

1 **Title: Plasma uptake of selected phenolic acids following New Zealand blackcurrant extract**  
2 **supplementation in humans**

3  
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33 **Abstract**

34 New Zealand blackcurrant (NZBC) extract is a rich source of anthocyanins and in order to exert physiological  
35 effects, the anthocyanin-derived metabolites need to be bioavailable *in vivo*. We examined the plasma uptake  
36 of selected phenolic acids following NZBC extract supplementation alongside maintaining a habitual diet (i.e.,  
37 not restricting habitual polyphenol intake). Twenty healthy volunteers (9 females, age: 28±7 years, height  
38 1.73±0.09 m, body mass 73±11 kg) consumed a 300 mg NZBC extract capsule (CurraNZ™; anthocyanin  
39 content 105 mg) following an overnight fast. Venous blood samples were taken pre and 1, 1.5, 2, 3, 4, 5, and  
40 6 h post-ingestion of the capsule. Plasma concentrations of vanillic acid (VA), gallic acid (GA), and  
41 protocatechuic acid (PCA) were analysed by reversed-phase high-performance liquid chromatography (HPLC)  
42 and the HPLC analysis identified two dihydroxybenzoic (VA and PCA) and one trihydroxybenzoic acids (GA)  
43 in plasma following NZBC extract supplementation. Habitual anthocyanin intake was 168 (95%CI:68–404)  
44 mg·day<sup>-1</sup> and no associations were observed between this and VA, PCA, and GA plasma uptake by the NZBC  
45 extract intake. Plasma time-concentration curves revealed that GA, and PCA were most abundant at 4, and  
46 1.5 h post-ingestion, representing a 261% and 320% increase above baseline, respectively, with VA remaining  
47 unchanged. This is the first study to demonstrate that an NZBC extract supplement increases the plasma  
48 uptake of phenolic acids GA, and PCA even when a habitual diet is followed in the days preceding the  
49 experimental trial, although inter-individual variability is apparent.

50

51 **Keywords.** Anthocyanin; Acute-Supplementation; Phenolic Acids; Systemic Appearance; Habitual Diet

52

53

54 **Introduction**

55 Within the last decade, consuming functional foods such as whole berry fruits or concentrated berry extracts  
56 has grown in popularity likely due to their reported sport and exercise performance benefits (Bell, McHugh,  
57 Stevenson & Howatson, 2014; Cook & Willems, 2018) attributed to the berry fruits rich polyphenol content  
58 (Bowtell & Kelly, 2019). Flavonoids are one of the most abundant naturally occurring polyphenols, which have  
59 been the focus of much research interest and can be divided into six subclasses: anthocyanins, flavonols,  
60 flavones, flavanones, flavanols and isoflavones (Martin & Appel, 2010).

61  
62 Blackcurrant is a rich source of anthocyanins; however, the anthocyanin content can vary depending on the  
63 berry fruits specific cultivar, cultivation site, temperature exposure, processing, storage and ripeness (Mikulic-  
64 Petkovsek, Koron, Zorenc & Veberic, 2017; Del Rio, Borges & Crozier, 2010; Chalker-Scott, 2008). The distinct  
65 purple colour of blackcurrants is due to the high levels of anthocyanins, which are glycosides generated from  
66 anthocyanidins and are the pigments often responsible also for the orange, red, and blue colours in fruits,  
67 vegetables, flowers and other storage tissues in plants (Blando, Gerardi & Nicoletti, 2004). Native cultivars of  
68 blackcurrants grown in New Zealand reportedly have a higher anthocyanin content than European cultivars  
69 grown in North America (Schrage et al., 2010; Moyer, Hummer, Finn, Frei & Wrolstad, 2002). For example,  
70 juice from the New Zealand cultivar, Ben Rua, containing 477 mg·100 mL<sup>-1</sup> anthocyanin compared to juice  
71 from the non-New Zealand cultivar, Ojebyn, contained 179 mg·100 mL<sup>-1</sup> anthocyanin (Schrage et al., 2010;  
72 Moyer, Hummer, Finn, Frei & Wrolstad, 2002).

73  
74 For anthocyanins to be able to exert any benefit *in vivo*, they would need to be bioavailable in sufficient  
75 quantities to produce systemic effects (Koli et al., 2010; Toutain & Bousquet-Melou, 2004). Of those  
76 anthocyanin pharmacokinetics data available, it has been suggested that the phenolic acids, such as  
77 protocatechuic acid (PCA), often appear in systemic circulation in much higher concentrations than that of their  
78 parent compounds and that they could be responsible for the associated exercise performance benefits of  
79 anthocyanins (Bowtell & Kelly, 2019; Fang, 2014). These phenolic acids provide unique taste, flavour, and  
80 health-promoting properties and are found in many fruits and vegetables (Tomas-Barberan & Espin, 2001).  
81 Previously, Sliemstad and Solheim (2002) identified ~15 different anthocyanins in Nordic-grown blackcurrant  
82 and observed that ~98% of the total anthocyanin content are made up of, delphinidin-3-O-glucoside,  
83 delphinidin-3-O-rutinoside, cyanidin-3-O-glucoside, and cyanidin-3-O-rutinoside. The phenolic acids, PCA and  
84 gallic acid (GA) are the most abundant degradation products of cyanidin and delphinidin, respectively, the two  
85 major parent anthocyanins detected in NZBC whole berry (Sliemstad & Solheim, 2002) and concentrate

86 (Matsumoto et al., 2001). Whereas VA is a reported breakdown phenolic acid of peonidin-3-O-rutinoside, which  
87 has been identified as a minor compound in blackcurrant (Matsumoto et al., 2001).  
88  
89 The high anthocyanin content within New Zealand blackcurrant (NZBC) has led to an increase in research  
90 interest in this particular cultivar. Lyall et al., (2009) first reported acute effects by intake of NZBC extract  
91 capsules (240 mg anthocyanins in total) immediately before and following 30 minutes of rowing on an  
92 ergometer in 10 healthy males and females (age: 48±2.5 years). The authors concluded that the anthocyanins  
93 in NZBC were able to mitigate exercise-induced oxidative stress and dampen the plasma creatine kinase (CK)  
94 response, a marker of micro and macro muscle damage, compared to the placebo group 24 h postexercise.  
95 Subsequent NZBC extract supplementation studies have utilised a repeated dosing strategy, whereby seven-  
96 days before the experimental visit, participants supplemented with NZBC each day, and on day seven,  
97 performed the set exercise task/assessment. Supplementation with NZBC extract has been shown to have a  
98 range of potential sport and exercise performance benefits, such as increased fat oxidation (Cook, Myers,  
99 Blacker & Willems, 2015; Cook, Myers, Gault, Edwards & Willems, 2017a; Şahin, Bilgiç, Montanari & Willems,  
100 2020; Strauss, Willems & Shepherd, 2018), improved cardiovascular response at rest and during exercise  
101 (Willems, Myers, Gault & Cook, 2015; Cook, Myers, Gault, Edwards & Willems, 2017b) and enhanced exercise  
102 performance (Cook, Myers, Blacker & Willems, 2015; Perkins, Vine, Blacker & Willems, 2015; Murphy et al.,  
103 2017; Godwin et al., 2017). These studies have used either commercially available NZBC extract in the form  
104 of capsules (CurraNZ™, Health Currancy Ltd, Surrey, UK) or NZBC powder dissolved in water (Sujon New  
105 Zealand blackcurrant, Gibb Holdings Ltd, New Zealand) at total anthocyanin doses of 105 mg·day<sup>-1</sup> (Perkins  
106 et al., 2015; Cook et al., 2015; Cook et al., 2017a; Cook et al., 2017b), 138.6 mg·day<sup>-1</sup> (Willems et al., 2015;  
107 Willems et al., 2017), 210 mg·day<sup>-1</sup> (Cook et al., 2017a; Cook et al., 2017b; Willems et al., 2016; Murphy et al.,  
108 2017; Godwin et al., 2017; Strauss, Willems & Shepherd, 2018; Şahin et al. 2020) and 315 mg·day<sup>-1</sup> (Cook et  
109 al., 2017a; Cook et al., 2017b). These doses are higher than the reported estimations of habitual anthocyanin  
110 dietary intake, which range from 19 to 65 mg·day<sup>-1</sup> in European countries (Zamora-Ros et al., 2011).  
111 Interestingly, Cook et al., (2017ab) explored the dose response effects of 7-days intake of NZBC extract  
112 supplementation during exercise and at rest, respectively. Cook et al. (2017b) observed that a dose response  
113 effect was apparent for cardiac output and stroke volume between control and 600 and 900 mg·day<sup>-1</sup>,  
114 respectively, in endurance-trained male cyclists during supine rest. In addition, total peripheral resistance  
115 decreased by 4±3, 5±9 and 3±4 mmHg·L<sup>-1</sup> between control and 600 mg·day<sup>-1</sup>, control and 900 mg·day<sup>-1</sup> and  
116 300 and 900 mg·day<sup>-1</sup>, respectively. Whereas Cook et al., (2017a) reported dose response effects for both fat  
117 and carbohydrate oxidation during 120-minute cycling bouts at 65%  $\dot{V}O_{2max}$  in endurance-trained male cyclists.

118 The dose response effect for mean fat oxidation revealed values of  $0.63\pm 0.21$ ,  $0.70\pm 0.17$ ,  $0.73\pm 0.19$ , and  
119  $0.73\pm 0.14$  g·min<sup>-1</sup> for control, 300, 600 and 900 mg·day<sup>-1</sup>, respectively, while dose dependent values in mean  
120 carbohydrate oxidation were  $1.78\pm 0.51$ ,  $1.65\pm 0.48$ ,  $1.57\pm 0.44$ , and  $1.56\pm 0.50$  g·min<sup>-1</sup> for control, 300, 600 and  
121 900 mg·day<sup>-1</sup>, respectively (Cook et al., 2017a). The authors attributed the dose response benefits to a possible  
122 build-up of anthocyanin-derived metabolites over the 7-day intake, where previously beneficial vascular  
123 responses following anthocyanin intake have been associated with a peak in phenolic metabolites such as  
124 vanillic acid (VA) and attributed to the phenolic metabolite's actions on neutrophil nicotinamide adenine  
125 dinucleotide phosphate oxidase activity (Rodriguez-Mateos et al., 2013). However, it is important to note that  
126 Rodriguez-Mateos et al., (2013) examined the effects of blueberry anthocyanins, which have a different  
127 anthocyanin profile to NZBC, and thus may present different plasma metabolite profiles. Furthermore, it is  
128 possible that the beneficial effects observed were the result of acute responses to the NZBC extract  
129 supplementation, where the last doses were ingested 2 h prior to the commencement of the study  
130 measurements (e.g., Şahin et al. 2020).

131

132 To date, none of the aforementioned NZBC extract supplementation studies have provided data on the plasma  
133 uptake of the phenolic acids of anthocyanins. Understanding the plasma uptake of both acute and repeated  
134 intakes of NZBC may help inform optimal dosing strategies and intake guidelines and move dosing protocols  
135 towards a more rigorous pharmacokinetic approach. However, most plasma uptake studies incorporate dietary  
136 polyphenol restriction in their design in an attempt to reduce the background noise that may be introduced by  
137 variation in dietary polyphenol intake (Matsumoto et al., 2001; Keane et al., 2016). This dietary restriction  
138 approach may also maximise the effects produced by polyphenol supplementation (Bowtell & Kelly, 2019).  
139 Therefore, it has been suggested that to ensure ecological validity, not restricting polyphenol intake in the lead-  
140 up to an experimental trial, would be the most appropriate approach to assessing the plasma uptake of  
141 phenolic acids by intake of anthocyanin-rich berries and their potential ability to exert a physiological effect.

142

143 Despite a number of studies indicating beneficial effects of NZBC extract supplementation, the plasma uptake  
144 of phenolic acids, VA, GA and PCA following NZBC extract ingestion when supplemented whilst consuming a  
145 habitual diet has yet to be investigated. Therefore, this investigation aims to examine the time course of VA,  
146 GA, and PCA following acute ingestion of a single dose of NZBC extract in individuals following a habitual diet.  
147 It was hypothesised that NZBC extract supplementation would increase plasma concentrations of VA, GA, and  
148 PCA in the hours following acute ingestion.

149

150 **Materials and methods**

151 Eleven healthy men and nine women (**Table 1**) volunteered to participate in the study. All participants were  
152 non-smokers, in apparent good health, with no known food allergies, and not currently using any nutritional  
153 supplementation. The study was approved by the University's Research Ethics Committee (Protocol Number  
154 1718\_02) and conducted in accordance with the Declaration of Helsinki (2013). Participants gave their written  
155 informed consent after an explanation of the experimental procedures.

156 **Study design**

157 Participants completed a self-report food diary in the three days leading up to the laboratory visit and refrained  
158 from strenuous exercise in the 48 h before their visit. In addition, participants completed a food frequency  
159 questionnaire to quantify habitual anthocyanin intake reflective of the past three months (Cook et al., 2017).  
160 On arrival to the laboratory after an overnight fast ( $\geq 12$  h), the participant's height and body mass were  
161 recorded, and a cannula (BD Venflon™ intravenous cannula with port, 18 G, Becton Dickinson, Wokingham,  
162 UK) was inserted into an antecubital vein. Resting venous blood samples were drawn pre and 1, 1.5, 2, 3, 4,  
163 5, and 6 h after ingestion of the NZBC extract capsule with water. Participants remained seated and rested for  
164 the duration of the blood sample time points to control for compartmental fluid shifts associated with changes  
165 in body posture (Hagan, Diaz & Horvath, 1978). Participants were permitted to only consume water *ab libitum*  
166 during their laboratory visit.

167

168 **Supplementation, dietary intake and habitual anthocyanin intake**

169 The NZBC extract capsules (CurraNZ™, Health Currancy Ltd, Surrey, UK) were stored at room temperature  
170 (~20 °C) in an opaque container before use to avoid UV light degradation (Fossen, Luis & Andersen, 1998).  
171 Participants consumed one NZBC extract capsule (300 mg containing 105 mg of anthocyanins, i.e., 35-50%  
172 delphinidin-3-O-rutinoside, 5-20% delphinidin-3-O-glucoside, 30-45% cyanidin-3-O-rutinoside, 3-10%  
173 cyanidin-3-O-glucoside) after the resting blood draw. Participants were instructed to maintain their normal diet  
174 before their trial visit to maintain study ecological validity (Bowtell & Kelly, 2019). Participants recorded their  
175 three-day dietary intake in food diaries which were analysed (Nutritics LTD, Dublin, Ireland) for carbohydrate,  
176 fat and protein, and total energy intake (kJ). The food frequency questionnaire detailed the amount and  
177 frequency of anthocyanin-containing foods eaten within the last three months from the Phenol Explorer  
178 database (Neveu et al., 2010). The intake of anthocyanin was then calculated as the sum of the consumption

179 frequency of each anthocyanin containing food, multiplied by the content of the anthocyanin content for the  
180 portion sizes.

181

### 182 ***Blood sampling***

183 Fasting whole blood samples were collected into two chilled 5 mL K3 EDTA tubes (Sarstedt, AG and Co,  
184 Kommanditgesellschaft, Germany), inverted to mix the anticoagulant and immediately centrifuged at 4100 rpm  
185 for 10 min at 4°C. Plasma was aspirated and pipetted into ~1 mL aliquots and then immediately stored at -  
186 80°C for subsequent analysis.

187

### 188 ***Plasma extraction***

189 A method previously described (Keane et al., 2016) was used for the extraction of phenolic compounds from  
190 the plasma. Briefly, 1 mL of plasma and 0.5 mL of propyl gallate (internal standard, 50 µg, 100 µL/mL) was  
191 mixed with 4 mL oxalic acid (10 mM) and 0.1 mL hydrochloric acid (HCl; 12.6 M) in 15 mL falcon tubes and  
192 centrifuged at 3000 rpm for 15 minutes. The supernatant was absorbed on to a primed (washed with 5 mL  
193 methanol (MeOH) with 0.2% trifluoroacetic acid (TFA) followed by 2×5 mL of water) solid-phase extraction  
194 cartridge (Waters Sep-Pak c17, 360 mg sorbent per cartridge, 55-105 µm). The sample was eluted with 3 mL  
195 of MeOH + 0.2% TFA and dried under N<sub>2</sub> at 45°C. Samples were then reconstituted in 400 µl of 0.1% formic  
196 acid in water: 2% HCl in MeOH (1:1) and filtered through a 0.2 µm polytetrafluoroethylene filter before HPLC  
197 analysis. Samples were analysed on a batch basis, where each batch included standards prepared in 0.1%  
198 formic acid in water: 2% HCl in MeOH (1:1), blank control, plasma samples, and fortified plasma samples at 1  
199 (low), 10 (medium), and 25 (high) µg/mL. The recovery ranges were 89-95%, 89-102%, and 90-103% for low,  
200 medium, and high fortified levels, respectively. The final results were collected for recovery at the low  
201 fortification level.

202

### 203 ***High-Performance Liquid Chromatography (HPLC) analysis***

204 A high-pressure liquid chromatography-diode array detector (DAD) method for the detection and quantitation  
205 of selected phenolic compounds in the plasma samples (pre-supplementation through to 6 h post-  
206 supplementation) was carried out using a Dionex UltiMate 3000 HPLC System (Dionex, Camberley, UK)  
207 equipped with an UltiMate 3000 RS pump, an UltiMate 3000 autosampler, and a 3000 RS UV/Vis Detector  
208 and a RS Fluorescence Detector (FLD). The filtered samples (20 µL) were injected on a Phenomenex Luna  
209 C<sub>18</sub>(2) (250×2.0 mm, 5 µm particle size) reverse-phase column thermostat-controlled at 30°C. The mobile  
210 phase consisted of water with 1% acetic acid (solvent A), and acetonitrile with 1% acetic acid (solvent B). After

211 a 5-minute equilibration with 20% A, the elution programme was as follows: 0-15 min, 20-100% B, (0.2 mL/min)  
212 followed by a washing stage (100% B, 15-18 min, 1.0 mL/min) and return at the initial conditions within 2  
213 minutes. Detection was performed at the following excitation/emission wavelengths:  $\lambda_{ex}$  =278 nm and  $\lambda_{em}$  =  
214 360 nm for PCA and propyl gallate (PG),  $\lambda_{ex}$  = 278 nm and  $\lambda_{em}$  = 366 for GA and  $\lambda_{ex}$  = 260 nm and  $\lambda_{em}$  =  
215 422 nm for VA, respectively. The identification and quantitation of PCA, GA, and VA content of plasma samples  
216 was based on a combination of retention time and spectral matching of reference standards. Final results are  
217 expressed as micrograms per millilitre ( $\mu\text{g/mL}$ ).

218

### 219 **Statistical analysis**

220 Statistical analyses were completed using Statistical Package for Social Sciences 23.0 (SPSS, Chicago,  
221 Illinois). All dependent variables were analysed using a treatment (300 mg NZBC dose) by time (0, 1, 1.5, 2,  
222 3, 4, 5, and 6 h) one-way, repeated-measures analysis of variance (ANOVA). Mauchly's test of sphericity was  
223 used to check homogeneity of variance for all variables; where necessary, any violations of the assumption  
224 were corrected using the Greenhouse-Geisser adjustment. Main effects for time were followed up using  
225 Bonferroni *post hoc* analysis. Further analysis was conducted to identify maximum group and mean of each  
226 individual plasma concentrations ( $C_{max}$ ) and times to achieve maximum plasma concentrations ( $t_{max}$ ), which  
227 were directly obtained from the plasma concentration-time profiles (Toutain & Bousquet-Melou, 2004). As a  
228 measure of overall plasma uptake of individual phenolic acids, the area under the plasma concentration-time  
229 curve ( $\text{AUC}_{0-6h}$ ) for each participant was estimated by using the linear trapezoidal rule, with the total sum and  
230 mean of each individual being reported. Pearson (r) correlation coefficients were calculated for the relationship  
231 between habitual anthocyanin intake and total sum  $\text{AUC}_{0-6h}$  for each metabolite (VA, GA, and PCA) and  
232 between each metabolite total sum  $\text{AUC}_{0-6h}$  (VA, GA, and PCA). Pearson (r) correlation coefficients of <0.3,  
233 <0.5 and  $\geq 0.5$  are interpreted as weak, moderate and strong correlations, respectively (Cohen, 1988). The  
234 alpha level for statistical significance was set at 0.05 *a priori* and Partial-eta<sup>2</sup> ( $\eta^2$ ) effect sizes (ANOVA) are  
235 reported to indicate the magnitude of observed effects (Lakens, 2013). Partial-eta<sup>2</sup> ( $\eta^2$ ) effect sizes of 0.01–  
236 0.06, 0.06–0.14 or  $\geq 0.14$  are considered small, medium and large changes, respectively (Lakens, 2013). Data  
237 in text and tables are reported as mean (95% confidence intervals) and data in figures as mean with individual  
238 data points (Weissgerber, Milic, Winham & Garovic, 2015).

239

### 240 **Results**



241 Vanillic acid (VA) results are based on  $n=17$  and PCA on  $n=18$  participants', due to their values falling below  
 242 the LOD during HPLC analysis. Gallic acid results are based on  $n=20$ . Table 1 reports data for the 72-h food  
 243 diaries and anthocyanin food frequency questionnaire.

Variable	Value
<b>Participant characteristics</b>	
Age (years)	28 (16-41)
Height (m)	1.73 (1.58-1.88)
Body mass (kg)	73 (55-92)
Body mass index ( $\text{kg}\cdot\text{m}^{-2}$ )	24 (20-29)
<b>Nutritional status</b>	
Total energy intake (kJ)	8175 (6034-10317)
Carbohydrate (g)	205 (118-292)
Fat (g)	75 (49-102)
Protein (g)	100 (49-150)
Habitual anthocyanin intake ( $\text{mg}\cdot\text{day}^{-1}$ )	168 (68-404)

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245

246 **Table 1** Mean (95%CI) descriptive data of the participants, absolute energy and macronutrient intake per day  
 247 72 h prior to the second experimental visit and habitual anthocyanin intake  
 248  $n=20$  (11 males).

249

## 250 Plasma time-concentration curves

### 251 Vanillic acid (VA)

252 There was no main effect of time on plasma VA plasma concentration ( $F=2.218$ ,  $p=0.109$ ,  $\eta^2=0.12$ ,  $\eta^2$   
 253 95%CI: 0–0.28; **Figure 1a**). Table 2 shows the  $t_{\text{max}}$ ,  $C_{\text{max}}$ , total  $\text{AUC}_{0-6\text{h}}$  and the mean of each individual  $\text{AUC}_{0-}$   
 254  $6\text{h}$  for VA.

255

### 256 Gallic acid (GA)

257 There was a main effect of time on plasma GA concentration ( $F=25.273$ ,  $p=0.001$ ,  $\eta^2=0.57$ ,  $\eta^2$  95%CI: 0.40–  
 258 0.66). Pairwise comparisons revealed that GA concentrations were increased at 1, 1.5, 2, 3, 4, 5 and 6 h

259 following supplementation when compared to 0 h ( $p<0.0001$ ; **Figure 1b**). Furthermore, plasma GA  
260 concentrations were elevated at 3, 4, 5 and 6 h compared to 1 h following supplementation ( $p<0.05$ ). GA levels  
261 in plasma were higher 4 and 5 h compared to 1.5 h following supplementation ( $p=0.004$  and  $p=0.011$ ,  
262 respectively). Table 2 shows the  $t_{max}$ ,  $C_{max}$ , total  $AUC_{0-6h}$  and the mean of each individual  $AUC_{0-6h}$  for GA.

263

#### 264 **Protocatechuic acid (PCA)**

265 Following supplementation, there was a main effect of time on PCA plasma levels ( $F=10.638$ ,  $p=0.001$ ,  
266  $\eta p^2=0.36$ ,  $\eta p^2$  95%CI: 0.11–0.52). Pairwise comparisons revealed that PCA concentrations were elevated at  
267 1, 1.5, 2, 3, 4, 5 and 6 h following supplementation when compared to 0 h ( $p<0.05$  **Figure 1c**). Table 2 shows  
268 the  $t_{max}$ ,  $C_{max}$ , total  $AUC_{0-6h}$  and the mean of each individual  $AUC_{0-6h}$  for PCA.

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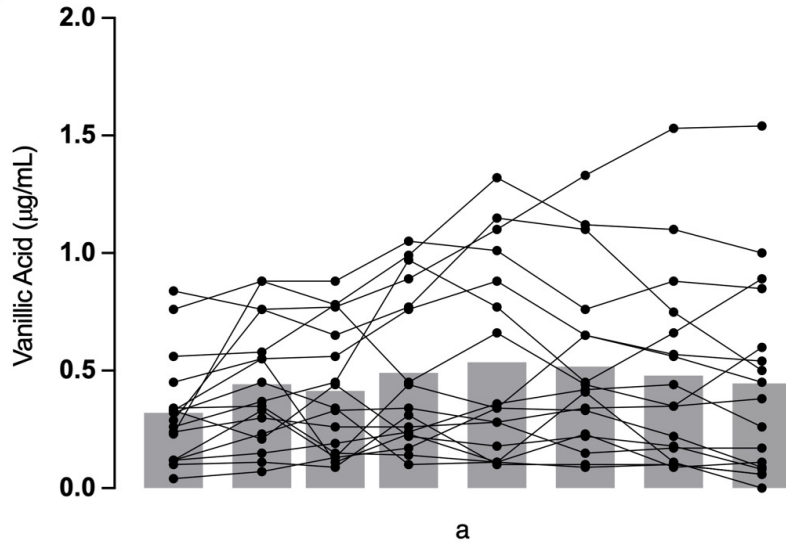
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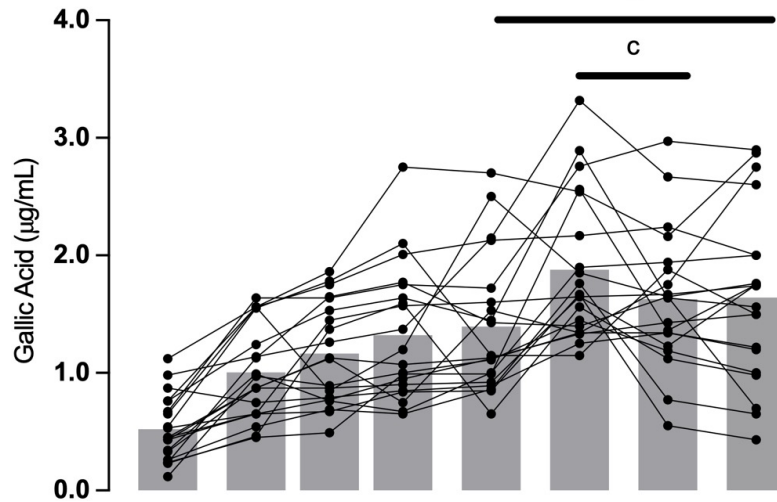
284 **Figure 1** (below) Vanillic acid (**a**,  $n=17$ ), gallic acid (**b**,  $n=20$ ) and protocatechuic acid (**c**,  $n=18$ ) responses  
285 from baseline to supplementation with 300 mg New Zealand Blackcurrant extract capsule. Absolute baseline  
286 values were 0.27 (95%CI: 0.13-0.68)  $\mu\text{g/mL}$ , 0.52 (95%CI: 0.12-0.92)  $\mu\text{g/mL}$  and 0.39 (95%CI: 0.40-1.19)  
287  $\mu\text{g/mL}$  for VA, GA and PCA, respectively. <sup>a</sup> indicates different from 0 h at all time points ( $p<0.0001$ ), <sup>b</sup> indicates  
288 different between 1 h and 3, 4, 5 and 6 h ( $p<0.05$ ), <sup>c</sup> indicates different between 1.5 h and 4 and 5 h ( $p<0.05$ ),  
289 <sup>d</sup> indicates different from 0 h at all time points ( $p<0.05$ ).

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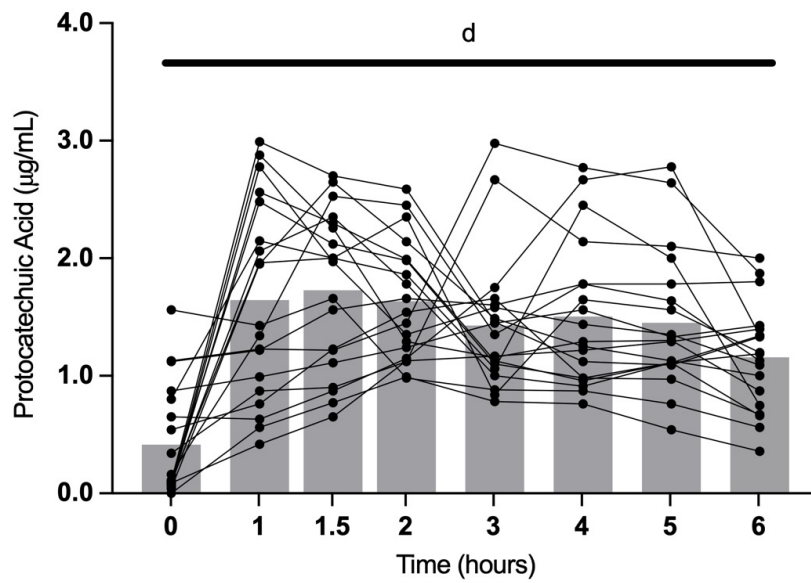
**1a**



**1b**



**1c**



**Table 2.** Mean (95%CI) vanillic acid, gallic acid and protocatechuic acid group and individual responses following intake of 300 mg of New Zealand blackcurrant extract.

Phenolic acid	Group $T_{max}$ (Time point following supplementation)	Individual mean $T_{max}$ (Time point following supplementation)	Group $C_{max}$ ( $\mu\text{g g/mL}^{-1}$ )	Individual mean $C_{max}$ ( $\mu\text{g g/mL}^{-1}$ )	Total $AUC_{0-6h}$ ( $\mu\text{g h/mL}^{-1}$ )	Individual mean $AUC_{0-6h}$ ( $\mu\text{g h/mL}^{-1}$ )
Vanillic acid	3 h	3 h (95%CI: 0-5 h)	0.49 (95%CI: 0.21-1.19)	0.71 (95%CI: 0.08-1.35)	48.0 (95%CI: 44.8–51.2)	2.8 (95%CI: 0.4–6.0)
Gallic acid	4 h	4 h (95%CI: 2-7 h)	1.88 (95%CI: 1.24-2.52)	2.12 (95%CI: 1.15-3.08)	166.2 (95%CI: 161.9-170.4)	8.3 (95%CI: 4.0-12.6)
Protocatechuic acid	1.5 h	3 h (95%CI: 0-5 h)	1.66 (95%CI: 0.43-2.89)	2.22 (95%CI: 1.13-3.30)	161.3 (95%CI: 158.0-164.6)	8.5 (95%CI: 5.2-11.8)

$t_{max}$ , time to maximum concentration;  $C_{max}$ , concentration maximum;  $AUC_{0-6h}$ , area under the curve 0 – 6 h.

**Table 3** Pearson's correlation coefficient matrix of habitual anthocyanin intake, total AUC<sub>0-6h</sub> vanillic acid, gallic acid and protocatechuic acid following intake of 300 mg of New Zealand blackcurrant extract.

	<b>Habitual anthocyanin intake</b>	<b>Vanillic Acid</b>	<b>Gallic Acid</b>	<b>Protocatechuic Acid</b>
<b>Habitual anthocyanin intake</b>		-0.107 0.681	-0.059 0.811	0.315 0.189
<b>Vanillic Acid</b>	-0.107 0.681		-0.396 0.129	0.599* 0.014
<b>Gallic Acid</b>	-0.059 0.811	-0.396 0.129		-0.240 0.322
<b>Protocatechuic Acid</b>	0.315 0.189	0.599* 0.014	-0.240 0.322	

Asterisk indicates statistically significant relationships between total AUC<sub>0-6h</sub> between each metabolite.

## 322 **Relationships between phenolic compounds and habitual anthocyanin intake**

323 There were no significant correlations between estimated habitual anthocyanin intake and the total AUC<sub>0-6h</sub> for  
324 VA, or PCA (**Table 3**). Table 3 shows that a moderate positive significant correlation was observed between  
325 VA and PCA. However, no significant correlations were observed for GA and PCA and GA and VA.

326

## 327 **Discussion**

328 This is the first study to demonstrate that a NZBC extract supplement increases the plasma uptake of phenolic  
329 acids GA, and PCA even when a habitual diet is followed in the days preceding the experimental trial (i.e., no  
330 restriction of polyphenol intake). The novel approach to this study was to investigate the appearance and 6 h  
331 time course of these phenolic acids in plasma following acute consumption of a NZBC extract supplement.

332

333 There is a limited understanding about the metabolism and absorption of VA, GA, and PCA following NZBC  
334 intake where only one pilot study has explored the plasma phenolic acid appearance following blackcurrant  
335 extract ingestion with participants on an anthocyanin-free diet 72 hrs before (Roehrig, Kirsch, Schipp, Galan  
336 & Richling, 2019). The present study demonstrated that PCA and GA are most bioavailable in plasma 1.5 and  
337 4 h post-NZBC extract ingestion, respectively (**Table 2**). An independent HPLC analysis on a batch of the  
338 NZBC extract capsules confirmed no presence of peonidin-3-O-rutinoside. However, in our study, the VA (a  
339 downstream metabolite of peonidin-3-O-rutinoside) was detectable in the plasma in 17 of the 20 participants,  
340 with transient increases from baseline observed in four out of 20 participants (**Figure 1a**), despite no group  
341 main effects of time being apparent. The transient increases in VA response were not uniformly observed  
342 within the cohort. As such we cannot determine if any change in this metabolite is a result of NZBC extract  
343 supplementation *per se* or an interaction between a dietary feature shared between these four participants,  
344 but absent from the others, or simply a noise/measurement artifact. Peonidin-3-O-rutinoside has been shown  
345 to be a minor anthocyanin compound in blackcurrant (Frøylog, Slimestad, & Andersen, 1998). The phenolic  
346 acid PCA, however, can be extensively metabolized to numerous metabolites such as VA, hippuric acid, ferulic  
347 acid, and 4-hydroxybenzaldehyde (Gao et al., 2006; de Ferrars et al., 2014). Therefore, it is possible that  
348 presence of VA in plasma over the 6 h was due to enterohepatic metabolism of PCA to VA. Thus, possibly  
349 being the reason as to why a positive relationship was observed in this present study between VA and PCA.  
350 However, it must be acknowledged that, despite participants completing an overnight fast before consuming  
351 the NZBC supplement, habitual dietary intake leading up to the experimental visit, could also be a contributing  
352 factor to the appearance of VA in plasma. High concentrations of VA *in vivo* have been linked to the abundance  
353 of anthocyanins in fruits and vegetables (Nurmi et al., 2009). Vanillic acid, GA, and PCA were present at

354 baseline in plasma for all participants before NZBC extract consumption, which is in accordance with previous  
355 research, where anthocyanin metabolites were still observable 48 h post- cyanidin 3-O-glucoside bolus  
356 ingestion (Czank et al., 2013). Thus, it is plausible that foods rich in anthocyanins consumed by the participants  
357 before the 12 h overnight fast, resulted in bioaccumulation of phenolic acids, which were then detectable in  
358 plasma on the morning of the laboratory visit. Despite the equivocal results for this metabolite, future research  
359 would benefit from retaining VA within the panel of metabolites measured to shed further light on the  
360 interindividual variability.

361

362 Slimestad and Solheim (2002) previously highlighted that the main pigments from whole blackcurrant berries  
363 are delphinidin and cyanidin and that these structures constitute ~97% of the extractable anthocyanin content  
364 from these blackcurrant berries. Post-NZBC extract ingestion, GA increased in plasma concentration across  
365 the 6 h time frame, achieving group  $t_{max}$  by 4 h, presented the greatest plasma uptake and had not returned to  
366 baseline at 6 h post-ingestion (**Figure 1b**). This finding is in contrast to previous work, which reported GA  $t_{max}$   
367 occurred at 1 h post-ingestion of a blackcurrant extract (Roehrig et al., 2019), however, this result was based  
368 on one participant. Gallic acid is a trihydroxybenzoic acid and the major stable phenolic acid derived from ring  
369 fission of the delphinidin skeleton, which is the most abundant anthocyanin in NZBC. A study that investigated  
370 the enzymatic potential for *Bifidobacteria* and *Lactobacillus*, two predominant members of the intestinal  
371 microflora, to convert delphinidin and malvidin glycosides into their phenolic acid degradation compounds,  
372 observed that the *Lactobacillus* strain, *L. casei*, resulted in the highest concentrations of GA after 24 h of  
373 incubation (Ávila et al., 2009). Furthermore, the authors highlighted that as delphinidin 3-O-glucoside  
374 underwent chemical degradation to form mainly GA, and concentrations of this phenolic acid were detected at  
375 1 h in samples, which increased throughout the time period concomitantly with delphinidin 3-O-glucoside  
376 clearance. Therefore, it is feasible that the appearance of GA in plasma over the 6 h in the present study was  
377 possibly a result of the gut microbiome profiles of the participants. Future research should consider studying  
378 faecal microflora samples before and following an anthocyanin-rich berry fruit supplementation to assess this  
379 relationship further (Tomas-Barberan, Selma & Espin, 2018).

380

381 Protocatechuic acid is the main degradation compound of cyanidin (Vitaglione et al., 2007). Previous research  
382 has reported observing a biphasic response in the serum of PCA metabolite kinetics, displaying an initial peak  
383 between 0 and 5 h and a second peak between 6 and 48 h following an isotopically labelled bolus of cyanidin  
384 3-O-glucoside (de Ferrars et al., 2014). A similar response was apparent for several participants in the present  
385 study, where an initial peak occurred around 1 h, followed by a second peak at 3 h (**Figure 1c**). The reason

386 for these biphasic profiles has been suggested to be a result of metabolism occurring in multiple tissues, such  
387 as the liver, and at different sites within the gastrointestinal tract (de Ferrars et al., 2014). Following blood  
388 orange consumption Vitaglione et al., (2007) observed a recovery of cyanidin 3-O-glucoside and PCA in the  
389 24 h faecal samples, which they suggested indicated *in vivo* production of PCA by intestinal microflora, and  
390 that this slow and continuous release of antioxidant compounds into systemic circulation may have  
391 physiological relevance to maintain the concentration of blood antioxidants over 24 h. With regards to tissue  
392 accumulation of anthocyanin, Kirakosyan et al., (2015), studied the effect of 3-weeks of anthocyanin-rich  
393 cherry supplementation in rats on tissue bioavailability and found that some tissues preferentially stored  
394 phenolic acids. However, when humans supplemented over a 12-week period with anthocyanin-rich elderberry  
395 extract, no differences were observed in the concentration of metabolites post-prandial in urine before and  
396 following the 12-weeks of repeated anthocyanin dosing (de Ferrars, Cassidy, Curtis & Kay, 2013). Therefore,  
397 further research is warranted on whether anthocyanin metabolites can be preferentially stored in tissue or  
398 whether bioaccumulation is limited to a short window of opportunity.

399

400 The individual participant uptake in plasma of VA, GA and PCA (**Figure 1a, b, c**) observed in the present  
401 study, supports previous observations by Keane et al. (2016) where large inter-individual variability was  
402 apparent following acute intake of 30 and 60 mL of Montmorency tart cherry concentrate. In an attempt to  
403 quantify the variation in individual participant plasma uptake in the present study, the mean of each individual  
404  $AUC_{0-6h}$  plasma time-concentration curves (linear trapezoidal model),  $t_{max}$  and  $C_{max}$  were estimated. Comparing  
405 the mean of each individual  $AUC_{0-6h}$ ,  $t_{max}$  and  $C_{max}$  responses to the mean total  $AUC_{0-6h}$ ,  $t_{max}$  and  $C_{max}$   
406 responses, it is apparent that some individuals present greater plasma uptake for each metabolite than others.  
407 This could explain why in some NZBC extract supplementation studies, the magnitude of the response (i.e. fat  
408 oxidation) to the intervention varies between participants (Cook et al., 2015; Cook et al., 2017; Strauss et al.,  
409 2018). Large inter-individual variations in the plasma concentrations of phenolic compounds following an 8-  
410 week mixed berry diet intervention have been observed previously (Koli et al., 2010), with these variations  
411 being attributed to differences within participant's intestinal microflora, which can be affected by dietary intake  
412 and genetic differences. However, no relationships were observed in the present study between estimated  
413 habitual anthocyanin intake and overall VA, GA, and PCA plasma uptake (quantified by total  $AUC_{0-6h}$ ).

414

415 Given the current focus on individualised sport and exercise nutrition (Betts & Gonzalez, 2016), it appears that  
416 when considering using an anthocyanin-rich supplement, such as NZBC, practitioners should take into  
417 consideration the large inter-individual response that can occur. For example, if taking the mean response



418 from this current study, it would seem plausible to suggest that most individuals will reach their peak plasma  
419 concentration for PCA at ~1.5 h, however, as can be observed in **Figure 1c and Table 2**, several participants  
420 did not achieve their peak plasma concentration until ~3 h. Future research should consider quantifying the  
421 plasma uptake of anthocyanin metabolites before and following berry fruit extract supplementation, alongside  
422 physiological performance measures to assess the relationship between them as has been suggested  
423 previously (Bowtell & Kelly, 2019).

424

425 The main limitation of the present study is the lack of a control group or condition; thus, we cannot infer whether  
426 any of the changes in the metabolites are due to the NZBC supplementation, or are natural fluctuations  
427 observed as a response of circadian variation over the 6 h. However, the changes in metabolites being due to  
428 natural fluctuations are highly unlikely, given the magnitude of change and that the participants had completed  
429 an overnight fast before the supplement was consumed. Additionally, the use of the 0 h timepoint does provide  
430 a control baseline of plasma VA, GA and PCA prior to the NZBC extract being ingested for comparative  
431 purposes. Furthermore, the analysis in the present study was not exhaustive and so no parent anthocyanins  
432 were analysed nor was every plasma metabolite was analysed; instead, the focus was on the degradation  
433 products of two of the main anthocyanins reported in NZBC extract and VA, the methylated degradation  
434 compound of PCA, that have previously been shown to exert positive effects on vascular function (Keane et  
435 al, 2016). Given that anthocyanins degrade and are extensively metabolised *in vivo*, it is possible that  
436 accumulation of multiple phenolic metabolites may ultimately be responsible for the reported bioactivity of  
437 anthocyanins (Kirakosyan et al., 2015). In addition, the present study utilised an acute dose of NZBC extract  
438 (105 mg anthocyanin) and so it is not possible to forecast the impact of a longer and/or repeated  
439 supplementation period on plasma phenolic uptake. Although previous NZBC research has utilised longer (7-  
440 day) supplementation periods, none have quantified the plasma uptake of anthocyanin phenolic aids such as  
441 VA, GA and PCA. For the first time, this study characterizes the appearance of VA, GA and PCA following an  
442 acute dose of NZBC extract. Future research is warranted to establish whether a repeated dose can provide  
443 additional physiological benefits with regards to plasma uptake or whether a ceiling effect exists in light of the  
444 dose response benefits observed by Cook et al., (2017a) and (2017b). It would also be of interest to examine  
445 the plasma uptake of phenolic acids with repeated intakes of NZBC extract during the day alongside normal  
446 food intake. Lastly, the timeframe of this current investigation lasted until 6 h, with VA, GA and PCA plasma  
447 levels still elevated at this time point. Previous studies focusing on the degradation products of cyanidin have  
448 reported a presence of VA in serum at ~42 h post isotopically labelled cyanidin 3-O-glucoside bolus ingestion  
449 (Czank et al., 2013) and so, it is probable that the phenolic acids in this study could have remained present in

450 plasma until ~42 h. Future research should incorporate a more extensive panel of parent anthocyanins along  
451 with possible gut microbial metabolites, utilise a control group and extend the study timeframe up to 48 h as  
452 has been done in previous pharmacokinetic studies (Czank et al., 2013) in order to be able to draw firm  
453 conclusions.

454

455 Nevertheless, this study presents new information regarding the presence *in vivo* of anthocyanin phenolic  
456 acids, VA, GA, and PCA from acute NZBC extract supplementation when a habitual diet is followed in the days  
457 preceding the experimental trial. The time course of phenolic acid accumulation peaks between 1.5 and 4 h  
458 post-NZBC extract ingestion depending on the phenolic acid of interest; however, large inter-individual  
459 variability is apparent. This information could inform future *in vivo* work that examines the sport and exercise  
460 related benefits with acute NZBC extract supplementation by indicating the times at which peak plasma  
461 concentrations are likely to occur and the overall plasma uptake of VA, GA, and PCA. This could in turn be  
462 used to suggest appropriate timings of supplementation before exercise intervention onset when a habitual  
463 diet is followed.

464

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468

#### 469 **Declaration of interest**

470 Health Currancy (United Kingdom) Ltd and CurraNZ (New Zealand) Ltd provided supplementation. However,  
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472 study and manuscript. The authors report no conflicts of interest.

473

474

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