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

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25 **ABSTRACT**

26 The aim of this study was to investigate the effects of different solvent and extraction
27 temperatures on the free and bound phenolic compounds and antioxidant activity of dried
28 blackcurrant skins (DBS). Apart from acetic acid buffer solution, different solvent systems
29 including water, methanol, and mixtures of methanol/water were also employed and the effects
30 of solvent and temperature (30 °C and 50 °C) on the free and bound forms of anthocyanins,
31 hydroxycinnamic acids and flavonols yield were assessed. **The results showed that amongst all**
32 **solvents, acetic acid buffer resulted in the highest free anthocyanin content (1712.3 ± 56.1**
33 **mg/100 g) (*p* < 0.05) after 2 h extraction at 50 °C from DBS, while lower amounts of bound**
34 **anthocyanins and anthocyanidins were detected after acid hydrolysis.** Acetic acid buffer extracts
35 exhibited the highest free hydroxycinnamic acid content (268.0 ± 4.5 mg/100 g), total phenolic
36 content (3702.2 ± 259.3 mg GAE/100 g) and DPPH activity (60.7 ± 2.0 % of inhibition).
37 However, their free flavonol content was slightly lower (60.2 ± 0.8 mg/100 g) compared to
38 100% methanol at 30 °C and 50 °C, 71.4 ± 1.5 mg/100 g and 71.5 ± 6.2 mg/100 g, respectively.
39 Two-way ANOVA indicated interactions between solvent and temperatures (*p* < 0.05), which
40 suggested that the relationship between solvent and phenolic compounds depends on the
41 extraction temperature.

42

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

47

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

50

51 **1. INTRODUCTION**

52 Blackcurrant (*Ribes nigrum* L.) is recognised as an economically important soft fruit
53 crop in Europe, Russia, Northern Asia, New Zealand and North America (Battino et al., 2009).
54 Nowadays there are approximately 50 growers that occupy 2,400 hectares in the British Isles
55 and produce around 11,000 tonnes of blackcurrant per year (IBA, 2018). Blackcurrants are
56 generally cultivated for food applications, as it can be used as natural colourants due to their
57 high content of anthocyanins and proanthocyanins, as preservatives and as sources of ascorbic
58 acid (~180 mg/100 g of berries) and phenolic compounds (500 – 1342 mg/100 g of berries); the
59 latter have been associated with health promoting effects in humans (Brennan & Graham, 2009;
60 Basegmez et al., 2017). Anthocyanins, flavonoids, hydroxycinnamic acids, *p*-coumaric acid,
61 myricetin, quercetin, kaempferol glycosides and isorhamnetin shape the phenolic compounds
62 profile of the blackcurrant fruit (Sójka & Król, 2009), that can be associated primarily with their
63 high antioxidant activities (Szajdek & Borowska, 2008). Blackcurrants are processed to produce
64 a range of functional ingredients, such as blackcurrant-pomace dietary fibers and defatted seeds,
65 which can be incorporated into jams, jellies, purées and teas (Varming, Peterson, & Poll, 2004).

66 In the UK, 75% of the total fresh blackcurrant production is processed into juice (Vagiri,
67 2014) with 15% is by-products (Pap et al., 2005) that equals to 1,650 tonnes per year. By-
68 products of the blackcurrant juice process (skins, seeds and stems) are collected during the
69 pressing stage. Only a small percentage of these by-products is recycled or upgraded, and the
70 majority is used as animal feed (although there are limitations in this regard due to their high
71 acidity and rancid taste) and composting material or is disposed through alternative routes (e.g.

72 land spreading) (Arvanitoyannis, 2010). Taking into account the fact that most phenolic
73 compounds are accumulated in the skin of fruits (Mäkilä et al., 2016), an additional value chain
74 can be created through the extraction of phenolic compounds from these by-products.

75 Extraction is the most important step in the isolation of phenolic compounds including
76 anthocyanins; however, there is no standardised method for their extraction. The initial step to
77 extract phenolic compounds from plant materials such as berries include grinding, drying and
78 soaking of the samples in extraction solvents such as water, organic solvents and acids
79 (Anderson & Markham, 2005). Dried plant materials are normally used as starting materials for
80 anthocyanin extraction in order to minimise the possibility of anthocyanin degradation due to
81 chemical reactions taking place in the wet material (Harbourne, Marete, Jacquier, & O'Riordan,
82 2013).

83 Due to the polarity of anthocyanins, polar solvents such as aqueous mixtures of
84 methanol, ethanol or acetone are often employed for their extraction (Kähkönen, Hopia, &
85 Heinonen, 2001). Also, since water can extract more polar compounds, and ethanol or methanol
86 can extract more hydrophobic compounds, the ratio of the water and methanol or ethanol
87 mixture can be adjusted according to the polarity and solubility of anthocyanins (Lapornik,
88 Prošek, & Wondra, 2005). Moreover, acidified methanol with hydrochloric or formic acid has
89 been commonly used as an extractant in order to disrupt the cell membrane as well as dissolve
90 the water-soluble anthocyanins (Rodriguez-Saona & Wrolstad, 2001; Amr & Al-Tamimi,
91 2007).

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

96 (Saura-Calixto, 2012; Durazzo, 2018). Anthocyanins and flavonols are examples of flavonoids
97 (Zhang & Tsao, 2016). In contrast, the non-extractable or bound phenolics are insoluble in
98 aqueous organic solvents, thus remain in the solid residues after extraction. They are bound to
99 protein or cell wall polysaccharides and can only be released by acid, alkaline or enzymatic
100 hydrolysis treatments. Examples of bound phenolics are high molecular weight of
101 proanthocyanidins and hydrolysable tannins, phenolic acids such as ferulic acid, caffeic acid
102 and sinapic acid (Saura-Calixto, 2012; Acosta-Estrada, Gutiérrez-Urbe, & Serna-Saldívar,
103 2014; Durazzo, 2018; Gulsunoglu, Karbancioglu-Guler, Raes, & Kilic-Akyilmaz, 2019).

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

112

113 **2. MATERIALS AND METHODS**

114 **2.1 Chemicals**

115 All solvents and chemicals used for extraction, including methanol (99.9%), ethanol
116 (99.8%), formic acid (95%) and acetic acid (99.7%), were of analytical grade and were
117 purchased from Sigma-Aldrich (UK). Folin-Ciocalteu reagent, sodium carbonate, potassium
118 chloride, sodium acetate, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) were also purchased from

119 Sigma-Aldrich (UK). Hydrochloric acid (37%) was purchased from Fisher Scientific
120 (Loughborough, UK).

121 A stock solution of 2 mM DPPH was prepared in methanol. Buffer solutions of
122 pH 1.0 (potassium chloride, 0.025 M) and pH 4.5 (sodium acetate, 0.4 M) were prepared as
123 described by Lee, Durst, & Wrolstad (2005). Purified water was used in all preparations,
124 obtained by a Purite reverse osmosis system (Oxon, UK). Anthocyanin standards of cyanidin-
125 3-O-glucoside (96%), cyanidin-3-O-rutinoside (96%), delphinidin-3-O-glucoside (95%), and
126 delphinidin-3-O-rutinoside (95%), cyanidin (96%), delphinidin (96%), kaempferol-3-O-
127 glucoside (99%), kaempferol-3-O-rutinoside (98%), quercetin-3-O-rutinoside (99%) and
128 myricetin-3-O-glucoside (99%) were obtained from ExtraSynthese Ltd (Genay, France). In
129 addition, quercetin (95%), myricetin (98%), kaempferol (99%), caffeic acid (98%), *p*-coumaric
130 (98%), ferulic acid (99%) and quercetin-3-O-glucoside (98%) were purchased from Sigma-
131 Aldrich (UK).

132

133 **2.2 Sample preparation of plant materials**

134 Dried blackcurrant pressed residues derived from a juice manufacturing process were
135 kindly supplied by Purn House Farm, (Bleadon, Weston-super-Mare, UK). Dried blackcurrants
136 were separated from the seeds by grinding in a coffee blender and passed through a 0.841 mm
137 (20 mesh) sieve; this constituted the dried blackcurrant skins (DBS) sample. Samples were
138 segregated in polyethylene bags and stored at -20 °C until further analysis.

139

140 **2.3 Preparation of dried blackcurrant skins (DBS) extracts**

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

143 [100% methanol, 100% water, and mixtures of methanol-water (50%, and 70%, v/v)] in a
144 shaking water bath (200 rpm) at 30 °C, 50 °C, 70 °C and 90 °C. The durations of the extraction
145 were set at 0.5, 1, 2, 4 and 6 h. Moreover, extractions using acetic acid buffer solutions at an
146 acidic pH value of 1.5 were also carried out in order to investigate the influence of solvent,
147 temperature and solvent-temperature interaction on anthocyanins and phenolics extraction.
148 Duran bottles were tightly closed using polybutylene terephthalate (PBT) caps with
149 polytetrafluoroethylene (PTFE) faced silicone cap liner in order to prevent the evaporation of
150 solvents during the extraction process. The obtained extracts were filtered using a vacuum filter
151 to remove the solid residues.

152

153 **2.4 Determination of total monomeric anthocyanin content**

154 In preliminary studies, total monomeric anthocyanin content of DBS was determined by
155 the differential pH method based on the property of anthocyanins to change colour at different
156 pH values (Lee et al., 2005). As such, 600 µL of anthocyanins extracts were mixed with 1.2 mL
157 of corresponding buffer (pH 1.0 and pH 4.5) and allowed to equilibrate for 20 min. The total
158 monomeric anthocyanin (mg cyanidin-3-O-glucoside equivalents/g dry weight) was calculated
159 as follows:

$$160 \quad C_{\text{anthocyanin}} = \frac{A \times MW \times DF \times 10^3}{\epsilon \times l} \quad \text{Eq.1}$$

161 where $C_{\text{anthocyanin}}$ = total monomeric anthocyanin concentration (cyanidin-3-O-glucoside
162 equivalents, mg/L); A (absorbance) = $(A_{520\text{nm}} - A_{700\text{nm}})_{\text{pH 1.0}} - (A_{520\text{nm}} - A_{700\text{nm}})_{\text{pH 4.5}}$; MW
163 (molecular weight) = 449.2 g/mol for cyanidin-3-O-glucoside; DF = dilution factor; l =
164 pathlength in cm; ϵ = 26900 molar extinction coefficient in L/mol/cm for cyanidin-3-O-
165 glucoside; and 10^3 = factor for conversion from g to mg (Lee et al., 2005). The anthocyanin
166 content was then calculated by Eq.2 as follows:

167
$$\text{Anthocyanin content} \left(\frac{\text{mg}}{\text{g}} \text{ of dry weight} \right) = \frac{C \text{ anthocyanin} \left(\frac{\text{mg}}{\text{L}} \right) \times \text{extract} \text{ (L)}}{\text{sample} \text{ (g)}} \text{ Eq. 2}$$

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

170

171 **2.5 HPLC analysis of phenolic compounds**

172 **Free phenolics.** HPLC analysis of free phenolics was based on a method by Kapasakalidis,
173 Rastall, & Gordon (2006) with slight modifications. HPLC analysis was performed in a 1200
174 Infinity HPLC system (Infinity 1200 series, Agilent Technologies, UK) equipped with a diode-
175 array detector (DAD) using a Zorbax C18 column (250 × 4.6 mm i.d., particle size 5 µm,
176 Agilent, UK) at 30 °C. The mobile phase consisted of 5% formic acid (v/v) (solvent A) and
177 100% (v/v) methanol (solvent B). The gradient elution protocol was: 15% (B) at 0 min,
178 increasing to 35% (B) at 15 min and to 60% (B) at 30 min, and reaching 80% (B) at 40 min
179 before decreasing back to 15% at 45 min. The flow rate was 1.0 mL/min and the injection
180 volume was 20 µL. The duration of the analysis was 50 min.

181 Free phenolic compounds were quantified in three subclasses: free anthocyanins
182 (consisting of cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, delphinidin-3-O-glucoside,
183 and delphinidin-3-O-rutinoside; detected at 520 nm), hydroxycinnamic acids (consisting of
184 caffeic acid, *p*-coumaric acid and ferulic acid; detected at 320 nm) and flavonols (consisting of
185 myricetin-3-O-glucoside, quercetin-3-O-glucoside, quercetin-3-O-rutinoside, myricetin,
186 kaempferol-3-O-glucoside, kaempferol-3-O-rutinoside, quercetin and kaempferol; detected at
187 360 nm). Briefly, 2.0 mg/mL of stock standard solutions were prepared separately followed by
188 dilution to give concentrations from 0.01 to 1.0 mg/mL (anthocyanins), 0.05 to 1.0 mg/mL
189 (hydroxycinnamic acids) and 0.001 to 0.05 mg/mL (flavonols). To obtain the standard curves,

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

192

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

203

204 **2.6 Determination of total free phenolics content**

205 The total free phenolics content was determined by the Folin–Ciocalteu method
206 (Waterman & Mole, 1994) with slight modifications. 20 µL of 4-fold diluted extracts were
207 added to 1.58 mL of distilled water and 100 µL of Folin–Ciocalteu reagent. After 8 min, 300
208 µL of sodium carbonate (75 g/L) were added. The absorbance of the samples was measured at
209 765 nm after 2 h of incubation at room temperature against a blank sample (water instead of
210 extract). Gallic acid (0 – 100 mg/L) was used as standard for the calibration curve. The results
211 were expressed as milligrams of gallic acid equivalents per 100 g of dried weight (mg GAE/100
212 g DW). Duplicate measurements were taken and mean values were calculated.

213

214 **2.7 Determination of total antioxidant activity**

215 The free radical scavenging activity of DBS extracts on the stable DPPH radical was
216 carried out according to the procedure described by Blois (1958) with slight modifications.
217 200 µL of 50-fold diluted extracts were mixed with 2 mL of 2 mM methanolic solution of DPPH.
218 The absorbance was measured at 517 nm after keeping the samples at 30 °C in the dark, for 30
219 min. The percentage of inhibition was calculated using the following equation:

220
$$\text{Inhibition (\%)} = \frac{A_o - A_e}{A_o} \times 100 \quad \text{Eq.3}$$

221

222 A_o = Absorbance of the control; A_e = Absorbance of the sample

223

224 **2.8 Colour measurement**

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

232

233
$$\text{Total colour difference } (\Delta E) = [(L^* - L_o)^2 + (a^* - a_o)^2 + (b^* - b_o)^2]^{1/2} \quad \text{Eq.4}$$

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

236

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

241

$$242 \quad \text{Chroma } (c) = \sqrt{(a^*)^2 + (b^*)^2} \quad \text{Eq. 5}$$

243

$$244 \quad \text{Hue angle } (h) = \text{ArcTan}\left(\frac{b^*}{a^*}\right) \quad \text{Eq. 6}$$

245

246 **2.9 Statistical analysis**

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

252

253 **3. RESULTS AND DISCUSSION**

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

260 According to the HPLC analysis, free and bound anthocyanins, including anthocyanidins
261 were detected at 520 nm (**Figure 1**). In addition, **Figure 2** and **Figure 3** show the HPLC
262 chromatograms of free and bound hydroxycinnamic acids and flavonols detected at 320 and 360
263 nm, respectively. Notably, anthocyanins can also be detected at 320 and 360 nm, but at lower
264 amounts.

265

266 **3.1 Free and bound anthocyanins**

267 Generally, among the four free anthocyanins in all DBS, delphinidin-3-O-rutinoside was
268 the predominant ($p < 0.05$) free anthocyanin (delphinidin-3-O-rutinoside; ~50.4% > cyanidin-
269 3-O-rutinoside; ~29.1% > delphinidin-3-O-glucoside; ~20.7% > cyanidin-3-O-glucoside;
270 ~8.6%) (**Figure 1a**). This trend was similar to the work reported by Borges, Degeneve, Mullen,
271 & Crozier (2009) with blackcurrant fruit extracted with methanol/formic acid (99:1, v/v) using
272 a homogeniser for 1 min.

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

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

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

288 Not only at 50 °C, extraction using acetic acid buffer at 30 °C also shows higher
289 concentrations of total free anthocyanins than in water (292.7 mg/100 g – 551.7 mg/100 g),
290 methanol (1109.4 mg/100 g – 1164.8 mg/100 g) and methanol/water (1135.7 mg/100 g – 1301.2
291 mg/100 g) extractions (**Table 1**). The pH value of the 100% methanol solution was ~4.3 to ~4.7;
292 the pH ranged between ~3.6 and ~3.8 in the 50% methanol extractions and increased slightly to
293 3.9 – 4.1 when higher concentrations of methanol/water (70%) were applied. It is apparent that
294 the application of extremely low pH (pH 1.5) enabled the efficient extraction of high amounts
295 of free anthocyanins. Acids are considered suitable extractants to extract phenolic compounds
296 as they may facilitate the disruption of the cell membrane and act as a dissolving medium of
297 water- soluble pigments as suggested by Amr and Al- Tamimi (2007).

298 As shown in **Table 1**, extraction using water at 50 °C showed significantly higher ($p <$
299 0.05) free anthocyanins (551.7 ± 0.5 mg/100 g) compared to 30 °C. Both water extracts showed
300 up to 3.0 times ($p < 0.05$) higher of anthocyanidins and bound anthocyanins than free
301 anthocyanins. Lower free anthocyanins but higher anthocyanidins content indicated that acid
302 hydrolysis helped to hydrolyse the remaining anthocyanins and proanthocyanidins in the DBS
303 residues. This is due to the fact that water was ineffective in extracting free anthocyanins due to
304 low diffusion rates and solubility of analytes compared to other solvents as suggested by
305 Oancea, Stoia, & Coman (2012). However, there is no interaction between solvent and
306 temperature on the yield of anthocyanidins.

307 Moreover, 100% methanol extractions at 30 °C showed relatively higher free and bound
308 anthocyanins and anthocyanidins (1164.8 ± 5.9 mg/100 g, 53.4 ± 5.2 mg/100 g and 814.1 ± 18.9
309 mg/100 g), as compared to counterpart at 50 °C. With regards to the absolute water or methanol
310 extractions used at 50 °C, significantly higher ($p < 0.05$) free anthocyanins were observed in the
311 methanol than in the water extracts. This suggests that methanol is a better extractant for free
312 anthocyanins compared to water. Boeing et al. (2014) also reported that methanol was the most
313 efficient solvent compared to ethanol, acetone and water in extractions carried out with black
314 mulberry, blackberry and strawberry.

315 In the extractions using 70% and 50% methanol, 50 °C exhibited a higher extraction
316 yield of free anthocyanins, equal to 1301.2 ± 5.3 mg/100 g and 1242.5 ± 1.5 mg/100 g,
317 respectively, compared to 30 °C. On the other hand, regarding the effect of the concentrations
318 of methanol/water used, 70% mixture showed relatively higher free anthocyanins than 50%
319 methanol at both low and high extraction temperatures.

320 Overall, in methanol extractions, 70% methanol resulted in relatively higher free
321 anthocyanins content than 100% and 50% methanol, whereas there was an increase in the yield
322 of free anthocyanins when water content of methanol/water solution was decreased.
323 Kapasakalidis et al. (2006) suggested that this difference occurred because anthocyanins are
324 polar and are more efficiently extracted in water-containing solvents. Moreover, according to
325 the mass transfer principles discussed by Spigno, Tramelli, & De Faveri (2007), higher solvent
326 to water ratios have an important role towards the efficiency of extraction.

327 As shown in **Table 1**, amongst all extraction conditions, the percent yield of delphinidin-
328 3-O-glucoside and cyanidin-3-O-glucoside were highest in the acetic acid buffer pH 1.5 extracts
329 (30 °C). Meanwhile, delphinidin-3-O-rutinoside and cyanidin-3-O-rutinoside were highest in
330 water at 30 °C of extraction. These suggested that extraction at 30 °C was capable to extract

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

336 Furthermore, except water, all DBS extracts had significantly ($p < 0.05$) higher ratio of
337 free anthocyanins to total anthocyanidins and bound anthocyanins, which differed from 1.4
338 (100% methanol at 30 °C) to 2.8 (acetic acid buffer pH 1.5 at 50 °C). Even though delphinidin-
339 3-O-rutinoside and cyanidin-3-O-rutinoside were two major free anthocyanins found in all
340 extracts, none of them was detected as bound anthocyanins (**Table 2**). Cyanidin-3-O-glucoside
341 was the lowest free anthocyanin found in the extracts, however, appeared as the most dominant
342 bound anthocyanin in all residues (83.8% – 100%). Other than that, delphinidin-3-O-glucoside
343 was also observed as bound anthocyanins, but only in DBS residues after water and 100%
344 methanol extractions at low concentrations (9.3% – 16.2%).

345

346 **3.2 Free and bound hydroxycinnamic acids**

347 Free hydroxycinnamic acid contents in DBS after 2 h of extraction are presented in **Table**
348 **3**. According to the results, *p*-coumaric acid (~61.6%) was the main free hydroxycinnamic acid
349 in all DBS extracts, followed by caffeic (~35.6%) and ferulic acid (~18.5%). Not only free
350 anthocyanins, acetic acid buffer was also efficient in the free hydroxycinnamic acid extraction
351 whereas higher ($p < 0.05$) concentrations were recorded at 50 °C (268.0 ± 4.5 mg/100 g) than
352 30 °C (206.0 ± 2.3 mg/100 g). At 50 °C, total free hydroxycinnamic acid was also significantly
353 higher ($p < 0.05$) compared to water and 50% methanol.

354 In the water extractions, significantly higher ($p < 0.05$) total free hydroxycinnamic acid
355 was detected at 50 °C extract (140.4 ± 0.2 mg/100 g) compared to 30 °C (78.8 ± 2.4 mg/100 g).
356 However, in 100% methanol extractions, 30 °C showed relatively higher total free
357 hydroxycinnamic acids (276.3 ± 9.0 mg/100 g) compared to 50 °C (264.4 ± 2.4 mg/100 g).
358 Moreover, in the extractions using mixtures of methanol/water, 70% methanol (50 °C and 30
359 °C) extracts showed significantly higher total free hydroxycinnamic acid concentrations (~ 254.4
360 mg/100 g) than 50% methanol (~ 228.7 mg/100 g).

361 The percent yield of caffeic acid was significantly higher ($p < 0.05$) in water (30 °C),
362 100% methanol (50 °C) and acetic acid buffer pH 1.5 (50 °C) extracts, whilst *p*-coumaric acid
363 was high in water and 100% methanol extracts at 50 °C. Also, ferulic acid showed a higher
364 percentage of yields in 50% methanol at 30 °C and 50 °C extractions. Overall, the application
365 of acetic acid buffer and 70% methanol at 50 °C, and 100% methanol at both temperatures
366 extracted higher total free hydroxycinnamic acids compared to other solvents used.

367 According to **Table 3**, *p*-coumaric acid was the only bound hydroxycinnamic acid
368 detected in all DBS residues. Amongst all DBS residues, significantly ($p < 0.05$) higher *p*-
369 coumaric acid was extracted in DBS residues after water (~ 47.8 mg/100 g) and 100% methanol
370 (~ 47.0 mg/100 g) extractions compared to acetic acid buffer pH 1.5 (~ 42.9 mg/100 g).
371 Moreover, DBS residue from 100% methanol extraction also exhibited higher yield of free and
372 bound hydroxycinnamic acids compared to other residues. This might indicate that the polarity
373 of methanol is suitable to extract both free and bound hydroxycinnamic acids. This finding was
374 in agreement with the study by Haminiuk et al. (2014), where absolute methanol was shown as
375 the most effective solvent for the extraction of phenolic acids and flavonols from *Eugenia*
376 *pyriformis* fruit. Gulsunoglu et al. (2019) also reported small amounts of *p*-coumaric acid (0.8
377 ± 0.1 mg/100 g) as bound hydroxycinnamic acid in black carrot pomace. However, it is

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

380

381 **3.3 Free and hydrolysed flavonols**

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

390 According to the free flavonol contents in the acid extracts as shown in the **Table 4**, the
391 application of higher extraction temperature (50 °C) resulted in significantly ($p < 0.05$) higher
392 total free flavonols (60.2 ± 0.8 mg/100 g) than 30 °C (47.4 ± 0.5 mg/100 g). On the other hand,
393 extraction using 100% methanol at both temperatures led to significantly ($p < 0.05$) higher total
394 free flavonol concentrations compared to other solvents including acetic acid buffer. In addition,
395 quercetin-3-O-glucoside appeared to be the major free flavonol in the acid extracts (26.4% –
396 27.6%), followed by myricetin-3-O-glucoside (~21.1% to ~21.9%).

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

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

409 Notably, **Table 4** demonstrates that the type of extractant influenced the individual free
410 flavonol yield, with myricetin-3-O-glucoside (~29.0%) and quercetin-3-O-glucoside (~25.4%)
411 being the main free flavonols ($p < 0.05$) detected in water, methanol/water and acetic acid buffer
412 pH 1.5 extracts. Whilst, 100% methanol contained higher amount ($p < 0.05$) of myricetin
413 (~25.0%). Upon acid hydrolysis as shown in **Table 4**, significantly ($p < 0.05$) higher myricetin
414 (~63.6%) was detected compared to quercetin (36.4%). Overall, the total content of free
415 flavonols were 1.1 to 3.0 times higher than their hydrolysed aglycones. Similar to
416 anthocyanidins, these flavonol aglycones were released by acid hydrolysis of the flavonols
417 glycosides that still remaining in cell vacuole after solvent extraction (**Figure 3b**). This was in
418 accordance with the findings by Moussa-Ayoub, El-Samahy, Kroh, & Rohn (2011) who
419 reported that acid hydrolysis of dried cactus peel for 1 h at 90 °C caused the degradation of
420 flavonols to form the respective aglycones.

421

422 **3.4 Total free phenolics and antioxidant activities of extracts**

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

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

430 According to **Figure 4**, the increase in the extraction temperature from 30 °C to 50 °C,
431 led to a concomitant increase in the total phenolic content of water extracts. This resulted in an
432 increase in the antioxidant activity from ~16.4% to ~39.4% inhibition during the first 2 h of
433 extraction. Even though anthocyanins were efficiently extracted at 30 °C (**Table 1**), the total
434 free phenolic content of methanol extracts at 50 °C in **Figure 4** was found to be significantly (p
435 < 0.05) higher (~2032.5 mg GAE/100 g), as did the antioxidant activity (~39.9% inhibition).
436 Compared to water, methanol was capable in extracting significantly ($p < 0.05$) higher amounts
437 of compounds including phenolics with higher antioxidant activities, hence most likely the
438 reason for this result.

439 Moreover, the extractions carried out at 50 °C using 70% and 50% methanol/water
440 mixtures showed significantly ($p < 0.05$) higher free phenolic contents, ranging between
441 ~3236.5 to ~3337.7 mg GAE/100 g, with higher antioxidant activity (~56.0% to ~57.7% of
442 inhibition) compared to 30 °C extracts (**Figure 4**). However, different methanol/water
443 concentrations (70% and 50%) did not show any significant difference on the total phenolics
444 and the antioxidant activity between the different extraction temperatures. As expected, 70%
445 methanol obtained higher free phenolics yield, with a higher antioxidant activity, than 100%
446 methanol. This is due to the fact that solvent/water mixtures can dissolve both polar and less
447 polar phenolic compounds, as suggested by Vagiri (2014). Overall, extraction at 50 °C using
448 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

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

475 Even though 50 °C of acid extract indicated a high amount of free anthocyanins after 2
476 h (**Table 1**), there was a significant ($p < 0.05$) low of a^* (redness) and chroma (colour intensity)
477 values indicating extraction at high temperature resulted in a decrease in redness and colour
478 intensity of extracts (**Table 5**). On the other hand, a^* values ($p < 0.05$) was significantly high in
479 30 °C water extract which led to high chroma. Meanwhile, extraction using 100% methanol
480 resulted in a significantly ($p < 0.05$) low of L^* and b^* values, representing an increase in
481 darkness and blueness, respectively. The combination of these values led to significantly ($p <$
482 0.05) low hue angle values of 100% methanol extracts compared to other solvents. Moreover,
483 no correlation was observed between anthocyanins content and chroma and redness. These
484 proved that rather than anthocyanins content, colour properties of the extracts were influenced
485 more by the pH values of the extraction solvents, as reported by Wrolstad (2004) and Pedro,
486 Granato, & Rosso (2016). Furthermore, statistical analysis using Two-way ANOVA shows the
487 significant interaction ($p < 0.05$) between solvent and extraction temperature on the L^* , a^* , b^* ,
488 Total Colour Difference and chroma values.

489

490 **4. CONCLUSIONS**

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

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

508

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514

515 **CONFLICTS OF INTEREST**

516 The authors declared no conflicts of interest.

517

518 **AUTHOR CONTRIBUTIONS**

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

521

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