)-C-3 (C-ring)-C-6 (B-ring) carbon skeleton (Harborne, 1998). In nature, the most common anthocyanidin aglycones include cyanidin (Cy; 50%), pelargonidin (Pg; 12%), delphinidin (Dp; 12%), petunidin (Pt; 7%), malvidin (Mv; 7%) and peonidin (Pn; 12%) (Fleschhut et al., 2006; Hou et al., 2005; McGhie et al., 2007; Kong, 2003). As shown in **Figure 2-12**, the basic anthocyanin structures vary according to the number of hydroxyl and methoxyl groups that occur at the 3' and 5' positions. The intensity and type of the anthocyanin colour varies among aglycones (**Table 2-5**); more hydroxyl groups result in more bluish colour, while more methoxyl groups result in redder colour (Heredia et al., 1998; Delgado-Vargas & Paredes-Lopez, 2002).

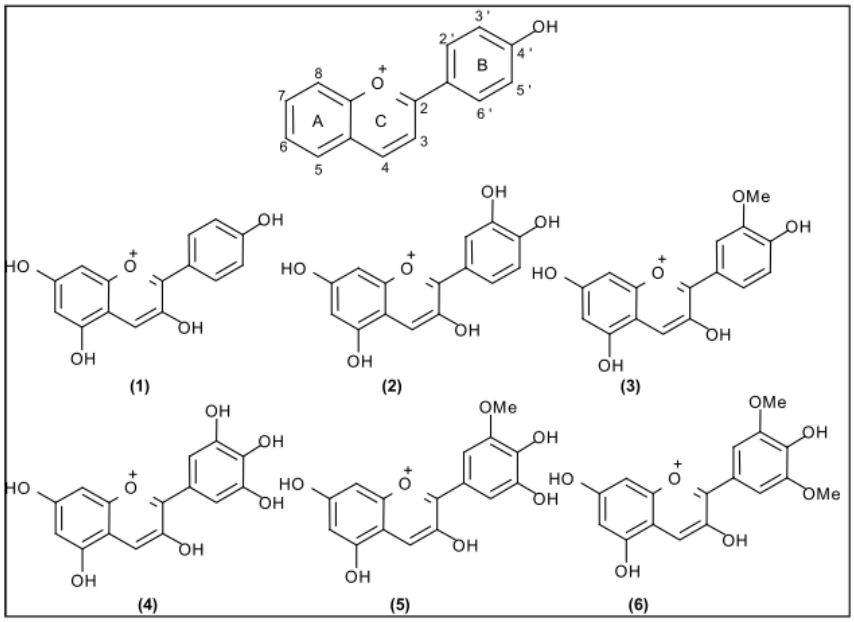

Figure 2-12. Chemical structures of the most common anthocyanidins. 1 - pelargonidin (Pg); 2 - cyanidin (Cy); 3 - peonidin (Pn); 4 - delphinidin (Dp); 5 - petunidin (Pt); 6 - malvidin (Mv) (Horbowicz et al., 2008).

Table 2-5. Differences in chemical structure, colour, and λ_{max} of the anthocyanidins most commonly found in nature (He & Giusti, 2010)

Anthocyanidins	Abv	Substitution		Colour	λ_{max} (nm)
		R1	R2		
Pelargonidin	Pg	H	H	Red	520
Cyanidin	Cy	OH	H	Magenta	535
Peonidin	Pn	OCH_3	H		532
Malvidin	Mv	OCH_3	OCH_3	Purple	542
Petunidin	Pt	OCH_3	OH		543
Delphinidin	Dp	OH	OH		546

In blackcurrant berries, the main types of anthocyanins are delphinidin-3-O-glucoside, delphinidin-3-O-rutinoside, cyanidin-3-O-glucoside, and cyanidin-3-O-rutinoside, which according to Slimestad & Solheim (2002) make up for more than 97% of the total anthocyanin content. Among these four types of anthocyanins, the main differences are the number of hydroxyl groups, the nature and number of sugars in their structure, the position of the sugar bond and the number of aliphatic or aromatic acids bonded to the sugars within the molecule (Kong, 2003). Structurally, the sugars linked to anthocyanidin aglycones are monosaccharides (glucose, galactose, rhamnose, and arabinose) and di- or tri-saccharides, formed by combination of the four monosaccharides by glycosidic bonds (Bureau et al., 2009). In nature, they are known as the anthocyanidins' glycoside forms, and include 3-monosides, 3-biosides, 3,5- and 3,7-diglucosides (Kong, 2003). When the aglycone (anthocyanidin) is glycosylated, it is known as anthocyanin.

The number, type and bond positioning of the sugars in the structure of anthocyanins affects considerably their solubility (Lapornik et al., 2005). The presence of sugars enhances the water solubility and leads to the stability and hydrophilicity of anthocyanins (Wrolstad, 2004; Watson, 2014). During wine and juice processing, anthocyanins, which are abundant in berry skins, remain in the husks; the latter are usually made into compost (Larrosa et al., 2002; Moure et al., 2001). The bright attractive colour and high water solubility of anthocyanins makes them suitable alternatives to synthetic food dyes (Malien-Aubert et al.,

2001; Morais et al., 2002). Additionally, their high antioxidant activity and promising health promoting properties, such as lowering the risk of coronary heart diseases, cancer and stroke, as well as their anti-inflammatory and antimicrobial properties (Wrolstad, 2004), have dramatically increased the interest of researchers and industrialists in these molecules.

In terms of their antioxidant activity, anthocyanins, as other phenolics, prevent radical formation by donating hydrogen to highly reactive radicals (Rice-Evans et al., 1996). Han et al. (2007) and Ramos (2008) also reported the beneficial action of anthocyanins in eye health and vision and their neuroprotective effects. Studies on animal models have shown that the oral intake of blackcurrant anthocyanins can prevent a myopic refractory shift partially through their relaxing effect on the bovine ciliary smooth muscles (Matsumoto et al., 2005).

2.4.2 Effect of pH

Unlike other flavonoids, anthocyanin molecules can undergo reversible structural transformations with changes in pH. Due to this, anthocyanins have been used as crude pH indicators (Forster, 1978). Structurally, anthocyanins exist in four major forms in equilibria: the red flavylium cation, the blue quinonoidal base, the colourless carbinol pseudobase and the colourless chalcone (Brouillard & Delaporte, 1977). **Figure 2-13** depicts the structural changes of the anthocyanin molecule in varying pH conditions (Wrolstad et al., 2005; He, 2008). In highly acidic environments (pH 1.0 – 3.0), anthocyanins exist in the flavylium cation form, carrying oxygen in the positive charge and produces a bright red to purple colour. At pH 4.5, anthocyanins exist in the colourless carbinol pseudobase form and the colourless chalcone form through hydration reactions at C-2 position, which result in colour fading. Between pH 6.0 to 7.0, the anthocyanin pigment exhibits a blue colour and it is present in its quinonoidal base form. Noteworthy, anthocyanins are mostly stable compounds at pH 1.0 where they provide their maximum colouration.

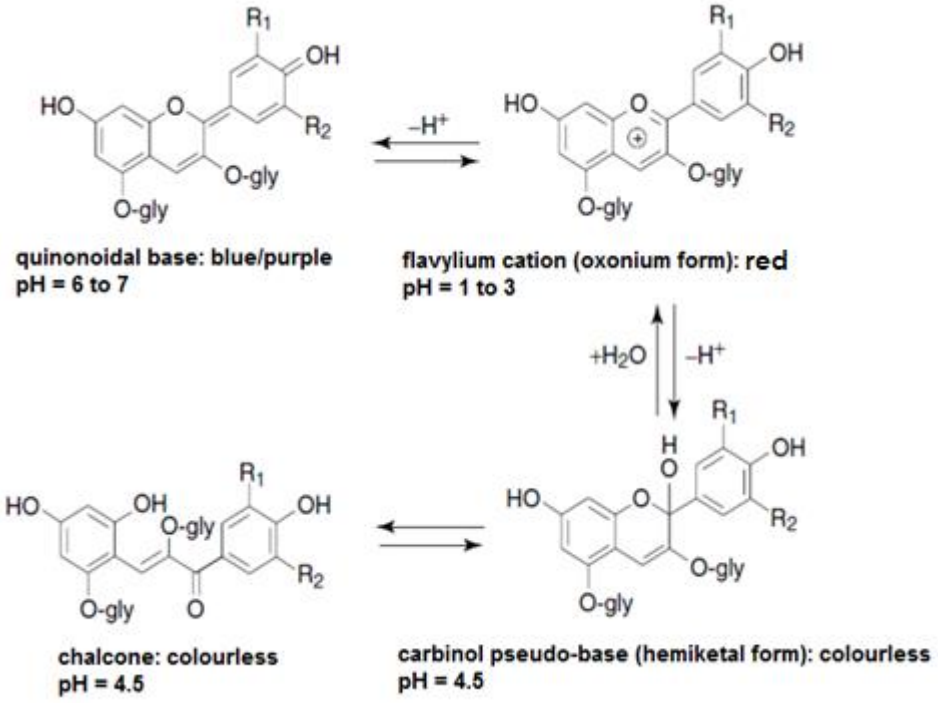

Figure 2-13. Anthocyanin chemical structures and their colours in various pH conditions (Ahmadiani, 2012).

2.4.3 Stability

The stability of the extracted anthocyanins during storage depends on oxygen, pH, temperature, light, metal ions, the presence of enzymes or ascorbic acid, and on the anthocyanin concentration (Mazza & Miniati, 1993). Besides storage at the optimum pH, complex patterns of glycosylation and acylation are also known to increase the stability of anthocyanin molecules (Chandra et al., 2001; Dao et al., 1998; Giusti & Wrolstad, 2003). The hydroxyl groups of the anthocyanin aglycone may be substituted by sugar moieties through glycosidic bonds and further linked to more sugar moieties (**Figure 2-14**), or may be acylated with cinnamic (*p*-coumaric, caffeic, ferulic, gallic and sinapic acids) or/and aliphatic acids (malonic, acetic, malic, succinic and oxalic acids) through ester bonds, as shown in **Figure 2-15** (He & Giusti, 2010).

The aglycones are rarely found in nature due to their poor stability. In nature, anthocyanins are commonly found with the attachment of one or more glycosylating and acylating groups (**Figure 2-15**) at the 3 and 5 positions (Wrolstad, 2004). However, in some anthocyanins, glycosylation also occurs at other positions in their structure. It has been also reported that glycosylation at the 3 position leads to an increase in spectral absorbance as compared to glycosylation at the 5 position and both the 3 and 5 positions (Harborne, 1967; Hong & Wrolstad, 1990; Giusti & Wrolstad, 1996).

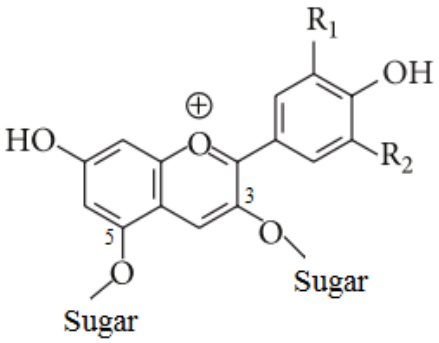

Figure 2-14. Common glycosylation of anthocyanin at 3 and 5 positions.

The most commonly found glycosylating groups bonded to an aglycone are glucose and rhamnose, but in some plant species, other sugar moieties, such as galactose, xylose and arabinose are found as well (He, 2008). These glycosylation and acylation groups have a major impact on the anthocyanin structure which can result in the plants' resistance to light, pH, heat and oxidation conditions (Manach et al., 2004). This is caused by changes in the anthocyanin spectral characteristics due to both glycosylation and acylation, which result also in changes in their molecules size and polarity. It has been shown that increased glycosylation increases anthocyanin polarity and water solubility, whereas an increased acylation reduces its polarity and water solubility (He & Giusti, 2010). Furthermore, glycosylation and acylation are noted to improve anthocyanin stability due to the formation of an intramolecular H-bonding network within the anthocyanin molecules (Borkowski et

al., 2005). Some very complex acylation groups that are linked to the different sugar moieties have been discovered; however these kinds of acylation patterns are rarely found in nature (Willig, 2009). For example, **Figure 2-15** shows the very complicated acylation pattern of an anthocyanin found in grape skin.

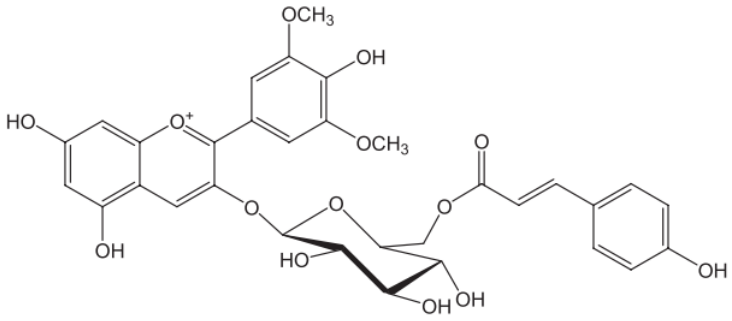

Figure 2-15. Complex chemical structure of an acylated anthocyanin [Malvidin-3-(p-coumaroyl) glucoside] found in grape skin (He & Giusti, 2010).

Acylation has an important function towards the colour of the anthocyanin pigment. Cyanidin anthocyanins with cinnamic acylations are known to increase the λ_{max} towards higher values through a bathochromic shift and shift the hue angle to purple colour, as well as increase the hyperchromic effect (Von Elbe & Schwartz, 1996; Giusti et al., 1999). Therefore, there has been an increased interest in using acylated anthocyanins as natural colourants for food applications. Examples of fruits and vegetables that contain high amounts of acylated pigments are red onion, red cabbage, red potatoes, black carrots, radishes, purple sweet potatoes and grapes (Giusti & Wrolstad, 2003).

In 2002, Slimestad and Solheim found two types of acyl groups attached to the blackcurrant anthocyanin, namely delphinidin 3-O-(6″-p-coumaroylglucoside) (Jose et al., 1990) and cyandin 3-O-(6″-p-coumaroylglucoside) (Saito et al., 1987); the latter has been given the name hyacinthin. This is the first report of acylated anthocyanins from blackcurrant berries. Although the amount of these compounds in berries is low (2%) (Sojka et al., 2009),

this study showed that the difference in distribution may be of importance for the absorption and metabolic route for such structures in the human body (Slimestad & Solheim, 2002).

Anthocyanins are thermo-labile compounds, a feature that constitutes a hurdle in the commercialisation of natural anthocyanin-based colourants. Processing and storage at elevated temperatures may degrade anthocyanins (Rubienskene et al., 2005; Patras et al., 2010). Decomposition can slowly occur during storage at ambient temperature (Hager et al., 2008), however, research by Svensson (2010) shows that heating the blackcurrant mash at 80 °C, 100 °C and 120 °C for 90 minutes caused a decrease in the anthocyanin content in all samples and rapid degradation was observed at the higher temperatures. However, Kirca et al. (2007) reported that anthocyanins from black carrots were reasonably stable during heating at 70 – 80 °C.

As shown in **Figure 2-16**, the thermal degradation of anthocyanins results in the formation of phenolic acids and aldehyde compounds (Fleschhut et al., 2006), thereby causing an overall reduction in their antioxidant activity (Patras et al., 2010). Spontaneous and irreversible degradation may occur when the pH is raised from 3 to 5 and this becomes faster at neutral and alkaline conditions (pH = 7 – 8), resulting in colour loss (Fleschhut et al., 2006; McGhie et al., 2007). This whole reversible conversion of flavylium ions into the chalcone forms is known to be a key-step in the overall mechanism of anthocyanin thermal degradation (Furtado et al., 1993). To circumvent this, refrigeration and an acidic environment are critical conditions to avoid the degradation of anthocyanin-containing materials.

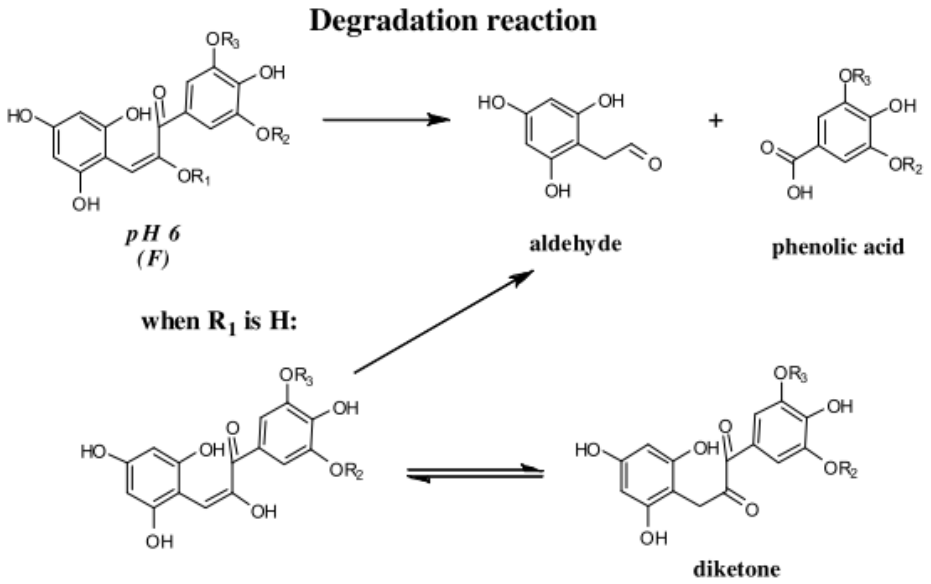

Figure 2-16. Degradation reaction for anthocyanins. Where R1 = H or saccharide, R2 and R3 = H or Methyl (Castañeda-Ovando et al., 2009).

2.5 Anthocyanin extraction

Extraction is the most important step for the isolation and identification of the polyphenols including anthocyanins; however, there is no standardised method for the extraction of phenolic compounds. A multi-step process is normally needed to extract phenolic compounds from plant materials consisting of grinding, drying, followed by soaking of the plant material with a suitable organic solvent (Merken & Beecher, 2000). However, these methods can also result in the co-extraction of compounds other than phenolic substances such as sugars, organic acids and proteins that require further purification processes, for example ultrafiltration and precipitation (Pap et al., 2005; Castañeda-Ovando et al., 2009).

Dried plant materials have been normally used as the starting material for the extraction of anthocyanins in order to minimise the possibility of anthocyanin degradation, which can take place in wet material due to metabolic activities (Harbourne et al., 2013). The conventional method to extract anthocyanins from plant material is a solid–liquid

extraction that includes Soxhlet extraction, maceration, and infusion of the berries in organic solvents (e.g., ethanol, methanol) containing a small concentration of acid (e.g., hydrochloric acid, formic acid). The factors that influence the extraction yield and the composition of individual anthocyanins depend on the plant species and the plant parts, but generally include the solvent type, temperature, particle size, solvent-to-solid ratio and extraction time. Other than the polarity and solubility of compounds to be extracted, the safety of the solvents also needs to be considered especially for inclusion in a food or beverage (Seidel, 2006). To this end, it is necessary to use food grade solvents (e.g. water, ethanol or mixtures of these) in the extraction process. Moreover, all solvent residues must undergo an evaporation step to remove the solvent from the extracts before the latter can be incorporated into food products. In addition, according to the European Parliament and Council of Europe (2011), it is stated under EU legislation that no health claims on the efficacy can be made for a food that contains more than 1.2% ethanol.

2.5.1 Chemical extraction

Due to the polarity of anthocyanins, polar solvents such as aqueous mixtures of methanol, ethanol or acetone are often employed for their extraction (Kähkönen et al., 2001). Since water can extract more polar compounds and ethanol or methanol can extract more hydrophobic compounds, the ratio of water and methanol or ethanol mixture can be adjusted according to the polarity of the targeted compounds. Normally, methanol is used as solvent because of its relatively high polarity and low boiling point. Since anthocyanins are unstable in neutral or alkaline conditions, acidified methanol with hydrochloric or formic acid has been commonly used as extractant in order to disrupt the cell membrane as well as dissolve the water-soluble pigments (Rodriguez-Saona & Wrolstad, 2001; Amr & Al-Tamimi, 2007).

According to Ignat et al. (2011), the extraction of anthocyanins from grape pulp using methanol was 20% more effective than using ethanol and 73% more effective than only water. In another study, Lee and Wrolstad (2004) found that acidified methanol blueberry extracts contained twice as high amounts of anthocyanins compared to aqueous acetone extracts. However, chemical extraction has some distinct disadvantages including long extraction time, requirement for large volumes of solvents and potential degradation of anthocyanins at high extraction temperatures (Harbourne et al., 2013).

2.5.2 Physical extraction

During recent years, various upcoming extraction techniques have been developed in order to overcome the drawbacks of the conventional extraction techniques and especially with a view to minimise the extraction time. For instance, the extraction of phytochemicals from plant materials can be carried out using ultrasound-assisted extraction, microwave-assisted extraction or supercritical fluid extraction (SFE). The ultrasound technology has been reported to reduce the extraction processing time (Zenker et al., 2003), as well as potential thermal degradation effects. Ultrasound involves applying soundwaves in a frequency of at least 20 kHz to disrupt the cell membrane in aqueous solutions. The mechanism of ultrasound can be divided into thermal and non-thermal. As shown in **Figure 2-17**, thermal effects occur when absorbed energy is transformed into heat, while non-thermal effects such as cavitation (implosion of gas bubbles) is caused by rapid changes of heating and pressure, as well as high shearing effects (Leighton, 1998). Extraction of anthocyanins using ultrasound processing is reported to decrease anthocyanin degradation and discolouration in juices (Tiwari et al, 2009). In addition, ultrasound processing has emerged as an alternative to pasteurisation of food products (O'Donnell et al., 2010).

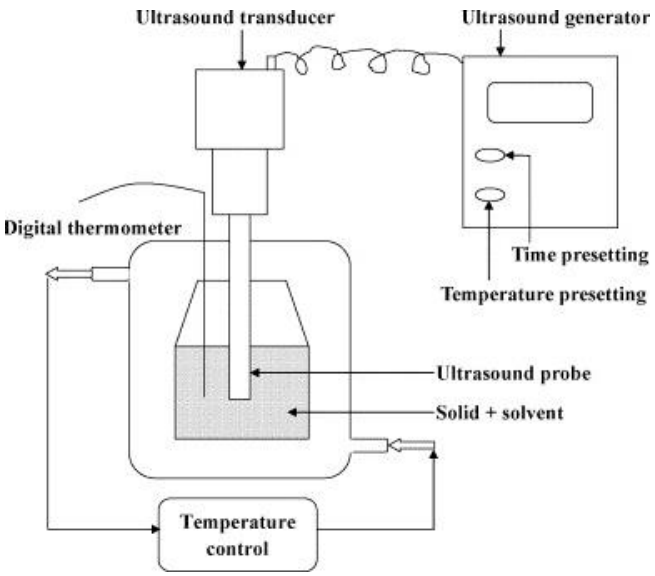

Figure 2-17. Schematic of an ultrasound assisted extraction (UAE) system (Zhao et al., 2007).

Microwave-assisted extraction also can be used to assist the extraction of anthocyanin compounds from berry juice processing by-products. During microwave irradiation (**Figure 2-18**), the cells are thermally stressed, an increase in the temperature and pressure within the cells eventually results in the rupture of the cell wall. The study by Pap et al. (2013) using blackcurrant marc showed that maximum yield of anthocyanins was obtained in laboratory-scale extraction experiments, conducted at pH 2.0 for 10 min of extraction, with a microwave power of 700 W. This technique was suggested as more efficient and resulted in a significant reduction in the extraction time compared to the conventional solvent extraction.

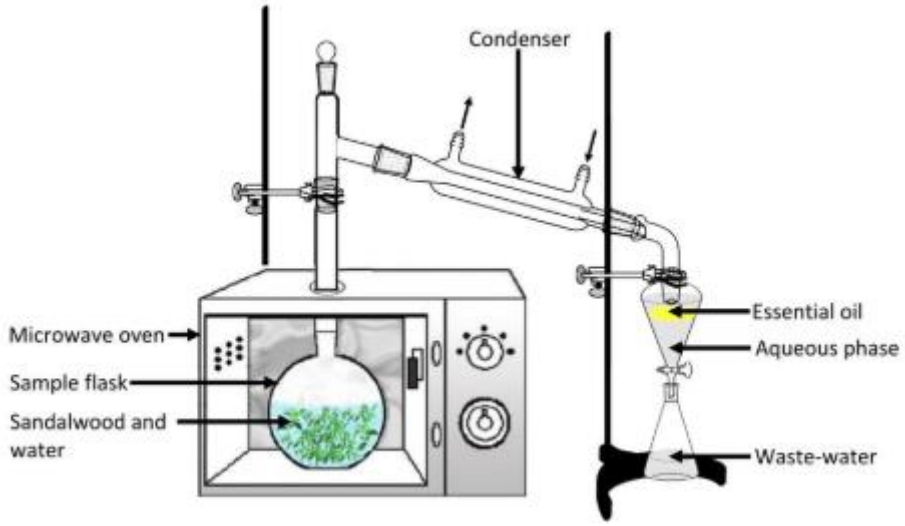

Figure 2-18. Schematic illustration of a laboratory microwave-assisted extraction system (Kusuma & Mahfud, 2016).

SFE (**Figure 2-19**) takes place at above the critical temperature and critical pressure of the solvent used. This critical temperature (highest temperature) causes an increase in pressure that can convert a gas to a liquid phase, meanwhile the critical pressure (highest pressure) can convert a liquid into a gas due to increase in temperature (Wijngaard et al., 2013). SFE has the potential for selective and efficient extraction by controlling the pressure and temperature, due to the enhancement of solvation power of CO_2, which is the solvent most commonly used (Paes et al., 2014). Generally, the major advantages of SFE are the elimination of residual solvents in the products, lower operating temperatures and prevention of oxidation during processing. SFE also provides an alternative method for replacing organic solvents such as hexane during the pre-treatment of plant materials. In experiments investigating the extraction of polyphenols from grape peel by Ghafoor et al. (2010), maximum extract yield (12.31%), total phenols (2.16 mg GAE/100 mL), antioxidants (1.63 mg/mL) and total anthocyanins (1.18 mg/mL) were obtained under optimum SFE conditions, i.e. 45 – 46 °C temperature, 160 – 165 kg/cm^2 pressure and 6 – 7% ethanol were used as solvent modifier. However, research using grape marc and elder berry showed that

SFE only improved the extraction of total polyphenols, while the anthocyanins yield was not influenced significantly (Vatai et al., 2009).

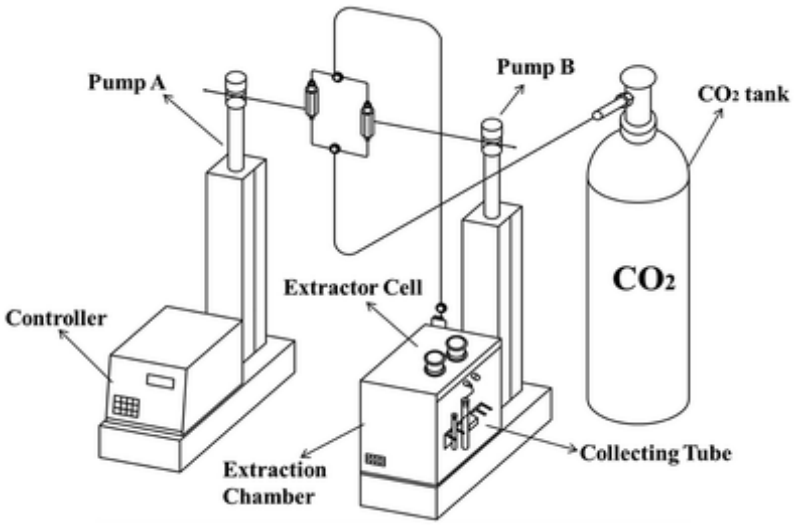

Figure 2-19. Schematic ilustration of the supercritical fluid extraction system (Chen et al., 2018).

2.5.3 Influence of temperature on extraction

In general, a high extraction temperature causes an increase in the rate of diffusion of the soluble plant compounds into the extraction solvent, thereby reducing the extraction time. In the extraction process, apart from the solvent to solid ratio, an increase in temperature can also cause an increase in the concentration of some phytochemicals, which is possibly due to an increase in the solubility of many of these bioactive compounds, or to the breakdown of cellular constituents resulting in their release (Lim & Murtijaya, 2007). In addition, high extraction temperatures may also inhibit the action of inherent plant enzymes that could otherwise act upon bioactive compounds. Marete et al. (2009) reported that an extraction temperature of more than 70 °C inactivated the activity of polyphenol oxidase, therefore resulted in a significant increase in the recovery of total phenols.

The temperature that can be used for extraction will be limited depending on the boiling point of the solvent employed. On the other hand, an exposure to high temperatures may result in a significant reduction in the anthocyanin content possibly due to accelerated chalcone formation (Pacheco-Palencia et al., 2009). To avoid this, temperature sensitive phytochemicals such as anthocyanins can be extracted at lower temperatures for a longer period of time. Moreover, the polarity of the water when under pressure may also vary with temperature. For instance, at lower temperatures the water has high polarity but at higher temperatures (≥250 °C), the pressurized water exhibits a similar polarity to that of polar organic solvents (Herrero et al., 2006).

2.6 Copigmentation

The term "copigmentation" is a special reaction term reserved for anthocyanins, which exist in red/purple colour in nature (Trouillas et al., 2016). A copigmentation reaction is functioning to stabilise and enhance the colour of anthocyanins (Rein, 2005). Naturally, anthocyanins are stabilised by natural compounds, namely copigments which exist in the cells of flowers, fruits and berries (Brouillard, 1982). Moreover, copigmentation can also be initiated by the addition of different plant extracts rich in copigments that are able to react with anthocyanins.

Chemically, copigmentation involves π-interactions between anthocyanins and copigments (Di Meo et al., 2012). It has been shown that there are several factors influencing the stability of anthocyanins such as changes in the pH of the medium, as well as processes such as hydroxylation, methylation, glycosylation and acylation (Harborne, 1967; Hoshino et al., 1980). Wilska-Jeszka & Korzuchowska (1996) reported that copigmentation is more efficient within berry juices rather for purified anthocyanin molecules, due to the presence of several compounds in the juice that play a role in the copigmentation reaction.

In food science, copigmentation is considered as an important interaction which has a direct impact on foods, and thus influences consumer acceptance (Eiro & Heinonen, 2002). Copigmentation can occur through several interactions as illustrated in **Figure 2-20**. The intermolecular and intramolecular interactions are the most common copigmentation reactions, with self-association and metal complexation taking place to a lesser extent (Rein, 2005).

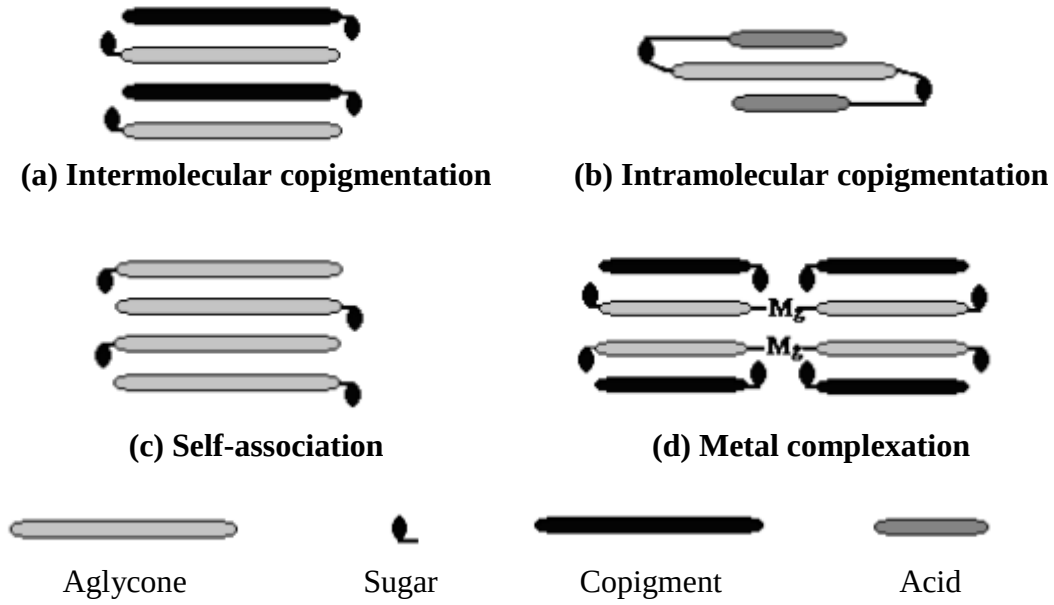

Figure 2-20. Anthocyanins interactions of (a) intermolecular copigmentation, (b) intramolecular copigmentation in acylated anthocyanins, (c) self-association and (d) metal complexion (Rein, 2005).

The stacking of anthocyanin molecules in the copigmentation complexes produces a sandwich configuration (**Figure 2-20**), which protects the flavylium chromophore from nucleophilic water attack, therefore limiting the formation of colourless hemiketal and chalcone forms (Brouillard, 1981). As a result, the colour of the anthocyanin solution become more intense depending on the pH and the copigment concentration. Copigmentation effect also results in an increase in the maximum absorbance (hyperchromic effect) and the maximum wavelength (bathochromic shift) as measured by UV-Visible spectroscopy (Malaj et al., 2013). **Figure 2-21** demonstrates the change in the absorption

maximum wavelength (bathochromic shift) and in the colour intensity (hyperchromic effect) for blackcurrant extract copigmented with ferulic acid at pH 3.0.

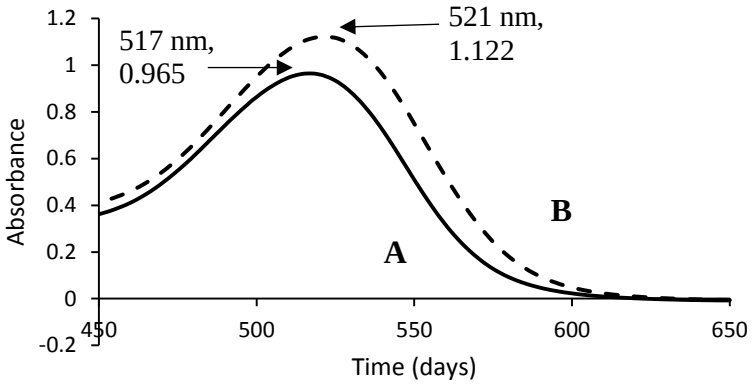

Figure 2-21. Copigmentation as hyperchromic effect and bathochromic shift at pH 3.0. (A) Blackcurrant extract and (B) Blackcurrant extract plus ferulic acid.

The main factors that influence the copigmentation effect include the concentration of anthocyanin and copigment, pH, temperature, solvent and the molecular structures (Lambert et al., 2011; Malaj et al., 2013; Zhao et al., 2017). The chemical structures of anthocyanins change according to the pH of the aqueous solution as shown and described in **Figure 2-13**. Most studies have reported that the enhancement of colour is due to the interactions between the flavylium ion and the copigments (Dangles et al., 1993; Bordignon-Luiz et al., 2007; Gras et al., 2017). Moreover, Gauche et al. (2010) suggested that the efficiency of copigments can be determined by measuring the anthocyanin stability and the loss of colour.

Copigments are normally colourless or slightly yellowish, and anthocyanins acylated with copigments existing naturally as coloured molecules in the cell vacuole (Brouillard et al., 1989). Until today, the most studied group of copigments in the intermolecular copigmentation include flavonoids, e.g. flavones, flavonols, flavanones and flavanols, and phenolic acids, e.g. hydroxycinnamic acids and hydroxybenzoic acids, which have shown a

prominent effect towards colour enhancement and stabilization of anthocyanins (Marković et al., 2000). On the other hand, aliphatic acids (e.g. acetic, oxalic, succinic and malic acids) and aromatic acids (e.g. *p*-hydroxybenzoic, gallic, *p*-coumaric, caffeic and ferulic acids) are among the copigments namely acyl groups that commonly involved in the intramolecular copigmentation (Zhou et al., 2017).

2.6.1 Intermolecular Copigmentation

Intermolecular copigmentation describes the noncovalent association between the colourless copigments and the planar polarisable nuclei of the coloured anthocyanins through weak hydrophobic forces (π-π stacking interactions) [**Figure 2-20 (a)**]. It is suggested that this type of interaction increases the stability of non-acylated anthocyanins. Hydrophobic interaction and ionic (electrostatic) interactions between the anthocyanins and the copigments have been suggested as the main mechanistic driving forces for intermolecular copigmentation (Asen et al., 1972; Brouillard et al., 1989). Intermolecular copigmentation reactions appear to contribute significantly to the colour enhancement of fruits, berries and their products (Eiro & Heinonen, 2002).

According to Eiro and Heninonen (2002) and Vanini et al. (2009), the increase in the copigment concentration enhances the copigmentation effect. However, it has been shown that increasing concentrations not only resulted in the increase in hyperchromic effects, but may also induce to the formation of precipitation (Trouillas et al., 2016). Furthermore, a high temperature (40 °C, 60 °C and 80 °C) hinders the intermolecular copigmentation phenomenon between malvidin-3-O-glucoside and *p*-coumaric, vanillic and syringic acids (Malaj et al., 2013).

Intermolecular interactions can involve the flavylium cation (pH 1 – 3) and the quinonoidal base forms (pH 6 – 7) (Brouillard et al., 1989). However, at pH values between

6 and 7 where the quinonoidal base form dominates, stronger copigmentation interactions take place compared to very low pH conditions (pH 1 – 3), where the flavylium is in its cation form (Williams & Hrazdina, 1979). Moreover, the intermolecular copigmentation through hydrophobic interactions between the anthocyanins and the copigments were dramatically weakened by the presence of organic solvents such as ethanol, methanol and acetone (Marpaung et al., 2017).

Bakowska et al. (2003) reported that amongst the flavonols, rutin and quercetin are the most efficient copigments producing strong intermolecular copigmentation. Among various phenolic acids, sinapic and ferulic acids were found to be the most efficient copigments, exhibiting a strong colour enhancement and a bathochromic shift (Eiro & Heinonen, 2002; Rein & Heinonen, 2004; Sharara et al., 2017). Notably, Marković, et al. (2000) found that methoxylated ferulic acid was a better copigment than nonmethoxylated caffeic acid, which contains only hydroxyl groups. Benzoic and coumaric acids are on the contrary quite weak copigments (Jamei & Babaloo, 2017; Sharara et al., 2017). **Table 2-6** provides a summary of key intermolecular copigmentation studies with anthocyanins at different conditions including types of buffers and concentrations.

Table 2-6. Examples of anthocyanins, copigments and experimental conditions and outcomes of different intermolecular copigmentation studies.

Anthocyanins and copigments	Buffer system	Comments	References
Anthocyanins: Purple sweet potato extract (Cyanidin and peonidin-based, non-acylated, mono-acylated and di-acylated anthocyanins) **Copigments:** Chlorogenic and rosmarinic acids, and food-grade apple and rosemary extracts **Ratio of anthocyanins to copigments:** -1 to 0.5, 1.25 and 5.0 (Chlorogenic and rosmarinic acids) (g/L) -1 to 0.7, 1.3, 6.3 and 0.3, 0.5, 2.4 (Apple and rosemary extract, respectively) (g/L)	-Acetic acid in water (pH 0.9 and 2.6) -Sodium acetate buffer (pH 3.6 and 4.6)	-pH 4.6 had the highest hyperchromic shift (~50.5%) at the absorption maximum. -Rosemary and apple extract showed higher bathochromic shifts (4 - 7 nm) than pure rosmarinic and chlorogenic acids. -Copigmentation effect ~43.8% rosmarinic acid and ~31.1% chlorogenic acid. -Phenolic acids and food-grade extracts showed higher hyperchromic shifts mainly on the di-acylated purple sweet potato anthocyanins (pH 3.6 and 4.6).	Gras et al. (2017)
Anthocyanins: Purple sweet potatoes (Cyanidin-3-glucoside) **Copigment:** Gallic, ferulic and caffeic acids **Ratio of anthocyanins to copigments:** Molar ratio of 1 to 100	-0.06 M sodium acetate and 0.02 M phosphoric acid (pH 3.2)	-Phenolic acids enhance the colour by 19% - 27%. -Anthocyanin degradation was faster than colour fading. -Colour fading during heating followed first-order reactions with half-lives of ~3.66 h (control), ~9.64 h (gallic), ~3.50 h (ferulic), and ~3.39 h (caffeic). -Anthocyanin degradation followed second-order reactions with half-lives of ~3.29 h (control), ~3.43 h (gallic), ~2.29 (ferulic), and ~2.72 h (caffeic).	Qian et al. (2017)

Anthocyanins: Roselle (Delphinidin-3-sambubioside and cyanidin-3-sambubioside) **Copigments:** Ferulic, cinnamic and coumaric acids **Ratio of anthocyanins to copigments:** Molar ratio of 1 to 100	-Roselle extract in water (pH 2.67 - 2.77)	-Copigments resulted in an increment of anthocyanin and colour stability during storage as well as hyperchromic effect and bathochromic shift. -The decrease in anthocyanin content were ~31.53% (control), ~20.48% (ferulic), ~9.31% (cinnamic) and ~5.52% (coumaric) after storage. -Copigmentation with roselle anthocyanin extract showed a noticeable antioxidant and antimicrobial activities. -Marshmallow prepared from copigmented roselle extracts as natural colourants showed high acceptability by panelists.	Sharara et al. (2017)
Anthocyanins: Blueberry extract expressed as malvidin-3-glucoside **Copigments:** Tannic, caffeic, benzoic, and coumaric acids **Ratio of anthocyanins to copigments:** 1 to 1 (Copigment 0, 0.12, 0.24, 0.48, and 0.96 g/L)	-Blueberry extract in water (pH 1.0, 2.0, 3.0 and 4.0)	-Caffeic acid was the best copigment, whilst benzoic acid was the weakest. -A positive correlation between the copigment concentrations and the stability of anthocyanin as well as hyperchromic and bathochromic changes. -Increase in the pH values degraded anthocyanin stability. -The strongest hyperchromic and bathochromic effects were observed in pH values 1 and 4, respectively.	Jamei & Babaloo (2017)
Anthocyanins: Malvidin-3-O-glucoside **Copigments:** *p*-coumaric, syringic and vanillic acids **Ratio of anthocyanins to copigments:** Molar ratio of 1 to 1, 10, 30 and 60	-0.02 M sodium acetate (pH 2.5 and 3.65)	-Di-O-methylated one (syringic acid) showed slightly more efficient copigment than the mono-O-methylated one (vanillic acid). -A methoxylated acids had stronger copigmentation effect than those with hydroxyl substituents. -The copigmentation process is more efficient at pH 3.65. -Increase in the temperature from 20 to 80 °C decreased the copigmentation efficiency.	Malaj et al. (2013)

Anthocyanins: Cabernet Sauvignon Grape skins crude extract expressed as malvidin-3-glucoside **Copigments:** Caffeic, ferulic, gallic and tannic acids **Ratio of anthocyanins to copigments:** 1 to 1 (v: w)	-Pottasium chloride buffer (pH 1.0 and 2.0) -Citrate buffer (pH 2.5, 3.0, 3.3, 3.5, 3.7, 4.0 and 4.5)	-The maximum copigmentation effect was at pH 3.3 with every acid used. -Tannic acid was the best copigment (~2,585 h of the estimated half-life time). -Addition of organic acids improved the anthocyanin stability except for the caffeic and ferulic acids that were suggested to form proanthocyanins with weak copigmentation effect.	Gauche et al. (2010)
Anthocyanins: Grapes crude extract expressed as malvidin-3-glucoside **Copigments:** Caffeic acid **Ratio of anthocyanins to copigments:** 1 to 0.5, 0.75 and 1.0 (v: w)	-100 mM hydrochloric acid and pottasium chloride (pH 3.0)	-The presence of light reduced the stability of anthocyanins. -A higher proportion of caffeic acid increased the half-life of this pigment, both in the presence and absence of light.	Vanini et al. (2009)
Anthocyanins: Cabernet Sauvignon grape extracts expressed as malvidin-3-glucoside **Copigments:** Caffeic acid **Ratio of anthocyanins to copigments:** 1 to 1 (v: w)	-0.1 M citric acid-sodium citrate (pH 3.0 and 4.0) -Buffer model system and yogurt model system	-The addition of caffeic acid significantly increased the stability of anthocyanins in both model and yoghurt systems. -In the model system the highest values for half-life were at pH 3.0, stored in the dark at of 4 ± 1 °C (~6.930 h). -In the yoghurt system the caffeic acid increased the half-life time of anthocyanins to ~6673 h.	Gris et al. (2007)

Anthocyanins: Strawberry, raspberry, lingonberry, and cranberry juices (pelargonidin 3-glucoside, cyanidin-3-glucoside, delphinidin-3-glucoside, peonidin-3-glucoside, petunidin-3-glucoside and malvidin-3-glucoside) **Copigments:** Sinapic, ferulic and rosmarinic acids **Ratio of anthocyanins to copigments:** Molar ratio of 1 to 10	-35% of strawberry (pH 3.5) and raspberry (pH 3.2) juices in water with added sugar -35% lingonberry (pH 2.6) and cranberry (pH 2.5) juices in water	-Sinapic acid induced the strongest colour (~104%) in strawberry juice. -Ferulic (~35%) and sinapic (~33%) acids improved raspberry colour; rosmarinic acid enhanced the colour of lingonberry (~48%) and cranberry (~42%) juices the most. -Sinapic and ferulic acids formed new intramolecular copigmentation compounds with berry anthocyanins, whilst rosmarinic acid stabilised anthocyanins intermolecularly.	Rein & Heinonen (2004)
Anthocyanins: Pelargonidin 3-glucoside, cyanidin 3-glucoside, malvidin 3-glucoside, cyanidin 3-O-(2″-O-β-xylopyranosyl-6″-O-β-glucopyranosyl)-β-galactoside and cyanidin 3-O-(2″-O-β-xylopyranosyl-6″-O-(6-O-((E)-coumaroyl)-β-glucopyranosyl))-β-galactoside **Copigments:** Rutin, quercetin, ferulic, caffeic, rosmarinic, chlorogenic, sinapic and gallic acids **Ratio of anthocyanins to copigments:** Molar ratio of 1 to 10, 50 and 100	-10% DMSO in 0.02 M ammonium acetate (pH 3.37)	-The best copigmentation reactions was in malvidin 3-glucoside solutions at ratio 1:100. -The strongest copigments were ferulic and rosmarinic acids, ~70% and ~80% of enhancement, respectively. -Rosmarinic acid with malvidin 3-glucoside had the biggest bathochromic shift (~19 nm) and the strongest hyperchromic effect (~260% enhancement). -The most unstable colour was induced by rosmarinic acid. -The colour intensity of pelargonidin 3-glucoside with ferulic and caffeic acids increased ~220% and ~190% during storage, respectively.	Eiro & Heinonen (2002)

2.6.2 Intramolecular Copigmentation

Intramolecular copigmentation **[Figure 2-20 (b)],** involves anthocyanin glycosyl reactions, and is a phenomenon in which the hydroxyl groups of anthocyanin glycosyls are esterified usually by aliphatic acids (e.g. acetic, oxalic, succinic and malic acids) or aromatic acids (e.g. *p*-hydroxybenzoic, gallic, *p*-coumaric, caffeic and ferulic acids) (Zhou et al., 2017). In intramolecular copigmentation, the copigment is covalently bound to the sugar moieties of the anthocyanin molecules (Brouillard, 1983). In nature, acylated anthocyanins can be found especially in the blue flowers (Malaj et al., 2013) and fruits such as black carrots, red radish and red cabbage (Giusti & Wrolstad, 2003). The mechanism of anthocyanin glycosyl acylation involves the π-π stacking (hydrohopic interactions) between the aromatic residues of the acyl groups of an acylated anthocyanin with the positively charged flavylium cation (**Figure 2-22**). This lowers the possibility of nucleophilic water attack at the anthocyanin molecule and prevents the occurrence of the colourless pseudobase or chalcone structures of anthocyanin molecules as pH of aqueous media changes from 4 to 6 (Zhao et al., 2017).

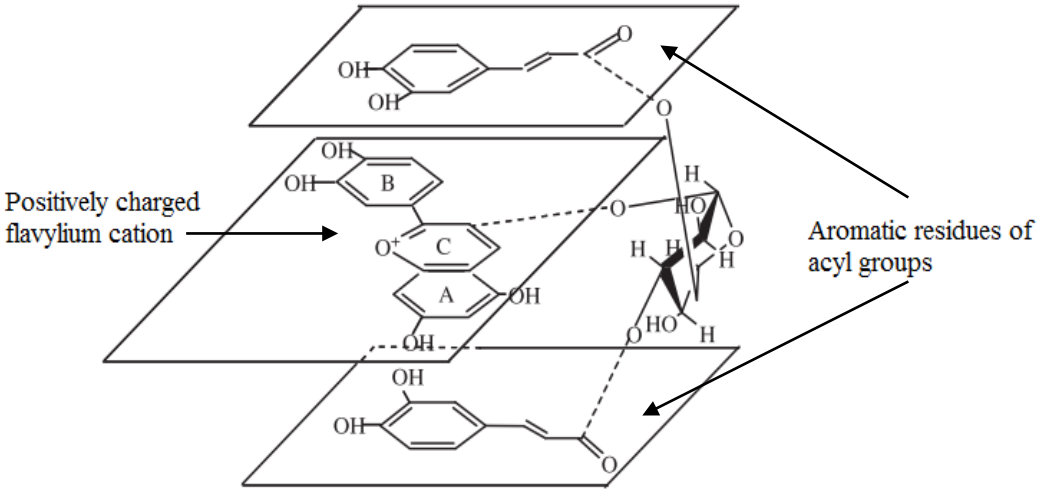

Figure 2-22. The π-π stacking interactions between the aromatic residues of the acyl groups of an acylated anthocyanin with the positively charged flavylium cation of (Zhao et al., 2017).

Intramolecular copigmentation is thought to be stronger and more effective than intermolecular copigmentation due to increased strengths of the covalent bonds which results in the enhancement of the colour and the stability of the anthocyanin molecules (Francis & Markakis, 1989; Dangles et al., 1993). Stintzing & Carle (2004) reported that aromatic acyl groups were more stable than aliphatic acyls, suggesting that the type of acyl donor affects the anthocyanin stability. In a similar line of work, good colour stability was achieved when the anthocyanins were acylated with aromatic or cinnamic (*p*-coumaric, sinapic, ferulic, and caffeic acids) acids than aliphatic ones (succinic, malic, malonic, oxalic and acetic acids) [**Figure 2-23**] (Giusti & Wrolstad, 2003).

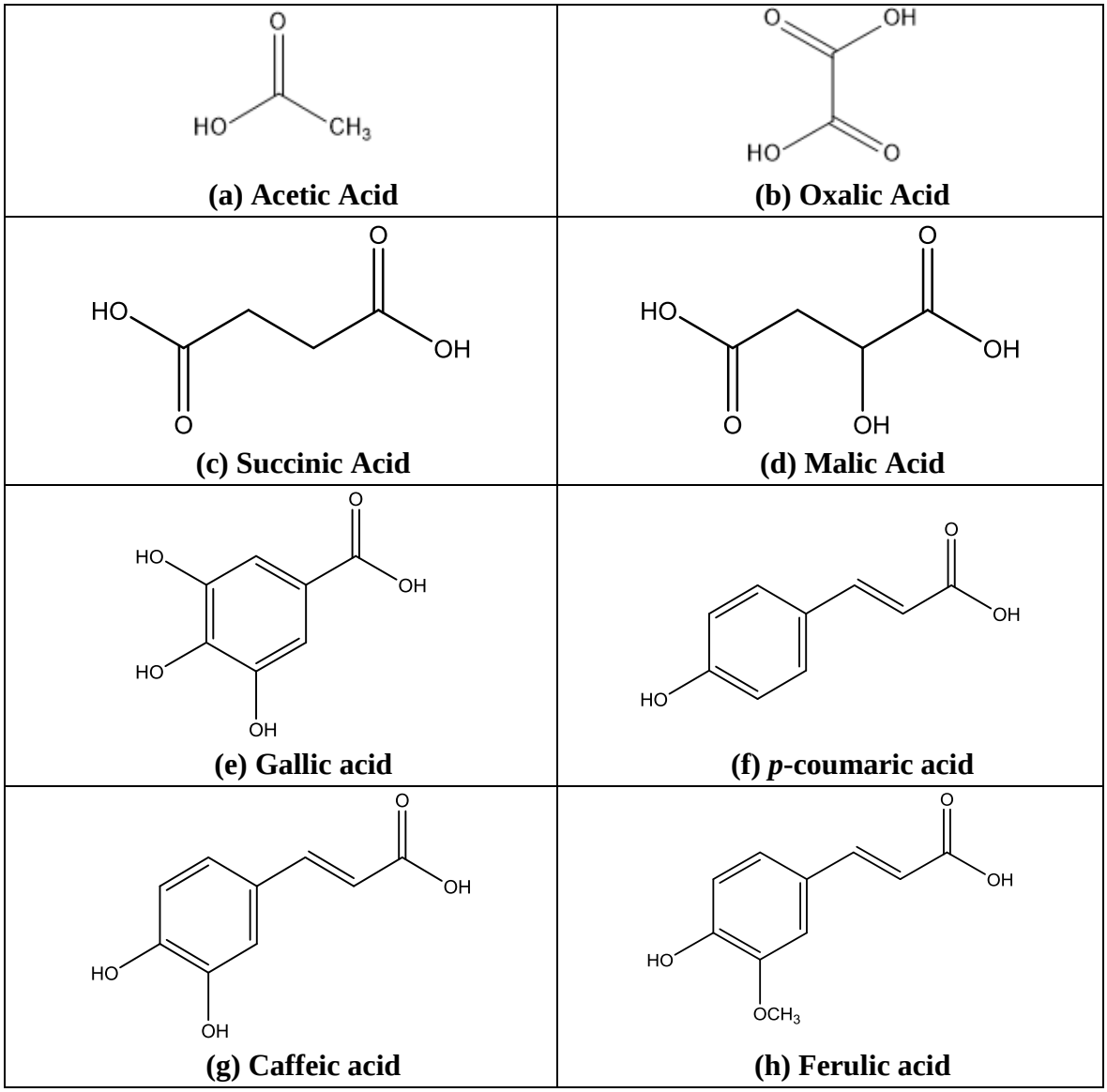

Figure 2-23. Examples of common aliphatic (a-d) and aromatic acids (e-h) attached to sugar moieties of anthocyanins in intramolecular copigmentation.

In vitro, the acylation of anthocyanins with phenolic acids as acyl donor can be achieved by using enzymes. As shown in the **Table 2-7**, there are a number of studies using *Candida antarctica* lipase as the biocatalyst to acylate flavonoid glycosides, including anthocyanins, with phenolic acids or fatty acids as acyl donors; the reactions take place in the solvent media in the presence of molecular sieves in order to absorb the excess of water which may decrease the lipase activity. Previous research has shown that enzymatic acylation resulted in an increase in the hydrophobicity of anthocyanins and other flavonoid glycosides, which most likely resulted in increased solubility in lipids (Cruz et al., 2017), bioavailability (Suda et al., 2002), antioxidant activity (Cruz et al., 2017) and thermostability (Yan et al., 2016). Zhao et al. (2017) and Bakowska-Barczak (2005) reported that the number of acyl groups in the anthocyanin chromophore, their structure and the position of sugar molecule to the glycosyl residues, also influence the intramolecular copigmentation effect. More specifically, the higher number of acyl groups (polyacylated anthocyanins) show better copigmentation effect than one acyl group (monoacylated anthocyanins). In term of structures, aromatic acyl groups were more stable than aliphatic acyl groups (Stintzing & Carle, 2004), whereas the acyl groups with larger sizes or have a higher hydrophobicity or more free -OHs can increase the stability on the anthocyanins (Figueiredo et al., 1999). Moreover, acylation at 3"-position in the sugar moiety resulted in better stability than that at the 6"-position (**Figure 2-24**) (Andersen & Fossen, 1995).

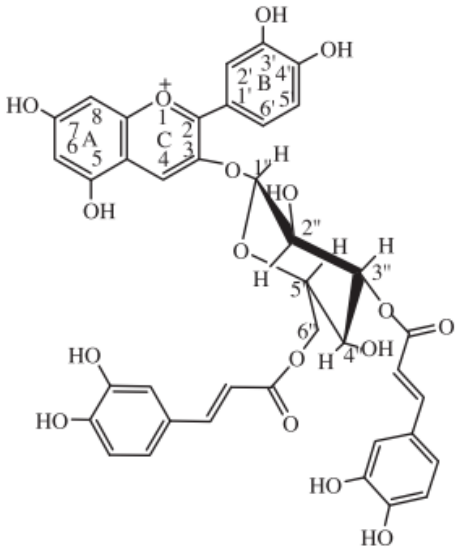

Figure 2-24. Chemical structure of cyanidin 3-O-(3"-O-E-caffeoyl-6"-O-E-caffeoyl-β-D-glucopyranoside (Zhao et al., 2017).

There are two types of enzymatic reactions that can be used for acylation, direct esterification and transesterification. Direct esterification (**Figure 2-25**) is the enzymatic acylation between a flavonoid glycoside with a free phenolic acid and produces acylated flavonoid as well as releases water to the medium. On the other hand, transesterification (**Figure 2-25**) is the reaction between a flavonoid glycoside with fatty acids or methyl, ethyl or vinyl phenolates and leads to the production of acylated flavonoid and alkyl alcohol as the co-product (Schär & Nyström, 2016; Cruz et al., 2016). An important factor that influences the efficiency and regioselectivity of the enzymatic acylation is the water content in the organic solvents, whereas the excess of water can lead to the hydrolysis, whilst adequate amount of water results in the synthesis reaction. In order to circumvent this problem and shift the equilibrium of the reaction towards acylation rather than hydrolysis, activated molecular sieves with a size of ~4 Å can be used to absorb the excess of water.

Chebil et al. (2007) reported that the type of solvent, the molar ratio of reactants, the enzyme origin and the flavonoid structure are among the factors that influence the conversion yield of the enzymatic acylation of flavonoids by lipases and the regioselectivity

of the enzyme. Previous studies have shown that the regioselectivity of the reaction was constant when using the immobilized lipase B from *Candida antarctica* (Novozym 435) as a biocatalyst; as the acylation normally yields only a monoester and takes place on the primary hydroxyl group present on the glycoside moiety of the molecule (C6″-OH). This can be confirmed by using ^{13}C NMR spectrum, which demonstrated that the C-6" position was detected between approximately ~62.9 and ~67.0 ppm, as opposed to ~60.4 ppm due to the resonance effect of the carbonyl of the synthesised ester (Yan et al., 2016; Cruz et al., 2016; Fernandes et al., 2015; McGhie et al., 2006).

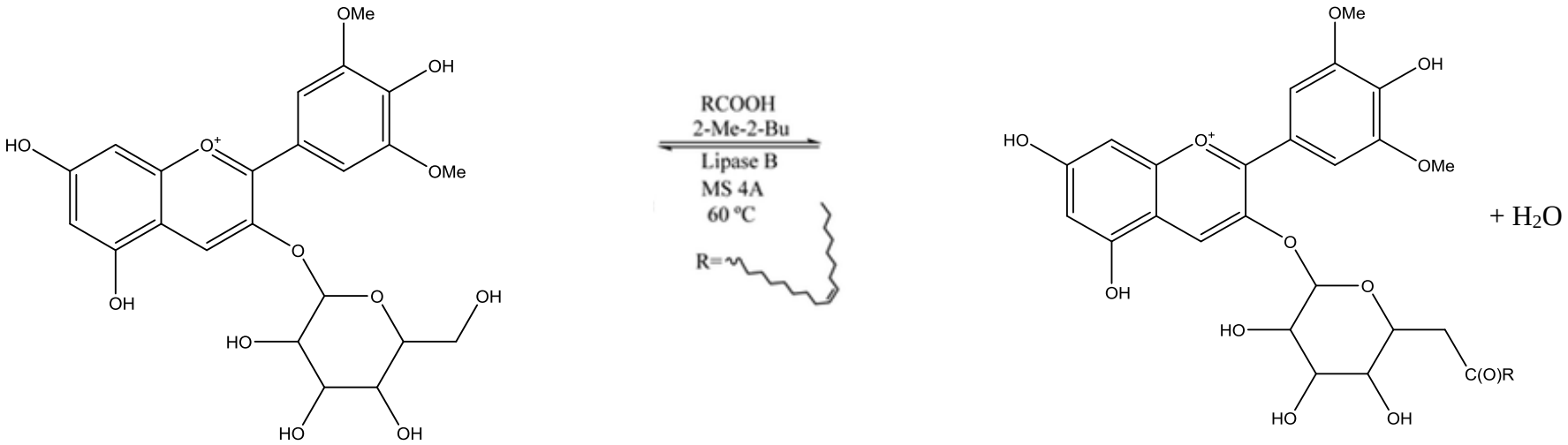

Figure 2-25. Direct enzymatic esterification reaction of malvidin-3-glucoside wine extract with oleic acid as acyl donor (Cruz et al., 2016).

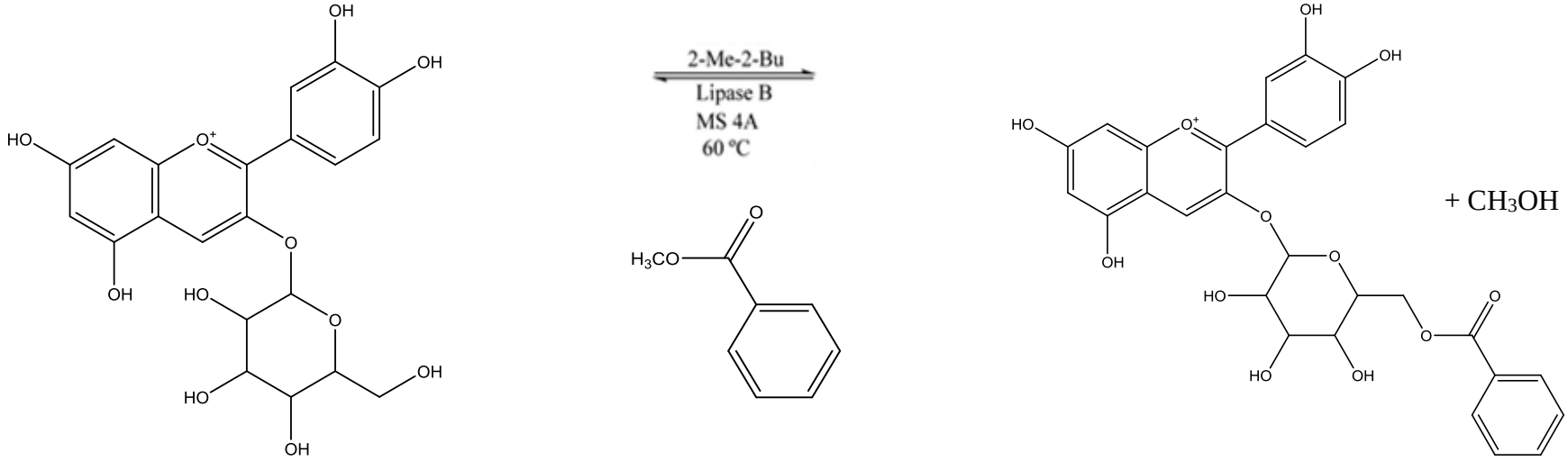

Figure 2-26. Transesterification reaction of cyanidin-3-O-glucoside with methyl benzoate as acyl donor (Yan et al., 2016).

Table 2-7. Examples of the enzyme-catalysed acylation of anthocyanins with phenolic acids or fatty acids as acyl donors.

Enzyme	Anthocyanin	Acyl donor	Acylated compounds	References
Lipase B from *C. antarctica* in anhydrous 2-methyl-2-butanol	Malvidin-3-glucoside from red wine extract	-Different saturated fatty acid chain lengths (C4 - C16)	Lipophilic anthocyanin (Mv3glc-C4, Mv3glc-C6, Mv3glc-C8, Mv3glc-C10, Mv3glc-C12, Mv3glc-C14, Mv3glc-C16) *(22 – 40%)	Cruz et al. (2017)
Lipase B from *C. antarctica* in anhydrous 2-methyl-2-butanol	Malvidin-3-glucoside from red wine extract	-Oleic acid	Malvidin-3-O-(6″-oleoyl) glucoside (Mv3glc–OA) *(1.9 – 21.2%)	Cruz et al. (2016)
Lipase B from *C. antarctica* immobilized on acrylic resin in pyridine	Cyanidin-3-O-glucoside from black rice (*Oryza sativa* L.)	-Methyl benzoate -Methyl salicylate -Methyl cinnamate	Cyanidin 3-(6″-benzoyl)-glucoside *(~91%), Cyanidin 3-(6″-salicyloyl)-glucoside *(~84%) and Cyanidin 3-(6″-cinnamoyl)-glucoside *(~61%)	Yan et al. (2016)
Lipase B from *C. antarctica* in 2-methyl-2-propanol, containing 0.2% (w/v) butylated hydroxy toluene (BHT)	Delphinidin-3-glucoside and cyanidin-3-glucoside from skin of jabuticaba fruits (*Myrciaria cauliflora*)	-Palmitic acid	Delphinidin-3-glucoside-palmitic monoester and Cyanidin-3-glucoside-palmitic monoester	Castro et al. (2014)
Lipase B from *C. antarctica* immobilized (Novozyme 435) in 2-methyl-2-propanol, containing 0.2% w/v butylated hydroxy toluene (BHT)	Delphinidin-3-O-glucoside, cyanidin-3-O-galactoside, petunidin-3-O- galactoside, Cy-3-O-glucoside, Pt-3-O-glucoside, malvidin-3-O-galactoside, and Malvidin-3-O-glucoside	-Palmitic acid -Cinnamic acid -Phenylpropionic acid (PPA) -2-hydroxy-, 4-hydroxy- and 3, 4-dihydroxyPPA	Delphinidin-3-galactoside PPA ester, Delphinidin-3-glucoside PPA ester, Cyanidin-3-galactoside PPA ester, Pentunidin-3-galactoside PPA ester, Cyanidin-3-glucoside PPA ester, Pentunidin-3-glucoside PPA ester, Malvidin-3-galactoside PPA ester, Malvidin-3-glucoside PPA ester, quercetin-3-galactoside PPA ester, and quercetin-3-glucoside (isoquercetin) PPA ester	Stevenson et al. (2006)

$$* \, Conversion\ yield(\%) = \frac{HPLC\ peak\ area\ of\ acylated\ anthocyanin}{HPLC\ peak\ area\ of\ nonacylated\ anthocyanin + acylated\ anthocyanin} \times 100$$

2.6.3 Self-association

Self-association of anthocyanin was first suggested by Asen et al (1972) when the nuclei of two or more anthocyanin molecules were associated through the hydrophobic π-π stacking interactions as shown in **Figure 2-20 (c).** González-Manzano et al. (2008) reported that the higher methoxyl group in the B-ring (**Figure 2-10**) resulted in the increase of self-association effect and colour appearance of anthocyanins. This structure offers molecular stability to the anthocyanin molecules by preventing water nucleophilic attack at the flavylium cation, which leads to the formation of a colourless pseudobase or chalcone. Notably, self-association of the neutral quinonoid bases (pH 6 – 7) is stronger than that of flavylium cations (pH 1 – 3) because the self-association is hindered by electrostatic repulsion between the flayvylium cations (Trouillas et al., 2016). Factors such as solvent, light, pH, temperature, structure and especially the ratio of anthocyanins to copigments significantly affect the association reactions (Cavalcanti et al., 2011).

The thermal resistance of anthocyanins was found to be higher in copigmentation through self-association compared to copigmentation with phenolic acids (Qian et al., 2017). This is likely due to the fact that the additional phenolic acids during the intermolecular copigmentation process interrupt the self-association between anthocyanins, thus accelerate anthocyanin degradation. To this end, the study by Qian et al. (2017) on the intermolecular copigmentation effects of gallic, ferulic and caffeic acids on the anthocyanins from purple sweet potatoes reported that colour stability was higher than anthocyanin stability due to self-association interruption. Furthermore, the colour of aged wines might be contributed by the self-associations of anthocyanins during wine aging process (González-Manzano et al., 2008).

2.6.4 Metal complexation

Metal complexation involves the metal chelation (bonding of ions and molecules to metal ions) between metal ions and the free hydroxyl group in the B-ring of the anthocyanin molecule **(Figure 2-10)**, as suggested by Osawa (1982). The most common metals involved in the formation of anthocyanin complexes include tin (Sn), copper (Cu), iron (Fe), aluminum (Al), magnesium (Mg), and potassium (K) (Markakis, 1982). In order to undergo the metal complexion reactions, more than one free hydroxyl group must be presented in the B-ring of the anthocyanin molecules, such as cyanidin, delphinidin and petunidin (Schreiber et al., 2010). Naturally, metals have been shown to stabilise the colour of different berry products, such as strawberry puree (Wrolstad & Erlandson, 1973), cranberry juice cocktail (Starr & Francis, 1974), and crowberry juice (Kallio et al., 1986). However, these reactions can also be potentially initiated through the addition of metals. For example, the study by Schreiber et al. (2010) showed that $Al3^{+}$ changes the colour of the anthocyanin pigment in hydrangea sepals in the acidic ethanol from red to blue as represented in two-step processes. Firstly, Al^{3+} replaces H^{+} ions from B-ring of delphinidin, thus transforming the red flavylium cation to form a blue quinodal base anion **[Figure 2-27 (b)]**. Secondly, this metal complexation is stabilised by the π–π stacking of another delphinidin as a flavylium cation on top of the metal chelated delphinidin [Al^{3+} – D^{-}] complex, as shown in **Figure 2-28**. The stacking interaction accentuated the bluing of hydrangea sepals as well as increased bathochromic shift in spectral absorbance.

Even though the use of artificial blue food colourants in the food industry has declined due to health concerns and consumer demand for the natural product (Sigurdson and Giusti, 2014), the metal complexation approach has not been used by the food industry due to undesired contamination of food products by metals (Rein, 2005).

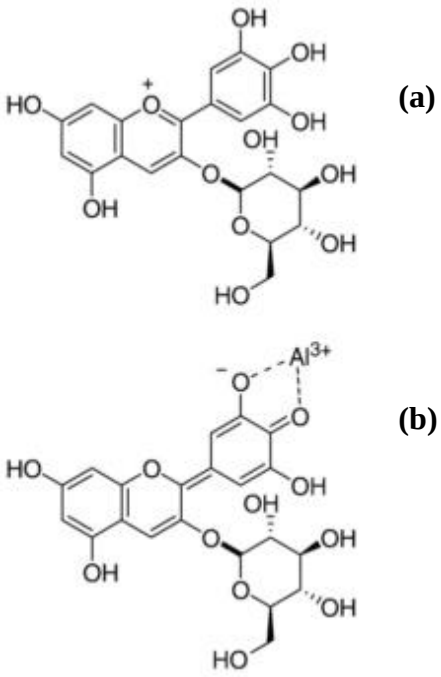

Figure 2-27. (a) Delphinidin-3-O-glucoside as in the red flavylium cation form and (b) the metal chelated delphinidin-3-O-glucoside with Al^{3+} in the blue quinoidal base form (Schreiber et al., 2010).

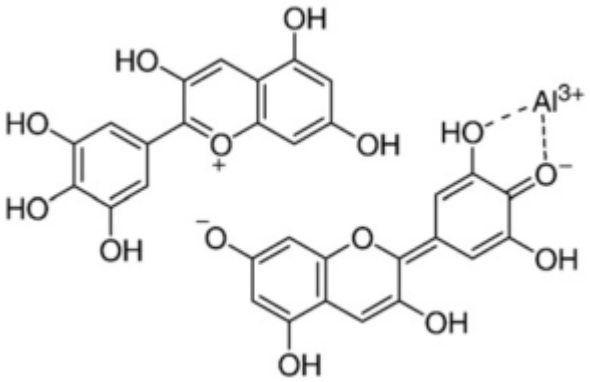

Figure 2-28. Flavylium cation of delphinidin associates (by charge transfer and π–π interaction) with the quinoidal base anion (Schreiber et al., 2010).

2.7 Colourimetry

Colourimetry is the science of colour measurement (Wrolstad and Smith, 2010), and it is a possible way for the observer to measure colour accurately (Konica Minolta, 2007). Munsell, CIE XYZ, Hunter LAB and Hunter CIELAB are the most common instruments widely used to provide colour data that correspond to how the human eye perceives colour (Wrolstad and Smith, 2010). Three important dimensions in colour are hue (*h**), lightness (*L**), and chroma (c). As shown in **Figure 2-29**, the hue component (*h** from 0-360°) in the colour wheel reflects the different colours, i.e. red, yellow, blue, etc. The lightness (*L**) of the colours changes vertically ranging from 0 (black) to 100 (white). Chroma (or saturation) indicates the intensity of the colour and describes its dullness or vividness (Konica Minolta, 2007). On the horizontal axis, positive *a** indicates redness, whilst negative is greenness. Meanwhile, on the vertical axis, positive and negative *b** indicate yellowness and blueness, respectively.

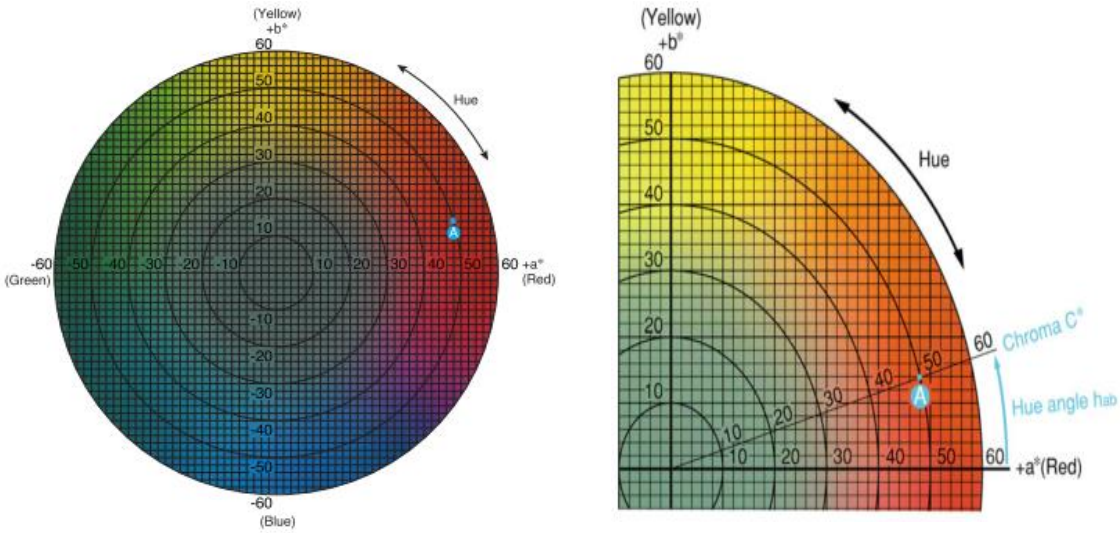

Figure 2-29. Colour space chromaticity diagram (Hue, Chroma and *L*a*b**) (Konica Minolta, 2007). *L** (lightness); *a** (redness/greenness); *b** (yellowness/blueness).

2.7.1 Anthocyanins as food colourants

In the food industry, there has been much interest in replacing synthetic food colourants with natural ones especially those originating from vegetable and fruit sources (Slimestad & Solheim, 2002). This is due to consumer concerns on the safety of synthetic dyes and consumer trends towards the consumption of natural products. E numbers are codes that cover the ranges of natural and synthetic food colourants as approved by Food Standards Agency (FSA) under European Union (EU) legislation. The E number for food colourants begins with 1, such as E 163 for anthocyanins (FSA, 2018).

Anthocyanins are natural products that could be considered as candidates for the replacement of synthetic dyes due to their bright and attractive colour (orange, red, purple), high water solubility and possible health benefits (Malien-Aubert et al., 2001; Morais et al., 2002). Anthocyanin-rich fruits such as blackcurrants, grapes, blueberries and cranberries, as well as their by-products (e.g. pulps, pomace, etc.) can be potentially used as natural colourants. The different types of anthocyanins correspond to different colours. For example, delphinidins are responsible for the purple, mauve and blue colours, pelargonidins are responsible for orange, pink and red colours, while magenta and crimson colours are due to the presence of cyanidins (Romero et al., 2008). However, the main challenges that need to be addressed before anthocyanins can be incorporated into food systems include the fact that as most of the natural colourings, they have poor stability during processing and storage (Socaciu, 2007).

Anthocyanins with acylating substituents are more stable during processing and storage, which makes them more suitable to be applied into food systems that have a low pH (e.g. juices, purees, jams, and syrups), as well as neutral and slightly alkaline food products (i.e. dairy products such as ice-cream and milk). Examples of fruits/vegetable extracts containing naturally acylated anthocyanins, which may impart desirable colour and stability

for commercial food products, include grapes, strawberries, elderberry, red cabbages, black carrots, purple sweet potatoes and radishes (Giusti & Wrolstad, 2003; He & Giusti, 2010). However, it must be noted that most commercial natural anthocyanin colourants (e.g. extracts) contain mixtures of both nonacylated and acylated anthocyanins. In this case, the main challenge is to maintain anthocyanin and consequently colour stability during processing and storage, particularly in neutral food systems, such as dairy products (ice cream and milk).

Researchers have tried to increase the stability of anthocyanins and colour by adding a copigments into the anthocyanin crude extract. In a study using an anthocyanin rich grape extracts carried out by Bordignon-Luiz et al. (2007), tannic acid was used as copigment with the ratio of anthocyanin to copigment was 1:1 (w/v). This copigmentation reaction was added to the yogurt system and kept at 4 ± 1 °C for 45 days. As results, the intermolecular copigmentation effect between the anthocyanin and tannic acid significantly influenced the colour stability of anthocyanins and increased their shelf life, thereby making them possible to be commercially applied in yogurt as colourants.

Moreover, Jing and Giusti (2005) incorporated an anthocyanin-rich extracts from purple corncobs (*Zea mays* L.) into milk and measured its stability. Colour and anthocyanins stability were evaluated using an accelerated stability test at a temperature close to typical pasteurization (70 °C) for 0, 30, 60, 90 and 120 min, and the results showed that the residual anthocyanins in the whole milk were 95.5, 92.0, 85.7, and 76.6%, respectively. Therefore, purple corncobs waste was suitable to be used as natural colourants into milk matrices, whereas acylated anthocyanin such as peonidin-3-(6″-malonylglucoside) were found more resistant to heat treatment. Besides that, proteins and fats in milk prevented the anthocyanins degradation when exposed to heat. However, to the best of our knowledge, no works have

been carried out investigating the use of enzymatically acylated anthocyanins as natural colourants within food matrices.

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CHAPTER 3 – Effect of different drying processes on the total free anthocyanins, total phenolics and antioxidant activity of blackcurrant (*Ribes nigrum* L.) skins

Abstract

The aim of this study was to investigate the effect of different drying processes (industrial rotary, laboratory oven and freeze drying) on the free anthocyanins, total phenolic content and antioxidant activity of dried blackcurrant skins (DBS) extracts. The effects of different drying parameters such as temperature, residence time, the ratio of air speed to drum rotor speed and sample particle size were evaluated. DBS produced using an industrial rotary dryer contained significantly ($p \leq 0.05$) higher total free anthocyanins (17.9 ± 0.4 mg/g) compared to laboratory oven drying, indicating the efficiency of industrial drying process in preventing skins from overheating which may lead to degradation of anthocyanin, especially on samples with smaller particle size distribution. In DBS produced using a laboratory oven, drying at 90 °C for 30 min resulted in 3.6 ± 0.1 % moisture content, with the highest free anthocyanin content (13.3 ± 0.5 mg/g). Very poor correlation was seen between anthocyanin and moisture content, indicating that changes in final moisture content (between ~7.6 to 9.0 %) had no influence in the anthocyanin content. Moreover, an increase in the ratio of drum speed to air speed increased the total phenolic content including anthocyanins (24.3 ± 0.5 mg/g) in the DBS extract. The highest free anthocyanin content and antioxidant activity were found in samples with particle sizes > 5 mm and < 5 mm, while the lowest anthocyanin content was detected in samples with < 800 μm particle size, indicating most probably that overheating of smaller particles had led to degradation of the anthocyanin content in DBS. In comparison to industrial rotary drying, the low free anthocyanins but high total phenols and antioxidant activity in DBS extracts from laboratory oven and freeze drying suggested that some other non-phenolic compounds were also extracted and that the stability of some phenolics compounds were preserved during the respective drying processes.

Keywords: Blackcurrant skins, drying process, particle size, free anthocyanins, phenolic compounds, antioxidant activity

3.1 Introduction

Waste management is an important factor in food processing, whereas the majority of plant-derived by-products is usually used as animal feed (Hui, 2006). An efficient utilisation of these by-products is required in order to minimise the adverse effects on the environment, due to production of high quantities of waste and pollutants (Szenes, 1995). Recently, the focus of waste management for these by-products is changing from simple disposal to the new alternatives, primarily as potential sources of value added ingredients (O'Shea et al., 2012). In some cases, waste streams, whilst inevitable, they do not necessarily reach their full potential in value if they are used as animal feed. Although alternative uses for such waste streams have been recently reported, they are not yet optimised.

Apart from the pomace, the blackcurrant skins obtained during juice processing are rich sources of polyphenol antioxidants, particularly the anthocyanins (250 mg/100 g of berries) (Vagiri, 2012). Anthocyanins and other phenolic compounds exist almost exclusively in the skins, with lesser amounts are usually found in the flesh and seeds of blackcurrant berries. However, their individual anthocyanin content can vary be varied according to genotype, light, temperature, and environment interactions factors (Horbowicz et al., 2008; Tiwari & Cummins, 2013). Blackcurrant solid residues are also a rich source of antioxidant compounds, which have potential uses in the manufacture of dietary supplements or food additives in addition to their nutritional value (Lapornik et al., 2005; Kapasakalidis et al., 2009).

In some certain cases, bioactive compounds are more effectively extracted from dried rather than fresh matter (Karam et al., 2016). This is due to the fact that phytochemical

degradation occurs more rapidly at higher moisture content. Therefore, it is recommended that instead of removing water completely, the moisture content of pigments should be kept below 5% (Cai et al., 2005) and between 6-11% (Yang et al., 2013) in order to enhance the stability of phytochemical in pigments, to inhibit the microbial growth, as well as to minimise browning reactions of enzymatic and non-enzymatic origin. Depending on the mechanisms of the heat transfer, drying is categorised into direct (convective), contact (cooled surface), radiative (infrared rays), and excitation (microwave) (Hui et al., 2006). Convective drying or direct drying is the application of hot air to the wet material and as a result, moisture transfers onto the surface of the material and then evaporates into the drying air. Rotary dryers are an example of convective drying and are commonly used in the industry to dry particulate solids of fruits or vegetables including their by-products (Keey, 2013). Commonly, the size of these dryers varies from 0.3 to 5 m in diameter and 2 to 90 m in length. The dryer usually slopes slightly towards the discharge end to allow the material to convey through the dryer under the air stream that is caused by the fan. During the drying process, solids with high moisture content (above 50%, w/w) enter on one end of the rotary drum that is rotating, and exit at the end when suitably dried as shown in **Figure 3-1 (a).** The drying medium is usually hot air or combustion gases (direct heated dryer) for water evaporation, which flow directly throughout the long cylindrical shell drum.

Rotary drum dryers contain flight lifters to lift the material away from the bottom of the dryer and drop them through the hot air to allow the effective contact between solids and the hot air [**Figure 3-1 (b)**] (Fakouri, 2013). The time spent in the dryer depends on the size of the particles. There is a variable retention time, so that a small (e.g. <200 μm) particle might become airborne after 1 second in the hot air stream and thus, will travel to the end of the drier in approximately 10-15 seconds. A larger particle (e.g. >5 mm and <5 mm) might spend some 4-5 min in the drum, and the dried skins are discharged out of the dryer by the air stream. However, denser (wetter) particles, will have to spend a disproportionately long

residence time in the drum, so that variable airspeeds can be used, as in the ‘triple-pass’ dryer.

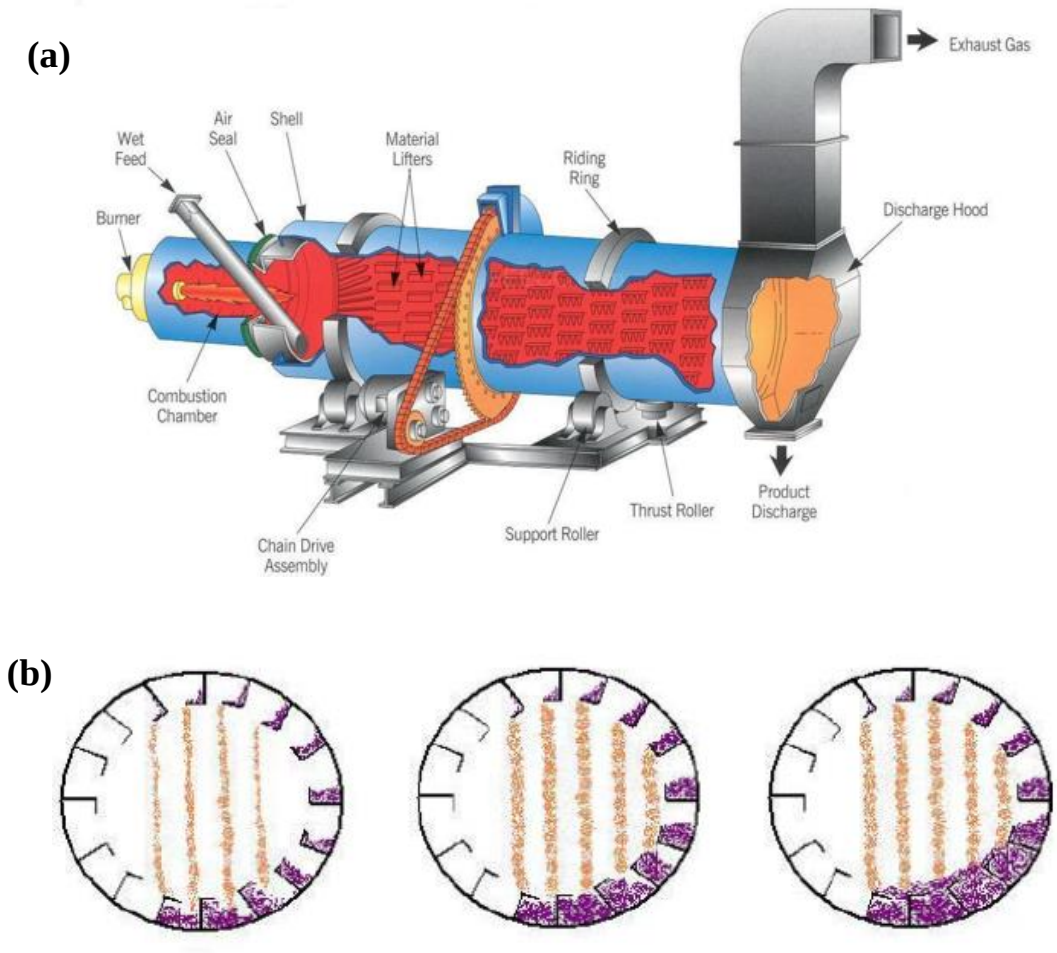

Figure 3-1. (a) Mechanical construction of a direct heat rotary dryer (KBW Machinery, 2017). (b) Illustration of the contact between the cascading particles and hot air in the rotary drum dryer (Fakouri et al., 2013).

Laboratory oven drying is also one of the convective thermal applications. It is generally based on fan circulating hot air and provides a uniform temperature throughout the drying process. According to Ratti (2001), drying using hot air produced dehydrated products with approximately one year of shelf life. However, this method affects the quality of the food matrices. Even though it is more costly and time consuming from a manufacturing point of view, vacuum freeze-drying can be alternatively used as a drying method, capable of preserving thermolabile compounds that are abundant in fresh fruits,

such as vitamins and certain phenolics as well as inhibit the microbial growth, thus produce an excellent quality of food product (Ciurzyńska & Lenart, 2011).

The aims of this study were to investigate the effect of different drying processes (industrial rotary, laboratory oven and freeze drying) and different drying parameters (temperature, residence time, the ratio of air speed to drum rotor speed and particle size) on total free anthocyanins, total phenolics and antioxidant activity of DBS extracts.

3.2 Materials and methods

3.2.1 Chemicals

All solvents and chemicals used for extraction included methanol (99.9%) and hydrochloric acid (HCl) (37%) were of analytical grade and were purchased from Sigma-Aldrich (UK) and Fisher Scientific (Loughborough, UK), respectively. Folin-Ciocalteu reagent, sodium carbonate, 1,1-diphenyl-2-picryl-hydrazyl (DPPH) were also purchased from Sigma-Aldrich (UK). A stock solution of 2 mM DPPH was prepared in methanol.

Purified water used in all preparation and analysis was supplied through a Purite reverse osmosis system (Oxon, UK). Anthocyanin standards of cyanidin-3-O-glucoside (96%), cyanidin-3-O-rutinoside (96%), delphinidin-3-O-glucoside (95%), and delphinidin-3-O-rutinoside (95%), kaempferol-3-O-glucoside (99%), kaempferol-3-O-rutinoside (98%), quercetin-3-O-rutinoside (99%) and myricetin-3-O-glucoside (99%) were obtained from ExtraSynthese Ltd (Genay, France). In addition, caffeic acid (98%), *p*-coumaric acid (98%), ferulic acid (99%), quercetin (95%), myricetin (98%), kaempferol (99%) and quercetin-3-O-glucoside (98%) were purchased from Sigma-Aldrich (UK).

3.2.2 Sample preparation of plant materials

Fresh and dried blackcurrant pressed residues from different drying processes, deriving from juice manufacturing process, were kindly supplied by A & R House (BCL) Ltd, (Bleadon, Weston-super-Mare, UK). Dried blackcurrant pressed residues were labelled according to the various drying parameters in the industry as shown in **Table 3-1**. Dried samples consisted of blackcurrant skins which were divided to <800 μm, <5 mm, >5 mm particle size, and mixtures of all particle sizes at a ratio of 15: 43: 41 (w/w/w). Both dried and fresh samples were kept in -20 °C until further analysis.

Table 3-1. Industrial DBS samples used in this study, dried using a rotary drum drier.

DBS	Drying parameters
315	Air on 450 °C/ Air off 97 °C; Drum speed/ air speed ratio decreased
915	Air on temp 475 °C/ Air off 97 °C; Drum speed/ air speed ratio increased
111-16	Air on temp 475 °C/ Air off 97 °C; Drum speed/ air speed ratio increased
132-16	Air on 450 °C/ Air off 98 °C; Drum speed/ air speed ratio increased
1611	Air on 475 °C/ Air off 97 °C; Drum speed/ air speed ratio decreased
240	Air on 475 °C/ Air off 97 °C; Drum speed/ air speed ratio decreased
245	Air on 475 °C/ Air off 97 °C; Drum speed/ air speed ratio decreased
250	Air on 475 °C/ Air off 97 °C; Drum speed/ air speed ratio decreased
255	Air on 475 °C/ Air off 97 °C; Drum speed/ air speed ratio decreased

*DBS: Dried blackcurrant skins contained mixtures of all particle sizes at a ratio of 15: 43: 41 (w/w/w).

Drying rates were experimentally determined for 40.0 g of fresh blackcurrant skin samples at six different temperatures (70, 80, 90, 100, 110, 120 °C) for various processing times (15, 30, 45, 60, 90, and 120 min) using a laboratory oven (SalvisLab Thermocenter TC-40T). For each time point, the moisture content of two replicates of 1.0 g ground DBS was analysed with Halogen Moisture Analyser (HE73, Mettler Toledo). Furthermore, 40.0 g of fresh blackcurrant skins were lyophilized in the freeze dryer (Virtis SP Scientific Model 2KBTES).

Dried blackcurrants were separated from seeds by grinding in a coffee blender to pass a 0.841 mm (20 mesh) sieve; this constituted the dried blackcurrant skins (DBS) fraction. Samples were segregated in polyethylene bags and stored at -20 °C until further analysis.

3.2.3 Determination of total free anthocyanins and phenolics

The total free anthocyanin and phenolic contents of the samples were determined according to the method by Bao et al. (2005), with slight modifications. Ground DBS (2.0 g) was accurately weighed. 20 mL of 1% (v/v) HCl in methanol were then added, and the sample was shaken (180 rpm) for 24 h at 30 °C. Then, the coloured liquid was filtered using a vacuum filter to separate the supernatants and the residues and was replaced with 20 mL of fresh solvent for another 24 h extraction. Supernatants were pooled together and the concentration of free anthocyanins and phenolics in the extracts were measured using High Performance Liquid Chromatography (HPLC) and quantified using external standards, as described in the following paragraph.

3.2.4 HPLC analysis of free anthocyanins and phenolics

HPLC analyses were performed on an 1260 Infinity HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array detector (DAD) using a Zorbax C18 column (250 × 4.6 mm i.d., particle size 5 µm, Agilent) at 30 °C. The mobile phase consisted of 5% formic acid (v/v) (solvent A) and 100% (v/v) methanol (solvent B). The gradient elution system was: 15% (B) at 0 min, increasing to 35% (B) at 15 min and to 60% (B) at 30 min, and reaching 80% (B) at 40 min. The flowrate was 1.0 mL/min and injection volume was 20 µL. The duration of analysis was 50 min.

Detections were carried out at wavelengths of 520, 360, 320 nm for free anthocyanins, flavonols and hydroxycinnamic acids, respectively. Briefly, 2.0 mg/mL of

stock standard solutions were prepared separately followed by dilution to give concentrations from 0.01 to 1.0 mg/mL (anthocyanin), 0.001 to 0.05 mg/mL (flavonol) and 0.05 to 1.0 mg/mL (hydroxycinnamic acid). To obtain the standard curves, the peak areas were plotted against the corresponding concentrations of the standard solutions injected into HPLC.

3.2.5 Determination of total phenols

The phenol content was determined by the Folin–Ciocalteu method with slight modifications (Waterman & Mole, 1994). 20 µL of 4-fold diluted extracts were added to 1.58 mL of distilled water and 100 µL of Folin–Ciocalteu reagent. After 8 min, 300 µL of sodium carbonate (75 g/L) were added. The absorbance of the samples was measured at 765 nm after 2 h of incubation at room temperature against a blank sample (water instead of extract). Gallic acid (0 – 100 mg/L) was used as standard for the calibration curve. The results were expressed as milligrams of gallic acid equivalents per 1 g of dried weight (mg GAE/g DW). Duplicate measurements were taken and mean values were calculated.

3.2.6 Determination of total antioxidant activity

The free radical scavenging activity of DBS extracts on the stable DPPH radical was carried out according to a procedure previously described (Blois, 1958) with slight modifications. 200 µL of 50-fold diluted extracts were mixed with 2 mL of 2 mM methanolic solution of DPPH. The absorbance was measured at 517 nm after keeping the samples at 30 °C in dark, for 30 min. The percentage of inhibition was calculated using the following equation:

$$Inhibition\ (\%) = \frac{A_o - A_e}{A_o} \times 100$$

A_o = Absorbance of the control; Ae = Absorbance of the sample

3.2.7 Statistical analysis

All statistical analyses were conducted by one-way analysis of variance (ANOVA). Tukey's multiple range tests were employed with the probability of $p \leq 0.05$. The linear Pearson correlation was also used to evaluate correlations between free anthocyanins, total phenolics and antioxidant activity. The software for statistical analysis was Minitab V.16 (Minitab Inc., State College, Pennsylvania, USA).

3.3 Results and discussion

3.3.1 Effect of drying temperature and time in a laboratory oven on the moisture content of DBS

In this study, the moisture content of fresh blackcurrant skins that were dried using a laboratory oven was investigated. Drying curves of moisture content versus drying time are showed in **Figure 3-2**. The curves of the drying rates can be divided into several parts, due to the inner mechanism and changes that occur during drying. These were influenced by the surface area, the temperature and moisture gradients between the surface and the surrounding air, and the heat (heat that is transferred to the skins to evaporate moisture) and mass transfer (mass that is transferred as vapour into the drying air) (Hui et al., 2006). Notably, the temperature increase of blackcurrant particles during drying in this study is a hypothesis since the actual temperature of the powder was not measured.

As shown in **Figure 3-2**, moisture transfer occurred during the first 15 min, falling rate drying between 15 min to 30 min and the drying rate became zero after 30 min at 100, 110 and 120 °C. There was a constant rate of moisture migration from the inner to the surface between 0 min to 15 min, which equals to the rate of the evaporation at the surface. The drying process reached the critical moisture content at 15 min, when the migration of water from inside to surface was reduced and became unsaturated for the entire surface. At this point, most samples still contained more than ~10% of moisture except for the skins dried at 110 °C and 120 °C. After 15 min, the temperature of the surface started to increase and this initiated the beginning of falling rate drying. Here, the rate of moisture migration to the surface is lower than the rate evaporation and the drying rate became zero for skins dried at 100, 110 and 120 °C after 30 min. However, the drying ended after 45 min at 80 and 90 °C, whilst it took 60 min at 70 °C for the skins to completely dry.

The moisture content decreased rapidly to less than ~10% after 30 min of drying, and most samples needed approximately 45 min in order for their moisture content to decrease from ~59.8% to less than ~0.1%. Overall, samples were dried more rapidly at 120 °C compared to 70 °C. Generally, these results are in agreement with Hui (2006), who indicated that an increase in the drying temperature accelerates the drying process. From these results, it was deemed interesting to further investigate the effect of drying temperatures on the extractability of free anthocyanins, total phenolics and antioxidant activity in DBS extracts. DBS extracts were obtained in a suspension by extraction using 1% (v/v) HCl in methanol at 30 °C for 48 h, followed by filtration.

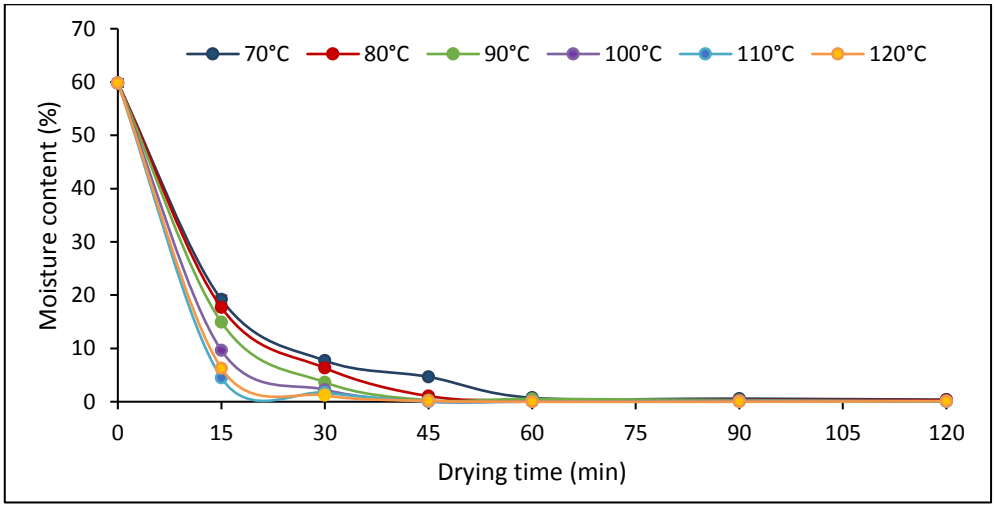

Figure 3-2. Drying curves of fresh blackcurrant skins dried in a laboratory oven at different drying temperatures as function of time. Error bars indicate ± standard deviation (SD).

3.3.2 Free anthocyanins and other phenolic content in DBS extracts

Supplementary Table 3-5 and **Supplementary Figure 3-6** present the typical concentrations and HPLC chromatograms, respectively, of the free anthocyanin profiles in the extracted DBS. Among the four free anthocyanins detected, delphinidin-3-O-rutinoside (~16.3 ± 0.2 mg/g) was the one found at the highest concentration in DBS (~51.4%). This was followed by cyanidin-3-O-rutinoside (~22.2%; ~7.0 ± 0.1 mg/g), delphinidin-3-O-glucoside (~20.2%; ~6.4 ± 0.0 mg/g) and cyanidin-3-O-glucoside (~6.2%; ~2.0 ± 0.0 mg/g).

In this study, delphinidin and cyanidin rutinoside (monosaccaharides glucose and rhamnose) were predominant compared to glucoside (glucose only), similar to anthocyanins in commercial blackcurrant juice processing as reported by Woodward et al. (2011). There are several factors that influence the type and quantity of anthocyanins in blackcurrant, including the genetic diversity of the cultivar, geographical variations, growing conditions, the use of fertilizers and other stress factors (Kähkönen et al., 2003).

As shown in **Supplementary Table 3-6** and **Supplementary Figure 3-7**, *p*-coumaric acid (~48.0%; ~1.0 ± 0.0 mg/g) was the main hydroxycinnamic acid detected in all extracts. In this study, *p*-coumaric together with caffeic (~27.0%; ~0.6 ± 0.0 mg/g) and ferulic acid (~25.0%; ~0.5 ± 0.0 mg/g) shaped the total hydroxycinnamic acid content. Furthermore, myricetin (~36.9%; 0.3 ± 0.0 mg/g) was the major flavonol, followed by quercetin (~20.8%; ~0.1 ± 0.0 mg/g) and myricetin-3-O-glucoside (~14.4%; 0.1 ± 0.0 mg/g) (**Supplementary Table 3-7** and **Supplementary Figure 3-8**). Most of phenolic compounds are accumulated in the outer layers of fruits and vegetables, i.e. the skins due to the biosynthesis of flavonoids by sunlight (ultraviolet radiation). This group of polyphenols is also responsible for the antioxidant capacity of fruit and vegetable extracts (Mäkilä et al., 2016).

3.3.3 Effect of different drying methods on the extractability of free anthocyanins and other phenolic compounds

The effect of drying on the yield of free anthocyanin and other phenolics was investigated, using fresh blackcurrant skins that were dried under different drying processes. The temperature of the drying air, moisture content, flow rate, direction of the drying air, and drying period are among the most important factors that influenced the drying process. The DBS from industrial rotary dryer was sourced from a commercial supplier that performs the drying process at gradient temperatures. Generally, the temperature in the rotary dryer

increase at the beginning of drying process and gradually decreased and maintain constant for a longer period (Hui et al., 2006). This is due to the rapid reduction of temperature by evaporation of freely available moisture at the surface of the matrix. However, this fast reduction of temperature is known not to be equal across particle sizes, but possibly proportional to the log of particle radius. For instance, the hot air in the rotary dryer fluctuating from 25 ℃, experiences a quick rise of in-rush 'air-on' of up to 450 ℃, and an 'air-off' temperature of around 97 ℃. The best temperature of 'air off' is thought to be correlated to the maximum yield of anthocyanin extracted from DBS particle.

Furthermore, freeze drying involves the dehydration process at low temperature (-45.0 ± 1.0 ℃) in high vacuum which leads to the removal of water in the form of ice by sublimation (Ratti, 2001). On the other hand, laboratory ovens generally apply uniform drying temperatures throughout the drying process, and at some point, overheat smaller particles. Among these three drying processes, industrially dried skins with moisture content of ~8.1% had the highest free anthocyanin content, accounting for 17.9 ± 0.4 mg/g (**Table 3-2**)**.** Among dried DBS samples from laboratory oven, those dried at 90 ℃ for 30 min with moisture content of ~3.6% had the highest free anthocyanin content (13.3 ± 0.5 mg/g). This can be explained by the fact that gradient temperatures and different residence times in the rotary dryer affected the contact between the cascading particles and hot air, whereas skins of all particle sizes had appropriate exposure to heat. However, it is possible that the particles which spent as little as 10-20 seconds in the hot air stream, may have endured higher temperatures than the 'air-off' temperature. Therefore, although particles of smaller size spent shorter time in the rotary dryer which prevented overheating of the skins, in fact they may have been also subjected to overheating.

As shown in **Table 3-2**, DBS that were dried at 70 ℃, 80 ℃, and 90 ℃ for 15 min in the laboratory oven contained more than ~14.9% of moisture and had lower free

anthocyanin content. However, as the temperature increased to 120 °C and the drying process went on for 30 min, the anthocyanin content decreased to 10.7 ± 0.3 mg/g, due to the thermal sensitivity of anthocyanins. Also, freeze drying resulted in relatively lower anthocyanin content (11.8 ± 0.7 mg/g), suggesting that a suitable thermal drying is required for an efficient anthocyanin extraction.

According to **Table 3-2,** DBS that were dried at 80 °C and 90 °C for 15 min in a laboratory oven contained significantly high ($p \leq 0.05$) amounts of total hydroxycinnamic acid (2.0 ± 0.0 mg/g and 2.1 ± 0.0 mg/g, respectively). However, different drying processes had no actual effect on flavonol content as presented in **Table 3-2**. Overall, higher total free anthocyaninns in skins dried using industrial rotary dryer also led to a higher total phenolic content (19.9 ± 0.5 mg/g) compared to laboratory oven and freeze dried samples. Based on the Pearson correlation results, moisture content showed an inversely weak correlation ($p \leq 0.05$) with total free anthocyanins and total phenolic content (R^2= -0.503 and R^2 = -0.448, respectively). These linked to the fact that the decrease of the moisture content in some DBS samples caused an increase in total free anthocyanin and phenolic content.

Table 3-2. Total free anthocyanins (mg/g), other phenolics (mg/g) and moisture content (%, w/w) of DBS using industrial rotary dryer, freeze dryer and laboratory oven.

DBS	Total Free ATC (mg/g)	Total HCA (mg/g)	Total Flavonols (mg/g)	*Total Phenolics (mg/g)	Moisture content (%)
Industrial rotary drying (315)	17.9 (0.4)^{a}	1.4 (0.0)^{i}	0.7 (0.0)^{a}	19.9 (0.5)^{a}	8.1 (0.2)^{e}
Freeze drying	11.8 (0.7)^{defg}	1.7 (0.0)^{ef}	0.7 (0.1)^{a}	14.2 (0.7)^{def}	8.4 (0.0)^{e}
Oven 70 °C-15 min	8.9 (0.0)^{i}	1.9 (0.0)^{b}	0.6 (0.0)^{a}	11.5 (0.0)^{h}	19.2 (0.8)^{a}
Oven 70 °C-30 min	11.1 (0.0)^{fgh}	1.6 (0.0)^{g}	0.7 (0.0)^{a}	13.4 (0.0)^{fg}	7.7 (0.1)^{e}
Oven 70 °C-45 min	12.3 (0.5)^{bcde}	1.8 (0.0)^{cd}	0.7 (0.0)^{a}	14.8 (0.5)^{bcd}	4.7 (0.0)^{g}
Oven 80 °C-15 min	10.9 (0.0)^{gh}	2.0 (0.0)^{a}	0.7 (0.0)^{a}	13.6 (0.0)^{efg}	17.7 (0.0)^{b}
Oven 80 °C-30 min	12.9 (0.0)^{bc}	1.8 (0.0)^{bc}	0.7 (0.0)^{a}	15.5 (0.0)^{abc}	6.3 (0.1)^{f}
Oven 90 °C-15 min	11.6 (0.1)^{efgh}	2.1 (0.0)^{a}	0.7 (0.0)^{a}	14.4 (0.0)^{cdef}	14.9 (0.2)^{c}
Oven 90 °C-30 min	13.3 (0.5)^{ab}	1.9 (0.0)^{b}	0.7 (0.0)^{a}	15.9 (0.5)^{ab}	3.6 (0.1)^{h}
Oven 100 °C-15 min	12.0 (0.1)^{cdef}	1.8 (0.0)^{bc}	0.7 (0.0)^{a}	14.6 (0.1)^{cdef}	9.6 (0.1)^{d}
Oven 100 °C-30 min	12.3 (0.1)^{bcde}	1.7 (0.0)^{f}	0.7 (0.0)^{a}	14.7 (0.1)^{cde}	2.3 (0.1)^{i}
Oven 110 °C-15 min	12.8 (0.1)^{bcd}	1.8 (0.0)^{bc}	0.7 (0.0)^{a}	15.4 (0.1)^{abc}	6.3 (0.2)^{gh}
Oven 110 °C-30 min	11.7 (0.2)^{efgh}	1.8 (0.0)^{de}	0.7 (0.0)^{a}	14.2 (0.2)^{def}	1.7 (0.0)^{ij}
Oven 120 °C-15 min	11.5 (0.1)^{efgh}	1.7 (0.0)^{ef}	0.7 (0.0)^{a}	14.0 (0.1)^{defg}	4.5 (0.2)^{f}
Oven 120 °C-30 min	10.7 (0.3)^{h}	1.5 (0.0)^{h}	0.7 (0.0)^{a}	12.9 (0.4)^{g}	1.2 (0.1)^{j}

*DBS: Dried blackcurrant skins; ATC: Anthocyanins; HCA: Hydroxycinnamic acid. * Total phenolics is the sum of total free anthocyanins, HCA and flavonols.
Error bars indicate ± standard deviation (SD). Values with the same letter ^{a,b} in each row are not significantly different ($p > 0.05$).

3.3.4 Effect of drying parameters on the extractability of free anthocyanins and other phenolic compounds during industrial rotary drying

Fresh blackcurrant skins underwent drying under various processing parameters in the industrial rotary dryer such as differences in the 'air-on' and 'air off' temperatures and in the ratio of rotary drum speed to air speed, which directly influenced the free anthocyanin and phenolic content in DBS, as summarised in **Table 3-1**. According to the results in **Table 3-3**, DBS 111-16 had significantly high ($p \leq 0.05$) free anthocyanin content (22.1 ± 0.5 mg/g), followed by DBS 915 (21.6 ± 1.1 mg/g) and 132-16 (20.4 ± 0.5 mg/g). Meanwhile, DBS 240, 245, 250 and 255 consisted of significantly low ($p \leq 0.05$) anthocyanin content, ranging between ~8.5 to ~9.7 mg/g. Furthermore, other than total free anthocyanins, DBS 111-16 also contained high concentrations of total hydroxycinnamic acids (1.4 ± 0.0 mg/g) and flavonols (0.8 ± 0.0 mg/g), which summed up to a high total phenolic content (24.3 ± 0.5 mg/g).

The results indicated that depending on the density and size of the skin particles, an increase in the drum and hot air speed in the DBS 111-16, 915 and 132-16 shortened the residence time within the drying drum and allowed an efficient heat transfer. These physical parameters inhibited the overheating of the particle, resulting in a high free anthocyanin and phenolic content. Furthermore, 'air on' temperature has the greatest effect towards the end moisture content of DBS and 'air off' temperature (Iguaz et al., 2003). For instance, according to **Table 3-3**, higher 'air on' temperature (475 °C) in DBS 111-16 provided a great amount of heat to vaporize the water in the skins. The skins' temperature only rose at the beginning of the drying process. The heat transfer decreased as the skins conveyed through the dryer. Then, the temperature started to drop as the skins stopped heating further until the 'air off' (97 °C).

According to Pearson correlation, an inversely weak correlation ($p \leq 0.05$) was noted between total free anthocyanins, total phenolics and moisture content (R^2 = -0.555 and R^2 = -0.551), suggesting that the decrease in moisture content in some DBS samples resulted in the increase of total free anthocyanins and phenolic content.

Table 3-3. Total free anthocyanins (mg/g), other phenolics (mg/g) and moisture content (%, w/w) in DBS from industrial rotary drying dried at various drying parameters.

DBS	Total Free ATC (mg/g)	Total HCA (mg/g)	Total Flavonols (mg/g)	*Total Phenolics (mg/g)	Moisture content (%)
315	17.9 (0.4)^{b}	1.4 (0.0)^{ab}	0.7 (0.0)^{ab}	19.9 (0.5)^{c}	8.1 (0.2)^{b}
915	21.6 (1.1)^{a}	1.1 (0.0)^{f}	0.7 (0.0)^{b}	23.4 (1.1)^{ab}	7.7 (0.1)^{b}
111-16	22.1 (0.5)^{a}	1.4 (0.0)^{a}	0.8 (0.0)^{a}	24.3 (0.5)^{a}	8.8 (0.1)^{a}
132-16	20.4 (0.5)^{a}	1.3 (0.0)^{d}	0.7 (0.1)^{ab}	22.3 (0.6)^{b}	8.0 (0.2)^{b}
1611	13.6 (0.1)^{c}	1.3 (0.0)^{c}	0.7 (0.0)^{ab}	15.7 (0.0)^{d}	7.6 (0.1)^{b}
240	9.7 (0.4)^{d}	1.3 (0.0)^{bc}	0.7 (0.0)^{b}	11.7 (0.3)^{e}	8.7 (0.0)^{a}
245	8.9 (0.1)^{d}	1.2 (0.0)^{e}	0.7 (0.0)^{b}	10.8 (0.1)^{e}	8.7 (0.1)^{a}
250	8.5 (0.0)^{d}	1.2 (0.0)^{e}	0.7 (0.0)^{b}	10.4 (0.0)^{e}	9.0 (0.2)^{a}
255	9.6 (0.1)^{d}	1.3 (0.0)^{d}	0.7 (0.0)^{b}	11.6 (0.1)^{e}	9.0 (0.1)^{a}

*DBS: Dried blackcurrant skins contained mixtures of all particle sizes at a ratio of 15: 43: 41 (w/w/w); ATC: Anthocyanins; HCA: Hydroxycinnamic acid.
*Total phenolics is the sum of total free anthocyanins, HCA and flavonols. Error bars indicate ± standard deviation (SD). Values with the same letter ^{a,b} in each row are not significantly different ($p > 0.05$).

3.3.5 Effect of industrial drum drying parameters on the extractability of free anthocyanin and other phenolic compounds as function of particle size

DBS samples coded 315 and 915 were dried using different industrial drying parameters as shown in **Table 3-1**. After the drying process, skins were separated according to the particle sizes of < 800 μm, > 5mm and < 5 mm. The 'mix' DBS shows mixtures of all particle sizes at a ratio of 15: 43: 41 (w/w/w). The DBS particle sizes as well as the drying parameters are thought likely to have an effect on the anthocyanin and other phenolics content. According to **Table 3-4**, significantly high ($p \leq 0.05$) total free anthocyanins was detected in skins with particle size >5 mm (31.6 ± 0.2 mg/g) from DBS 915, followed by

DBS 315 (24.1 ± 0.3 mg/g). Besides, DBS 915 also showed significantly high ($p \leq 0.05$) total free anthocyanins in < 800 µm and < 5 mm particle size distribution; the same trend was seen in the mixed sample.

According to the data presented in **Table 3-4,** total hydroxycinnamic acid was highest ($p \leq 0.05$) in DBS 315 (mix) (1.4 ± 0.0 mg/g). Moreover, high total flavonols were found in DBS samples of particle size >5 mm (coded as 315 and 915). The highest content of total phenolics which consisted of total free anthocyanins, hydroxycinnamic acids and flavonols in DBS 915 with the particle size > 5 mm and < 5 mm were 33.7 ± 0.2 mg/g and 24.4 ± 0.2 mg/g, respectively (**Table 3-4**).

Table 3-4. Total free anthocyanins (mg/g), other phenolics (mg/g) and moisture content (%, w/w) in different particle sizes of DBS from industrial rotary drying dried under various drying parameters.

DBS	Total Free ATC (mg/g)	Total HCA (mg/g)	Total Flavonol (mg/g)	*Total Phenolics (mg/g)	Moisture content (%)
>5 mm-315	24.1 (0.3)^{b}	1.1 (0.0)^{d}	0.8 (0.0)^{a}	26.0 (0.3)^{b}	8.3 (0.1)^{b}
<5 mm-315	18.5 (0.2)^{d}	1.1 (0.0)^{d}	0.8 (0.0)^{ab}	20.4 (0.2)^{d}	8.8 (0.1)^{a}
<800 µm-315	12.7 (0.3)^{f}	1.1 (0.0)^{e}	0.6 (0.0)^{c}	14.4 (0.3)^{f}	7.0 (0.1)^{e}
315 (Mix)	17.9 (0.4)^{d}	1.4 (0.0)^{a}	0.7 (0.0)^{bc}	19.9 (0.5)^{d}	8.1 (0.2)^{bc}
>5 mm-915	31.6 (0.2)^{a}	1.2 (0.0)^{b}	0.8 (0.0)^{a}	33.7 (0.2)^{a}	7.6 (0.0)^{d}
<5 mm-915	22.4 (0.2)^{bc}	1.2 (0.0)^{c}	0.8 (0.0)^{ab}	24.4 (0.2)^{ab}	7.8 (0.1)^{cd}
<800 µm-915	14.6 (0.2)^{e}	1.1 (0.0)^{e}	0.5 (0.0)^{d}	16.2 (0.2)^{e}	7.9 (0.2)^{bcd}
915 (Mix)	21.6 (1.1)^{c}	1.1 (0.0)^{d}	0.7 (0.0)^{c}	23.4 (1.1)^{c}	7.7 (0.1)^{cd}

*DBS: Dried blackcurrant skins from industrial rotary drying; Mix: Dried blackcurrant skins contained mixtures of all particle sizes at a ratio of 15: 43: 41 (w/w/w); ATC: Anthocyanins; HCA: Hydroxycinnamic acid.

*Total phenolics is the sum of total free anthocyanins, HCA and flavonols. Error bars indicate ± standard deviation (SD). Values with the same letter ^{a,b} in each row are not significantly different ($p > 0.05$).

Even though the > 5mm and < 5 mm particle size fractions in DBS 315 had endured a much higher residence time (decreased ratio of air to drum speed) compared to DBS 915, they had reserved higher moisture content within the particle mass. To support this evidence, moisture content had a significant correlation ($p \leq 0.05$) with total free anthocyanins and phenolic content in all particle sizes of DBS 315 ($R^2 = 0.711$ and $R^2 = 0.717$, respectively). This fact suggested that skin particles with higher moisture content also had higher amounts of free anthocyanins and phenolics.

In DBS 915, the increased in the 'air in' temperature and ratio of drum speed to air speed allowed bigger particle sizes (e.g. >5 mm and <5 mm) to undergo efficient mass and heat transfer compared to small particles (<800 μm) which might have been overheated. Furthermore, it was thought likely that smaller particles were unable to reduce their temperature by offering up moisture to the heating air, due to the fact that the moisture had already left the surface of the particle. The residual temperature of the smaller particles therefore might have risen higher than the 'air-off' temperature of the average of the product in the process. Based on the Pearson correlation, moisture content in DBS 915 was inversely correlated with total free anthocyanins and phenolic content ($R^2 = -0.774$ and $R^2 = -0.773$), indicating that higher content of phenolic compounds were found in the skin particle with lower moisture content.

3.3.6 Effect of drying method on the extractability of total phenolic compounds and antioxidant activity

Drying represents a processing factor that influences not only total free anthocyanins, but also total phenols and inevitably the antioxidant activity of the processed sample. As

shown in **Figure 3-3**, DBS samples processed in the industrial rotary drier contained significantly high ($p \leq 0.05$) total free anthocyanins (**Table 3-2**), but significantly low ($p \leq 0.05$) total phenols content and antioxidant activity, compared to DBS extracts from laboratory oven and freeze dryer. Similarly, Spigno et al. (2007) reported that freeze drying did not lead into the degradation of total phenolic compounds and into reduction of antioxidant activity in grape marc. The dehydration by sublimation at low temperatures inhibits the deterioration of food product and slows down significantly the rate of microbiological reactions, leading into an excellent quality of the final product. Besides, minimal shrinkage (from 5% to 15% for berries) and negligible collapse (less than 10%) can be seen during the solid state of water during freeze drying process (Ratti, 2001; Argyropoulos et al., 2011).

Furthermore, other than phenolic compounds, the overheating of samples in the laboratory oven might lead to the extraction of reducing sugars, proteins and organic acids which can also react with the Folin-Ciocalteu reagent (Kapasakalidis et al., 2006; Everette et al., 2010). This can be seen from the correlation results whereby very poor and statistically insignificant ($p < 0.05$) correlations were observed between free anthocyanins and total phenolics. Moreover, total free anthocyanins were inversely weakly correlated with antioxidant activity ($R^2 = -0.404$), which suggests that DBS extracts with low free anthocyanins also contributed towards high antioxidant activity. However, the Pearson correlation between the total phenolic content and the antioxidant activity in the obtained DBS extracts was strong, with $R^2 = 0.971$ ($p \leq 0.05$), due compounds other than free anthocyanins in extracts from DBS dried in laboratory oven and freeze drying which were also most likely responsible for the high antioxidant activities. Besides, the release of

compounds from ruptured skins cells during freeze drying process was shown to contribute to high antioxidant activity of Rabbiteye blueberry extracts, as reported by Vuthijumnok et al. (2013).

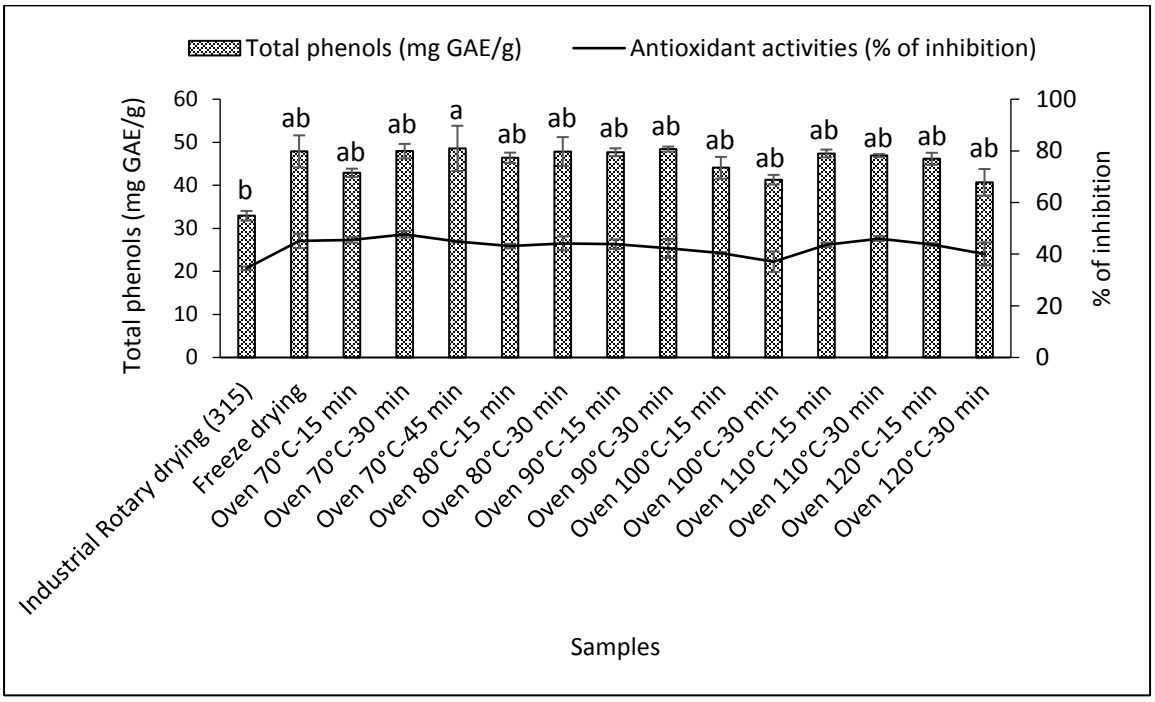

Figure 3-3. Total phenols (mg GAE/g) and antioxidant activity (% inhibition) of DBS dried using industrial dryer, freeze dryer and laboratory oven. Error bars indicate ±standard deviation (SD).

According to the **Table 3-3**, DBS that were dried industrially under different parameters had high amounts of total free anthocyanins (DBS 315, 915, 111-16, 132-16 and 16-11) as well as high total phenols and antioxidant activity (**Figure 3-4**). The Pearson correlation between the total free anthocyanin content and the total phenols in the obtained DBS extracts was weak, with $R^2 = 0.463$ ($p \leq 0.05$). Apart from anthocyanins, ~50% of total phenols consisted of non-phenolics and other compounds such as hydroxycinnamic acids (*p*-coumaric, ferulic and caffeic acids) and flavonols (quercetin, myricetin and kaempferol) that also reacted with the Folin-Ciocalteu reagent. However, the correlation between the

antioxidant activity with total free anthocyanins and total phenols were R^2 = 0.708 and R^2 = 0.854, respectively. It is apparent that the high total free anthocyanin content in DBS extracts was most likely responsible for the high antioxidant activities of the extracts, in line with the report by Kapasakalidis et al. (2006) in the extraction of polyphenols from blackcurrant residues. Also, Kähkönen and Heinonen (2003) also suggested that anthocyanins and their aglycones are powerful antioxidants and possess high antioxidant activity.

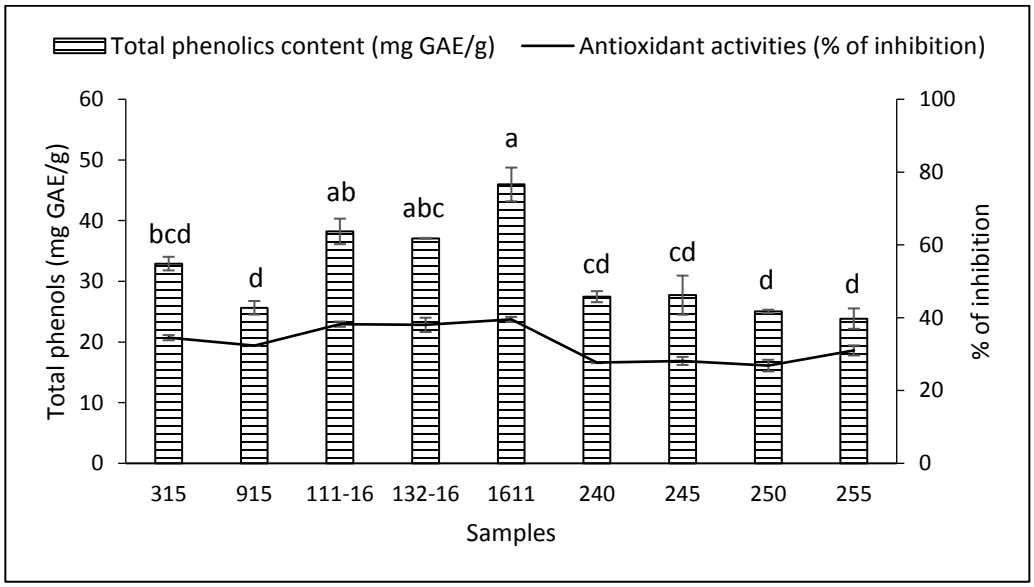

Figure 3-4. Total phenols (mg GAE/g) and antioxidant activity (% inhibition) of DBS dried industrially under different drying parameters.

Samples of DBS with varying particle size distribution of DBS contained different total free anthocyanin contents (**Table 3-4**) and total phenols (**Figure 3-5**) due to the exposure of surface area to the hot air in the rotary drier. Overall, particle size of DBS 915 samples with particle diameter > 5 mm and < 5 mm, not only had the highest total free anthocyanins (**Table 3-4**) but also the highest phenol content (**Figure 3-5**), followed by <5 mm particle from DBS 315. Total phenols measured using Folin-Calcioteu method

indicated very poor correlation with total free anthocyanins and total phenolics as determined by HPLC. However, the total phenols were well correlated to antioxidant activity (R^2 = 0.718). This is linked to the fact that compounds such as proteins were also present in the DBS 315 and 915 extracts that reacted with the Folin-Ciocalteu reagent and were responsible for their high antioxidant activity.

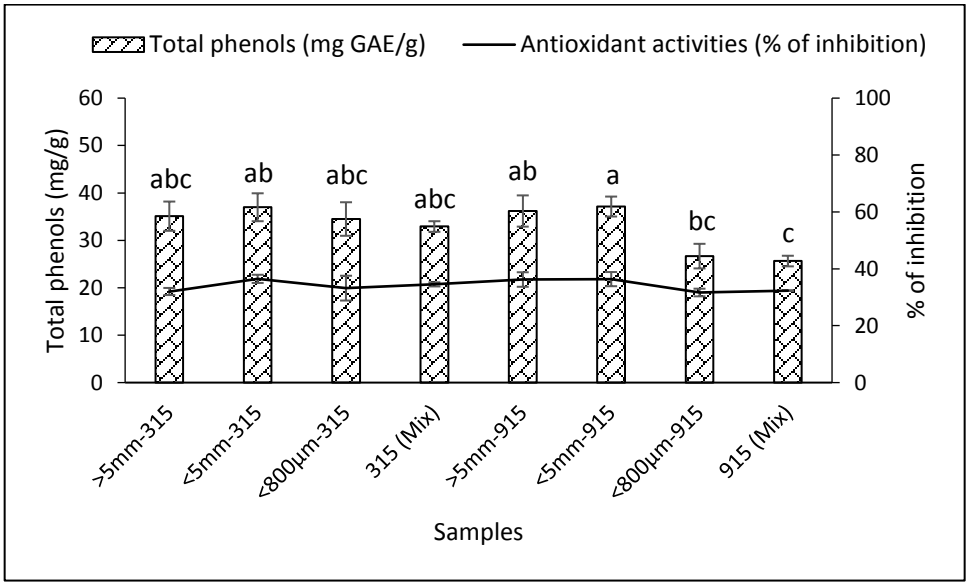

Figure 3-5. Total phenols (mg GAE/g) and antioxidant activity (% inhibition) in various particle size distribution fractions of DBS that underwent different drying process.

3.4 Conclusions

In this study, it was demonstrated that gradient temperatures in the rotary dryer are more efficient in protecting free anthocyanins in blackcurrant skins compared to drying using freeze dryer and laboratory oven, under constant temperature. However, hydroxycinnamic acids were best extracted in the DBS from laboratory oven, whilst flavonols were not influenced by the drying process. During the drying process in the industrial rotary dyer, the free anthocyanin and phenolic content of DBS extracts were affected by various factors such as the 'air-on', 'air off', gradient of temperature, ratio of rotary drum speed to air speed and particle size of blackcurrant skins. Among these parameters, the increase in the 'air on' temperature and the ratio of drum speed to air speed were the best conditions for the efficient extraction of phenolics and especially of free anthocyanins. Moreover, the exposure to high temperatures for longer time in rotary dryer can potentially damage the smaller particles as opposed to the larger ones, leading into degradation of phenolics compounds including free anthocyanins, as well as the antioxidant activity. Apart from free anthocyanins, laboratory oven and freeze drying processes were deemed efficient for the extraction of other phenolic and non-phenolic compounds with high antioxidant activity. In the next chapter, a representative DBS (Sample 315) from industrial rotary drying was used for further investigation on the extraction of anthocyanins and total phenolics content.

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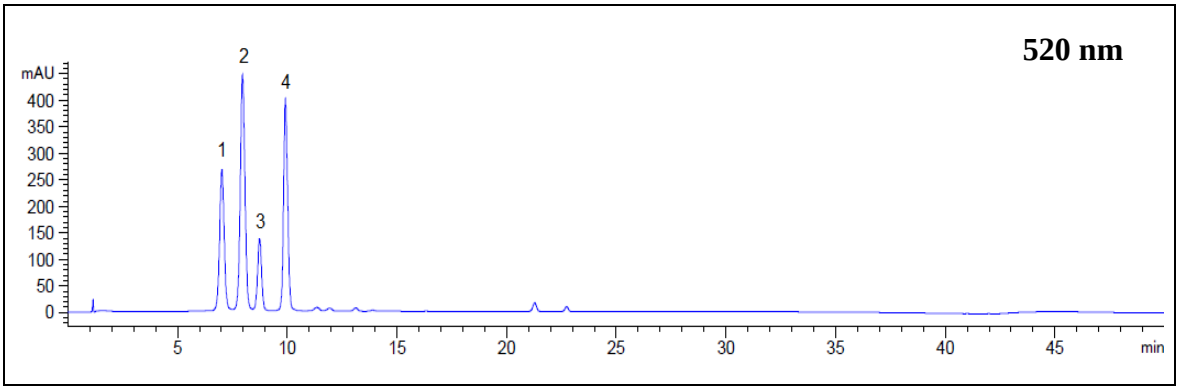
Supplementary material

Supplementary Table 3-5. Typical free anthocyanin content (in mg/g dry weight) found in DBS extract.

Free Anthocyanins	**Concentration (mg/g dry weight)**	**Percentage (%)**
Delphinidin-3-O-glucoside	6.4 ± 0.0[c]	20.2 ± 0.0[c]
Delphinidin-3-O-rutinoside	16.3 ± 0.2[a]	51.4 ± 0.1[a]
Cyanidin-3-O-glucoside	2.0 ± 0.0[d]	6.18 ± 0.0[d]
Cyanidin-3-O-rutinoside	7.0 ± 0.1[b]	22.2 ± 0.1[b]
Total free anthocyanins	31.6 ± 0.2	100.0 ± 0.0

Values with the same letter [a,b] in each row are not significantly different ($p > 0.05$)

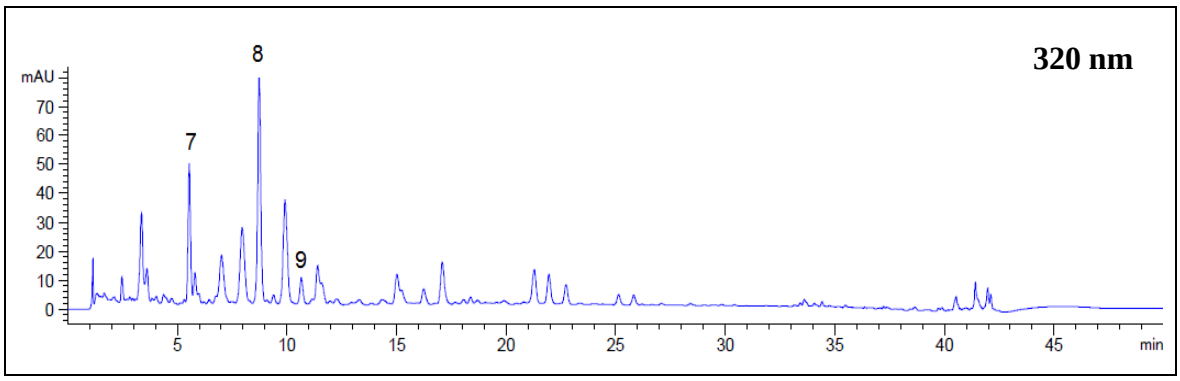


Supplementary Figure 3-6. Typical HPLC chromatograms of DBS extract showing the detected free anthocyanins at 520 nm. (A) Delphinidin-3-O-glucoside, (B) Delphinidin-3-O-rutinoside, (C) Cyanidin-3-O-glucoside and (D) Cyanidin-3-O-rutinoside.

Supplementary Table 3-6. Typical hydroxycinnamic acid content (in mg/g dry weight) found in DBS extract.

Hydroxycinnamic acid	**Concentration (mg/g dry weight)**	**Percentage (%)**
Caffeic acid	0.6 ± 0.0[b]	27.0 ± 0.1[b]
p-Coumaric acid	1.0 ± 0.1[a]	48.0 ± 0.1[a]
Ferulic acid	0.5 ± 0.0[c]	25.0 ± 0.0[c]
Total hydroxycinnamic acids	2.1 ± 0.1	100 ± 0.0

Values with the same letter [a,b] in each row are not significantly different ($p > 0.05$)

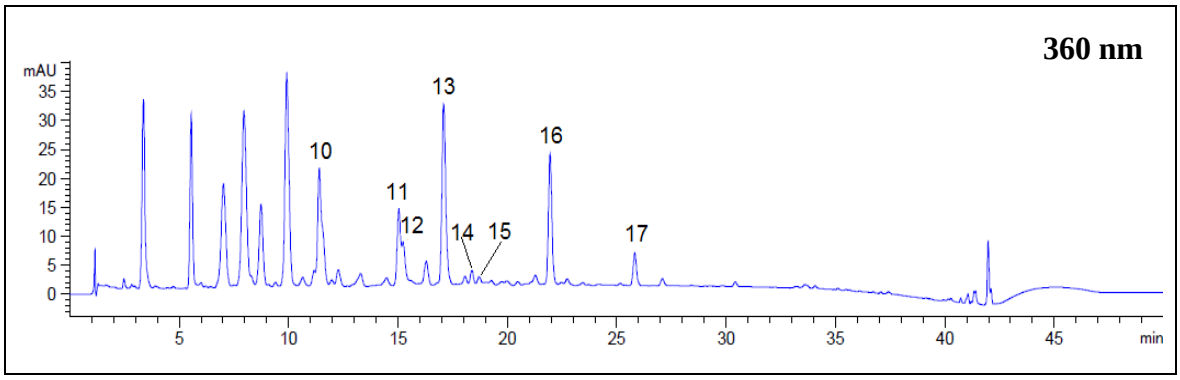


Supplementary Figure 3-7. Typical HPLC chromatograms of DBS extract showing the detected hydroxycinnamic acid at 320 nm. (7) Caffeic acid, (8) *p*-Coumaric acid and (9) Ferulic acid.

Supplementary Table 3-7. Typical flavonol content (in mg/g dry weight) found in DBS extract.

Flavonols	**Concentration (mg/g dry weight)**	**Percentage (%)**
Myricetin-3-O-glucoside	0.1 ± 0.0[c]	14.4 ± 0.1[c]
Quercetin-3-O-glucoside	0.1 ± 0.0[d]	8.0 ± 0.1[d]
Quercetin-3-O-rutinoside	0.1 ± 0.0[d]	8.4 ± 0.0[d]
Myricetin	0.3 ± 0.0[a]	36.9 ± 0.4[a]
Kaempferol-3-O-glucoside	0.0 ± 0.0[e]	4.1 ± 0.0[e]
Kaempferol-3-O-rutinoside	0.0 ± 0.0[f]	2.0 ± 0.1[f]
Quercetin	0.2 ± 0.0[b]	20.8 ± 0.2[b]
Kaempferol	0.0 ± 0.0[e]	5.4 ± 0.1[e]
Total flavonols	0.8 ± 0.0	100 ± 0.0

Values with the same letter [a,b] in each row are not significantly different ($p > 0.05$)



Supplementary Figure 3-8. Typical HPLC chromatograms of DBS extract showing the detected flavonols at 360 nm (10) Myricetin 3-O-glucoside, (11) Quercetin 3-O-glucoside, (12) Quercetin 3-O-rutinoside, (13) Myricetin, (14) Kaempferol-3-O-glucoside, (15) Kaempferol-3-O-rutinoside (16) Quercetin and (17) Kaempferol.

CHAPTER 4 – Extraction of anthocyanins and phenolics from dried blackcurrant (*Ribes nigrum* L.) skins

Abstract

The aim of this study was to develop a process for the extraction of anthocyanins and phenolics from dried blackcurrant skins (DBS) and investigate the stability and antioxidant activity of the extracts. Water, organic solvents (methanol and ethanol), mixtures of methanol/water and acetic acid buffer solutions were employed and the effects of solvent, temperature, time and pH on the anthocyanin extraction yield was assessed. Acetic acid buffer (pH 1.5) resulted in the highest free anthocyanin and total phenolic content [16.6 mg/g and 37.0 mg gallic acid equivalent (GAE)/g, respectively] after 2 h extraction at 50 °C from DBS amongst all solvents. Delphinidin-3-O-rutinoside and *p*-coumaric acid were the main types of free anthocyanins and hydroxycinnamic acids in all extracts. Acetic acid buffer extracts exhibited high antioxidant activity according to the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (~60.1% inhibition for extracts containing 37.0 mg GAE/g of total phenolics), not only due to the presence of phenolic compounds, but most likely due to other non-phenolic compounds. With the exception of water, all other solvents showed no difference in the total colour difference (TCD) for up to 6 h of extraction, indicating the colour stability of the extracts. However, very poor correlation was observed between the total free anthocyanin content and TCD. Overall, this work demonstrated that the acetic acid buffer was the best among the other solvents used for extracting anthocyanins and phenolic compounds from DBS, resulting in extracts with high antioxidant activities and high colour intensity and stability.

Keywords: Dried blackcurrant skins, anthocyanins, extraction, antioxidant activity, acetic acid buffer

4.1 Introduction

Blackcurrant (*Ribes nigrum* L.) is recognised as a temperate soft fruit crop in Europe, Russia, Northern Asia, New Zealand and North America (Battino et al., 2009). Data from 2017 reported that the commercial production in United Kingdom covers 2,400 hectares which producing around 11,000 tonnes of blackcurrant per year (IBA, 2018). Due to their high content of anthocyanins and proanthocyanins, blackcurrant can be used as natural colourants and preservatives, as well as a source of ascorbic acid (~180 mg/100g of berries) and phenolic compounds (500 – 1342 mg/100g of berries); the latter have a wide range of health-promoting properties in humans (Brennan & Graham, 2009). The documented biological activities of phenolic compounds such as anthocyanins, procyanidins, flavonols and phenolic acids contributed to their high antioxidant activities (Szajdek & Borowska, 2008). The polyphenol profile of the blackcurrant fruit consisting of anthocyanins, flavonoids, hydroxycynnamic acids, *p*-coumaric acid, myrcetin, quercetin, kaempferol glycosides and isorhamnetin (Sójka & Król, 2009). Apart from their consumption as fresh fruits, blackcurrants are mainly cultivated for juice and beverage production. In the UK, 75% of the total fresh blackcurrant production is processed into juice (Vagiri, 2014). Blackcurrants are also processed to produce a range of functional ingredients, such as blackcurrant-pomace dietary fibres and defatted seeds, which can be incorporated into jams, jellies, purées and teas (Varming et al., 2004).

The by-products of blackcurrant juice-pressing process containing mainly of skins, seeds and stems. However, only a small percentage of these by-products is recycled or upgraded, while the majority is used as animal feed (although there are limitations in this due to their high acidity and rancid taste) and composting material or is disposed through alternative routes (e.g. land spreading) (Arvanitoyannis, 2010). Taking into account the fact that most phenolic compounds are accumulated in the skin of fruits (Mäkilä et al., 2016), an

additional value chain can be created through the extraction of phenolic compounds from these by-products. Many studies have highlighted that extracts containing phenolic compounds could be potentially used as functional food ingredients including as antioxidants and preservatives (Basegmez et al., 2017). This is in line with the fact that natural antioxidants such as tocopherol and tocotrienols, ascorbic acid, carotenoids and phenolic compounds can been used as alternatives to chemically synthesised antioxidants such as butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), tert-butylhydroquinone (TBHQ) and propyl gallate (PG) (Puganen et al., 2018).

There is no uniform or standardised procedure that is suitable for isolation and identification of polyphenols including anthocyanins. The initial steps to extract phenolic compounds from plant materials such as berries include grinding, drying and soaking of the samples in extraction solvents such as water, organic solvents and acids (Anderson & Markham, 2005). In order to reduce the possibility of anthocyanin degradation due to chemical reactions taking place in the wet material, dried plant materials are normally used as starting materials for anthocyanin extraction (Harbourne et al., 2013). Several conventional method employing organic solvent (e.g. ethanol, methanol) containing small amounts of acid (e.g., hydrochloric acid, formic acid) are efficient for the extraction of anthocyanins from berries (Harbourne et al., 2013).

In the extraction of anthocyanins from grape pulps, methanol was the best extractant being 20% more effective than using ethanol and 73% more effective than only water (Ignat et al., 2011). Anthocyanins are soluble in polar solvents; therefore, polar solvents such as aqueous mixtures of methanol, ethanol or acetone are often employed for their extraction (Kähkönen et al., 2001). Also, since water can extract more polar compounds, and ethanol or methanol can extract more hydrophobic compounds, the ratio of the water and methanol or ethanol mixture can be adjusted according to the polarity and solubility of anthocyanins

(Lapornik et al., 2005). Moreover, extraction using acidic aqueous solvents have been commonly used as an extractant in order to disrupt the cell membrane as well as dissolve the water-soluble anthocyanins (Rodriguez-Saona & Wrolstad, 2001; Amr & Al-Tamimi, 2007).

This study aimed to investigate the effect of the process conditions on the extraction of anthocyanins, hydoxycinnamic acids and flavonols from dried blackcurrant skins (DBS) and the properties of the extracted phenolic compounds. To this end, various solvents and pH conditions were employed and the obtained extracts were characterised in terms of their composition, antioxidant activity, as well as colour stability.

4.2 Materials and methods

4.2.1 Chemicals

All solvents and chemicals used for extraction, including methanol (99.9%), ethanol (99.8%), formic acid (95%) and acetic acid (99.7%), were of analytical grade and were purchased from Sigma-Aldrich (UK). Folin-Ciocalteu reagent, sodium carbonate, potassium chloride, sodium acetate, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) were also purchased from Sigma-Aldrich (UK). Hydrochloric acid (37%) was purchased from Fisher Scientific (Loughborough, UK).

A stock solution of 2 mM DPPH was prepared in methanol. Buffer solutions of pH 1.0 (potassium chloride, 0.025M) and pH 4.5 (sodium acetate, 0.4M) were prepared as described by Lee et al. (2005). Purified water was used in all preparations, obtained by a Purite reverse osmosis system (Oxon, UK). Anthocyanin standards of cyanidin-3-O-glucoside (96%), cyanidin-3-O-rutinoside (96%), delphinidin-3-O-glucoside (95%), and delphinidin-3-O-rutinoside (95%), cyanidin (96%), delphinidin (96%), kaempferol-3-O-glucoside (99%), kaempferol-3-O-rutinoside (98%), quercetin-3-O-rutinoside (99%) and myricetin-3-O-glucoside (99%) were obtained from ExtraSynthese Ltd (Genay, France). In

addition, quercetin (95%), myricetin (98%), kaempferol (99%) and quercetin-3-O-glucoside (98%) were purchased from Sigma-Aldrich (UK).

4.2.2 Sample preparation of plant materials

Dried blackcurrant pressed residues derived from a juice manufacturing process were kindly supplied by Purn House Farm, (Bleadon, Weston-super-Mare, UK) in March 2015. Dried blackcurrant used was a representative sample (315) from industrial rotary drying with high anthocyanins content as shown in Chapter 3. Dried blackcurrants were separated from the seeds by grinding in a coffee blender and passed through a 0.841 mm (20 mesh) sieve; this constituted the dried blackcurrant skins (DBS) sample. Samples were segregated in polyethylene bags and stored at -20 °C until further analysis.

4.2.3 Preparation of dried blackcurrant skins (DBS) extracts and total monomeric anthocyanin analysis

DBS (2.5 g) were extracted with 25 mL solvents [100% organic solvent (methanol and ethanol), 100% water, or mixtures of methanol-water (20%, 35%, 50%, and 70%, v/v)] in a shaking water bath at 30 °C, 50 °C, 70 °C, and 90 °C. The duration of the extraction was 6 h. The obtained extracts were filtered using a vacuum filter to remove the solid residues. The total monomeric anthocyanin content of DBS was determined by the differential pH method based on the property of anthocyanins to change colour at different pH values (Lee et al., 2005). 600 µL of anthocyanins extracts were mixed with 1.2 mL of corresponding buffer (pH 1.0 and pH 4.5) and allowed to equilibrate for 20 minutes. The total monomeric anthocyanin (mg cyanidin-3-O-glucoside equivalents/g dry weight) was calculated as follows:

$$C_{anthocyanin} = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times 1}$$

where $C_{anthocyanin}$ = Total monomeric anthocyanin concentration (cyanidin-3-O-glucoside equivalents, mg/L); A (absorbance) = ($A_{520nm} - A_{700nm}$) pH 1.0 – ($A_{520nm} - A_{700nm}$) pH 4.5; MW (molecular weight) = 449.2 g/mol for cyanidin-3-O-glucoside; DF = dilution factor; l = pathlength in cm; ε = 26900 molar extinction coefficient in L/mol/cm for cyanidin-3-O-glucoside; and 10^3 = factor for conversion from g to mg (Lee et al., 2005). The anthocyanin content was then calculated by:

$$Anthocyanin\ content(\frac{mg}{g}of\ dry\ weight) = \frac{C\ anthocyanin\ (\frac{mg}{L}) \times extract\ (L)}{sample\ (g)}$$

The absorbance of the samples at 520 nm and 700 nm was determined using a spectrophotometer (Thermo Electron Corporation, USA). The extractions were carried out in duplicate.

4.2.4 HPLC analysis of anthocyanins

Free anthocyanin content. The method was as described in Chapter 3.

Bound anthocyanin content. For the determination of bound anthocyanins, a method described by Hertog et al. (1992) was used. After free anthocyanin extraction, the remaining residues were hydrolysed to release the cell wall-bound anthocyanins. A 7 mL volume of acidified (with hydrochloric acid 2 M) 60% (v/v) aqueous methanol was added to each residue and placed in an oven at 90 °C for 90 min. Samples were allowed to cool down, and supernatants were filtered through a 0.22 μm syringe filter. Stock standard solutions (2.0 mg/mL) of cyanidin (96%) and delphinidin (96%) were prepared separately and were diluted to give working solutions with concentrations ranging from 0.01 to 1.0 mg/mL. Calibration curves were obtained by plotting the peak areas against the corresponding concentrations of the standard solutions injected into the HPLC.

4.2.5 Determination of total phenolic content

The method was as described in Chapter 3.

4.2.6 Determination of total antioxidant activity

The method was as described in Chapter 3.

4.2.7 Colour measurement

The colour of the extracts was determined using a Hunter-Lab colourimeter (Hunter Lab, ColourQuest, Hunter Associates Laboratory, Virginia, USA) based on three colour coordinates, *L**, *a**, and *b** at D65 standard illuminant and 10° standard observer angle. The instrument was calibrated using a black card. The colour was measured by the Hunter Lab units, *L** (Lightness/darkness; 0 – 100), *a** (positive = redness/negative = greenness) and *b** (positive = yellowness/negative = blueness). The total colour difference (ΔE) between two samples was calculated according to the following formula:

$$Total\ colour\ difference\ (\Delta E) = [(L^* - L_o)^2 + (a^* - a_o)^2 + (b^* - b_o)^2]^{1/2}$$

where, L_o, a_o, b_o = blank values of control samples extracted at 30 minutes, according to free anthocyanins content

Chroma (C) is the quantitative attribute of colour intensity and hue (h°) gives a qualitative attribute of colours which are defined as reddish, greenish, yellowish and bluish. The chroma and hue angle can be calculated using the *a** and *b** values according to the equations below (Wrolstad and Smith, 2010).

$$Chroma\ (c) = \sqrt{(a^*)^2 + (b^*)^2}$$

$$Hue\ angle\ (h) = ArcTan(\frac{b^*}{a^*})$$

4.2.8 Statistical analysis

All statistical analyses were conducted by one-way analysis of variance (ANOVA). Tukey's multiple range tests were employed with a probability of $p \leq 0.05$. The linear Pearson correlation was also used to evaluate correlations between free anthocyanin, total phenolics, antioxidant activity and TCD. The software for statistical analysis was Minitab V.16 (Minitab Inc., State College, Pennsylvania, USA).

4.3 Results and discussion

Extraction of total monomeric anthocyanins using water and organic solvents (100% ethanol, 100%, 70%, 35% and 20% of methanol in water) was conducted at different temperatures (30 °C, 50 °C, 70 °C, 90 °C) and for different times (0.5, 1, 2, 4 and 6 h) to identify the conditions that favour better extraction of anthocyanins (**Supplementary Table 4-2**). According to these results, high anthocyanin contents in the extracts were observed in the extraction conditions of water (90 °C and 70 °C), 100% ethanol (70 °C), 100% methanol (70 °C and 50 °C), and 70% and 50% of methanol in water (50 °C and 30 °C). Therefore, these extraction conditions were used to carry out extractions for 6 h, and the extracts were then subjected to HPLC analysis to identify the type of bound and free anthocyanins, flavonols and hydroxycinnamic acids. Moreover, extractions using acetic acid buffer solutions at an acidic pH value of 1.5 were also carried out at 50 °C and 30 °C, in order to investigate the influence of low pH on anthocyanins and phenolics extraction.

Supplementary Figure 4-5 shows the HPLC chromatograms of (a) free and (b) bound anthocyanins detected at 520 nm. In addition, **Supplementary Figure 4-6** shows the HPLC chromatograms of hydroxycinnamic acids and flavonols detected at 320 and 360 nm, respectively. Notably, anthocyanins can also be detected at 320 and 360 nm, but at lower amounts.

Furthermore, the concentration of total phenolics in the extracts was determined using the Folin-Ciocalteu method. The Pearson correlation between the total phenolic content and the free anthocyanin content in the obtained extracts was weak, with $R^2 = 0.581$ ($p \leq 0.05$), indicating that besides free anthocyanins, there were other phenolic compounds that also reacted with the Folin reagent. Moreover, a study by Häkkinen et al. (1999) showed that phenolic compounds such as hydroxycinnamic acids (*p*-coumaric, ferulic and caffeic acids) and flavonols (quercetin, myricetin and kaempferol) were also found in blackcurrant

fruit extracts. Moreover, Karseno et al. (2018) and Everette et al. (2014) reported that the Folin reagent is significantly reactive towards other compounds besides phenols such as proteins, organic acids, vitamins and reducing sugars.

Generally, the yield of phenolics compounds including free and bound anthocyanins, hydroxycinnamic acids and flavonols started to decrease over time, especially in the case of free anthocyanins which showed rapid degradation in the hot water extraction at 90 °C and 70 °C (**Supplementary Figure 4-7**). Therefore, due to the thermal sensitivity of free anthocyanins, 2 h seemed to be the best extraction time whereas no significant difference was observed within the first 2 h of extraction in the case of all solvent and acid extractions. In the next sections, the results from the 2 h extraction are presented in **Figure 4-1**, **Figure 4-2** and **Figure 4-3** with the aim being to compare the different extraction methodologies in terms of their ability to extract anthocyanins and phenolic compounds.

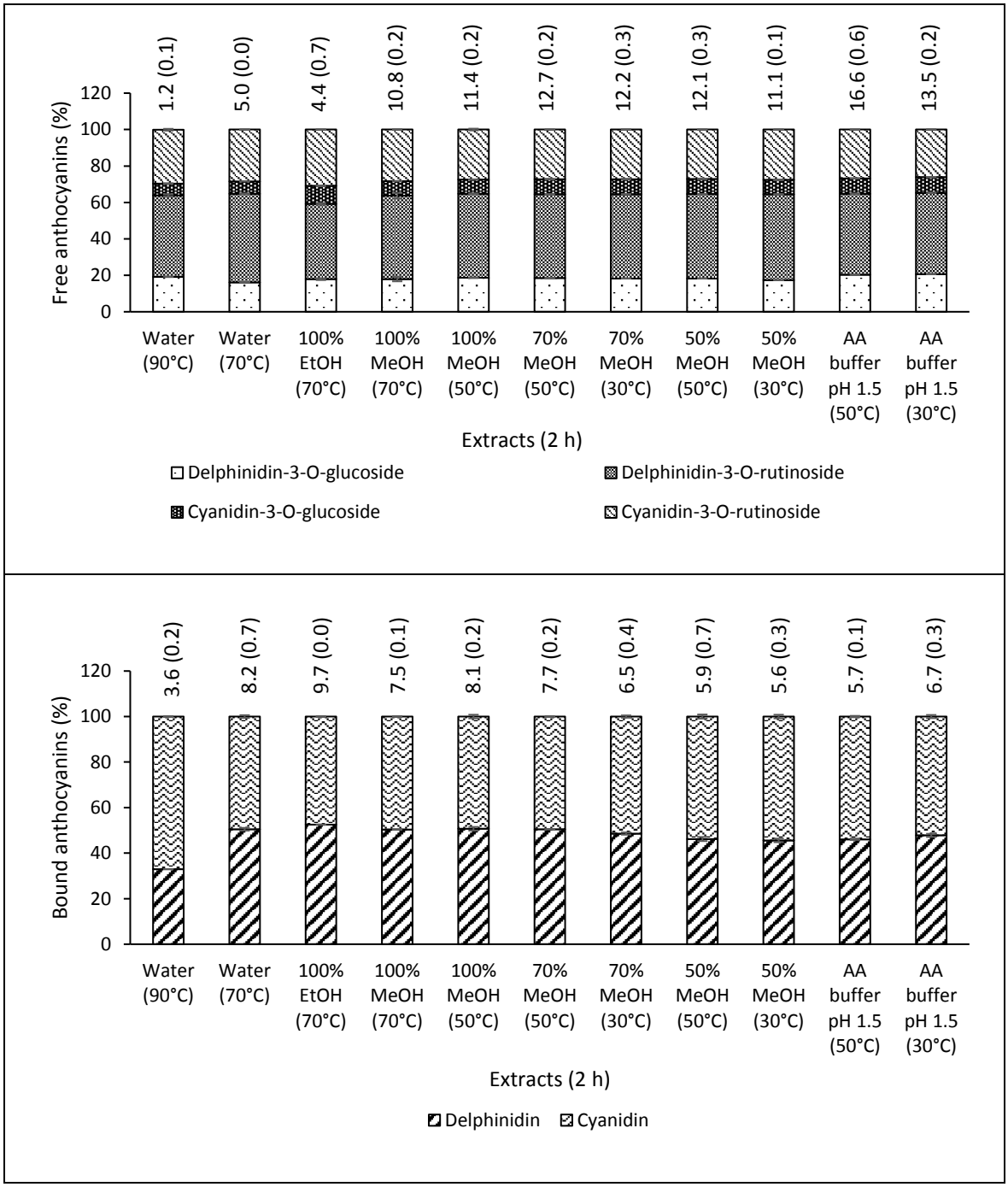


Figure 4-1. Free and bound anthocyanin contents in DBS extracts obtained after 2 h of extraction as quantified using HPLC. *EtOH: Ethanol; MeOH: Methanol; AA: Acetic acid. Figures on the top of bar charts indicate the total free and bound anthocyanins in mg/g. Figures in parentheses indicate the standard deviation.

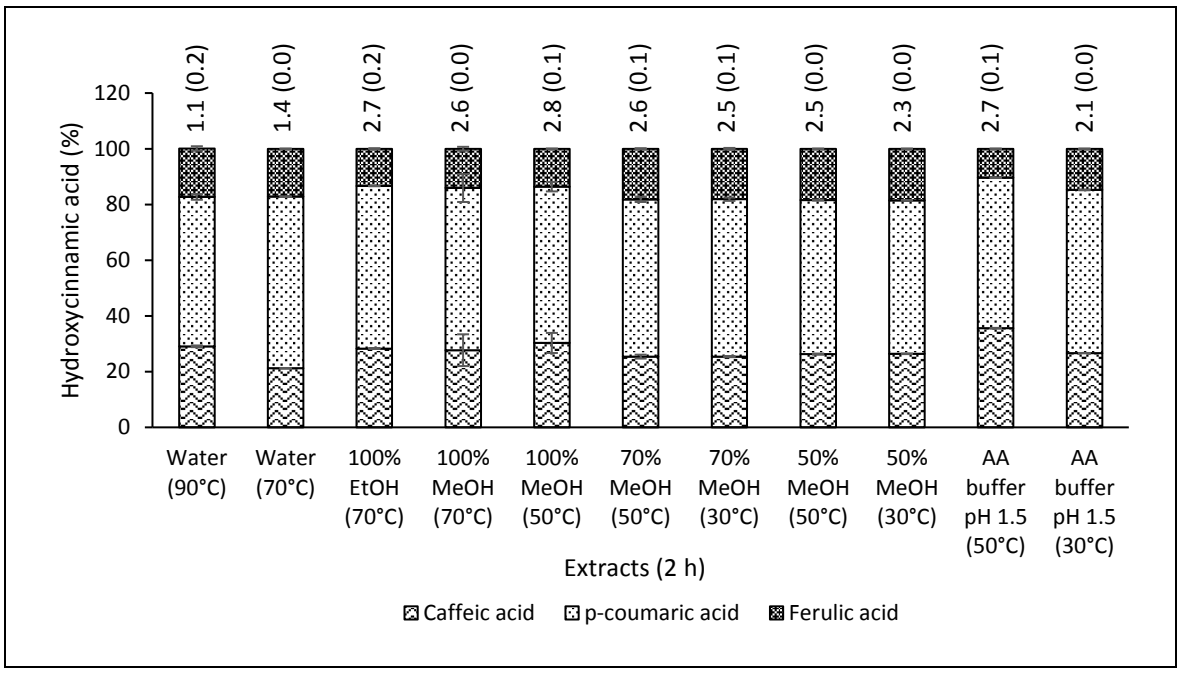


Figure 4-2. Hydroxycinnamic acid content in DBS extracts obtained after 2 h of extraction as quantified using HPLC. *EtOH: Ethanol; MeOH: Methanol; AA: Acetic acid. Figures on the top of bar charts indicate the total hydroxycinnamic acids in mg/g. Figures in parentheses indicate the standard deviation.

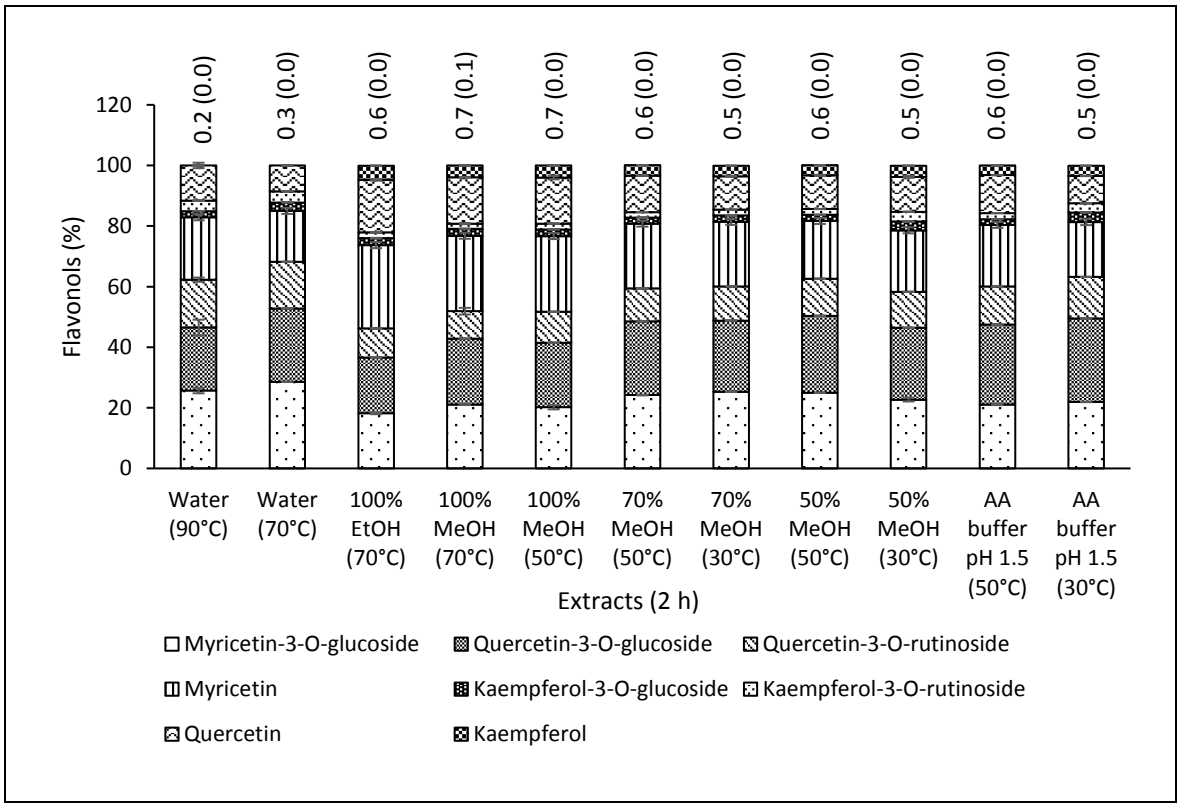


Figure 4-3. Flavonol contents in DBS extracts obtained after 2 h extraction as quantified using HPLC. *EtOH: Ethanol; MeOH: Methanol; AA: Acetic acid. Figures on the top of bar charts indicate the total flavonols in mg/g. Figures in parentheses indicate the standard deviation.

4.3.1 Water extraction of anthocyanins and phenolics

Figure 4-1 shows the comparison between the free and bound anthocyanin contents (mg/g of dry weight) of DBS after 2 h of extraction in water. At 90 °C and 70 °C, delphinidin-3-O-rutinoside was predominant ($p \leq 0.05$) among all free anthocyanins, i.e. found at ~44.7% and ~48.7%, respectively. This was followed by cyanidin-3-O-rutinoside and delphinidin-3-O-glucoside, while cyanidin-3-O-glucoside was the lowest free anthocyanin detected in both water extracts.

Furthermore, at 90 °C, cyanidin was found as the major ($p \leq 0.05$) bound anthocyanin (~67.0%), however, there was no significant difference between the concentrations of delphinidin and cyanidin at 70 °C. The ratios of total free to bound anthocyanin were significantly ($p \leq 0.05$) lower in both 90 °C and 70 °C water extracts than in the mixtures of methanol/water and acetic acid buffer extracts. Overall, water extraction at 70 °C showed significantly ($p \leq 0.05$) higher total free and bound anthocyanins, i.e. 5.0 ± 0.0 mg/g and 8.2 ± 0.7 mg/g, respectively, compared to 90 °C extraction.

Figure 4-2 presents the hydoxycinnamic acid content after 2 h of water extraction. At 90 °C and 70 °C, a significantly ($p \leq 0.05$) high content of *p*-coumaric acid was observed, ranging from ~53.6% to ~61.6%, compared to caffeic acid, while ferulic acid was the lowest. However, there was no significant difference in total hydroxycinnamic acid between the 90 °C and 70 °C of water extracts.

As shown in **Figure 4-3**, myricetin-3-O-glucoside was the main flavonol ($p \leq 0.05$) in both 90 °C and 70 °C water extracts (~25.7% to ~28.6%), followed by quercetin-3-O-glucoside; no kaempferol was detected. However, there was no significant difference ($p > 0.05$) in the total

flavonol content between these two extraction temperatures. Laaksonen et al. (2014) reported similar trends for flavonols obtained in blackcurrant juices produced by pressing blackcurrants at an industrial scale.

Compared to 90 °C extraction, the extraction carried out at 70 °C in water resulted in higher concentrations of total free anthocyanins (5.0 ± 0.0 mg/g), bound anthocyanins (8.2 ± 0.7 mg/g), hydroxycinnamic acids (1.4 ± 0.0 mg/g) and flavonols (0.3 ± 0.0 mg/g). However, the application of high extraction temperatures (70 °C and 90 °C) did not seem to favour the extraction of phenolic compounds, especially that of anthocyanins at 90 °C, due to the high heat sensitivity of anthocyanins. According to Kirca et al. (2007) who studied the stability of anthocyanins extracted from black carrots at 70 °C, 80 °C and 90 °C, anthocyanins showed lower stability as the temperatures increased; the extraction process involved 10 h incubation in a water bath.

4.3.2 Solvent extraction of anthocyanins and phenolics

Organic solvents such as ethanol and methanol were also evaluated as extraction media for the extraction of anthocyanins from DBS. In the methanol extraction data shown in **Figure 4-1**, 50 °C showed relatively higher anthocyanin contents (11.4 ± 0.2 mg/g), as compared to 70 °C (10.8 ± 0.2 mg/g). However at 70 °C, ethanol extracted significantly ($p \leq 0.05$) lower amounts of anthocyanin (4.4 ± 0.7 mg/g) than methanol. Similar to water extraction, delphinidin-3-O-rutinoside was the main free anthocyanin found in the methanol and ethanol extracts. Furthermore, delphinidin (bound anthocyanin) was present in higher ($p \leq 0.05$) amounts than cyanidin in the ethanol and methanol extracts at 70 °C, but there was no significant difference between delphinidin and cyanidin in the 50 °C of methanol extracts. Methanol

extracts at 70 °C and 50 °C showed significantly ($p \leq 0.05$) higher ratio of free to bound anthocyanins, however, ethanol extraction at 70 °C resulted in the opposite. Similar to the hot water extracts (90 °C and 70 °C), the ethanol extract (70 °C) had a significantly ($p \leq 0.05$) lower free anthocyanin content but higher bound anthocyanin content than methanol, indicating that the acid hydrolysis helps to hydrolyse the remaining anthocyanin left in the cell membranes. Notably, the application of acidic media might also have caused the hydrolysis of the acyl and glycosidic bonds (Markham, 1982), such as those present in delphinidin-3-O-rutinoside, and most likely resulted in higher delphinidin concentration.

According to the results in **Figure 4-2**, *p*-coumaric acid was the main hydroxycinnamic acid in the solvent extractions, followed by caffeic and ferulic acid. However, no significant differences were observed in the hydroxycinnamic acid content between both solvents and temperatures used. Moreover, as shown in **Figure 4-3,** the 70 °C and 50 °C methanol extracts exhibited significantly higher ($p \leq 0.05$) flavonol content (~0.7 mg/g) compared to the ethanol extracts (~0.6 mg/g). Also, in solvent extractions, myricetin was the dominant flavonol rather than myricetin-3-O-glucoside, as in the case of hot water extractions. This was followed by quercetin-3-O-glucoside and myricetin-3-O-glucoside, whilst kaempferol was the lowest ($p \leq 0.05$) flavonol (~0.03 mg/g) detected in the solvent extractions.

Overall, methanol extracts showed relatively higher free anthocyanin contents at 50 °C compared to 70 °C. With regards to the solvent used at 70 °C, significantly higher ($p \leq 0.05$) free anthocyanins and flavonols contents were observed in the methanol than in the ethanol extracts. This suggests that methanol is a better extractant for free anthocyanins and phenolic compounds compared to ethanol. Boeing et al. (2014) also reported that methanol was the most efficient solvent compared to ethanol, acetone and water in extractions carried out with

black mulberry, blackberry and strawberry. In comparison to hot water extraction, methanol showed the highest ($p \leq 0.05$) free anthocyanin, hydroxycinnamic acid and flavonol, whilst bound anthocyanin was the highest ($p \leq 0.05$) in the ethanol extracts.

4.3.3 Solvent/water extraction of anthocyanins and phenolics

In this study, 70% and 50% methanol were used to extract DBS at 50 °C and 30 °C as shown in **Figure 4-1**. Regarding the effect of temperature, extractions at 50 °C using 70% and 50% methanol exhibited a higher extraction yield of free anthocyanins, equal to 12.7 ± 0.2 mg/g and 12.1 ± 0.3 mg/g, respectively, compared to 30 °C. Regarding the effect of the concentrations of the methanol/water used, 70% showed relatively higher free anthocyanins than 50% methanol. Also, 70% methanol at 50 °C and 30 °C had significantly ($p \leq 0.05$) higher bound anthocyanins, ranging between ~6.5 mg/g to ~7.7 mg/g, compared to 50% methanol. Furthermore, the ratio of free to bound anthocyanins were significantly ($p \leq 0.05$) high, with delphinidin-3-O-rutinoside being the predominant free anthocyanins at both temperatures and methanol/water concentrations. Moreover, delphinidin was the main bound anthocyanin in the 70% methanol extract at 50 °C, whilst cyanidin was the major bound anthocyanin in the 50% methanol extract obtained at 30 °C and 50 °C.

According to the **Figure 4-2**, *p*-coumaric was the major hydroxycinnamic acid in the methanol/water extractions, ranging between ~55.1% to ~56.5%, followed by caffeic and ferulic acids. The total hydroxycinnamic acid concentrations were higher in the 70% methanol (50 °C and 30 °C) and 50% methanol (50 °C) extractions (~2.5 mg/g to ~2.6 mg/g) compared to 50% methanol at 30 °C (~2.3 mg/g). Furthermore, 70% methanol extraction at 50 °C (**Figure 4-3**) resulted in relatively higher flavonol content (0.6 ± 0.0 mg/g) than 70% methanol at 30 °C and

50% methanol at 50 °C and 30 °C. Myricetin-3-O-glucoside and quercetin-3-O-glucoside were the main flavonols detected in all extractions, i.e. with different methanol/water ratios and extraction temperatures. Furthermore, in terms of the solvent used, extractions using 100% methanol at 70 °C and 50 °C showed significantly ($p \leq 0.05$) higher total flavonols contents compared to water and the mixtures of methanol/water.

Overall, the application of methanol/water at 50 °C exhibited a higher extraction yield than at 30 °C. In terms of free anthocyanin extraction, 70% methanol at 50 °C resulted in relatively higher free anthocyanins content than 100% and 50% methanol. There was an increase in the yield of free anthocyanins when the water content of the methanol/water solution was decreased. Kapasakalidis et al. (2006) suggested that this difference occurred because anthocyanins are polar and are more efficiently extracted in water-containing solvents. Moreover, according to the mass transfer principles discussed by Spigno et al. (2007), higher solvent to water ratios have an important role towards the efficiency of extraction.

4.3.4 Acid extraction of anthocyanins and phenolics

Extraction using acetic acid/buffer was carried out at two different extraction temperatures (30 °C and 50 °C) to investigate the effect of acid on anthocyanin and phenolic extraction (**Figure 4-1)**. Significantly higher ($p \leq 0.05$) free anthocyanins (16.6 ± 0.6 mg/g) but lower bound anthocyanins (2.9 ± 0.1 mg/g) were found in the acetic acid buffer at pH 1.5 when higher extraction temperature (50 °C) was applied compared to 30 °C. **Figure 4-1** shows that the concentrations of total free anthocyanins in the acid extraction at 30 °C were higher than in water (1.2 mg/g – 5.0 mg/g), ethanol and methanol (4.4 mg/g – 11.4 mg/g) and methanol/water (11.1 mg/g – 12.7 mg/g) extractions. The pH in the 100% methanol and ethanol

extractions were ~4.3 to ~4.7; the pH ranged between ~3.6 and ~3.8 in the 50% methanol extractions, and increased slightly to 3.9 – 4.1 when higher concentration of methanol/water (70%) was applied. It is apparent that the application of low pH (pH 1.5) enabled the efficient extraction of high amounts of free anthocyanins. Acids are considered suitable extractants as they may facilitate the disruption of the cell membrane and act as a dissolving medium of water-soluble pigments as suggested by Amr and Al- Tamimi, (2007). Delphinidin-3-O-rutinoside and cyanidin were the main ($p \leq 0.05$) free and bound anthocyanins in the acid extracts. Acid extractions at both 30 °C and 50 °C had significantly ($p \leq 0.05$) high ratio of free to bound anthocyanins.

Significantly higher ($p \leq 0.05$) total hydroxycinnamic acids were recorded at 50 °C (2.7 ± 0.1 mg/g) than at 30 °C (2.1 ± 0.0 mg/g) (**Figure 4-2**). Similar to the water and solvent extractions, *p*-coumaric acid (~54.0% to ~58.5%) was the major ($p \leq 0.05$) hydroxycinnamic acid in acid extractions, followed by caffeic and ferulic acid. According to the flavonol contents in the acid extracts shown in the **Figure 4-3**, the application of higher extraction temperature (50 °C) resulted in significantly ($p \leq 0.05$) higher total flavonols (0.6 ± 0.0 mg/g) than 30 °C (0.5 ± 0.0 mg/g). However, extraction using 100% methanol led to significantly ($p \leq 0.05$) higher total flavonol concentrations compared to the other solvents including acetic acid buffer. In addition, quercetin-3-O-glucoside appeared to be the major flavonol in the acid extracts (26.4% – 27.6%), followed by myricetin-3-O-glucoside (~21.1% to ~21.9%).

Generally, among the four free anthocyanins in all DBS extracts which were analysed by HPLC (**Figure 4-1**), delphinidin-3-O-rutinoside was the predominant ($p \leq 0.05$) free anthocyanin (delphinidin-3-O-rutinoside; ~48.7% > cyanidin-3-O-rutinoside; ~30.9% > delphinidin-3-O-glucoside; ~20.7% > cyanidin-3-O-glucoside; ~9.9%), while cyanidin and

delphinidin were present as cell-bound anthocyanins. This trend was similar to the work reported by Borges et al. (2009) with blackcurrant fruit extracted with methanol/formic acid (99:1, v/v) using a homogeniser for 1 min.

Furthermore, as shown in **Figure 4-2**, *p*-coumaric acid was the main hydroxycinnamic acid detected in all DBS extracts (*p*-coumaric acid; ~61.6% > caffeic acid; ~35.6% > ferulic acid; ~18.5%). Moreover, caffeic, *p*-coumaric and ferulic acid showed higher yields in acetic acid buffer pH 1.5 (50 °C), 100% ethanol (70 °C) and 70% methanol (50 °C) compared to the other extraction conditions. Notably, **Figure 4-3** demonstrates that the type of extractant influenced the flavonol yield, with myricetin-3-O-glucoside and quercetin-3-O-glucoside (~25.4%) being the main flavonols ($p \leq 0.05$) detected in the methanol/water extracts. Hot water, absolute solvent (methanol and ethanol) and acetic acid buffer pH 1.5 extracts contained high amounts ($p \leq 0.05$) of myricetin-3-O-glucoside (~28.6%), myricetin (~27.5%) and quercetin-3-O-glucoside (~27.6%), respectively.

Overall, different conditions showed different levels of effectiveness in terms of the type of phenolic compounds extracted. For example, acetic acid pH 1.5 buffer was the best extractant for free anthocyanins (~13.5 mg/g to ~16.6 mg/g). Also, except water, total hydroxycinnamic acids were more efficiency extracted at higher temperatures (70 °C and 50 °C), which yielded between 2.5 mg/g to 2.8 mg/g. Moreover, 100% methanol at 70 °C and 50 °C were the most suitable extraction conditions to obtain the highest concentration of flavonol (~0.7 mg/g) compared to water, ethanol, methanol/water and acid.

4.3.5 Antioxidant activities of extracts

In the blackcurrant skins, the phenolic compounds present include anthocyanins, phenolic acids, flavonols, and flavan-3-ols (Karjalainen et al., 2008). Other than acting as food preservatives, these are potent antioxidants and exhibit promising health promoting properties, such as lowering the risk of cancer and heart diseases, as well as anti-inflammatory and antimicrobial properties (Wrolstad, 2004). The biosynthesis of flavonoids is stimulated by sunlight (ultraviolet radiation), therefore higher concentrations of flavonoids can typically be found in the outer layers of fruits and vegetables, i.e. the skins. This group of phenolic compounds is also responsible for the antioxidant capacity of such plant extracts (Mäkilä et al., 2016).

Kähkönen and Heinonen (2003) also suggested that anthocyanins and their aglycones are powerful antioxidants and possess high antioxidant activity. However, from the observations from the up to 6 h data (**Supplementary Figure 4-8**), a moderate correlation ($R^2 = 0.704$) with $p \leq 0.05$ was found between the anthocyanin content and antioxidant activity suggesting that besides anthocyanins, ~30% of the antioxidant activity could be attributed to other phenolics such as hydroxycinnamic acids and flavonols, and potentially to non-phenolic compounds. This is supported by the significant correlation ($p \leq 0.05$) between the total phenolic content in the DBS extracts and antioxidant activity ($R^2 = 0.935$).

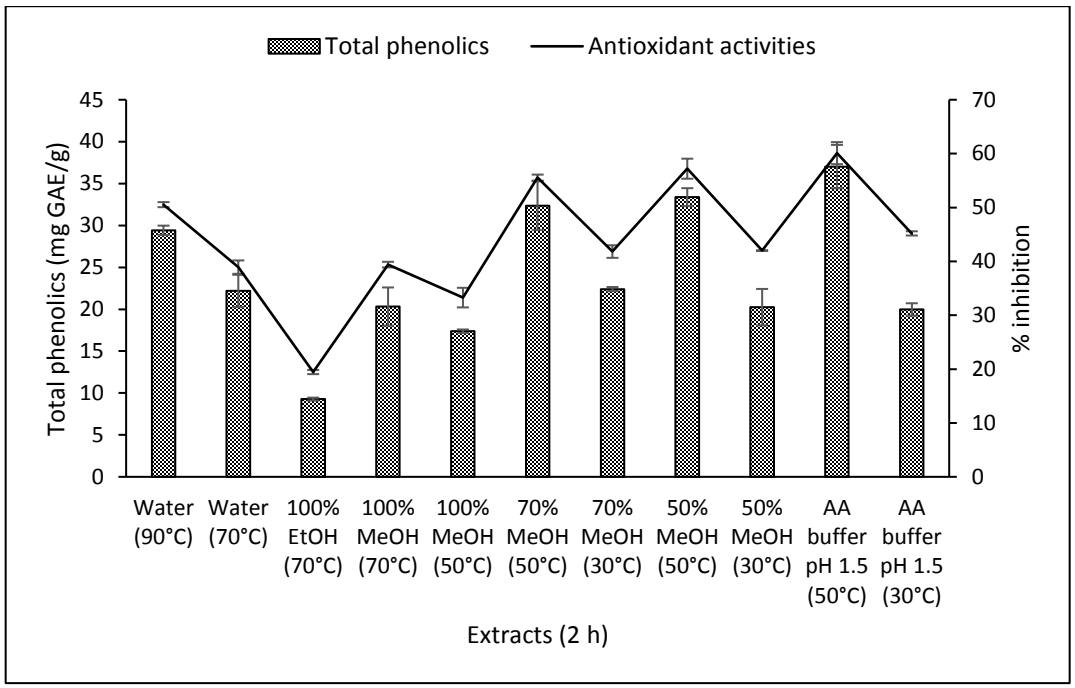


Figure 4-4. Total phenolic contents (mg GAE/g) and antioxidant activity (% inhibition) in DBS extracts after 2 h of extraction as measured using Folin-Ciocalteu and the DPPH method. *EtOH: Ethanol; MeOH: Methanol; AA: Acetic acid.

According to **Figure 4-4**, the increase in the extraction temperature from 70 °C to 90 °C, led to a concomitant increase in the total phenolic content of the water extracts. This resulted in an increase in the antioxidant activity from ~38.9% to ~50.5% during the first 2 h of extraction. Interestingly, the 90 °C water extract had lower free anthocyanin content than the 70 °C water extract but contained higher amount of total phenolics. Similarly, Ramirez-Rodrigues et al. (2011) reported that in hot water extractions (90°C) of Roselle (*Hibiscus sabdariffa*), the high concentrations of phenolic compounds, in addition to anthocyanins, also contributed to the high antioxidant activity.

Even though anthocyanins were efficiently extracted at 50 °C (**Figure 4-1**), the total phenolic content of methanol extracts at 70 °C in **Figure 4-4** was found to be significantly ($p \leq 0.05$) high (~20.3 mg GAE/g), as did the antioxidant activity (~39.4 % inhibition). Compared to ethanol, methanol was capable in extracting significantly ($p \leq 0.05$) higher

amounts of compounds including phenolics with higher antioxidant activities, hence most likely the reason for this result.

Moreover, the extractions carried out at 50 °C using 70% and 50% methanol/water mixtures showed significantly ($p \leq 0.05$) higher phenolic contents, ranging between ~32.4 to ~33.4 mg GAE/g, with higher antioxidant activity (~55.6% to ~57.2% of inhibition) compared to the 30 °C extracts (**Figure 4-4**). However, different methanol/water concentrations (70% and 50%) did not show any significant difference on the total phenolics and the antioxidant activity between the different extraction temperatures. As expected, 70% methanol obtained higher phenolics yield, with a higher antioxidant activity, than 100% methanol. This is due to the fact that solvent/water mixtures can dissolve both polar and less polar phenolic compounds, as suggested by Vagiri (2014).

In the acid extraction using acid buffer pH 1.5 at 50 °C, higher amount of total phenolics (~37.0 mg GAE/g) and antioxidant activities (~60.1% inhibition) were detected compared to 30 °C (**Figure 4-4**). It is apparent that the low pH of the acetic acid buffer was capable in extracting other compounds besides phenolics and anthocyanins which most likely contributed to the high antioxidant activities. Overall, extraction at 50 °C using methanol/water (70% and 50%) and acetic acid buffer pH 1.5 resulted in the highest phenolics content (~32.4 to ~37.0 mg GAE/g) and antioxidant activity (~55.6% to ~60.1% of inhibition) compared to other extraction conditions.

4.3.6 Colour of extracts

Many studies on anthocyanins from fruits investigate their use as natural colourants (He & Giusti, 2010). In this study, colour measurement aimed to investigate the effect of the

extraction conditions to the colour of the extracts. **Supplementary Figure 4-9** to **Supplementary Figure 4-12**, presents the changes in colour, representing the colour stability after 6 h extraction at different extraction conditions; these were reported in terms of colour values (L^*, a^*, b^*) and Total Colour Difference (TCD), as a comparison between the colour of the extracts and the controls (30 min extraction).

In this study, the rate of colour changes up to 6 h in the hot water extracts followed a similar trend as anthocyanin degradation. Rapid degradation of anthocyanin was observed at high temperatures (90 °C and 70 °C) [**Supplementary Figure 4-7 (a)]**, due to the heat sensitivity of anthocyanins, which also resulted in a loss of colour stability [**Supplementary Figure 4-9 (a)**]. Markakis (2012) also reported that high extraction temperatures increased the structural transformation of anthocyanins to hemiketal and chalcone (colourless) and eventually led to anthocyanin degradation. Furthermore, even though the 90 °C water extract indicated a low amount of free anthocyanins after 2 h (**Figure 4-1**), the L^* and a^* values were significantly ($p \leq 0.05$) high (**Table 4-1**), indicating a decrease in darkness and an increase in redness. This led to the increase in chroma (colour intensity) compared to 70 °C water extract, which might be due to the formation of coloured derivatives other than anthocyanins at higher extraction temperatures.

Table 4-1. Colour characteristics of DBS extracts obtained after 2 h of extraction, measured using a Hunter Lab colourimeter.

Extracts (2 h)	Total colour difference (ΔE)	*L**	*a**	*b**	Chroma (C)	Hue angle (h°)
Water (90 °C)	9.2 (1.5)^{a}	18.0 (0.4)^{bc}	18.5 (1.4)^{d}	0.0 (0.4)^{de}	18.5 (1.4)^{c}	0.1 (1.4)^{ab}
Water (70 °C)	5.1 (0.4)^{ab}	14.8 (0.3)^{ef}	13.9 (0.3)^{de}	-1.0 (0.1)^{e}	13.9 (0.3)^{cd}	-4.0 (0.3)^{b}
100% EtOH (70 °C)	5.8 (2.8)^{ab}	13.5 (1.2)^{f}	13.3 (4.0)^{de}	-0.2 (1.5)^{de}	13.4 (3.9)^{cd}	-1.7 (6.9)^{ab}
100% MeOH (70 °C)	1.9 (1.1)^{b}	20.2 (0.4)^{a}	9.7 (2.2)^{e}	-5.1 (0.9)^{f}	11.0 (1.6)^{d}	-28.4 (9.6)^{c}
100% MeOH (50 °C)	6.0 (0.7)^{ab}	17.2 (0.3)^{cd}	9.6 (0.5)^{e}	-3.5 (0.4)^{f}	10.2 (0.3)^{d}	-20.3 (3.2)^{c}
70% MeOH (50 °C)	1.5 (0.6)^{b}	15.6 (0.2)^{de}	27.9 (0.5)^{bc}	5.0 (0.2)^{b}	28.4 (0.6)^{b}	10.2 (0.2)^{ab}
70% MeOH (30 °C)	2.0 (0.2)^{b}	17.9 (0.0)^{bc}	33.8 (0.2)^{ab}	7.0 (0.1)^{ab}	34.5 (0.2)^{a}	11.8 (0.1)^{a}
50% MeOH (50 °C)	2.1 (0.3)^{b}	14.7 (0.1)^{ef}	30.2(0.2)^{abc}	6.4 (0.1)^{ab}	30.8 (0.2)^{ab}	11.9 (0.1)^{a}
50% MeOH (30 °C)	2.0 (0.8)^{b}	16.9 (0.4)^{cd}	34.4 (0.7)^{a}	7.6 (0.2)^{a}	35.3 (0.7)^{a}	12.5 (0.2)^{a}
AA buffer pH 1.5 (50 °C)	4.0 (0.0)^{ab}	19.1 (0.0)^{ab}	25.3 (0.1)^{c}	2.0 (0.0)^{cd}	25.4 (0.1)^{b}	4.5 (0.1)^{ab}
AA buffer pH 1.5 (30 °C)	3.2 (0.8)^{ab}	19.1 (0.1)^{ab}	27.2 (0.8)^{c}	2.6 (0.2)^{c}	27.3 (0.8)^{b}	5.5 (0.2)^{ab}

*EtOH: Ethanol; MeOH: Methanol; AA: Acetic acid.
Figures in parenthesis indicate the standard deviation.
Values with the same letter ^{a,b} in each column are not significantly different ($p > 0.05$).

The data in **Supplementary Figure 4-10 (a)** show that at 70 °C the ethanol extract had higher colour changes compared to methanol. Although the ethanol extracts contained a low phenolic content and low antioxidant activity (**Figure 4-4**), they exhibited higher *a** (increase in redness) and lower *L** (increase in darkness) values after 2 h compared to water and methanol extracts (**Table 4-1**). The combination of these values increased the TCD and chroma values to 5.8 ± 2.8 and 13.4 ± 3.9, respectively, indicating a darker and more intense colour for the ethanol

extract. However, extraction using 100% methanol resulted in a significantly ($p \leq 0.05$) low of *b** value, representing an increase in blueness.

Supplementary Figure 4-11 presents the colour characteristics of the 50% and 70% methanol extracts, showing that 50 °C extracts had lower values of *L** (darker colour), *a** (decrease in redness), *b** (increase in blueness) and chroma than 30 °C extracts after 6 h of extraction. Focusing at the 2 h extraction data in **Table 4-1**, 70% methanol (30 °C) and 50% methanol (50 °C and 30 °C) extracts had higher *a** (increase in redness), *b** (decrease in blueness), chroma (increase in colour intensity) and hue angle values. Furthermore, lower *L** values were obtained at 50 °C for the 70% and 50% methanol extracts, indicating the darker colour of these extracts. However, the combination of these values led to no significant ($p \leq 0.05$) difference between TCD values of the 70% and 50% methanol extracts. This fact suggested that the increase in the ratio of methanol to water and higher extraction temperatures had no effect on the colour changes.

As shown in **Supplementary Figure 4-12**, extraction for 6 h using acetic acid buffer pH 1.5 at 50 °C resulted in lower *L** (increase in darkness), *a** (decrease in redness) and *b** (increase in blueness) values, and decreased the colour intensity (chroma) compared to 30 °C. Also, acetic acid buffer pH 1.5 at 50 °C caused more changes in the colour of the extract than at 30 °C, as can be seen in the higher TCD values of the former. However, the data for the acid extraction at 2 h in **Table 4-1** indicated no significant difference in the *L**, *a**, chroma, hue angle and TCD values between acid extraction at 50 °C and 30 °C. The only difference was a lower *b** value (increase in blueness) in the 50 °C extract.

Moderate correlations (R^2 = 0.562 and 0.521) were observed between anthocyanin content and chroma and redness, due to the fact that colour intensity, redness and anthocyanins content depended considerably on the extraction conditions. Moreover, an increase in polarity of methanol/water extracts also added to the redness and colour intensity of the extracts due to fact that anthocyanin is very soluble in aqueous mixtures of methanol and acetic acid buffers. Consequently, the different pH, solvent and extraction temperature influenced each of the *L**, *a** and *b** values, and changed the overall TCD and colour intensity. On the other hand, the blueness increased in the 100% methanol extracts, which had higher pH values (4.5 – 4.7). Finally, high extraction temperatures decreased the colour stability due to the heat sensitivity of anthocyanins.

4.4 Conclusions

The extraction yields of anthocyanin from dried blackcurrant skins was affected by various extraction parameters including the pH, solvent type, extraction temperature and time. Overall, the extracts contained relatively high amounts of total phenolic compounds and exhibited high antioxidant activity, as well as good colour stability. The ratio of free to bound anthocyanins depended considerably on the type of solvent used. To this end, the application of low pH (pH 1.5) at 50 °C enabled the efficient extraction of higher amounts of free anthocyanins, total phenolic content and antioxidant activity compared to water and solvent extractions. The amount of hydroxycinnamic acids were higher in the solvents (ethanol and methanol) and acid extractions at 50 °C and 70°C, while higher amounts of flavonols were obtained in the 100% methanol at 50 °C and 70 °C extractions.

In terms of colour, the redness and colour intensity were enhanced in the 50% and 70% methanol, as well as in acetic acid buffer extracts, as these which exhibited higher polarity and solubility of anthocyanins. The most stable total colour change was observed in the ethanol, methanol, mixtures of methanol/water, and acetic acid buffer pH 1.5 extracts, with no apparent loss of colour throughout the 6 h extraction, at both low and high temperatures.

Overall, the yield of total phenolics and anthocyanins were strongly correlated with high antioxidant activity. On the other hand, colour intensity and redness of extracts were moderately correlated with anthocyanin contents, suggesting that other than anthocyanins, other extraction conditions also influenced the colours of extracts. It is important to note that generally, the extractions using acetic acid/buffer seemed to give the best results in terms of anthocyanin extraction yields, antioxidant activity and colour properties and are regarded as safer compared

to the use of organic solvents, especially when the obtained extracts are to be used for applications in food matrices as natural colourants.

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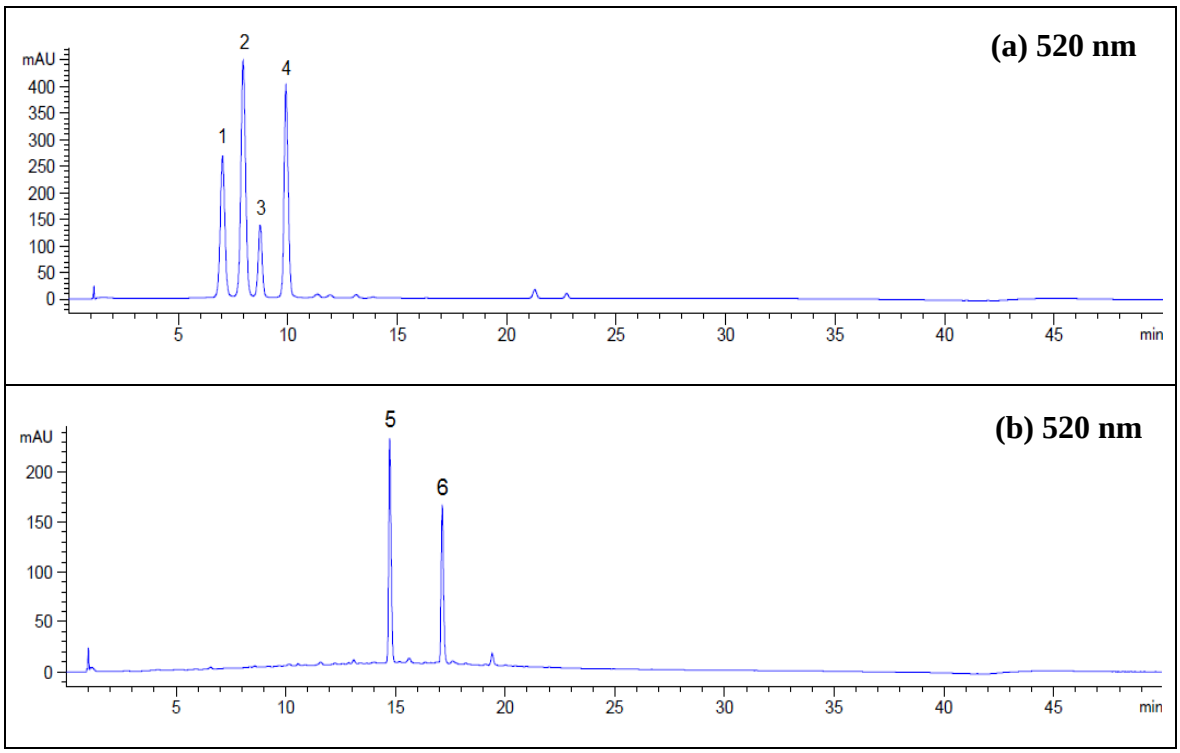
Supplementary Materials

Supplementary Table 4-2. Total monomeric anthocyanins (mg/g) obtained in DBS extracts after 6 h of extraction using different solvents and temperatures as quantified by spectrophotometer.

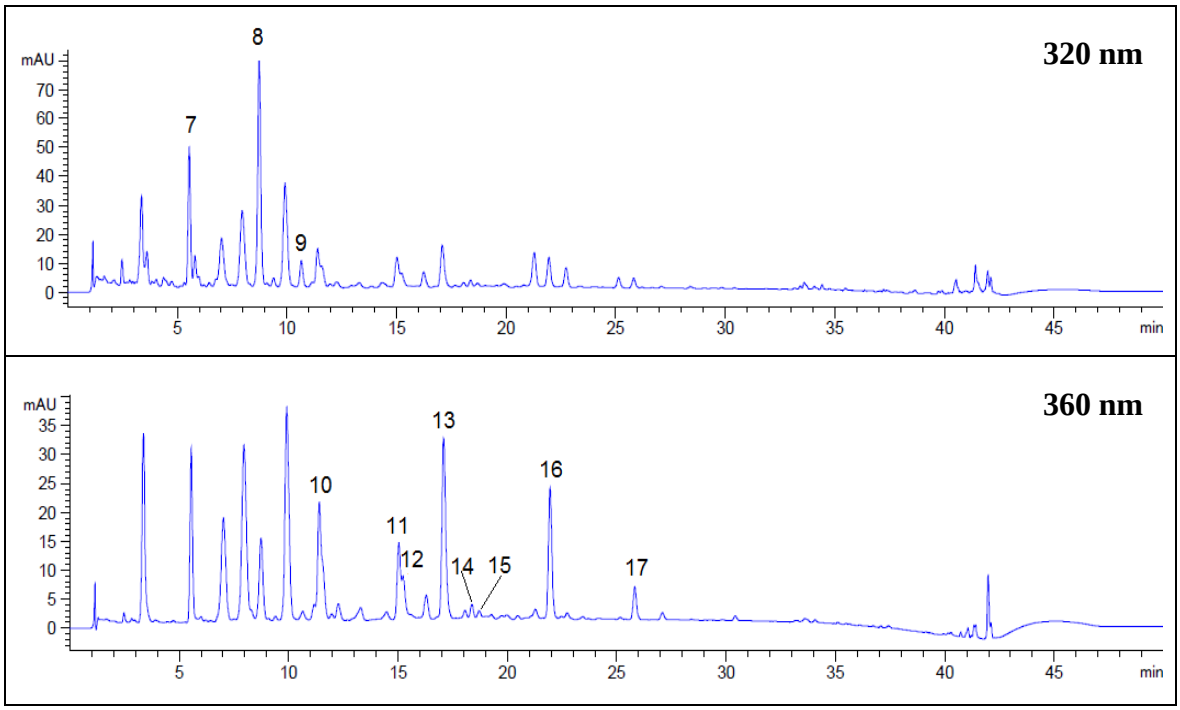
Solvents	Temperature (°C)	Total monomeric anthocyanins content (mg/g)				
		Extraction time (h)				
		0.5	1	2	4	6
Water	30	2.0 (0.0)^{b}	1.9 (0.0)^{b}	2.0 (0.0)^{ab}	2.0 (0.0)^{ab}	2.2 (0.0)^{a}
	50	3.9 (0.3)^{a}	3.9 (0.9)^{a}	4.2 (0.6)^{a}	3.9 (0.3)^{a}	3.7 (0.0)^{a}
	70	5.5 (0.0)^{a}	5.4 (0.2)^{a}	4.9 (0.1)^{b}	3.9 (0.1)^{c}	2.9 (0.0)^{d}
	90	5.3 (0.0)^{a}	4.1 (0.1)^{b}	2.5 (0.0)^{c}	1.1 (0.0)^{d}	0.3 (0.0)^{e}
100% EtoH	30	0.5 (0.0)^{c}	0.5 (0.1)^{c}	0.6 (0.0)^{bc}	0.7 (0.0)^{b}	0.9 (0.0)^{a}
	50	1.3 (0.0)^{d}	1.7 (0.3)^{cd}	2.2 (0.2)^{bc}	2.5 (0.3)^{ab}	3.2 (0.5)^{a}
	70	3.8 (0.1)^{b}	3.9 (0.6)^{b}	5.5 (0.0)^{a}	4.9 (0.0)^{ab}	4.3 (0.6)^{ab}
	90	3.3 (0.6)a	2.5 (0.6)^{ab}	2.4 (0.3)^{abc}	2.0 (0.1)^{bc}	1.4 (0.0)^{c}
100% MeOH	30	5.5 (0.5)^{d}	5.9 (0.3)^{cd}	6.6 (0.7)^{bc}	7.4 (0.2)^{b}	9.3 (0.4)^{a}
	50	8.1 (0.5)^{b}	9.0 (0.3)^{ab}	8.7 (0.3)^{ab}	9.6 (0.7)^{ab}	10.2 (0.8)^{a}
	70	9.5 (0.3)^{a}	9.0 (0.5)^{a}	10.0 (0.4)^{a}	10.0 (0.2)^{a}	9.6 (0.8)^{a}
	90	7.4 (0.4)^{a}	6.6 (1.0)^{a}	5.8 (0.6)^{a}	3.5 (0.4)^{b}	2.9 (0.1)^{b}
70% MeOH	30	9.3 (0.0)^{a}	9.8 (0.6)^{a}	10.8 (0.5)^{a}	10.3 (0.0)^{a}	9.3 (0.6)^{a}
	50	11.6 (0.4)^{a}	11.1(0.1)^{ab}	10.6 (0.8)^{abc}	10.2 (0.2)^{bc}	9.6 (0.4)^{c}
	70	9.3 (0.0)^{ab}	9.5 (0.1)^{a}	9.1 (0.1)^{b}	8.4 (0.0)^{c}	7.6 (0.1)^{d}
	90	10.9 (0.1)^{a}	8.5 (0.3)^{b}	6.8 (0.5)^{c}	5.0 (0.5)^{d}	3.4 (0.0)^{d}
50% MeOH	30	9.2 (0.9)^{a}	9.6 (0.6)^{a}	10.8 (0.6)^{a}	11.2 (1.2)^{a}	10.5 (0.3)^{a}
	50	10.5 (0.2)^{a}	10.3 (0.2)^{a}	11.1 (0.7)^{a}	10.6 (0.2)^{a}	11.2 (0.6)^{a}
	70	8.5 (0.0)^{a}	8.6 (0.1)^{a}	7.9 (0.2)^{b}	7.1 (0.0)^{c}	6.0 (0.0)^{d}
	90	8.0 (0.0)^{a}	7.2 (0.0)^{b}	5.9 (0.2)^{c}	4.1 (0.2)^{d}	3.0 (0.0)^{e}
35% MeOH	30	7.2 (0.1)^{a}	7.2 (0.1)^{a}	8.0 (0.7)^{a}	8.7 (0.7)^{a}	7.6 (0.2)^{a}
	50	7.8 (0.1)^{b}	7.9 (0.1)^{b}	7.5 (0.2)^{b}	7.6 (0.0)^{b}	9.4 (0.0)^{a}
	70	9.1 (0.1)^{a}	9.1 (0.1)^{a}	8.0 (0.1)^{b}	6.7 (0.0)^{c}	5.8 (0.0)^{d}
	90	11.6 (0.6)^{a}	8.0 (0.7)^{b}	7.7 (0.0)^{b}	3.7 (0.1)^{c}	4.0 (0.2)^{c}

20% MeOH	30	4.9 (0.6)[a]	4.4 (0.0)[a]	5.3 (0.6)[a]	5.4 (0.5)[a]	4.8 (0.3)[a]
	50	6.7 (0.8)[a]	6.5 (0.9)[a]	6.3 (0.2)[a]	6.6 (0.3)[a]	6.6 (0.8)[a]
	70	8.1 (0.3)[a]	7.6 (0.5)[ab]	6.8 (0.1)[b]	5.0 (0.0)[c]	4.8 (0.1)[c]
	90	8.3 (0.1)[a]	7.3 (0.0)[b]	6.3 (0.1)[c]	4.1 (0.1)[d]	1.6 (0.0)[e]

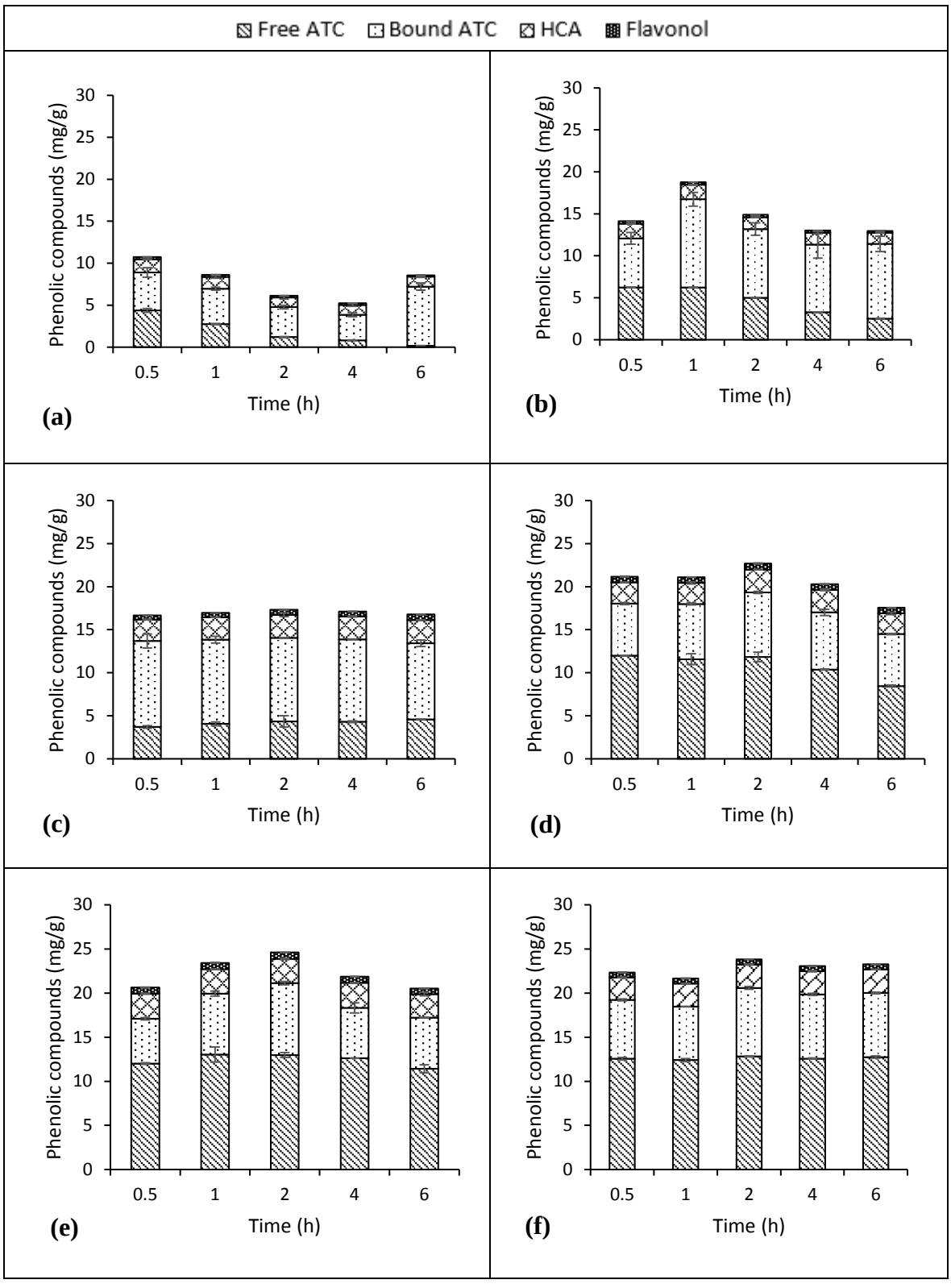
EtOH: Ethanol; MeOH: Methanol.
Figures in parenthesis indicate the standard deviation.
Values with the same letter [a,b] in each column are not significantly different ($p > 0.05$).

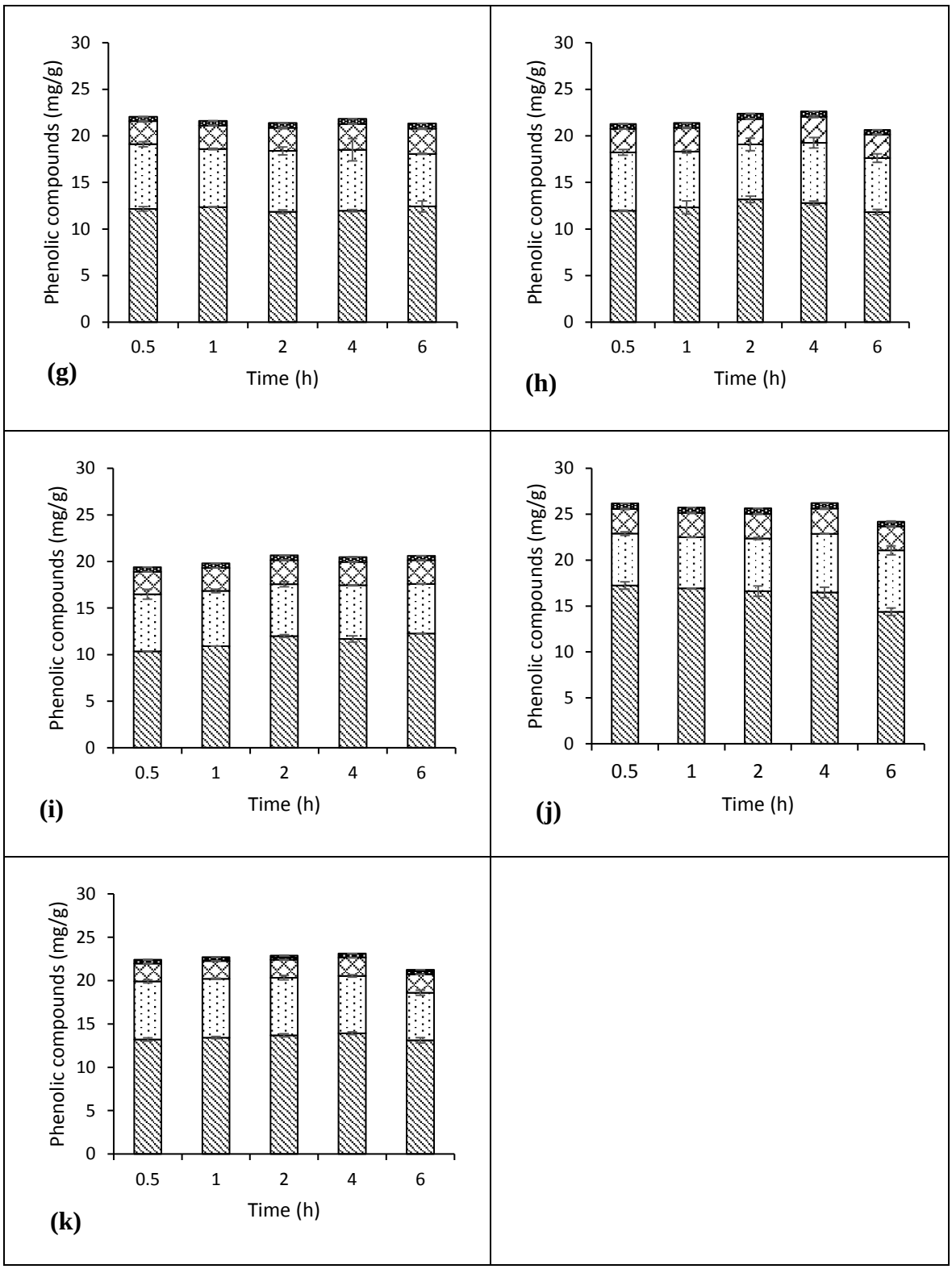


Supplementary Figure 4-5. Typical HPLC chromatograms of dried blackcurrant skins (DBS) extract showing the detected (at 520 nm) (a) free anthocyanin and (b) bound anthocyanins. (1) Delphinidin-3-O-glucoside, (2) Delphinidin-3-O-rutinoside, (3) Cyanidin-3-O-glucoside, (4) Cyanidin-3-O-rutinoside, (5) Delphinidin and (6) Cyanidin.

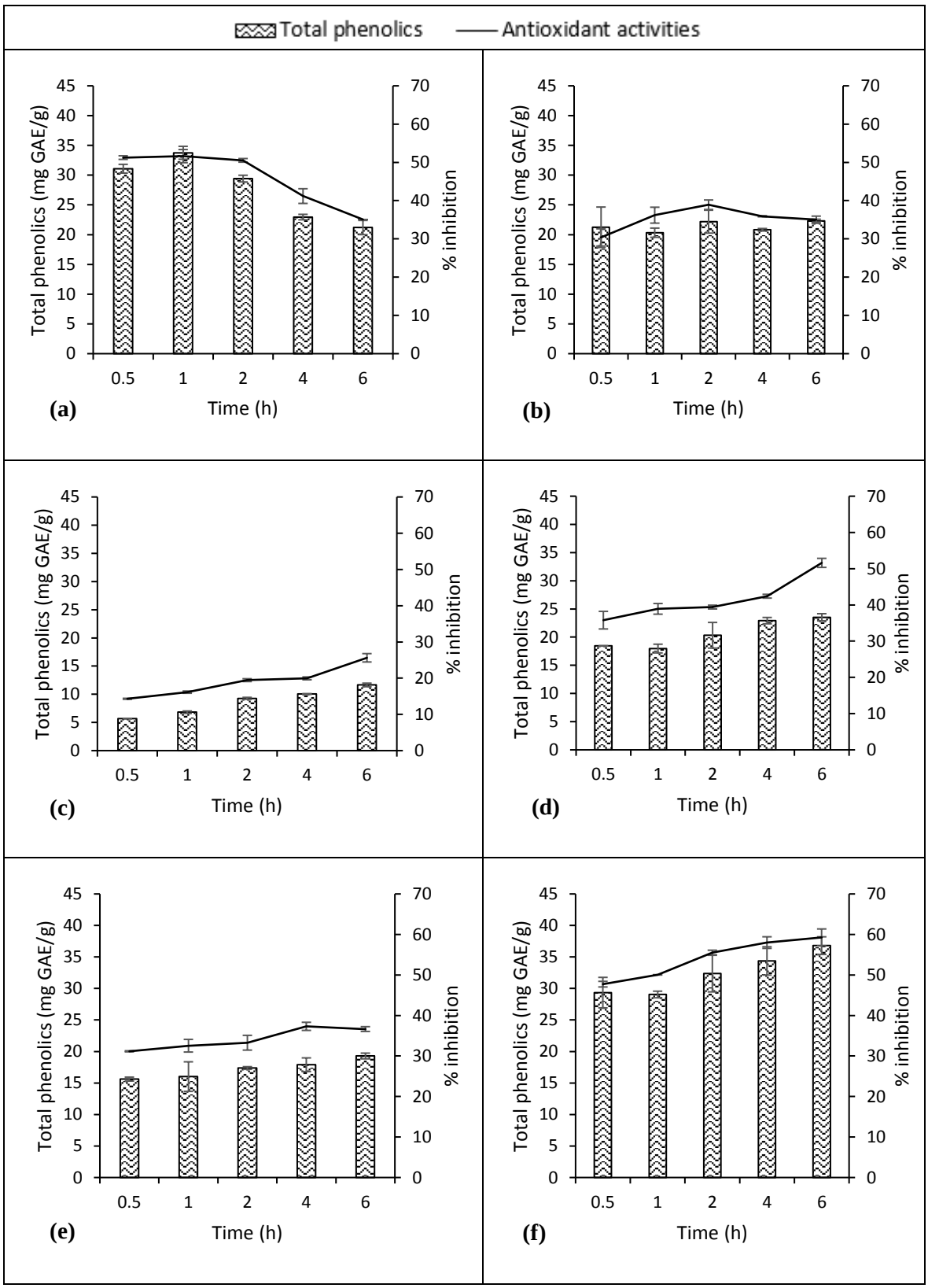


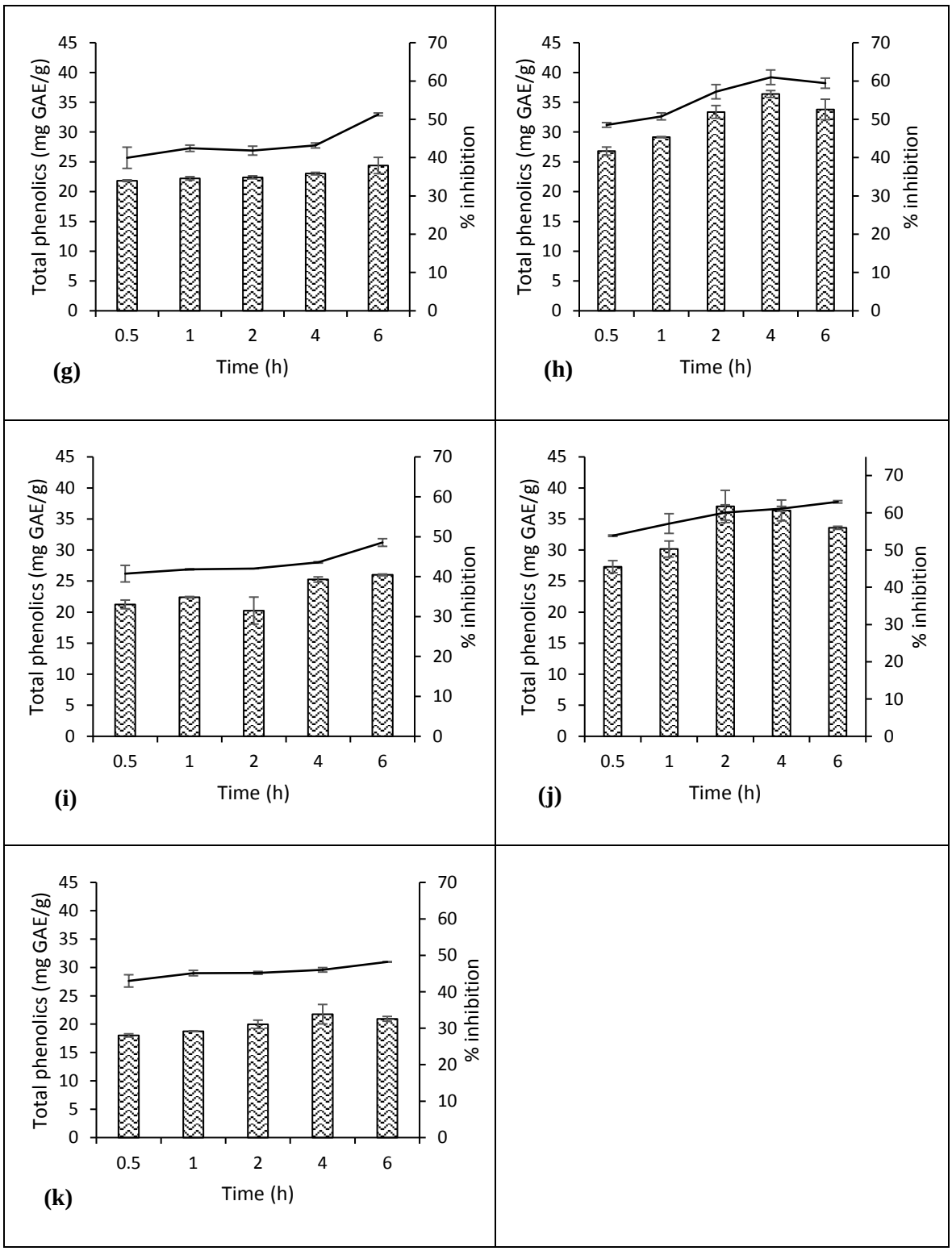
Supplementary Figure 4-6. Typical HPLC chromatograms of dried blackcurrant skins (DBS) extract showing the detected hydroxycinnamic acids (at 320 nm) and flavonols (at 360 nm) (7) Caffeic acid, (8) *p*-Coumaric acid, (9) Ferulic acid, (10) Myricetin 3-O-glucoside, (11) Quercetin 3-O-glucoside, (12) Quercetin 3-O-rutinoside, (13) Myricetin, (14) Kaempferol-3-O-glucoside, (15) Kaempferol-3-O-rutinoside (16) Quercetin and (17) Kaempferol.


Free ATC
Bound ATC
HCA
Flavonol
Phenolic compounds (mg/g)
Time (h)
0.5
1
2
4
6
(a)
(b)
(c)
(d)
(e)
(f)

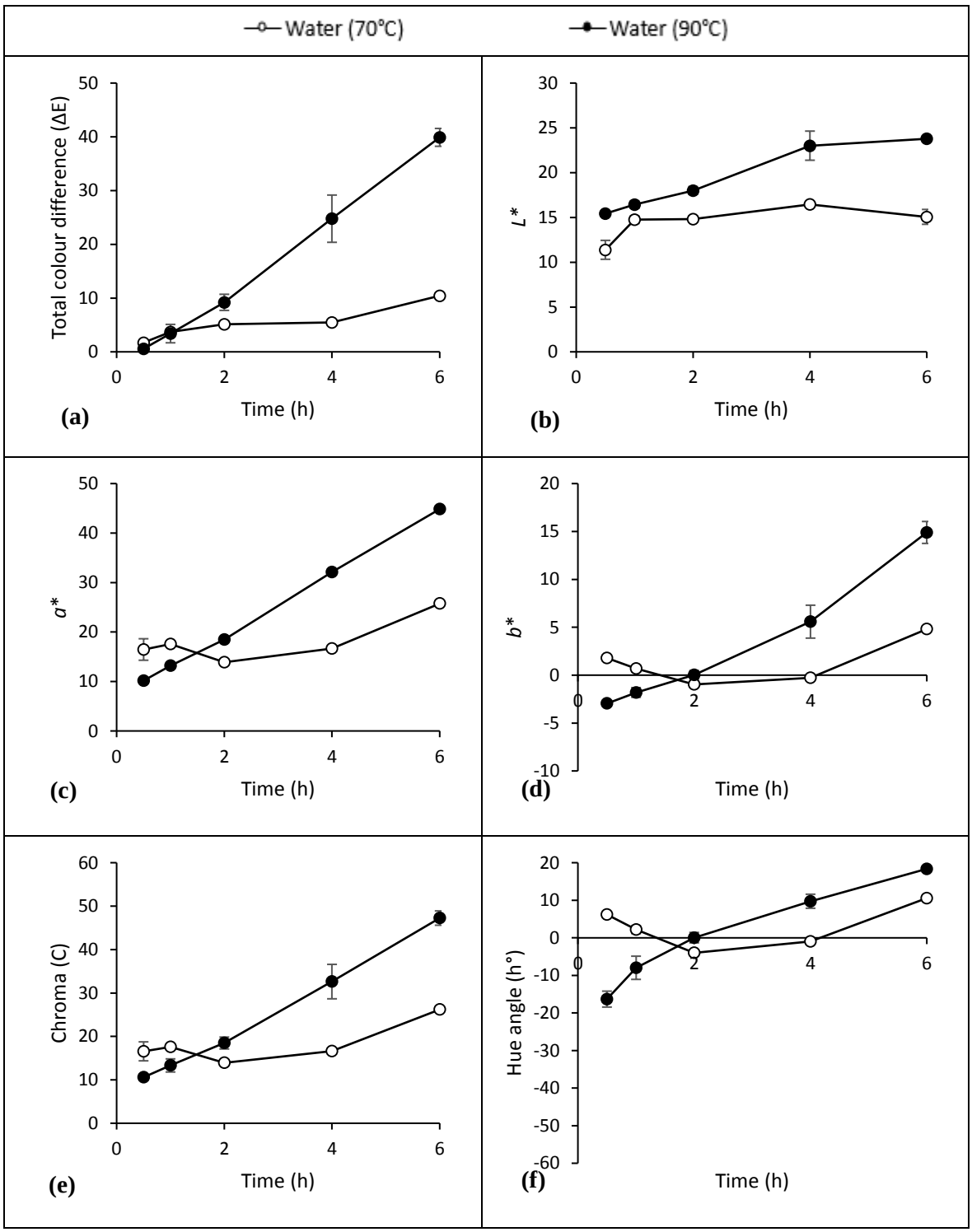


Supplementary Figure 4-7. Phenolic compounds in (a) water (90 °C), (b) water (70 °C), (c) 100% ethanol (70 °C), (d) 100% methanol (70 °C), (e) 100% methanol (50 °C), (f) 70% methanol (50 °C), (g) 70% methanol (30 °C), (h) 50% methanol (50 °C), (i) 50% methanol (30 °C), (j) acetic acid buffer pH 1.5 (50 °C) and (k) acetic acid buffer pH 1.5 (30 °C) after 6 h of extraction as quantified by HPLC.*ATC: Anthocyanin; HCA: Hydroxycinnamic acid.

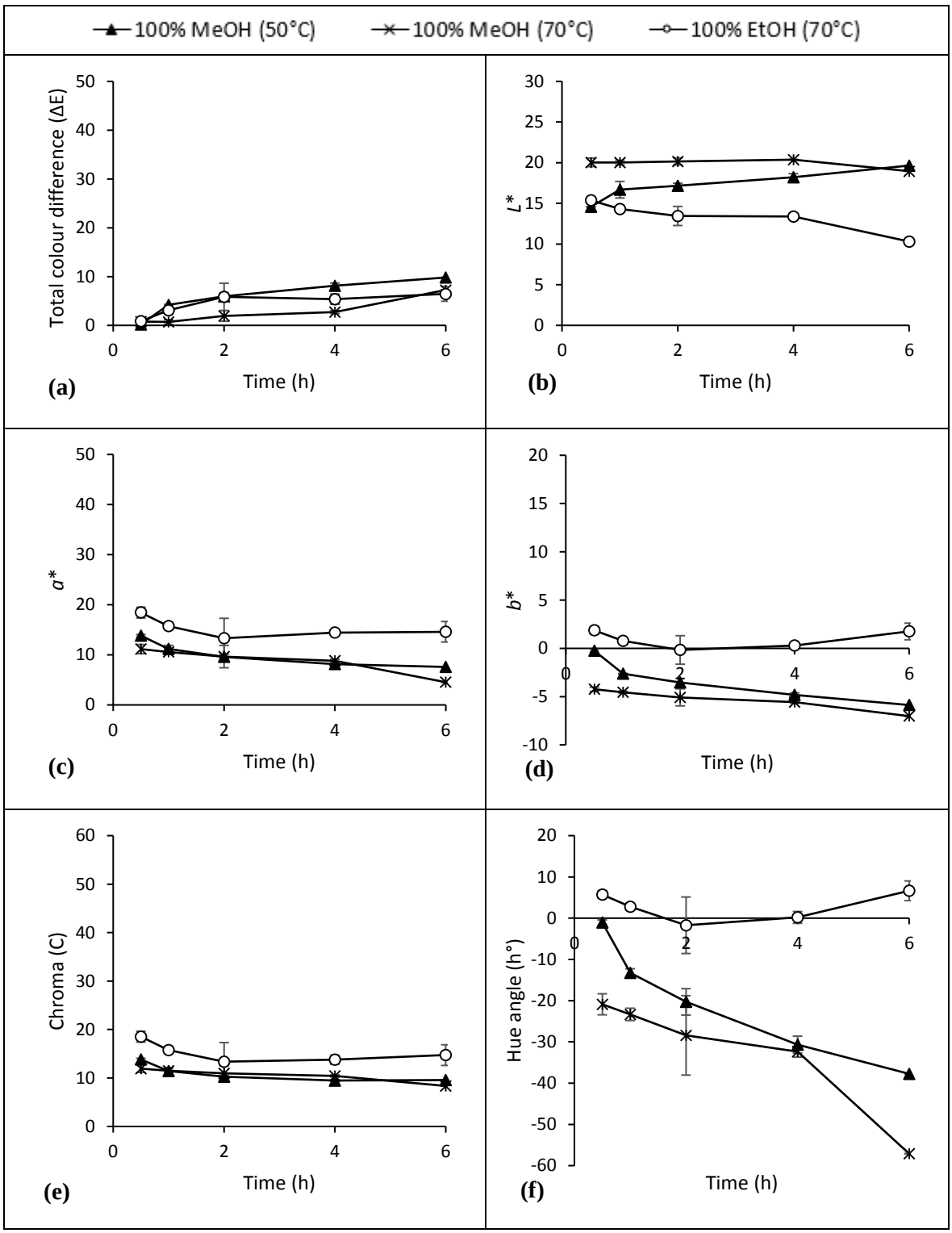

Total phenolics
Antioxidant activities
Total phenolics (mg GAE/g)
% inhibition
Time (h)
0.5
1
2
4
6
(a)
(b)
(c)
(d)
(e)
(f)



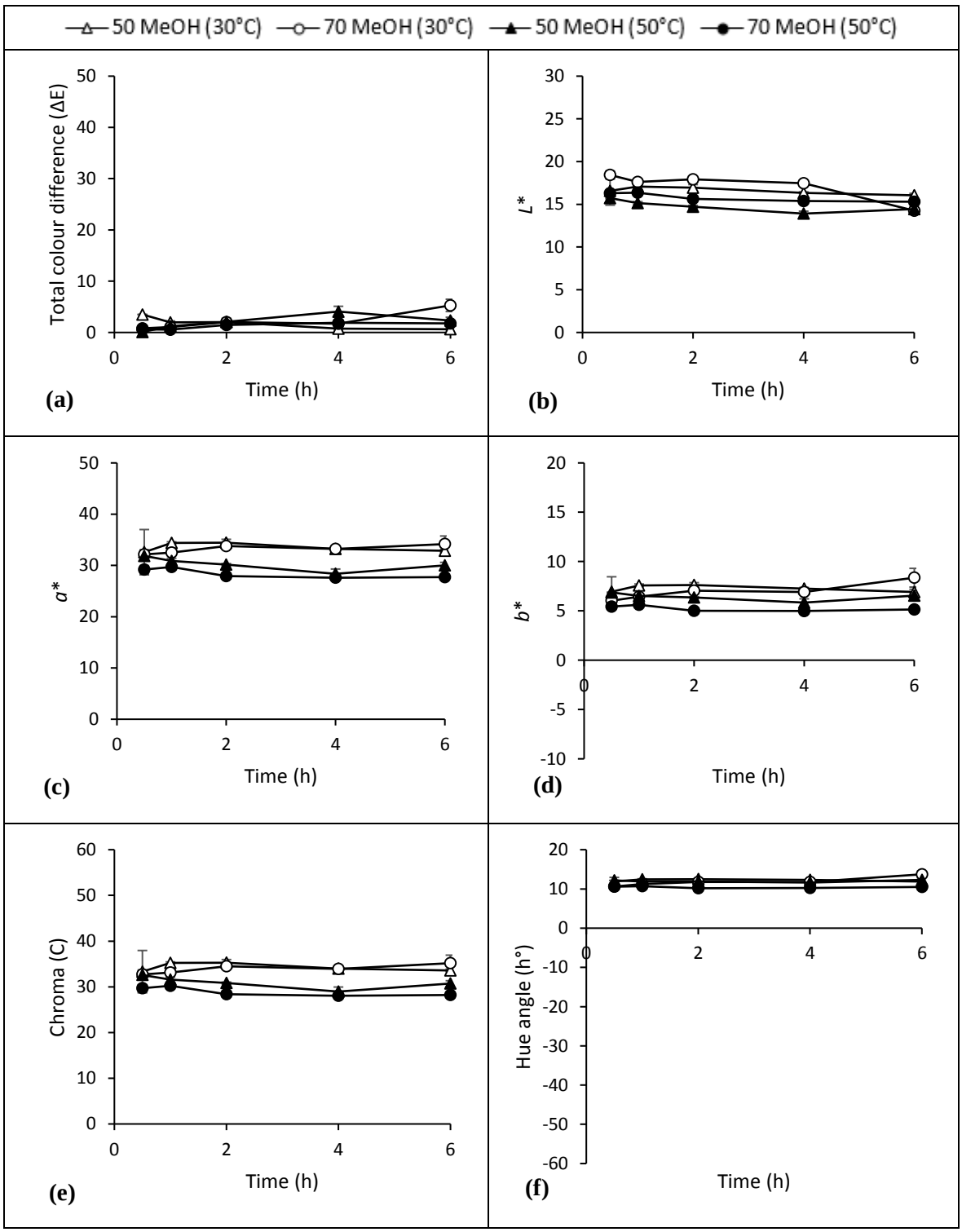
Supplementary Figure 4-8. Total phenolic and antioxidant activities based on the Folin-Ciocalteu and DPPH methods in (a) water (90 °C), (b) water (70 °C), (c) 100% ethanol (70 °C), (d) 100% methanol (70 °C), (e) 100% methanol (50 °C), (f) 70% methanol (50 °C), (g) 70% methanol (30 °C), (h) 50% methanol (50 °C), (i) 50% methanol (30 °C) (j) acetic acid buffer pH 1.5 (50 °C) and (k) acetic acid buffer pH 1.5 (30 °C) after 6 h of extraction as quantified by spectrophotometer.



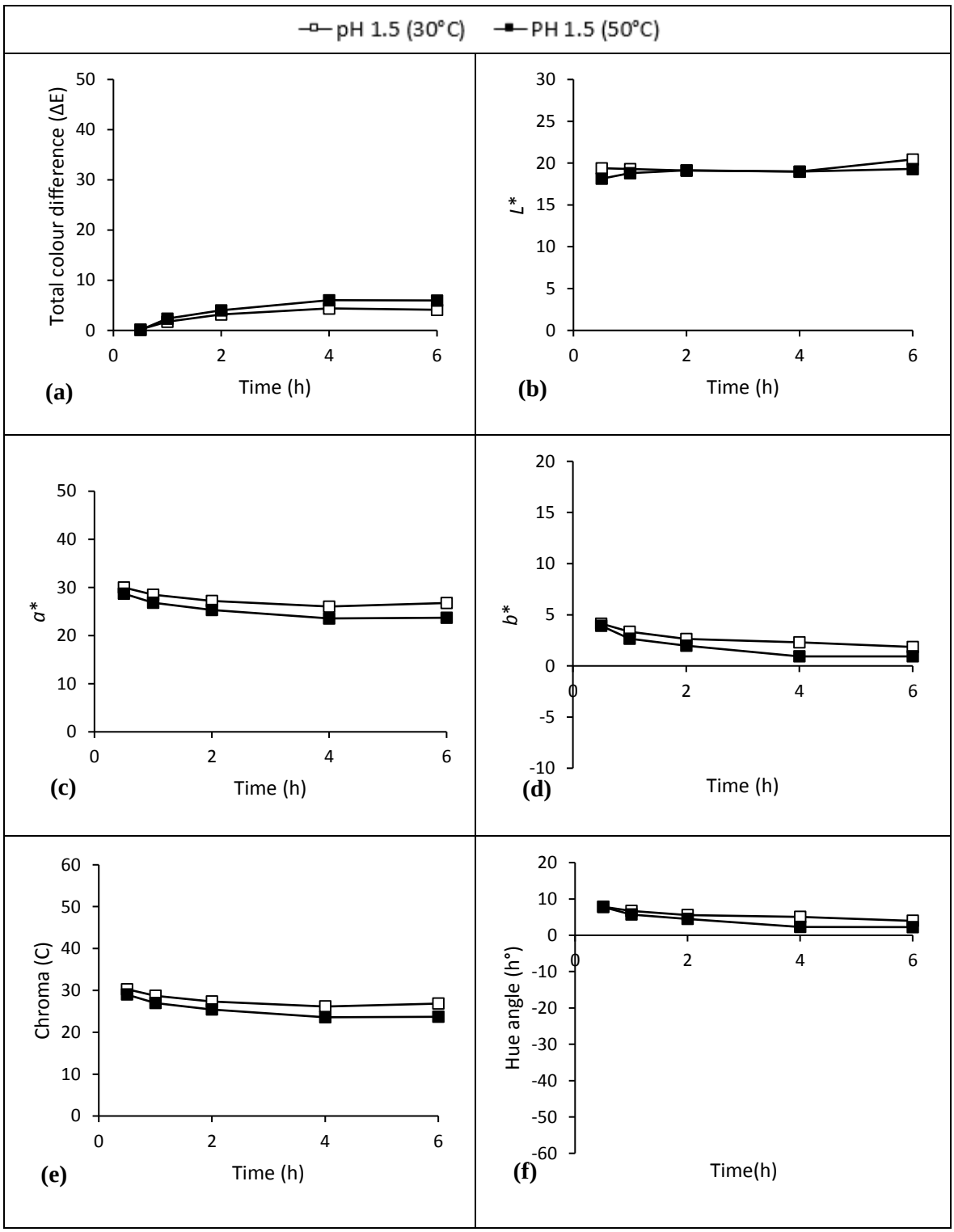
Supplementary Figure 4-9. (a) Total colour difference, (b) *L**, (c) *a**, (d) *b**, (e) chroma and (f) hue angle of DBS in water at 90 °C and 70 °C extracts after 6 h of extraction as measured using a Hunter-Lab colorimeter.



Supplementary Figure 4-10. (a) Total colour difference, (b) *L**, (c) *a**, (d) *b**, (e) chroma and (f) hue angle of DBS in 100% ethanol at 70 °C and 100% methanol (70 °C and 50 °C) extracts after 6 h of extraction as measured using a Hunter-Lab colorimeter.



Supplementary Figure 4-11. (a) Total colour difference, (b) *L**, (c) *a**, (d) *b**, (e) chroma and (f) hue angle of DBS in 70% methanol (50 °C and 30 °C) and 50% methanol (50 °C and 30 °C) after 6 h of extraction as measured using a Hunter-Lab colorimeter.



Supplementary Figure 4-12. (a) Total colour difference, (b) *L**, (c) *a**, (d) *b**, (e) chroma and (f) hue angle of DBS in acetic acid buffer pH 1.5 at 30 °C and 50 °C extracts after 6 h of extraction as measured using a Hunter-Lab colorimeter.

CHAPTER 5 – Stability of anthocyanins from blackcurrant (*Ribes nigrum* L.) skins during storage

Abstract

Blackcurrant contains non-acylated anthocyanins, which are generally not very stable during processing and storage. Intermolecular copigmentation is an approach that can be used to protect the anthocyanin chromophore (flavylium cation form) against nucleophilic attack of water molecules which consequently may result in the formation of colourless hydrated forms (chalcone or carbinol pseudobase). This is hypothesised to improve the stability, shelf life and colour of food products containing anthocyanins. In this study, these reactions were investigated using five pure phenolic acids as copigments, namely ferulic, caffeic, chlorogenic, tannic and rosmarinic acid, at pH 3.0 and pH 6.0 in buffer and solvent/buffer solutions. The molar ratio of copigment to anthocyanin extract was 5:1 and the samples were stored at 20 °C and 4 °C and their anthocyanin content and colour stability measured. Stronger hyperchromic and bathochromic effects were observed for the day 0 samples at pH 6.0 as opposed to pH 3.0, while overall, temperature and pH significantly influenced the copigmentation effect and colour stability during storage. At pH 3.0, ferulic acid showed a strong hyperchromic effect on the day of preparation, with 14.1 – 16.4 % increase in colour absorbance compared to the control, and was capable of maintaining very high levels of anthocyanin stability at 4 °C, (estimated half shelf life of 504.8 ± 13.0 days), whereas at 20 °C this was much lower (59.4 ± 2.0 days). Copigmentation with chlorogenic acid demonstrated the longest estimated half shelf life (117.1 ± 3.0 days) after storage at 20 °C. Rosmarinic acid at pH 6.0 showed the strongest hyperchromic effect among the phenolic acids at day 0, although after storage at 20 °C and 4 °C, the control sample exhibited higher half shelf life compared to the copigmented one (21.6 ± 0.7 over 52.9 ± 3.6 days, respectively). Caffeic acid proved to be the weakest copigment for both pH values. At

20 °C, browning took place faster most likely due to the activity of the enzyme polyphenol oxidase, especially at pH 6.0. Mixtures of solvent and buffer extended the anthocyanin shelf life and colour stability compared to buffer only samples. Overall, copigmentation at low storage temperature is recommended for applications in low pH, neutral and slightly alkaline food systems such as dairy products (e.g. ice-cream and milk).

Keywords: Blackcurrant skins, intermolecular copigmentation, pH, stability, storage, phenolic acids, anthocyanins

5.1 Introduction

There has been considerable interest by the food industry to replace synthetic food colourants with natural ones, especially those originating from vegetable and fruit sources (Slimestad & Solheim, 2002). This is due to consumer concerns on the safety of synthetic dyes and the increasing preference towards natural products. Anthocyanins are natural products that could be considered as candidates for the replacement of synthetic dyes due to their bright, attractive colours (orange, red, purple), high water solubility and possible health benefits (Malien-Aubert et al., 2001; Morais et al., 2002; Bueno et al., 2012; Cortez et al., 2017). However, the main challenges that need to be tackled before anthocyanins can be incorporated into food systems rely on the fact that as natural colourings, they have poor stability, dull shades and exhibit rapid colour fading when exposed to light, oxygen, high temperature, pH and enzymes such as polyphenoloxidases and peroxidases (Kammerer et al., 2007).

A particular problem with anthocyanins is their sensitivity to pH changes, since anthocyanins lose their colour at pH values of more than 3.0, and therefore this limits their use as natural colourants only into acidic food products (Haslam, 1998). Meanwhile, at higher pH values such as 6.0, anthocyanins are converted from their flavylium cation form

to their purple quinonoidal base form. At pH values of 6.0 and above, anthocyanins are labile and easily converted into colourless carbinol pseudobases and chalcone psuedobases by water nucleophilic attack (Parisa et al., 2007) (**Figure 5-1**).

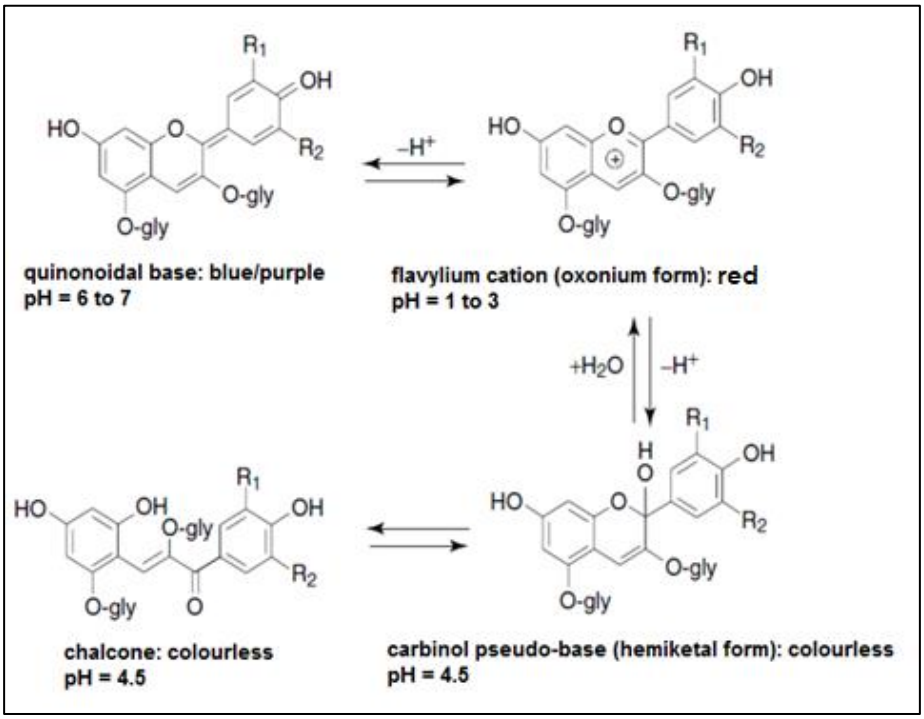


Figure 5-1. Different structures and colours of anthocyanins at various pH values (Ahmadiani, 2012).

The stability of anthocyanins can be improved by copigmentation. The types of copigmentation reactions include self-association, metal complex formation, as well as intermolecular and intramolecular copigmentation (Harborne, 2013). The term "intermolecular copigmentation" is especially reserved for anthocyanins, where a pigment (anthocyanin) and a copigment (flavonoid) are mixed directly by non-covalent bonds in a solution, as shown in **Figure 5-2**. The copigmentation is called "self-association" when two or more anthocyanin molecules are associated through stacking-like interactions, and was first suggested by Asen et al. (1972). The copigmentation reactions can improve the colour stability of anthocyanins and increase their shelf life, thereby making them possible to be commercially applied as natural colourants in food matrices. Previous studies have shown

that intermolecular copigmentation is influenced by the type of anthocyanins, the copigment structure and its concentration, the pH of the medium, temperature, storage conditions and the presence of organic co-solvents in the medium (Wilska-Jeszka & Korzuchowska, 1996). Phenolic acids, flavanols, alkaloids, metals and anthocyanins themselves (through self-association) are potent copigments for copigmentation reactions.

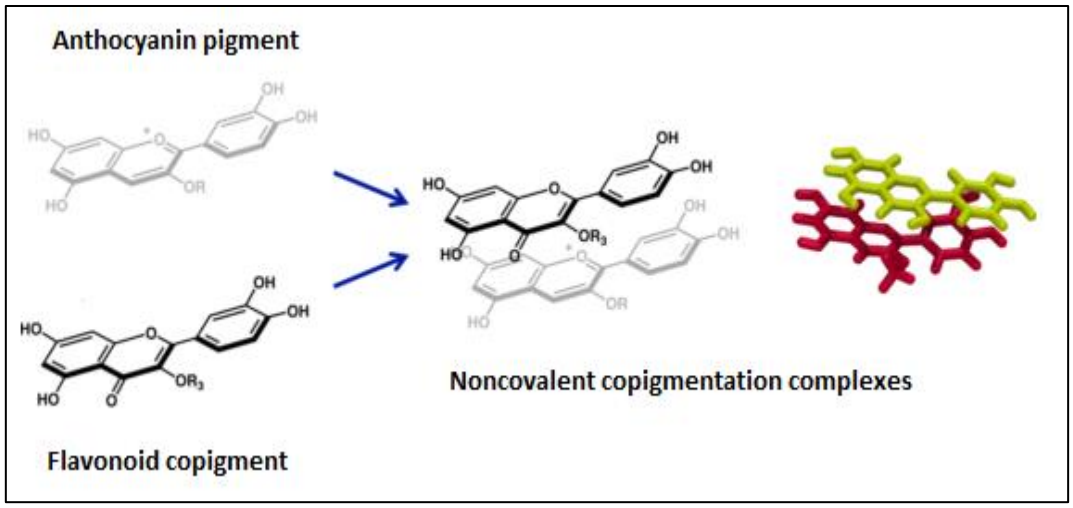


Figure 5-2. Copigmentation complexes between anthocyanin pigment and flavonoid copigment (intermolecular copigmentation). R and R_3 = H or sugar (Trouillas et al. 2016).

Hydrophobic and "π–π"-interactions between electron-rich copigments and electron-poor flavylium ions present in the anthocyanin molecules protect the anthocyanin chromophore (flavylium cation form) against nucleophilic attack of the water molecules, which may result in the formation of colourless hydrated forms (chalcone or carbinol pseudobase). Non-covalent complexes between copigments and anthocyanins form a parallel stacking phenomenon (**Figure 5-2**), and not only exert a protective effect on anthocyanins but also contribute to the colour stabilisation of the system (Brouillard et al., 1982; Mazza & Brouillard, 1987). This results in an increased absorbance of the copigmentation complex (hypherchromic effect; ΔA) whereas the wavelength of the maximum absorption shifts toward higher wavelengths (bathocromic shift, Δλ), which in turn increases the colour intensity.

Overall, this study had three main objectives: (i) to investigate the effect of low (pH 3.0) and high pH (pH 6.0) on the intermolecular copigmentation of anthocyanins extracted from dried blackcurrant skins (DBS); (ii) to evaluate the effect of buffer and solvent/buffer mixtures on copigmentation, and (iii) to assess the stability of the copigmentation complexes during storage at 20 °C and 4 °C.

5.2 Materials and methods

5.2.1 Chemicals

All solvents and chemicals used for extraction including methanol (99.9%) and ethyl acetate (99%), were of analytical grade and were purchased from Sigma-Aldrich (UK). Potassium chloride, potassium sorbate, sodium acetate, citric acid monohydrate and sodium phosphate were also purchased from Sigma-Aldrich (UK). Hydrochloric acid (37%) was purchased from Fisher Scientific (Loughborough, UK).

The buffers used in this study, i.e. pH 1.0 (potassium chloride, 0.025M) and pH 4.5 (sodium acetate, 0.4M) were prepared as described by Lee et al. (2005). Purified water, purified through a Purite reverse osmosis system (Oxon, UK), was used for buffer preparations as well as analyses. The copigments used included ferulic, caffeic, chlorogenic, tannic and rosmarinic acids and were purchased from Sigma-Aldrich (UK).

5.2.2 Sample preparation of plant materials

Dried blackcurrant pressed residues from a juice manufacturing process were kindly supplied by A & R House (BCL) Ltd, (Bleadon, Weston-super-Mare, UK). Dried blackcurrant used was a representative sample (315) from industrial rotary drying with high anthocyanins content as shown in Chapter 3. The seeds, which were present in the residue samples were separated by initially grinding the samples in a coffee blender and

subsequently passing them through a 0.841 mm (20 mesh) sieve; the obtained fraction constituted the dried blackcurrant skins (DBS) fraction. DBS samples were segregated in polyethylene bags and stored at -20 °C until further analysis.

5.2.3 Extraction of anthocyanins from DBS

Anthocyanins were extracted from DBS (50.0 g) according to the protocol by Stevenson et al. (2006) which involved steeping the DBS sample in 250 mL of 0.1% (v/v) HCl in methanol for 1 h. The obtained extracts were vacuum filtered to separate the supernatants and solid residues and the latter underwent a second extraction with 250 mL of fresh solvents for another 1 h. The extraction process was repeated 3 times and the supernatants were pooled together and evaporated under vacuum by a rotary evaporator to remove methanol. 100 mL of distilled water were then added to the residue; this constituted the crude anthocyanin extract sample.

5.2.4 Purification of anthocyanin extract

The anthocyanin-containing extract was introduced into a Strata® C18-U (55 µm, 70 Å, 500 mg / 6 mL) cartridge (Phnenomenex Ltd., Macclesfield, UK) after 5-fold dilution with distilled water. The extract was then washed with 12.0 mL of 0.01% (v/v) HCl in water and subsequently with 12.0 mL of ethyl acetate in order to remove non-phenolic compounds as well as phenolic compounds other than anthocyanins, respectively (Rodriguez-Saona & Wrolstad, 2001). Finally, anthocyanins were eluted with 0.01% (v/v) HCl in methanol, followed by evaporation to remove the solvents and then 100 mL of distilled water were used to solubilise the residue. The purified crude extract was stored at -20 °C until further analysis.

5.2.5 HPLC analysis of anthocyanins

HPLC analyses were performed on an 1260 Infinity HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array detector (DAD) using a Zorbax C18 column (250 × 4.6 mm i.d., particle size 5 µm, Agilent) at 30 °C. The mobile phase consisted of 5% (v/v) formic acid (solvent A) and 100% (v/v) methanol (solvent B). The gradient elution system was: 15% (B) at 0 min, increasing to 35% (B) at 15 min and to 60% (B) at 30 min, and reaching 80% (B) at 40 min. The flowrate was 1.0 mL/min and the injection volume was 20 µL. The duration of analysis was 50 min.

Detections were carried out at a wavelength of 520 nm for anthocyanins. Briefly, 2.0 mg/mL of stock standard solutions were prepared separately followed by dilution to give concentrations from 0.01 to 1.0 mg/mL. To obtain the standard curves, the peak areas were plotted against the corresponding concentrations of the standard solutions injected into HPLC.

5.2.6 Preparation of buffer and solvent/buffer solutions

Buffer solutions of pH 3.0 and 6.0 were prepared in accordance with **Table 5-1**. The pH value of each buffer solution was adjusted with 5 M HCl or 5 M NaOH and measured with a pH meter (Mettler Toledo Seven Easy, China). The pH meter was calibrated with standard solutions of pH 7.0 and 4.0 (standards, Nalgon).

Table 5-1. Solvent proportions (v/v) used to prepare citric acid-sodium phosphate buffer solutions (Sigma, 2018).

pH	0.1 M Citric acid (mL)	0.2 Sodium phosphate (mL)
3.0	79.45	20.55
6.0	36.85	63.15

Mixtures of solvent/buffer solutions were made up by replacing 20% (v/v) of each buffer solution with ethanol. Then, the pH values of the mixtures were adjusted with either 5 M HCl or 5 M NaOH, as described above.

5.2.7 Copigmentation reactions and stability studies

Concentration of anthocyanins solution. Different concentrations of purified extracts ranging from 0.002-0.016 (v/v) and 0.008-0.18 (v/v) were prepared in buffer and solvent/buffer solutions at pH 3.0 and pH 6.0, respectively. Calibration curves were obtained by plotting the absorbance at λ_{max} measured by UV-Vis spectrophotometer as a function of the anthocyanin concentration. A maximum absorbance value close to 1.000 was used to determine the concentration of anthocyanin extract to be added into the buffer and solvent/ buffer solutions.

Copigmentation reactions. The molar ratio of copigments to anthocyanins extract (expressed as cyanidin-3-O-glucoside) was set to 5:1 based on a previous study by Shikov et al. (2008). Five phenolic acids were used for the copigmentation studies, namely ferulic, caffeic, chlorogenic, tannic and rosmarinic acid (**Figure 5-3** depicts their chemical formulae). The weight of copigments (phenolic acids) to be added into the buffer and solvent/buffer solutions was calculated to achieve a 5:1 molar ratio of copigment to anthocyanin extract. All phenolic acids were used as copigments in the buffer solutions, whereas only chlorogenic acid was used in the case of the solvent/buffer solution.

Both control samples (purified anthocyanin extract without phenolic acids) and test samples (copigmentation of anthocyanins with phenolic acids) were prepared in amber bottles with lids (25 ml) in either buffer or solvent/buffer solutions, containing 0.05% potassium sorbate (w/v) to inhibit potential microbial growth. The pH of each sample was

adjusted using 5 M HCl or 5 M NaOH. The solutions were shaken for 30 minutes and left to rest for 2 h in the dark at room temperature to reach equilibrium (Fuleki and Francis, 1968).

Stability studies. The samples were kept in the dark at 20 °C and 4 °C. For the pH 3.0 and 6.0, samples were analysed until 140 days or until the time point were at least 50% of the anthocyanins were degraded. The stability experiments were carried out in duplicate.

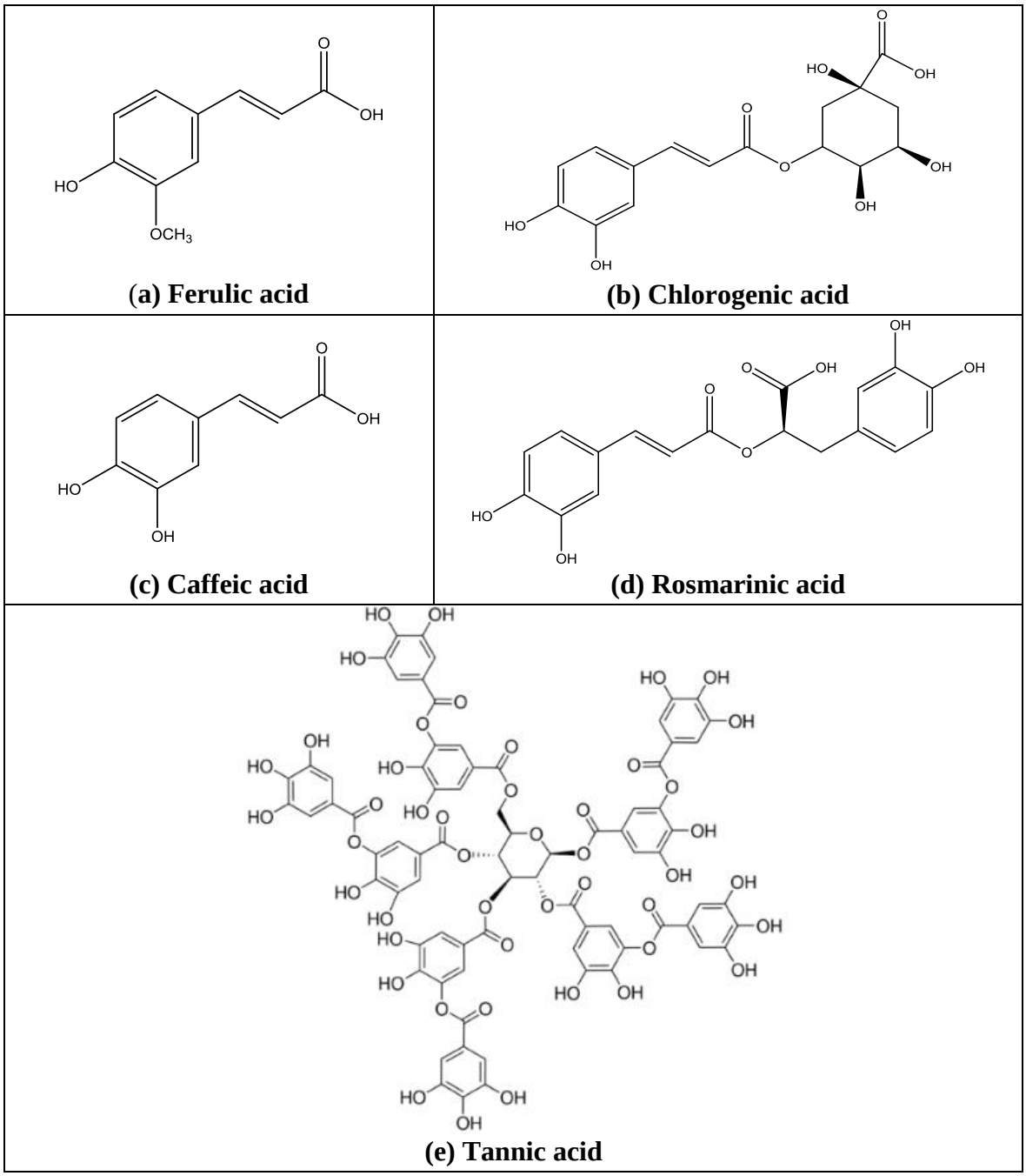


Figure 5-3. Chemical structures of phenolic acids used as copigment in intermolecular copigmentation complexes. Structures plotted using the ChemDraw website (2018).

5.2.8 UV-Vis spectrophotometry and colour measurements

Spectrophotometric measurements were carried out using a UV-Vis spectrophotometer (Perkin Elmer Lambda 20, USA). The spectra were recorded in 1-cm path length disposable cuvettes at wavelength from 450 to 700 nm. Copigmentation phenomena were related with a bathochromic shift, i.e. a shift of the maximum absorption wavelength (λ_{max}) and a hyperchromic effect, i.e. an increase in the absorbance value (ΔA) at λ_{max}. Each spectroscopic measurement was performed in duplicate. The percentage of colour retention at λ_{max} was used to understand the colour degradation at different pH over time and was calculated according to the following formula:

$$\text{Colour Retention (\%)} = \frac{At}{Ao} \times 100$$

where A_0 is the initial absorbance at time zero and A_t is the absorbance at time t (Katsaboxakis et al., 1998).

Sample colour was measured using a Hunter-Lab colourimeter (Hunter Lab, ColourQuest, Hunter Associates Laboratory, Virginia, USA) based on three colour coordinates L^*, a^*, and b^* at D65 standard illuminant and 10° standard observer. The instrument was calibrated using a black card. Colour was expressed in Hunter Lab units L^* (Lightness/ darkness; 0-100), a^* (positive = redness/negative = greenness) and b^* (positive = yellowness/negative = blueness). The total colour difference (TCD) between two samples was calculated according to the following formula:

$$Total\ colour\ difference\ (TCD) = [(L^* - L_o)^2 + (a^* - a_o)^2 + (b^* - b_o)^2]^{1/2}$$

where, L_o, a_o, b_o = blank values of control samples extracted at day 0

Chroma (c) is the quantitative attribute of colour intensity and hue (h) is the qualitative attribute of colours which are defined as reddish, greenish, yellowish and bluish. The chroma and hue angle were calculated using the a^* and b^* values according to the equations below (Wrolstad & Smith, 2010).

$$Chroma\ (c) = \sqrt{(a^*)^2 + (b^*)^2}$$

$$Hue\ angle\ (h) = ArcTan(\frac{b^*}{a^*})$$

5.2.9 Determination of total monomeric anthocyanins

The total anthocyanin content of DBS was determined by the differential pH method based on the property of anthocyanin pigments to change colour in different pH values. 600 µL of anthocyanins-containing solutions were mixed with 1.2 mL of corresponding buffer (pH 1.0 and pH 4.5) and allowed to equilibrate for 20 minutes. Total monomeric anthocyanins (mg cyanidin-3-O-glucoside equivalents/g DW) were calculated as follows:

$$C_{anthocyanin} = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times 1}$$

where $C_{anthocyanin}$ = Total monomeric anthocyanin (cyanidin-3-O-glucoside equivalents, mg/L); A (absorbance) = ($A_{520nm} - A_{700nm}$) at pH 1.0 – ($A_{520nm} - A_{700nm}$) at pH 4.5; MW (molecular weight) = 449.2 g/mol for cyanidin-3-O-glucoside; DF = dilution factor; l = pathlength in cm; ε = 26900 molar extinction coefficient in L/mol/cm for cyanidin-3-O-glucoside; and 10^3 = factor for conversion from g to mg (Lee et al., 2005). The anthocyanin content was then calculated by:

$$Anthocyanin\ content(mg/g) = \frac{C\ anthocyanin\ \left(\frac{mg}{L}\right) \times extract\ (L)}{sample\ (g)}$$

The absorbance of the samples at pH 1.0 and pH 4.5 buffer, respectively, was determined at both 520 and 700 nm using a spectrophotometer (Thermo Electron Corporation, USA). The absorbance of the samples was read against distilled water (blank).

5.2.10 Kinetic study

The zero order reaction rate constants (k) and half-lives ($t_{1/2}$) describing anthocyanin degradation during storage were calculated using the following equations:

$$C_t = C_0 - k*t$$

$$t_{1/2} = \frac{Co}{2k}$$

where C_0 is the initial anthocyanin concentration and C_t is the anthocyanin concentration at time t (Remini et al., 2015).

5.2.11 Statistical analysis

All statistical analyses were conducted by one-way analysis of variance (ANOVA). Tukey's multiple range tests were employed with a probability of $p \leq 0.05$. The linear Pearson correlation was also used to evaluate correlations between anthocyanin half shelf life and percentage of colour retention. The software used for statistical analysis was Minitab V.16 (Minitab Inc., State College, Pennsylvania, USA).

5.3 Results and discussion

The DBS extracts contained four types of non-acylated anthocyanins, namely delphinidin-3-O-glucoside, cyanidin-3-O-glucoside, delphinidin-3-rutinoside and cyanidin-3-O-rutinoside (**Supplementary Figure 5-6**). The anthocyanin content in the purified extracts was calculated as 9.0 ± 0.1 mg/g, corresponding to 88.1 ± 0.6 % of anthocyanin recovery from the original DBS samples. According to the HPLC results, the concentrations of purified anthocyanins extract (expressed as cyanidin-3-O-glucoside) was 9.3×10^{-3} M. Therefore, the concentrations of purified anthocyanins extract used to obtain an initial absorbance close to 1.000 in buffer solutions at pH 3.0 and 6.0 were 0.009 (v/v) (8.9×10^{-5} M) and 0.057 (v/v) (5.4×10^{-4} M), respectively. The weight of copigments (phenolic acids) added to 25 mL of buffer and solvent/buffer solutions are shown in **Table 5-2**.

Table 5-2. Amount of phenolic acids (mg) added into 25 mL of buffer and solvent/buffer solutions at pH 3.0 and 6.0. Molar ratio of copigment to anthocyanin extract (5:1).

Copigments	pH 3.0 (mg)	pH 6.0 (mg)
Ferulic acid	2.2	13.0
Caffeic acid	4.0	23.7
Chlorogenic acid	2.0	12.1
Tannic acid	19.0	113.9
Rosmarinic acid	4.0	24.1

5.3.1 Effect of copigmentation on UV-Vis absorption spectra

Copigmentation effect in buffer solutions at pH 3.0 and pH 6.0. In this study, the pH values of the buffer solutions were set to pH 3.0 and 6.0, in order to compare the effect of low and high pH on copigmentation. The copigmentation of anthocyanins with phenolic acids in buffer solutions resulted in an increase in the maximum absorption wavelength (bathochromic effect, $\Delta\lambda_{max}$) and absorbance (hyperchromic effect, ΔA_{max}), which in turn

increased the colour intensity of the anthocyanin solutions on the day of preparation (day 0) as shown in **Figure 5-4**.

As it can be seen in **Figure 5-4 (a)** and **Table 5-3 (a and b)**, ferulic acid had the highest percentage of hyperchromic effect (ΔA = 14.1 – 16.4%) and bathochromic shift (Δλ = 4 – 6) in buffer solutions at pH 3.0, followed by rosmarinic acid (12.6 – 14.0%; Δλ = 3 – 4), caffeic acid (12.2 – 12.3%; Δλ = 3 – 5), chlorogenic acid (8.8 – 12.3%; Δλ = 1 – 2) and tannic acid (7.4 – 9.1%; Δλ = 1), as compared to the control (no copigment). This is due to the interactions between the flavylium cation and the copigment which increased the hyperchromic effect to a maximum absorbance (Parisa et al., 2007). In addition, ferulic acid contains methoxylated derivatives that induce a stronger copigmentation effect than phenolic acids with hydroxyl substituents such as rosmarinic, chlorogenic, caffeic and tannic acid (**Figure 5-3**) (Marković et al., 2000).

Marković, et al. (2000) also reported that the copigmentation of malvidin 3, 5-diglucoside with ferulic acid in buffer solution at pH 2.50 and pH 3.65 showed higher bathocromic and hyperchromic effects compared to caffeic acid. Furthermore, Eiro and Heinonen (2002) demonstrated that at pH 3.37 in a 1:100 molar ratio of cyanidin-3-glucoside to phenolic acid, rosmarinic and ferulic acid were the best copigments enhancing the colour of the complex by 80% and 70%, respectively. Similarly, rosmarinic acid was proved a better copigment than chlorogenic acid in mixtures of purple sweet potato anthocyanins (Trouillas et al., 2016). Rosmarinic acid exhibits two aromatic ring systems, each substituted with two hydrogen bond donor/acceptor OH-groups, while chlorogenic acid only contains a single system of delocalised π-conjugated electrons (**Figure 5-3**). Thus, rosmarinic acid may be able to form stronger π-π stacking interactions with anthocyanin molecules, thereby resulting in colour enhancement.

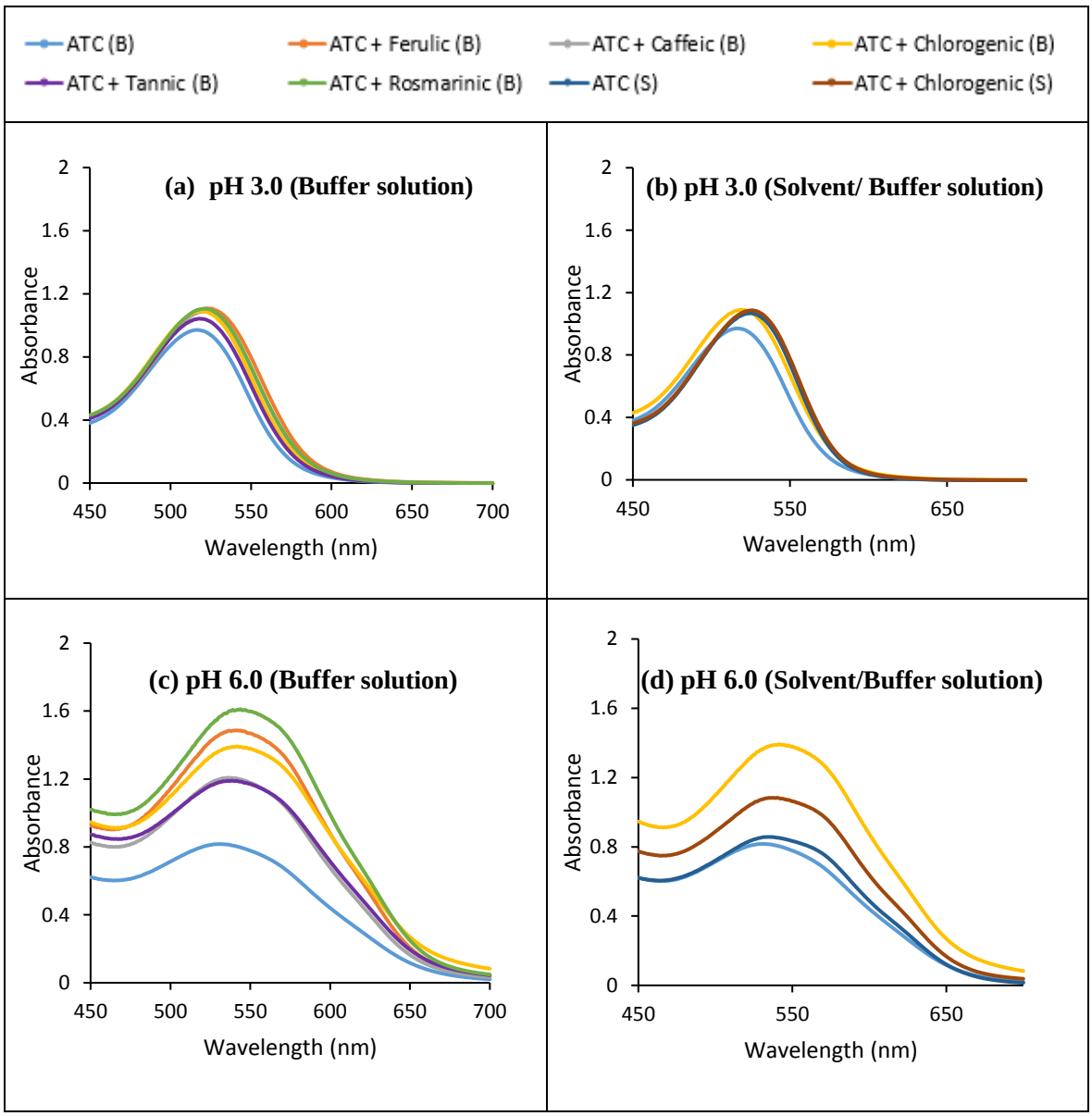


Figure 5-4. Hyperchromic effect and bathochromic shift of copigmented anthocyanins from DBS in: (a) buffer solution pH 3.0, (b) solvent/buffer solution pH 3.0, (c) buffer solution pH 6.0 and (d) solvent/buffer solution pH 6.0 at Day 0. (copigment: anthocyanin molar ratio 5:1). *Anthocyanins (ATC); Buffer solution (B); Solvent/Buffer solution (S).

In the case of a more neutral pH environment, as shown in **Figure 5-4 (c)** and **Table 5-3 (c** and **d)**, there was a significant ($p \leq 0.05$) increase in the percentage of hyperchromic effect ($\Delta A = 96.6 – 97.3\%$) and bathochromic shift ($\Delta\lambda = 10 – 14$) at pH 6.0 especially when using rosmarinic acid as copigment. On the other hand, caffeic and tannic acid had the lowest increase ($\Delta A = 47.5 – 55.7\%$; $\Delta\lambda = 3 – 5$ and $\Delta A = 45.6 – 59.4\%$; $\Delta\lambda = 4 – 9$, respectively). The increase in the λ_{max} and A_{max} values suggested that copigmentation also occurred at

pH 6.0, in agreement with Boulton (2001) who confirmed that copigmentation showed greater intensity in higher pH with neutral quinonoidal bases.

Copigmentation effect in solvent/buffer solutions at pH 3.0 and 6.0. In order to investigate the effect of the solvent on copigmentation, chlorogenic acid was added into solvent/buffer solutions and the latter were set to pH 3.0 and 6.0. Chlorogenic acid when used as copigment in the buffer solutions, showed a consistent reading in the maximum absorption wavelength (λ_{max}) during storage in the preliminary test. After the addition of chlorogenic acid at pH 3.0 and 6.0, significantly higher ($p \leq 0.05$) percentages of hyperchromic effect and bathochromic shift were noted (ΔA = 1.8 – 1.9 %; $\Delta\lambda$ = 1 nm and ΔA = 26.4 – 27.8%; $\Delta\lambda$ = 2 – 6, respectively) compared to the control sample without copigment (**Table 5-3**). According to the studies by Wilska-Jeszka & Korzuchowska (1996) and Kemp (1991), even the addition of 5% ethanol (higher polarity) tends to increase the exothermicity of the copigmentation reaction, which interrupts the physical association in copigmentation and modifies the wavelength of the maximum absorbance. As a result, the maximum absorbance (A_{max}) and wavelength (λ_{max}) in the solvent/buffer solutions were substantially higher than in the buffer solutions, especially at pH 3.0.

Table 5-3. Effect of intermolecular copigmentation on the maximum absorption wavelength (λ_{max}) and maximum absorbance (A_{max}) of anthocyanin solutions of DBS extracts with phenolic acids as copigments in buffer and solvent/buffer solutions at Day 0 (copigment: anthocyanin molar ratio 5:1).

Samples	(a) pH 3.0 (20 °C)			(b) pH 3.0 (4 °C)			(c) pH 6.0 (20 °C)			(d) pH 6.0 (4 °C)		
	λ_{max} (nm)	**A_{max}**	**ΔA (%)**	**λ_{max} (nm)**	**A_{max}**	**ΔA (%)**	**λ_{max} (nm)**	**A_{max}**	**ΔA (%)**	**λ_{max} (nm)**	**A_{max}**	**ΔA (%)**
ATC (B)	517	0.9641	–	517	0.9702	–	531	0.8237	–	530	0.8169	–
ATC + Ferulic (B)	521	1.1219	16.4 (0.0)^{a}	523	1.1075	14.1 (0.1)^{a}	539	1.4891	80.8 (0.5)^{b}	541	1.4860	81.9 (0.0)^{b}
ATC + Caffeic (B)	520	1.0829	12.3 (0.6)^{ab}	522	1.0885	12.2 (0.0)^{b}	536	1.2823	55.7 (0.2)^{d}	533	1.2047	47.5 (0.0)^{d}
ATC + Chlorogenic (B)	518	1.0485	8.8 (0.3)^{b}	519	1.0893	12.3 (0.0)^{b}	539	1.4167	72.0 (0.2)^{c}	542	1.3914	70.3 (0.3)^{c}
ATC + Tannic (B)	518	1.0518	9.1 (0.3)^{b}	518	1.0419	7.4 (0.1)^{c}	535	1.3127	59.4 (4.5)^{d}	539	1.1893	45.6 (0.2)^{d}
ATC + Rosmarinic (B)	520	1.0859	12.6 (1.3)^{ab}	521	1.1058	14.0 (0.0)^{a}	541	1.6195	96.6 (0.3)^{a}	544	1.6113	97.3 (0.1)^{a}
ATC (S)	524	1.0989	–	525	1.0675	–	535	0.8977	–	534	0.8568	–
ATC + Chlorogenic (S)	525	1.1200	1.9 (0.0)^{c}	526	1.0864	1.8 (0.0)^{d}	537	1.1470	27.8 (0.6)^{e}	540	1.0830	26.4 (0.0)^{e}

*Absorbance (A); Absorbance increase (ΔA); Anthocyanins (ATC); Buffer solution (B); Solvent/Buffer solution (S).
Figures in parentheses indicate the standard deviation.
Values with the same letter ^{a,b} in each column are not significantly different ($p > 0.05$).

5.3.2 Stability of total monomeric anthocyanins

In the subsequent stability tests, the DBS anthocyanin copigment complexes were kept at 20 ± 1 °C and 4 ± 1 °C and the half shelf life and colour stability of the anthocyanin solutions in buffer and solvent/buffer solutions at pH 3.0 and 6.0 were evaluated for up to 140 days and until at least 50% of the pigments were degraded, respectively. **Figure 5-5** shows that the degradation of anthocyanins followed zero order reaction kinetics, with a correlation coefficient (R^2) of greater than 0.900 in all cases.

A high percentage of colour retention, measured by UV-Vis spectrophotometry, reflects a high colour stability of the anthocyanin solution and consequently low colour degradation over time. The changes in the colour of the samples were expressed in terms of colour values (*L**, *a**, *b**) by Hunter Lab. In addition, a high chroma value indicates a high colour intensity, which is accompanied with an increase in absorbance in the visible range (hyperchromic shift) and a shift in the wavelength of the maximum absorbance toward higher values (bathochromic shift) (Hurtado et al., 2009). At day 0 in pH 3.0 and 6.0, copigmentation in buffer solutions and especially with ferulic and rosmarinic acid appeared to have a significant ($p \leq 0.05$) impact on the colour of the solutions. In particular, the *L** (darker colour) and *b** (increase in blueness) values decreased, whereas the *a** (decrease in redness) and chroma values increased, indicating the enhancement of colour intensity (**Supplementary Figure 5-8** to **Supplementary Figure 5-11**).

Copigmentation stability in buffer solutions at pH 3.0. As shown in **Table 5-4 (a)**, at 20 °C the presence of chlorogenic acid significantly increased ($p \leq 0.05$) the half shelf life of anthocyanin in buffer solutions to 117.1 ± 3.0 days, followed by rosmarinic acid (chlorogenic acid > rosmarinic acid > control > tannic acid > ferulic acid > caffeic acid). This is due to the

fact that chlorogenic and rosmarinic acids prevented the hydration of the flavylium cation on the anthocyanin structure which could form the colourless carbinol pseudobase, and consequently stabilised the anthocyanin better than the other copigments. Surprisingly, anthocyanin without any copigment (control) in the buffer solution had a significantly ($p \leq 0.05$) higher estimated half shelf life (93.2 ± 1.2 days) compared to the anthocyanin solutions that contained ferulic and caffeic acid.

Table 5-4. Effect of intermolecular copigmentation on the estimated half-life time values (t ½, days) of anthocyanins from DBS extracts with phenolic acids as copigments in buffer and solvent/buffer solutions.

Samples	(a) pH 3.0 (20 °C)			(b) pH 3.0 (4 °C)			(c) pH 6.0 (20 °C)			(d) pH 6.0 (4 °C)		
	k	**$t_{1/2}$ (days)**	**R^2**	**k**	**$t_{1/2}$ (days)**	**R^2**	**k**	**$t_{1/2}$ (days)**	**R^2**	**k**	**$t_{1/2}$ (days)**	**R^2**
ATC (B)	0.1223	93.2 (1.2)^{c}	0.9940	0.0205	569.2 (25.6)^{a}	0.9818	0.5046	21.6 (0.7)^{b}	0.9921	0.2259	52.9 (3.6)^{b}	0.9570
ATC + Ferulic (B)	0.1996	59.4 (2.0)^{d}	0.9949	0.0246	504.8 (13.0)^{ab}	0.9489	0.9965	9.7 (0.5)^{d}	0.9722	0.3336	31.8 (1.3)^{c}	0.9869
ATC + Caffeic (B)	0.2831	43.0 (1.6)^{d}	0.9375	0.0659	172.1 (4.6)^{d}	0.9625	2.2056	4.8 (0.2)^{d}	0.9826	0.6563	16.3 (0.6)^{e}	0.9958
ATC + Chlorogenic (B)	0.1180	117.1 (3.0)^{b}	0.9537	0.0244	490.8 (28.9)^{bc}	0.9732	0.7331	15.3 (1.3)^{c}	0.9858	0.5254	21.1 (0.7)^{de}	0.9998
ATC + Tannic (B)	0.1351	94.3 (8.0)^{c}	0.9840	0.0257	460.0 (10.1)^{bc}	0.9638	1.2917	8.2 (0.6)^{d}	0.9895	0.5139	22.7 (0.2)^{d}	0.9991
ATC + Rosmarinic (B)	0.1244	98.5 (4.7)^{bc}	0.9832	0.0269	423.9 (23.4)^{c}	0.9605	1.1364	8.8 (0.5)^{d}	0.9963	0.6628	18.2 (1.1)^{e}	0.9696
ATC (S)	0.0817	162.2 (11.5)^{a}	0.9895	0.0260	486.3 (14.9)^{bc}	0.9137	0.3503	31.6 (1.2)^{a}	0.9179	0.1512	75.8 (1.3)^{a}	0.9937
ATC + Chlorogenic (S)	0.1141	111.5 (1.5)^{b}	0.9885	0.0281	424.6 (5.3)^{c}	0.9690	0.4118	26.2 (3.1)^{b}	0.9255	0.2135	53.5 (1.2)^{b}	0.9864

*Estimated half-life time ($t_{½}$); Zero order reaction rate constants (k); Correlation coefficient (R^2); Anthocyanins (ATC); Buffer solution (B); Solvent/Buffer solution (S).

Figures in parentheses indicate the standard deviation.

Values with the same letter ^{a,b} in each column are not significantly different ($p > 0.05$).

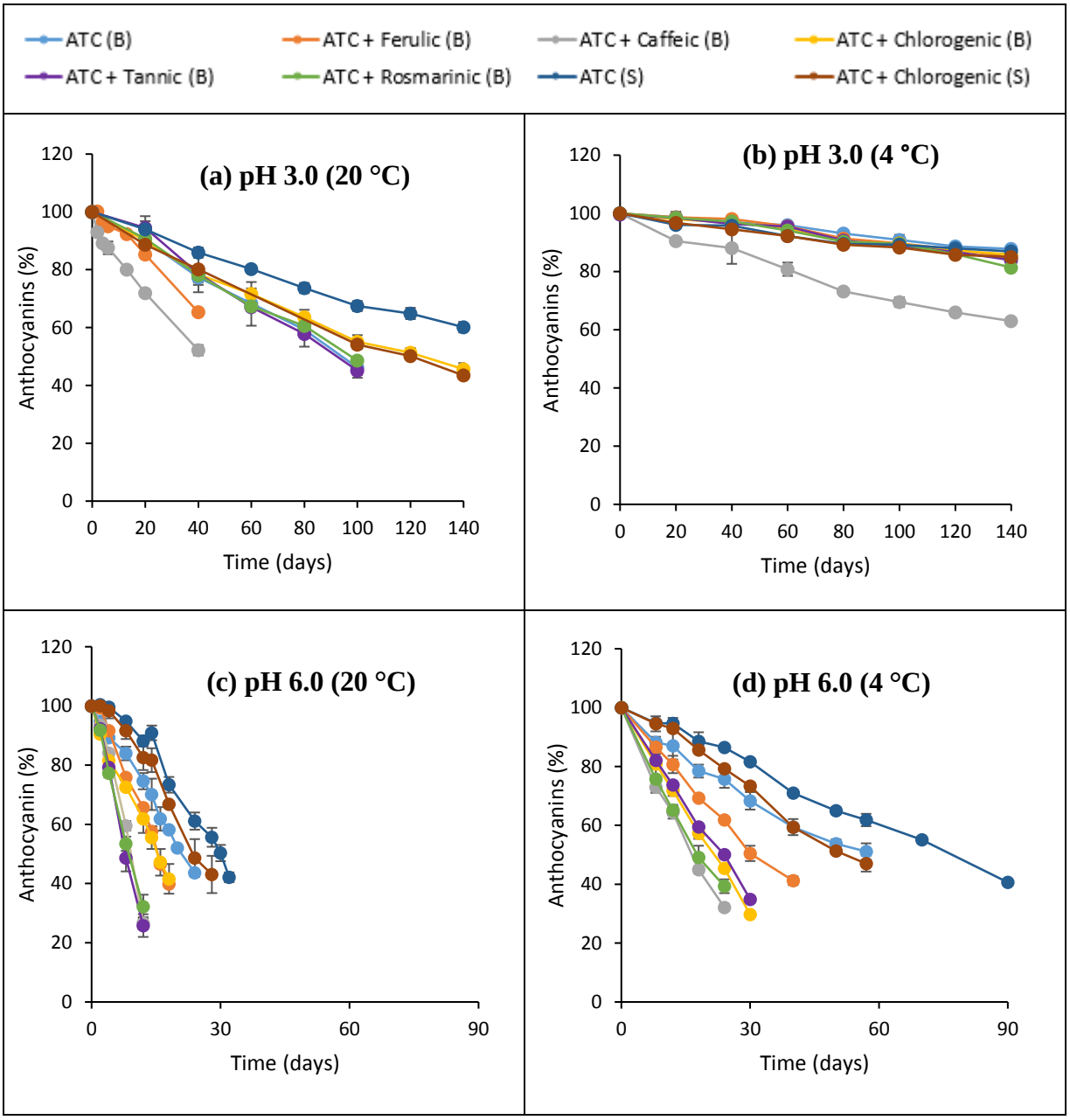


Figure 5-5. Degradation kinetics of anthocyanin content (%) of DBS extracts with phenolic acids as copigments during storage at 20 °C and 4 °C in buffer and solvent/buffer solutions. *Anthocyanins (ATC); Buffer solution (B); Solvent/Buffer solution (S).

According to **Table 5-4 (b),** after 140 days of storage at low temperature (4 °C), the samples with intermolecular copigmentation with ferulic acid exhibited slightly shorter estimated anthocyanin half shelf life (504.8 ± 13.0 days) compared to the control sample (569.2 ± 25.6 days). On the other hand, chlorogenic (~490.8 days), tannic (~460.0 days) and rosmarinic

acid (~423.9 days) exhibited lower stability. Similar to the results with the storage studies at 20 °C, caffeic acid had significantly lower estimated anthocyanin half shelf life compared to the other copigments during storage at 4 °C.

The observation that the control samples showed a longer half shelf life compared to some of the copigmentation samples can be possibly attributed to the fact that molecular complexes with other anthocyanins (self-association) are more stable compared to other colourless compounds (intermolecular copigmentation) as suggested by Trouillas et al. (2016). Moreover, it has been reported that self-associated copigmentation provides anthocyanin extracts with enhanced thermal resistance against the heat that is introduced during processing and storage as opposed to copigmentation with phenolic acids via π-π complexes (Qian et al., 2017). It is likely that the addition of certain phenolic acids during the copigmentation process interrupts the association between self-stacking anthocyanins and accelerates anthocyanin degradation.

The stability of copigmentation during storage was also measured based on the colour retention (%). However, the best copigmentation complexes as seen at day 0 were unable to maintain their colour stability throughout their storage at 20 °C. For example at pH 3.0, even though ferulic and caffeic acid were among the best colour enhancers, the anthocyanin contents [**Figure 5-5 (a)**] and the wavelength of the maximum absorbance ($\lambda_{max)}$ [**Supplementary Figure 5-7 (a)**] gradually decreased during storage (hypsochromic shift). As shown in **Table 5-4 (a)**, ferulic and caffeic acid showed significantly low ($p \leq 0.05$) estimated half shelf life, 59.4 ± 2.0 and 43.0 ± 1.6 days, respectively, indicating the instability of both copigments.

Except for ferulic and caffeic acid as copigment, a moderate correlation ($R^2 = 0.837$, $p \leq 0.05$) was seen between the anthocyanin half shelf life and colour retention, suggesting that

the anthocyanin content correlates with colour stability. Even though after 140 days high percentage of colour retention for ferulic (55.3 ± 3.3 %) and caffeic acid (55.3 ± 2.6 %) was seen in the UV-Vis spectrum [**Table 5-5 (a)],** both these copigments had low estimated anthocyanin half shelf life. The high colour retention was probably due to the formation of new pigments as suggested by Trouillas et al. (2016), which led to the yellowish/orange tonalities. This is supported by the hypsochromic shift seen in **Supplementary Figure 5-7 (a)** and the significantly higher ($p \leq 0.05$) *b and hue angle values (**Supplementary Figure 5-8**) compared to the other copigments. Similarly, Qian et al. (2017) determined the effects of ferulic and caffeic acids on the anthocyanin stability and colour intensification, and reported that their addition accelerated the degradation of anthocyanins, since the formed copigment complexes did not protect anthocyanins from thermal degradation. Also, Rein (2005) found that ferulic acid in blackcurrant syrups became yellowish during storage. Overall, after 140 days of storage at 20 °C, anthocyanins with chlorogenic acid in a buffer solution demonstrated better colour retention [**Table 5-5 (a)**], indicating higher colour stability, with lower Total Colour Difference, L^*, b^* and hue angle values, as well as higher a^* and chroma values, followed by the control sample, rosmarinic and tannic acid complexes [**Supplementary Figure 5-8**].

Throughout 4 °C storage, a moderate correlation ($R^2 = 0.810$, $p \leq 0.05$) was found between anthocyanin half shelf life and colour retention [**Table 5-5 (b)]**. Moreover, self-association of anthocyanin at pH 3.0 and low temperature was more efficient than intermolecular copigmentation with caffeic, chlorogenic, tannic and rosmarinic acid. However, only 18.6 to 34.1% of total anthocyanins were degraded within 140 days of the copigmentation stability study at pH 3.0 and 4 °C of storage [**Figure 5-5** (**b**)]. This suggests that longer storage periods are needed in order to reach a 50% of total anthocyanins degradation and obtain more

accurate results with regards to anthocyanin shelf life and colour stability under the abovementioned storage conditions.

Table 5-5. Effect of intermolecular copigmentation on colour range retention during storage at (a) 20 °C and (b) 4 °C in buffer and solvent/buffer solutions at pH 3.0.

(a) Storage Temp (20 °C)	ATC (B)	Ferulic (B)	Caffeic (B)	Chlorogenic (B)	Tannic (B)	Rosmarinic (B)	ATC (S)	Chlorogenic (S)
Day 0								
Day 60								
Day 140								
Colour Retention (%) (Day 140)	44.5 (6.4)^{Bbc}	55.3 (3.3)^{Bab}	55.3 (2.6)^{Bab}	50.4 (0.6)^{Bab}	38.7 (0.2)^{Bc}	44.1 (2.0)^{Bbc}	60.1 (0.1)^{Ba}	53.2 (1.1)^{Bab}
(b) Storage Temp (4 °C)	**ATC (B)**	**Ferulic (B)**	**Caffeic (B)**	**Chlorogenic (B)**	**Tannic (B)**	**Rosmarinic (B)**	**ATC (S)**	**Chlorogenic (S)**
Day 0								
Day 60								
Day 140								
Colour Retention (%) (Day 140)	89.0 (0.0)^{Aa}	89.3 (0.2)^{Aa}	81.5 (0.2)^{Ae}	86.6 (0.0)^{Ac}	85.4 (0.2)^{Ad}	81.6 (0.2)^{Ae}	88.4 (0.0)^{Ab}	85.7 (0.1)^{Ad}

Conversion of *L**, *a**, and *b** values to RGB (Red, Green, Blue) colour model for visualisation of anthocyanin colour. Anthocyanins (ATC); Buffer solution (B); Solvent/Buffer solution (S). Figures in parentheses indicate the standard deviation. Values with the same letter ^{a,b} in each column are not significantly different ($p > 0.05$). Values with the same letter ^{A,B} in each row are not significantly different ($p > 0.05$).

Moreover, potassium sorbate is commonly used as preservative in food products due to its antimicrobial properties. In this study, potassium sorbate was added in both the control and copigmentation samples. However, the presence of potassium sorbate most likely resulted in faster degradation of the anthocyanins in the copigmentation samples compared to control. Moldovan & David, (2014) investigated the storage stability of anthocyanins from Cornelian cherry fruits (*Cornus mas* L.) at different temperatures (2 °C, 25 °C and 75 °C) at pH 3.02, in the presence of sodium benzoate and potassium sorbate. A higher stability was observed in the anthocyanin extract without any added preservatives, followed by sodium benzoate, whilst potassium sorbate was the lowest. This indicated that the addition of preservatives interrupted the anthocyanin self-association, leading to their degradation.

Copigmentation stability in buffer solutions at pH 6.0. At pH 6.0, the addition of phenolic acids did not significantly increase the stability of anthocyanins, and in fact it induced anthocyanin degradation, especially during storage at 20 °C. According to **Table 5-4 (c)**, the control sample showed significantly ($p \leq 0.05$) high estimated half shelf life (21.6 ± 0.7 days) (control > chlorogenic acid > ferulic acid > rosmarinic acid > tannic acid > caffeic acid). At pH 6.0, quinoidal bases (**Figure 5-1**) are formed and decreased the amount of the carbinol pseudobase in the solution. This suggests that self-association of the neutral quinonoid bases is stronger than that of flavylium cations, since the self-association of flavylium cation interferes due to electrostatic repulsion as reported by Trouillas et al. (2016). Also, the interaction between anthocyanins and copigments causes an increase in temperature (exothermic reaction) and leads to the formation of colourless compounds that can cause the dissociation of copigmentation complexes (Mazza & Brouillard, 1990). High temperatures enhance the solubility of most copigments, thus increasing the interaction between anthocyanins and copigment complexes.

Thermodynamically, however, they also favour the dissociation of copigmented forms and may actually attenuate colour (Trouillas et al., 2016). At lower storage temperature, pigment precipitation can occur which usually results in a significant loss of colour solution (Boulton, 2001).

Storage at 20 °C was found to greatly influence the colour stability whereas copigmented anthocyanins produced relativity quickly a yellowish/browning colour during storage. Rapid degradation of the anthocyanins was also noted. This is further supported by the significant correlation ($p \leq 0.05$) between the anthocyanin half shelf life and their colour retention ($R^2 = 0.884$). Also, a significant ($p \leq 0.05$) increase in b^* and hue angle values (**Supplementary Figure 5-10**) was observed, as well as gradual degradation of the bathochromic shift [**Supplementary Figure 5-7 (c)**] to 450 nm, as a result of the yellow tonalities especially in caffeic, rosmarinic and tannic acid samples after 16 days of storage at 20 °C. Gras et al. (2017) also reported that in copigmented anthocyanins from sweet potato, apple and rosemary extracts at pH 2.6, 3.6 and 4.6, the absorption of the complexes ranged between 400 to 480 nm, indicative of a yellowish or brownish of colour of the copigment.

Furthermore, Welch et al. (2008) reported that anthocyanins can also be degraded by oxidative mechanisms such as the action of the enzyme polyphenol oxidase (PPO). PPO is responsible for the browning of blueberries, strawberries, grapes, and cherries juices. Jaiswal et al. (2010) reported that oven drying of fresh pomegranate arils (*Punica granatum* L.) at 90 °C resulted in a 68% loss of PPO activity, suggesting that drying process did not fully denaturate or inactivate the PPO enzyme. Moreover, drying processes that kept the moisture content between 6 to 11% were only capable to minimise browning reactions of enzymatic and non-enzymatic origin (Yang et al., 2013). However, PPO cannot oxidise anthocyanins on its own.

Another substrate with o-diphenolic compounds (caffeic, ferulic and chlorogenic acid) must be present for the first step of polyphenolic oxidation, in which the acid is oxidised into its o-quinone form. Then, the anthocyanin with an o-diphenolic B ring (cyanidin and delphinidin) is degraded by the o-quinone form of acid and forms brown polymers (Kader et al., 1997). It is likely that at 20 °C, the PPO action was responsible for the decrease in anthocyanin stability [**Table 5-4 (c)**] as well as for the change in the copigment complex colour [**Table 5-6 (a)**] to yellowish/brownish compared to the control sample. This was indicated by the high percentage of colour retention [**Table 5-6 (a)**], due to the formation of brown condensation. Moreover, it is worth noticing that the increase in the copigmentation concentration, not only increases the hyperchromic effect, but also may have led to the production of larger aggregates and precipitates, that can affect the optical and spectrophotometry behaviour (Trouillas et al., 2016). However, in this study, the hypothesis on the action of the enzyme PPO is not supported by experimental data.

Similar to 20 °C, in the storage trials at 4 °C, the control sample had a significantly higher estimated half shelf life of anthocyanins (52.9 ± 3.6 days) compared to the addition of phenolic acids in the copigmentation reaction (control > ferulic acid > tannic acid > chlorogenic acid > rosmarinic acid > caffeic acid) [**Table 5-4 (d)]**. Notably, ferulic and tannic acid showed improvement (3 – 3.5-fold) in the half shelf life during storage at 4 °C compared to 20 °C.

Table 5-6. Effect of intermolecular copigmentation on colour range and retention during storage at (a) 20 °C and (b) 4 °C in buffer and buffer/solvent solutions at pH 6.0.

(a) Storage Temp (20 °C)	**ATC (B)**	**Ferulic (B)**	**Caffeic (B)**	**Chlorogenic (B)**	**Tannic (B)**	**Rosmarinic (B)**	**ATC (S)**	**Chlorogenic (S)**
Day 0								
Day 12								
Day 24								
Colour Retention (%) (Day 24)	77.2 (4.0)^{Bb}	62.3 (0.1)^{Bc}	52.3 (0.1)^{Bd}	63.6 (3.3)^{Bc}	52.1 (0.1)^{Bd}	36.6 (3.1)^{Be}	88.3 (1.3)^{Aa}	71.1 (3.4)^{Bbc}
(b) Storage Temp (4 °C)	**ATC (B)**	**Ferulic (B)**	**Caffeic (B)**	**Chlorogenic (B)**	**Tannic (B)**	**Rosmarinic (B)**	**ATC (S)**	**Chlorogenic (S)**
Day 0								
Day 12								
Day 24								
Colour Retention (%) (Day 24)	82.5 (0.0)^{Ac}	91.8 (0.4)^{Aa}	77.2 (0.2)^{Ae}	71.8 (0.1)^{Af}	79.6 (0.2)^{Ad}	63.4 (0.2)^{Ag}	88.1 (0.0)^{Ab}	83.5 (0.1)^{Ab}

Conversion of *L**, *a**, and *b** values to RGB (Red, Green, Blue) colour model for visualisation of anthocyanin colour. Anthocyanins (ATC); Buffer solution (B); Solvent/Buffer solution (S). Figures in parentheses indicate the standard deviation. Values with the same letter ^{a,b} in each column are not significantly different ($p > 0.05$). Values with the same letter ^{A,B} in each row are not significantly different ($p > 0.05$).

As shown in **Table 5-6 (b)**, the colour of all samples became brighter during storage at 4 °C. According to **Supplementary Figure 5-11**, ferulic and tannic acid had a lower Total Colour Difference and *L** values which explain the higher colour stability and colour darkness after 24 days of storage, respectively. Meanwhile, caffeic acid copigmentation showed significantly high ($p \leq 0.05$) *b** and hue angle values after 24 days of storage, with browning/yellowish in appearance, suggesting the copigmentation instability. However, there was non-significant ($p > 0.05$) correlation between anthocyanin half shelf life and colour stability. For example, during storage copigmentation with ferulic acid resulted in significantly ($p \leq 0.05$) lower anthocyanin half shelf life than that of the control sample [**Table 5-4 (d)**], but higher percentage of colour retention [**Table 5-6 (b)**]**.** This is due to the efficiency of ferulic acid as a copigment in maintaining colour stability rather than anthocyanin stability at low temperature. Welch et al. (2008) also reported that copigmentation complexes are only stable at low temperature within cool and dark environments, as the presence of sunlight and high temperatures result in the loss of copigmentation.

Copigmentation stability in solvent/buffer solutions at pH 3.0 and 6.0. According to the data from the stability tests with chlorogenic acid as copigment in solvent/buffer environment at both pHs and temperatures, the estimated half shelf life of anthocyanins was significantly lower ($p \leq 0.05$) compared to the control. Moreover, the temperature influenced the stability of copigmentation; storage at 4 °C resulted in 2-fold higher estimated half shelf life of anthocyanins compared to 20 °C (**Table 5-4**). In the study by Brouillard et al. (1991) investigating the effect of copigmentation between malvin and chlorogenic acid, the presence of organic solvents such as methanol, ethanol and acetone weakened the copigmentation effect

compared to buffer solutions. Organic solvents tend to disrupt copigmentation stacks due to the weakening of hydrophobic interactions between anthocyanins and copigments (Boulton, 2001).

Also, the Hunter Lab results presented in **Supplementary Figure 5-8** to **Supplementary Figure 5-11** demonstrated a colour enhancement in the solvent environment after the addition of chlorogenic acid. However, there is a significant increase ($p \leq 0.05$) in the Total Colour Difference and L^* values, as well as significant decrease ($p \leq 0.05$) in the a^* and chroma values in the chlorogenic acid samples, indicating the degradation of colour intensity and instability of copigmentation throughout the storage. This is supported by the significant correlation ($p \leq 0.05$) between the anthocyanin half shelf life and colour stability ($R^2 = \sim 0.994$) in the copigmentation in solvent/buffer solution, with the exception of the pH 6.0 (20 °C) samples. This is in agreement with a previous study by Guisti et al. (1999) who stated that the solvent/buffer systems have more effect on the colour characteristics of anthocyanins, but the copigmentation is dramatically weakened by the presence of organic co-solvents (Brouillard et al., 1991).

However, these results are significantly lower ($p \leq 0.05$) than that of the copigmentation effect in the buffer solution. Copigmentation with chlorogenic acid in buffer gave significantly higher ($p \leq 0.05$) colour intensity than in the solvent/buffer solution as demonstrated by lower L^* (darker colour) and higher chroma values, which indicated an increase in colour intensity. This is in agreement with Brouillard et al. (1991), whereby copigmentation was more efficient in buffers than in solvent/buffer mixtures when sufficient water existed.

5.4 Conclusions

Factors such as temperature, pH, copigment concentration and type, the presence of solvent as well as enzyme activity, substantially influenced the copigmentation complex of anthocyanin solutions from DBS and also resulted in colour changes. The addition of phenolic acids imparted strong copigmentation effects (hyperchromic shift) on the anthocyanins and led into colour enhancement especially at higher pH.

In this study, ferulic acid and rosmarinic acid were superior over other phenolic acids in terms of colour enhancement at pH 3.0 and pH 6.0, respectively, but failed to maintain anthocyanin stability during storage at 20 °C. Moreover, anthocyanin self-association of the neutral quinonoid bases at pH 6.0 was stronger than intermolecular copigmentation with phenolic acids. Therefore, monomeric anthocyanin degradation was faster than colour fading due to self-association.

Overall, maintaining the anthocyanins copigmentation reactions at low temperature is recommended, especially in high pH environment, in order to assure the stability of colour in the products. Anthocyanins from DBS copigmented with food grade ferulic acid are considered applicable as natural colourants in low pH foods (jelly) and in high pH foods (ice cream and dairy products), but must be stored at low temperatures in order to achieve long shelf-life. On the other hand, during storage at room temperature, anthocyanin copigment complexes with food grade chlorogenic acid are suitable to be used as natural colourants, especially in low pH foods. For high pH foods which require room temperature storage, intermolecular copigmentation is not recommended due to the reaction of the enzyme PPO with the phenolic acid, which might alter the colour of the anthocyanin pigment. Furthermore, in order to investigate the influence of copigmentation on anthocyanin and colour stability, the interaction

between anthocyanins and the copigments through non-covalent bonding in the case of intermolecular copigmentation and covalent bonding in the enzymatic copigmentation were compared in the next chapter.

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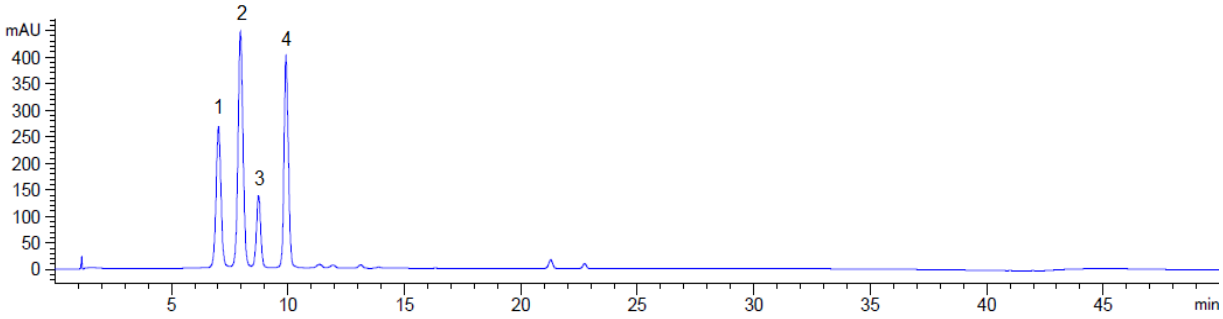
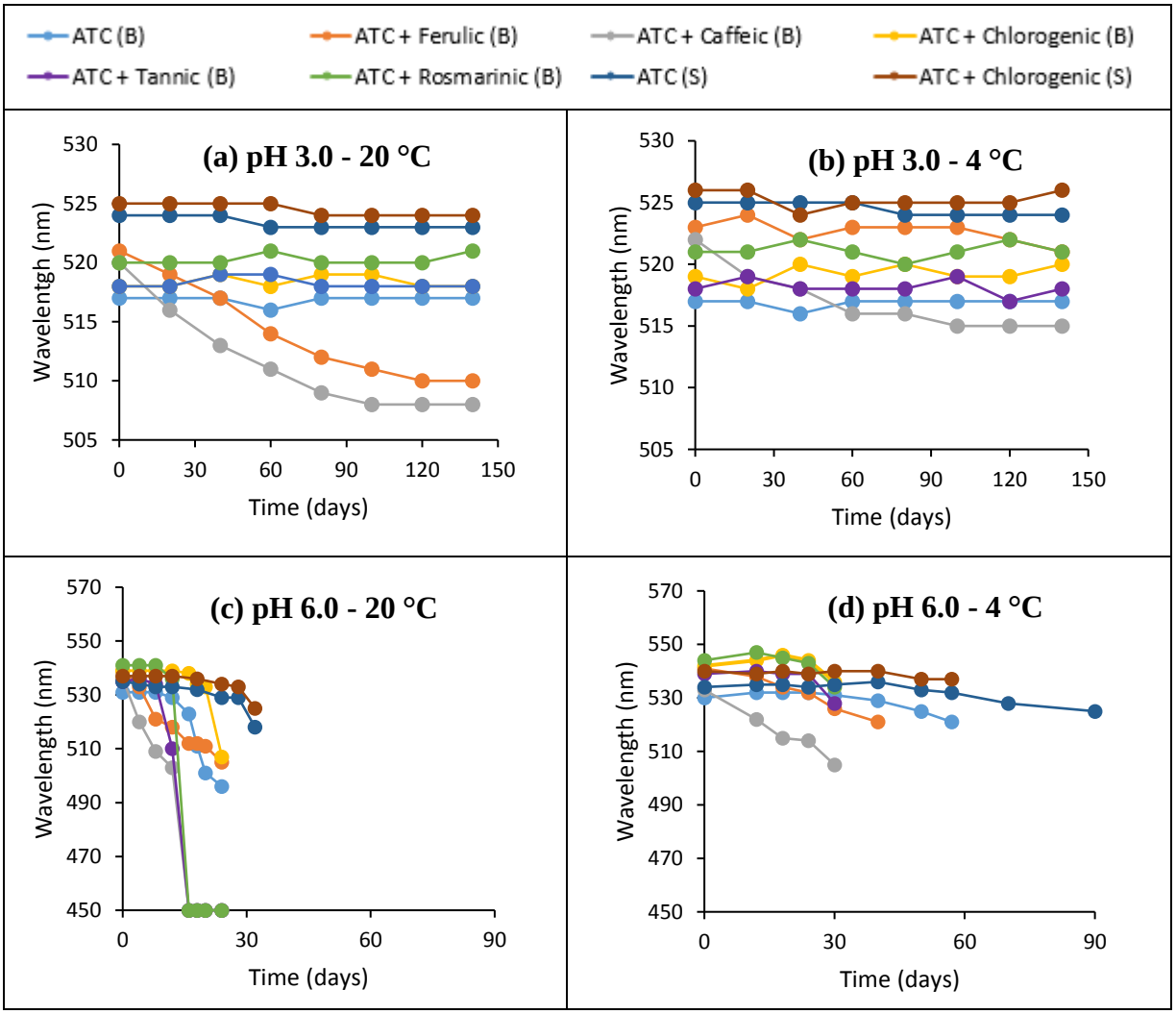
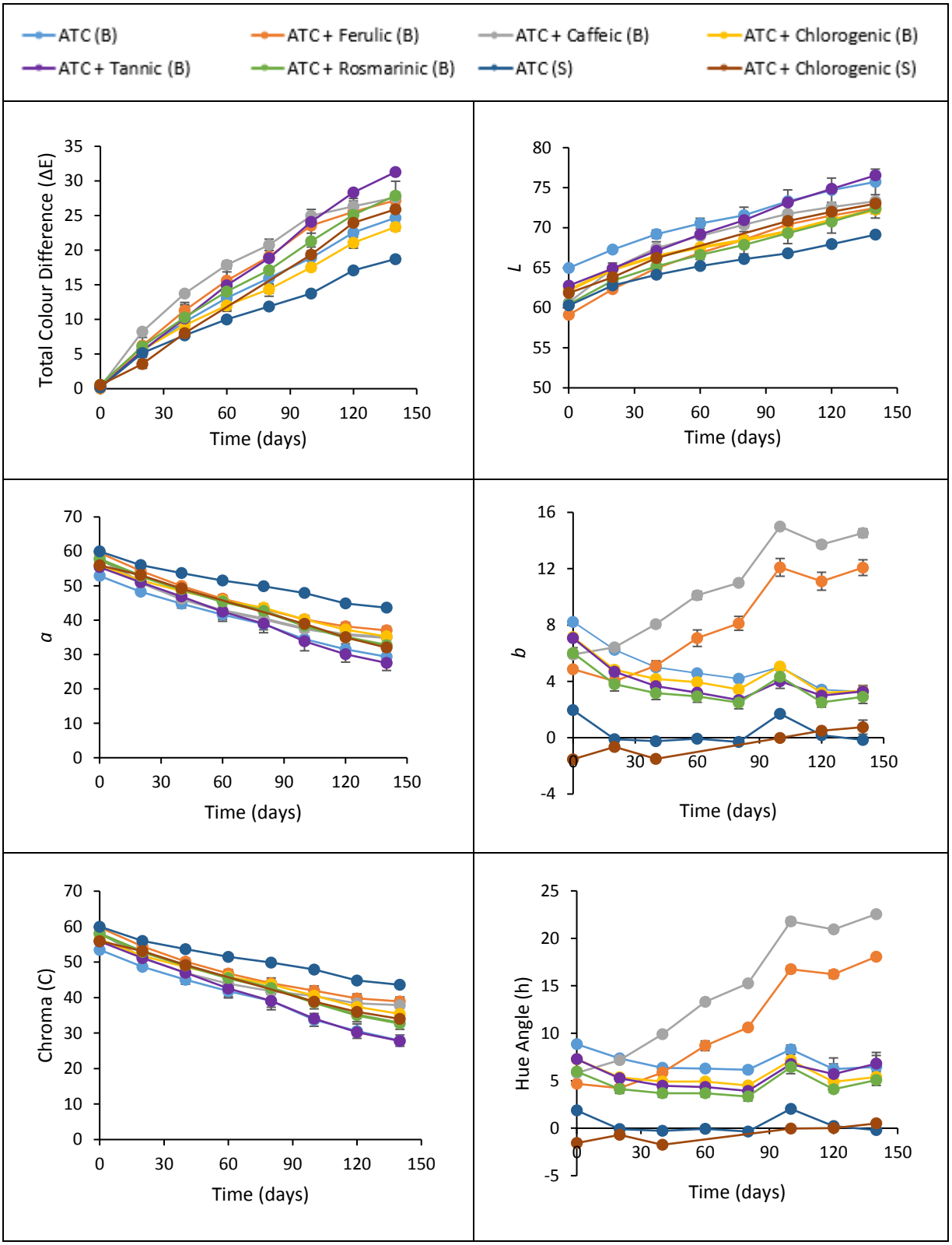
Supplementary Materials



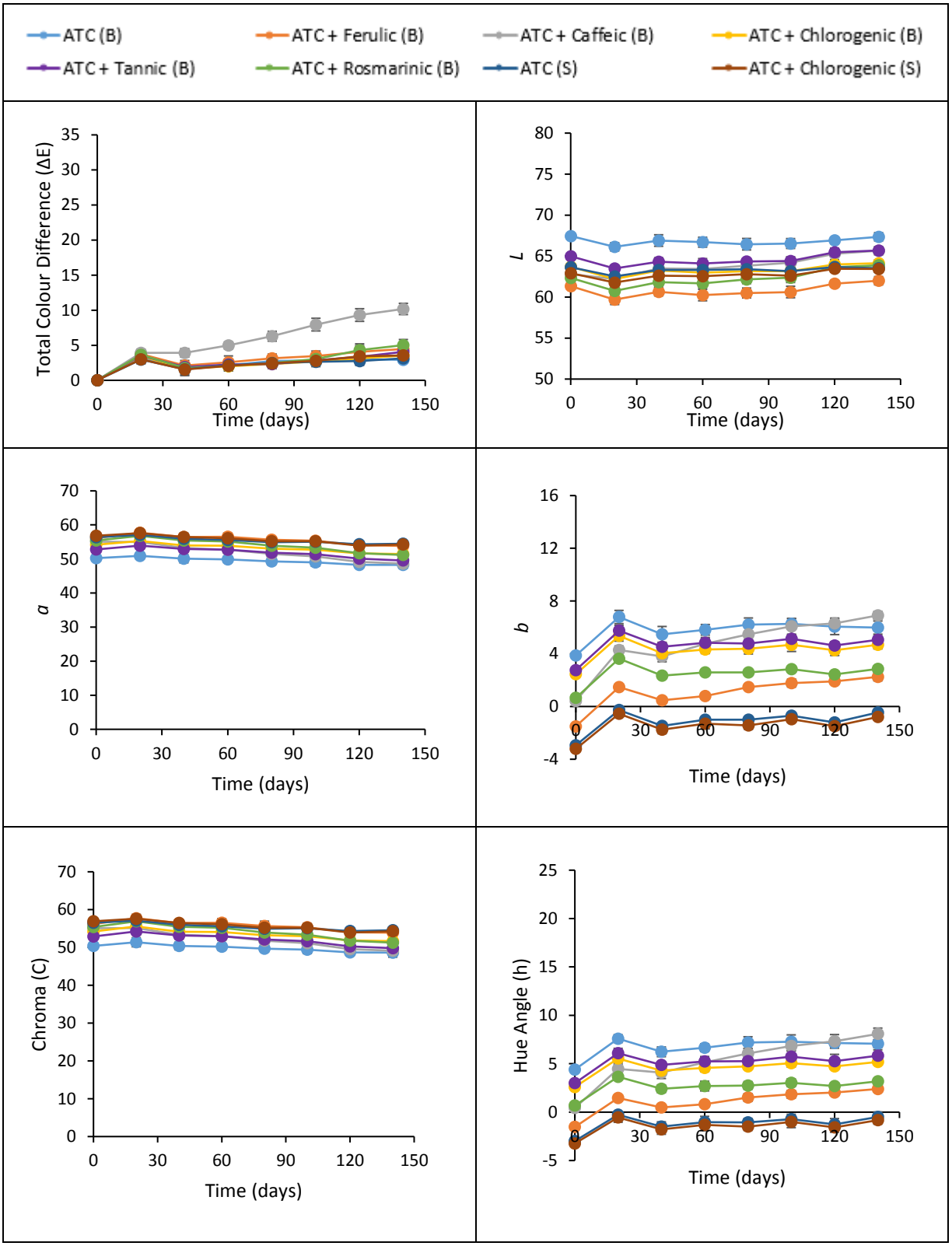
Supplementary Figure 5-6. Typical HPLC chromatograms of DBS showing the detected free anthocyanins at 520 nm. (1) Delphinidin-3-O-glucoside, (2) Delphinidin-3-O-rutinoside, (3) Cyanidin-3-O-glucoside and (4) Cyanidin-3-O-rutinoside.



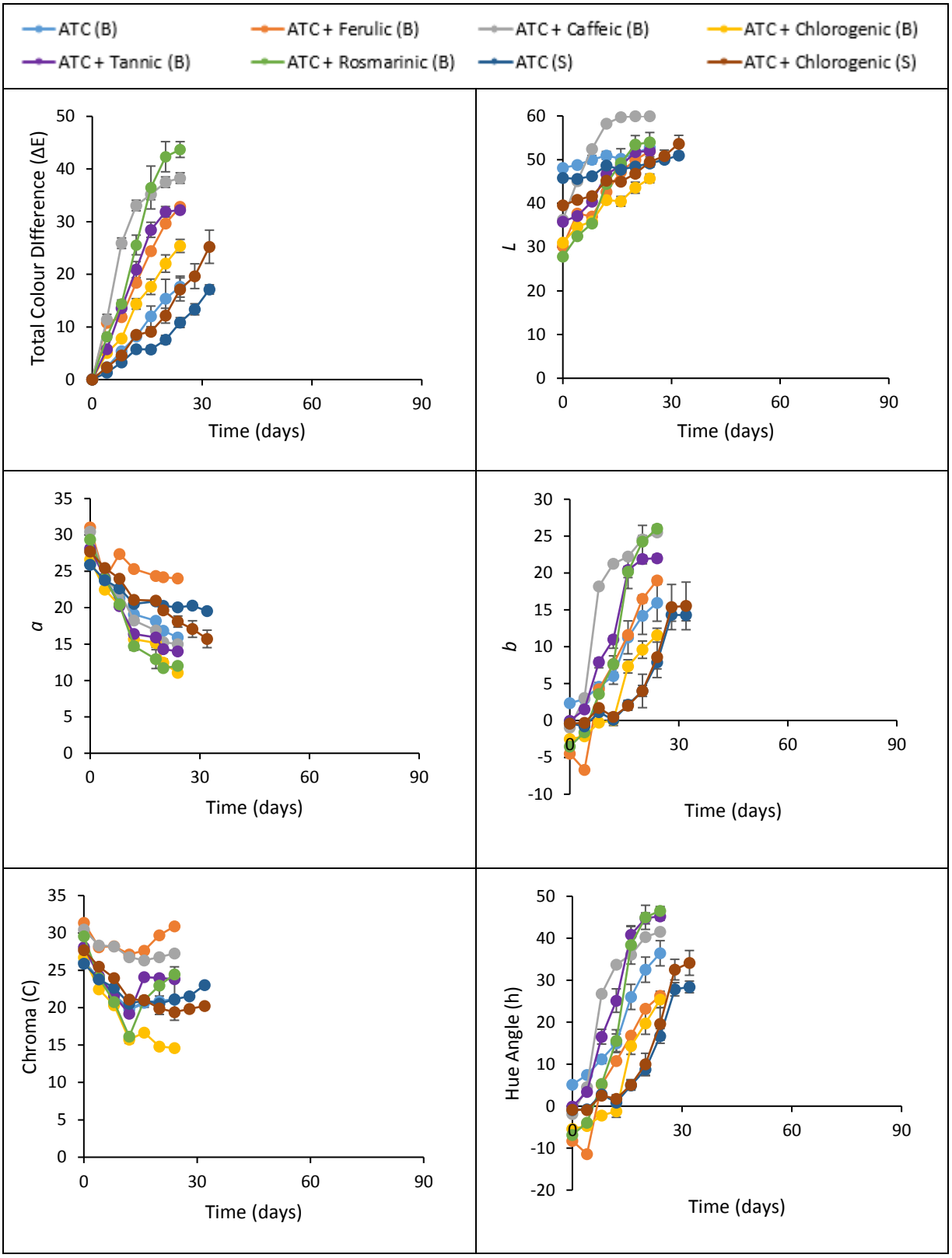
Supplementary Figure 5-7. Effect of intermolecular copigmentation on maximum absorption wavelength (λ_{max}) (hypsochromic shift) throughout storage in buffer and solvent/buffer solutions at (a) pH 3.0; 20 °C, (b) pH 3.0; 4 °C, (c) pH 6.0; 20 °C and (d) pH 6.0; 4 °C. *Anthocyanins (ATC); Buffer solution (B); Solvent/Buffer solution (S).



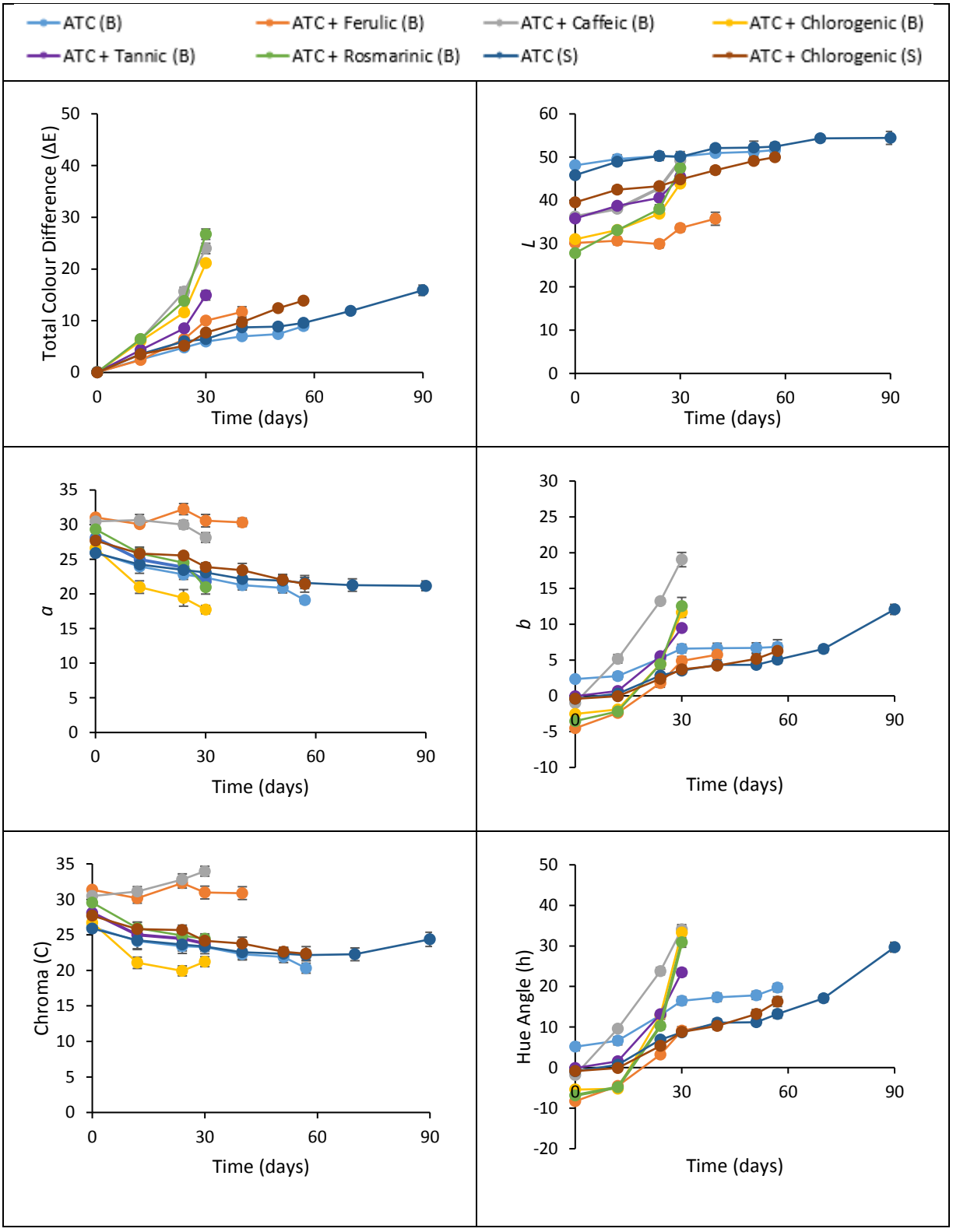
Supplementary Figure 5-8. Effect of intermolecular copigmentation on total colour difference, *L**, *a**, *b**, chroma and hue angle during storage at 20 °C in pH 3.0 buffer and solvent/ buffer solutions. *Anthocyanins (ATC); Buffer solution (B); Solvent/Buffer solution (S); *L** (lightness); *a** (redness/greenness); *b** (yellowness/blueness).



Supplementary Figure 5-9. Effect of intermolecular copigmentation on total colour difference, *L**, *a**, *b**, chroma and hue angle during storage at 4 °C in pH 3.0 buffer and solvent/ buffer solutions. *Anthocyanins (ATC); Buffer solution (B); Solvent/Buffer solution (S); *L** (lightness); *a** (redness/greenness); *b** (yellowness/blueness).



Supplementary Figure 5-10. Effect of intermolecular copigmentation on total colour difference, *L**, *a**, *b**, chroma and hue angle during storage at 20 °C in pH 6.0 buffer and solvent/ buffer solution. *Anthocyanins (ATC); Buffer solution (B); Solvent/Buffer solution (S); *L** (lightness); *a** (redness/greenness); *b** (yellowness/blueness).



Supplementary Figure 5-11. Effect of intermolecular copigmentation on total colour difference, *L**, *a**, *b**, chroma and hue angle during storage at 4 °C in pH 6.0 buffer and solvent/ buffer solution. *Anthocyanins (ATC); Buffer solution (B); Solvent/Buffer solution (S); *L** (lightness); *a** (redness/greenness); *b** (yellowness/blueness).

CHAPTER 6 – Enzymatic acylation and stability of anthocyanins from dried blackcurrant (*Ribes nigrum* L.) skins with methyl cinnamate as acyl donor

Abstract

The aim of this study was to investigate the effect of enzymatic acylation of anthocyanins from blackcurrant (*Ribes nigrum* L.*)* skins with aromatic acid methyl esters, namely methyl cinnamate and methyl ferulate as acyl donors. *Candida antarctica* lipase B was used as biocatalyst at different concentrations and incubation temperatures. The highest conversion yields of cyanidin 3-(6″- cinnamoyl)-glucoside (~15.1%) and delphinidin 3-(6″- cinnamoyl)-glucoside (~10.1%) were obtained with cinnamic acid methyl ester as the acyl donor. No reaction was observed when ferulic acid methyl ester was used as the acyl donor. HPLC-MS evidenced the formation of cinnamic monoesters with the delphinidin-3-O-glucoside and cyanidin-3-O-glucoside fractions. NMR showed that the acylation took place at the 6"-OH group of the glucose moiety of the anthocyanin. The enzymatically acylated anthocyanins demonstrated higher molecular stability and colour stability during storage at pH 3.0 (4 °C) compared to both intermolecular copigmented anthocyanin (through non-covalent interaction with methyl cinnamate) and the control sample (anthocyanin without added methyl cinnamate). This is the first evidence showing the enzymatic acylation of two anthocyanins (delphinidin-3-O-glucoside and cyanidin-3-O-glucoside) occurring simultaneously in a blackcurrant skin extract using methyl aromatic esters as acyl donors. Moreover, the enzymatic acylation of delphinidin-3-O-glucoside has not been reported in previous studies. Stability studies demonstrated that acylated anthocyanins have the potential to be used as natural colourants in acidic (e.g. jelly and yogurt) and neutral food matrices (e.g. ice cream and milk).

Keywords: Blackcurrant skins, anthocyanin, intramolecular copigmentation, *Candida antarctica* lipase B, enzyme acylation

6.1 Introduction

Blackcurrant skins that are left behind as residue during juice processing are a rich source of polyphenols and especially anthocyanins (250 mg/100 g of berries) (Vagiri, 2012). Anthocyanins exist almost exclusively in the skins, while lesser amounts are usually found in the flesh and seeds of blackcurrant berries. The bright attractive colour and high water solubility of anthocyanins make them suitable alternatives to synthetic food dyes (Malien-Aubert et al., 2001; Morais et al., 2002). Additionally, their high antioxidant activity and promising health promoting properties, such as lowering the risk of coronary heart diseases, cancer and stroke, as well as their anti-inflammatory and antimicrobial properties (Wrolstad, 2004), have significanlty increased the interest of anthocyanins for applications in the food, cosmetic and pharmaceutical industries.

The term "copigmentation" is commonly reserved for anthocyanins (Trouillas et al., 2016), where the colour of anthocyanins can be stabilised and enhanced by copigmentation reactions (Rein, 2005). Commonly, acylated anthocyanins naturally exist in some flowers fruits and berries which improve and stabilise the colour of the plant extract. Anthocyanin glycosyl acylation is a phenomenon in which the hydroxyl groups of anthocyanin glycosyls are esterified by aliphatic acid (e.g. acetic, oxalic, succinic and malic acids) or aromatic acids (e.g. gallic acid, *p*-coumaric acid, caffeic and ferulic acids) (Zhou et al., 2017). However, many studies report that acylated anthocyanin can be also produced *in vitro* through the enzymatic acylation of anthocyanin and other flavonoid glycosides with an acyl donor; the immobilised enzyme *Candida antarctica* lipase B (Novozym 435®) is commonly used as the biocatalyst with the reaction taking place in organic solvents at low water activity (Yan et al., 2016; Cruz et al., 2016; Cruz et al., 2017).

The copigment can attach to anthocyanin through covalent bonding leading to the formation of an intramolecular copigmentation complex, and consequently to increased

anthocyanin and colour stability the (Brouillard, 1982). The acylation of flavonoid glycosides such as anthocyanins can be carried out through two types of reactions, i.e. direct esterification and transesterification. In direct esterification fatty acids or phenolic acids are used as acyl donors in organic solvents at low water activity, and the by-product of water is removed by molecular sieves (Chebil et al., 2007). In transesterification, fatty acids and aromatic carboxylic acid of methyl, ethyl or vinyl esters can be used as acyl donors. However, these acyl donors need to be synthesised in advance in biocatalytic reactions with enzymes such as lipases, with alcohol being produced as by-product (Schär & Nyström, 2016). Villeneuve (2007) reported that the transesterification rate is usually greater than that of direct esterification, due to the fact that in the latter case the produced water forms a layer surrounding the catalyst particles which decreases the ability of the acyl donor to interact with the lipase active side (Humeau et al., 1998).

The enzymatic acylation of anthocyanins is less reported compared to other flavonoid glycosides such as rutin, isoquercitrin, rutin, and naringin. Yan et al. (2016) reported that anthocyanins isolated from black rice (*Oryza sativa* L.) were acylated enzymatically by lipase B Novozym 435 with different aromatic carboxylic acid methyl esters (methyl benzoate, methyl salicylate and methyl cinnamate) as acyl donors. Three new acylated compounds were successfully synthesised, with the highest conversion yield being for cyanidin 3-(6″-benzoyl)-glucoside (~91%), followed by cyanidin 3-(6″-salicyloyl)-glucoside (~84%), and finally cyanidin 3-(6″- cinnamoyl)-glucoside (~68%). In another study by Cruz et al. (2016), *Candida antarctica* lipase B (Novozym 435) was used to acylate malvidin-3-glucoside from red wine with oleic acid (fatty acid) as acyl donor. A modified anthocyanin of a pure malvidin-3-glucoside–oleic acid ester derivative was produced with improved solubility in lipophilic as well as aqueous media, a feature that could allow applications in the food, medical and cosmetic sectors.

Examples of fruits and vegetables that contain high amounts of naturally acylated pigments are red onion, red cabbage, red potatoes, black carrots, radishes, purple sweet potatoes and grapes (Giusti & Wrolstad, 2003). Slimestad and Solheim (2002) found two types of acyl groups attached to the blackcurrant anthocyanin, namely delphinidin 3-O-(6″-p-coumaroylglucoside) (Jose et al., 1990) and cyandin 3-O-(6″-p-coumaroylglucoside) (Saito et al., 1987). However, the amount of these compounds in the berries is too low (2%) (Sójka et al., 2009) to have an effect on the stability of the berry extracts, in terms of their total phenolic content and antioxidant activity. To the best of our knowledge, no information is available in the literature on the enzymatic acylation of anthocyanins derived from blackcurrant skins. Moreover, limited information is available on the anthocyanin degradation kinetics during storage at different pH environments. This knowledge is essential for predicting the changes that may occur in food products containing these acylated anthocyanins.

The enzymatic acylation was expected to increase the stability of anthocyanins and colour, as well as extending the shelf life of anthocyanins compared to chemical acylation. In the present study, the enzymatic acylation of anthocyanins was investigated using aromatic carboxylic acid methyl esters as acyl donor and immobilised lipase (Novozym 435) as the biocatalyst, aiming to obtain a high conversion yield. The stability of the acylated anthocyanins during storage in acetate buffer at different pH values (pH 3.0 and 6.0) and temperatures (20 °C and 4 °C) was also investigated.

6.2 Materials and methods

6.2.1 Chemicals

All solvents and chemicals used for either extraction or enzymatic synthesis were of analytical grade. More specifically, methanol (99%) and acetonitrile (99%) were purchased from Fisher Scientific (Loughborough, UK). Formic acid (95%), hydrochloric acid (HCl) (37%), deuterium chloride solution (DCl) (35%), heptane (96%), ethyl acetate (99%), 2-methyl-2-butanol (99.9%), 4 Å molecular sieves, methyl cinnamate (99%), methyl ferulate (99%), Lipase B from *Candida antarctica* (CALB) immobilized on acrylic resin and Toyopearl® HW-40 Size Exclusion Media were purchased from Sigma-Aldrich (UK). Deuterated methanol (CD_3OD) (99.8%) was purchased from Cambridge Isotope Laboratories, Inc (UK). Strata® C18-U (55 µm, 70 Å) 500 mg / 6 mL cartridges were purchased from Phenomenex (Queens Avenue, UK).

Potassium chloride, potassium sorbate, sodium acetate, citric acid monohydrate and sodium phosphate, used for the stability experiments were purchased from Sigma-Aldrich (UK). The buffers used in this study, i.e. pH 1.0 (potassium chloride, 0.025M) and pH 4.5 (sodium acetate, 0.4M) were prepared as described by Lee et al. (2005). Purified water was used in all preparation and analysis and was obtained using a Purite reverse osmosis system (Oxon, UK). Anthocyanin standards of cyanidin-3-O-glucoside (96%), cyanidin-3-O-rutinoside (96%), delphinidin-3-O-glucoside (95%), and delphinidin-3-O-rutinoside (95%) were obtained from ExtraSynthese Ltd (Genay, France) (**Figure 6-1** depicts their molecular structures).

6.2.2 Sample preparation of plant materials

Dried blackcurrant pressed residues from a juice manufacturing process were kindly supplied by A & R House (BCL) Ltd, (Bleadon, Weston-super-Mare, UK). Dried blackcurrant used was a representative sample (315) from industrial rotary drying with high anthocyanins content as shown in Chapter 3. The seeds, which were present in the residue samples, were separated by initially grinding the samples in a coffee blender and subsequently passing them through a 0.841 mm (20 mesh) sieve; the obtained fraction constituted the dried blackcurrant skins (DBS) fraction. DBS samples were segregated in polyethylene bags and stored at -20 °C until further analysis.

6.2.3 Extraction of anthocyanin from DBS

Anthocyanins were extracted from DBS (50.0 g) according to the protocol by Stevenson et al. (2006) which involved steeping the DBS sample in 250 mL of 0.1% (v/v) HCl in methanol for 1 h. The obtained extracts were vacuum filtered to separate the supernatants and solid residues and the latter underwent a second extraction with 250 mL of fresh solvents for another 1 h. The extraction process was repeated 3 times and the supernatants were pooled together and evaporated under vacuum by a rotary evaporator to remove methanol. 100 mL of distilled water were then added to the residue; this constituted the crude anthocyanin extract sample.

6.2.4 Purification of anthocyanin extracts

The anthocyanin-containing extract was introduced into a Strata® C18-U (55 µm, 70 Å, 500 mg / 6 mL) cartridge (Phnenomenex Ltd., Macclesfield, UK) after 5-fold dilution with distilled water. The extract was then washed with 12.0 mL of 0.01% (v/v) HCl in water and subsequently 12.0 mL of ethyl acetate in order to remove non-phenolic compounds as well as phenolic compounds other than anthocyanins, respectively (Rodriguez-Saona &

Wrolstad, 2001). Finally, anthocyanins were eluted with 12.0 mL of 0.01% (v/v) HCl in methanol, followed by evaporation to remove the solvents. 100 mL of distilled water were then added to the residue and further dried by freeze-drying. The freeze-dried purified crude extract was stored at -20 °C until further analysis.

6.2.5 Enzymatic acylation

Enzymatic acylation was performed according to Cruz et al. (2016), with minor modifications. Freeze-dried purified blackcurrant skin extracts (6.0 mg) containing anthocyanins were combined with 100 molar equivalent of methyl esters (methyl cinnamate and methyl ferulate, chemical structures depicted in **Figure 6-1**) used as acyl donors in 2.0 mL Eppendorf tubes. In order to calculate the molarity of the anthocyanin extract, it was considered equivalent to the molecular weight of delphinidin-3-O-rutinoside (611.53 g/mol), which is the most dominant anthocyanin in the extract. Then, 1 mL of 2-methyl-2-butanol was added as the solvent medium due to its benign effect on enzymes (Ren & Lamsal, 2017), and the mixtures were left to dissolve by shaking at room temperature in a thermomixer (Eppendorf ThermoMixer C, Eppendorf UK Limited).

An important factor that influences the efficiency and regioselectivity of the enzymatic acylation is the water content in the organic solvents, whereas the excess of water can lead to the hydrolysis, whilst adequate amount of water results in the synthesis reaction. In order to circumvent this problem and shift the equilibrium of the reaction towards acylation rather than hydrolysis, activated molecular sieves with a size of ~4 Å can be used to absorb the excess of water.

A number of acylation reactions between blackcurrant skins extract and methyl esters were carried out (Reaction 1 to 8) as shown in **Table 6-1**. The reactions were initiated by adding 100 g/L of molecular sieves (4 Å) and lipase enzyme (25 and 50 g/L) and the mixtures

were shaken thoroughly at 45 °C and 60 °C, respectively, for 52 hours at 1300 rpm. These parameters and their values were selected taking into account preliminary studies (data not shown) and previous research works (Stevenson et al., 2006; Cruz et al., 2016). Samples (10 µL) were taken every 4 hours, dried under nitrogen gas and diluted with mobile phase A [acetonitrile/water/formic acid; 5: 92: 3 (v/v/v)], followed by analysis using HPLC.

Table 6-1. Reaction conditions for the enzymatic acylation of dried blackcurrant skins extract with methyl esters acyl donors for 52 h with different lipase enzyme concentrations and at different incubation temperatures (45 °C and 60 °C).

Reaction	Extract (mg)	Methyl ester acyl donor (Molar eq.)	Volume of 2-methyl-2-butanol (mL)	Molecular sieves (4 Å) (g/L)	Lipase (g/L)	Incubation temperature (°C)
1	6	100 (MC)	1	100	25	45
2	6	100 (MC)	1	100	25	60
3	6	100 (MC)	1	100	50	45
4	6	100 (MC)	1	100	50	60
5	6	100 (MF)	1	100	25	45
6	6	100 (MF)	1	100	25	60
7	6	100 (MF)	1	100	50	45
8	6	100 (MF)	1	100	50	60
9 (intermolecular copigmentation)	6	100 (MC)	1	–	–	60
10 (control)	6	–	1	–	–	60

*Methyl cinnamate (MC); Methyl ferulate (MF).

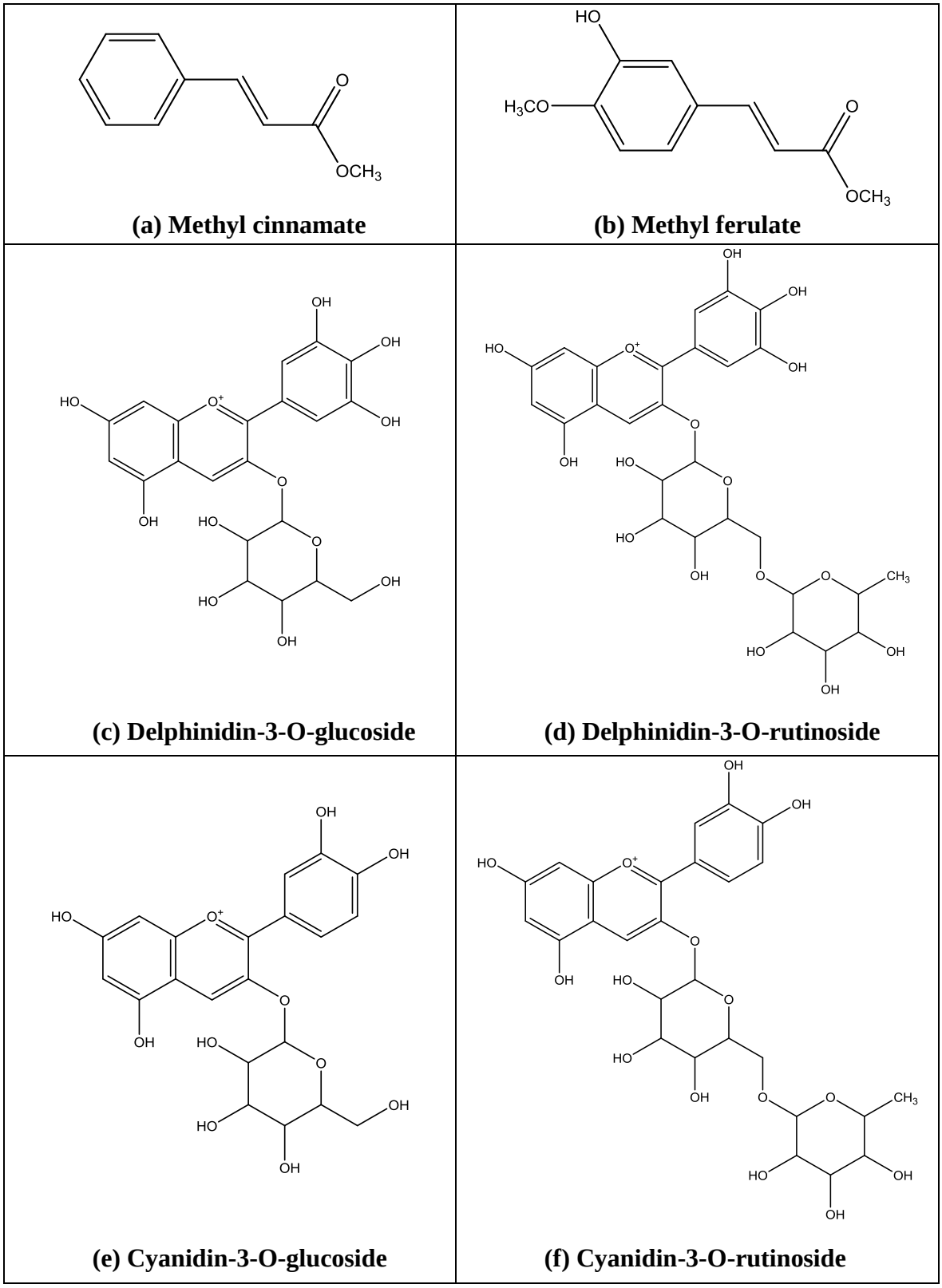


Figure 6-1. Chemical structures of methyl cinnamate (a) and methyl ferulate (b), and key anthocyanins present in dried blackcurrant skins (c – f). Structures plotted using the ChemDraw website (2018).

6.2.6 HPLC analysis of anthocyanins

HPLC analyses were performed on an 1260 Infinity HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array detector (DAD) using a XBridge BEH Phenyl column (100 × 4.6 mm i.d., particle size 3.5 µm, Waters) at 25°C. The mobile phase consisted of acetonitrile/water/formic acid; 5: 92: 3 (v/v/v) (solvent A) and 0.1% of formic acid in acetonitrile (solvent B). The gradient elution system was: 0% (B) at 0 min, increasing to 20% (B) at 20 min, 30% (B) at 26 min, 50% (B) at 28.50 min, and reaching 95% (B) at 32 min before decreased to 0% between 36 and 42 min. The flowrate was 1.0 mL/min and injection volume was 20 µL. The duration of analysis was 42 min.

Detections were carried out at wavelengths of 520 nm for anthocyanins. Briefly, 2.0 mg/mL of stock standard solutions were prepared separately followed by dilution to give concentrations from 0.01 to 1.0 mg/mL. To obtain the standard curves, the peak areas were plotted against the corresponding concentrations of the standard solutions injected into HPLC.

The conversion yield of the produced acylated anthocyanin was calculated according to the following formula:

$$\frac{HPLC\ area\ of\ acylated\ anthocyanin\ A}{HPLC\ area\ of\ initial\ non-acylated\ anthocyanin\ A} \quad (1)$$

$$\frac{100\%\ Mass\ fraction\ conversion\ of\ 1\ mol\ acylated\ anthocyanin\ A}{100\%\ Mass\ fraction\ conversion\ of\ 1\ mol\ non-acylated\ anthocyanin\ A} \quad (2)$$

$$Conversion\ yield\ (\%) = \frac{(1)}{(2)} \times 100$$

where *A* is a type of anthocyanin.

6.2.7 Purification of acylated anthocyanins

The enzymatic acylation reaction was stopped by filtration (53 μm pore size sieve; Fisher Scientific, UK) to remove the molecular sieves and the immobilised lipase B followed by solvent evaporation under vacuum. The residue was re-dissolved in 0.1% HCl in methanol and any excess of methyl aromatic acids and some impurities were extracted using a separation funnel with heptane for several times [**Figure 6-2 (a)**]. Subsequently, the methanol fraction was evaporated, the residue was dissolved in 85% methanol/water and loaded onto a Toyopearl HW-40S column (30 × 1.6 cm) at a flow rate of 1 mL/min as shown in [**Figure 6-2 (b)]**. The starting material was recovered with acetonitrile/water/acetic acid (15:79:6), followed by acetonitrile/water/acetic acid (30:64:6) for 30 min each, and the desired acylated compounds were eluted using 90% ethanol for 30 min. The fractions were analysed by HPLC and the acylated anthocyanins peaks that formed in the chromatogram were fractionated. Then, the solvents were evaporated and the pigments were freeze-dried; these were then analysed by liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) as described in the following sections.

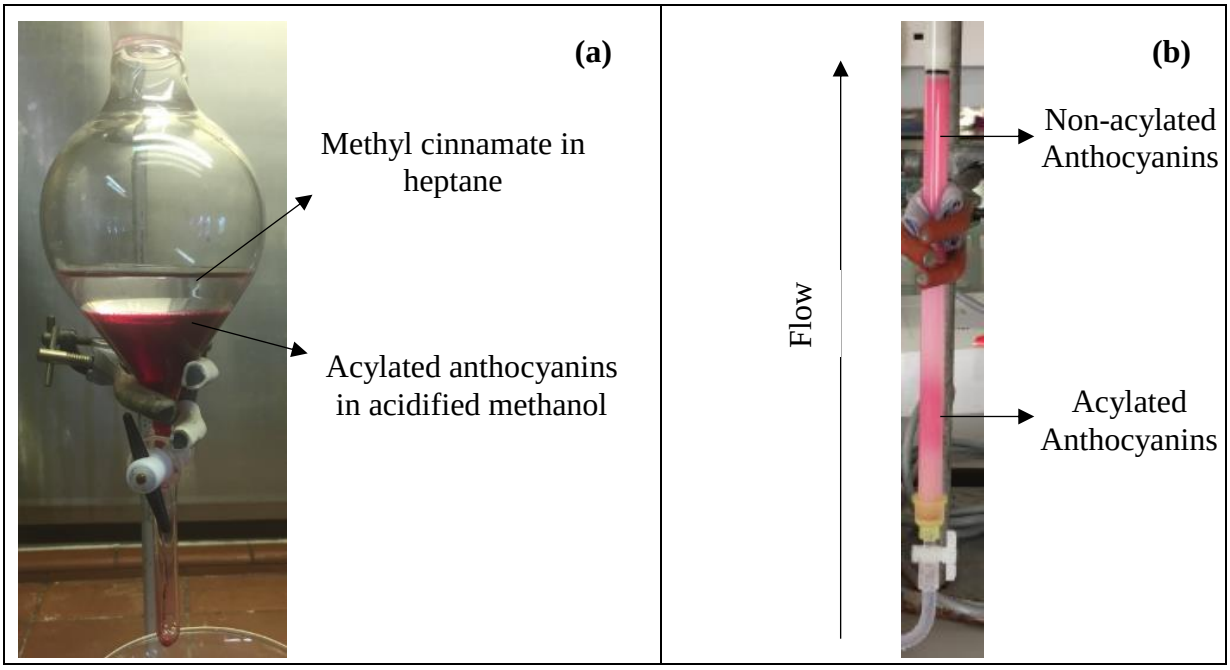


Figure 6-2. (a) Extraction of excess phenolic acids from the acylation reaction mixture. (b) Purification of acylated anthocyanins using Toyopearl HW-40S column (30 x 1.6 cm).

6.2.8 Liquid chromatography-mass spectrometry (LC-MS) analysis

A Thermo Scientifc Accela HPLC system with a photo diode array (PDA) detector interfaced to a Thermo Scientific LTQ Orbitrap XL mass spectrometer was used in this study. The chromatographic separation was conducted by using an XBridge BEH Phenyl column (100 × 4.6 mm i.d., particle size 3.5 µm, Waters). The mobile phase consisted of eluent A (acetonitrile/water/formic acid; 5: 92: 3; v/v/v) and eluent B (0.1% formic acid in acetonitrile). The gradient elution system was; 0−20 min, from 5 to 25% B; 20−26 min, from 25 to 35% B; 26−28.5 min, from 35 to 55% B; 28.5−32 min, from 55 to 95% B; 32−42 min, from 95 to 5% B. The flow rate was 1.0 mL/min. The temperature of the column oven was set at 25 °C and the injection volume was 10 µL. 520 nm wavelength was used for the detection of anthocyanins. A post PDA splitter was used that diverted approximately 75% of the analysed sample to waste. The remaining 25% was directed into the MS that was operated using the Orbitrap detector in positive ion mode scanning from m/z 85 to m/z 2000 at a scan resolution of 100,000. These samples were also directly infused into the same MS using similar acquisition settings and the ions of interested were subjected to MS^2 in an effort to gain more conformation data. The data were analysed using MS analysis Qual Browser of Xcalibur software (Thermo Scientific, USA).

6.2.9 NMR Analysis

The freeze-dried sample of purified acylated anthocyanins (20 mg) were dissolved in 0.7 mL of 0.1 % DCl in CD_3OD as solvent. The sample temperature was set at 25 °C. The structures of the acylated anthocyanins were characterized by ^1H and ^{13}C NMR spectrometry using a Bruker high-resolution AVANCE III 700NMR spectrometer. All the ^1H signals resonances were assigned through the 2D correlation spectroscopy (COSY) method. The correlation between ^1H and ^{13}C NMR were then assigned by the heteronuclear multiple-quantum coherence (HMQC) and heteronuclear multiple-bond correlation (HMBC)

methods, in addition to comparing the data to literature data. The data were analysed using MestReNova software (Mestrelab Research, S.L., USA).

6.2.10 Determination of acylated anthocyanin stability during storage at different pH values and temperatures

Anthocyanin acylation reactions. The following reactions (from **Table 6-1** were carried out for 24 h at 60 °C, i.e. Reaction 4 (enzymatic acylation/ intramolecular copigmentation), Reaction 9 (intermolecular copigmentation) and Reaction 10 (control). After 24 h of incubation, the reactions were stopped by filtering the immobilised enzyme (through 53 μm pore size sieve; Fisher Scientific, UK) and the solvents were removed by solvent evaporation under vacuum; the residues was then replaced with 1 mL of water, reflecting the acylated anthocyanins used in the stability tests described below (**Table 6-3**).

Buffer solutions. Buffer solutions of pH 3.0 and 6.0 were prepared in accordance with **Table 6-2**. The pH value of each buffer solution was adjusted with 5 M HCl or 5 M NaOH and measured with a pH meter (Mettler Toledo Seven Easy, China). The pH meter was calibrated with standard solutions of pH 7.0 and 4.0 (standards, Nalgon).

Table 6-2. Solvent proportions (v/v) used to prepare citric acid-sodium phosphate buffer solutions (Sigma, 2018).

pH	0.1 M Citric acid (mL)	0.2 Sodium phosphate (mL)
3.0	79.45	20.55
6.0	36.85	63.15

Stability tests. Appropriate volumes of Reactions 4, 9 and 10 were added into 25 mL of buffer solutions containing 0.05% pottasium sorbate (g/L) to inhibit microbial growth; the solutions were contained in amber bottles with lids. The aim was to obtain initial absorbance values close to 1.000; these were determined using a UV-vis spectrophotometer

(**Table 6-3**). The final pH value of each sample was adjusted using 5M HCl or 5M NaOH. The solutions were shaken for 30 minutes and left to rest for 2 h in the dark at room temperature to reach equilibrium (Fuleki & Francis, 1968). The samples were kept in the dark at 20 °C and 4 °C. For the pH 3.0 and 6.0, samples were analysed until 110 days or until at least 50% of the anthocyanins were degraded. The stability experiments was carried out in duplicate.

Table 6-3. Volumes of total acylated DBS-extracted anthocyanin (Reaction 4 in **Table 6-1**), intermolecular copigmented DBS-extracted anthocyanins (Reaction 9) and control DBS-extracted anthocyanin (Reaction 10) in water added into 25 mL of buffer solutions (at pH 3.0 and 6.0) for testing stability during storage.

Buffer solutions	**Enzymatic acylation/ Intramolecular copigmentation (Reaction 4)**	**Intermolecular copigmentation (Reaction 9)**	**Control (Reaction 10)**
pH 3.0 (mL)[a]	0.83	0.57	0.53
pH 6.0 (mL)[a]	4.76	4.00	3.57

[a]Volumes of reaction 4, 9 and 10 in water used to obtain initial absorbance close to 1.000. Maximum absorption wavelength (λ_{max}). Dried blackcurrant skins (DBS).

Determination of total monomeric anthocyanins using pH differential method. The total anthocyanin content of DBS was determined by the differential pH method based on the property of anthocyanin pigments to change colour in different pH values. 600 µL of anthocyanins-containing solutions were mixed with 1.2 mL of corresponding buffer (pH 1.0 and pH 4.5) and allowed to equilibrate for 20 minutes. Total monomeric anthocyanins (mg cyanidin-3-O-glucoside equivalents/g DW) were calculated as follows:

$$C_{anthocyanin} = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times 1}$$

where $C_{anthocyanin}$ = Total monomeric anthocyanin (cyanidin-3-O-glucoside equivalents, mg/L); A (absorbance) = (A_{520nm} – A_{700nm}) at pH 1.0 – (A_{520nm} – A_{700nm}) at pH 4.5; MW

(molecular weight) = 449.2 g/mol for cyanidin-3-O-glucoside; DF = dilution factor; l = pathlength in cm; ε = 26900 molar extinction coefficient in L/mol/cm for cyanidin-3-O-glucoside; and 10^3 = factor for conversion from g to mg (Lee et al., 2005). The anthocyanin content was then calculated by:

$$Anthocyanin\ content(mg/g) = \frac{C\ anthocyanin\ \left(\frac{mg}{L}\right) \times extract\ (L)}{sample\ (g)}$$

The absorbance of the samples at pH 1.0 and pH 4.5 buffer, respectively, was determined at both 520 and 700 nm using a spectrophotometer (Thermo Electron Corporation, USA). The absorbance of the samples was read against distilled water (blank).

UV-Vis Spectrophotometry. Spectrophotometric measurements were carried out using a UV-Vis spectrophotometer (Perkin Elmer Lambda 20, USA). The spectra were recorded in 1 cm path length disposable cuvettes at wavelength from 450 to 700 nm. Copigmentation phenomena were related with a bathochromic shift, i.e. a shift of the maximum absorption wavelength (λ_{max}) and a hyperchromic effect, i.e. an increase in the absorbance value (ΔA) at λ_{max}. Each spectroscopic measurement was performed in duplicate. The percentage of colour retention at λ_{max} was used to evaluate the colour degradation at different pH over time and was calculated according to the following formula:

$$\text{Colour Retention (\%)} = \frac{At}{Ao} \times 100$$

where A_0 is the initial absorbance at time zero and A_t is the absorbance at time t (Katsaboxakis et al., 1998).

Colour measurement. The sample colour was measured using a Hunter-Lab colourimeter (Hunter Lab, ColourQuest, Hunter Associates Laboratory, Virginia, USA) based on three colour coordinates *L**, *a**, and *b** at D65 standard illuminant and 10° standard observer. The instrument was calibrated using a black card. Colour was expressed in Hunter Lab units *L** (Lightness/ darkness; 0-100), *a** (positive = redness/negative = greenness) and *b** (positive = yellowness/negative = blueness). The total colour difference (TCD) between two samples was calculated according to the following formula:

$$Total\ colour\ difference\ (TCD) = [(L^* - L_o)^2 + (a^* - a_o)^2 + (b^* - b_o)^2]^{1/2}$$

where, L_o, a_o, b_o = blank values of control samples extracted at day 0

Chroma (c) is the quantitative attribute of colour intensity and hue (h) is the qualitative attribute of colours which are defined as reddish, greenish, yellowish and bluish. The chroma and hue angle were calculated using the *a** and *b** values according to the equations below (Wrolstad & Smith, 2010).

$$Chroma\ (c) = \sqrt{(a^*)^2 + (b^*)^2}$$

$$Hue\ angle\ (h) = ArcTan(\frac{b^*}{a^*})$$

Kinetic study. The zero order reaction rate constants (k) and half-lives ($t_{1/2}$) were calculated using the following equations:

$$C_t = C_0 - k*t$$

$$t_{1/2} = \frac{Co}{2k}$$

where C_0 is the initial anthocyanin concentration and C_t is the anthocyanin concentration at the reaction time t (Remini et al., 2015).

6.2.11 Statistical analysis

All statistical analyses were conducted by one-way analysis of variance (ANOVA). Tukey's multiple range tests were employed with probability of $p \leq 0.05$. The software used or statistical analysis was Minitab V.16 (Minitab Inc., State College, Pennsylvania, USA).

6.3 Results and discussion

6.3.1 Enzymatic acylation of anthocyanins

According to the HPLC analysis, the total anthocyanin content in the purified DBS extract was 9.0 ± 0.1 mg/g (of which delphinidin-3-O-rutinoside; D3R, ~34.6% > cyanidin-3-O-rutinoside; C3G, ~26.0% > delphinidin-3-O-glucoside; D3G, ~19.2% > cyanidin-3-O-glucoside; C3G, ~8.3%) [**Figure 6-3 (a**)]. In the case of the acylation reactions with methyl ferulate (Reactions 5 – 8 in **Table 6-1**) no additional HPLC peaks were observed indicating that acylation of anthocyanins did not take place. On the other hand, in the case the acylation reactions with methyl cinnamate (Reactions 1 – 4 in **Table 6-1**), two new peaks around 22 and 24 min were observed; these are shown in **Figure 6-3 (b)** (peaks E and F), which depicts a representative chromatograph after 24 h of reaction. The fact that no peak was observed in the acylation reaction of anthocyanins with methyl ferulate suggests that the selectivity of lipase B was affected by the structure of the acyl donor and the anthocyanins present. The structure of methyl ferulate [**Figure 6-1 (b)**] is more complex, with a methoxyl group in the benzene ring, compared to methyl cinnamate [**Figure 6-1 (a)**].

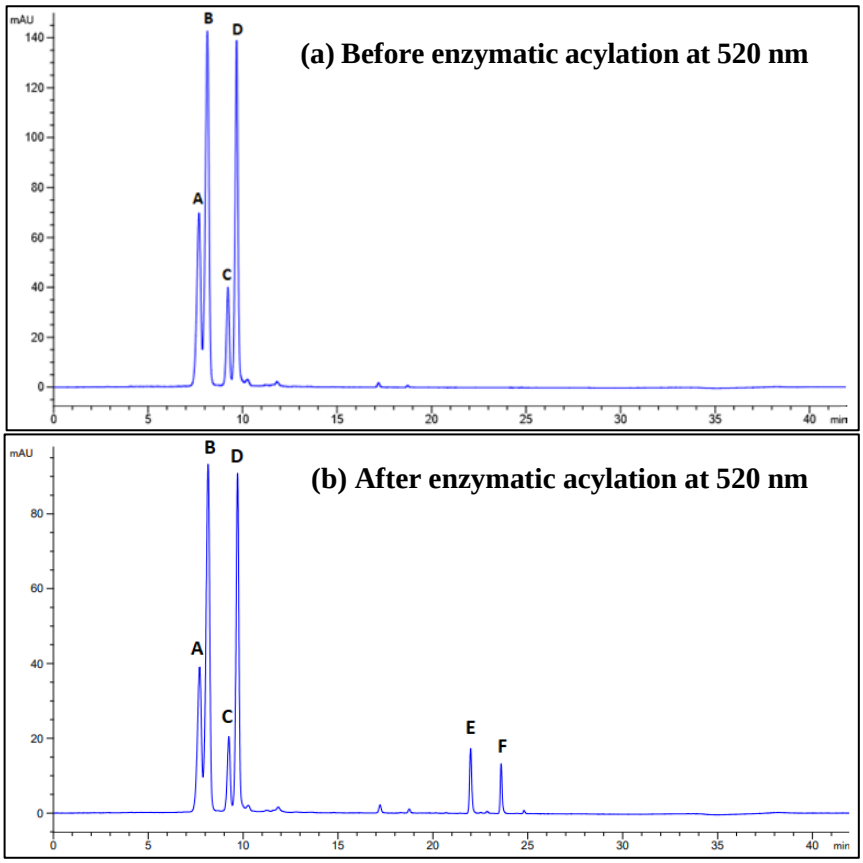


Figure 6-3. HPLC chromatogram of anthocyanins and acylated anthocyanins after 24 h reaction of purified anthocyanin mixture with methyl cinnamate at 60 °C with 50 mg/ml of lipase enzyme. (A) Delphinidin-3-O-glucoside; (B) Delphinidin-3-O-rutinoside; (C) Cyanidin-3-O-glucoside; (D) Cyanidin-3-O-rutinoside; (E) Acylated delphinidin 3-O-glucoside; (F) Acylated cyanidin 3-O-glucoside. Peaks E and F were confirmed after HPLC/MS and NMR analysis.

Theoretical ions for peak E (acylated D3G) and F (acylated C3G) in **Figure 6-3 (b)** were calculated and the extracted ion chromatograms (EICs) generated for these two masses ($[M+H]^+$) were m/z 595.1446 and 579.1497, respectively [**Figure 6-5 (b)** and **Figure 6-6 (b)]**. Furthermore, **Figure 6-5 (b)** and **Figure 6-6 (b)** present the LCMS data of the new peaks that were obtained by in HPLC analysis, which were consistent as being D3G and C3G molecules attached to the methyl cinnamate. The MS^2 data from the direct infusion analyses showed a characteristic fragment ion at m/z 303.0499 in the D3G sample which corresponds to delphinidin from the parent ion m/z 595.1446. Similarly, in the C3G sample, a characteristic fragment ion at m/z 287.0550 for cyanidin from the parent ion m/z 579.1497

was observed. These are both in agreement with the D3G and C3G aglycone structures, reflecting the loss of the glucose residue attached to the cinnamic acid group.

Also, these results suggested that the selectivity of the lipase enzyme is also modulated by the nature of the sugar moiety of the flavonoid (Ardhaoui et al., 2004), whereas the regioselectivity of the lipase enzyme is towards primary hydroxyl groups that are present on the glycoside moiety of the anthocyanin molecule [**Figure 6-4 (a)**], thus monoacylated products were synthesised. Therefore, no acylation was detected in the case of the cyanidin-3-O-rutinoside and delphinidin-3-O-rutinoside due to the unavailability of primary hydroxyl groups on the glucoside moieties in these cases, as shown in [**Figure 6-4 (b)**].

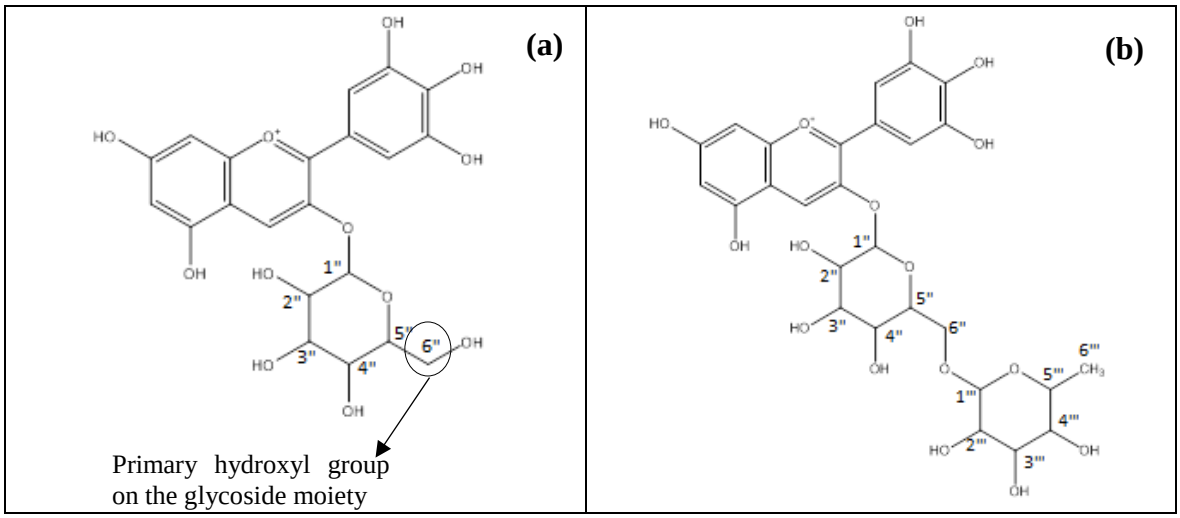


Figure 6-4. Illustration of primary hydroxyl groups of glucose moieties on (a) delphinidin-3-O-glucoside and (b) delphinidin-3-O-rutinoside. Structures plotted using the ChemDraw website (2018).

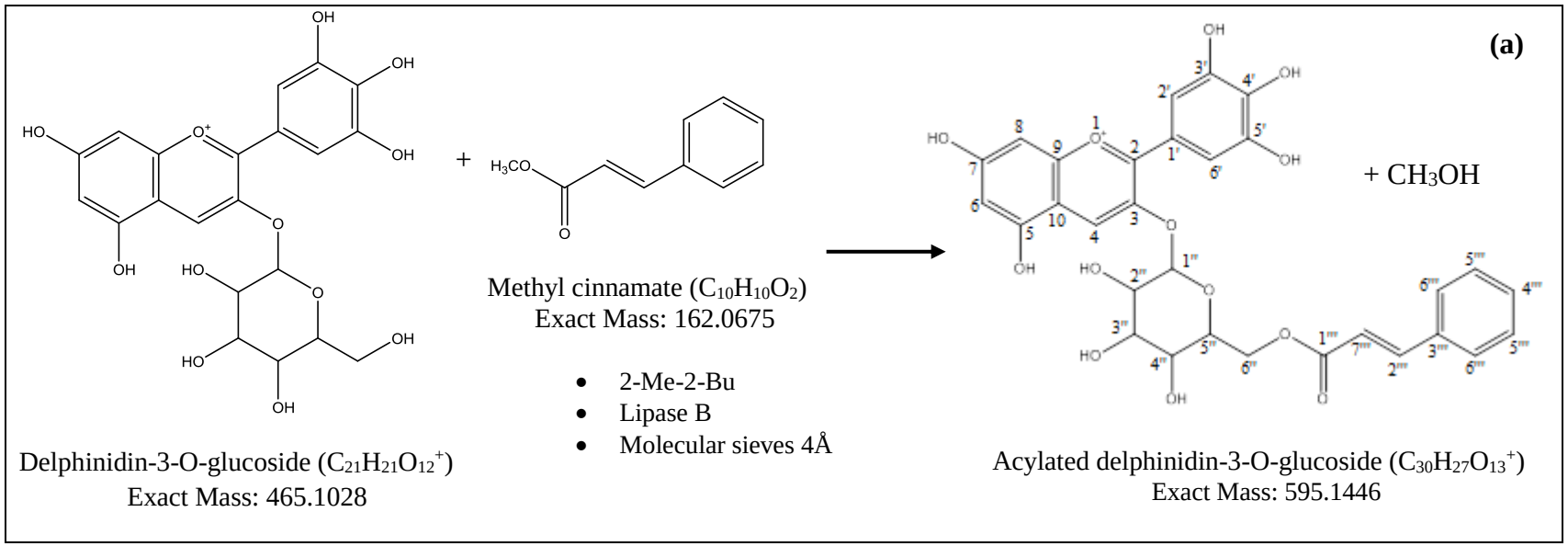


Figure 6-5. Enzymatic acylation reaction of (a) delphinidin-3-O-glucoside from dried blackcurrant skins extract with methyl cinnamate; (b) MS and MS^2 of acylated delphinidin-3-O-glucoside. Structures plotted using the ChemDraw website (2018).

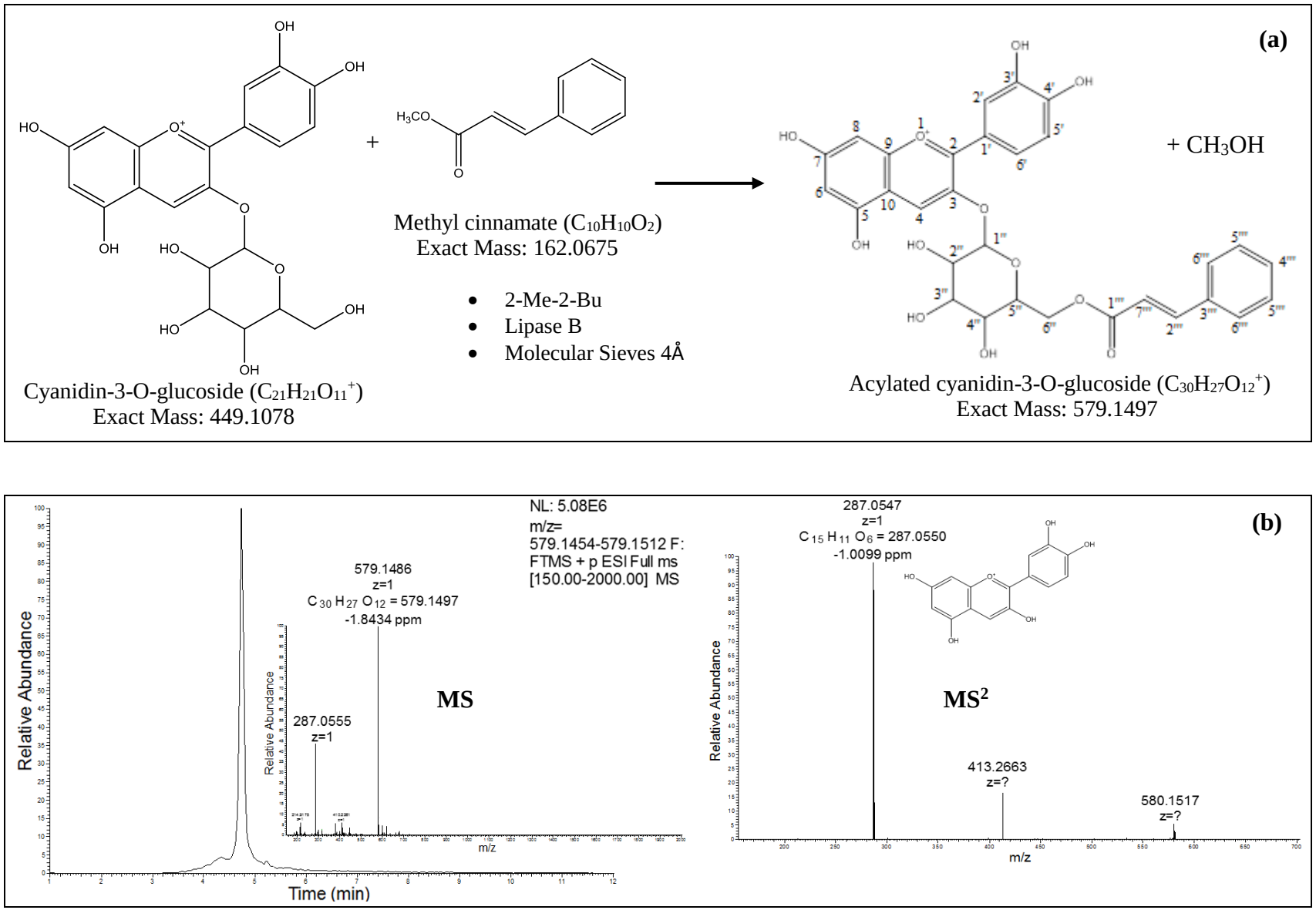


Figure 6-6. Enzymatic acylation reaction of (a) cyanidin-3-O-glucoside from dried blackcurrant skins extract with methyl cinnamate; (b) MS and MS^2 of acylated cyanidin 3-O-glucoside. Structures plotted using the ChemDraw website (2018).

6.3.2 Conversion yields of enzymatic acylation of anthocyanins

Figure 6-7 depicts the kinetic data of the enzymatic acylation of D3G and C3G with methyl cinnamate as the acyl donor, corresponding to Reactions 1 – 4 in **Table 6-1**. The highest conversion yield was obtained with 50 g/L of lipase B after 24 h of incubation at 60°C [**Figure 6-7 (d)** corresponding to Reaction 4 in **Table 6-1**]. This indicated that a higher temperature (60 °C vs 45 °C) resulted in higher lipase activity and most likely better solubility of the anthocyanins, leading to high conversion yields. As shown in **Figure 6-7 (d)**, the acylation equilibrium was reached between 20 and 24 h at 60 °C. After 24 h, C3G had significantly ($p \leq 0.05$) higher conversion yield (~15.1%) compared to D3G (~10.1%). The lower conversion yield obtained for D3G might be due to the presence of three OH groups on the aglycone compared to two for C3G [**Figure 6-1 (c** and **e)**].

When methyl cinnamate was used as acyl donor under the same incubation temperature (60°C) but lower lipase B concentration (25 g/L) [**Figure 6-7 (c)]**, the conversion yield of the reaction after 24 h was significantly decreased ($p \leq 0.05$) to ~10.4% for C3G and ~4.8% for D3G. Moreover, at 50 g/L of lipase B concentration and 45 °C of incubation [**Figure 6-7 (b)**], the maximum conversion yields of the C3G and D3G acylation reactions were only ~8.6% and ~4.0%, respectively. Overall, the results demonstrated that a higher incubation temperature (60°C) and lipase B concentration (50 g/L) led to higher conversion yields for both D3G and C3G.

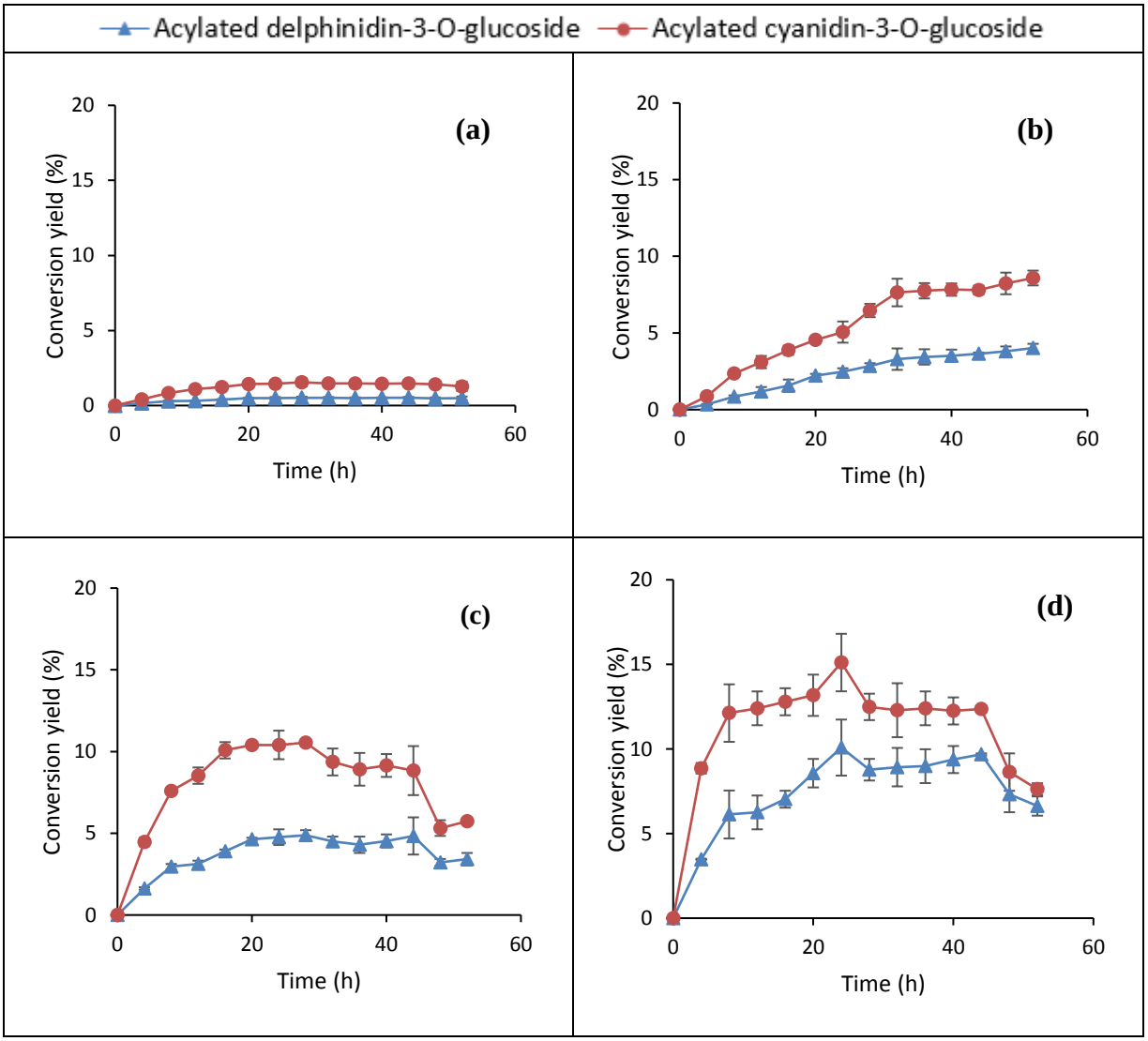


Figure 6-7. Conversion yields (%) of acylated anthocyanins in the presence of methyl cinnamate as acyl donor at different reaction temperatures and lipase concentrations. (a) 45 °C, 25 g/L lipase, (b) 45 °C, 50 g/L lipase, (c) 60 °C, 25 g/L lipase and (d) 60 °C, 50 g/L lipase.

The enzymatic acylation between anthocyanins extracted from blackcurrant skins and aromatic acid methyl esters (transesterification) is a relatively a new research approach, as previous studies by Kontogianni et al. (2003), Ardahoui et al. (2004) and Stevenson et al. (2006) used carboxylic acids (e.g. cinnamic, hydrocinnamic and benzoic acids) and fatty acids (e.g. palmitic and decanoic acids) as acyl donors for the direct esterification of flavonoids and anthocyanins. However, in a bioconversion study carried out by Yan et al. (2016) using

extracted C3G from black rice and methyl cinnamate as the acyl donor, higher conversion yields were obtained (68.0% vs 15.1% in this study). This might be due to the anthocyanin composition of black rice, in which C3G was the predominant anthocyanin (~80.8%) in black rice, whereas in the blackcurrant skins extract four types of anthocyanins are present (D3R; ~34.6%, C3G; ~26.0%, D3G; ~19.2%, C3G; ~8.3%). Moreover, that study used 100 g/L of starting material in pyridine as the solvent medium, 200 g/L of lipase (Novozym 435) and the reaction was incubated at 40 °C and stirred at 30 rpm under vacuum of 900 mbar. These different enzymatic acylation parameters most likely also influenced the conversion yield of acylated anthocyanins.

6.3.3 Structural identification of acylated anthocyanins by NMR spectroscopy

Whilst the LC-MS data provide the data in order to verify the formation of acylated anthocyanins, NMR is the most important tool for complete structure interpretation. Also, the NMR method is primarily used to determine the nature of the sugar moieties and the sites of acyl and sugar substitutions. As summarised in **Table 6-4** and depicted **Supplementary Figure 6-9** and **Supplementary Figure 6-10**, the number of methyl cinnamates linked to the anthocyanin molecules and their position on either the glycosidic moiety or the aglycon moiety were determined by analysing their ^{1}H NMR and ^{13}C NMR spectra. Moreover, these structures were compared to literature data and led to the confirmation of the structures proposed in **Figure 6-5 (a)** and **Figure 6-6 (a)**.

In the ^{13}C NMR spectrum of the acylated D3G, the signal for C″-6 of the glucose moiety shifted by ~0.65 ppm (from 65.36 to 66.01 ppm) compared with that of the acylated C3G due to the resonance effect towards the carbonyl groups of the different esters that have been formed. The difference between acylated D3G and acylated C3G was that acylated D3G consisted of one more hydroxyl group at the C-5´ of the delphinidin molecule, therefore, no proton signal

was observed at the C-5′ position in the ^{1}H NMR spectrum compared to acylated C3G (**Table 6-4**). This is the first report of an NMR spectrum for the enzymatic acylation of D3G from blackcurrant skins using methyl cinnamate as acyl donors and lipase as biocatalyst.

The data in **Table 6-4** are in agreement with most studies, where the acylation of glycosylated flavonoids (including anthocyanins) by immobilized *C. Antartica* lipase B occurs preferentially on the primary hydroxyl group on the glucoside moiety of the molecule (C6″-OH). For example, in the study by Cruz et al. (2016), the acylation of malvidin-3-glucoside with oleic acid and *C. Antartica* Lipase B carried out in anhydrous 2-methyl-2-butanol, led to the enzymatic synthesis of malvidin-3-O-(6″-oleoyl) glucoside). Furthermore, the location of the acylated site of anthocyanins at the C6″-OH group of malvidin-3-O-glucoside and cyanidin-3-O-glucoside have been well documented in previous studies to be in the range between 62.9 to 67.0 ppm of the ^{13}C NMR spectrum as opposed to ~60.8 ppm for non-acylated anthocyanins (Yan et al., 2016; Cruz et al., 2016; Fernandes et al., 2015 and McGhie et al., 2006). Therefore, the NMR data in this study verified the hypothesis that acylation took place at the C6″-OH group. As such, the acylated delphinidin-3-O-glucoside and acylated cyanidin-3-O-glucoside were delphinidin 3-(6″- cinnamoyl)-glucoside and cyanidin 3-(6″- cinnamoyl)-glucoside, respectively.

Table 6-4. ^{13}C NMR data of the delphinidin 3-(6″- cinnamoyl)-glucoside and cyanidin 3-(6″-cinnamoyl)-glucoside in CD_3OD containing 0.1% DCl.

Position	δ (^{13}C)	δ (^{1}H); *J* (Hz)
Delphinidin		
2	167.2	-
3	137.4	-
4	132.6	6.71; m
5	162.8	-
6	101.4	6.59; s
7	167.1	-
8	99.9	6.61; s
9	166.9	-
10	117.0	-
1'	127.6	-
2'	104.0	7.66; s
3'	145.1	-
4'	134.3	-
5'	145.1	-
6'	104.0	7.68; s
Glucose		
1''	101.4	5.70; s
2''	70.7	3.86; m
3''	76.4	3.80; m
4''	69.7	3.67; m
5''	74.3	3.54; m
6''a	65.4	4.47; d, 7.0
6''b	65.4	4.56; d, 7.0
Cinnamic acid		
1'''	172.7	-
2'''	145.1	7.56; s
3'''	134.1	-
4'''	130.2	7.62; m
5'''	128.6	7.49; m
6'''	127.8	7.75; m
7'''	117.3	6.60; d, 7.0

Position	δ (^{13}C)	δ (^{1}H); *J* (Hz)
Cyanidin		
2	167.4	-
3	137.8	-
4	133.7	6.57; m
5	167.2	-
6	101.2	6.45; s
7	167.3	-
8	99.7	6.45; s
9	167.3	-
10	117.8	-
1'	128.1	-
2'	105.7	7.52; s
3'	145.6	-
4'	134.1	-
5'	145.6	7.49; m
6'	106.6	7.56; s
Glucose		
1''	101.4	5.39; d, 7.0
2''	70.7	3.44; m
3''	84.2	3.72; m
4''	69.9	3.53; m
5''	73.9	3.67; m
6''a	66.0	4.33; d, 7.0
6''b	66.0	4.42; d, 7.0
Cinnamic acid		
1'''	173.2	-
2'''	145.4	7.43; s
3'''	134.0	-
4'''	129.5	7.49; m
5'''	129.4	7.36; m
6'''	128.6	7.63; m
7'''	118.0	6.47; d, 7.0

*Singlet (s); Doublet (d); Multiplet (m). Deuterated methanol (CD_3OD); Deuterium chloride solution (DCl).

6.3.4 Stability of the acylated anthocyanins

The stability of the enzymatically acylated anthocyanins during storage at different pH (pH 3.0 and 6.0) and temperatures (4 °C and 20 °C) was investigated and compared to intermolecular copigmented anthocyanins with methyl cinnamate (Reaction 9 in **Table 6-1**) and the control sample (Reaction 10 in **Table 6-1**). The anthocyanin extract that was used in this study was the reaction mixtures after the acylation reactions and included the acylated anthocyanins, i.e. delphinidin 3-(6″- cinnamoyl)-glucoside and cyanidin 3-(6″- cinnamoyl)-glucoside, as well as the no-acylated anthocyanins.

The percentage of anthocyanin content (%) versus storage time (t) was plotted (**Figure 6-8**); all samples followed zero order reaction kinetics throughout storage with a correlation coefficient (R^2) greater than 0.900. The zero order reaction rate constant (k) was calculated from the graphs to obtain the estimated half-life time ($t_{1/2}$); the results are shown **Table 6-5**. The colour changes were also monitored during storage and these were reported in terms of changes in the maximum absorption wavelength (λ_{max}) (**Supplementary Figure 6-11**), colour values (*L**, *a**, *b**) (**Supplementary Figure 6-12** to **Supplementary Figure 6-15**) and percentage of colour retention (**Table 6-6**).

A high percentage of colour retention, measured by UV-Vis spectrophotometry reflects a high colour stability of the anthocyanin solution and consequently low colour degradation over time. The changes in the colour of the samples were expressed in terms of colour values (*L**, *a**, *b**) by Hunter Lab. In addition, a high chroma value indicates a high colour intensity, which is accompanied with an increase in absorbance in the visible range (hyperchromic shift) and a shift in the wavelength of the maximum absorbance toward higher values (bathochromic shift) (Hurtado et al., 2009).

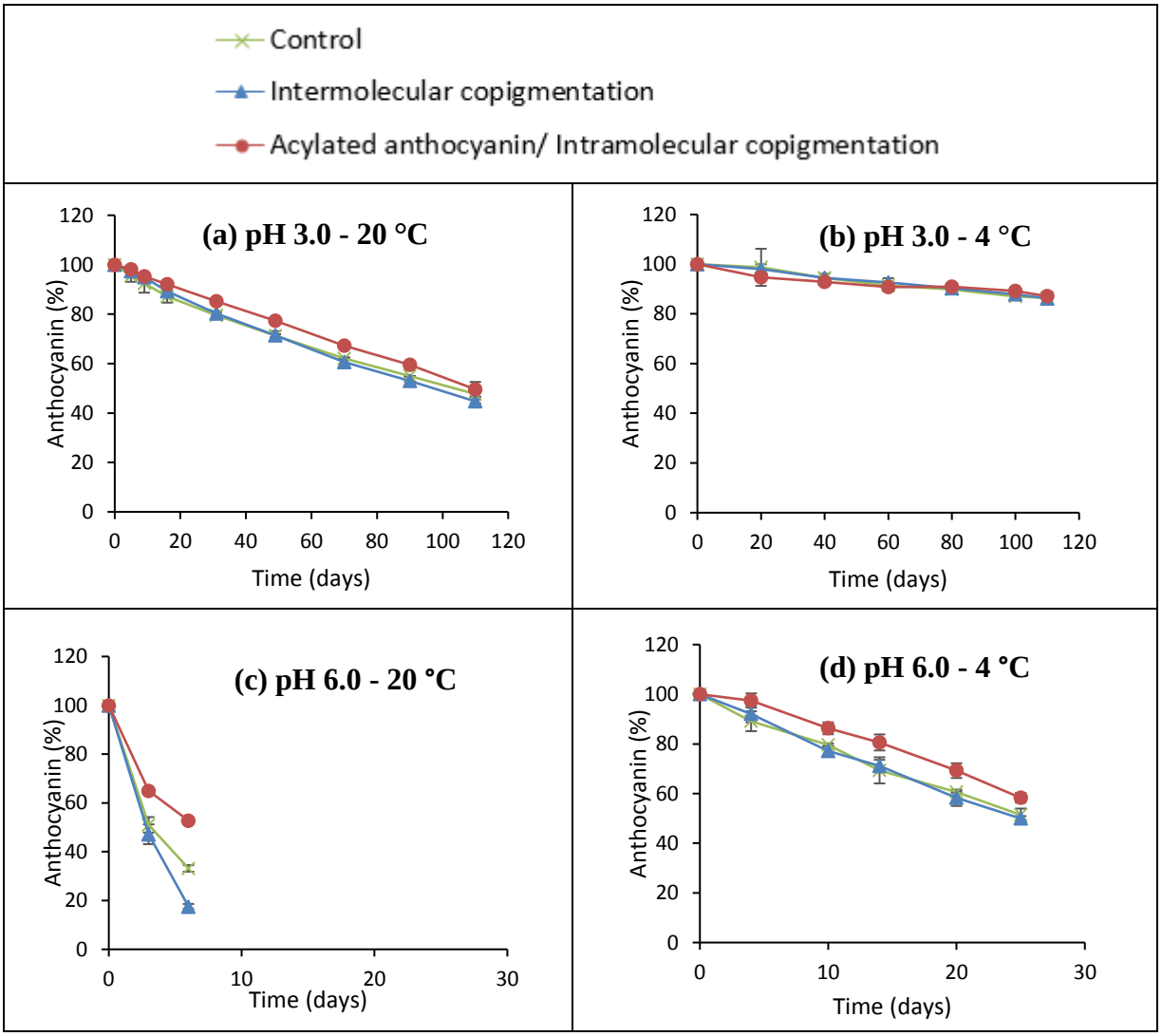


Figure 6-8. Degradation kinetics (% content) of total acylated DBS-extracted anthocyanin (Reaction 4 in **Table 6-1**), intermolecular copigmented DBS-extracted anthocyanins (Reaction 9) and control DBS-extracted anthocyanin (Reaction 10) during storage in buffer solutions at (a) pH 3.0; 20 °C, (b) pH 3.0; 4 °C, (c) pH 6.0; 20 °C and (d) pH 6.0; 4 °C. *DBS (Dried blackcurrant skins).

Copigmentation stability in buffer solution at pH 3.0. As shown in **Table 6-5 (a)**, the presence of acylated anthocyanins at 20 °C significantly increased ($p \leq 0.05$) the half shelf life of anthocyanins to 108.8 ± 1.5 days, compared to the control and intramolecular copigmentation samples. Acylated anthocyanins showed higher stability than non-acylated anthocyanins, which consequently prevented the hydration of the flavylium cation on the anthocyanin structure that would otherwise form a colourless carbinol pseudobase. At 4 °C the acylated anthocyanins

showed a significantly high ($p \leq 0.05$) half shelf life, i.e. 442.9 ± 1.9 days compared to the control and intramolecular copigmentation samples. This represents approximately a 5-fold increase compared to storage at 20 °C [**Table 6-5 (b)**]. However, only 12.9 to 13.8 % of total anthocyanins were degraded within 110 days storage at 4 °C of storage [**Figure 6-8** (**b**)]. Therefore, longer storage periods are needed in order to reach at least 50% of total anthocyanins degradation and thus obtain more accurate results with regards to anthocyanin shelf life and colour stability under the abovementioned storage conditions.

Table 6-5. Estimated half-life time values (t ½) of total acylated DBS-extracted anthocyanin (Reaction 4 in **Table 6-1**), intermolecular copigmented DBS-extracted anthocyanins (Reaction 9) and control DBS-extracted anthocyanin (Reaction 10) during storage in buffer solutions at (a) pH 3.0; 20 °C, (b) pH 3.0; 4 °C, (c) pH 6.0; 20 °C and (d) pH 6.0; 4 °C.

Samples	**(a) pH 3.0 - 20 °C**			**(b) pH 3.0 - 4 °C**		
	k	**$t_{1/2}$ (days)**	**R^2**	**k**	**$t_{1/2}$ (days)**	**R^2**
ATC (Control)	0.1304	97.4 (0.6)^{b}	0.9992	0.0310	386.8 (4.8)^{c}	0.9413
ATC + MC (Intermolecular Copigmentation)	0.1284	94.2 (2.9)^{b}	0.9885	0.0299	404.4 (2.0)^{b}	0.9443
ATC + MC + Lipase + MS (Acylated ATC/ Intramolecular Copigmentation)	0.1212	108.8 (1.5)^{a}	0.9680	0.0288	442.9 (1.9)^{a}	0.9521
	(c) pH 6.0 - 20 °C			**(d) pH 6.0 - 4 °C**		
ATC (Control)	6.0481	4.1 (0.1)^{b}	0.9187	1.0122	25.0 (1.6)^{b}	0.9860
ATC + MC (Intermolecular Copigmentation)	7.9894	3.4 (0.1)^{c}	0.9689	1.0965	24.3 (1.0)^{b}	0.9959
ATC + MC + Lipase + MS (Acylated ATC/ Intramolecular Copigmentation)	3.1658	5.8 (0.0)^{a}	0.9138	0.5047	32.3 (2.9)^{a}	0.9770

*Estimated half life time ($t_{½}$); Anthocyanins (ATC); Methyl cinnamate (MC); Molecular Sieves 4Å (MS); Dried blackcurrant skins (DBS).Figures in parenthesis indicate the standard deviation. Values with the same letter ^{a,b} in each column are not significantly different ($p > 0.05$).

The stability of copigmentation during storage was also assessed by measuring the percentage of colour retention (%). After 110 days of storage at 20 °C, acylated anthocyanin and the control sample showed slightly higher but statistically significant ($p \leq 0.05$) percentage of colour retention, (55.1 ± 0.9 % and 54.7 ± 0.5 %, respectively) compared to intermolecular copigmented anthocyanin (51.5 ± 0.7 %) [**Table 6-6 (a)**]. Similarly, after 110 days of storage at 4 °C, acylated anthocyanin showed slightly higher but statistically significant ($p \leq 0.05$) percentage of colour retention, (93.6 ± 0.9 %) compared to intermolecular copigmented anthocyanin and the control samples (90.5 ± 0.7 % and 90.7 ± 0.1 %, respectively) [**Table 6-6 (b)**]. In both cases, a slightly better colour retention was observed in the case of the acylated anthocyanin at pH 3.0. Moreover, the colour retention of the anthocyanin at pH 3.0 was supported by a constant λ_{max} reading over storage time, shown in **Supplementary Figure 6-11 (a** and **b)**.

Table 6-6. Changes of visual colour and colour retentions (%) of total acylated DBS-extracted anthocyanin (Reaction 4 in **Table 6-1**), intermolecular copigmented DBS-extracted anthocyanins (Reaction 9) and control DBS-extracted anthocyanin (Reaction 10) during storage in buffer solutions at (a) pH 3.0; 20 °C and (b) pH 3.0; 4 °C.

ATC	ATC + MC	ATC + MC + Lipase + MS	ATC	ATC + MC	ATC + MC + Lipase + MS
(a) pH 3.0 – 20 °C			**(b) pH 3.0 – 4 °C**		
Day 0			**Day 0**		
					
Day 50			**Day 50**		
					
Day 110			**Day 110**		
					
Colour Retention (%) at Day 110			**Colour Retention (%) at Day 110**		
54.7 (0.5)[a]	51.5 (0.7)[b]	55.1 (0.9)[a]	90.7 (0.1)[b]	90.5 (0.7)[b]	93.6 (0.9)[a]

Conversion of *L**, *a**, and *b** values to RGB (Red, Green, Blue) colour model for visualisation of anthocyanin colour. Anthocyanins (ATC); Methyl cinnamate (MC); Molecular Sieves 4Å (MS); Dried blackcurrant skins (DBS). Figures in parenthesis indicate the standard deviation. Values with the same letter [a,b] in each column are not significantly different ($p > 0.05$).

According to the Hunter Lab results shown in (**Supplementary Figure 6-12**), acylated anthocyanin exhibited slightly lower *L** (darker colour), total colour difference and hue angle, whilst higher *a** (increase in redness), *b** (decrease in blueness) and chroma values compared to intermolecular copigmented and control samples, indicating an enhancement of colour stability throughout storage at 20 °C. These values however, were not different to the values of the samples during storage at 4 °C (**Supplementary Figure 6-13**).

Copigmentation stability in buffer solution at pH 6.0. Acylated anthocyanins at pH 6.0 and stored at 20 °C as shown in **Table 6-5 (c)** had significantly higher half life time

(5.8 ± 0.0 days). Also, acylated anthocyanin showed the highest half life time (32.3 ± 2.9 days) during storage at lower temperature (4 °C), which was 6-fold higher compared to 20 °C [**Table 6-5 (d)**].

The hue and *b* value of acylated anthocyanin with cinnamic acids decreased as the pH was increased to pH 6.0, towards a more purplish hue (**Supplementary Figure 6-14** and **Supplementary Figure 6-15**), similar as reported by Giusti and Wrolstad (2003) in a radish pigment containing di-acylated anthocyanin. However, as shown in **Table 6-7**, acylated anthocyanin showed lower percentage of colour retention at both storage temperatures (4 °C and 20 °C) compared to non-acylated anthocyanins. Especially at 20 °C, the temperature greatly influenced the colour stability of non-acylated anthocyanin, whereas the higher percentage colour retention was actually due to the formation of new pigments that led to yellowish/orange tonalities, as suggested by Trouillas et al. (2016). This is supported by the gradual decrease of hypsochromic shift during storage as shown in **Supplementary Figure 6-11 (c** and **d)**.

According to Giusti et al. (1999), acylated anthocyanins with cinnamic acid from strawberries, radishes and red-fleshed potatoes caused higher bathochromic shift which translated to higher chroma. Moreover, the hue angle and lightness of the samples were clearly decreased by acylation in the aqueous buffer solutions. In this study, acylated anthocyanins showed the lowest *b* (increase in blueness) and *a* values (decrease in redness) on the day of preparation (**Supplementary Figure 6-14** and **Supplementary Figure 6-15**). Throughout storage, even though acylated anthocyanins could prevent browning taking place, the colour degradation was rapid suggesting the instability of purple colour in the alkaline region. These can be seen from the significant ($p \leq 0.05$) increase in the total colour difference, b^* and hue

angle values especially during storage at 20 °C. The chroma values also significantly ($p \leq 0.05$) decreased throughout the storage indicating the decrease in the colour intensity.

Table 6-7. Changes of visual colour and colour retentions (%) of total acylated DBS-extracted anthocyanin (Reaction 4 in **Table 6-1**), intermolecular copigmented DBS-extracted anthocyanins (Reaction 9) and control DBS-extracted anthocyanin (Reaction 10) during storage in buffer solutions at (a) pH 6.0; 20 °C and (b) pH 6.0; 4 °C.

ATC	**ATC + MC**	**ATC + MC + Lipase + MS**	**ATC**	**ATC + MC**	**ATC + MC + Lipase + MS**
(a) pH 6.0 – 20 °C			**(b) pH 6.0 – 4 °C**		
Day 0			**Day 0**		
					
Day 3			**Day 10**		
					
Day 6			**Day 25**		
					
Colour Retention (%) at Day 6			**Colour Retention (%) at Day 25**		
96.8 (0.7)[a]	75.7 (0.8)[b]	46.1 (1.5)[c]	70.5 (0.0)[a]	71.8 (0.6)[a]	49.7 (0.1)[b]

Conversion of *L**, *a**, and *b** values to RGB (Red, Green, Blue) colour model for visualisation of anthocyanin colour. Anthocyanins (ATC); Methyl cinnamate (MC); Molecular Sieves 4Å (MS); Dried blackcurrant skins (DBS). Figures in parenthesis indicate the standard deviation. Values with the same letter [a,b] in each column are not significantly different ($p > 0.05$).

6.4 Conclusions

This work reported the acylation of delphinidin-3-O-glucoside and cyanidin-3-O-glucoside with methyl cinnamate by enzymatic acylation of anthocyanins extracted from dried blackcurrant skins extract. The highest conversion yields (acylated delphinidin-3-O-glucoside; ~10.1 % and acylated cyanidin-3-glucoside; ~15.1 %) were obtained after 24 h incubation at 60 ℃ using 50 g/L of lipase B. No acylation was observed for delphinidin-3-O-rutinoside and cyanidin-3-O-rutinoside indicating the regioselectivity of the lipase towards primary hydroxyl groups that are present on the glycoside moiety of the anthocyanin molecule. Also enzymatic acylation depends on the structure of the acyl donor, therefore, no acylation with methyl ferulate was detected. The MS data confirmed the expected mass of acylated delphinidin-3-O-glucoside (m/z 595.1446) and acylated cyanidin-3-O-glucoside (m/z 579.1497). Moreover, the NMR spectra indicated that the 6″-OH group of delphinidin-3-O-glucoside and cyanidin-3-O-glucoside was acylated.

Acylated anthocyanins were found to be stable, since during storage at pH 3.0 and 6.0, the new acylated anthocyanin compounds had longer estimated shelf life than the non-enzymatically copigmentated anthocyanins. Moreover, the colour stability of the acylated anthocyanins increased during storage at pH 3.0, and also acylation was capable of inhibit browning at pH 6.0. Overall, this work indicated the potential of using enzymatically produced anthocyanin compounds as natural colourants in acidic and alkaline food matrices. Moreover, the approach of using anthocyanin-containing extracts as the starting material for the acylation reaction is potentially economically viable although further work is needed to optimise the enzymatic synthesis process and assess its scalability.

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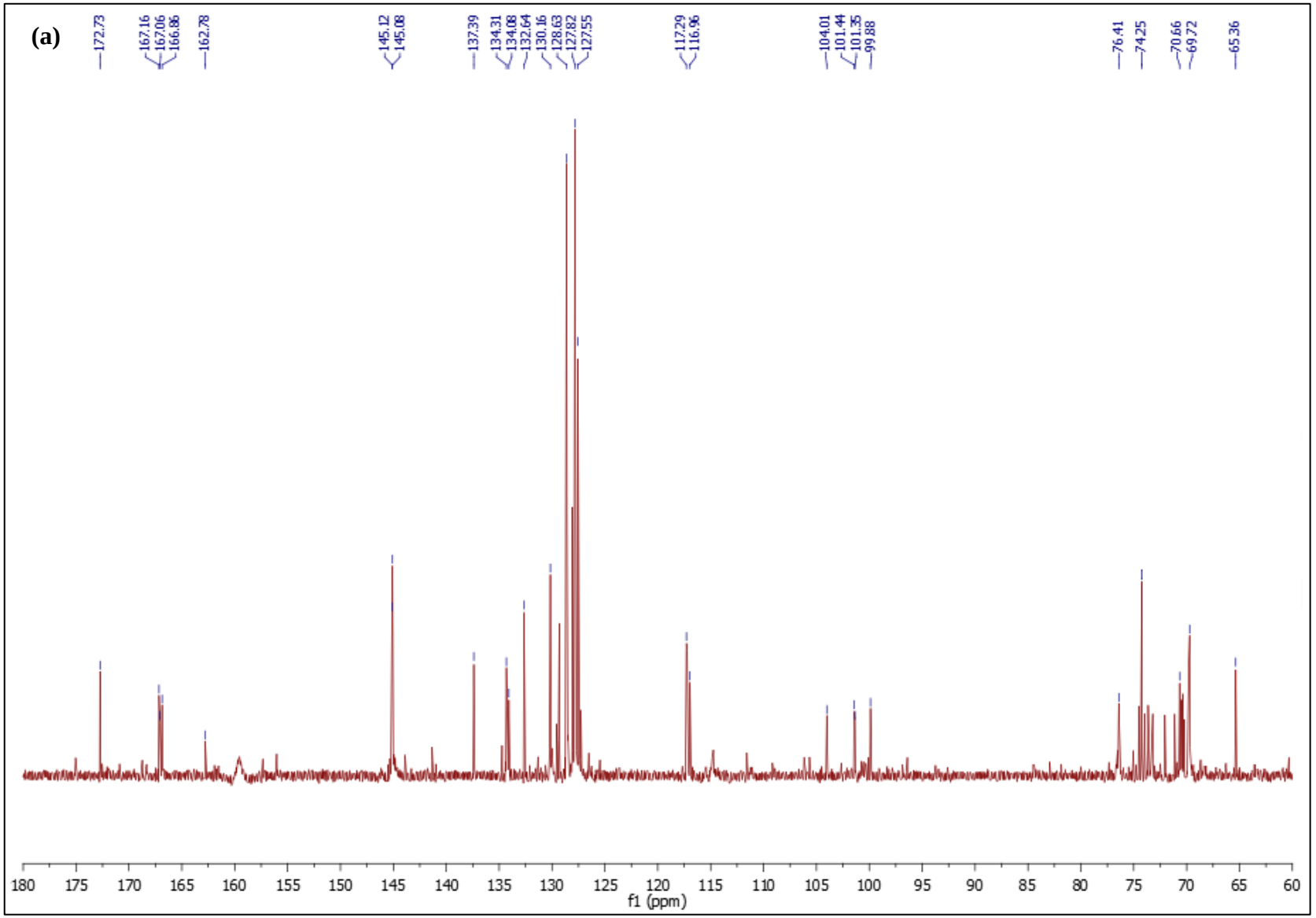
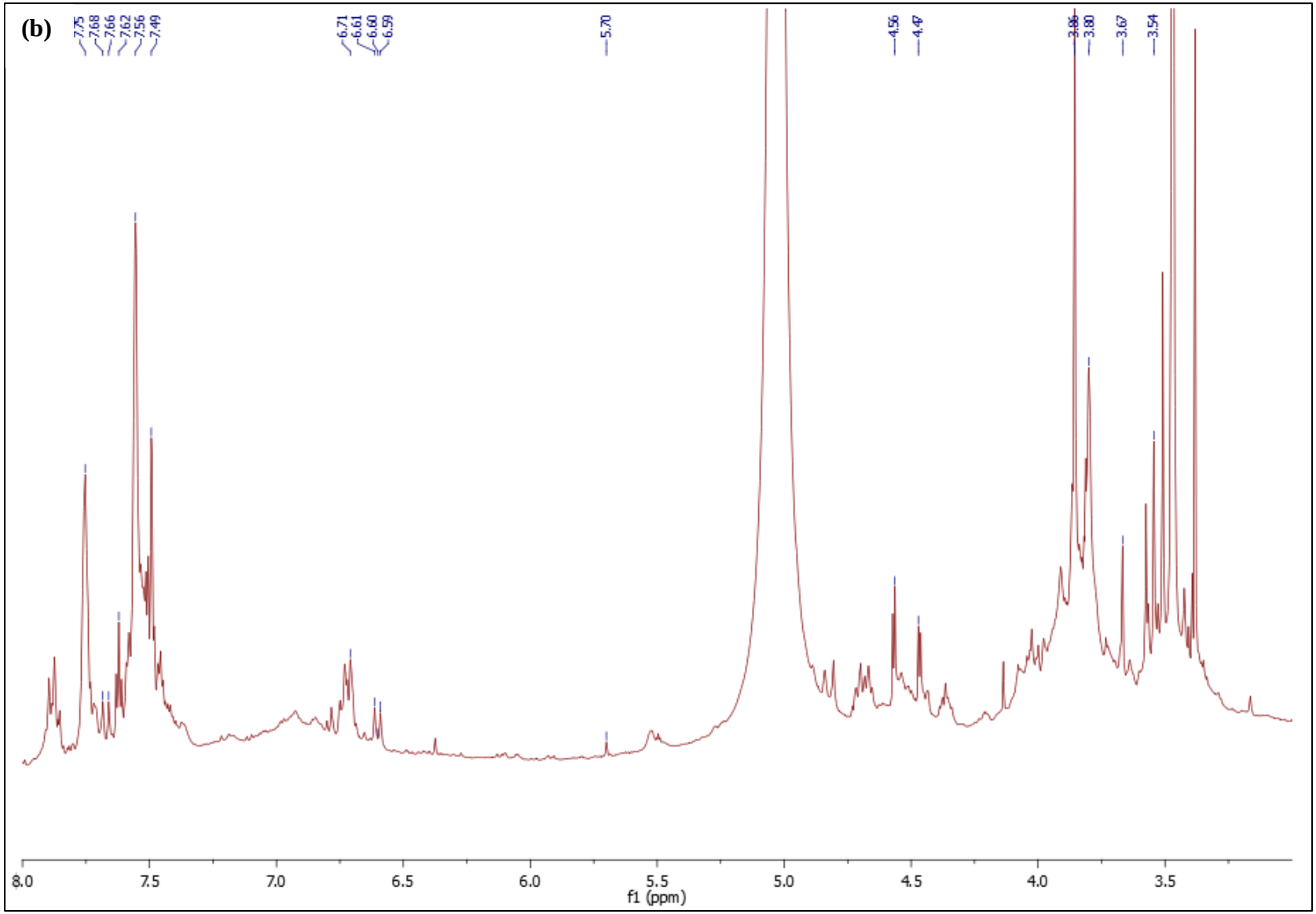
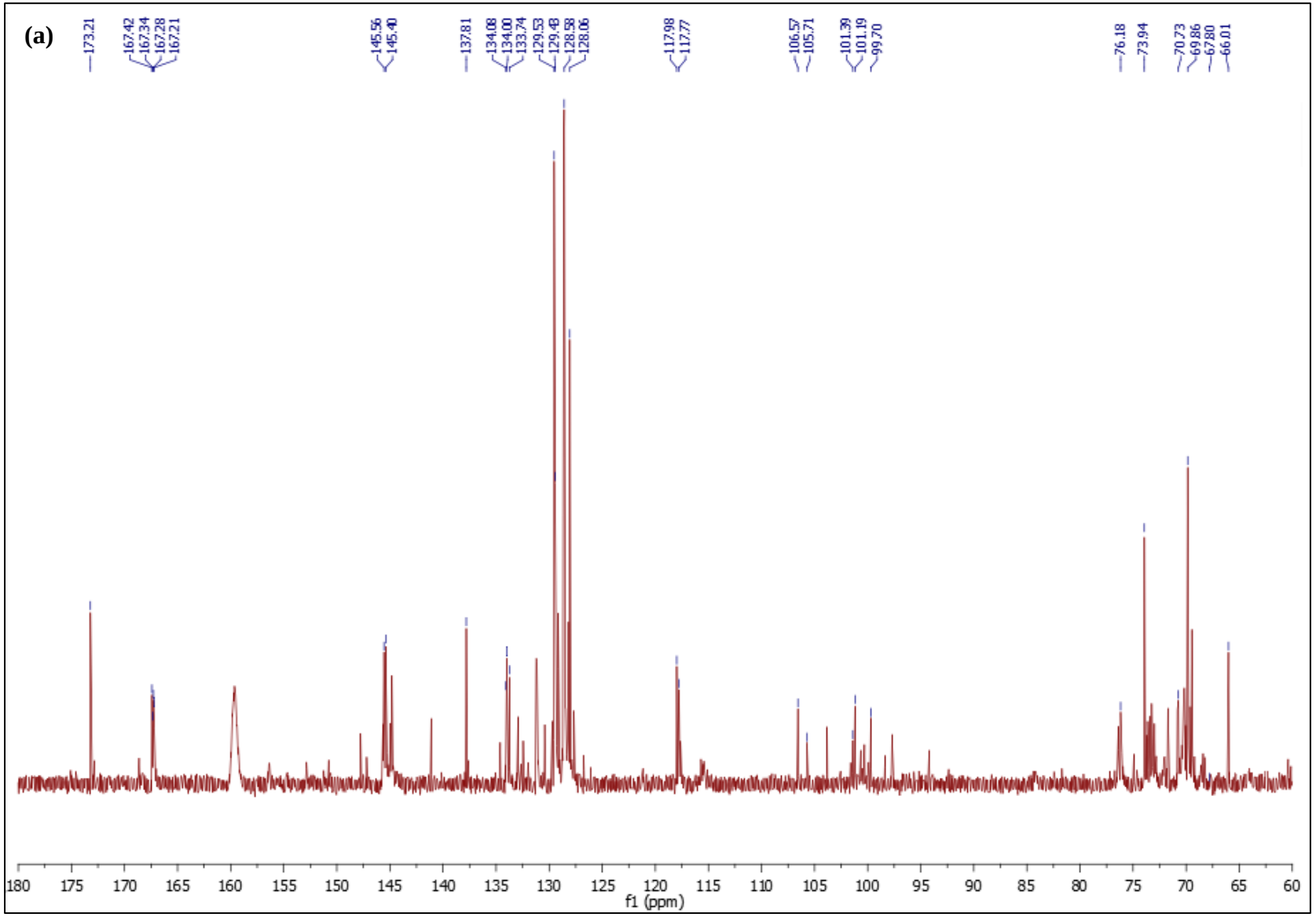
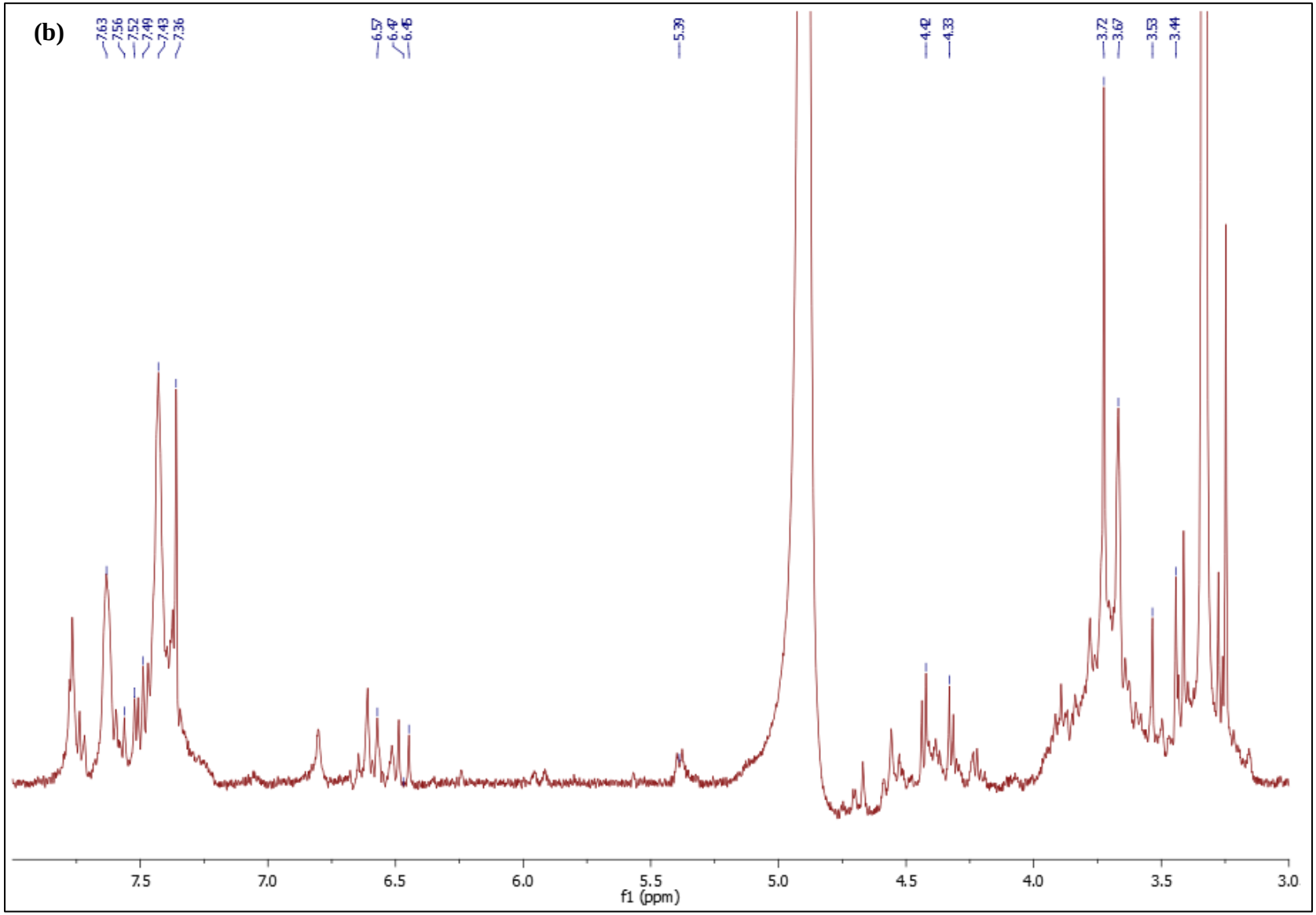
Supplementary Material



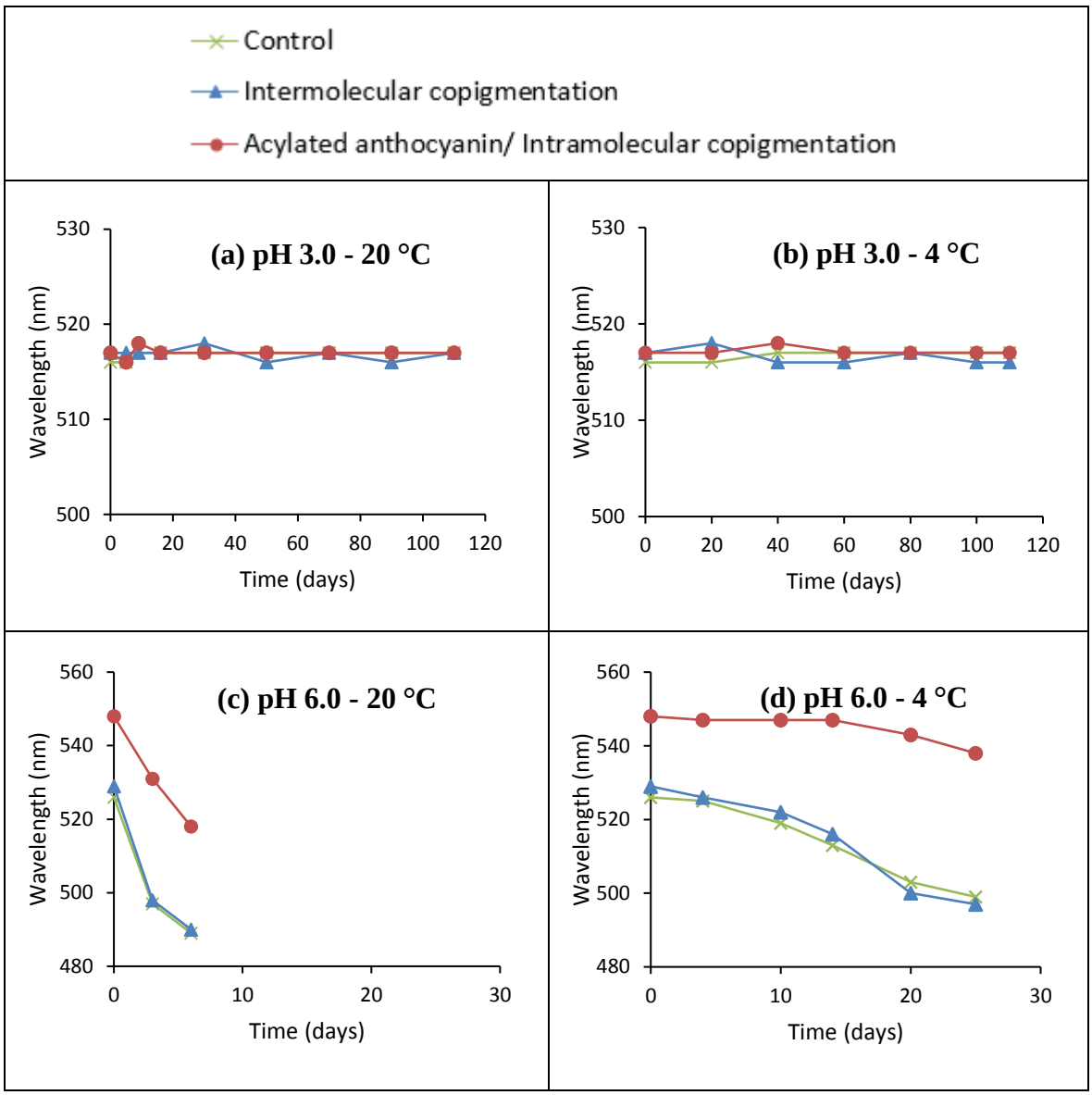


Supplementary Figure 6-9. (a) ^{13}C-NMR and (b) ^{1}H-NMR spectra of acylated delphinidin-3-O-glucoside from dried blackcurrant skins extract.

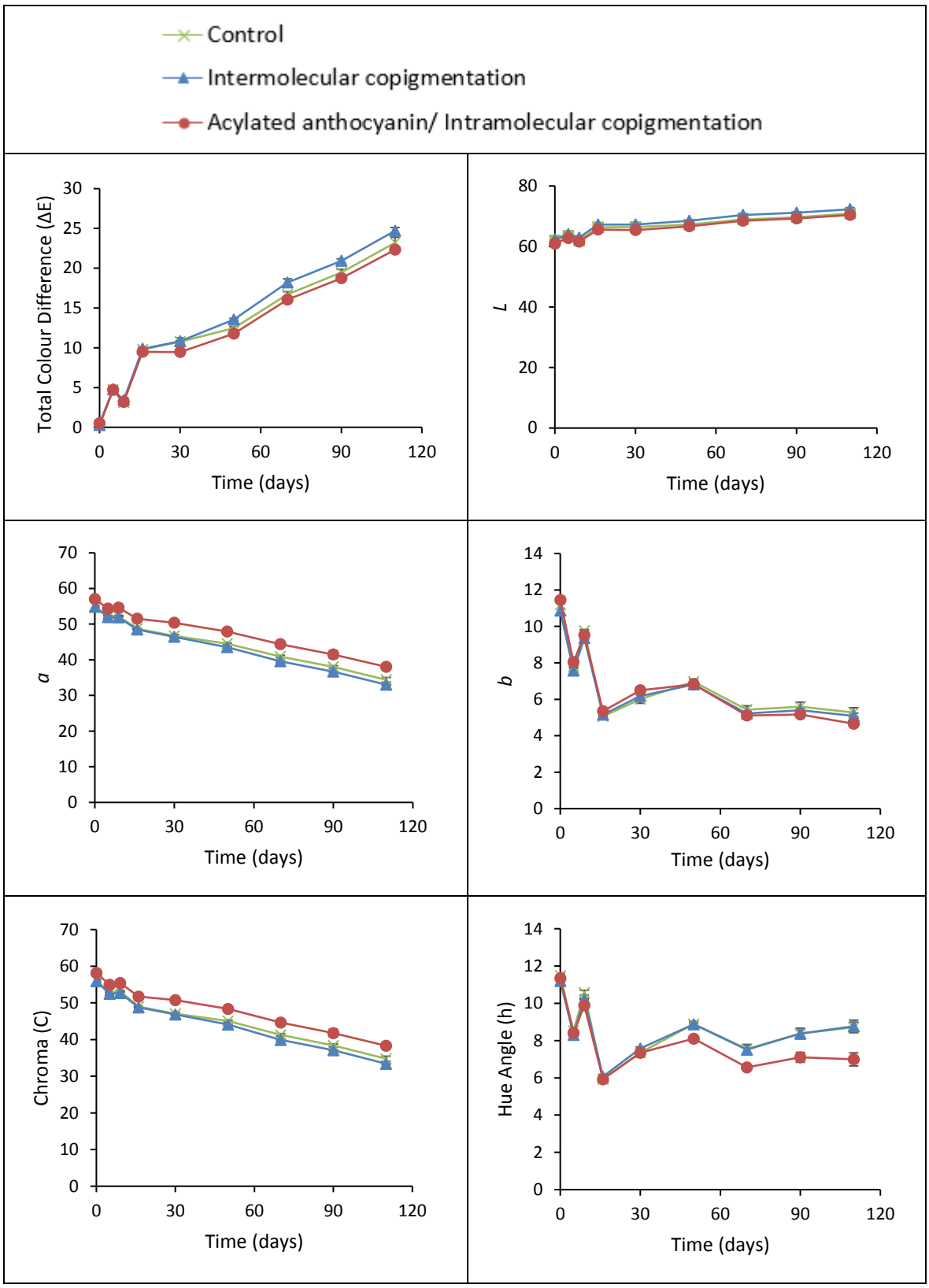

(a)
173.21
167.42
167.34
167.28
167.21
145.56
145.40
137.81
134.08
134.00
133.74
129.53
129.48
128.58
128.06
117.98
117.77
106.57
105.71
101.39
101.19
99.70
76.18
73.94
70.73
69.86
67.80
66.01
180 175 170 165 160 155 150 145 140 135 130 125 120 115 110 105 100 95 90 85 80 75 70 65 60
f1 (ppm)



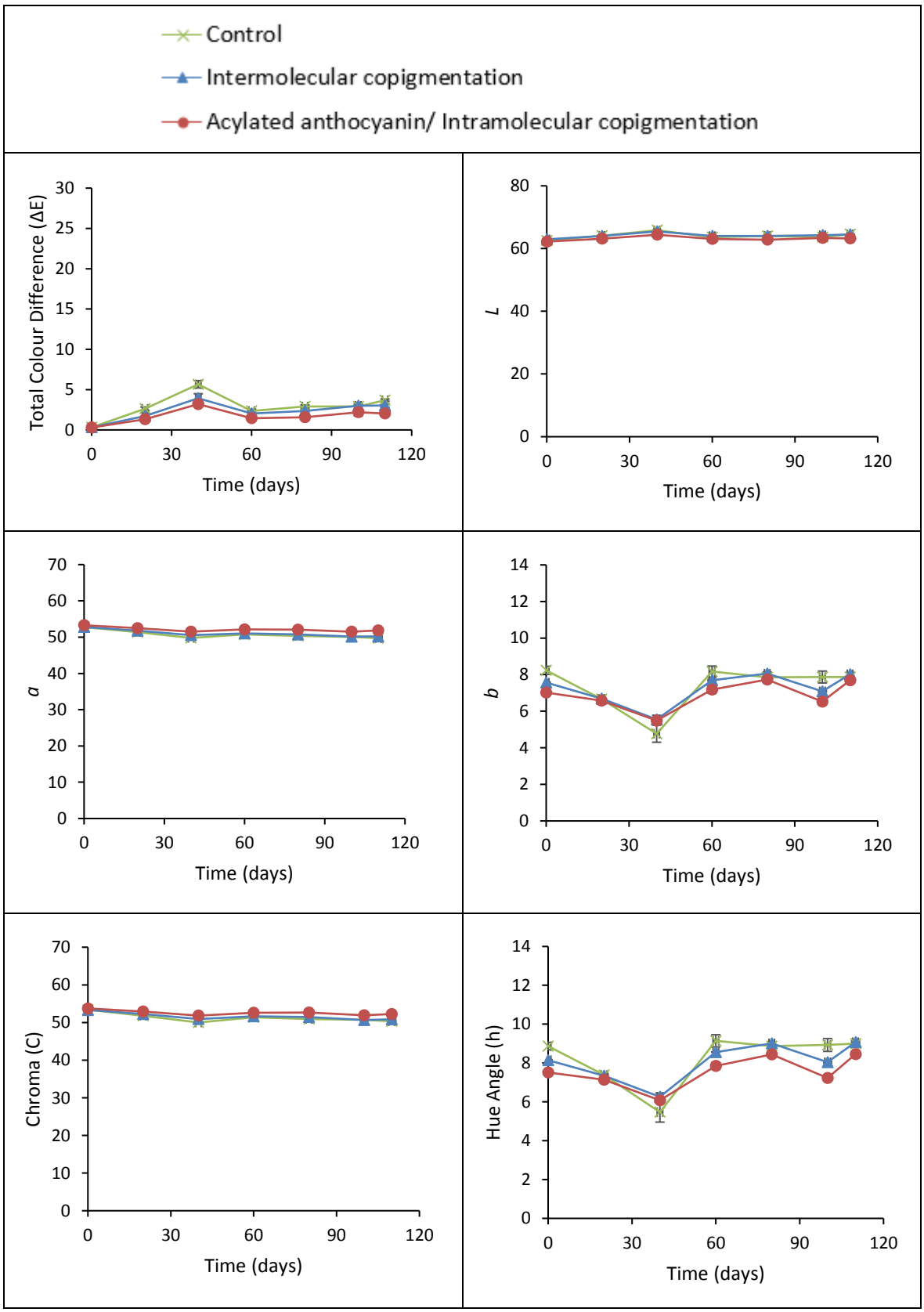
Supplementary Figure 6-10. (a) ^{13}C-NMR and (b) ^{1}H-NMR spectra of acylated cyanidin-3-O-glucoside from dried blackcurrant skins extract.



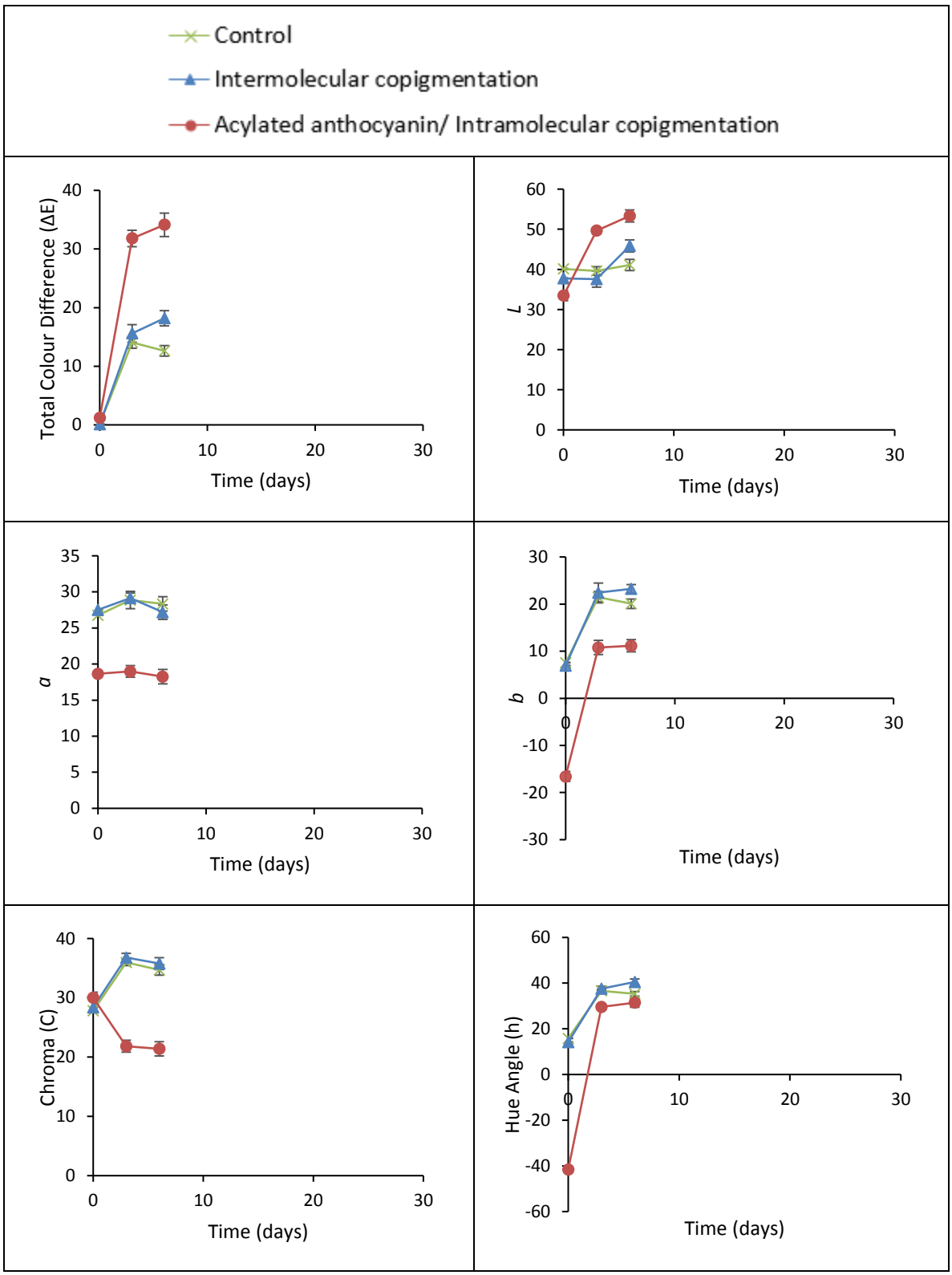
Supplementary Figure 6-11. Changes in the maximum absorption wavelength (λ_{max}) (hypsochromic shift) of total acylated DBS-extracted anthocyanin (Reaction 4 in **Table 6-1**), intermolecular copigmented DBS-extracted anthocyanins (Reaction 9) and control DBS-extracted anthocyanin (Reaction 10) during storage in buffer solutions at (a) pH 3.0; 20 °C, (b) pH 3.0; 4 °C, (c) pH 6.0; 20 °C and (d) pH 6.0; 4 °C. Dried blackcurrant skins (DBS).



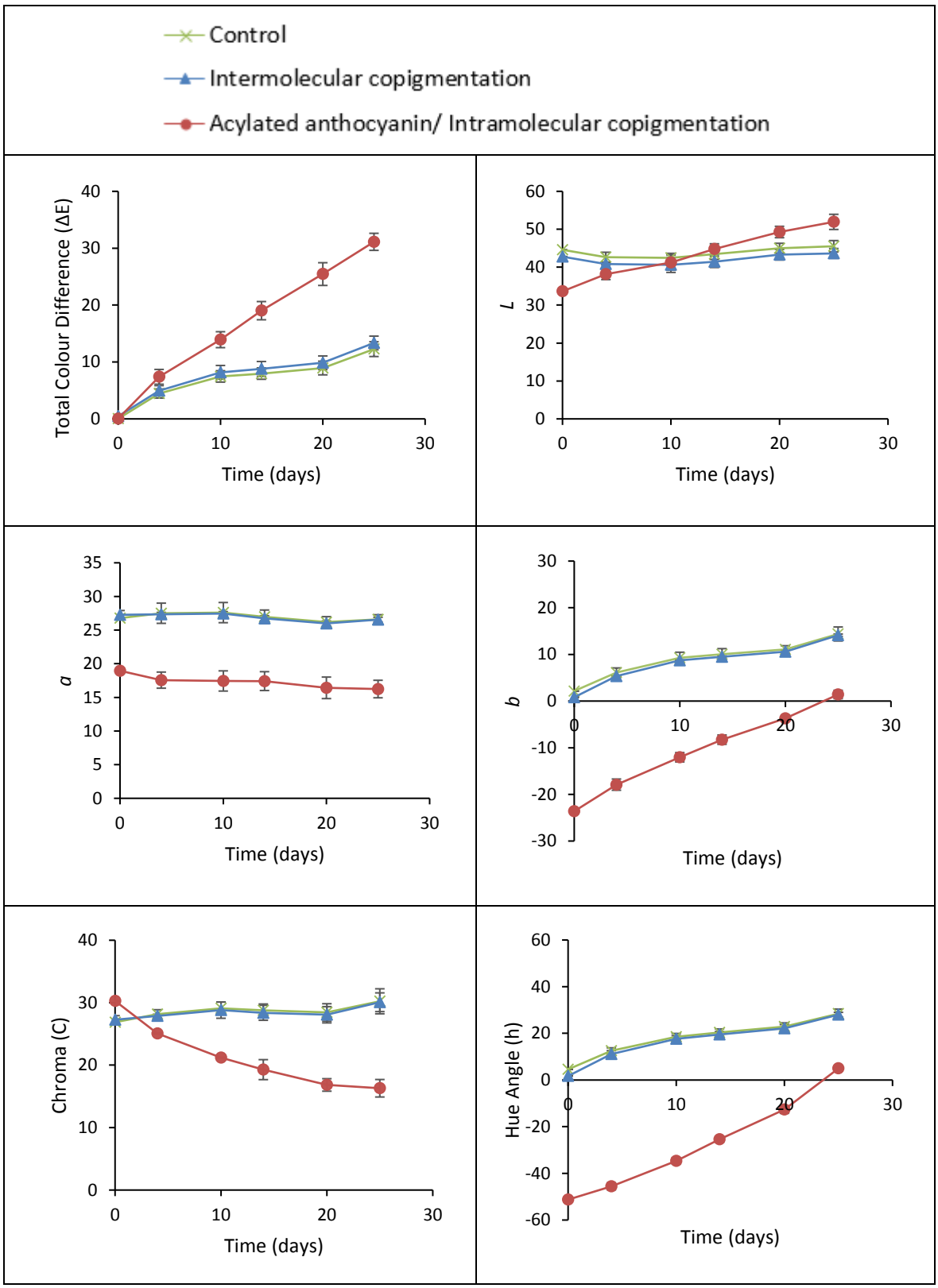
Supplementary Figure 6-12. Total colour difference, *L**, *a**, *b**, chroma and hue angle of total acylated DBS-extracted anthocyanin (Reaction 4 in **Table 6-1**), intermolecular copigmented DBS-extracted anthocyanins (Reaction 9) and control DBS-extracted anthocyanin (Reaction 10) during storage at 20 °C in buffer solution at pH 3.0. Dried blackcurrant skins (DBS); *L** (lightness); *a** (redness/greenness); *b** (yellowness/blueness).



Supplementary Figure 6-13. Total colour difference, *L**, *a**, *b**, chroma and hue angle of total acylated DBS-extracted anthocyanin (Reaction 4 in **Table 6-1**), intermolecular copigmented DBS-extracted anthocyanins (Reaction 9) and control DBS-extracted anthocyanin (Reaction 10) during storage at 4 °C in buffer solution pH 3.0. Dried blackcurrant skins (DBS); *L** (lightness); *a** (redness/greenness); *b** (yellowness/blueness).



Supplementary Figure 6-14. Total colour difference, *L**, *a**, *b**, chroma and hue angle of total acylated DBS-extracted anthocyanin (Reaction 4 in **Table 6-1**), intermolecular copigmented DBS-extracted anthocyanins (Reaction 9) and control DBS-extracted anthocyanin (Reaction 10) during storage at 20 °C in buffer solution pH 6.0. Dried blackcurrant skins (DBS); *L** (lightness); *a** (redness/greenness); *b** (yellowness/blueness).



Supplementary Figure 6-15. Total colour difference, *L**, *a**, *b**, chroma and hue angle of total acylated DBS-extracted anthocyanin (Reaction 4 in **Table 6-1**), intermolecular copigmented DBS-extracted anthocyanins (Reaction 9) and control DBS-extracted anthocyanin (Reaction 10) during storage at 4 °C in buffer solution pH 6.0. Dried blackcurrant skins (DBS); *L** (lightness); *a** (redness/greenness); *b** (yellowness/blueness).

CHAPTER 7 – GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Anthocyanins extracted from blackcurrant skins have considerable potential to be applied as food ingredients in a range of food matrices due to their natural origin, attractive colour, water solubility and potential health promoting properties. This work demonstrated that in order to develop an economically viable commercial supply chain based on the valorisation of blackcurrant skins, investigating key process stages is critical, including the drying process of fresh blackcurrant skins, the process used to extract anthocyanins and the stabilisation of the extracted anthocyanins within food matrices. This thesis generated new scientific knowledge in relevance to both the academic and industrial communities, more specifically in: (i) demonstrating that industrial drum drying under optimal conditions does not seem to degrade anthocyanins, hydroxycinnamic acids and flavonols and seems to be more effective compared to more expensive operations such as freeze drying (at a laboratory scale); (ii) identifying a low pH acetate buffer system as the optimum extraction system for the recovery of free anthocyanins and phenolic compounds, with delphinidin-3-O-rutinoside and *p*-coumaric acid being the main components, respectively; and (iii) generating new knowledge in the area of copigmentation, and more specifically in terms of the mechanisms of interaction of native and acylated anthocyanins with a number of copigments, including phenolic acids (e.g. ferulic, caffeic, chlorogenic, tannic and rosmarinic acids) and aromatic acids such as methyl cinnamate, as well as their influence on anthocyanin and colour stability.

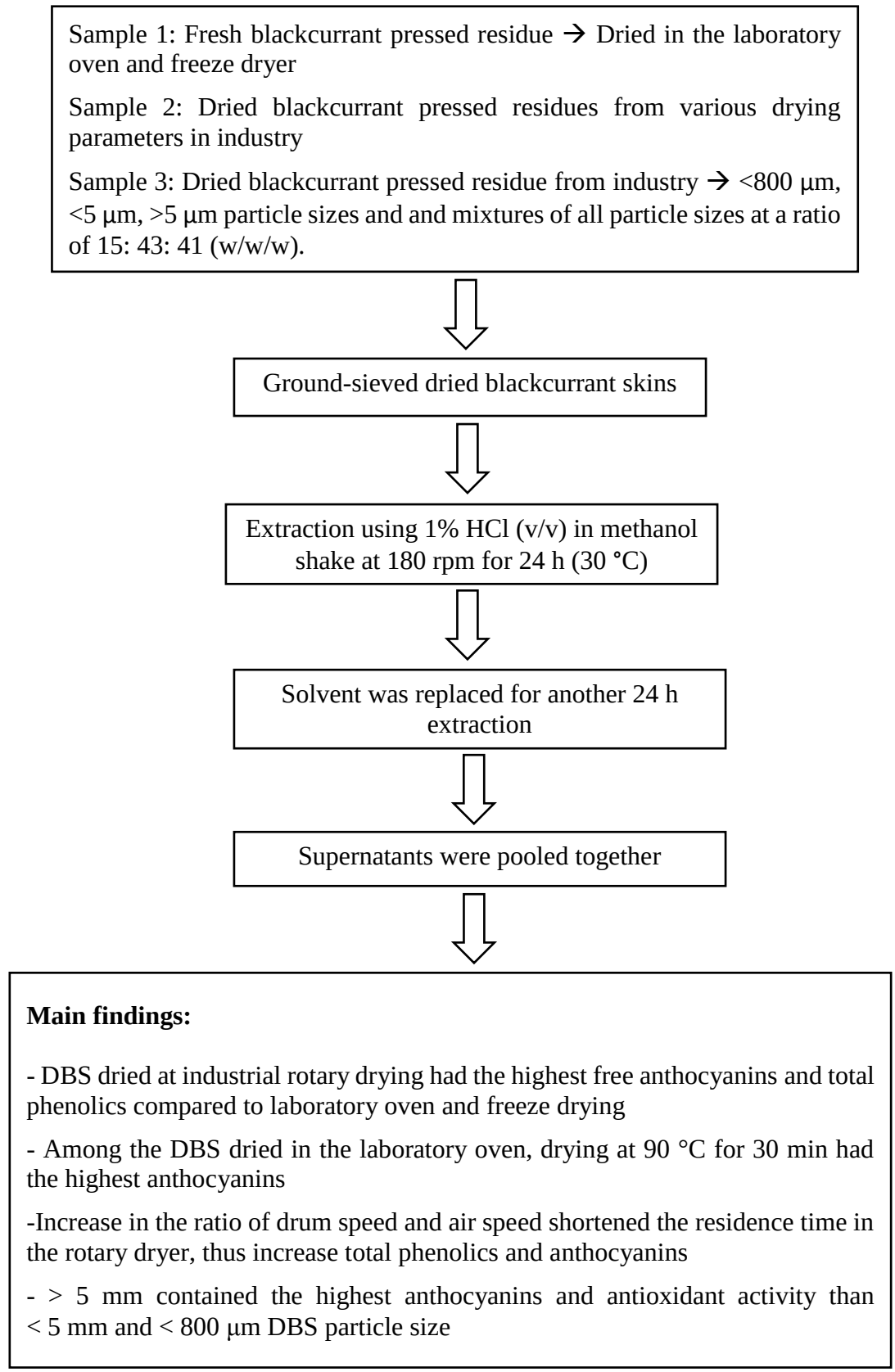


Figure 7-1. A flow diagram with all the process steps and suggested conditions in drying chapter (Chapter 4). *DBS: Dried blackcurrant skins.

In terms of drying as shown in **Figure 7-1**, industrial rotary dryer is a standard industrial drying process that provides efficient drying of particulate materials. It offers advantages over conventional oven drying and has considerable potential to be utilised for the commercial drying of blackcurrant by-products, as part of large scale valorisation process. Notably, this work demonstrated that the particle size of the blackcurrant by-products should be higher than 800 μm in order to avoid degradation of anthocyanins, which are thermally sensitive. Key process parameters such as the temperature of inlet and outlet air, the ratio of the drum speed to air flow speed could be optimised further to minimise anthocyanin degradation and improve process efficiency. Such further research should help in developing an industrial sector that focuses on drying and stabilisation of agri-food by-products at large scale as well as on the extraction of useful phytochemicals (e.g. anthocyanins, hydroxycinnamic acids and flavonols); this at the moment is very small and most work is done at semi-pilot scale. Worth mentioning though that such facilities would need to be food grade as the dried products/extracts would be applied in the food sector. Overall, further R&D in this area would provide a significant opportunity to the UK drying industry to develop new markets for dried by-products and phytochemical-rich extracts, and potentially export these products to their countries (e.g. China, Italy, France) which have been showing commercial interest.

The research carried out as part of this PhD work, demonstrated that the main advantages of extracting anthocyanins using acidic buffer solutions of acetic acid compared to the use of solvents are this approach is simple, rapid, potentially scalable and economically viable, as well as more environmentally friendly. In the case of standard solvent extraction, there is a requirement for large amounts of solvents such as methanol and acetone, which are potential environmental pollutants and health hazards, and they often requires long extraction times.

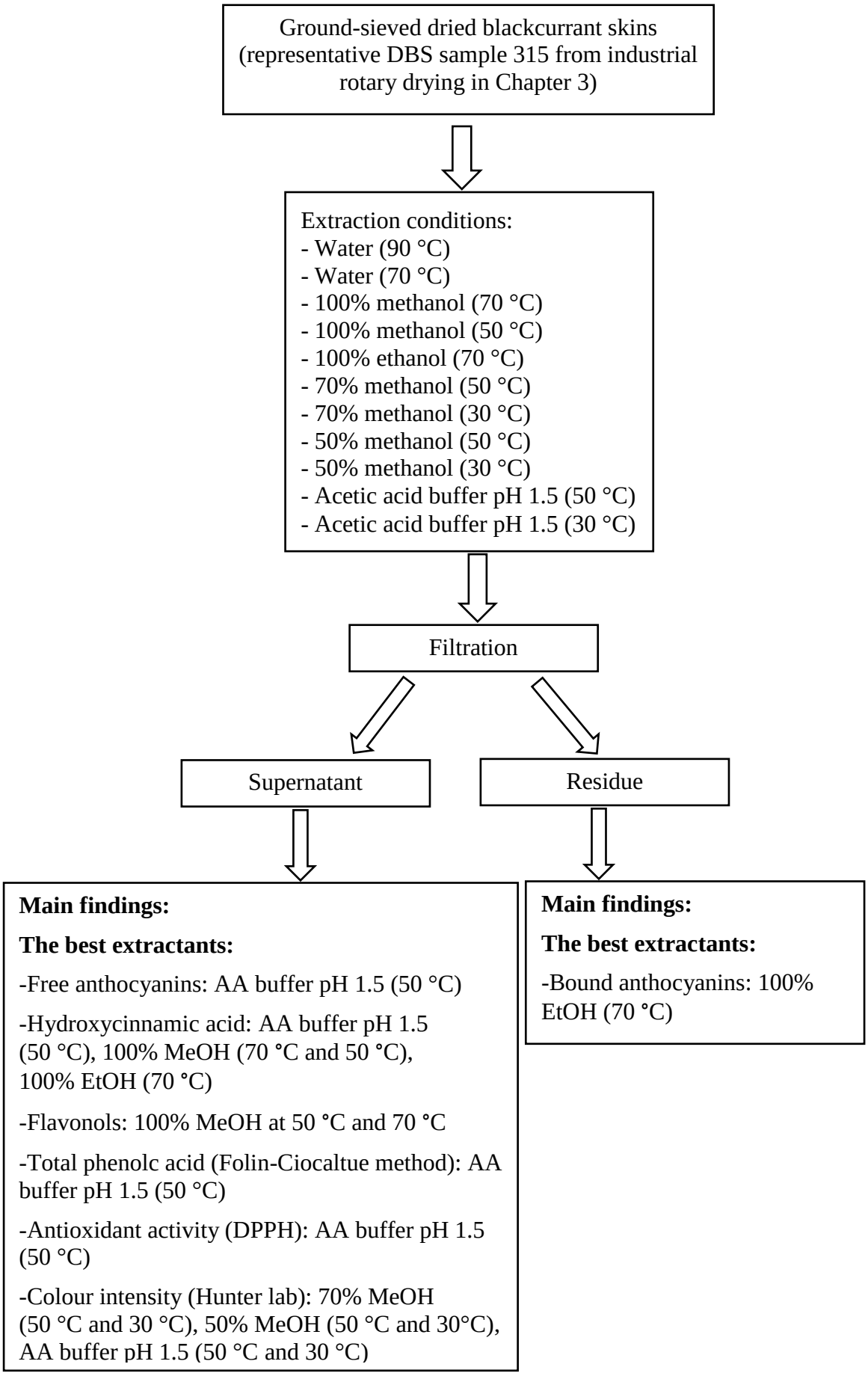
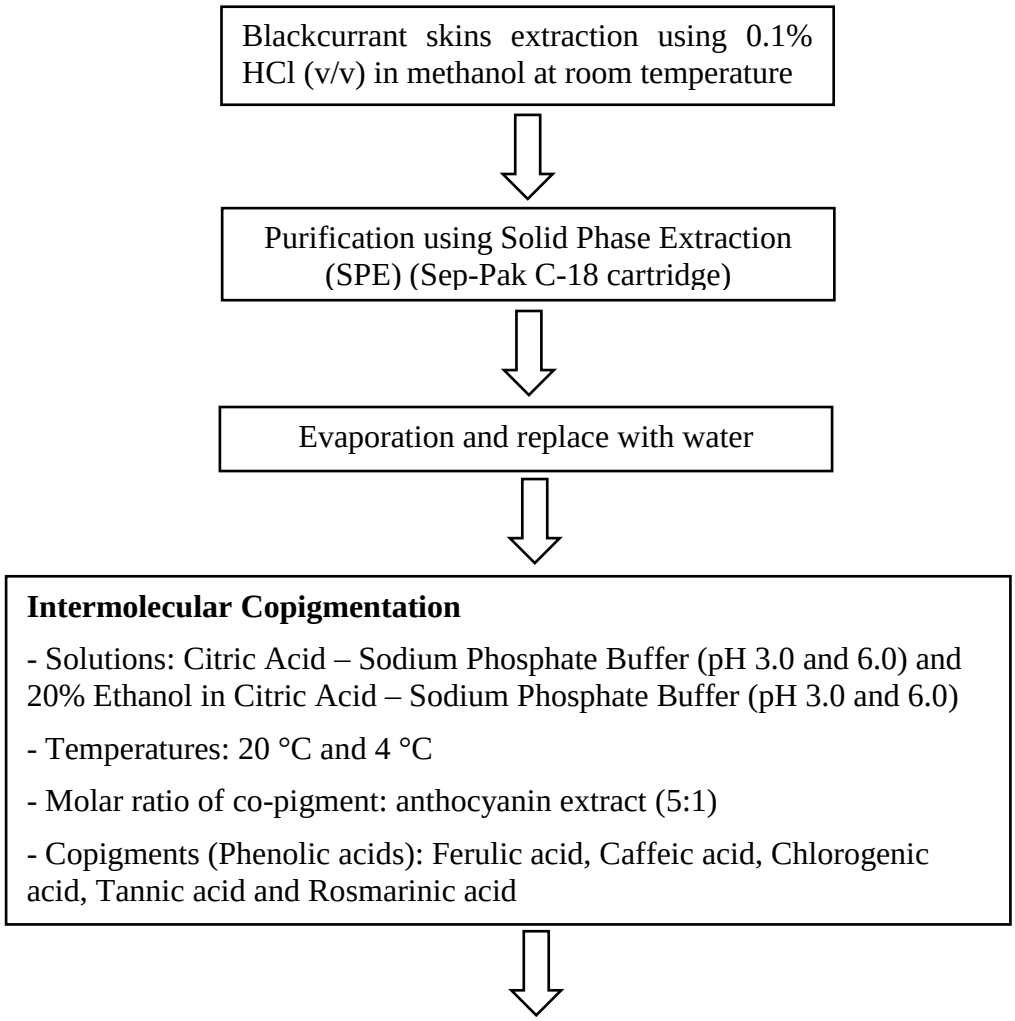


Figure 7-2. A flow diagram with all the process steps and suggested conditions in extraction chapter (Chapter 3). *EtOH: Ethanol; MeOH: Methanol; AA: Acetic acid.

The anthocyanins extracted by the proposed acetic acid buffer method can be characterised as natural and 'clean label' food-grade products that can be practically utilised as functional ingredients in a variety of food matrices, which is in-line with market trends and consumer demands for natural products. The experiments demonstrated that acidic buffer solutions of acetic acid were able to extract anthocyanins, hydroxycinnamic acids and flavonols at high yields (higher than a standard solvent-based method), with the extracts exhibiting high colour intensity and antioxidant activity as well (**Figure 7-2**). However, acetic acid has a distinctive sour taste and pungent smell, therefore, acetic acid should be removed (potentially be evaporation) and this should then be followed by a purification process aiming to increase anthocyanin purity in the extracts. Research in the downstream processing of the extracts needs to be intensified in order to ensure that the produced extracts are of the appropriate quality needed to be used in foods. Moreover, life cycle assessment (LCA) is necessary to be conducted to assess the environmental impact of a potential acetic acid-based extraction method compared to standard solvent-based extraction processes normally used for the extraction of phytochemicals from plant resources. From an industrial perspective, there is considerable potential to scale up the developed extraction process with acetic acid buffer solutions in an industrial setting, i.e. by using an industrial extractor that could be operated as a continuous flow process. This could potentially handle a large proportion of the UK output of dried blackcurrant by-products. The advantages include lower capital investment and energy requirements compared to solvent extraction. To this end, the use and retrofitting of existing plant which are used for other popular processes in the de-juicing business, could potentially reduce the capital requirement considerably. However, more R&D work is needed for developing a continuous flow extraction process and should focus on a better understanding the heat and mass transfer phenomena during acetic acid extraction as well as on process design and process intensification.

The incorporation of anthocyanin as natural colourants in food and beverages has become an attractive option to the food industry and is driven by customer demands for natural products. Anthocyanins are promising candidates to replace synthetic colourants due to their characteristic intense colours and potential health benefits. In recent years, the market of natural food colourants has increased considerably and it is expected to continue to grow by around 10% to 15% annually; on the other hand the market for synthetic food colourants is dropping consistently (Institute of Food Technologists, 2018). Providing the food grade drying, extraction and purification processes are established, anthocyanins have the potential to be sued as natural colourants in a range of food matrices, particularly acidic ones. This is due to the fact that, as shown in this study and other published works, anthocyanins are stable at low pH, where they can exert an intense red colour to the food matrix. This relates to the structure of anthocyanins which is pH dependent, i.e. at pH 1.0 - 3.0 they are in the red flavylium cation form, at pH ~ 4.5 they are in the colourless carbinol pseudobase or colourless chalcone forms, and at pH 6.0 - 7.0 they are in the blue quinonoidal base form. In line with the above, anthocyanins can be applied as natural colourants in acidic food such as jellies and jams, but their incorporation into neutral foods such as dairy products (e.g. ice cream and milk) has been explored less, due to their instability especially during processing and storage at such conditions.



Main findings:

Effect of intermolecular copigmentation	**pH 3.0**		**pH 6.0**	
	20 °C	**4 °C**	**20 °C**	**4 °C**
The highest colour enhancement at Day 0 (ΔA %)	Ferulic acid		Rosmarinic acid	
The longest half shelf life ($t_{1/2}$ days)	Chlorogenic acid	Ferulic acid	Control	Control
Colour Retention (%)	Chlorogenic acid	Ferulic acid	Control	Ferulic acid
The weakest copigment	Caffeic acid		Caffeic acid	

Figure 7-3. A flow diagram with all the process steps and suggested conditions in intermolecular copigmentation chapter (Chapter 5).

In this work it was shown that the stability of natural non-acylated anthocyanins extracted from dried blackcurrant skins can be enhanced through intermolecular copigmentation reactions, taking place between anthocyanins and phenolic acids used as copigments (**Figure 7-3**). The main stabilisation mechanism is most likely based on hydrophobic interactions between the anthocyanins and phenolic acids. The research showed that pH, storage temperature and the structure of the copigments are most likely the most important factors influencing copigmentation. Anthocyanins/ferulic acid complexes at pH 3.0 exhibited the strongest interaction and resulted in the highest colour and anthocyanin stability during storage at 4 °C for 140 days in buffer solutions, most likely because ferulic acid contains methoxylated derivatives that induce a stronger copigmentation effect than the other phenolic acids used in this study (caffeic, chlorogenic, tannic and rosmarinic acids), containing only hydroxyl groups. Such a copigmentation approach could be applied in refrigerated acidic (e.g. juices, jellies and yogurts) and neutral (e.g. ice cream and milk) food products, as the copigmentation complexes showed better stability compared to storage at 20 °C. In order to minimise the cost of adding pure phenolic acids as copigments, ferulic acid from corn extracts (*Zea mays*. L) could be potentially used as it has a generally recognised as safe (GRAS) status. In order to demonstrate the applicability of this copigmentation approach, further needs to be done with food systems, encompassing both processing and storage studies.

The work also investigated a much less explored area, that of intramolecular copigmentation, with the aim being to synthesis acylated anthocyanins through the enzymatic acylation of anthocyanins using methyl cinnamate as the acyl donor and immobilised lipase B from *Candida antarctica* (Novozym 435) as the biocatalyst, as shown in **Figure 7-4**. The advantage of this copigmentation approach is based on the interaction between anthocyanins and the copigments through covalent bonding compared to the interaction through non-covalent bonding in the case of intermolecular copigmentation.

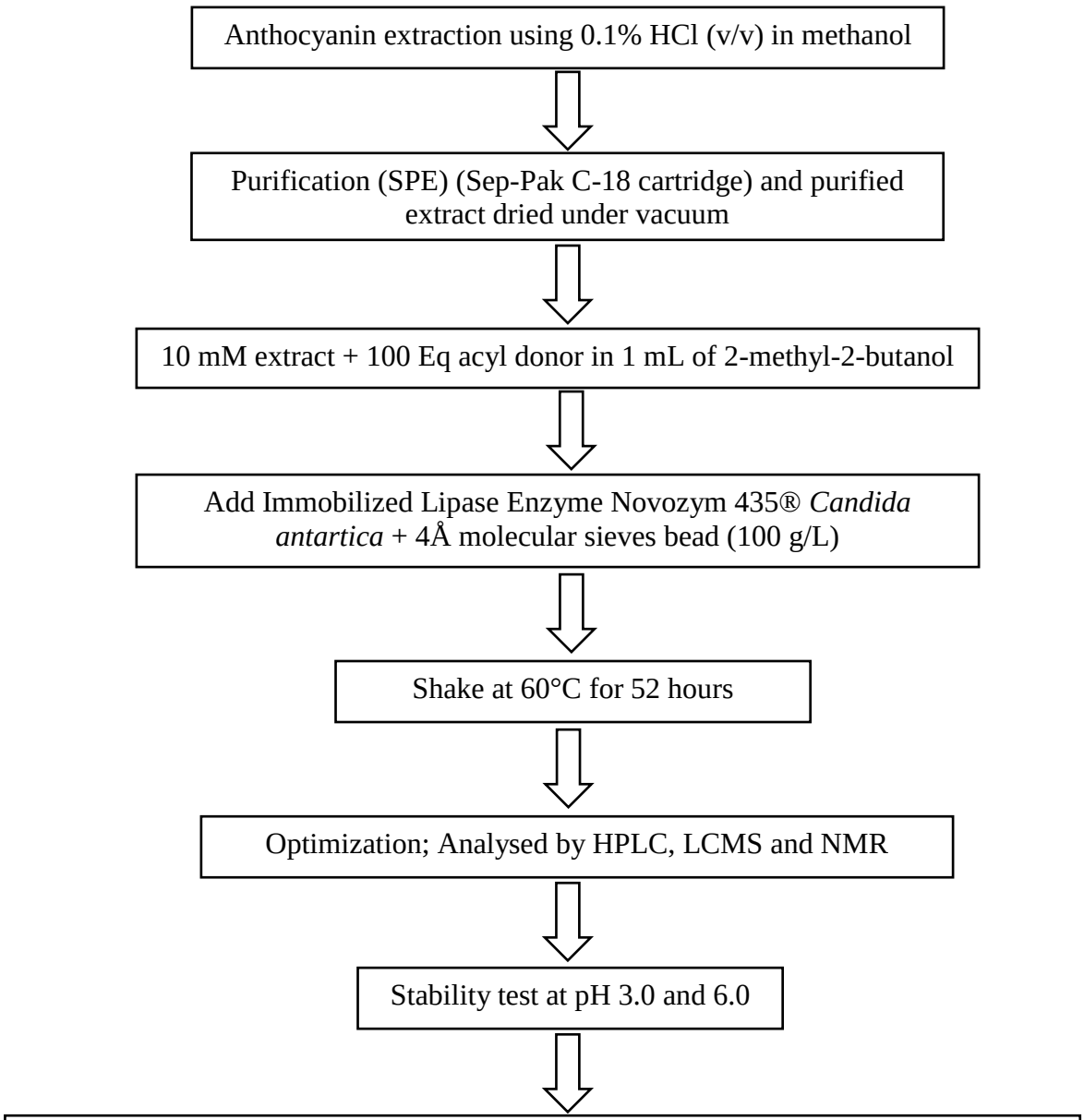


Figure 7-4. A flow diagram with all the process steps and suggested conditions in enzymatic acylation chapter (Chapter 6).

It was shown that the enzymatic acylation process could be conducted effectively using semi-purified anthocyanin extracts of dried blackcurrant skins, giving good acylation yields (10 - 20%). However, the synthesis favoured the acylation of delphinidin 3-O-glucoside and cyanidin 3-O-glucoside rather than that of delphinidin-3-O-rutinoside and cyanidin-3-O-rutinoside, which were also present in the extracts. LC-MS and NMR analysis verified that the synthesis of the acylated anthocyanins did indeed occur, and that the acylation occurred preferentially on the primary hydroxyl group on the glucoside moiety of the anthocyanins (C6″-OH), most likely due to the regioslectivity of the lipase enzyme and the nature/structure of the substrates (anthocyanins). It was also shown that the enzymatically acylated anthocyanins demonstrated higher molecular stability and colour stability during storage at in buffer systems pH 3.0 and pH 6.0 for up to period of ~100 days, compared to both intermolecular copigmented anthocyanins (through non-covalent interaction with methyl cinnamate) and the control sample (anthocyanin without added methyl cinnamate). This is a very important finding as it demonstrates that acylated anthocyanins could be potentially incorporated into acidic and neutral food matrices, although the latter, which potentially include dairy products (e.g. ice cream and milk), are much more challenging systems compared to acidic foods. Further research is needed aiming to: (i) optimise the enzymatic acylation process (e.g. through improved enzymes, optimisation of reaction conditions, bioreactor design, etc.) in order to achieve higher conversion yields, (ii) assess the scalability of the process, (iii) develop a scalable downstream process for the recovery of the acylated anthocyanins, and (iv) understand the influence of food components (e.g. protein and fats) on the stabilisation of acylated anthocyanins and colour during processing and storage. Moreover, from a more commercial perspective, addressing the regulatory hurdles for utilising acylated anthocyanins as food ingredients is critical.

As mentioned above, investigating the performance of intermolecular copigmented anthocyanins or acylated anthocyanins in food matrices, particularly neutral matrices such as dairy products, is a key next step for this research work. A study has been started investigating the effect of intermolecular copigmentation on the colour and anthocyanin stability of re-formulated ice cream. The results were not included as the work was not completed by the thesis submission date, however the findings thus far indicate that the anthocyanin and colour (blue) stability of re-formulated ice cream were enhanced (at the point of production as well as during storage for 1 month at -18 °C) compared to control ice cream due to the intermolecular copigmentation between anthocyanins and food-grade ferulic acid, which were added in the ice cream mix. This is a novel approach as the copigmentation reaction is implemented at a neutral pH (pH ~6.8) of a food product such as ice cream. The results demonstrate that copigmentation prevents the degradation of anthocyanins which can lead to colour fading, especially when the ice cream is served at room temperature. The short-term aim is to complete all this experimental work and long-term to design and perform a similar study with ice cream using enzymatically acylated anthocyanins. The possibility of performing sensory trials in order to assess the organoleptic properties of the anthocyanin-containing ice creams will also be explored.

References

Institute of Food Technologists (2018). https://www.ift.org/?gclid=EAIaIQobChMI5NWqt4383QIVCxHTCh3begQmEAAYASAAEgIvXvD_BwE. Retrieved on June 2018.

APPENDIX I

DATA LOGGING (STORAGE AT 20 ± 1°C)

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12/12/17	00:00	19.3	17/12/17	00:00	19.3	22/12/17	00:00	19.8	27/12/17	00:00	19.4
12/12/17	03:00	19.3	17/12/17	03:00	19.7	22/12/17	03:00	19.9	27/12/17	03:00	19.2
12/12/17	06:00	19.2	17/12/17	06:00	19.1	22/12/17	06:00	20.1	27/12/17	06:00	19.1
12/12/17	09:00	19.3	17/12/17	09:00	19.7	22/12/17	09:00	20.0	27/12/17	09:00	19.0
12/12/17	12:00	19.5	17/12/17	12:00	20.4	22/12/17	12:00	20.0	27/12/17	12:00	19.1
12/12/17	15:00	19.6	17/12/17	15:00	19.9	22/12/17	15:00	19.9	27/12/17	15:00	19.2
12/12/17	18:00	19.2	17/12/17	18:00	19.8	22/12/17	18:00	19.9	27/12/17	18:00	19.4
12/12/17	21:00	19.4	17/12/17	21:00	19.1	22/12/17	21:00	20.0	27/12/17	21:00	19.4
13/12/17	00:00	19.4	18/12/17	00:00	19.2	23/12/17	00:00	20.0	28/12/17	00:00	19.2
13/12/17	03:00	19.3	18/12/17	03:00	19.0	23/12/17	03:00	20.0	28/12/17	03:00	19.0
13/12/17	06:00	19.4	18/12/17	06:00	19.1	23/12/17	06:00	20.0	28/12/17	06:00	19.1
13/12/17	09:00	19.5	18/12/17	09:00	19.3	23/12/17	09:00	19.9	28/12/17	09:00	19.2
13/12/17	12:00	19.6	18/12/17	12:00	19.3	23/12/17	12:00	19.7	28/12/17	12:00	19.4
13/12/17	15:00	19.5	18/12/17	15:00	19.3	23/12/17	15:00	19.9	28/12/17	15:00	19.3
13/12/17	18:00	19.4	18/12/17	18:00	19.4	23/12/17	18:00	19.8	28/12/17	18:00	19.2
13/12/17	21:00	19.4	18/12/17	21:00	19.9	23/12/17	21:00	19.9	28/12/17	21:00	19.2
14/12/17	00:00	19.4	19/12/17	00:00	19.1	24/12/17	00:00	19.8	29/12/17	00:00	19.1
14/12/17	03:00	19.4	19/12/17	03:00	19.3	24/12/17	03:00	19.9	29/12/17	03:00	19.1
14/12/17	06:00	19.3	19/12/17	06:00	19.4	24/12/17	06:00	19.8	29/12/17	06:00	19.2
14/12/17	09:00	19.3	19/12/17	09:00	19.4	24/12/17	09:00	19.6	29/12/17	09:00	19.2
14/12/17	12:00	19.2	19/12/17	12:00	19.4	24/12/17	12:00	19.6	29/12/17	12:00	19.2
14/12/17	15:00	19.1	19/12/17	15:00	19.4	24/12/17	15:00	19.7	29/12/17	15:00	19.2
14/12/17	18:00	19.0	19/12/17	18:00	19.3	24/12/17	18:00	19.8	29/12/17	18:00	19.1
14/12/17	21:00	18.9	19/12/17	21:00	19.3	24/12/17	21:00	19.9	29/12/17	21:00	19.1

Date	Time	T (°C)
30/12/17	00:00	19.1
30/12/17	03:00	19.2
30/12/17	06:00	19.2
30/12/17	09:00	19.2
30/12/17	12:00	19.1
30/12/17	15:00	19.1
30/12/17	18:00	19.1
30/12/17	21:00	19.2
31/12/17	00:00	19.5
31/12/17	03:00	19.4
31/12/17	06:00	19.3
31/12/17	09:00	19.2
31/12/17	12:00	19.1
31/12/17	15:00	19.2
31/12/17	18:00	19.3
31/12/17	21:00	19.4
1/1/18	00:00	19.1
1/1/18	03:00	19.1
1/1/18	06:00	19.2
1/1/18	09:00	19.2
1/1/18	12:00	19.2
1/1/18	15:00	19.2
1/1/18	18:00	19.1
1/1/18	21:00	19.1
2/1/18	00:00	19.5
2/1/18	03:00	19.4
2/1/18	06:00	19.3
2/1/18	09:00	19.2
2/1/18	12:00	19.1
2/1/18	15:00	19.2
2/1/18	18:00	19.3
2/1/18	21:00	19.4
3/1/18	00:00	19.3
3/1/18	03:00	19.3
3/1/18	06:00	19.2
3/1/18	09:00	19.2
3/1/18	12:00	19.2
3/1/18	15:00	19.4
3/1/18	18:00	19.4
3/1/18	21:00	19.4

Date	Time	T (°C)
4/1/18	00:00	19.3
4/1/18	03:00	19.3
4/1/18	06:00	19.2
4/1/18	09:00	19.2
4/1/18	12:00	19.2
4/1/18	15:00	19.3
4/1/18	18:00	19.3
4/1/18	21:00	19.3
5/1/18	00:00	19.3
5/1/18	03:00	19.3
5/1/18	06:00	19.2
5/1/18	09:00	19.2
5/1/18	12:00	19.3
5/1/18	15:00	19.4
5/1/18	18:00	19.4
5/1/18	21:00	19.3
6/1/18	00:00	19.3
6/1/18	03:00	19.4
6/1/18	06:00	19.3
6/1/18	09:00	19.2
6/1/18	12:00	19.2
6/1/18	15:00	19.2
6/1/18	18:00	19.4
6/1/18	21:00	19.4
7/1/18	00:00	19.3
7/1/18	03:00	19.3
7/1/18	06:00	19.2
7/1/18	09:00	19.2
7/1/18	12:00	19.2
7/1/18	15:00	19.3
7/1/18	18:00	19.3
7/1/18	21:00	19.3
8/1/18	00:00	18.5
8/1/18	03:00	18.9
8/1/18	06:00	18.8
8/1/18	09:00	18.9
8/1/18	12:00	18.9
8/1/18	15:00	18.8
8/1/18	18:00	18.8
8/1/18	21:00	19.1

Date	Time	T (°C)
9/1/18	00:00	19.3
9/1/18	03:00	19.2
9/1/18	06:00	19.1
9/1/18	09:00	19.0
9/1/18	12:00	19.0
9/1/18	15:00	19.2
9/1/18	18:00	19.2
9/1/18	21:00	19.4
10/1/18	00:00	19.1
10/1/18	03:00	19.0
10/1/18	06:00	19.1
10/1/18	09:00	19.3
10/1/18	12:00	19.3
10/1/18	15:00	19.3
10/1/18	18:00	19.1
10/1/18	21:00	19.1
11/1/18	00:00	19.1
11/1/18	03:00	19.2
11/1/18	06:00	19.3
11/1/18	09:00	19.3
11/1/18	12:00	19.2
11/1/18	15:00	19.1
11/1/18	18:00	19.1
11/1/18	21:00	19.0
12/1/18	00:00	19.3
12/1/18	03:00	19.3
12/1/18	06:00	19.3
12/1/18	09:00	19.3
12/1/18	12:00	19.5
12/1/18	15:00	19.4
12/1/18	18:00	19.4
12/1/18	21:00	19.4
13/1/18	00:00	19.9
13/1/18	03:00	19.9
13/1/18	06:00	19.9
13/1/18	09:00	19.9
13/1/18	12:00	19.9
13/1/18	15:00	19.8
13/1/18	18:00	19.8
13/1/18	21:00	19.9

Date	Time	T (°C)
14/1/18	00:00	19.1
14/1/18	03:00	19.2
14/1/18	06:00	19.2
14/1/18	09:00	19.2
14/1/18	12:00	19.1
14/1/18	15:00	19.1
14/1/18	18:00	19.1
14/1/18	21:00	19.2
15/1/18	00:00	19.3
15/1/18	03:00	19.4
15/1/18	06:00	19.4
15/1/18	09:00	19.5
15/1/18	12:00	19.6
15/1/18	15:00	19.6
15/1/18	18:00	19.5
15/1/18	21:00	19.5
16/1/18	00:00	19.3
16/1/18	03:00	19.7
16/1/18	06:00	19.1
16/1/18	09:00	19.7
16/1/18	12:00	20.4
16/1/18	15:00	19.9
16/1/18	18:00	19.8
16/1/18	21:00	19.1
17/1/18	00:00	19.3
17/1/18	03:00	19.7
17/1/18	06:00	19.1
17/1/18	09:00	19.7
17/1/18	12:00	20.4
17/1/18	15:00	19.9
17/1/18	18:00	19.8
17/1/18	21:00	19.1
18/1/18	00:00	19.8
18/1/18	03:00	19.9
18/1/18	06:00	20.1
18/1/18	09:00	20.0
18/1/18	12:00	20.0
18/1/18	15:00	19.9
18/1/18	18:00	19.9
18/1/18	21:00	20.0

Date	Time	T (°C)
19/1/18	00:00	19.6
19/1/18	03:00	19.8
19/1/18	06:00	19.7
19/1/18	09:00	19.7
19/1/18	12:00	19.7
19/1/18	15:00	19.7
19/1/18	18:00	19.7
19/1/18	21:00	19.7
20/1/18	00:00	19.4
20/1/18	03:00	19.4
20/1/18	06:00	19.3
20/1/18	09:00	19.3
20/1/18	12:00	19.2
20/1/18	15:00	19.1
20/1/18	18:00	19.0
20/1/18	21:00	18.9
21/1/18	00:00	19.2
21/1/18	03:00	19.0
21/1/18	06:00	19.1
21/1/18	09:00	19.3
21/1/18	12:00	19.3
21/1/18	15:00	19.3
21/1/18	18:00	19.4
21/1/18	21:00	19.9
22/1/18	00:00	19.8
22/1/18	03:00	19.6
22/1/18	06:00	19.6
22/1/18	09:00	19.7
22/1/18	12:00	19.8
22/1/18	15:00	19.9
22/1/18	18:00	19.8
22/1/18	21:00	19.7
23/1/18	00:00	19.2
23/1/18	03:00	19.0
23/1/18	06:00	19.1
23/1/18	09:00	19.3
23/1/18	12:00	19.3
23/1/18	15:00	19.3
23/1/18	18:00	19.4
23/1/18	21:00	19.9

Date	Time	T (°C)
24/1/18	00:00	19.8
24/1/18	03:00	19.9
24/1/18	06:00	19.8
24/1/18	09:00	19.8
24/1/18	12:00	19.8
24/1/18	15:00	19.7
24/1/18	18:00	19.5
24/1/18	21:00	19.5
25/1/18	00:00	19.4
25/1/18	03:00	19.3
25/1/18	06:00	19.4
25/1/18	09:00	19.5
25/1/18	12:00	19.6
25/1/18	15:00	19.5
25/1/18	18:00	19.4
25/1/18	21:00	19.4
26/1/18	00:00	19.1
26/1/18	03:00	19.3
26/1/18	06:00	19.4
26/1/18	09:00	19.4
26/1/18	12:00	19.4
26/1/18	15:00	19.4
26/1/18	18:00	19.3
26/1/18	21:00	19.3
27/1/18	00:00	19.6
27/1/18	03:00	19.6
27/1/18	06:00	19.5
27/1/18	09:00	19.5
27/1/18	12:00	19.4
27/1/18	15:00	19.5
27/1/18	18:00	19.4
27/1/18	21:00	19.4
28/1/18	00:00	19.8
28/1/18	03:00	19.9
28/1/18	06:00	19.8
28/1/18	09:00	19.8
28/1/18	12:00	19.8
28/1/18	15:00	19.7
28/1/18	18:00	19.5
28/1/18	21:00	19.5

Date	Time	T (°C)
29/1/18	00:00	19.6
29/1/18	03:00	19.6
29/1/18	06:00	19.5
29/1/18	09:00	19.5
29/1/18	12:00	19.4
29/1/18	15:00	19.5
29/1/18	18:00	19.4
29/1/18	21:00	19.4
30/1/18	00:00	18.9
30/1/18	03:00	19.0
30/1/18	06:00	19.1
30/1/18	09:00	19.0
30/1/18	12:00	18.9
30/1/18	15:00	18.9
30/1/18	18:00	18.8
30/1/18	21:00	18.8
31/1/18	00:00	19.4
31/1/18	03:00	19.4
31/1/18	06:00	19.4
31/1/18	09:00	19.4
31/1/18	12:00	19.4
31/1/18	15:00	19.3
31/1/18	18:00	19.3
31/1/18	21:00	19.3
1/2/18	00:00	19.8
1/2/18	03:00	19.6
1/2/18	06:00	19.6
1/2/18	09:00	19.7
1/2/18	12:00	19.8
1/2/18	15:00	19.9
1/2/18	18:00	19.8
1/2/18	21:00	19.7
2/2/18	00:00	19.6
2/2/18	03:00	19.8
2/2/18	06:00	19.7
2/2/18	09:00	19.7
2/2/18	12:00	19.7
2/2/18	15:00	19.7
2/2/18	18:00	19.7
2/2/18	21:00	19.7

Date	Time	T (°C)
3/2/18	00:00	19.4
3/2/18	03:00	19.4
3/2/18	06:00	19.4
3/2/18	09:00	19.4
3/2/18	12:00	19.4
3/2/18	15:00	19.3
3/2/18	18:00	19.3
3/2/18	21:00	19.3
4/2/18	00:00	18.8
4/2/18	03:00	18.9
4/2/18	06:00	18.9
4/2/18	09:00	18.8
4/2/18	12:00	18.8
4/2/18	15:00	19.1
4/2/18	18:00	19.5
4/2/18	21:00	19.4
5/2/18	00:00	19.3
5/2/18	03:00	19.3
5/2/18	06:00	19.3
5/2/18	09:00	19.2
5/2/18	12:00	19.2
5/2/18	15:00	19.3
5/2/18	18:00	19.3
5/2/18	21:00	19.4
6/2/18	00:00	19.9
6/2/18	03:00	20.0
6/2/18	06:00	20.0
6/2/18	09:00	19.9
6/2/18	12:00	19.7
6/2/18	15:00	19.9
6/2/18	18:00	19.8
6/2/18	21:00	19.9
7/2/18	00:00	19.4
7/2/18	03:00	19.4
7/2/18	06:00	19.3
7/2/18	09:00	19.3
7/2/18	12:00	19.2
7/2/18	15:00	19.1
7/2/18	18:00	19.0
7/2/18	21:00	18.9

Date	Time	T (°C)
8/2/18	00:00	19.3
8/2/18	03:00	19.3
8/2/18	06:00	19.3
8/2/18	09:00	19.2
8/2/18	12:00	19.2
8/2/18	15:00	19.3
8/2/18	18:00	19.3
8/2/18	21:00	19.4
9/2/18	00:00	19.0
9/2/18	03:00	20.0
9/2/18	06:00	20.0
9/2/18	09:00	19.9
9/2/18	12:00	20.0
9/2/18	15:00	19.8
9/2/18	18:00	19.9
9/2/18	21:00	19.8
10/2/18	00:00	19.4
10/2/18	03:00	19.3
10/2/18	06:00	19.4
10/2/18	09:00	19.5
10/2/18	12:00	19.6
10/2/18	15:00	19.5
10/2/18	18:00	19.4
10/2/18	21:00	19.4
11/2/18	00:00	19.4
11/2/18	03:00	19.4
11/2/18	06:00	19.4
11/2/18	09:00	19.4
11/2/18	12:00	19.4
11/2/18	15:00	19.3
11/2/18	18:00	19.3
11/2/18	21:00	19.3
12/2/18	00:00	18.9
12/2/18	03:00	19.0
12/2/18	06:00	19.1
12/2/18	09:00	19.0
12/2/18	12:00	18.9
12/2/18	15:00	18.9
12/2/18	18:00	18.8
12/2/18	21:00	18.8

Date	Time	T (°C)
13/2/18	00:00	19.3
13/2/18	03:00	19.3
13/2/18	06:00	19.2
13/2/18	09:00	19.1
13/2/18	12:00	19.3
13/2/18	15:00	19.5
13/2/18	18:00	19.4
13/2/18	21:00	19.4
14/2/18	00:00	19.3
14/2/18	03:00	19.3
14/2/18	06:00	19.3
14/2/18	09:00	19.2
14/2/18	12:00	19.2
14/2/18	15:00	19.3
14/2/18	18:00	19.3
14/2/18	21:00	19.4
15/2/18	00:00	19.9
15/2/18	03:00	19.9
15/2/18	06:00	19.9
15/2/18	09:00	19.9
15/2/18	12:00	19.9
15/2/18	15:00	19.8
15/2/18	18:00	19.8
15/2/18	21:00	19.9
16/2/18	00:00	19.3
16/2/18	03:00	19.3
16/2/18	06:00	19.2
16/2/18	09:00	19.1
16/2/18	12:00	19.3
16/2/18	15:00	19.5
16/2/18	18:00	19.4
16/2/18	21:00	19.4
17/2/18	00:00	19.6
17/2/18	03:00	19.8
17/2/18	06:00	19.7
17/2/18	09:00	19.7
17/2/18	12:00	19.7
17/2/18	15:00	19.7
17/2/18	18:00	19.7
17/2/18	21:00	19.7

Date	Time	T (°C)
18/2/18	00:00	19.3
18/2/18	03:00	19.3
18/2/18	06:00	19.2
18/2/18	09:00	19.3
18/2/18	12:00	19.5
18/2/18	15:00	19.6
18/2/18	18:00	19.0
18/2/18	21:00	19.4
19/2/18	00:00	19.6
19/2/18	03:00	19.6
19/2/18	06:00	19.5
19/2/18	09:00	19.5
19/2/18	12:00	19.4
19/2/18	15:00	19.5
19/2/18	18:00	19.4
19/2/18	21:00	19.4
20/2/18	00:00	19.3
20/2/18	03:00	19.3
20/2/18	06:00	19.2
20/2/18	09:00	19.2
20/2/18	12:00	19.2
20/2/18	15:00	19.4
20/2/18	18:00	19.4
20/2/18	21:00	19.4
21/2/18	00:00	19.5
21/2/18	03:00	19.5
21/2/18	06:00	19.5
21/2/18	09:00	19.6
21/2/18	12:00	19.6
21/2/18	15:00	19.6
21/2/18	18:00	19.6
21/2/18	21:00	19.6
22/2/18	00:00	19.4
22/2/18	03:00	19.4
22/2/18	06:00	19.3
22/2/18	09:00	19.3
22/2/18	12:00	19.2
22/2/18	15:00	19.1
22/2/18	18:00	19.0
22/2/18	21:00	18.9

Date	Time	T (°C)
23/2/18	00:00	19.9
23/2/18	03:00	19.9
23/2/18	06:00	19.9
23/2/18	09:00	19.9
23/2/18	12:00	19.9
23/2/18	15:00	19.8
23/2/18	18:00	19.8
23/2/18	21:00	19.9
24/2/18	00:00	19.3
24/2/18	03:00	19.7
24/2/18	06:00	19.1
24/2/18	09:00	19.7
24/2/18	12:00	20.4
24/2/18	15:00	19.9
24/2/18	18:00	19.8
24/2/18	21:00	19.1
25/2/18	00:00	19.4
25/2/18	03:00	19.3
25/2/18	06:00	19.4
25/2/18	09:00	19.5
25/2/18	12:00	19.6
25/2/18	15:00	19.5
25/2/18	18:00	19.4
25/2/18	21:00	19.4
26/2/18	00:00	19.8
26/2/18	03:00	19.9
26/2/18	06:00	19.8
26/2/18	09:00	19.6
26/2/18	12:00	19.6
26/2/18	15:00	19.7
26/2/18	18:00	19.8
26/2/18	21:00	19.9
27/2/18	00:00	19.3
27/2/18	03:00	19.3
27/2/18	06:00	19.2
27/2/18	09:00	19.3
27/2/18	12:00	19.5
27/2/18	15:00	19.6
27/2/18	18:00	19.1
27/2/18	21:00	19.4

Date	Time	T (°C)
28/2/18	00:00	19.8
28/2/18	03:00	19.9
28/2/18	06:00	20.1
28/2/18	09:00	20.0
28/2/18	12:00	20.0
28/2/18	15:00	19.9
28/2/18	18:00	19.9
28/2/18	21:00	20.0
1/3/18	00:00	19.3
1/3/18	03:00	19.3
1/3/18	06:00	19.2
1/3/18	09:00	19.1
1/3/18	12:00	19.3
1/3/18	15:00	19.5
1/3/18	18:00	19.4
1/3/18	21:00	19.4
2/3/18	00:00	19.3
2/3/18	03:00	19.3
2/3/18	06:00	19.3
2/3/18	09:00	19.3
2/3/18	12:00	19.5
2/3/18	15:00	19.4
2/3/18	18:00	19.4
2/3/18	21:00	19.4
3/3/18	00:00	19.3
3/3/18	03:00	19.3
3/3/18	06:00	19.3
3/3/18	09:00	19.2
3/3/18	12:00	19.2
3/3/18	15:00	19.3
3/3/18	18:00	19.3
3/3/18	21:00	19.4
4/3/18	00:00	20.0
4/3/18	03:00	20.0
4/3/18	06:00	20.0
4/3/18	09:00	19.9
4/3/18	12:00	19.7
4/3/18	15:00	19.9
4/3/18	18:00	19.8
4/3/18	21:00	19.9

Date	Time	T (°C)
5/3/18	00:00	19.9
5/3/18	03:00	19.8
5/3/18	06:00	19.6
5/3/18	09:00	19.6
5/3/18	12:00	19.7
5/3/18	15:00	19.8
5/3/18	18:00	19.9
5/3/18	21:00	19.8
6/3/18	00:00	19.4
6/3/18	03:00	19.4
6/3/18	06:00	19.3
6/3/18	09:00	19.3
6/3/18	12:00	19.2
6/3/18	15:00	19.1
6/3/18	18:00	19.0
6/3/18	21:00	18.9
7/3/18	00:00	19.3
7/3/18	03:00	19.3
7/3/18	06:00	19.2
7/3/18	09:00	19.2
7/3/18	12:00	19.2
7/3/18	15:00	19.3
7/3/18	18:00	19.3
7/3/18	21:00	19.3
8/3/18	00:00	19.3
8/3/18	03:00	19.4
8/3/18	06:00	19.4
8/3/18	09:00	19.5
8/3/18	12:00	19.6
8/3/18	15:00	19.6
8/3/18	18:00	19.5
8/3/18	21:00	19.5
9/3/18	00:00	18.8
9/3/18	03:00	18.9
9/3/18	06:00	18.9
9/3/18	09:00	18.8
9/3/18	12:00	18.8
9/3/18	15:00	19.1
9/3/18	18:00	19.1
9/3/18	21:00	18.9

Date	Time	T (°C)
10/3/18	00:00	19.2
10/3/18	03:00	19.0
10/3/18	06:00	19.1
10/3/18	09:00	19.2
10/3/18	12:00	19.4
10/3/18	15:00	19.3
10/3/18	18:00	19.2
10/3/18	21:00	19.2
11/3/18	00:00	19.3
11/3/18	03:00	19.4
11/3/18	06:00	19.3
11/3/18	09:00	19.2
11/3/18	12:00	19.2
11/3/18	15:00	19.2
11/3/18	18:00	19.4
11/3/18	21:00	19.4
12/3/18	00:00	19.3
12/3/18	03:00	19.3
12/3/18	06:00	19.2
12/3/18	09:00	19.3
12/3/18	12:00	19.5
12/3/18	15:00	19.6
12/3/18	18:00	19.1
12/3/18	21:00	19.4
13/3/18	00:00	19.5
13/3/18	03:00	19.5
13/3/18	06:00	19.5
13/3/18	09:00	19.6
13/3/18	12:00	19.6
13/3/18	15:00	19.6
13/3/18	18:00	19.6
13/3/18	21:00	19.6
14/3/18	00:00	19.4
14/3/18	03:00	19.3
14/3/18	06:00	19.4
14/3/18	09:00	19.5
14/3/18	12:00	19.6
14/3/18	15:00	19.5
14/3/18	18:00	19.4
14/3/18	21:00	19.4

Date	Time	T (°C)
15/3/18	00:00	18.9
15/3/18	03:00	19.0
15/3/18	06:00	19.1
15/3/18	09:00	19.0
15/3/18	12:00	18.9
15/3/18	15:00	18.9
15/3/18	18:00	18.8
15/3/18	21:00	18.8
16/3/18	00:00	19.3
16/3/18	03:00	19.3
16/3/18	06:00	19.2
16/3/18	09:00	19.2
16/3/18	12:00	19.3
16/3/18	15:00	19.4
16/3/18	18:00	19.4
16/3/18	21:00	19.3
17/3/18	00:00	19.5
17/3/18	03:00	19.4
17/3/18	06:00	19.3
17/3/18	09:00	19.2
17/3/18	12:00	19.1
17/3/18	15:00	19.2
17/3/18	18:00	19.3
17/3/18	21:00	19.4
18/3/18	00:00	19.3
18/3/18	03:00	19.3
18/3/18	06:00	19.2
18/3/18	09:00	19.3
18/3/18	12:00	19.5
18/3/18	15:00	19.6
18/3/18	18:00	28.1
18/3/18	21:00	19.4
19/3/18	00:00	19.2
19/3/18	03:00	19.0
19/3/18	06:00	19.1
19/3/18	09:00	19.3
19/3/18	12:00	19.3
19/3/18	15:00	19.3
19/3/18	18:00	19.4
19/3/18	21:00	19.9

Date	Time	T (°C)
20/3/18	00:00	19.1
20/3/18	03:00	19.0
20/3/18	06:00	19.1
20/3/18	09:00	19.3
20/3/18	12:00	19.3
20/3/18	15:00	19.3
20/3/18	18:00	19.1
20/3/18	21:00	19.1
21/3/18	00:00	19.8
21/3/18	03:00	19.9
21/3/18	06:00	19.8
21/3/18	09:00	19.6
21/3/18	12:00	19.6
21/3/18	15:00	19.7
21/3/18	18:00	19.8
21/3/18	21:00	19.9
22/3/18	00:00	19.4
22/3/18	03:00	19.2
22/3/18	06:00	19.1
22/3/18	09:00	19.0
22/3/18	12:00	19.1
22/3/18	15:00	19.2
22/3/18	18:00	19.4
22/3/18	21:00	19.4
23/3/18	00:00	19.9
23/3/18	03:00	19.9
23/3/18	06:00	19.9
23/3/18	09:00	19.9
23/3/18	12:00	19.9
23/3/18	15:00	19.8
23/3/18	18:00	19.8
23/3/18	21:00	19.9
24/3/18	00:00	19.1
24/3/18	03:00	19.3
24/3/18	06:00	19.4
24/3/18	09:00	19.4
24/3/18	12:00	19.4
24/3/18	15:00	19.4
24/3/18	18:00	19.3
24/3/18	21:00	19.3

Date	Time	T (°C)
25/3/18	00:00	19.3
25/3/18	03:00	19.3
25/3/18	06:00	19.3
25/3/18	09:00	19.2
25/3/18	12:00	19.2
25/3/18	15:00	19.3
25/3/18	18:00	19.3
25/3/18	21:00	19.4
26/3/18	00:00	19.4
26/3/18	03:00	19.3
26/3/18	06:00	19.4
26/3/18	09:00	19.5
26/3/18	12:00	19.6
26/3/18	15:00	19.5
26/3/18	18:00	19.4
26/3/18	21:00	19.4
27/3/18	00:00	19.9
27/3/18	03:00	19.8
27/3/18	06:00	19.6
27/3/18	09:00	19.6
27/3/18	12:00	19.7
27/3/18	15:00	19.8
27/3/18	18:00	19.9
27/3/18	21:00	19.8
28/3/18	00:00	19.2
28/3/18	03:00	19.0
28/3/18	06:00	19.1
28/3/18	09:00	19.2
28/3/18	12:00	19.4
28/3/18	15:00	19.3
28/3/18	18:00	19.2
28/3/18	21:00	19.2
29/3/18	00:00	19.3
29/3/18	03:00	19.3
29/3/18	06:00	19.2
29/3/18	09:00	19.1
29/3/18	12:00	19.3
29/3/18	15:00	19.5
29/3/18	18:00	19.4
29/3/18	21:00	19.4

Date	Time	T (°C)
30/3/18	00:00	19.1
30/3/18	03:00	19.1
30/3/18	06:00	19.2
30/3/18	09:00	19.2
30/3/18	12:00	19.2
30/3/18	15:00	19.2
30/3/18	18:00	19.1
30/3/18	21:00	19.1
31/3/18	00:00	19.0
31/3/18	03:00	20.0
31/3/18	06:00	20.0
31/3/18	09:00	19.9
31/3/18	12:00	20.0
31/3/18	15:00	19.8
31/3/18	18:00	19.9
31/3/18	21:00	19.8
1/4/18	00:00	19.3
1/4/18	03:00	19.2
1/4/18	06:00	19.1
1/4/18	09:00	19.0
1/4/18	12:00	19.0
1/4/18	15:00	19.2
1/4/18	18:00	19.2
1/4/18	21:00	19.4
2/4/18	00:00	19.2
2/4/18	03:00	19.0
2/4/18	06:00	19.1
2/4/18	09:00	19.3
2/4/18	12:00	19.3
2/4/18	15:00	19.3
2/4/18	18:00	19.4
2/4/18	21:00	19.9
3/4/18	00:00	18.9
3/4/18	03:00	19.0
3/4/18	06:00	19.1
3/4/18	09:00	19.0
3/4/18	12:00	18.9
3/4/18	15:00	18.9
3/4/18	18:00	18.8
3/4/18	21:00	18.8

Date	Time	T (°C)
4/4/18	00:00	19.3
4/4/18	03:00	19.7
4/4/18	06:00	19.1
4/4/18	09:00	19.7
4/4/18	12:00	20.4
4/4/18	15:00	19.9
4/4/18	18:00	19.8
4/4/18	21:00	19.1
5/4/18	00:00	19.8
5/4/18	03:00	19.9
5/4/18	06:00	20.1
5/4/18	09:00	20.0
5/4/18	12:00	20.0
5/4/18	15:00	19.9
5/4/18	18:00	19.9
5/4/18	21:00	20.0
6/4/18	00:00	19.4
6/4/18	03:00	19.2
6/4/18	06:00	19.1
6/4/18	09:00	19.0
6/4/18	12:00	19.1
6/4/18	15:00	19.2
6/4/18	18:00	19.4
6/4/18	21:00	19.4
7/4/18	00:00	19.4
7/4/18	03:00	19.2
7/4/18	06:00	19.1
7/4/18	09:00	19.0
7/4/18	12:00	19.1
7/4/18	15:00	19.2
7/4/18	18:00	19.4
7/4/18	21:00	19.4
8/4/18	00:00	19.1
8/4/18	03:00	19.2
8/4/18	06:00	19.2
8/4/18	09:00	19.2
8/4/18	12:00	19.1
8/4/18	15:00	19.1
8/4/18	18:00	19.1
8/4/18	21:00	19.2

Date	Time	T (°C)
9/4/18	00:00	19.1
9/4/18	03:00	19.2
9/4/18	06:00	19.3
9/4/18	09:00	19.3
9/4/18	12:00	19.2
9/4/18	15:00	19.1
9/4/18	18:00	19.1
9/4/18	21:00	19.0
10/4/18	00:00	19.3
10/4/18	03:00	19.3
10/4/18	06:00	19.2
10/4/18	09:00	19.2
10/4/18	12:00	19.2
10/4/18	15:00	19.4
10/4/18	18:00	19.4
10/4/18	21:00	19.4
11/4/18	00:00	19.6
11/4/18	03:00	19.8
11/4/18	06:00	19.7
11/4/18	09:00	19.7
11/4/18	12:00	19.7
11/4/18	15:00	19.7
11/4/18	18:00	19.7
11/4/18	21:00	19.7
12/4/18	00:00	19.3
12/4/18	03:00	19.2
12/4/18	06:00	19.1
12/4/18	09:00	19.0
12/4/18	12:00	19.0
12/4/18	15:00	19.2
12/4/18	18:00	19.2
12/4/18	21:00	19.4
13/4/18	00:00	19.0
13/4/18	03:00	20.0
13/4/18	06:00	20.0
13/4/18	09:00	19.9
13/4/18	12:00	20.0
13/4/18	15:00	19.8
13/4/18	18:00	19.9
13/4/18	21:00	19.8

Date	Time	T (°C)
14/4/18	00:00	19.1
14/4/18	03:00	19.2
14/4/18	06:00	19.2
14/4/18	09:00	19.2
14/4/18	12:00	19.1
14/4/18	15:00	19.1
14/4/18	18:00	19.1
14/4/18	21:00	19.2
15/4/18	00:00	19.1
15/4/18	03:00	19.3
15/4/18	06:00	19.4
15/4/18	09:00	19.4
15/4/18	12:00	19.4
15/4/18	15:00	19.4
15/4/18	18:00	19.3
15/4/18	21:00	19.3
16/4/18	00:00	19.8
16/4/18	03:00	19.9
16/4/18	06:00	19.8
16/4/18	09:00	19.6
16/4/18	12:00	19.6
16/4/18	15:00	19.7
16/4/18	18:00	19.8
16/4/18	21:00	19.9
17/4/18	00:00	20.0
17/4/18	03:00	20.0
17/4/18	06:00	20.0
17/4/18	09:00	19.9
17/4/18	12:00	19.7
17/4/18	15:00	19.9
17/4/18	18:00	19.8
17/4/18	21:00	19.9
18/4/18	00:00	18.9
18/4/18	03:00	18.8
18/4/18	06:00	19.0
18/4/18	09:00	19.3
18/4/18	12:00	19.0
18/4/18	15:00	18.9
18/4/18	18:00	19.1
18/4/18	21:00	19.2

DATA LOGGING (STORAGE AT 4 ± 1°C)

Date	Time	T (°C)
4/1/18	00:00	3.7
4/1/18	03:00	3.7
4/1/18	06:00	3.7
4/1/18	09:00	3.6
4/1/18	12:00	3.6
4/1/18	15:00	3.6
4/1/18	18:00	3.7
4/1/18	21:00	3.8
5/1/18	00:00	3.7
5/1/18	03:00	3.7
5/1/18	06:00	3.7
5/1/18	09:00	3.5
5/1/18	12:00	3.5
5/1/18	15:00	3.5
5/1/18	18:00	3.5
5/1/18	21:00	3.6
6/1/18	00:00	3.5
6/1/18	03:00	3.6
6/1/18	06:00	3.5
6/1/18	09:00	3.6
6/1/18	12:00	3.6
6/1/18	15:00	3.5
6/1/18	18:00	3.5
6/1/18	21:00	3.5
7/1/18	00:00	3.5
7/1/18	03:00	3.5
7/1/18	06:00	3.6
7/1/18	09:00	3.6
7/1/18	12:00	3.6
7/1/18	15:00	3.5
7/1/18	18:00	3.6
7/1/18	21:00	3.6
8/1/18	00:00	3.5
8/1/18	03:00	3.6
8/1/18	06:00	3.5
8/1/18	09:00	3.5
8/1/18	12:00	3.6
8/1/18	15:00	3.6
8/1/18	18:00	3.8
8/1/18	21:00	3.8

Date	Time	T (°C)
9/1/18	00:00	3.9
9/1/18	03:00	3.5
9/1/18	06:00	3.5
9/1/18	09:00	3.7
9/1/18	12:00	3.6
9/1/18	15:00	3.6
9/1/18	18:00	3.5
9/1/18	21:00	3.6
10/1/18	00:00	3.6
10/1/18	03:00	3.6
10/1/18	06:00	3.5
10/1/18	09:00	3.8
10/1/18	12:00	3.6
10/1/18	15:00	3.8
10/1/18	18:00	3.6
10/1/18	21:00	3.6
11/1/18	00:00	3.7
11/1/18	03:00	3.8
11/1/18	06:00	3.6
11/1/18	09:00	3.7
11/1/18	12:00	3.8
11/1/18	15:00	3.7
11/1/18	18:00	3.7
11/1/18	21:00	3.7
12/1/18	00:00	3.4
12/1/18	03:00	3.5
12/1/18	06:00	3.8
12/1/18	09:00	3.4
12/1/18	12:00	3.4
12/1/18	15:00	3.4
12/1/18	18:00	3.3
12/1/18	21:00	3.4
13/1/18	00:00	3.4
13/1/18	03:00	3.5
13/1/18	06:00	3.5
13/1/18	09:00	3.5
13/1/18	12:00	3.5
13/1/18	15:00	3.6
13/1/18	18:00	3.6
13/1/18	21:00	3.6

Date	Time	T (°C)
14/1/18	00:00	3.6
14/1/18	03:00	3.6
14/1/18	06:00	3.6
14/1/18	09:00	3.7
14/1/18	12:00	3.7
14/1/18	15:00	3.6
14/1/18	18:00	3.7
14/1/18	21:00	3.7
15/1/18	00:00	3.6
15/1/18	03:00	3.5
15/1/18	06:00	3.6
15/1/18	09:00	3.5
15/1/18	12:00	3.6
15/1/18	15:00	3.5
15/1/18	18:00	3.7
15/1/18	21:00	3.3
16/1/18	00:00	3.6
16/1/18	03:00	3.4
16/1/18	06:00	3.5
16/1/18	09:00	3.8
16/1/18	12:00	3.7
16/1/18	15:00	3.5
16/1/18	18:00	3.6
16/1/18	21:00	3.5
17/1/18	00:00	3.4
17/1/18	03:00	3.7
17/1/18	06:00	3.3
17/1/18	09:00	3.3
17/1/18	12:00	3.5
17/1/18	15:00	3.5
17/1/18	18:00	3.5
17/1/18	21:00	3.4
18/1/18	00:00	3.6
18/1/18	03:00	3.6
18/1/18	06:00	3.5
18/1/18	09:00	3.6
18/1/18	12:00	3.5
18/1/18	15:00	3.4
18/1/18	18:00	3.5
18/1/18	21:00	3.6

Date	Time	T (°C)
19/1/18	00:00	3.4
19/1/18	03:00	3.5
19/1/18	06:00	3.3
19/1/18	09:00	3.4
19/1/18	12:00	3.4
19/1/18	15:00	3.4
19/1/18	18:00	3.4
19/1/18	21:00	3.5
20/1/18	00:00	3.3
20/1/18	03:00	3.2
20/1/18	06:00	3.3
20/1/18	09:00	3.4
20/1/18	12:00	3.5
20/1/18	15:00	3.4
20/1/18	18:00	3.5
20/1/18	21:00	3.5
21/1/18	00:00	3.5
21/1/18	03:00	3.3
21/1/18	06:00	3.5
21/1/18	09:00	3.5
21/1/18	12:00	3.7
21/1/18	15:00	3.6
21/1/18	18:00	3.7
21/1/18	21:00	3.7
22/1/18	00:00	3.8
22/1/18	03:00	3.7
22/1/18	06:00	3.7
22/1/18	09:00	3.6
22/1/18	12:00	3.8
22/1/18	15:00	3.6
22/1/18	18:00	3.7
22/1/18	21:00	3.9
23/1/18	00:00	3.6
23/1/18	03:00	3.8
23/1/18	06:00	3.6
23/1/18	09:00	3.6
23/1/18	12:00	3.8
23/1/18	15:00	3.6
23/1/18	18:00	3.7
23/1/18	21:00	3.8

Date	Time	T (°C)
24/1/18	00:00	3.8
24/1/18	03:00	3.7
24/1/18	06:00	3.7
24/1/18	09:00	3.7
24/1/18	12:00	3.8
24/1/18	15:00	3.8
24/1/18	18:00	3.7
24/1/18	21:00	3.7
25/1/18	00:00	4.0
25/1/18	03:00	3.8
25/1/18	06:00	3.7
25/1/18	09:00	3.7
25/1/18	12:00	3.8
25/1/18	15:00	3.7
25/1/18	18:00	3.7
25/1/18	21:00	3.8
26/1/18	00:00	3.6
26/1/18	03:00	3.7
26/1/18	06:00	3.5
26/1/18	09:00	3.7
26/1/18	12:00	3.7
26/1/18	15:00	3.7
26/1/18	18:00	3.7
26/1/18	21:00	3.5
27/1/18	00:00	3.5
27/1/18	03:00	3.5
27/1/18	06:00	3.6
27/1/18	09:00	3.7
27/1/18	12:00	3.6
27/1/18	15:00	3.6
27/1/18	18:00	3.5
27/1/18	21:00	3.7
28/1/18	00:00	3.5
28/1/18	03:00	3.6
28/1/18	06:00	3.7
28/1/18	09:00	3.6
28/1/18	12:00	3.7
28/1/18	15:00	3.6
28/1/18	18:00	3.6
28/1/18	21:00	3.7

Date	Time	T (°C)
29/1/18	00:00	3.6
29/1/18	03:00	3.7
29/1/18	06:00	3.7
29/1/18	09:00	3.7
29/1/18	12:00	3.6
29/1/18	15:00	3.7
29/1/18	18:00	3.7
29/1/18	21:00	3.6
30/1/18	00:00	3.8
30/1/18	03:00	3.9
30/1/18	06:00	3.7
30/1/18	09:00	3.6
30/1/18	12:00	3.8
30/1/18	15:00	3.6
30/1/18	18:00	3.6
30/1/18	21:00	3.7
31/1/18	00:00	3.7
31/1/18	03:00	3.6
31/1/18	06:00	3.7
31/1/18	09:00	3.4
31/1/18	12:00	3.6
31/1/18	15:00	3.5
31/1/18	18:00	3.6
31/1/18	21:00	3.8
1/2/18	00:00	3.6
1/2/18	03:00	3.6
1/2/18	06:00	3.4
1/2/18	09:00	3.5
1/2/18	12:00	3.6
1/2/18	15:00	3.5
1/2/18	18:00	3.4
1/2/18	21:00	3.3
2/2/18	00:00	3.5
2/2/18	03:00	3.6
2/2/18	06:00	3.5
2/2/18	09:00	3.5
2/2/18	12:00	3.6
2/2/18	15:00	3.6
2/2/18	18:00	3.8
2/2/18	21:00	3.8

Date	Time	T (°C)
3/2/18	00:00	3.6
3/2/18	03:00	3.6
3/2/18	06:00	3.5
3/2/18	09:00	3.8
3/2/18	12:00	3.6
3/2/18	15:00	3.8
3/2/18	18:00	3.6
3/2/18	21:00	3.6
4/2/18	00:00	3.4
4/2/18	03:00	3.5
4/2/18	06:00	3.8
4/2/18	09:00	3.4
4/2/18	12:00	3.4
4/2/18	15:00	3.4
4/2/18	18:00	3.3
4/2/18	21:00	3.4
5/2/18	00:00	3.6
5/2/18	03:00	3.6
5/2/18	06:00	3.5
5/2/18	09:00	3.6
5/2/18	12:00	3.5
5/2/18	15:00	3.4
5/2/18	18:00	3.5
5/2/18	21:00	3.6
6/2/18	00:00	3.5
6/2/18	03:00	3.3
6/2/18	06:00	3.5
6/2/18	09:00	3.5
6/2/18	12:00	3.7
6/2/18	15:00	3.6
6/2/18	18:00	3.7
6/2/18	21:00	3.7
7/2/18	00:00	4.0
7/2/18	03:00	3.8
7/2/18	06:00	3.7
7/2/18	09:00	3.7
7/2/18	12:00	3.8
7/2/18	15:00	3.7
7/2/18	18:00	3.7
7/2/18	21:00	3.8

Date	Time	T (°C)
8/2/18	00:00	3.6
8/2/18	03:00	3.6
8/2/18	06:00	3.6
8/2/18	09:00	3.7
8/2/18	12:00	3.7
8/2/18	15:00	3.6
8/2/18	18:00	3.7
8/2/18	21:00	3.7
9/2/18	00:00	3.6
9/2/18	03:00	3.4
9/2/18	06:00	3.5
9/2/18	09:00	3.8
9/2/18	12:00	3.7
9/2/18	15:00	3.5
9/2/18	18:00	3.6
9/2/18	21:00	3.5
10/2/18	00:00	3.3
10/2/18	03:00	3.2
10/2/18	06:00	3.3
10/2/18	09:00	3.4
10/2/18	12:00	3.5
10/2/18	15:00	3.4
10/2/18	18:00	3.5
10/2/18	21:00	3.5
11/2/18	00:00	3.6
11/2/18	03:00	3.8
11/2/18	06:00	3.6
11/2/18	09:00	3.6
11/2/18	12:00	3.8
11/2/18	15:00	3.6
11/2/18	18:00	3.7
11/2/18	21:00	3.8
12/2/18	00:00	3.6
12/2/18	03:00	3.7
12/2/18	06:00	3.7
12/2/18	09:00	3.7
12/2/18	12:00	3.6
12/2/18	15:00	3.7
12/2/18	18:00	3.7
12/2/18	21:00	3.6

APPENDIX II

List of conferences/seminars, rewards/awards, professional memberships and publications during PhD program:

A. Conferences/ Seminars:

1. Oral Presentation at Department of Food and Nutritional Sciences Seminar on 15th November 2017 in University of Reading, under title "Stability of anthocyanins from blackcurrant (*Ribes nigrum* L.) by-products: effect of low and high pH on intermolecular and intramolecular copigmentation during storage".

2. Poster Presentation at Total Food 2017 Conference on October 2017 in Norwich, UK, under title "Effect of drying on anthocyanins from blackcurrant (*Ribes nigrum* L.) skins".

3. Oral Presentation at 11th World Congress on Polyphenol Applications on 20th June 2017 in Vienna, Austria, under title "Stability of anthocyanins from blackcurrant (*Ribes nigrum* L.) by-products: effect of low and high pH on intermolecular and intramolecular copigmentation during storage".

4. Oral Presentation at 10th World Congress on Polyphenol Applications on 29th June 2016 in Porto, Portugal, under title "Extraction of anthocyanins from blackcurrant (*Ribes nigrum* L.) skins".

5. Poster Presentation at SCI Young Researchers in Agri-Food: Food Quality and Sustainability from Plough to Plate on 12th May 2016 in University of Reading, UK, under title "Extraction of anthocyanins from blackcurrant (*Ribes nigrum* L.) skins".

6. Conference at 5th International Blackcurrant Association on 16th June 2016 in Kent, UK.

7. Oral Presentation at Department of Food and Nutritional Sciences Seminar on 5th May 2016 in University of Reading, UK, under title "Extraction and application of anthocyanins from blackcurrant (*Ribes nigrum* L.) skins".

8. Proposal Presentation at Department of Food and Nutritional Sciences Seminar on 10th July 2015 in University of Reading, UK, under title "Extraction of anthocyanin from blackcurrant (*Ribes nigrum* L.) waste".

B. Rewards/ Awards

1. Netherfield Travel Award for attending the 11th World Congress on Polyphenol Applications in Vienna on 20th June 2017
2. Graduate School Travel Grant, University of Reading
3. Bursary Fund for attending a Poster Presentation at Total Food 2017 Conference on October 2017 in Norwich, UK.

C. Professional Membership

1. Associate Member of Royal Society of Chemistry (RSC)
2. Member of FoodWasteNet, UK