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Acetic acid buffer as extraction medium for free and bound phenolics from dried blackcurrant (*Ribes nigrum* L.) skins

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ABSTRACT

The aim of this study was to investigate the effects of different solvent and extraction temperatures on the free and bound phenolic compounds and antioxidant activity of dried blackcurrant skins (DBS). Apart from acetic acid buffer solution, different solvent systems including water, methanol, and mixtures of methanol/water were also employed and the effects of solvent and temperature (30 °C and 50 °C) on the free and bound forms of anthocyanins, hydroxycinnamic acids and flavonols yield were assessed. The results showed that amongst all solvents, acetic acid buffer resulted in the highest free anthocyanin content (1712.3 ± 56.1 mg/100 g) ($p < 0.05$) after 2 h extraction at 50 °C from DBS, while lower amounts of bound anthocyanins and anthocyanidins were detected after acid hydrolysis. Acetic acid buffer extracts exhibited the highest free hydroxycinnamic acid content (268.0 ± 4.5 mg/100 g), total phenolic content (3702.2 ± 259.3 mg GAE/100 g) and DPPH activity (60.7 ± 2.0 % of inhibition). However, their free flavonol content was slightly lower (60.2 ± 0.8 mg/100 g) compared to 100% methanol at 30 °C and 50 °C, 71.4 ± 1.5 mg/100 g and 71.5 ± 6.2 mg/100 g, respectively. Two-way ANOVA indicated interactions between solvent and temperatures ($p < 0.05$), which suggested that the relationship between solvent and phenolic compounds depends on the extraction temperature.

Practical Application: Overall, acetic acid buffer is more environmentally friendly, efficient and cost-effective than other solvents, thus offering an improved extraction method for phytochemicals as valuable ingredients for nutraceutical applications, from under-utilised dried blackcurrant skins (DBS).

Keywords: dried blackcurrant skins, anthocyanins, HPLC, antioxidant activity, acetic acid buffer

1. INTRODUCTION

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). Nowadays there are approximately 50 growers that occupy 2,400 hectares in the British Isles and produce around 11,000 tonnes of blackcurrant per year (IBA, 2018). Blackcurrants are generally cultivated for food applications, as it can be used as natural colourants due to their high content of anthocyanins and proanthocyanins, as preservatives and as sources of ascorbic acid (~180 mg/100 g of berries) and phenolic compounds (500 – 1342 mg/100 g of berries); the latter have been associated with health promoting effects in humans (Brennan & Graham, 2009; Basegmez et al., 2017). Anthocyanins, flavonoids, hydroxycinnamic acids, *p*-coumaric acid, myricetin, quercetin, kaempferol glycosides and isorhamnetin shape the phenolic compounds profile of the blackcurrant fruit (Sójka & Król, 2009), that can be associated primarily with their high antioxidant activities (Szajdek & Borowska, 2008). Blackcurrants are processed to produce a range of functional ingredients, such as blackcurrant-pomace dietary fibers and defatted seeds, which can be incorporated into jams, jellies, purées and teas (Varming, Peterson, & Poll, 2004).

In the UK, 75% of the total fresh blackcurrant production is processed into juice (Vagiri, 2014) with 15% is by-products (Pap et al., 2005) that equals to 1,650 tonnes per year. By-products of the blackcurrant juice process (skins, seeds and stems) are collected during the pressing stage. Only a small percentage of these by-products is recycled or upgraded, and the majority is used as animal feed (although there are limitations in this regard 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.

Extraction is the most important step in the isolation of phenolic compounds including anthocyanins; however, there is no standardised method for their extraction. The initial step 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). Dried plant materials are normally used as starting materials for anthocyanin extraction in order to minimise the possibility of anthocyanin degradation due to chemical reactions taking place in the wet material (Harbourne, Marete, Jacquier, & O'Riordan, 2013).

Due to the polarity of anthocyanins, polar solvents such as aqueous mixtures of methanol, ethanol or acetone are often employed for their extraction (Kähkönen, Hopia, & Heinonen, 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, Prošek, & Wondra, 2005). Moreover, acidified methanol with hydrochloric or formic acid has 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).

Phenolics compounds that can be extracted by aqueous organic solvents from plant materials are known as extractable phenolics or free phenolic compounds. Free phenolics are mainly deposited in the plant vacuoles and have relatively low molecular weight such as extractable proanthocyanidins, hydrolysable tannins, hydroxycinnamic acids and flavonoids

(Saura-Calixto, 2012; Durazzo, 2018). Anthocyanins and flavonols are examples of flavonoids (Zhang & Tsao, 2016). In contrast, the non-extractable or bound phenolics are insoluble in aqueous organic solvents, thus remain in the solid residues after extraction. They are bound to protein or cell wall polysaccharides and can only be released by acid, alkaline or enzymatic hydrolysis treatments. Examples of bound phenolics are high molecular weight of proanthocyanidins and hydrolysable tannins, phenolic acids such as ferulic acid, caffeic acid and sinapic acid (Saura-Calixto, 2012; Acosta-Estrada, Gutiérrez-Uribe, & Serna-Saldívar, 2014; Durazzo, 2018; Gulsunoglu, Karbancioglu-Guler, Raes, & Kilic-Akyilmaz, 2019).

Until today, many studies have reported free phenolics extraction from juice, marc and pressed residues of blackcurrant by-products but very few from dried blackcurrant skins, especially focusing on both free and bound phenolic compounds. Therefore, this study aimed to evaluate the effects of different solvents and extraction temperatures on the free and bound phenolic compounds content and the antioxidant activity of blackcurrant using HPLC analysis and DPPH assay. In addition, the correlation between free anthocyanins, free phenolics, antioxidant activity and colour intensity, as well as the interaction between solvent and extraction temperature on the extraction yield of phenolic compounds will be evaluated.

2. MATERIALS AND METHODS

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, 0.025 M) and pH 4.5 (sodium acetate, 0.4 M) were prepared as described by Lee, Durst, & Wrolstad (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%), caffeic acid (98%), *p*-coumaric (98%), ferulic acid (99%) and quercetin-3-O-glucoside (98%) were purchased from Sigma-Aldrich (UK).

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). 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.

2.3 Preparation of dried blackcurrant skins (DBS) extracts

Preliminary experiments were performed to identify the conditions that favour better extraction of anthocyanins. DBS (2.5 g) were independently extracted with 25 mL solvents

[100% methanol, 100% water, and mixtures of methanol-water (50%, and 70%, v/v)] in a shaking water bath (200 rpm) at 30 °C, 50 °C, 70 °C and 90 °C. The durations of the extraction were set at 0.5, 1, 2, 4 and 6 h. Moreover, extractions using acetic acid buffer solutions at an acidic pH value of 1.5 were also carried out in order to investigate the influence of solvent, temperature and solvent-temperature interaction on anthocyanins and phenolics extraction. Duran bottles were tightly closed using polybutylene terephthalate (PBT) caps with polytetrafluoroethylene (PTFE) faced silicone cap liner in order to prevent the evaporation of solvents during the extraction process. The obtained extracts were filtered using a vacuum filter to remove the solid residues.

2.4 Determination of total monomeric anthocyanin content

In preliminary studies, 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). As such, 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 min. The total monomeric anthocyanin (mg cyanidin-3-O-glucoside equivalents/g dry weight) was calculated as follows:

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

where $C_{anthocyanin}$ = otal 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 Eq.2 as follows:

$$Anthocyanin\ content\left(\frac{mg}{g}\ of\ dry\ weight\right) = \frac{C\ anthocyanin\ \left(\frac{mg}{L}\right) \times extract\ (L)}{sample\ (g)}$$ Eq. 2

The absorbance of the samples at 520 nm and 700 nm was determined using a spectrophotometer (Thermo Electron Corporation, USA).

2.5 HPLC analysis of phenolic compounds

Free phenolics. HPLC analysis of free phenolics was based on a method by Kapasakalidis, Rastall, & Gordon (2006) with slight modifications. HPLC analysis was performed in a 1200 Infinity HPLC system (Infinity 1200 series, Agilent Technologies, UK) equipped with a diode-array detector (DAD) using a Zorbax C18 column (250 × 4.6 mm i.d., particle size 5 µm, Agilent, UK) 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 protocol 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 before decreasing back to 15% at 45 min. The flow rate was 1.0 mL/min and the injection volume was 20 µL. The duration of the analysis was 50 min.

Free phenolic compounds were quantified in three subclasses: free anthocyanins (consisting of cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, delphinidin-3-O-glucoside, and delphinidin-3-O-rutinoside; detected at 520 nm), hydroxycinnamic acids (consisting of caffeic acid, *p*-coumaric acid and ferulic acid; detected at 320 nm) and flavonols (consisting of myricetin-3-O-glucoside, quercetin-3-O-glucoside, quercetin-3-O-rutinoside, myricetin, kaempferol-3-O-glucoside, kaempferol-3-O-rutinoside, quercetin and kaempferol; detected at 360 nm). 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 (anthocyanins), 0.05 to 1.0 mg/mL (hydroxycinnamic acids) and 0.001 to 0.05 mg/mL (flavonols). To obtain the standard curves,

the peak areas were plotted against the corresponding concentrations of the standard solutions injected into HPLC.

***Bound phenolics*.** For the determination of bound phenolics, a method described by Hertog, Hollman, & Venema (1992) was used. After free phenolics extraction, the remaining residues were hydrolysed to release the cell wall-bound phenolics. 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 anthocyanidins (cyanidin and delphinidin) 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.

2.6 Determination of total free phenolics content

The total free phenolics content was determined by the Folin–Ciocalteu method (Waterman & Mole, 1994) with slight modifications. 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 100 g of dried weight (mg GAE/100 g DW). Duplicate measurements were taken and mean values were calculated.

2.7 Determination of total antioxidant activity

The free radical scavenging activity of DBS extracts on the stable DPPH radical was carried out according to the procedure described by 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 the dark, for 30 min. The percentage of inhibition was calculated using the following equation:

$$Inhibition\ (\%) = \frac{A_o - A_e}{A_o} \times 100 \qquad \text{Eq.3}$$

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

2.8 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:

$$Total\ colour\ difference\ (\Delta E) = [(L^* - L_o)^2 + (a^* - a_o)^2 + (b^* - b_o)^2]^{1/2} \qquad \text{Eq.4}$$

where, L_o, a_o, b_o = blank values of control samples extracted at 30 min, 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).

$$Chroma\ (c) = \sqrt{(a^*)^2 + (b^*)^2} \qquad \text{Eq. 5}$$

$$Hue\ angle\ (h) = ArcTan(\frac{b^*}{a^*}) \qquad \text{Eq. 6}$$

2.9 Statistical analysis

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

3. RESULTS AND DISCUSSION

Based on the results of total monomeric anthocyanin content during preliminary studies (**Supplementary Table 1**) and due to the thermal sensitivity of free anthocyanins, extractions at 30 °C and 50 °C for 2 h were chosen as the best conditions, whereas no significant difference was observed in the extraction of total monomeric anthocyanins within the first 2 h in the case of all solvent and acid extractions. Consequently, these extracts were then subjected to HPLC analysis to identify the types of anthocyanins, flavonols and hydroxycinnamic acids present.

According to the HPLC analysis, free and bound anthocyanins, including anthocyanidins were detected at 520 nm (**Figure 1**). In addition, **Figure 2** and **Figure 3** show the HPLC chromatograms of free and bound 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.

3.1 Free and bound anthocyanins

Generally, among the four free anthocyanins in all DBS, delphinidin-3-O-rutinoside was the predominant ($p < 0.05$) free anthocyanin (delphinidin-3-O-rutinoside; ~50.4% > cyanidin-3-O-rutinoside; ~29.1% > delphinidin-3-O-glucoside; ~20.7% > cyanidin-3-O-glucoside; ~8.6%) (**Figure 1a**). This trend was similar to the work reported by Borges, Degeneve, Mullen, & Crozier (2009) with blackcurrant fruit extracted with methanol/formic acid (99:1, v/v) using a homogeniser for 1 min.

According to Padayachee et al. (2013), bound anthocyanins and phenolic acids bonded to the cell wall cellulose and pectin through hydrogen bonding and/or hydrophobic interactions. In this study, bound anthocyanins were detected as delphinidin-3-O-glucoside and cyanidin-3-O-glucoside based on the comparison of the retention time as shown in **Figure 1b.** Interestingly, not only bound anthocyanins, but also proanthocyanidins and anthocyanins in the DBS residue were also hydrolysed to form anthocyanidins, namely delphinidin and cyanidin. Similar results were observed in the study by Kapasakalidis et al. (2006), whereas acid hydrolysis of solvent extracted blackcurrant pomace residue produced approximately 200 – 700 mg/100 g of total bound anthocyanins and anthocyanidins (delphinidin and cyanidin).

Extraction using extremely low pH of acetic acid buffer (pH 1.5) was carried out at two different extraction temperatures (30 °C and 50 °C) to investigate the effect of strong acid on

anthocyanin and phenolic extraction (**Table 1**). As expected, significantly higher ($p < 0.05$) free anthocyanins (1712.3 ± 56.1 mg/100 g) was obtained compared to 30 °C (1397.5 ± 21.5 mg/100 g). This is higher than that reported by Lapornik et al. (2005), whereas 1360.0 ± 10.0 mg/100 g of free anthocyanins was extracted when using 70% methanol for 24 h at room temperature.

Not only at 50 °C, extraction using acetic acid buffer at 30 °C also shows higher concentrations of total free anthocyanins than in water (292.7 mg/100 g – 551.7 mg/100 g), methanol (1109.4 mg/100 g – 1164.8 mg/100 g) and methanol/water (1135.7 mg/100 g – 1301.2 mg/100 g) extractions (**Table 1)**. The pH value of the 100% methanol solution was ~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 concentrations of methanol/water (70%) were applied. It is apparent that the application of extremely low pH (pH 1.5) enabled the efficient extraction of high amounts of free anthocyanins. Acids are considered suitable extractants to extract phenolic compounds 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).

As shown in **Table 1**, extraction using water at 50 °C showed significantly higher ($p < 0.05$) free anthocyanins (551.7 ± 0.5 mg/100 g) compared to 30 °C. Both water extracts showed up to 3.0 times ($p < 0.05$) higher of anthocyanidins and bound anthocyanins than free anthocyanins. Lower free anthocyanins but higher anthocyanidins content indicated that acid hydrolysis helped to hydrolyse the remaining anthocyanins and proanthocyanidins in the DBS residues. This is due to the fact that water was ineffective in extracting free anthocyanins due to low diffusion rates and solubility of analytes compared to other solvents as suggested by Oancea, Stoia, & Coman (2012). However, there is no interaction between solvent and temperature on the yield of anthocyanidins.

Moreover, 100% methanol extractions at 30 °C showed relatively higher free and bound anthocyanins and anthocyanidins (1164.8 ± 5.9 mg/100 g, 53.4 ± 5.2 mg/100 g and 814.1 ± 18.9 mg/100 g), as compared to counterpart at 50 °C. With regards to the absolute water or methanol extractions used at 50 °C, significantly higher ($p < 0.05$) free anthocyanins were observed in the methanol than in the water extracts. This suggests that methanol is a better extractant for free anthocyanins compared to water. 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 the extractions using 70% and 50% methanol, 50 °C exhibited a higher extraction yield of free anthocyanins, equal to 1301.2 ± 5.3 mg/100 g and 1242.5 ± 1.5 mg/100 g, respectively, compared to 30 °C. On the other hand, regarding the effect of the concentrations of methanol/water used, 70% mixture showed relatively higher free anthocyanins than 50% methanol at both low and high extraction temperatures.

Overall, in methanol extractions, 70% methanol resulted in relatively higher free anthocyanins content than 100% and 50% methanol, whereas there was an increase in the yield of free anthocyanins when water content of 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, Tramelli, & De Faveri (2007), higher solvent to water ratios have an important role towards the efficiency of extraction.

As shown in **Table 1**, amongst all extraction conditions, the percent yield of delphinidin-3-O-glucoside and cyanidin-3-O-glucoside were highest in the acetic acid buffer pH 1.5 extracts (30 °C). Meanwhile, delphinidin-3-O-rutinoside and cyanidin-3-O-rutinoside were highest in water at 30 °C of extraction. These suggested that extraction at 30 °C was capable to extract

higher amount of some free anthocyanins compared to 50 °C. Approximately ~73.9% of free anthocyanins were recovered in acetic acid extraction, while 1.4% and 33.5% of bound anthocyanins and anthocyanidins, respectively were released by acid hydrolysis of DBS residue. The yield of bound anthocyanins was in agreement with previous studies, reporting 1.0% of total bound anthocyanins from black carrot puree (Padayachee et al., 2013).

Furthermore, except water, all DBS extracts had significantly ($p < 0.05$) higher ratio of free anthocyanins to total anthocyanidins and bound anthocyanins, which differed from 1.4 (100% methanol at 30 °C) to 2.8 (acetic acid buffer pH 1.5 at 50 °C). Even though delphinidin-3-O-rutinoside and cyanidin-3-O-rutinoside were two major free anthocyanins found in all extracts, none of them was detected as bound anthocyanins (**Table 2**). Cyanidin-3-O-glucoside was the lowest free anthocyanin found in the extracts, however, appeared as the most dominant bound anthocyanin in all residues (83.8% – 100%). Other than that, delphinidin-3-O-glucoside was also observed as bound anthocyanins, but only in DBS residues after water and 100% methanol extractions at low concentrations (9.3% – 16.2%).

3.2 Free and bound hydroxycinnamic acids

Free hydoxycinnamic acid contents in DBS after 2 h of extraction are presented in **Table 3**. According to the results, *p*-coumaric acid (~61.6%) was the main free hydroxycinnamic acid in all DBS extracts, followed by caffeic (~35.6%) and ferulic acid (~18.5%). Not only free anthocyanins, acetic acid buffer was also efficient in the free hydroxycinnamic acid extraction whereas higher ($p < 0.05$) concentrations were recorded at 50 °C (268.0 ± 4.5 mg/100 g) than 30 °C (206.0 ± 2.3 mg/100 g). At 50 °C, total free hydroxycinnamic acid was also significantly higher ($p < 0.05$) compared to water and 50% methanol.

In the water extractions, significantly higher ($p < 0.05$) total free hydroxycinnamic acid was detected at 50 °C extract (140.4 ± 0.2 mg/100 g) compared to 30 °C (78.8 ± 2.4 mg/100 g). However, in 100% methanol extractions, 30 °C showed relatively higher total free hydroxycinnamic acids (276.3 ± 9.0 mg/100 g) compared to 50 °C (264.4 ± 2.4 mg/100 g). Moreover, in the extractions using mixtures of methanol/water, 70% methanol (50 °C and 30 °C) extracts showed significantly higher total free hydroxycinnamic acid concentrations (~254.4 mg/100 g) than 50% methanol (~228.7 mg/100 g).

The percent yield of caffeic acid was significantly higher ($p < 0.05$) in water (30 °C), 100% methanol (50 °C) and acetic acid buffer pH 1.5 (50 °C) extracts, whilst *p*-coumaric acid was high in water and 100% methanol extracts at 50 °C. Also, ferulic acid showed a higher percentage of yields in 50% methanol at 30 °C and 50 °C extractions. Overall, the application of acetic acid buffer and 70% methanol at 50 °C, and 100% methanol at both temperatures extracted higher total free hydroxycinnamic acids compared to other solvents used.

According to **Table 3**, *p*-coumaric acid was the only bound hydroxycinnamic acid detected in all DBS residues. Amongst all DBS residues, significantly ($p < 0.05$) higher *p*-coumaric acid was extracted in DBS residues after water (~47.8 mg/100 g) and 100% methanol (~47.0 mg/100 g) extractions compared to acetic acid buffer pH 1.5 (~42.9 mg/100 g). Moreover, DBS residue from 100% methanol extraction also exhibited higher yield of free and bound hydroxycinnamic acids compared to other residues. This might indicate that the polarity of methanol is suitable to extract both free and bound hydroxycinnamic acids. This finding was in agreement with the study by Haminiuk et al. (2014), where absolute methanol was shown as the most effective solvent for the extraction of phenolic acids and flavonols from *Eugenia pyriformis* fruit. Gulsunoglu et al. (2019) also reported small amounts of *p*-coumaric acid (0.8 ± 0.1 mg/100 g) as bound hydroxycinnamic acid in black carrot pomace. However, it is

noteworthy to mention that acid hydrolysis at high temperature might also denature hydroxycinnamic acids in the residue.

3.3 Free and hydrolysed flavonols

Flavonols are a subgroup of flavonoids and can typically be found in the vacuole of fruits and vegetables. Myricetin, quercetin, kaempferol and fisetin are among the most studied flavonols (Panche, Diwan, & Chandra, 2016). As shown in **Table 4**, myricetin-3-O-glucoside was the main free flavonol ($p < 0.05$) detected in both 50 °C and 30 °C water extracts (~28.6% to ~29.0%), followed by quercetin-3-O-glucoside; no kaempferol was detected. There was no significant difference ($p > 0.05$) in the total free flavonol content between these two extraction temperatures. Laaksonen et al. (2014) reported similar trends for free flavonols obtained in blackcurrant juices produced by pressing blackcurrants at an industrial scale.

According to the free flavonol contents in the acid extracts as shown in the **Table 4**, the application of higher extraction temperature (50 °C) resulted in significantly ($p < 0.05$) higher total free flavonols (60.2 ± 0.8 mg/100 g) than 30 °C (47.4 ± 0.5 mg/100 g). On the other hand, extraction using 100% methanol at both temperatures led to significantly ($p < 0.05$) higher total free flavonol concentrations compared to other solvents including acetic acid buffer. In addition, quercetin-3-O-glucoside appeared to be the major free flavonol in the acid extracts (26.4% – 27.6%), followed by myricetin-3-O-glucoside (~21.1% to ~21.9%).

Moreover, there is no significance difference in the free flavonol content between 100% methanol at 50 °C and 30 °C extractions. Also, in methanol extractions, myricetin was the dominant free flavonol rather than myricetin-3-O-glucoside, as in the case of water extractions. This was followed by quercetin-3-O-glucoside and myricetin-3-O-glucoside, whilst kaempferol was the lowest ($p < 0.05$) free flavonol (~2.8 mg/100 g) detected in the methanol extractions.

Furthermore, 70% and 50% methanol extractions at 50 °C (**Table 4**) resulted in relatively higher free flavonol content (~58.5 mg/100 g) than at 30 °C (~53.6 mg/100 g). Myricetin-3-O-glucoside and quercetin-3-O-glucoside were the main free flavonols detected in all extractions, i.e. with different methanol/water ratios and extraction temperatures. Furthermore, in terms of the solvent used, 100% methanol at 50 °C and 30 °C were the most suitable extraction systems/ conditions to obtain the highest concentration of free flavonol (~71.5 mg/100 g) compared to water, methanol/water and acid.

Notably, **Table 4** demonstrates that the type of extractant influenced the individual free flavonol yield, with myricetin-3-O-glucoside (~29.0%) and quercetin-3-O-glucoside (~25.4%) being the main free flavonols ($p < 0.05$) detected in water, methanol/water and acetic acid buffer pH 1.5 extracts. Whilst, 100% methanol contained higher amount ($p < 0.05$) of myricetin (~25.0%). Upon acid hydrolysis as shown in **Table 4**, significantly ($p < 0.05$) higher myricetin (~63.6%) was detected compared to quercetin (36.4%). Overall, the total content of free flavonols were 1.1 to 3.0 times higher than their hydrolysed aglycones. Similar to anthocyanidins, these flavonol aglycones were released by acid hydrolysis of the flavonols glycosides that still remaining in cell vacuole after solvent extraction (**Figure 3b**). This was in accordance with the findings by Moussa-Ayoub, El-Samahy, Kroh, & Rohn (2011) who reported that acid hydrolysis of dried cactus peel for 1 h at 90 °C caused the degradation of flavonols to form the respective aglycones.

3.4 Total free phenolics and antioxidant activities of extracts

Total free phenolics concentration and antioxidant activity of the extracts were determined using the Folin-Ciocalteu method and DPPH assay, respectively. In the acid extraction using acid buffer pH 1.5 at 50 °C, significantly higher ($p < 0.05$) amount of total free

phenolics (~3702.2 mg GAE/100 g) and antioxidant activities (~60.7% inhibition) were detected compared to extraction at 30 °C (**Figure 4**). It is apparent that the low pH of the acetic acid buffer and high temperature were capable in extracting other compounds besides free phenolics and anthocyanins which most likely contributed to the high antioxidant activities.

According to **Figure 4**, the increase in the extraction temperature from 30 °C to 50 °C, led to a concomitant increase in the total phenolic content of water extracts. This resulted in an increase in the antioxidant activity from ~16.4% to ~39.4% inhibition during the first 2 h of extraction. Even though anthocyanins were efficiently extracted at 30 °C (**Table 1**), the total free phenolic content of methanol extracts at 50 °C in **Figure 4** was found to be significantly ($p < 0.05$) higher (~2032.5 mg GAE/100 g), as did the antioxidant activity (~39.9% inhibition). Compared to water, methanol was capable in extracting significantly ($p < 0.05$) 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 ($p < 0.05$) higher free phenolic contents, ranging between ~3236.5 to ~3337.7 mg GAE/100 g, with higher antioxidant activity (~56.0% to ~57.7% of inhibition) compared to 30 °C extracts (**Figure 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 free 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). Overall, extraction at 50 °C using methanol/water (70% and 50%) and especially acetic acid buffer pH 1.5 resulted in the highest

449 phenolics content (~3236.5 to ~3702.2 mg GAE/100 g) and antioxidant activity (~56.0% to
450 ~60.7% of inhibition) compared to other extraction conditions.

451 The Pearson correlation between the total phenolics content and the free anthocyanin
452 content in the obtained extracts was weak, with $R^2 = 0.628$ ($p < 0.05$), indicating that besides
453 free anthocyanins, there were other phenolic compounds that also reacted with the Folin reagent.
454 Moreover, a study by Häkkinen et al. (1999) showed that phenolic compounds such as
455 hydroxycinnamic acids (*p*-coumaric, ferulic and caffeic acids) and flavonols (quercetin,
456 myricetin and kaempferol) were also found in blackcurrant fruit extracts. Moreover, Karseno,
457 Yanto, Setyowati, & Haryanti (2018) and Everette et al. (2014) reported that the Folin reagent
458 is significantly reactive towards other compounds besides phenols such as proteins, organic
459 acids, vitamins and reducing sugars.

460 Kähkönen and Heinonen (2003) also suggested that anthocyanins and their aglycones
461 are powerful antioxidants and possess high antioxidant activity. Therefore, a strong correlation
462 ($R^2 = 0.802$) with $p < 0.05$ was found between the anthocyanin content and antioxidant activity
463 suggesting that besides anthocyanins other phenolics might have also contributed to this
464 attribute. However, ~20% of the antioxidant activity could be attributed to other phenolics such
465 as hydroxycinnamic acids and flavonols, and potentially to non-phenolic compounds. This is
466 supported by the significant correlation ($p < 0.05$) between the total phenolics content in the
467 DBS extracts and antioxidant activity ($R^2 = 0.935$).

468

469 **3.5 Colour of extracts**

470 Many studies on anthocyanins from fruits investigate their use as natural colourants (He
471 & Giusti, 2010). In this study, colour measurement aimed to investigate the effect of the
472 extraction conditions to the colour of the extracts. The changes in colour were reported in terms

of colour values (*L**, *a**, *b**), Total Colour Difference, chroma and hue angle (h°) as a comparison between the colour of the extracts and the controls (30 min extraction).

Even though 50 ℃ of acid extract indicated a high amount of free anthocyanins after 2 h (**Table 1**), there was a significant ($p < 0.05$) low of *a** (redness) and chroma (colour intensity) values indicating extraction at high temperature resulted in a decrease in redness and colour intensity of extracts (**Table 5**). On the other hand, *a** values ($p < 0.05$) was significantly high in 30 ℃ water extract which led to high chroma. Meanwhile, extraction using 100% methanol resulted in a significantly ($p < 0.05$) low of *L** and *b** values, representing an increase in darkness and blueness, respectively. The combination of these values led to significantly ($p < 0.05$) low hue angle values of 100% methanol extracts compared to other solvents. Moreover, no correlation was observed between anthocyanins content and chroma and redness. These proved that rather than anthocyanins content, colour properties of the extracts were influenced more by the pH values of the extraction solvents, as reported by Wrolstad (2004) and Pedro, Granato, & Rosso (2016). Furthermore, statistical analysis using Two-way ANOVA shows the significant interaction ($p < 0.05$) between solvent and extraction temperature on the *L**, *a**, *b**, Total Colour Difference and chroma values.

4. CONCLUSIONS

The extraction yields of free phenolic compounds (including free anthocyanins) from dried blackcurrant skins were mainly affected by the choice of solvent and extraction temperature. Generally, higher extraction temperature (50 °C) was more effective than 30 °C. The application of low pH of acetic acid buffer (pH 1.5) at 50 ℃ efficiently extracted higher amounts of free anthocyanins, free hydroxycinnamic acids, total free phenolic content and antioxidant activity compared to water and solvent extractions. The extracts contained relatively

high amounts of total free phenolic compounds and exhibited superior antioxidant activity, as well as good colour intensity. Moreover, HPLC profiling results show that anthocyanins, hydroxycinnamic acids and flavonols are dependent on the type of solvent used. In this study, acid hydrolysis method produced bound anthocyanins, anthocyanidins, bound hydroxycinnamic acid (*p*-coumaric acid) and flavonol aglycones from dried blackcurrant skins residue. The yields of these bound phenolic compounds were mainly lower than that of their free forms. Overall, the yield of total free phenolics and free anthocyanins were strongly correlated with high antioxidant activity. However, colour properties did not correlate with the free anthocyanin contents but depended considerably on solvent type and extraction temperature used. Also, in order to reduce the cost of extraction, liquid-liquid extraction should be conducted to recover and recycle the acetic acid.

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CONFLICTS OF INTEREST

The authors declared no conflicts of interest.

AUTHOR CONTRIBUTIONS

EMA conducted the research, interpreted the results, and drafted the manuscript. DC and AC conceptualized, designed the study and reviewed- finalized the manuscript.

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