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

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1 **Acetic acid buffer as extraction medium for free and bound phenolics from dried**
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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

2

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.

3

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

4

(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

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

[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:

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

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; 1 = 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:

7

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

8

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

221

222 A_o = Absorbance of the control; Ae = 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 $$Total\ colour\ difference\ (\Delta E) = [(L^* - L_o)^2 + (a^* - a_o)^2 + (b^* - b_o)^2]^{1/2} \qquad Eq.4$$

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

243

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

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

14

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 hydoxycinnamic 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 ℃ (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.

15

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     $\pm$ 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.

17

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

18

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

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.

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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