

1 **Acute supplementation with blackcurrant extracts modulates cognitive functioning and**
2 **inhibits monoamine oxidase-B in healthy young adults.**

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22 **Abbreviations**

23 BCA- Bovine serum albumin
24 DHPG - Dihydroxyphenylglycine
25 EDTA - Ethylenediaminetetraacetic acid
26 H₂O₂ - Hydrogen peroxide
27 HVA- Homovanillic acid
28 LH- Lithium heparin
29 MAO- Monoamine oxidase
30 MRM- Multiple reaction method
31 MSEC – Milliseconds
32 PBS- Phosphate buffered saline solution
33 PEA- Phenylethylamine
34 RVIP – Rapid visual information processing
35 CY-GLU - Cyanidin glucoside
36 DEL-GLU - Delphinidin glucoside
37 CY- RUT - Cyanidin rutinoside
38 DEL- RUT - Delphinidin rutinoside

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41

42 **Abstract**

43 **Background:** Berry fruit have been shown to convey a number of benefits in animal models;
44 including improvements in cognitive performance, slowing of cognitive decline and
45 neuroprotection. These findings, along with epidemiological evidence and data showing
46 modulation of factors related to brain function, suggest a potential role for berry
47 polyphenols in improving cognitive performance.

48 **Objective:** The current study assessed the effects of two blackcurrant extracts on cognitive
49 outcomes, mood, blood glucose profile and peripheral monoamine levels. Anthocyanin
50 bioavailability was also assessed.

51 **Design:** A randomised, double-blind, placebo-controlled, crossover study was conducted in
52 36 healthy young participants (18-35y). Following a 10 minute baseline assessment,
53 participants consumed sugar, flavour and colour matched drinks containing no polyphenols
54 (control) or 525 +/- 5mg of polyphenols per 60kg body weight from either an anthocyanin
55 enriched powdered blackcurrant extract (Delcyan™) or cold pressed blackcurrant juice
56 (cultivar Blackadder) in counterbalanced order on separate days. A 70-minute computerised
57 cognitive assessment (COMPASS) designed to be attentionally demanding and mentally
58 fatiguing was then completed following a 60-minute resting absorption period. Blood
59 platelet monoamine oxidase-B (MAO-B), plasma anthocyanin levels, plasma prolactin and
60 plasma monoamines and associated metabolites were also investigated in a subsection of
61 the cohort at 2.5 hours post-consumption.

62 **Results:** When compared to control, both blackcurrant extracts improved attention task
63 performance. The juiced extract reduced reaction times during the digit vigilance task,
64 whereas the powdered extract increased accuracy during a rapid visual information
65 processing task. Following the juiced Blackadder extract, platelet MAO-B was inhibited by
66 96%, dihydroxyphenylglycol (DHPG) was reduced and normetadrenalin was increased in
67 blood plasma, and a rapid decline in blood glucose levels was significantly attenuated, when
68 compared to control.

69 **Conclusion:** This is the first illustration of a cognitive benefit of acute blackcurrant
70 supplementation in healthy young humans and the first description of a clinically significant
71 inhibition of MAO-B and MAO-A using a commonly consumed fruit. These data also illustrate
72 that compounds other than anthocyanins are important to observe *in vivo* MAO inhibition
73 and that the degree of processing and cultivar of blackcurrant fruit used substantially alters
74 the neuroendocrinological and cognitive benefits conveyed.

75

76 **Introduction**

77 Epidemiological evidence suggests a relationship between flavonoid intake and cognitive
78 decline/dementia [1, 2], with a specific benefit indicated for berries (strawberry and
79 blueberry) that was not observed with other individual foods (Devore et al. 2012). Support
80 for this comes from literature demonstrating a slowing or reversal of natural cognitive
81 decline in berry-fed rats. Several different mechanisms of action have been proposed and
82 investigated in an attempt to explain improvements to memory in animal models, including
83 anti-inflammatory and antioxidant responses and improvements to neural signalling (see
84 Spencer 2010 for review). Of particular relevance to the current study, anthocyanins, their
85 aglycones and phenolic acid metabolites have been shown to have monoamine oxidase
86 (MAO) inhibitory effects *in vitro*. As MAO metabolises monoamines, inhibition of this
87 enzyme could reduce oxidative stress associated with this process and lead to increased
88 levels of these neurotransmitters, essential for normal cognitive function. Indeed MAO-B
89 inhibitors are used in the treatment of neurodegenerative symptoms associated with
90 Parkinson's disease. The use of MAO-A inhibitors for several decades in the treatment of
91 mood disorders also suggests that this inhibition, if demonstrated *in vivo*, could lead to
92 enhanced mood [3, 4].

93 Only three published peer reviewed intervention studies have demonstrated positive
94 effects of berry consumption on human behaviour, impacting verbal memory and spatial
95 memory after supplementation of concord grape juice [5, 6] and blueberry juice [7] in adults
96 with age related memory decline. There is, however, no published evidence pertaining to
97 modulation of cognitive performance in healthy young adults.

98 One naturally rich source of anthocyanins that has been neglected in the literature is
99 blackcurrant (*Ribes nigrum*). Intact glucosides, galactosides and arbinosides of the berry

100 anthocyanins and their associated metabolites have been found at levels ranging from 0.2 to
101 1.5ng/L in the blood and urine of humans after oral ingestion of flavonoid rich berries such
102 as blackcurrants, blueberries and boysenberries [3, 4]. Bioavailability of these compounds is,
103 therefore, low. As well as anthocyanins, blackcurrant also contains an abundance of other
104 phenolic structures in smaller quantities, which are able to exert physiological changes, such
105 as the rate and pattern of glucose uptake from the small intestine [8-10], and improved
106 vascular function [11] and gut microbiota profile [12], which all have the potential to
107 modulate human behaviour.

108 The aim of the present study was to explore the effects of acute supplementation of
109 two blackcurrant extracts, with matched quantities of polyphenols and sugars but differing
110 phenolic profiles, on attention, subjective mood, peripheral monoamines, prolactin and
111 blood glucose. Anthocyanin bioavailability was also assessed at 2.5 hours post-dose in
112 plasma.

113 **Materials and methods**

114 **Participants**

115 Thirty six participants were recruited from Auckland, New Zealand using opportunity
116 sampling and received \$120NZ to recompense them for any expense they may have
117 incurred to participate in the trial. Before participants were enrolled in the study they
118 attended a 90 minute screening session. During this session, participants gave their written
119 informed consent to participate in the study and were screened for any contraindications to
120 the study. In brief, all participants reported themselves to be healthy, not pregnant, non-
121 tobacco users. Participants were not using dietary supplements or prescribed, over the
122 counter or recreational drugs (excluding the contraceptive pill), did not have any
123 sensitivities to any of the study treatments and had a body mass index below 35kg/m².
124 Participants also completed three repetitions of the study day tasks to ensure they met the
125 required minimum standards (internally set) to participate in the study and to minimise
126 practice effects.

127 **Treatments**

128 Participants received three treatment drinks in an order dictated by random allocation to a
129 counterbalancing (Williams Latin Square) schedule with at least one week washout between
130 visits. Extracts were assessed for the phytochemical constituents using the method
131 described by Schrage *et al* [13]. Anthocyanin stability of the Blackadder juice extract at -20
132 degrees was confirmed via HPLC. Over the eight week period no significant loss due to
133 storage was observed. Intervention drinks contained either 0mg of polyphenols (control) or
134 525±5mg of polyphenols per 60kg of bodyweight from an anthocyanin enriched
135 blackcurrant extract, (1.66g of Just the Berries, New Zealand (Delcyan™)) or from 142ml of a

136 cold pressed blackcurrant fruit juice, (Blackadder cultivar, cultivated and processed by Plant
137 and Food Research Ltd, New Zealand (Blackadder juice)). One hundred and forty two
138 millilitres of juice was yielded from approximately 150g of fresh fruit, an amount which
139 could realistically be consumed in one serving. The phytochemical content of each
140 treatment can be seen in table 1. The Blackadder juice was frozen in 50ml aliquots at -20°C
141 until the day of use. The naturally occurring sugars in the Blackadder juice were quantified
142 via HPLC and the same levels were supplemented to the control and Delcylan™ treatments.
143 The total volume of the drink was then made up to 200ml (for a 60kg person) with cold
144 drinking water. All drinks quantities were calculated per kilo of body weight resulting in
145 differing volumes. In each case all drinks contained; 0.78g of glucose, 0.13g of fructose,
146 0.09g of Splenda® sweetener and 3.34µl blackcurrant flavouring (NI #12220, Formula foods
147 NZ) per kilogram of bodyweight. Drinks were coded and prepared fresh from frozen each
148 morning by a third party who had no further part in the running of the study. No member of
149 the investigation team was aware of the coding of the drinks until a blind-data review was
150 completed.

Table 1: Phytochemical constituents of Blackadder juice (mg/100ml of raw juice & mg supplemented per 60kg of body weight) and Delcyan™ extract (mg/g of raw powder & mg supplemented per 60kg of body weight)

Compound	Blackadder juice mg/100ml	Delcyan™ extract mg/g	Blackadder juice mg/60kg	Delcyan™ extract mg/60kg
Caffeoyl quinate	6.3	0.1	9	0.1
Caffeic acid glucoside	1.9	0.2	2.4	0.0
<i>p</i> -Coumaroyl quinate	3.6	0.4	5.4	0.7
Epigallocatechin	8.6	0	12	0
Delphinidin glucoside	24.1	44.6	34.2	73.8
Delphinidin rutinoside	115.9	107.4	164.4	178.2
Cyanidin glucoside	13.6	28.8	19.2	47.4
Cyanidin rutinoside	150.9	149	214.2	247.2
Myricetin rutinoside	15.6	4.5	22.2	7.2
Myricetin glucoside	2.1	0	3	0
Quercetin rutinoside	3.4	1.2	4.8	1.8
Quercetin glucoside	1.9	2.3	2.4	3.6
Quercetin pentoside	1.4	5.5	1.8	9
Myricetin	0.2	0.6	0.0	0.6
Vitamin C	168	0	100.8	0

151

152 **Cognitive and mood measures**

153 All cognitive measures and mood scales were delivered using the Computerised Mental
 154 Performance Assessment System (COMPASS, University of Northumbria), a purpose
 155 designed software application for the flexible delivery of randomly generated parallel
 156 versions of standard and novel cognitive assessment tasks, which has previously been
 157 shown to be sensitive to a range of nutritional interventions [14-16]. For the purpose of
 158 behavioural analysis, three tasks were selected with the intention that attention
 159 performance and cognitive flexibility could be assessed. Seven repetitions of the digit
 160 vigilance task, Stroop task and rapid visual information task were completed in a fashion
 161 similar to that of the cognitive demand battery [17] where subsequent repetitions of a ten
 162 minute battery are shown to incrementally induce mental fatigue. Mood scales were used
 163 at baseline, between each post-dose repetition of digit vigilance, Stroop and rapid visual
 164 information tasks and at the end of the cognitive tasks. The logical reasoning task was used
 165 at baseline and after the attentionally demanding cognitive battery to assess executive
 166 functioning.

167 **Study tasks**

168 *Digit vigilance:* The digit vigilance task is a measure of sustained attention involving accurate
169 selection of target stimuli. It focuses on alertness and vigilance while placing minimal
170 demands on two other components of attention: selectivity and capacity. A single target
171 digit was randomly selected and constantly displayed to the right of the screen. A series of
172 single digits were presented in the centre of the screen at the rate of 80 per minute. The
173 participant was required to press the response key on the computer keyboard as quickly as
174 possible every time the digit in the series matched the target digit. The task lasted two
175 minutes and there were 30 stimulus-target matches. Task outcomes were accuracy (%),
176 reaction time for correct responses (msec) and number of incorrect responses (false
177 alarms).

178 *Stroop:* The Stroop test is a measure of attention, inhibition and cognitive flexibility.
179 Participants were presented with a colour name. The colour name presented was written in
180 a coloured ink which could be the same as the colour name or different. Participants had to
181 respond to the colour of the ink using the peripheral mouse and corresponding colour
182 response buttons. Participants were presented with 60 stimuli. Task measures were
183 accuracy (percent correct) and reaction time (msec).

184 *Rapid visual information processing (RVIP):* The RVIP task is a measure of sustained
185 attention and working memory. The participant was required to monitor a continuous series
186 of digits for targets of three consecutive odd or three consecutive even single digits. The
187 single digits were presented at the rate of 100 per minute and the participant responded to
188 the detection of a target string by pressing the response key on the computer keyboard as
189 quickly as possible. The task was continuous and lasted for 5 minutes, with 8 correct target
190 strings being presented in each minute. The task was scored for percentage of target strings

191 correctly detected, average reaction time for correct detections (msec), and number of
192 incorrect responses (false alarms).

193 *Logical reasoning:* The logical reasoning test requires the participant to think
194 logically and analytically and is a measure of cognitive flexibility. A series of statements
195 referring to the relationships between two letters appeared on the screen one at a time
196 (e.g. “a precedes b: ba”). Participants were required to decide if each statement correctly
197 described the order of the 2 letters that followed it by pressing the designated response
198 keys on the computer keyboard. There were 24 stimuli. Mean reaction times were
199 measured in msec, and accuracy of responses were recorded as percentages.

200 **Mood**

201 *Bond-Lader visual analogue scales:* Bond-Lader visual analogue mood scales [18] which have
202 been shown to be sensitive to a number of nutritional intervention studies were employed
203 [14-16]. The reliability and validity of these visual analogue scales has been demonstrated
204 [19]. The scales comprise a total of sixteen 100mm lines anchored at either end by
205 antonyms (e.g. alert-drowsy, calm-excited) on which participants mark their current
206 subjective position. Scores from the 16 Bond-Lader visual analogue scales were combined as
207 recommended by the authors to form three mood factors: ‘alert’, ‘calm’ and ‘content’ [18].

208 *Visual analogue scales:* Following each repetition of the attentional demand battery,
209 participants were asked to subjectively rate how mentally fatigued they felt and how
210 difficult they found the cognitive tasks. The electronic visual analogue scales were anchored
211 “not at all” on the left hand side of the scale and “extremely” on the right, with higher
212 scores representing more mental fatigue/higher difficulty.

213 **Study procedure**

214 Each participant was required to attend a total of three study days which were conducted at
215 least seven days apart to ensure a sufficient wash out between conditions. During the week
216 before, and throughout their participation in the study, participants were asked to abstain
217 from berry consumption. Cognitive testing took place in a laboratory with participants
218 visually and auditorily isolated from each other. On arrival at their first session, participants
219 were randomly allocated to a treatment regime using a Latin square design that
220 counterbalanced the order of treatments across the three active days of the study. On all
221 three study days participants arrived at the lab in the morning (8:30am), after an overnight
222 fast, and firstly gave a 10ml venous blood sample. Heart rate, blood pressure and blood
223 glucose were then measured. Participants then completed one repetition of the ten minute
224 baseline cognitive assessment comprising of the digit vigilance task, the Stroop task, the
225 RVIP task, mood scales and the logical reasoning task. This constituted the baseline measure
226 for that day. Participants were then supplemented with one of the study treatments in the
227 form of a drink, which they were given five minutes to consume. Drinks were served chilled
228 and in a dark brown 300ml plastic bottle with a straw to minimise the possibility of the
229 participant recognising subtle differences in taste, look and mouth-feel between the
230 treatments. After a 60 minute resting absorption period, in which participants read in a
231 waiting area, participants' blood pressure and heart rate were measured again and a second
232 blood glucose reading was taken by finger prick. Participants then completed the post-dose
233 cognitive assessment 65 minutes post consumption of the study treatments, a time when
234 anthocyanins are known to be detectable in plasma after blackcurrant consumption [20].
235 This paradigm consisted of seven repetitions of the attention tasks (digit vigilance, Stroop,
236 RVIP) and mood scales. This lasted 70 minutes and was followed by the logical reasoning

237 central executive task. Participants then gave a third blood pressure reading and a third
238 blood glucose reading before providing a second and final venous blood sample. A diagram
239 of the study visit running order can be seen in figure 1.

240 The study received ethical approval from the New Zealand Regional Northern X Ethics board
241 and was conducted according to the Declaration of Helsinki (1964).

242 ***(Place figure 1 here)***

243 **Biochemical analysis**

244 Venous blood samples (2x5ml) were collected at baseline and 150 minutes after
245 supplementation of treatments, which coincided with the end of the tasks. Samples were
246 collected in 5ml BD vacutainers© (Becton, Dickinson and company, Plymouth, New
247 Zealand). Both receptacles were treated with anticoagulants, one with lithium heparin (LH)
248 and one with ethylenediaminetetraacetic acid (EDTA).

249 Whole blood samples treated with LH were immediately centrifuged (4°C, 5000rpm,
250 10 minutes) (Hitachi Himac preparative ultracentrifuge model CP100MX). Plasma was then
251 extracted and aliquoted into 1ml eppendorf© tubes. Aliquots were spiked with 200µl of 5%
252 trifluoroacetic acid for the purpose of measuring plasma anthocyanin content. Plasma
253 samples were stored at -80°C until analysis was performed.

254 Whole blood samples treated with EDTA were used to isolate blood platelets using
255 the method reported by Snell *et al* [21]. Three and a half millilitres of whole blood were
256 added to 2ml of phosphate buffered saline (PBS) containing 2g of glucose per litre of
257 solution (PBS solution) and gently inverted. The solution was then centrifuged at (22°C,
258 600g, 3 minutes) and the supernatant placed on ice. The volume of the residual red cell
259 pellet was restored to 7ml with PBS solution, gently inverted to mix and centrifuged again

260 (22°C, 600g at 22°C, 3 minutes); the supernatant was removed and pooled with the first
261 supernatant fraction. This procedure was performed 5 times. The pooled supernatant
262 fractions were then centrifuged (4°C, 2000g, 10 minutes) and decanted leaving the platelet
263 pellet which was stored at -80°C until the MAO-B analysis was performed.

264 **Monoamine oxidase-B (MAO-B) activity analysis**

265 A subset of eight participants provided sufficient blood samples for all the study time points
266 to allow for MAO-B analysis.

267 The isolated platelet pellet was slowly thawed on ice, re-suspended in 1ml of PBS
268 solution, sonicated with a probe sonicator (Microson ultrasonic cell disruptor, model
269 XL2005) for 15 seconds on ice and centrifuged (4°C, 36,000g, 10 minutes) at. Sonication and
270 centrifugation were then repeated after which the supernatant was removed and the pellet
271 consisting of lysed platelets was re-suspended in sodium phosphate buffer. The protein
272 concentration of the lysed platelet solution was determined against a bicinchoninic acid
273 standard curve by using the Pierce BCA protein assay (ThermoFischer Scientific New Zealand
274 Ltd) as per manufacturer's instructions. Each sample was measured in triplicate and the
275 average protein concentration was used. The lysed platelet solution was re-suspended in
276 sodium phosphate buffer to a final concentration of 150µg/ml of protein.

277 Determination of MAO-B activity was conducted using the Amplex[®] Red Monoamine
278 Oxidase-B Assay Kit (A12214 Invitrogen), as per manufacturer's instructions. One hundred
279 microlitres of the diluted lysed platelet solution was added to a 96 well plate in triplicate.
280 Two microlitres of the MAO-A inhibitor clorgyline were then added to each well that
281 contained platelet membranes and incubated for 30 minutes at room temperature. During
282 the incubation, 100µl of H₂O₂ standards and the negative control were then added to the

283 micro-plate in triplicate. After the 30 minute incubation, 100µl of the amplex red working
284 solution were added to each well. The plate was immediately placed into the microplate
285 reader (FLUOstar Omega Plate reader, BMG labtech) and set to incubate at 37°C with an
286 excitation wavelength of 530-560nm and an emission wavelength of 590nm. The micro-
287 plate reader was programmed to take a reading every five minutes for one hour (13
288 readings in total). The 30 minute reading was used to compare platelet MAO-B activity
289 between treatments.

290 **Glucose**

291 Blood glucose was measured with the use of an Accu-Check (Roche Healthcare, NZ) blood
292 glucose monitor via a finger prick blood sample at baseline, 60 minutes and 150 minutes
293 post supplementation. All 35 participants who completed the study gave all required finger
294 prick blood samples.

295 **Prolactin analysis**

296 Prolactin was measured by diagnostic Medlab, Auckland, New Zealand. Prolactin was
297 analysed in 300µL of blood plasma collected in LH treated vacutainers. Due to technical
298 issues, only 20 sets of blood samples were available for prolactin analysis (8 control, 7
299 Delcylan™, 5 Blackadder juice). For this reason, prolactin analysis was between subjects.

300 **LCMS analysis**

301 The phytochemical composition of the blackcurrant extracts (Blackadder juice, Delcylan™)
302 and the control sample were determined by liquid chromatography mass spectrometry
303 (LCMS) using a Shimadzu 2020 single-quadrupole mass spectrometer coupled to a Shimadzu
304 20-Series UFLC system (Auckland, New Zealand) using the method described by Schrage *et*
305 *al* [13].

306 Levels of anthocyanins and monoamines in plasma at defined time points
307 throughout the study were determined by LCMS using a 5500 QTrap triple
308 quadrupole/linear ion trap (QqLIT) mass spectrometer equipped with a Turbolon-Spray™
309 interface (AB Sciex, Concord, Ontario, Canada) coupled to an Ultimate 3000 UHPLC (Dionex,
310 Sunnyvale, California, USA).

311 **LCMS materials**

312 Formic acid (Riedel-de Haën), ammonium formate and acetic anhydride (Fluka), and Hunig's
313 base were purchased from Sigma Aldrich (Auckland, New Zealand). Optima LC/MS grade
314 acetonitrile (Fisher Scientific) was purchased from ThermoFisher (Auckland, New Zealand).
315 Water was of Milli-Q grade. Analytical standards, dopamine, normetadrenalin, noradrenalin,
316 adrenalin, 3,4-dihydroxyphenylglycol (DHPG), serotonin and homovanillic acid (HVA) were
317 purchased from Sigma-Aldrich, phenylethylamine (PEA) from Acros Organics (Geel,
318 Belgium), cyanidin 3-glucoside, cyanidin 3-rutinoside, delphinidin 3-glucoside and
319 delphinidin 3-rutinoside from Polyphenols Laboratories (Sandnes, Norway) and malvidin 3-
320 galactoside chloride from Extrasynthese (Genay Cedex, France). Deuterated acetic
321 anhydride [d6] was purchased from Sigma-Aldrich and deuterated dopamine [d4] from CDN
322 Isotopes (Quebec, Canada). Phree™ Phospholipid removal plates were purchased from
323 Phenomenex (Torrance, CA, USA).

324 **Anthocyanin analysis in plasma**

325 A subset of 17 participants provided sufficient blood samples for all of the study time points
326 for anthocyanin analysis to be conducted.

327 Plasma samples (1ml) were further acidified (1:4 6N HCl:5% formic acid_{aq}, 250µl) and
328 then spiked with malvidin galactoside (5ng) as an internal standard. Samples were

329 centrifuged (4°C, 16100 RCF, 5 minutes) and proteins removed by precipitation via addition
330 of acetone (1:3) to an aqueous aliquot (600µl). The samples were then chilled at -80°C for 30
331 minutes prior to re-centrifuging (4°C, 16,100 RCF, 5 minutes) and the acetone removed via
332 evaporation. Further clean-up to minimise the presence of phospholipids was achieved via
333 liquid-liquid partition with hexane versus the aqueous sample. A final protein precipitation
334 cleanup of the aqueous aliquot (400µl) with chloroform was performed prior to centrifuging
335 (4°C, 16100 RCF, 5 minutes). Two hundred microlitres of the aqueous phase was transferred
336 to an autosampler vial for immediate analysis by LCMS.

337 Anthocyanin separation was achieved on a Zorbax SB-C18 Rapid Resolution HD
338 2.1x100mm ID 1.8 micron column (Agilent Technologies, Santa Clara, CA, USA) maintained
339 at 70°C. Solvents were (A) 5:3:92 acetonitrile/formic acid:water v/v/v and (B) 99.9:0.1
340 acetonitrile/formic acid v/v and the flow rate was 600µL/min. The initial mobile phase,
341 100% A was held isocratically for 0.5 minutes, then ramped linearly to 10% B at 5 minutes,
342 followed by another linear ramp to 90% B at 5.1 minutes and held for 1.9 minutes before
343 resetting to the original conditions. Sample injection volume was 20µl. MS data was
344 acquired in the positive mode using a multiple reaction monitoring (MRM) method. The
345 transitions monitored (Q1 and Q3), along with their optimised parameters (declustering
346 potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential
347 (CXP)) are listed in Table 2.

348 *Table 2: Multiple reaction monitoring transitions used for blackcurrant anthocyanins*

Q1	Q3	Name	DP	EP	CE	CXP
465	303	Delphinidin glucoside	70	10	30	10
611	303	Delphinidin rutinoside	130	10	50	30
449	287	Cyanidin glucoside	110	10	35	20
595	287	Cyanidin rutinoside	50	10	55	1
493	331	Malvidin galactoside (IS)	50	10	45	25

349

350 Other operating parameters were as follows: dwell time, 100ms; ionspray voltage,
351 2500V; ion source temperature, 700°C; ion source gas one, 60psi; ion source gas two, 90psi
352 and curtain gas, 40psi.

353 Quantification was performed using the internal standard ratio method using
354 MultiQuant software v3.0 (AB Sciex).

355 **Monoamine analysis in plasma**

356 Eight monoamines were analysed by LCMS from blood plasma following derivatisation.
357 These were; serotonin, dopamine, phenylethylamine, adrenalin, noradrenalin,
358 normetadrenalin, 3,4-dihydroxyphenylglycol (DHPG) and homovanillic acid (HVA).

359 Plasma samples were treated to remove proteins and phospholipids and derivatised
360 in two stages to acetylate alcohol and amine functional groups and alkylate free carboxylic
361 acids with Hunig's base prior to LCMS analysis. A double derivatisation was found to be
362 necessary to acetylate the less reactive alkyl hydroxyl groups. The schematic in figure 2
363 summarises the derivatisation of the different functional groups and the synthesis and use
364 of labelled internal standards for each analyte to facilitate quantitation and to correct for
365 matrix effects during analysis.

366 ***(Place figure 2 here)***

367 Briefly, each plasma sample (200µl) was added to an individual well of a Phree™
368 Phospholipid removal plate already containing cold 600µl acetonitrile, 100µl acetic
369 anhydride and 1ng dopamine-d4 [internal standard (IS)]. The Phree™ plate was centrifuged
370 at 500g for 30 minutes, a further 200µl acetonitrile added to each well, and the plate
371 centrifuged at 500g for a further 10 minutes. The filtrate was transferred to a 2ml micro
372 tube, 100µl acetic anhydride added and heated at 50°C. After 30 minutes 20µl Hunig's base

373 was added to each sample, vortexed then heated for a further 60 minutes. Samples were
 374 then evaporated to near dryness with nitrogen at 40°C. Samples were re-derivatised; 100µl
 375 acetonitrile, 100µl acetic anhydride and 10µl Hunig's base heated for 40 minutes at 50°C.
 376 Finally, to each sample 1ng of the derivatised labelled internal standard monoamine mixture
 377 (d-IS) was added, and the sample made up to 1ml with water and transferred to an
 378 autosampler vial ready for analysis.

379 Monoamine separation was achieved on an Atlantis® T3 150x2.1mm 3 micron
 380 column (Waters Corp., Milford, MA, USA), maintained at 40°C. Solvents were (A) MilliQ
 381 water +0.03% ammonium formate + 0.1 % formic acid and (B) acetonitrile + 0.1 % formic
 382 acid and the flow rate was 0.6ml/min. The initial mobile phase, 98% A, was held for 4
 383 minutes then ramped linearly to 70% A at 11 minutes, 20% A at 14 minutes, and 0% A at
 384 14.5 minutes and held for 5 minutes before resetting to the original conditions. Sample
 385 injection volume was 100µl.

386 MS data was acquired in the positive mode using a scheduled MRM method. In some
 387 cases the ammonium adduct was the most abundant ion observed for Q1. The transitions
 388 monitored (Q1 and Q3), along with their optimised DP, EP, CE and CXP parameters are listed
 389 in Table 3.

390 *Table 3 Multiple reaction monitoring transitions used for monoamines and their isotopically labelled internal standard*
 391 *analogues*

Q1	Q3	Time	Name	DP	EP	CE	CXP
164	105	10.6	PEA	30	10	25	10
167	105	10.6	PEA [d3]	30	10	25	10
280	137	11.1	Dopamine	70	6.1	35	15
289	139	11.1	Dopamine [d9]	70	6.1	35	15
284	141	11.1	Dopamine [d4]	70	10	37	15
293	143	11.1	Dopamine [d4] [d9]	70	10	37	15
261	160	11.1	Serotonin	10	5	25	1
267	161	11.1	Serotonin [d6]	10	5	25	1
250	166	11.6	Normetadrenalin	50	9	25	15
256	168	11.5	Normetadrenalin [d9]	50	9	25	15
355	194	11.6	Noradrenalin	10	10	30	1
367	199	11.5	Noradrenalin [d12]	10	10	30	1
292	250	12.5	Adrenalin	170	10	20	1
301	257	12.5	Adrenalin [d12]	170	10	20	1

356.	237	13.4	DHPG	90	13	20	20
368	244	13.3	DHPG [d12]	90	13	20	20
308	224	13.9	HVA	110	10	25	20
311	225	13.9	HVA [d3]	110	10	25	20

392

393 Other operating parameters were as follows: ionspray voltage, 2500V; ion source
394 temperature, 700°C; ion source gas one, 40psi; ion source gas two, 50psi and curtain gas,
395 50psi.

396 Quantification was performed using the internal standard ratio method using
397 MultiQuant software v3.0 (AB Sciex).

398 **Statistics**

399 Mood, cognitive scores and the physiological measures were analysed as ‘change from
400 baseline’ using the SPSS 18 statistics package. Baseline differences were calculated for all
401 measures using a one way (treatment) ANOVA.

402 Two way repeated measures ANOVAs (General linear model) (Treatment [control,
403 Delcyan™, juice] X completion [1 to 7] for attentional tasks and visual analogue scale
404 outcomes OR Treatment [control, Delcyan™, juice] X completion [1 to 2] for blood glucose
405 and Bond-Lader) were conducted. Logical reasoning performance, platelet Monoamine
406 Oxidase B activity, plasma monoamines and plasma anthocyanins levels were analysed by
407 one-way (treatment) repeated measures ANOVA. Blood plasma prolactin was analysed
408 using a one way (treatment) between subjects ANOVA. In all instances Mauchly’s test of
409 sphericity was used to assess equality of the variances of the differences between factors.
410 Where sphericity had been violated, Huynh-Feldt corrections for non-sphericity were
411 implemented. Pairwise comparisons were conducted on all treatment-related effects with a
412 p value <0.05 on the initial ANOVA to ascertain any differences between treatments for the
413 whole session or, in the case of interactions, at each task repetition. Partial Bonferroni

414 corrections were applied to protect for error against multiple comparisons, therefore, the p
415 value was multiplied by the number of treatments being compared to control. All post hoc p
416 values are reported after corrections for multiple comparisons have been applied (x3 for
417 anthocyanins and MAO-B and x2 for all other comparisons).

418 **Results**

419 Prior to analysis of change from baseline data, mean pre-dose scores for all three
420 treatments (control, Delcyan™, Juice) for each outcome were subjected to a one way
421 repeated measures ANOVA. The only significant difference found was between active
422 treatments for homovanillic acid. Data tables for all outcomes can be found in the
423 supplementary materials.

424 **Cognitive performance**

425 A one way ANOVA was conducted on control change from baseline scores for the outcome
426 fatigue to ensure the sustained attention cognitive paradigm was indeed mentally fatiguing.
427 A significant effect of repetition [$F(6,192)=16.44$, $p<0.0001$] confirmed that each subsequent
428 repetition of the tasks caused an increase in rating of mental fatigue.

429 **Digit vigilance**

430 There was a significant treatment × repetition interaction on digit vigilance reaction time [F
431 $(12,384)=1.82$, $p=0.044$] without any effect upon accuracy. Pairwise comparisons revealed an
432 increase in speed of response after supplementation of the juice treatment at repetition 1
433 ($p=0.028$), 4 ($p=0.011$) and 7 ($p=0.038$). See Figure 3a. There were no effects on any digit
434 vigilance outcomes after supplementation with Delcyan™.

435 **RVIP**

436 There was a significant main effect of treatment on RVIP accuracy [F (2,62)=5.87, p=0.005].
437 Pairwise comparisons showed an attenuation in the reduction of RVIP accuracy after
438 supplementation of the Delcyan™ extract when compared to control (p=0.011), irrespective
439 of repetition. There were no significant effects on reaction time or false alarms. See Figure
440 3b. There were no effects on any RVIP outcomes after supplementation with juice.

441 *(Place figure 3 here)*

442 **Blood glucose**

443 There was a significant main effect of treatment on blood glucose [F(2,68)=8.89, p<0.001].
444 Pairwise comparisons showed significantly higher blood glucose levels following
445 supplementation of the juice treatment when compared to control (p=0.002), irrespective
446 of repetition. See figure 4a. There were no significant effects following supplementation of
447 Delcyan™.

448 **Platelet MAO-B**

449 There was a significant effect of treatment on blood platelet MAO-B activity [F (2,16)=15.20
450 p<0.001]. Pairwise comparisons showed a decrease in platelet MAO-B activity after
451 supplementation with the juice treatment when compared to control (p<0.001). See figure
452 4b. There were no significant differences between active treatment groups. There was no
453 effect of the Delcyan™ treatment on blood platelet MAO-B.

454 **Monoamines**

455 The repeated measures ANOVA revealed a significant effect of treatment [F (2,32)=12.18
456 p<0.001] on plasma levels of normetadrenalin. Pairwise comparisons showed levels of

457 normetadrenalin were significantly higher after supplementation of the juice treatment
458 when compared to the control ($p < 0.001$) and Delcyan™ ($p < 0.001$). There were no effects of
459 Delcyan™. See figure 4c.

460 The repeated measures ANOVA revealed a significant main effect of treatment [F
461 (2,32)=21.30 $p < 0.001$] on plasma levels of DHPG. Pairwise comparisons revealed levels of
462 DHPG were significantly higher after supplementation of the juice treatment when
463 compared to the control ($p < 0.001$) and Delcyan™ ($p < 0.001$). There were no effects of
464 Delcyan™ versus control. See figure 4d.

465 *(Place figure 4 here)*

466 **Blood plasma anthocyanin levels**

467 The repeated measures ANOVA revealed a significant effect of treatment on plasma levels
468 of CY-GLU, CY-RUT, DEL-GLU and DEL-RUT [F(2,34)=27.5 ($p < 0.001$)], [F(2,34)=33.7
469 ($p < 0.001$)], [F(2,34)=112.51 $p < 0.001$], [F(1.45,25.25)=96.26 $p < 0.001$] respectively.

470 Plasma CY-GLU, CY-RUT, DEL-GLU and DEL-RUT levels were significantly higher after
471 supplementation with the Delcyan™ treatment when compared to control ($p < 0.001$,
472 $p = 0.005$, $p < 0.001$, $p < 0.001$) and the Blackadder juice treatment ($p < 0.001$, $p < 0.001$, $p < 0.003$,
473 $p < 0.001$) respectively.

474 Plasma CY-GLU, CY-RUT, DEL-GLU and DEL-RUT levels were also significantly higher
475 after supplementation of the Blackadder juice treatment when compared to control
476 ($p < 0.001$), ($p < 0.001$), ($p = 0.003$) and ($p < 0.001$) respectively. A graphical representation of
477 anthocyanin levels in blood plasma can be seen in figure 5a.

478 Following supplementation, the repeated measures ANOVA revealed a significant
479 effect of the treatment on combined levels of CY-GLU, CY-RUT, DEL-GLU and DEL-RUT in

480 blood plasma (combined anthocyanin level is the total amount of anthocyanins measured in
 481 blood plasma after supplementation of the study treatment) after consumption of the study
 482 treatments, [F (2,32)=105.34, p<0.0001]. Pairwise comparisons revealed that combined
 483 anthocyanin levels were significantly higher after consumption of the Delcyan™ (p<0.001)
 484 and juice (p<0.001) treatment when compared to control. Levels were also significantly
 485 higher after supplementation of the Delcyan™ extract when compared to the Blackadder
 486 juice extract (p<0.001). A graphical representation of combined anthocyanin levels in blood
 487 plasma can be seen in figure 5b.

488

489 **(Place figure 5 here)**

490

Table 3 Means and SD for the amount of measured anthocyanins given to the participants and the amount found in blood plasma (change from baseline) 150 minutes post supplementation of the Delcyan™ and Blackadder juice treatments

Treatment	Anthocyanin	Amount supplemented mg/kilo body weight	Amount supplemented mg/60kg of body weight	Average amount in plasma (nM)
Delcyan™	Cyanidin glucoside	0.8	48	0.4 ± 0.3
	Delphinidin glucoside	1.2	73.8	1.3 ± 0.9
	Cyanidin rutinoside	4.1	247.2	8.6 ± 2.9
	Delphinidin rutinoside	2.9	178.2	11.7 ± 4.5
Blackadder juice	Cyanidin glucoside	0.3	19.2	0.2 ± 0.1
	Delphinidin glucoside	0.5	34.2	0.8 ± 0.6
	Cyanidin rutinoside	3.5	214.2	6.4 ± 2.3
	Delphinidin rutinoside	2.7	164.4	7.8 ± 2.5
Control	Cyanidin glucoside	0	0	0.0 ± 0.1
	Delphinidin glucoside	0	0	0.0 ± 0.01
	Cyanidin rutinoside	0	0	0.0 ± 0.1
	Delphinidin rutinoside	0	0	0.0 ± 0.1

491

492

493

494 **Discussion and conclusion**

495 The current study has outlined evidence of positive modulation of behaviour following
496 administration of two blackcurrant extracts when compared to control, with no negative
497 effects of either active extract. Improvements in RVIP accuracy were found after
498 supplementation of the Delcyan™ extract and improvements in reaction time on the digit
499 vigilance task were found after supplementation of the Blackadder juice extract. Although
500 there were no other significant effects on behaviour, the Blackadder juice treatment
501 demonstrated a number of physiological effects not present following Delcyan™. These
502 comprised of an inhibition of platelet MAO-B activity (96%) and a significant reduction in
503 plasma normetadrenalin (60%) and increase in DHPG (~35.5%) when measured 2.5 hours
504 after supplementation. The Blackadder blackcurrant juice treatment also showed a
505 significantly sustained (over both time points) increased blood glucose when compared to
506 control at 60 and 150 minutes, despite being sugar matched.

507 An increase in accuracy was shown during the RVIP task after supplementation with
508 the Delcyan™ treatment, irrespective of task repetition, with no evidence of slowed
509 reaction times. In regards to the juice treatment, there was evidence of an attenuation of
510 the increase of digit vigilance reaction times seen with repeated testing, with no evidence of
511 decreased accuracy. This improvement was seen during repetitions one, four and seven (70,
512 100 and 140 minutes post-supplementation, respectively). Further evidence for a
513 modulation of behaviour following the blackcurrant extracts comes from non-significant
514 trends observed on the treatment*repetition ANOVAs for Bond-Lader alertness ratings and
515 mental fatigue visual analogue scales showed. These indicated a pattern of attenuation in
516 decreased self-reported alertness and increased ratings of fatigue following
517 supplementation of the Delcyan™ treatment but only reached statistical significance after

518 the final repetition of the 70 minute attentionally demanding cognitive battery. Graphical
519 representations of fatigue and alert ratings can be found in the supplementary materials.
520 There is evidence of direct cellular and molecular interactions of flavonoids on rodent brains
521 [22] and changes in central [23] and peripheral [20] vascular function in humans after
522 consumption of flavonoid-rich fruits. However, definitive mechanisms driving the
523 behavioural effects in the present study are currently unknown, especially after
524 supplementation of the Delcyan™ treatment, which had no significant effect upon any of
525 the physiological outcomes measured.

526 As expected, blood plasma anthocyanins DEL-GLU, DEL-RUT, CY-GLU and CY-RUT
527 were significantly increased 2.5 hours after supplementation of both blackcurrant
528 treatments when compared to control. Measured blood plasma anthocyanins were also
529 greater after supplementation of the Delcyan™ treatment when compared to the juice
530 treatment. When all four measured anthocyanins were combined there was a 30% increase
531 in plasma concentration following Delcyan™ when compared to the juice treatment.
532 However, this extract contained 20% more of the measured anthocyanins than the juice
533 drink. Although there was a significant difference in blood plasma anthocyanins between
534 the two blackcurrant treatments, in line with past published research [20, 24, 25],
535 anthocyanin quantities found in blood plasma were less than one percent of that ingested.
536 It must be noted that vitamins and minerals, other than L-ascorbic were not quantified in
537 any of the study treatments. However, to our knowledge there are no reported cognitive or
538 behavioural effects of acute vitamin or mineral supplementation. Given that the major
539 phenolic constituents of each treatment were anthocyanins and both extracts affected
540 attention based tasks, this may indicate that the effects of blackcurrant upon attention
541 processing are directly related to their anthocyanin content; an acute effect which has

542 previously been indicated in children aged 7-9 years [26]. The specific demands of the two
543 attention tasks are, however, not equal, with a higher demand both in processing, and
544 duration of the RVIP task when compared to the digit vigilance task. The RVIP contains a
545 higher working memory element than the digit vigilance task, potentially indicating changes
546 in working memory processing as well as attention, a cognitive outcome which has
547 previously been shown to be sensitive to flavonoid-rich cocoa [27] and ginkgo biloba [28].
548 However, until replication of the behavioural effects presented in the current study has
549 been achieved, it is difficult to elaborate further at this point.

550 In terms of MAO-B activity, this is the first demonstration of a clinically significant
551 inhibition of platelet MAO-B following blackcurrant supplementation. Central MAO-B
552 inhibitors have been used for several decades for the treatment of depressive disorders and
553 neurodegenerative diseases [29] and have also been shown to improve cognitive processing
554 when given to non-demented Parkinson patients [30]. MAO-B inhibitors also have the
555 potential to attenuate the breakdown of endogenous neurotransmitters, reducing levels of
556 H₂O₂ associated with deamination of dopamine [31]. Although the current study only
557 measured MAO-B inhibition in peripheral tissue, if the inhibition can be shown to be
558 centrally active, the clinical applications of a MAO inhibitor from a commonly consumed
559 fruit could be vast. Potential applications include attenuating cognitive decline associated
560 with natural ageing, as well as in clinical populations, including those suffering from early
561 stage Parkinson's disease, whom are known to respond favourably to MAO inhibitors [30].
562 DHPG, a metabolite largely determined by MAO-A dependent metabolism of noradrenalin
563 [32], which is a marker for reduced MAO-A activity after administration of pharmacological
564 MAO-A inhibitors [33], was also found to be reduced after consumption of the Blackadder
565 blackcurrant juice extract in the current study. This effect was not seen after consumption

566 of the Delcyan™ extract, highlighting that, in addition to MAO-B inhibition, the Blackadder
567 juice treatment possesses MAO-A inhibitory properties. These changes in DHPG did not
568 coincide with an accumulation of adrenalin or noradrenalin in the current study, which is in
569 line with previous research investigating acute supplementation of MAO inhibitors in
570 humans [34]. In addition to decreased levels of DHPG, indicating MAO-A inhibition, we also
571 observed an increase in normetadrenalin, a metabolite of noradrenalin via catechol-O-
572 methyl transferase (COMT). This increase is potentially indicative of increased noradrenalin
573 breakdown through COMT as a result of inhibition of the MAO-A enzyme. A diagram
574 depicting potential inhibition pathways can be found in figure 6. Also related to this MAO
575 inhibitory effect is a non-significant modulation of plasma prolactin observed in the current
576 study where post-dose prolactin was lower after consumption of the Blackadder juice
577 extract when compared to control. Although gamma-aminobutyric acid (GABA), serotonin,
578 adrenalin and noradrenalin are slight rate limiting factors of prolactin secretion, dopamine is
579 the most important hypothalamic prolactin inhibiting factor[35], indicating that general and
580 potentially central dopaminergic tone could have been affected by supplementation of the
581 Blackadder juice drink. Although these prolactin findings are hindered by a small sample size
582 and between subjects design, they illuminate the need for further research.

583

584 ***Place figure 6 here***

585

586 The blackcurrant juice treatment also showed a significant (over both time points)
587 increase in blood glucose when compared to control, despite being sugar matched; an effect
588 not seen with supplementation of the Delcyan™ treatment. Blood glucose was elevated by
589 0.53mmo/L at 60 minutes and 0.23mmol/L at 150 minutes post supplementation of the

590 juice treatment. Although these results must be interpreted with caution as there were only
591 two post-dose blood glucose measurements, this result shows a clear effect of the juice
592 treatment on blood glucose. Based upon the current findings, the effect on glucose appears
593 to resemble the pattern after supplementation of berry puree where the peak in blood
594 glucose levels following a glucose load when combined with a berry puree is reduced
595 resulting in a higher blood glucose reading one hour after supplementation [36]. The main
596 difference between the study treatments was phenolic acids at 0mg in the Delcyan™
597 treatment and 61mg in the juice treatment per 60kg of bodyweight, which could provide
598 further evidence of the slowing of glucose transport from the gut via direct inhibition of
599 intestinal epithelial glucose transporters by phenolic acids as described by Manzano and
600 Williamson [8]. A more thorough investigation needs to be completed to ascertain a full
601 post supplementation blood glucose profile.

602 The findings of the present study demonstrate, for the first time, a positive
603 modulation of behaviour in a young and healthy adult cohort after supplementation of a
604 blackcurrant extract. This is also the first evidence of a clinically significant reduction in MAO
605 activity following ingestion of a commonly consumed fruit. The results suggest that the MAO
606 inhibition found in this study cannot be wholly responsible for the behavioural effects
607 observed as both active conditions positively influenced attention based cognitive tasks,
608 whereas only the juice treatment inhibited MAO-A and MAO-B. The finding of more robust
609 effects on attention following Delcyan™, containing higher levels of anthocyanins, may
610 indicate that these effects are attributable to the anthocyanin content and that any effects
611 on MAO are independent of these. The possibility that a MAO-A and MAO-B inhibiting
612 blackcurrant drink will exert favourable effects on cognitive modulation of clinical and non-
613 clinical populations deserves further investigation. More exploration therefore needs to be

614 undertaken to ascertain if other cognitive paradigms, especially those which have previously
615 been shown to be sensitive to flavonoid-rich nutritional interventions in rats and humans,
616 specifically memory tasks and paradigms sensitive to changes in levels of dopamine, are
617 modulated after supplementation of a MAO inhibiting blackcurrant juice.

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624

625 **Conflict of interests**

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630

631

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