DOI 10.1002/mnfr.201300322 *Mol. Nutr. Food Res.* 2014, *58*, 490–502

RESEARCH ARTICLE

Phenolic metabolites of anthocyanins following a dietary intervention study in post-menopausal women

Rachel M. de Ferrars, Aedín Cassidy, Peter Curtis and Colin D. Kay

Department of Nutrition, Norwich Medical School, University of East Anglia, Norwich, UK

Received: May 2, 2013
Revised: July 28, 2013
Accepted: July 31, 2013

Scope: Numerous studies feeding anthocyanin-rich foods report limited bioavailability of the parent anthocyanins. The present study explores the identity and concentration of the phenolic metabolites of anthocyanins in humans.

Methods and results: Anthocyanin metabolites were quantified in samples collected from a previously conducted 12-wk elderberry intervention study in healthy post-menopausal women. Individual 1-, 2- and 3-h post-bolus urine samples and pooled plasma samples following acute (single bolus) and chronic (12-wk supplementation) anthocyanin consumption (500 mg/day) were analysed using HPLC-ESI-MS/MS. Twenty-eight anthocyanin metabolites were identified in urine and 21 in plasma (including sulfates of vanillic, protocatechuic and benzoic acid). Phenolic metabolites reached peak concentrations of 1237 nM in plasma, while anthocyanin conjugates only reached concentrations of 34 nM. Similarly, in urine, phenolic metabolites were detected at concentrations of 33 185 ± 2549 nM/mM creatinine, while anthocyanin conjugates reached concentrations of 548 ± 219 nM/mM creatinine. There was no evidence that chronic exposure had any impact on either the profile or quantity of metabolites recovered relative to acute exposure.

Conclusion: An extensive range of phenolic metabolites of anthocyanin was identified following elderberry consumption in humans, including 11 novel metabolites, which were identified at much higher concentrations than their parent compounds.

Keywords:
Anthocyanins / Elderberries / Glucuronide / Phenolic acids / Sulfate

1 Introduction

A number of epidemiological [1–3] and in vitro [4, 5] studies have linked high consumption of anthocyanin-rich foods with a reduced risk of cardiovascular disease. However, ambiguity has arisen concerning the absorption, distribution, metabolism and elimination of anthocyanins, as their recovery from biological samples has been poor, with studies rarely identifying above 1% of the ingested dose [6–8]. This raises questions regarding the metabolic fate of anthocyanins and similarly the compounds/metabolites responsible for their potential cardioprotective effects.

Correspondence: Dr. Colin D. Kay, Department of Nutrition, Norwich Medical School, University of East Anglia, Norwich, NR4 7TJ, UK
E-mail: Colin.Kay@uea.ac.uk
Fax: +44-0-1603-593752

Abbreviations: C3G, cyanidin-3-glucoside; **P3G**, peonidin-3-glucoside; **PCA**, protocatechuic acid; **PGA**, phloroglucinaldehyde; **PFP**, pentafluorophenol; **VA**, vanillic acid

Due to their unstable nature, significant quantities of anthocyanins are unlikely to be present in the circulation in their parent forms [9]. The degradation of anthocyanins to their phenolic acid and aldehyde constituents has been well established in vitro [10, 11]; yet few human studies have detected these degradation products within biological samples [12]. Human intervention studies feeding anthocyanin-rich foods and extracts including cranberry, strawberry and bilberry-loganberry have identified potential metabolites of anthocyanins including hippuric acid, benzoic acid, 4-hydroxybenzoic acid, protocatechuic acid (PCA), dihydroxybenzoic acids, caffeic acid, *p*-courmaric acid, ferulic acid, sinapic acid, 2-hydroxyphenylacetic acid, homovanillic acid, vanillic acid (VA) and 4-hydroxyphenylacetic acid [13–17].

Our recent study feeding a pure ^{13}C-labeled anthocyanin (500 mg) to human volunteers confirmed many of these structures, in addition to identifying novel metabolites; 25 individual ^{13}C-labeled phenolic metabolites were recovered, attaining a total peak concentration of 5.97 ± 2.14 μM in the serum and 10.77 ± 4.52 μM in the urine [18]. The present study aimed to confirm the formation of these anthocyanin-derived metabolites following the consumption of an

anthocyanin-rich dietary source (elderberries; *Sambucus nigra*), as opposed to a pure synthetic anthocyanin preparation. We used stored samples from a previously completed 12-wk anthocyanin intervention, where healthy post-menopausal women were fed 500 mg/day of anthocyanins derived from 2 g of elderberry extract [19]. The present study also investigated the impact of a single 500 mg bolus (acute post-prandial effect) and repeated daily 500 mg doses (post-prandial effect after 12-wk chronic supplementation) on metabolite excretion to establish if sustained exposure altered either the quantity or profile of metabolites eliminated. To the best of our knowledge, this is the first study to describe the impact of acute versus chronic anthocyanin exposure on anthocyanin-derived phenolic metabolites.

2 Materials and methods

2.1 Chemicals and materials

Cyanidin-3-glucoside (C3G), peonidin-3-glucoside (P3G) and cyanidin-3-sambubioside were obtained from Extrasynthese (Genay, France). Phase II conjugates of phenolic acids (PCA-3-glucuronide, PCA-4-glucuronide, VA-4-glucuronide, benzoic acid-4-glucuronide, isoVA-3-glucuronide, isoVA-3-sulfate, PCA-4-sulfate, VA-4-sulfate, PCA-3-sulfate and benzoic acid-4-sulfate) were synthesised in the School of Chemistry and Centre for Biomolecular Sciences, University of St. Andrews (UK) via recently published methods [20]. All other metabolites (Fig. 1) were purchase from Sigma-Aldrich (Dorset, England). Strata-X^{TM} SPE columns (6 mL, 500 mg), Kinetex pentafluorophenol (PFP) HPLC column (2.6 μm, 100 × 4.6 mm, 100 Å) and SecurityGuard® cartridges (PFP, 4.0 × 2.0 mm) were purchased from Phenomenex (UK). HPLC grade methanol, acetonitrile and hydrochloric acid (HCl) were purchased from Fisher Scientific (Loughborough, UK). Discovery® DSC-18 SPE columns (6 mL, 1 g) and Acrodisc 13 mm, 0.45 μm PTFE syringe filters and all other chemicals were purchased from Sigma-Aldrich. All water utilised was of Milli-Q grade (18.2 MΩ/cm).

2.2 Analysis of elderberry extract

The anthocyanin content of the elderberry (*S. nigra*) extract (standardised to 25% anthocyanins) obtained from Artemis International (Fort Wayne, Indianapolis, IN, USA) was previously established as 500 mg anthocyanins (C3G and cyanidin-3-sambubioside, quantified as C3G equivalents) per 2 g extract [19]. The phenolic profile was presently established through HPLC-ESI-MS/MS analysis, where prior to analysis, 500 mg of elderberry extract was dissolved in 2 mL of 0.1% HCl and 5 mL acidified methanol (0.1% v/v HCl) and sonicated for 30 min followed by syringe filtration.

2.3 Study design

We previously conducted a parallel-designed, randomised, placebo-controlled study feeding elderberry extract or placebo to 52 (26 in treatment and 26 in placebo group) healthy (age, 58.2 ± 5.6 years; BMI, 24.7 ± 0.6 kg/m^2) post-menopausal women [19], which explored the effect of anthocyanins on cardiovascular bioactivity and liver function. Participants underwent a 7-day washout period (avoiding anthocyanin-rich foods and limiting tea and chocolate intake), followed by the consumption of identically coloured opaque elderberry or placebo capsules each day for 12 wk. Dietary restrictions were continued throughout the duration of the 12-wk intervention. The elderberry capsules provided a daily total of 2 g elderberry extract, containing 500 mg total anthocyanins while the placebo capsules contained 500 mg of cellulose. Participants were assessed following a single bolus dose (wk 0, acute) and following 12 wk of continuous supplementation (wk 12, chronic), where blood and urine samples were collected in the fasting period (baseline) and over 3 h following the consumption of a 500 mg oral bolus dose of anthocyanins. An anthocyanin- and caffeine-free breakfast of cereal (Weetabix^{TM} (Weetabix, UK) or Cornflakes (Kelloggs^{TM}, UK)), white toast with margarine/butter, milk and water was provided ad libitum on the intervention days. Post-prandial blood and urine samples were collected for 3 h (t = 1, 2, 3 h) following ingestion. Blood was collected in EDTA tubes and plasma obtained by centrifugation at 1500 × 3 *g* for 15 min, where urine and plasma samples were acidified with HCl (to a final concentration of 1%) prior to storage at –80°C until analysis. The study protocol was approved by the Norfolk Research Ethics Committee (ClinicalTrials.gov registry identifier NCT00574574), conducted at the University Clinical Research and Trials Unit (UEA, UK) and all participants provided written informed consent. In the present investigation, a random subset of the banked samples from 15 of the 26 participants on the anthocyanin treatment was analysed to explore the effect of 12-wk repeated dosing on anthocyanin metabolism (the placebo group was not explored).

2.4 Creatinine analysis

Urine creatinine concentrations were quantified on an iLab 650 Chemistry Analyzer using the IL Test Creatinine-0018257240 kit (Instrumentation Laboratory, Italy). Quality control samples indicated a CV = 4.24%, n = 6.

2.5 Sample preparation

Urine and plasma samples were extracted using preconditioned DSC-18 (for urine) and Strata-X^{TM} (for plasma) SPE cartridges, evaporated to approximately 50 μL using a Thermo speedvac and diluted with 200 μL of 10% formic acid v/v in

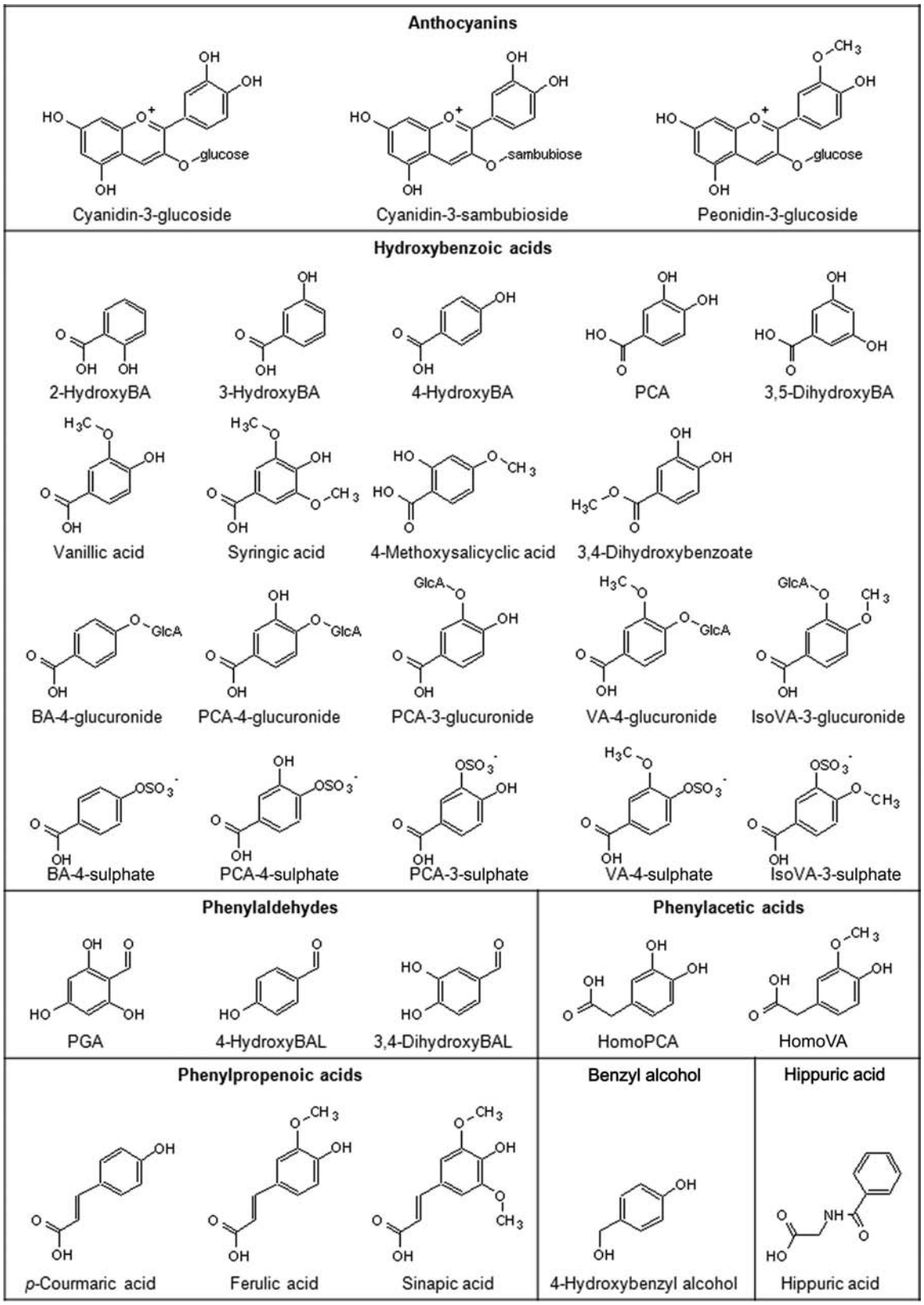

Figure 1. Structure of anthocyanins, degradation products and putative metabolites. BA, benzoic acid; BAL, benzaldehyde; GlcA, glucuronide; PCA, protocatechuic acid; PGA, phloroglucinaldehyde; VA, vanillic acid.

water (pH 2.4). A total of 10 μL of 1 mg/mL scopoletin was added as the volume control standard post-extraction and samples were then syringe filtered. Individual analysis of the urine samples was carried out to establish metabolite variation between participants, while plasma samples were pooled prior to extraction, by taking 66.7 μL of plasma from each participant ($n = 15$; for a total volume 1 mL), thus providing a "snapshot" of the plasma metabolic profile. The extraction efficiency of the SPE methods for C3G and the 17 phenolic metabolites identified in urine and plasma was established as 88.0 ± 5.4% (CV, 8.6 ± 5.4%) for urine and 86.9 ± 9.1% (CV, 8.2 ± 5.9%) for plasma, for $n = 3$ replicates of each analyte.

2.6 HPLC-MS/MS analysis

HPLC-MS/MS analysis was carried out on an Agilent 1200 series HPLC system coupled with an AB Sciex 3200 Q-trap MS/MS, with samples injected onto a Kinetex PFP column. The mobile phase consisted of 0.1% formic acid v/v in water (A) and 0.1% formic acid v/v in acetonitrile (B), at a flow rate of 1.5 mL/min at 0 min, 1 mL/min at 7–14 min and 1.5 mL/min at 14–28 min. The gradient consisted of 1% B at 0 min, 7.5% B at 7 min, 7.6% B at 14 min, 10% B at 17 min, 12% B at 18.5 min, 12.5% B at 20 min, 30% B at 24 min and 90% B at 25–28 min. MS/MS source parameters included curtain gas 40 psi, ionspray voltage –4000 V/+5500 V, temperature 700°C, nebulizer gas and auxiliary gas 60 psi. The system was controlled by Analyst software (v. 1.5, Applied Biosystems/MDS Sciex). Multiple reaction monitoring scans were performed using conditions optimised for the detection of pure standards (Table 1). Metabolites were confirmed on the basis of retention time and the presence of two or more ion transitions. The peak area of the most intense ion transition was determined from the chromatograph and used for quantification. Seven-point calibration curves ranging from 0 to 10 μM for the analysis of the anthocyanin conjugates and from 0 to 50 μM for the analysis of phenolic metabolites were utilised for quantification. The calibration curves were prepared by spiking a standard mixture of the compounds into a matched matrix (pooled blank/baseline urine or plasma from all participants) and were baseline adjusted to account for any interference from analytes present in baseline samples, such as phenolic compounds. For anthocyanin glucuronide and sulfate conjugates, where the corresponding standards were not available, compounds were quantified relative to C3G or P3G (where appropriate).

The HPLC-MS/MS methods were validated for linearity and precision across all metabolites. Six-point standard curves ranging from 1.25 to 20 μM were constructed from analytical standards and injected six times. The precision was established as 1.4–8.2% and linearity of the standard curves was established as $r^2 = 0.991$–1.000 (CV, 0.2%) across the identified metabolites. In addition, there was a between run variation of 9.17% CV for quality control standards injected between every eight samples run.

2.7 Statistics

All statistical analyses were performed using SPSS for Windows (statistical software package version 18.0). Within-subject comparisons were analysed using a repeated-measures general linear model, with Greenhouse-Geisser correction for non-spherical data and Bonferroni adjustment (within-subjects, baseline, 0–1 h, 1–2 h, 2–3 h) for pairwise comparisons. Between-group comparisons were analysed using repeated-measures general linear model, with Bonferroni adjustment (within-subjects, wk 0 and wk 12 at baseline, 0–1 h, 1–2 h, 2–3 h). Unless otherwise stated, results are expressed as mean ± SD and $p \leq 0.05$ was considered significant. Baseline refers to the fasting samples collected prior to the consumption of the anthocyanin bolus. No statistical analyses were possible for the pooled plasma samples ($n = 1$; 15 participants plasma pooled at each individual time point).

3 Results

3.1 Elderberry extract composition

Independent analysis of the commercially available elderberry-derived anthocyanin extract identified 15 phenolic acids, aldehydes or benzoates (hereafter referred to as phenolics) present, accounting for 202.90 mg total phenolics per gram of elderberry extract (Table 2). PCA was identified in the highest concentrations, constituting 68.3% of the total phenolics identified, followed by phloroglucinaldehyde (PGA) (7.7%) and caffeic acid (7.3%). The anthocyanin content of the elderberry bolus was previously established as 250 mg anthocyanin per gram extract [19], predominantly consisting of C3G (53.5%) and cyanidin-3-sambubioside (39.5%) [21]. Elderberries are also reported to contain low amounts of cyanidin-3-sambubioside-5-glucoside (6%) and cyanidin-3,5-diglucoside (1%) [21]. A total proximate analysis of the extract was not conducted to establish the composition of the remaining 55% by weight; however, elderberries have a reported relative composition of 18.4% carbohydrate, 0.7% protein, 0.5% fat, 0.5% organic acids and 6.2% quercetin [21–23].

3.2 Metabolites in urine and plasma

Twenty-eight metabolites were identified in the urine following acute anthocyanin consumption, of which 17 were phenolics and 11 were anthocyanin conjugates (Table 3). Cumulatively, urinary phenolic metabolite concentrations were 60-fold higher (ranging from 145 ± 86 to 7382 ± 5927 nM/mM creatinine at 3-h) than the parent anthocyanins

Table 1. HPLC-MS/MS parameters for the identification of anthocyanins, anthocyanin conjugates and phenolics in human urine after the ingestion of 500 mg elderberry anthocyanins

Compound	Rt (min)	MW	Precursor ion/MS2 fragments (*m/z*)	CE (V)	DP (V)	EP (V)	CXP (V)
Anthocyanins							
Cyanidin-3-glucoside (C3G)	11.6	449.4	449/287[a], 241, 213, 137	+30	+60	+5	+2.5
Cyandin-3-sambubioside	12.0	581.5	581/287[a]	+60	+30	+5	+2.5
Cyanidin-glucuronide	11.7	463.4	463/287[a]	+60	+30	+5	+2.5
Cyanidin-glucuronide	16.5	463.4	463/287[a]	+60	+30	+5	+2.5
C3G-glucoside-disulfate	21.1	609.5	609/287[a]	+60	+30	+5	+2.5
Cyanidin-diglucuronide	9.3	639.5	639/287[a]	+60	+30	+5	+2.5
Peonidin-3-glucoside (P3G)	16.9	463.4	463/301[a]	+60	+30	+5	+2.5
Peonidin-glucuronide	17.0	477.4	477/301[a]	+60	+30	+5	+2.5
P3G-glucuronide	5.8	639.5	639/301[a]	+60	+30	+5	+2.5
P3G-glucuronide	8.2	639.5	639/301[a]	+60	+30	+5	+2.5
P3G-glucuronide	9.4	639.5	639/301[a]	+60	+30	+5	+2.5
Hydroxybenzoic acids							
2-Hydroxybenzoic acid	16.7	138.1	137/93[a], 75, 65	−25	−30	−7	−2
3-Hydroxybenzoic acid	8.1	138.1	137/93[a], 65	−18	−40	−9.5	0
4-Hydroxybenzoic acid	6.5	138.1	137/93[a], 65	−20	−35	−5	0
Protocatechuic acid (PCA)	4.3	154.1	153/108[a], 91, 81	−31	−42	−10	0
3,5-Dihydroxybenzoic acid	4.4	154.1	153/109[a], 67, 65	−16	−35	−9.5	0
Methoxybenzoic acids							
Vanillic acid (VA)	9.4	168.1	167/152[a], 123, 108	−20	−30	−2	0
Methyl-3,4-dihydroxybenzoate	12.9	168.1	167/108[a], 91	−28	−45	−8	0
4-Methoxysalicyclic acid[b]	20.1	168.1	167/123, 108[a], 80	−15	−34	−3	−2
Syringic acid	12.1	198.2	199/155, 140[a], 125, 77	+21	+34	+5	+6
Cinnamic acids							
Caffeic acid	10.4	180.2	179/135[a], 106, 89	−20	−45	−6	−2
Sinapic acid	20.6	224.2	223/208[a], 193, 164, 149	−18	−38	−2	−2
p-Coumaric acid	15.1	164.2	163/119[a], 117, 93	−20	−30	−10	0
Ferulic acid	18.8	194.2	193/178, 149, 134[a]	−20	−30	−6	−2
Aldehydes							
Phloroglucinaldehyde	16	154.1	153/151[a], 125, 107, 83	−20	−88	−7	−2
4-Hydroxybenzaldehyde	8.5	122.1	121/108[a], 92, 65	−31	−47	−7	−10
3,4-Dihydroxybenzaldehyde	5.8	138.1	137/108[a], 92, 81	−30	−40	−10	0
Phenylacetic acids							
Homovanillic acid	9.6	182.2	181/137[a], 122	−10	−43	−2	−4
Homoprotocatechuic acid[c]	4.6	168.1	167/123[a], 108, 95	−20	−20	−8	−2
Conjugated metabolites							
Benzoic acid-4-glucuronide	3	314.2	313/175, 137, 113, 93[a]	−54	−54	−7	−2
PCA-3-glucuronide	4.4	330.2	329/175,153[a], 113, 109	−25	−45	−5	−3
PCA-4-glucuronide	3.3	330.2	329/175, 153[a], 113, 109	−25	−45	−5	−3
VA-4-glucuronide	4.9	344.3	343/175, 167, 152, 113[a]	−20	−60	−4	−2
IsoVA-3-glucuronide	6.2	344.3	343/175, 167, 152, 113[a]	−20	−66	−4	−2
Benzoic acid-4-sulfate	7.5	218.2	217/173, 137[a]	−21	−31	−3	−2
PCA-sulfate	6.3	234.2	233/189, 153, 109[a], 97	−30	−38	−3	−2
VA-sulfate	8.8	248.2	247/167[a], 152, 123, 108	−20	−33	−4	−2
Benzyl alcohols							
4-Hydroxybenzyl alcohol	4.4	124.1	123/105, 77[a]	−20	−24	−10	−2
Hippuric acids							
Hippuric acid	6.3	179.2	178/134[a], 132, 77	−18	−40	−3	0
Internal standards							
Phloridzin	24.1	436.4	435/273[a], 167, 123, 81	−26	−40	−4	−2
Scopoletin	18.4	192.2	191/148, 120, 104[a]	−34	−35	−10	0
			193/133[a], 122, 94	+27	+56	+4	+4

A dwell time of 50 ms was used across all transitions.
a) MRM transition used for quantitation.
b) Also commonly known as 3,4-dihydroxyphenylacetic acid.
c) Also commonly known as 2-hydroxy-4-methoxybenzoic acid.
C3G, cyanidin-3-glucoside; CE, collision energy corresponding to parent/daughter fragments used for MRM quantitation; DP, declustering potential; MW, molecular weight; P3G, peonidin-3-glucoside; PCA, protocatechuic acid; Rt, retention time; VA, vanillic acid; V, Volts.

Table 2. Phenolics present within 1 g elderberry extract (25% anthocyanin content, from Artemis International)

Phenolic compounds	Concentration (mg/g extract)
Protocatechuic acid	138.59 ± 4.48
Phloroglucinaldehyde	15.65 ± 1.18
Caffeic acid	14.74 ± 1.10
4-Hydroxybenzoic acid	11.28 ± 0.28
p-Courmaric acid	9.43 ± 0.32
Vanillic acid	2.70 ± 0.13
4-Hydroxybenzaldehyde	2.51 ± 0.01
Methyl-3,4-dihydroxybenzoate	1.85 ± 0.14
Gallic acid	1.54 ± 1.18
Ferulic acid	1.39 ± 0.08
3,4-Dihydroxybenzaldehyde	1.37 ± 0.02
Sinapic acid	1.10 ± 0.22
Homoprotocatechuic acid	0.38 ± 0.03
Isovanillic acid	0.30 ± 0.01
Methyl gallate	0.04 ± 0.00

Data represented as mean ± SD, $n = 3$.

(which ranged from 0.1 ± 0.3 to 309 ± 673 nM/mM creatinine at 3 h). In plasma, 21 metabolites were identified, of which 17 were phenolics and four were anthocyanin conjugates (Table 4), and similarly, the cumulative phenolic metabolites were present at 45-fold higher concentrations (at a range of 2–358 nM at 3 h) than their parent anthocyanins (at a range of 2–12 nM at 3 h).

The metabolites identified differed substantially between urine and plasma; plasma contained aldehydes (4-hydroxybenzaldehyde and 3,4-dihydroxybenzaldehyde), benzoic acids (4-methoxysalicyclic acid (also known as 2-hydroxy-4-methoxybenzoic acid) and benzoic acid-4-sulfate) and methyl derivatives (methyl-3,4-dihydroxybenzoate and syringic acid), all of which were absent from urine. By comparison, urine contained more anthocyanin conjugates (sulfate, glucuronide and methyl conjugates), cinnamic acids (*p*-courmaric and sinapic acid), phenylacetic acids (homoVA and homoPCA (also known as 3,4-dihydroxyphenylacetic acid)), 3,5-dihydroxybenzoic acid and 4-hydroxybenzyl alcohol, and all of these conjugates were absent from the plasma samples.

Anthocyanins reached maximal urinary concentrations of 548 ± 219 nM/mM creatinine 3 h following intake of the elderberry extract (Table 3). In 3-h post intake urine samples, C3G was present at concentrations of 309 ± 673 nM/mM creatinine and cyanidin-3-sambubioside was identified at concentrations reaching 99 ± 116 nM/mM creatinine. In addition, nine conjugates of cyanidin were putatively identified in urine samples: two cyanidin-glucuronides, cyanidin-diglucuronide, C3G-disulfate, P3G, peonidin-glucuronide and three P3G-glucuronides, reaching cumulative concentrations of 140 ± 33 nM/mM creatinine 3-h post-bolus (Table 3). Within pooled plasma samples, anthocyanins reached a maximum concentration of 34 nM 2-h post-bolus, where C3G and cyanidin-3-sambubioside were present at concentrations of 7 and 16 nM, respectively. Only two anthocyanin conjugates, namely cyanidin-glucuronide and peonidin-glucuronide, were identified within the plasma and were found at maximum concentrations of 2 and 9 nM at 2- and 3-h post-bolus, respectively (Table 4).

The maximum cumulative concentration of urinary phenolic metabolites was observed at 3 h, where they were present at concentrations of 33 185 ± 2549 nM/mM creatinine, representing a 23 448 nM/mM creatinine increase from baseline values. A high variability was observed in the concentrations of individual phenolic metabolites, where homoVA and VA reached the highest concentrations at 3 h, representing levels of 7382 ± 5927 and 4345 ± 3205 nM/mM creatinine, respectively, while PCA-4-glucuronide and *p*-courmaric acid were present in the lowest concentrations, reaching 145 ± 86 and 203 ± 144 nM/mM creatinine, respectively (Table 3). Within plasma, total concentrations of metabolites cumulatively reached 1238 nM, 3-h post-bolus, representing an 847 nM increase from baseline values (Table 4); where PCA-sulfate reached the highest concentration (358 nM), while 4-methoxysalicyclic acid was identified at the lowest concentration (2 nM) within plasma. The maximum plasma concentration for the majority of the compounds was at 2 h; however, PCA, 3,5-dihydroxybenzoic acid, 4-methoxysalicyclic acid, PCA-4-glucuronide, VA-4-glucuronide, PCA-3-, and 4-sulfate and benzoic acid-4-sulfate had not reached maximum concentration by 3 h.

There was no significant difference in the concentration of metabolites in post-prandial urine collected prior to (wk 0) and following 12-wk repeated anthocyanin dosing (Fig. 2).

4 Discussion

To the best of our knowledge, the identified anthocyanin conjugates (Tables 3 and 4) represent the largest number of metabolic conjugates of elderberry anthocyanins identified to date [24–27]. In addition, despite creatinine adjustment, the concentration of total anthocyanins quantified in urine (548 ± 219 nM/mM creatinine) is amongst the highest reported [8, 24, 28–32]. A total of 28 metabolites were identified in urine (11 anthocyanin conjugates and 17 phenolic acid metabolites, Table 3) and 21 in plasma (four anthocyanin conjugates and 17 phenolic acid metabolites, Table 4). The most predominant conjugation reaction for anthocyanins was glucuronidation, followed by methylation, with many anthocyanins undergoing multiple conjugation reactions. Peonidin was not present in the elderberry extract and therefore its identification is indicative of the methylation of cyanidin.

PCA and PGA, the degradation products of C3G, were detected in both urine and plasma. PCA reached concentrations of 1534 ± 1232 nM/mM creatinine and 24 nM after 3 h in urine and plasma, respectively (Tables 3 and 4). PCA is

Table 3. Concentration of anthocyanins, anthocyanin conjugates and phenolics in urine samples following acute anthocyanin intake (500 mg anthocyanins)

Compound	Concentration (nM/mM creatinine) (% of total metabolites)[a]					Previously described in urine
	Baseline	*t* (0–1 h)	*t* (1–2 h)	*t* (2–3 h)	Total	
Cyanidin-3-glucoside (C3G)	ND	5 ± 7 (0.05)	168 ± 317 (0.76)	309 ± 673 (0.93)	481 ± 385	[18, 24–27, 31, 32]
Cyanidin-3-sambubioside	ND	2 ± 4 (0.02)	42 ± 55 (0.19)	99 ± 116 (0.30)	142 ± 75	[24–27, 31, 32]
Cyanidin-glucuronide[b]	ND	2 ± 5 (0.02)	25 ± 44 (0.12)	41 ± 39 (0.12)	68 ± 33	[18]
Cyanidin-glucuronide[b]	ND	0.1 ± 0.2 (0)	1 ± 2 (0.01)	4 ± 4 (0.01)	5 ± 3	[18]
Cyanidin-diglucuronide[b]	ND	0 ± 0 (0)	0.1 ± 0.3 (0)	0.1 ± 0.3 (0)	0.3 ± 0.2	
C3G-disulfate[b]	ND	ND	1 ± 1 (0)	1 ± 2 (0)	2 ± 1	
Peonidin-3-glucoside (P3G)	ND	1 ± 1 (0)	9 ± 10 (0.04)	18 ± 21 (0.05)	27 ± 13	[18, 24]
Peonidin-glucuronide[c]	ND	2 ± 5 (0.02)	35 ± 57 (0.16)	69 ± 61 (0.21)	105 ± 50	[18, 24]
P3G-glucuronide[c]	ND	0 ± 0.1 (0)	1 ± 2 (0.01)	3 ± 3 (0.01)	4 ± 2	[18]
P3G-glucuronide[c]	ND	0 ± 0.1 (0)	1 ± 1 (0)	1 ± 1 (0)	2 ± 1	[18]
P3G-glucuronide[c]	ND	0.1 ± 0.3 (0)	2 ± 2 (0.01)	4 ± 4 (0.01)	6 ± 3	[18]
4-Hydroxybenzoic acid	904 ± 694 (9.28)	1386 ± 941 (13.44)	1897 ± 1026 (8.54)[d]	1985 ± 985 (5.89)[d]	6172 ± 998	[13, 14, 18]
Protocatechuic acid (PCA)	70 ± 52 (0.72)	155 ± 209 (1.50)	802 ± 768 (3.61)	1534 ± 1232 (4.55)[d]	2560 ± 929	[12–14, 17, 18]
3,5-Dihydroxybenzoic acid	258 ± 261 (2.65)	327 ± 220 (3.17)	1015 ± 556 (4.57)	1862 ± 1016 (5.52)[d]	3462 ± 878	
Vanillic acid (VA)	215 ± 145 (2.21)	321 ± 275 (3.11)	2110 ± 2031 (9.51)	4345 ± 3205 (12.88)[d]	6991 ± 2511	[13, 14, 17, 18]
Phloroglucinaldehyde	3 ± 3 (0.03)	37 ± 81 (0.35)	309 ± 368 (1.39)[d]	602 ± 632 (1.79)[d]	951 ± 433	[18]
HomoVA	3695 ± 2647(37.95)	3508 ± 2390 (34.03)	5403 ± 3880 (24.34)	7382 ± 5927 (21.88)[e]	19 989 ± 4172	[13, 17]
HomoPCA	870 ± 521 (8.93)	1095 ± 902 (10.62)	1453 ± 1023 (6.55)	1945 ± 1302 (5.77)[d]	5363 ± 1037	[13, 17]
4-Hydroxybenzyl alcohol	716 ± 321 (7.35)	790 ± 420 (7.67)	1074 ± 564 (4.84)	1397 ± 700 (4.14)[d]	3977 ± 575	
p-Courmaric acid	77 ± 82 (0.79)	76 ± 70 (0.74)	132 ± 94 (0.59)	203 ± 144 (0.6)[d]	488 ± 112	[13, 14, 17]
Ferulic acid	141 ± 131 (1.44)	408 ± 754 (3.95)	1338 ± 1171 (6.03)[d]	1517 ± 1344 (4.5)[d]	3404 ± 1116	[13, 14, 17, 18]
Sinapic acid	110 ± 176 (1.13)	210 ± 232 (2.03)	1120 ± 903 (5.05)[d]	1205 ± 943 (3.57)[d]	2645 ± 826	[14]
PCA-3-glucuronide	32 ± 32 (0.33)	30 ± 32 (0.29)	163 ± 157 (0.73)	349.9 ± 190 (1.04)[d]	576 ± 179	[18]
PCA-4-glucuronide	44 ± 33 (0.45)	36 ± 37 (0.34)	91 ± 71 (0.41)[d]	145.4 ± 86 (0.43)[d]	315 ± 74	[18]
IsoVA-3-glucuronide	271 ± 479 (2.79)	230 ± 352 (2.23)	660 ± 615 (2.97)[d]	1226.3 ± 774 (3.64)[d]	2387 ± 692	[18]
VA-4-glucuronide	427 ± 307 (4.38)	306 ± 157 (2.97)	1154 ± 750 (5.2)	2296 ± 946 (6.81)[d]	4183 ± 1005	[18]
PCA-sulfate[f]	183 ± 291 (1.88)	153 ± 182 (1.48)	885 ± 1215 (3.99)	2014 ± 1765 (5.97)[d]	3235 ± 1303	[18]
VA-sulfate[g]	1723 ± 1418 (17.69)	1231 ± 980 (11.94)	2314 ± 2114 (10.42)	3176 ± 2478 (9.41)[e]	8444 ± 1938	[18]
Total anthocyanins	0 ± 0	12 ± 4	283 ± 107	548 ± 219	842 ± 123	
Total phenolic metabolites	9737 ± 1173	10 298 ± 1110	21 920 ± 1796	33 185 ± 2549	75 141 ± 1843	

2- and 3-hydroxybenzoic acid, benzoic acid-4-glucuronide and hippuric acid were identified but showed no increase from baseline concentrations.
a) Values represent mean concentrations ± SD with percentage of total metabolites (across each time point) represented in brackets, $n = 15$; all samples have been adjusted for creatinine concentration.
b) No standard available, quantified relative to C3G.
c) No standard available, quantified relative to P3G.
d) Significantly increased compared to baseline values (repeated measures, $p < 0.05$).
e) Significance indicated by ANOVA with Greenhouse–Geisser adjustment but post hoc tests did not reach significance ($p > 0.05$).
f) PCA-3-sulfate co-elutes with PCA-4-sulfate and concentrations represent the combined total.
g) Vanillic acid-4-sulfate co-elutes with isovanillic acid-3-sulfate and concentrations represent the combined total.
C3G, cyanidin-3-glucoside; ND, not detected; P3G, peonidin-3-glucoside; PCA, protocatechuic acid; VA, vanillic acid.

Table 4. Concentration of anthocyanins, anthocyanin conjugates and phenolics in pooled plasma samples following acute anthocyanins intake (500 mg anthocyanins)

Compound	Concentration (nM) (% of total metabolites)[a)]					Previously described in plasma
	Baseline	1 h	2 h	3 h	Total	
Cyanidin-3-glucoside[d)]	ND	4 (0.63)	7 (0.82)	5 (0.38)	16	[18, 25]
Cyanidin-3-sambubioside[d)]	ND	10 (1.44)	16 (1.85)	12 (0.91)	38	[18, 25]
Cyanidin-glucuronide[b),d)]	ND	ND	2 (0.27)	2 (0.18)	5	
Peonidin-glucuronide[c),d)]	ND	2 (0.24)	8 (0.96)	9 (0.71)	19	
Protocatechuic acid (PCA)[d)]	11 (2.71)	12 (1.72)	14 (1.65)	24 (1.93)	61	[14, 17, 18, 33]
3,5-Dihydroxybenzoic acid[d)]	20 (5.05)	18 (2.67)	39 (4.39)	49 (3.92)	126	
Vanillic acid (VA)[d)]	6 (1.64)	35 (5.13)	62 (7.03)	58 (4.60)	161	[14, 17, 18]
Syringic acid[d)]	5 (1.16)	17 (2.44)	22 (2.56)	15 (1.20)	59	
4-Methoxysalicyclic acid[d)]	1 (0.23)	0 (0.05)	1 (0.07)	2 (0.13)	3	
Methyl-3,4-dihydroxybenzoate[d)]	3 (0.71)	2 (0.33)	6 (0.68)	6 (0.48)	17	[18]
Phloroglucinaldehyde[d)]	4 (1.05)	21 (3.02)	103 (11.69)	72 (5.66)	199	[18]
3,4-Dihydroxybenzaldehyde[d)]	17 (4.43)	18 (2.65)	23 (2.62)	21 (1.68)	80	
4-Hydroxybenzaldehyde[d)]	97 (24.74)	146 (21.29)	182 (20.80)	150 (11.85)	575	
Ferulic acid[d)]	8 (1.97)	27 (3.92)	28 (3.23)	21 (1.70)	84	[15, 18]
PCA-3-glucuronide[d)]	3 (0.79)	8 (1.10)	15 (1.75)	15 (1.22)	41	[18]
PCA-4-glucuronide[d)]	6 (1.43)	4 (0.52)	10 (1.14)	14 (1.11)	33	[18]
IsoVA-3-glucuronide[d)]	10 (2.65)	14 (1.97)	24 (2.71)	22 (1.71)	69	
VA-4-glucuronide[d)]	16 (4.21)	33 (4.85)	78 (8.91)	120 (9.49)	248	
PCA-sulfate[d),e)]	92 (23.55)	74 (10.78)	115 (13.17)	358 (28.31)	640	[18]
VA-sulfate[d),f)]	27 (6.79)	161 (23.43)	23 (2.67)	93 (7.36)	304	[18]
Benzoic acid-4-sulfate[d)]	66 (16.89)	81 (11.82)	97 (11.02)	196 (15.48)	439	
Total anthocyanins	ND	16	34	28	78	
Total phenolic metabolites	391	670	842	1238	3140	

2-, 3- and 4-hydroxybenzoic acid, benzoic acid-4-glucuronide and hippuric acid were identified but showed no increase from baseline concentrations.
a) Values represent concentrations of a pooled sample ($n = 1$) from the samples of 15 participants, with percentage of total metabolites across each timepoint represented in brackets.
b) No standard available, quantified relative to cyanidin-3-glucoside.
c) No standard available, quantified relative to peonidin-3-glucoside.
d) Visual increase in concentration compared to baseline values (no statistical analysis possible as a result of using a pooled sample).
e) PCA-3-sulfate co-elutes with PCA-4-sulfate and concentrations represent the combined total.
f) Vanillic acid-4-sulfate co-elutes with isovanillic acid-3-sulfate and concentrations represent the combined total.
PCA, protocatechuic acid; ND, not detected; VA, vanillic acid.

naturally present in many fruits, including elderberries, and in the present study was the most abundant phenolic in the intervention foods, present at concentrations of 138.59 mg/g extract (Table 2). The presence of PCA in the background diet may have therefore led to an overestimation of anthocyanin-derived PCA in this and previous interventions [12–14]. PGA was identified at maximum concentrations of 602 ± 632 nM/mM creatinine at 3 h and 103 nM at 2 h in urine (Table 3) and plasma (Table 4), respectively. To the best of our knowledge, this is the first study where PGA has been identified in human blood or urine following a fruit-derived anthocyanin intervention.

The degradation products of anthocyanins appeared to undergo further phase I and II metabolism as apparent by the identification of various methylated (VA), glucuronidated (PCA-3-glucuronide, PCA-4-glucuronide, VA-4-glucuronide and isoVA-3-glucuronide), sulfated (PCA-sulfate and VA-sulfate) and de/hydroxylated (4-hydroxybenzoic acid and 3,5-dihydroxybenzoic acid) products. VA-4-glucuronide demonstrated the largest increase from baseline values in urine, reaching concentrations of 2296 ± 946 nM/mM creatinine 3-h post-bolus (Table 3) and PCA-sulfate demonstrated the largest increase in concentration in plasma, reaching 358 nM 3 h following intake (Table 4). 4-Hydroxybenzoic acid and VA have previously been identified within biological samples following bilberry-lingonberry purée [13], cranberry juice [15] and strawberry consumption [33], while to the best of our knowledge, the glucuronidated and sulfated metabolites of PCA, VA and hydroxybenzoic acid have not been previously identified following a fruit-derived anthocyanin intervention. Anthocyanins also appeared to be metabolised to phenylpropenoic, phenylacetic and phenylalcohol structures, as homoVA, homoPCA, 4-hydroxybenzyl alcohol, *p*-courmaric acid, ferulic acid and sinapic acid were established

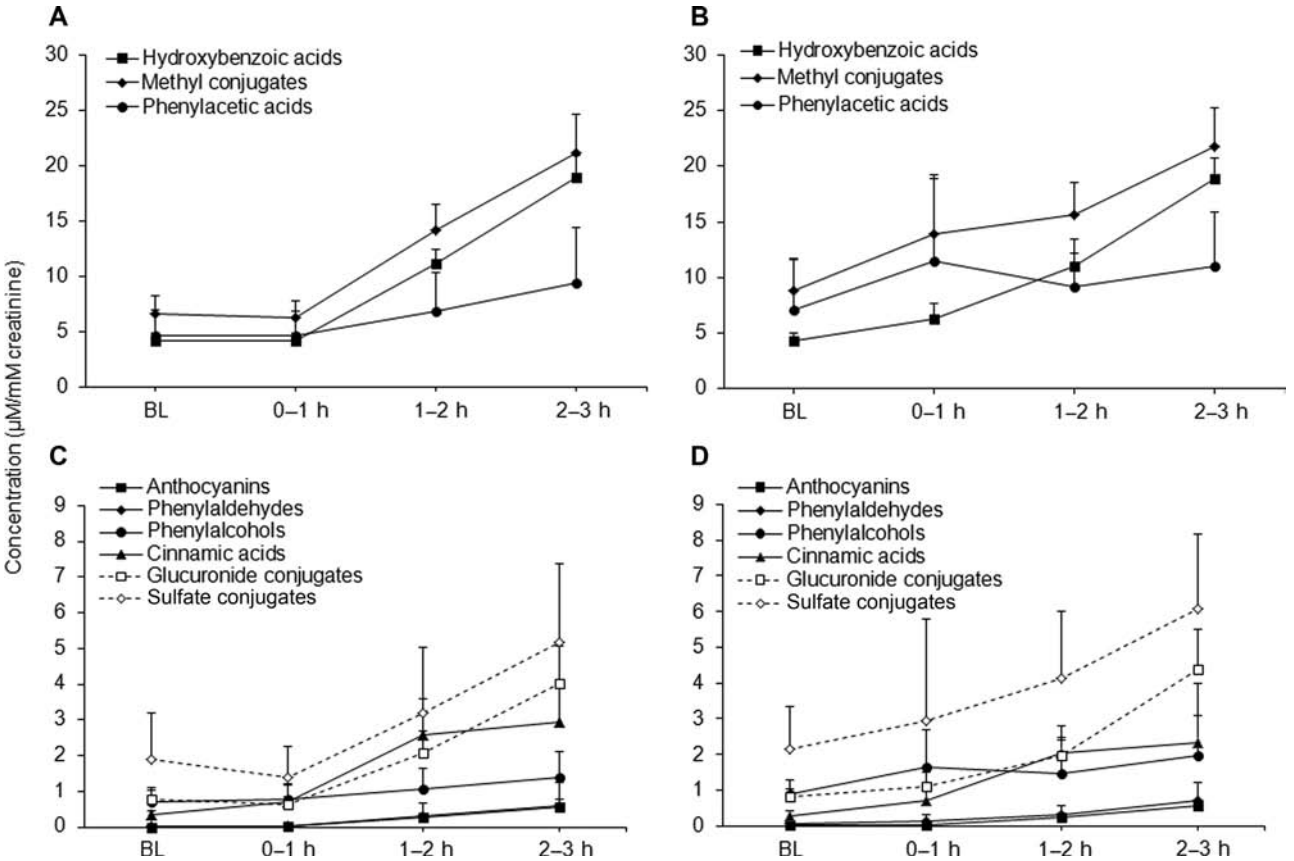

Figure 2. Baseline (BL) and post-bolus (0–3 h) anthocyanin and metabolite concentrations in urine. (A) Concentration of major anthocyanin metabolites (>8 μM/mM creatinine) identified following a single bolus and (B) following 12-wk supplementation; (C) concentration of minor anthocyanin metabolites (<8 μM/mM creatinine) identified following a single bolus and (D) following 12-wk supplementation; metabolites are classified by conjugation/structure where hydroxybenzoic acids include 4-hydroxybenzoic acid, protocatechuic acid (PCA), 3,5-dihydroxybenzoic acid, vanillic acid (VA), PCA-3-glucuronide, PCA-4-glucuronide, VA-4-glucuronide, isoVA-3-glucuronide, PCA-sulfate and VA-sulfate; methyl conjugates include VA, homoVA, ferulic acid, sinapic acid, VA-4-glucuronide, isoVA-3-glucuronide and VA-sulfate; phenylacetic acids include homoPCA and homoVA; phenylpropenoic acids include *p*-courmaric acid, ferulic acid and sinapic acid; anthocyanins include cyanidin-3-glucoside (C3G), cyanidin-3-sambubioside, cyanidin-glucuronides, cyanidin-diglucuronide, C3G-disulfate, peonidin-3-glucoside (P3G), peonidin-glucuronide and P3G-glucuronides; phenylaldehydes include phloroglucinolaldehyde; phenylalcohols include 4-hydroxybenzyl alcohol; glucuronide conjugates include PCA-3-glucuronide, PCA-4-glucuronide, VA-4-glucuronide and isoVA-3-glucuronide; and sulfate conjugates include PCA-sulfate and VA-sulfate.

to increase from baseline concentrations in post-prandial urine samples (Table 3). Similarly, syringic acid, 4-methoxysalicyclic acid, methyl-3,4-dihydroxybenzoate, 3,4-dihydroxybenzaldehyde, 4-hydroxybenzaldehyde and ferulic acid were detected at increased concentrations in post-prandial plasma samples (Table 4). Of these, homoVA, homoPCA, *p*-courmaric acid, ferulic acid and sinapic acid have been previously detected within urine [13,14], and ferulic acid has previously been identified within plasma [15] following anthocyanin interventions feeding phenolic-rich products.

The phenylacetic structures, homoVA and homoPCA, which together provided a 4762 nM/mM creatinine increase from baseline urinary levels, appeared to be major anthocyanin metabolites in urine but were absent from plasma. Similarly, *p*-courmaric acid, sinapic acid, 3,5-dihydroxybenzoic acid and 4-hydroxybenzyl alcohol were also identified within urine, but were absent from the plasma. The presence of metabolites in the urine but not the plasma suggests some compounds were below the detection limit of the methodology but became detectable as they were concentrated by the kidneys and excreted into the urine.

The plasma contained 4-hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, 4-methoxysalicyclic acid, benzoic acid-4-sulfate, methyl-3,4-dihydroxybenzoate and syringic acid, which were absent from urine samples. The appearance of metabolites in the plasma but not in the urine suggests that some metabolites may have been metabolised further in various tissues, or were eliminated by the biliary route, and either metabolised further by colonic bacteria and reabsorbed or excreted. This is supported by the identification of methyl-3,4-dihydroxybenzoate and 4-methoxysalicyclic acid within faecal samples following our recent ^{13}C-C3G study [18]. Conjugates

of phenolic degradation products of anthocyanins appear in the circulation as early as 1-h post-consumption (Table 4) [18], suggesting they do not follow the commonly reported elimination profiles of other flavonoids, such as quercetin and epicatechin, which require bacterial catabolism to liberate free phenolic intermediates, and therefore appear much later in the circulation [6, 34–38]. The early appearance of phenolic metabolites in the present study likely results from the instability of anthocyanins at physiological pH (pH 5–7) [10, 11, 38], resulting in their rapid degradation and metabolism within the proximal and distal small intestine. In addition, the presence of bacteria within the small intestine will also contribute to the appearance of these metabolites [39].

In the present study, a number of unconjugated metabolites, including PCA, *p*-courmaric acid and homoPCA, were identified. Although flavonoids are generally reported in the circulation as conjugated metabolites [40], unconjugated phenolic acids have been reported at levels similar to those detected within the present intervention study [13, 14, 18].

Several other putative phenolic metabolites were explored (such as hippuric acid, 2-, 3- and 4-hydroxybenzoic acid and benzoic acid-4-glucuronide) as they have been previously reported as possible anthocyanin- or flavonoid-derived metabolites [13–15, 18, 33]; however, these compounds were not shown to increase in concentration from baseline levels in the present study, and are therefore likely predominantly derived from other source in the "background" diet or endogenous protein turnover [41]. Anthocyanin metabolism may however contribute to their concentrations in biofluids to some extent.

The present study is the first study to the best of our knowledge to look at post-prandial phenolic metabolite excretion following sustained anthocyanin intake. A previous flavonoid intervention found that daily quercetin supplementation over 2 wk resulted in increased plasma quercetin concentrations [42], suggesting reduced clearance following repeated dosing. However, there was no significant impact of 12-wk daily intake of anthocyanins on urinary metabolite excretion in the present investigation (Fig. 2). This is in accordance with a previous 8-wk berry intervention that found no effect of sustained dosing on fasting polyphenol concentrations [17], which suggests there is little risk of bioaccumulation or toxicity associated with the metabolism and clearance of anthocyanins. There was an increase in plasma anthocyanins (78 nM at wk 0 versus 100 nM at wk 12) and decrease in hydroxybenzoic acids (3 nM at wk 0 versus 2 nM at wk 12) following sustained dosing (Fig. 3); however, no statistical comparisons were possible due to the use of pooled plasma samples ($n = 1$). This observation was not apparent in the urine analysis.

In contrast to our recent intervention study where we fed pure, ^{13}C-labeled synthetic C3G [18], here we administered a dose that more closely mimics a dietary source of anthocyanins, such as that found in a single serving of some berries [19, 21]. While the present study therefore provides a comprehensive account of the metabolism of anthocyanins following acute and chronic consumption of elderberries, there are certain limitations with this work. Primarily, although the use of a food source of anthocyanins, as opposed to a pure chemical, better represents habitual dietary intake, it complicates the elucidation of anthocyanin-derived metabolites.

Phenolic compounds are ubiquitous within plants [43, 44] and are present in quantities of 0.04–138.59 mg/g within the elderberry extract (Table 2) and breakfast provided. As the intervention meal was fed ad libitum, and intakes were not reported, the phenolic intake could not be quantitatively established with accuracy. However, database analysis of the main ingredients present within the breakfast (unprocessed wheat, barley and maize) indicated that the foods likely contained six of the metabolites identified in the present study, at concentrations ranging from 0.01 to 0.15 mg/100 g FW [21], presuming the phenolics are not degraded during processing. In addition, some of the metabolites identified in the present study are typically associated with the colonic metabolites of other phenolic compounds [11] and could therefore be derived from prior consumption of a range of polyphenolic compounds other than anthocyanins. For example, propenoic acids and phenylacetic acids are reported metabolites of flavonols and procyanidins [34, 45]. However, only metabolites that increased from fasted/baseline levels were reported presently and in addition, 19 and 11 of the metabolites identified in urine and plasma, respectively, including ferulic acid and phase II conjugates of PCA and VA, were recently reported in a ^{13}C-labeled anthocyanin feeding study [18]. Together this suggests that the metabolites identified within the present study were derived, at least in part, from the consumed anthocyanins. Nevertheless, without the use of a labeled anthocyanin, it is not possible to categorically define the origin of all phenolics within the diet, and therefore the contribution of phenolic acids from other sources consumed prior to the fast cannot be definitively excluded.

The samples collected in the present study were limited to 3-h post-bolus sampling, and longer duration sampling following acute exposure would have allowed us to conduct a more comprehensive pharmacokinetic analysis. In addition, there are a number of substances known to interfere with the creatinine assay [46]; however, we found no reports in the literature of anthocyanins interfering with creatinine measurements. Anthocyanins do absorb at the same wavelength as the assay substrate (510/570 nm), however they would have minimal absorbance intensity under the alkaline conditions of the assay [47] and are therefore likely to have minor impact on assay sensitivity. The collection and measurement of complete urine voids would have allowed total recoveries to be calculated as opposed to reporting creatinine-adjusted concentrations. Finally, as polyphenol metabolites are generally more concentrated in the urine, and urinary clearance generally reflects the circulating plasma pool of polar metabolites, plasma samples were pooled to reduce analytical costs. This may be seen as a further limitation of the present analysis

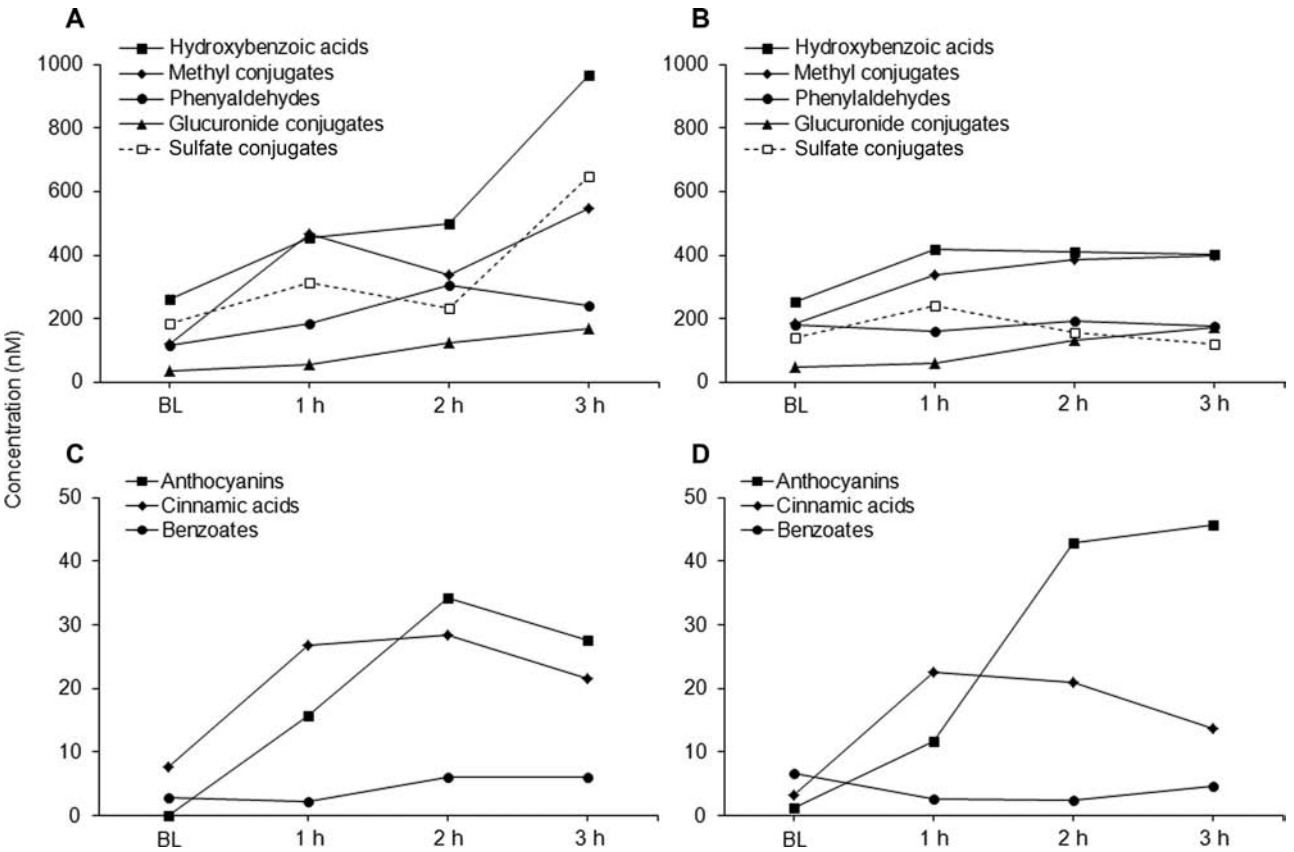

Figure 3. Baseline (BL) and post-bolus (0–3 h) anthocyanin and metabolite concentrations in plasma. (A) Concentration of major anthocyanin metabolites (>150 nM) identified following a single bolus and (B) following 12-wk supplementation; (C) concentration of minor anthocyanin metabolites (<150 nM) identified following a single bolus and (D) following 12-wk supplementation; metabolites are classified by conjugation/structure where hydroxybenzoic acids include protocatechuic acid (PCA), 3,5-dihydroxybenzoic acid, vanillic acid (VA), syringic acid, 4-methoxysalicyclic acid, PCA-3-glucuronide, PCA-4-glucuronide, VA-4-glucuronide, isoVA-3-glucuronide, PCA-sulfate and VA-sulfate; methyl conjugates include VA, syringic acid, 4-methoxysalicyclic acid, methyl-3,4-dihydroxybenzoate, ferulic acid, VA-4-glucuronide, isoVA-3-glucuronide and VA-sulfate; phenylaldehydes include phloroglucinaldehyde, 4-hydroxybenzaldehyde and 3,4-dihydroxybenzaldehyde; glucuronide conjugates include PCA-3-glucuronide, PCA-4-glucuronide VA-3-glucuronide and isoVA-3-glucuronide; sulfate conjugates include PCA-sulfate, VA-sulfate and benzoic acid-4-sulfate; anthocyanins include cyanidin-3-glucoside, cyanidin-3-sambubioside, cyanidin-glucuronide and peonidin-glucuronide; cinnamic acids include ferulic acid; and benzoates include methyl-3,4-dihydroxybenzoate.

as it reduces the intrinsic variability in clearance kinetics between participants. In order to determine whether there was an increase in anthocyanin recovery and decrease in hydroxybenzoic acid recovery following sustained dosing (Fig. 3), the present study would need to have analysed participant samples individually.

In conclusion, the present study identified a greater number of anthocyanin metabolites and higher metabolite concentrations than previously reported, with phenolic metabolites identified at 60- and 45-fold higher concentrations than their parent compounds in urine and plasma, respectively. No significant difference in metabolism or clearance was identified following chronic anthocyanin consumption, suggesting there is no alteration in metabolic processing following 12 wk intake versus acute exposure. These results therefore indicate that anthocyanins degrade and are extensively metabolised in vivo, suggesting that the accumulation of multiple phenolic metabolites may ultimately be responsible for the reported bioactivity of anthocyanins.

The authors would like to thank the participants for taking part in the study and Nigel Botting, Qingzhi Zhang, K. Saki Raheem and David O'Hagan for their work on the chemical synthesis of the phenolic conjugate standards (the synthesis work was supported by a UK Biotechnology and Biological Sciences Research Council Diet and Health Research Industry Club grant; BB/I006028/1).

The authors have declared no conflict of interest.

5 References

[1] Cassidy, A., O'Reilly, É. J., Kay, C., Sampson, L. et al., Habitual intake of flavonoid subclasses and incident hypertension in adults. *Am. J. Clin. Nutr.* 2011, *93*, 338–347.

[2] Mink, P. J., Scrafford, C. G., Barraj, L. M., Harnack, L. et al., Flavonoid intake and cardiovascular disease mortality: a prospective study in postmenopausal women. *Am. J. Clin. Nutr.* 2007, *85*, 895–909.

[3] Cassidy, A., Mukamal, K. J., Liu, L., Franz, M. et al., High anthocyanin intake is associated with a reduced risk of myocardial infarction in young and middle-aged women. *Circulation* 2013, *127*, 188–196.

[4] Xu, J.-W., Ikeda, K., Yamori, Y., Cyanidin-3-glucoside regulates phosphorylation of endothelial nitric oxide synthase. *FEBS Lett.* 2004, *574*, 176–180.

[5] Edirisinghe, I., Banaszewski, K., Cappozzo, J., McCarthy, D. et al., Effect of black currant anthocyanins on the activation of endothelial nitric oxide synthase (eNOS) in vitro in human endothelial cells. *J. Agric. Food Chem.* 2011, *59*, 8616–8624.

[6] Manach, C., Williamson, G., Morand, C., Scalbert, A. et al., Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* 2005, *81*, 230S–242S.

[7] Williamson, G., Manach, C., Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *Am. J. Clin. Nutr.* 2005, *81*, 243S–255S.

[8] Kay, C. D., Mazza, G. J., Holub, B. J., Anthocyanins exist in the circulation primarily as metabolites in adult men. *J. Nutr.* 2005, *135*, 2582–2588.

[9] Kay, C. D., The future of flavonoid research. *Br. J. Nutr.* 2010, *104*, S91–S95.

[10] Gonzalez-Barrio, R., Christine, E., Crozier, A., Colonic catabolism of ellagitannins, ellagic acid and raspberry anthocyanins: in vivo and in vitro studies. *Drug Metab. Dispos.* 2011, *39*, 1680–1688.

[11] Williamson, G., Clifford, M. N., Colonic metabolites of berry polyphenols: the missing link to biological activity? *Br. J. Nutr.* 2010, *104*(Suppl 3), S48–S66.

[12] Vitaglione, P., Donnarumma, G., Napolitano, A., Galvano, F. et al., Protocatechuic acid is the major human metabolite of cyanidin-glucosides. *J. Nutr.* 2007, *137*, 2043–2048.

[13] Nurmi, T., Mursu, J., Heinonen, M., Nurmi, A. et al., Metabolism of berry anthocyanins to phenolic acids in humans. *J. Agric. Food Chem.* 2009, *57*, 2274–2281.

[14] Russell, W. R., Scobbie, L., Labat, A., Duthie, G. G., Selective bio-availability of phenolic acids from Scottish strawberries. *Mol. Nutr. Food Res.* 2009, *53*, S85–S91.

[15] Zhang, K., Zuo, Y., GC-MS determination of flavonoids and phenolic and benzoic acids in human plasma after consumption of cranberry juice. *J. Agric. Food Chem.* 2004, *52*, 222–227.

[16] Valentova, K., Stejskal, D., Bednar, P., Vostalova, J. et al., Biosafety, antioxidant status, and metabolites in urine after consumption of dried cranberry juice in healthy women: a pilot double-blind placebo-controlled trial. *J. Agric. Food Chem.* 2007, *55*, 3217–3224.

[17] Koli, R., Erlund, I., Jula, A., Marniemi, J. et al., Bioavailability of various polyphenols from a diet containing moderate amounts of berries. *J. Agric. Food Chem.* 2010, *58*, 3927–3932.

[18] Czank, C., Cassidy, A., Zhang, Q., Morrison, D. J. et al., Human metabolism and elimination of the anthocyanin, cyanidin-3-glucoside: a 13C-tracer study. *Am. J. Clin. Nutr.* 2013, *97*, 995–1003.

[19] Curtis, P. J., Kroon, P. A., Hollands, W. J., Walls, R. et al., Cardiovascular disease risk biomarkers and liver and kidney function are not altered in postmenopausal women after ingesting an elderberry extract rich in anthocyanins for 12 weeks. *J. Nutr.* 2009, *139*, 2266–2271.

[20] Zhang, Q., Raheem, K. S., Botting, N. P., Slawin, A. M. Z. et al., Flavonoid metabolism: the synthesis of phenolic glucuronides and sulfates as candidate metabolites for bioactivity studies of dietary flavonoids. *Tetrahedron* 2012, *68*, 4194–4201.

[21] Neveu, V., Perez-Jiménez, J., Vos, F., Crespy, V. et al., Phenol-Explorer: an online comprehensive database on polyphenol contents in foods. *Database* 2010, doi: 10.1093/database/bap024.

[22] Veberic, R., Jakopic, J., Stampar, F., Schmitzer, V., European elderberry (*Sambucus nigra* L.) rich in sugars, organic acids, anthocyanins and selected polyphenols. *Food Chem.* 2009, *114*, 511–515.

[23] Saxholt, E., Christensen, A. T., Møller, A., Hartkopp, H. B. et al., Danish Food Composition Databank, revision 7. Department of Nutrition, National Food Institute, Technical University of Denmark. 2008. Available at http://www.foodcomp.dk/.

[24] Wu, X., Cao, G., Prior, R. L., Absorption and metabolism of anthocyanins in elderly women after consumption of elderberry or blueberry. *J. Nutr.* 2002, *132*, 1865–1871.

[25] Milbury, P. E., Cao, G., Prior, R. L., Blumberg, J., Bioavailablility of elderberry anthocyanins. *Mech. Ageing Dev.* 2002, *123*, 997–1006.

[26] Mülleder, U., Murkovic, M., Pfannhauser, W., Urinary excretion of cyanidin glycosides. *J. Biochem. Biophys. Methods* 2002, *53*, 61–66.

[27] Murkovic, M., Mülleder, U., Adam, U., Pfannhauser, W., Detection of anthocyanins from elderberry juice in human urine. *J. Sci. Food Agric.* 2001, *81*, 934–937.

[28] Kay, C. D., Mazza, G., Holub, B. J., Wang, J., Anthocyanin metabolites in human urine and serum. *Br. J. Nutr.* 2004, *91*, 933–942.

[29] Felgines, C., Talavera, S., Texier, O., Gil-Izquierdo, A. et al., Blackberry anthocyanins are mainly recovered from urine as methylated and glucuronidated conjugates in humans. *J. Agric. Food Chem.* 2005, *53*, 7721–7727.

[30] Garcia-Alonso, M., Minihane, A.-M., Rimbach, G., Rivas-Gonzalo, J. C. et al., Red wine anthocyanins are rapidly absorbed in humans and affect monocyte chemoattractant protein 1 levels and antioxidant capacity of plasma. *J. Nutr. Biochem.* 2009, *20*, 521–529.

[31] Bitsch, R., Netzel, M., Sonntag, S., Strass, G. et al., Urinary excretion of cyanidin glucosides and glucuronides in healthy humans after elderberry juice ingestion. *J. Biomed. Biotechnol.* 2004, *2004*, 343–345.

[32] Cao, G., Muccitelli, H. U., Sánchez-Moreno, C., Prior, R. L., Anthocyanins are absorbed in glycated forms in elderly women: a pharmacokinetic study. *Am. J. Clin. Nutr.* 2001, *73*, 920–926.

[33] Azzini, E., Vitaglione, P., Intorre, F., Napolitano, A. et al., Bioavailability of strawberry antioxidants in human subjects. *Br. J. Nutr.* 2010, *104*, 1165–1173.

[34] Selma, M. V., Espín, J. C., Tomás-Barberán, F. A., Interaction between phenolics and gut microbiota: role in human health. *J. Agric. Food Chem.* 2009, *57*, 6485–6501.

[35] Winter, J., Moore, L., Dowell, V., Bokkenheuser, V., C-ring cleavage of flavonoids by human intestinal bacteria. *Appl. Environ. Microbiol.* 1989, *55*, 1203–1208.

[36] Aura, A. M., O'Leary, K. A., Williamson, G., Ojala, M. et al., Quercetin derivatives are deconjugated and converted to hydroxyphenylacetic acids but not methylated by human fecal flora in vitro. *J. Agric. Food Chem.* 2002, *50*, 1725–1730.

[37] Griffiths, L., Barrow, A., Metabolism of flavonoid compounds in germ-free rats. *Biochem. J.* 1972, *130*, 1161–1162.

[38] Mullen, W., Rouanet, J. M., Auger, C., Teissedre, P. L. et al., Bioavailability of [2-(14)C]quercetin-4′-glucoside in rats. *J. Agric. Food Chem.* 2008, *56*, 12127–12137.

[39] Berg, R. D., The indigenous gastrointestinal microflora. *Trends Microbiol.* 1996, *4*, 430–435.

[40] Hollman, P. C. H., Absorption, bioavailability, and metabolism of flavonoids. *Pharm. Biol.* 2004, *42*, 74–83.

[41] Toromanović, J., Kovač-Bešović, E., Šapčanin, A., Tahirović, I. et al., Urinary hippuric acid after ingestion of edible fruits. *Bosnian J. Basic Med. Sci.* 2008, *8*, 38–43.

[42] Egert, S., Wolffram, S., Bosy-Westphal, A., Boesch-Saadatmandi, C. et al., Daily quercetin supplementation dose-dependently increases plasma quercetin concentrations in healthy humans. *J. Nutr.* 2008, *138*, 1615–1621.

[43] Schmitzer, V., Veberic, R., Slatnar, A., Stampar, F., Elderberry (*Sambucus nigra* L.) wine: a product rich in health promoting compounds. *J. Agric. Food Chem.* 2010, *58*, 10143–10146.

[44] Dykes, L., Rooney, L. W., Phenolic compounds in cereal grains and their health benefits. *Cereal Foods World.* 2007, *52*, 105–111.

[45] Del Rio, D., Rodriguez-Mateos, A., Spencer, J. P., Tognolini, M. et al., Dietary (poly) phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxid. Redox Signal.* 2013, *18*, 1818–1892.

[46] Weber, J., Van Zanten, A., Interferences in current methods for measurements of creatinine. *Clin. Chem.* 1991, *37*, 695–700.

[47] Fossen, T., Cabrita, L., Andersen, O. M., Colour and stability of pure anthocyanins influenced by pH including the alkaline region. *Food Chem.* 1998, *63*, 435–440.

