Methods for isolating, identifying and quantifying anthocyanin metabolites in clinical samples

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ABSTRACT The metabolic fate of anthocyanins until recently was relatively unknown, primarily as a result of their instability at physiological pH and a lack of published methods for isolating and identifying their metabolites from biological samples. The aim of the present work was to establish methods for the extraction and quantification of anthocyanin metabolites present in urine, serum and fecal samples. 35 commercial and 10 synthetic analytes, including both known and predicted human and microbial metabolites of anthocyanins were obtained as reference standards. HPLC and MS/MS conditions were optimized for organic modifier, ionic modifier, mobile phase gradient, flow rate, column type and MS source and compound dependent parameters. The impact of sorbent, solvent, acid, preservative, elution and evaporation on SPE extraction efficiency was also explored. The HPLC-MS/MS method validation demonstrated acceptable linearity (r^2, 0.997 ± 0.002) and sensitivity (LODs: urine, 100 ± 375 nM; serum, 104 ± 358 nM and feces 138 ± 344 nM) and the final SPE methods provided recoveries of 88.3 ± 17.8% for urine, 86.5 ± 11.1% for serum and 80.6 ± 20.9% for feces. Final methods were applied to clinical samples derived from an anthocyanin intervention study, where 36 of the 45 modeled metabolites were detected within urine, plasma or fecal samples. The described methods provide suitable versatility for the identification and quantification of an extensive series of anthocyanin metabolites for use in future clinical studies exploring absorption, distribution, metabolism and elimination.

Introduction

Epidemiological evidence suggests an association between the consumption of foods rich in anthocyanins and a decreased risk of cardiovascular disease [1-3]. However, until recently there was a considerable lack of information regarding the bioavailability and metabolic fate of anthocyanins in humans [4,5]. A limited number of previous human studies have reported the degradation of anthocyanins into phenolic acids and aldehydes and their subsequent methyl, glucuronide and sulfate conjugation, yet there are still conflicting reports in the literature regarding the identity and prevalence of the major metabolites present following the consumption of anthocyanin-rich foods [4-10].

The major challenges associated with the recovery and detection of anthocyanins relate to their instability under neutral pH [11], their extensive metabolic conjugation *in vivo* [12] and their probable catabolism by intestinal microflora [13]. It is therefore likely that after consumption of anthocyanin rich foods, a complex mixture of intact anthocyanins, phenolic degradation products, phase II metabolic conjugates and colonic metabolites exist in tissues and biological fluids [4]. Whilst a number of methods have been developed for the analysis of flavonoids and flavonoid derived phenolics [14-16], the vast majority of reported methods for anthocyanin analysis (in particular) have concentrated on quantification of parent/precursor forms or their respective metabolic conjugates (methyl, glucuronide and sulfate derivatives of anthocyanins). There are limited reports where quantitative analytical methods have been optimized for the analysis of anthocyanins and their phenolic acid and aldehyde degradation products and metabolites together [9,13,17]. Understandably, developing an appropriate method for this purpose presents many challenges, as there is an extremely large diversity of metabolic by-products whose structures and physicochemical properties make extraction and quantification in complex matrices problematic. In addition, many phase II conjugates of

phenolic acids are not commercially available for use as reference standards. In order to facilitate development of a suitable method, in-house synthesis is often necessary.

The objective of the present study was to ultimately validate methods suitable for determining the absorption, distribution, metabolism and elimination (ADME) of anthocyanins, including the clearance of their degradation products and metabolites in clinical samples. In addition, HPLC and MS variables such as organic modifier, ionic modifier concentration, mobile phase gradient, flow rate, column, ion source and multiple reaction monitoring (MRM) parameters were optimized to increase analytical performance. Analytical methods were validated for linearity, precision and accuracy using the U.S. Department of Health and Human Services Food and Drug Administration (FDA) guidance for industry bioanalytical methods validation [18], for the 45 analytes and confirmed using clinically derived urine, serum and fecal samples. The analytes explored include anthocyanins, their degradation products, phase II conjugates and probable colonic metabolites [13] (**Supplementary Information Table 1**). As many factors can influence analyte recoveries in biological matrices, the present study also aimed to validate the impact of several commonly utilized variables (i.e., sorbent, solvent, acid type, preservative, elution and sample evaporation) [19-28] on the extraction efficiency by solid phase extraction (SPE) of the 45 analytes spiked into biological matrices (urine, serum and fecal homogenate). The present study therefore describes methods for the identification and quantification of an extensive series of anthocyanin metabolites and the validation of these methods demonstrates suitability for use in future clinical intervention studies.

Experimental

Materials and reagents

Strata-X™ SPE cartridges (6 mL, 500 mg, 88 Å), HPLC columns [Kinetex pentafluorophenol (PFP) reverse phase (RP) (2.6 µm, 100 × 4.6 mm, 100 Å), Synergi Max RP (4 µm, 250 × 4.6 mm, 80 Å), Luna C18 (2) RP (4 µm, 250 × 4.6 mm, 100 Å), Synergi Polar RP (4 µm, 250 × 4.6 mm, 80 Å)] and SecurityGuard® cartridges (PFP and C18, 4 × 2.0 mm) were purchased from Phenomenex (Macclesfield, UK). The Eclipse XDB-C18 HPLC (5 µm, 150 × 4.6 mm, 80 Å) column and Bond Elute C18 SPE cartridges (20 mL, 5 g, 70 Å) were from Agilent (Wokingham, UK). HPLC grade methanol and acetonitrile were purchased from Fisher Scientific (Loughborough, UK). All water utilized was of Milli-Q grade (18.2 MΩ cm^{-1}). Sterile filtered human male serum was from AB plasma, Discovery® DSC-18 SPE cartridges (6 mL, 1 g) and Acrodisc PTFE syringe filters (13 mm, 0.45 µm) were purchased from Sigma-Aldrich (Dorset, UK). Human feces and urine were collected following internal protocols, approved by the Norfolk Research Ethics Committee (Norfolk, UK). A complete list of all analytical standards and their makeup is provided in the supplementary materials.

Metabolite modeling

Target compounds for method development were chosen based on previously published studies and known phase II conjugation pathways and colonic metabolism of other similarly structured flavonoids. Further details of the modeling protocol are provided in the supplemental materials. Of the modeled compounds, 35 were commercially available and ten were chosen for synthesis [29] and utilized in the present study (Supplementary Information Table 1).

HPLC-MS/MS conditions

HPLC-MS/MS analysis was conducted using an Agilent 1200 series HPLC-DAD (Wokingham, UK), attached to an ABSciex 3200 series Q-trap MS/MS (Warrington, UK), as described in detail in the supplementary information. The final HPLC-MS/MS analysis utilized a mobile phase consisting of 0.1% formic acid (v/v) in water and 0.1% formic acid (v/v) in acetonitrile, with ion spray voltage (IS) -4000 V/+5500 V and temperature 700ºC. Optimized MRM parameters were established for each analyte with the final method analyzed separately in both positive and negative ionization mode.

SPE conditions

The SPE procedure was conducted as described in detail in the supplementary information. Briefly, the final SPE method consisted of samples being loaded onto either DSC-18 (6 mL, 1 g, urine), Strata-X™ (6 mL, 500 mg, serum) or Bond Elute C18 (20 mL, 5 g, feces) SPE cartridges, washed with two column volumes of 1% formic acid in water, eluted under gravity with 1% formic acid in methanol and concentrated using a Speedvac® centrifugal evaporator.

Method Validation

Validation of the HPLC-MS/MS method was carried out in terms of the linearity, precision and accuracy of compounds spiked into mobile phase, using the guidelines set out by the FDA for Bioanalytical methods validation (2001) [18]. LODs were established by calculating the concentration of analyte yielding a peak height signal-to-noise ratio of 3:1 (signal-to-noise method) when the analyte was spiked into urine, serum and fecal matrices post extraction. Where the analytes of interest were endogenously present in the fasting urine, serum or fecal samples, peak heights were corrected for the endogenous analyte

concentration. All data are given as mean ± SD of three replicates and where stated, statistical comparisons were undertaken using t-tests ($p<0.05$, n=3) in SPSS 18 (IBM, UK).

The extraction methods were validated by calculating the extraction efficiencies of the standards spiked in urine, serum and fecal homogenates prior to SPE, relative to matrix-matched (urine, serum, feces) control samples spiked with the same mixture of the standards post SPE.

The final urine and plasma methods were applied to samples derived from a previous clinical intervention trial feeding participants (n=15) a 500 mg bolus of elderberry derived anthocyanins, where samples were collected for 3 hours post bolus[30]. The fecal method was applied to samples derived from a study feeding participants (n=8) a 500 mg bolus dose of pure ^{13}C-labelled cyanidin-3-glucoside, where samples were collected for 48 hours post bolus[4]. The analytes were quantified using the optimized extraction and detection methods and the lowest and highest urinary, plasma and fecal concentrations identified are presented.

Results

HPLC-MS/MS

Five HPLC columns commonly utilized in flavonoid analysis (Eclipse XDB C18, Kinetex PFP, Synergi Max, Luna C18 and Synergi Polar) were selected to establish the chromatographic separation of the target compounds. Here, the PFP column provided the optimal peak resolution, where flow rate was optimal at 1.5 mL min^{-1} (40% reduced peak width relative to 1 mL min^{-1}; data not shown). The Eclipse and Luna C18 columns resulted in poor resolution and separation of the sulfated compounds. Whilst the Synergi Max and Synergi Polar columns resulted in slightly improved separation, the PFP column provided the greatest resolution, with a two to tenfold improvement in peak intensity relative to the other

stationary phases (data not shown). No column tested was capable of sufficiently separating the isomers of protocatechuic acid (PCA)-sulfate under the explored conditions.

A flow rate of 1.5 mL min^{-1} decreasing to 1 mL min^{-1} from 7 to 14 min achieved optimal separation while staying within the pressure limitations of the HPLC system utilized (<400 bar) and there was no apparent difference in ion intensity when comparing mobile phase solvents methanol relative to acetonitrile, however, acetonitrile slightly improved the separation of the analytes at 0.1% formic acid (data not shown). Optimized source parameters were established at a curtain gas (CUR) of 40 psi, which prevented solvent entering the orifice, a temperature of 700 °C and gas flows of 60 psi (nebulizer and auxiliary gas) for optimal nebulization of the solvent. The MRM related parameters, were optimized for each individual compound separately (**Supplementary Information Table 2**) and MRM analysis of the mixed standards at 50 µM was used to verify the final parameters in positive and negative mode (**Figure 1**).

The final HPLC-MS/MS method was validated for linearity, precision and accuracy using six-point calibration curves constructed in 5% methanol, 0.1% formic acid (aqueous), following six repeat injections. All calibration curves were linear over the concentration ranges (1.25 to 20 µM) (**Table 1**) and the HPLC-MS/MS LODs ranged from 1 nM for phloridzin to 2604 nM for homovanillic acid in urine, 0.3 nM for phloridzin to 2340 nM for homovanillic acid in serum and 1 nM for phloridzin to 2238 nM for 4-hydroxyphenylacetic acid in feces (Table 1).

Extraction

The addition of a preservative (10% ascorbate w/v in 0.5 mM EDTA) prior to SPE and the change of acid modifier from formic acid to HCl during SPE had little impact on recovery ($p>0.05$, $n=3$; data not shown). The lowest elution volume which provided the maximum

retention of a range of representative analytes was 7 mL and complete evaporation of the eluent to dryness reduced the recovery of some compounds significantly (including anthocyanins and phloroglucinaldehyde (PGA); data not shown). Therefore, samples were dried to approximately 50 µL, reconstituted with 200 µL acidified water and a volume marker (scopoletin) was added to allow calculation of the exact volume.

Following solid phase extraction, the mean recovery of the 45 analytes from urine, serum and feces was 88.3 ± 17.8%, 86.5 ± 11.1% and 80.6 ± 20.9% respectively (**Table 2**). Of the total 45 analytes, 34, 34, and 26 compounds were recovered with greater than 80% efficiency in urine, serum and feces, respectively. Poor recoveries (<50%) were exhibited for 4-methoxysalicyclic acid, gallic acid and benzoic acid (BA)-4-glucuronide in the feces, whilst the extraction efficiencies of the analytes within urine and serum matrices were all >60% (Table 2). The coefficient of variation (CV) of the extraction efficiency of the analytes averaged 7.9 ± 5.3% for urine, 6.8 ± 5.0% for serum and 14.1 ± 7.9% for feces.

Validation using human samples.

Thirty six of the 45 putative metabolites were detected in the human samples; 26 within urine, 25 within plasma and 24 with feces, with the minimum concentrations identified ranging from 0.4 nM for vanillic acid (VA)-sulfate to 127,899 nM for hippuric acid in urine, 2 nM for methyl-3,4-dihydroxybenzoate to 5,771 nM for hippuric acid in plasma and 0.3 nM for BA-4-glucuronide to 6,974 nM for 2,3-dihydroxyBA in feces (**Table 3**). The maximum concentrations identified within participant samples ranged from 3,103,601 nM in urine to 10,106 nM in plasma for hippuric acid and 211,194 nM for ferulic acid in feces.

Discussion

The objective of the present study was to develop methods suitable for establishing the ADME of anthocyanins, including the clearance of their degradation products and

metabolites. The investigation strategy was to: (1) model putative metabolites of anthocyanins to establish a range of targets for method validation; (2) synthesize glucuronide and sulfate conjugates of common anthocyanin degradation products; and (3) establish fit for purpose methods for extracting and quantifying the anthocyanin metabolites.

Achieving suitable chromatographic separation is challenging when large mixtures of analytes are present in a complex matrix and is further complicated by the presence of isomers (e.g: PCA-3-sulfate, PCA-4-sulfate). The five columns described in the present study were selected for analysis based on their frequent use within flavonoid research[31-34]. The majority of studies using RP-HPLC to analyse anthocyanins have utilised C18 packing materials[31-34]. However, more recently interest has grown in newer Kinetex phases such as the PFP stationary phase, which incorporates fluorine atoms on the periphery of a phenyl ring. This enables chromatographic separation via dipole-dipole interactions, hydrogen bonding and π- π interactions in addition to the hydrophobic and shape selectivity retention mechanisms utilised by more typical C18 columns[35]. Of the five HPLC columns tested, the present study identified the Kinetex PFP column as providing the greatest chromatographic separation efficiency under the present conditions and also demonstrated superior resolution of the sulfated conjugates. None of the columns tested were able to effectively separate the isomers of PCA sulfate under the explored conditions. In addition, changing the mobile phase from methanol to acetonitrile and decreasing the flow rate to 1 mL min^{-1} from 7 to 14 min within the run-time significantly improved the separation. A column temperature of 37 °C was selected to reduce the system backpressure, whilst staying within a physiologically relevant temperature range for these analytes as their stability is uncertain at higher temperatures. A high source temperature (700 °C) and gas flow (60 psi) appeared particularly important to ensure ionization of the compounds at this relatively high flow rate. It should be noted that HPLC and MS optimization is instrument specific and the presented values (Table

2) should only be used as a guide for method development or validation, with lower flow rates likely proving optimal with other instruments and column dimensions. An unconventional HPLC method, where the flow rate was decreased from 1.5 mL/min to 1 mL/min from 7 to 14 min was selected for use in the current study, as it enabled optimal separation of isomeric compounds eluting within this timeframe.

The LODs of the final method ranged from 0.3 nM (for phloridzin in serum) to 2604 nM (for homoVA in urine) (Table 1). The LODs of the majority of the compounds were below 100 nM; however, the LODs of 8, 7 and 15 compounds in urine, serum and feces respectively were above 100 nM, generally a result of a high 'baseline noise' (background mass spectrum) or poor ionization. The LOD for homoVA was extremely high as a result of its high fasting endogenous analyte concentration and poor ionization; suggesting the described HPLC-MS/MS method is not optimal for the detection of low levels of the metabolite in biological samples. Yet despite this, it was still quantifiable, as it was present at such high concentrations within urine. In these cases where ionization is poor, derivatization and quantification via GC-MS should be considered. Furthermore, the goal of the present study was to develop a single method for detecting an extensive range of anthocyanins and phenolic metabolites within a single HPLC-MS method and mobile phase, for the processing of large numbers of clinical samples. Sensitivity could be improved for certain analytes, by optimizing flow rates, mobile phases and source parameters separately for anthocyanins, phenolic acids, and polar phenolic metabolites. However this would require multiple methods and mobile phases. For example, increasing the acid content of the mobile phase would improve the chromatographic resolution of anthocyanins, but this would have deleterious effects on the ionisation of other analytes.SPE is often the preferred extraction method when utilizing HPLC-MS/MS as it removes salts that may affect ionization [36]. It should be noted however that dilution, acidification and syringe filtration [23-25], protein precipitation [22-25,27,37]

and liquid-liquid extraction [27,28] are also often commonly used techniques in the analysis of polyphenols [23-25]. These methods were also initially and exhaustively explored, however, due to issues with poor recovery, extremely high variability (inter- and intra-extraction) and insufficient chromatographic resolution of some analytes (data not shown), they were abandoned in favor of SPE. Thus, SPE was the optimal method for the extraction of anthocyanin metabolites, resulting in mean extraction efficiencies of 88.3 ± 17.8% for urine, 86.5 ± 11.1% for serum and 80.6 ± 20.9% for feces for the 45 modeled metabolites. In addition, the methods provided acceptable reproducibility for the established extraction efficiencies (Table 2). For urine extraction, the DSC-18 and Strata-X™ SPE cartridges yielded similar recoveries for C3G, PGA and the internal standard (taxifolin), however the binding characteristics of the polymeric divinylbenzene Strata-X™ sorbent allowed large amounts of polar compounds in the matrix to remain bound to the column after the aqueous wash, resulting in poor resolution of PCA and PCA-4-glucuronide from other analytes when using UVvis detection. Therefore, DSC-18 SPE cartridges, were selected as they gave superior recoveries for compounds from urine. However, when using MRM as a single detection method, co-elution of background analytes may be of limited significance, thus permitting the use of Strata-X™. Within serum, the more selective Strata-X™ cartridges were optimal for the extraction of target analytes, as they afforded higher extraction efficiencies and improved repeatability under the explored conditions. The SPE recoveries of the 45 analytes of interest ranged from 10.2 ± 4.6% to 121 ± 41.5% with a high mean recovery of 80.6 ± 20.9%, demonstrating that despite the complexity of the fecal matrix, the presented method is suitable for the recovery of the target analytes.

Validation of the methods for use with clinical samples [4,30], was carried out using matrix-matched standard curves rather than standard curves prepared in mobile phase (a common approach), which provides an extra degree of precision as the ionization efficiencies of the

sample and standards are more similar compared to using mobile phase alone. The methods described herein were successful in identifying 36 metabolites, 26 analytes within urine, 25 within serum, and 24 within feces; 19 of which have now been confirmed as anthocyanin metabolites in a recent ^{13}C-labelled anthocyanin study [4]. The lowest concentrations of the metabolites identified in the urine samples ranged from 0.4 nM for VA-4-sulfate to 127,899 nM for hippuric acid, while concentrations in the plasma ranged from 2 nM for methyl-3,4-dihydroxybenzoate to 5,771 nM for hippuric acid and concentrations in the feces ranged from 0.3 nM for BA-4-glucuronide to 6,974 nM for 2,3-dihydroxyBA (Table 3). The identification of these metabolites within clinical samples demonstrates that the methods are suitable and have acceptable LODs for the detection of anthocyanin metabolites in clinically relevant samples.

Conclusion

The present study describes the validation of analytical methods that are suitable for the quantification of a large number of structurally diverse anthocyanin metabolites, thus providing a valuable tool for future studies of ADME and bioactivity.

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Supporting Information Available

Supporting information containing a list of all analytical standards, their structures and makeup and a complete description of the analytical methods, including the optimized MRM parameters is provided. This information is available free of charge via the Internet at http://pubs.acs.org/.

ABBREVIATIONS

C3G, cyanidin-3-glucoside; LLE, liquid-liquid extraction; ADME, absorption distribution metabolism elimination; DMSO, dimethyl sulfoxide; PCA, protocatechuic acid; PGA, phloroglucinaldehyde; PFP, pentafluorophenyl; RP, reverse phase; SPE, solid phase extraction; MRM, multiple reaction monitoring; DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, collision exit potential; CUR, curtain gas; CV, coefficient of variation; LOD, limit of detection.

Figure 1. Multiple reaction monitoring (MRM) chromatogram of standard compounds, illustrating optimized negative (A) and positive (B) HPLC-MS/MS methods. (A) [1]Gallic acid, [2]Benzoic acid (BA)-4-glucuronide, [3]PCA-4-glucuronide, [4]PCA-3-glucuronide, [5]Vanillic acid-4-glucuronide, [6]Protocatechuic acid (PCA),[7]3,5-DihydroxyBA, [8]PCA-3 and 4-sulfate, [9]4-Hydroxybenzyl alcohol, [10]Homoprotocatechuic acid, [11]Isovanillic acid-3-glucuronide, [12]BA-4-sulfate, [13]3,4-Dihydroxybenzaldehyde, [14]Isovanillic acid-3-sulfate, [15]4-HydroxyBA, [16]Hippuric acid, [17]4-Hydroxyphenylacetic acid, [18]Vanillic acid-4-sulfate, [19]Methylgallate, [20]3-HydroxyBA, [21]2,3-DihydroxyBA, [22]4-Hydroxybenzaldehyde, [23]Vanillic acid (VA), [24]2,4-DihydroxyBA, [25]Homovanillic acid, [26]Caffeic acid, [27]7,8-Dihydroxycourmarin, [28]4-Methylhippuric acid, [29]Methyl 3,4-dihydroxybenzoate, [30]3-Methylhippuric acid, [31]*p*-

Coumaric acid, [32]Phloroglucinaldehyde, [33]Ferulic acid, [34]2-HydroxyBA, [35]Sinapic acid, [36]Taxifolin, [37]4-Methoxysalicylic acid, [38]6-Methoxysalicyclic acid, [39]Phloridzin. (B) [1]3-Methylgallic acid, [2]Isovanillic acid, [3]Cyanidin-3-glucoside, [4]Syringic acid, [5]2-MethoxyBA, [6]Pelargonidin-3-glucoside, [7]Methyl vanillate, [8]Methyl-3,4-dimethoxybenzoate.

Table 1. Precision data and limits of detection for the optimized urine, serum and fecal methods.

Analyte	Ionization Mode	Slope (a)[a] Mean ± SD	CV (%)	R^2	LOD (nM)		
					Urine	Serum	Feces
Cyanidin-3-glucoside	+	594,473 ± 39,888	6.71	0.998	2	4	4
Pelargonidin-3-glucoside	+	1,034,231 ± 98,822	9.56	0.996	2	1	2
2-Hydroxybenzoic acid	-	189,231 ± 4334	2.29	0.998	116	86	136
3-Hydroxybenzoic acid	-	60,138 ± 2698	4.49	0.999	173	191	214
4-Hydroxybenzoic acid	-	167,981 ± 7078	4.21	0.995	139	74	86
2,3-Dihydroxybenzoic acid	-	78,108 ± 1819	2.33	0.999	92	29	126
2,4-Dihydroxybenzoic acid	-	19,681 ± 746	3.79	0.999	98	68	112
Protocatechuic acid (PCA)	-	15,128 ± 760	5.02	0.997	41	1	9
PCA-3-glucuronide	-	174,408 ± 5711	3.27	0.999	6	1	4
PCA-4-glucuronide	-	102,003 ± 4943	4.85	0.999	5	2	4
PCA-3 and 4-sulfate	-	378,495 ± 13,989	3.70	0.998	1	2	1
3,5-Dihydroxybenzoic acid	-	234,649 ± 8897	3.79	0.996	24	31	150
Gallic acid	-	34,012 ± 907	2.67	0.997	15	29	20
2-Methoxybenzoic acid	+	400,431 ± 20,139	5.03	0.996	16	23	22
4-Methoxysalicylic acid	-	300,116 ± 9224	3.07	0.998	23	29	375
6-Methoxysalicyclic acid	-	15,687 ± 583	3.72	0.999	116	25	333
Vanillic acid (VA)	-	11,322 ± 337	2.97	0.999	53	16	45
VA-4-glucuronide	-	56,069 ± 2416	4.31	0.999	37	6	11
VA-4-sulfate	-	214,295 ± 3041	1.42	0.999	2	3	3
IsoVA	+	30,715 ± 1362	4.44	0.998	47	77	94
IsoVA-3-glucuronide	-	67,871 ± 3294	4.85	1.000	9	5	9
IsoVA-3-sulfate	-	308,646 ± 7864	2.55	0.999	2	3	3
Syringic acid	+	61,287 ± 2709	4.42	0.998	16	45	21
3-Methylgallic acid	-	1584 ± 69	4.37	0.997	360	121	400
Benzoic acid-4-glucuronide	-	66,978 ± 2529	3.78	0.998	4	10	21
Benzoic acid-4-sulfate	-	143,079 ± 4552	3.18	0.998	1	4	2
Methyl-3,4-dihydroxybenzoate	-	230,527 ± 10,659	4.62	0.996	2	3	6
Methyl-3,4-dimethoxybenzoate	+	93,306 ± 5946	6.37	0.999	8	23	22
Methyl vanillate	+	33,814 ± 3204	9.47	0.999	43	68	36
Methyl gallate	-	257,934 ± 19,070	7.39	1.00	1	2	3
4-Hydroxybenzyl alcohol	-	963 ± 40	4.16	0.995	62	246	424
p-Coumaric acid	-	111,853 ± 8169	7.30	0.994	46	51	30
Caffeic acid	-	333,099 ± 8610	2.58	0.992	67	122	100
Ferulic acid	-	40,588 ± 1939	4.78	1.000	14	9	67
Sinapic acid	-	7806 ± 638	8.17	0.999	39	7	20
Phloroglucinaldehyde	-	23,907 ± 1126	4.71	0.992	13	5	40
4-Hydroxybenzaldehyde	-	1235 ± 63	5.11	0.998	25	40	14

3,4-Dihydroxybenzaldehyde	-	43,960 ± 1288	2.93	0.993	10	4	2
4-Hydroxyphenylacetic acid	-	30,535 ± 1017	3.33	0.998	116	938	2238
HomoPCA	-	2146 ± 65	3.03	0.999	108	117	413
HomoVA	-	23,907 ± 1126	4.71	0.992	2604	2340	727
Hippuric acid	-	92,688 ± 3528	3.81	0.996	4	0.4	70
3-Methylhippuric acid	-	160,138 ± 5016	3.13	0.999	99	43	105
4-Methylhippuric acid	-	99,126 ± 4512	4.55	0.999	77	43	122
		Internal standards					
Phloridzin	-	292,333 ± 6420	2.20	0.991	1	0.3	1
Scopoletin	-	10,817 ± 536	4.96	0.999	23	11	30
Taxifolin	-	124,601 ± 3176	2.55	0.995	5	5	6
7,8-Dihydroxycourmarin	-	28,696 ± 1308	4.56	0.994	19	20	3

[a]Linear regression analysis with a regression equation of $y = ax + b$, where x is the concentration in μM, b is equal to 0 and y is the peak area; LOD, the limit of detection (Signal/Noise = 3); R^2, correlation coefficient of regression equations.

Table 2. SPE extraction efficiencies of analytes in urine, serum and faecal matrices as determined using HPLC-ESI-MS/MS.

Analyte	Extraction Efficiency (% recovery)		
	Urine	Serum	Faeces
Cyanidin-3-glucoside	97.8 ± 6.2	72.1 ± 4.6	55.4 ± 9.0
Pelargonidin-3-glucoside	101.9 ± 6.0	74.0 ± 3.4	64.5 ± 11.0
2-Hydroxybenzoic acid	89.6 ± 1.1	81.2 ± 3.0	71.2 ± 7.0
3-Hydroxybenzoic acid	85.8 ± 10.5	92.9 ± 7.6	82.9 ± 10.1
4-Hydroxybenzoic acid	86.2 ± 2.0	86.6 ± 9.8	121.1 ± 41.5
2,3-Dihydroxybenzoic acid	86.8 ± 3.0	73.9 ± 4.4	86.6 ± 7.8
2,4-Dihydroxybenzoic acid	79.1 ± 10.0	97.4 ± 3.0	79.9 ± 6.4
Protocatechuic acid (PCA)	89.7 ± 10.7	89.3 ± 6.4	80.4 ± 4.3
PCA-3-glucuronide	94.9 ± 5.0	92.0 ± 6.3	78.4 ± 5.8
PCA-4-glucuronide	91.8 ± 5.1	93.2 ± 1.3	84.8 ± 23.4
PCA-3 and 4-sulfate	92.9 ± 1.9	67.4 ± 11.5	111.0 ± 13.6
3,5-Dihydroxybenzoic acid	91.3 ± 16.9	80.9 ± 16.7	82.0 ± 13.7
Gallic acid	72.8 ± 3.9	85.0 ± 7.6	10.2 ± 4.6
2-Methoxybenzoic acid	90.6 ± 3.5	89.6 ± 6.3	93.7 ± 10.9
4-Methoxysalicylic acid	92.7 ± 2.2	85.3 ± 3.6	9.3 ± 2.7
6-Methoxysalicyclic acid	95.9 ± 1.9	85.3 ± 3.1	67.7 ± 3.0
Vanillic acid (VA)	91.6 ± 3.9	87.3 ± 3.7	76.0 ± 8.2
VA-4-glucuronide	74.5 ± 12.8	93.6 ± 2.6	85.9 ± 12.8
VA-4-sulfate	87.3 ± 7.1	92.6 ± 2.6	96.2 ± 16.8
IsoVA	110.7 ± 10.2	101.8 ± 5.8	79.4 ± 11.6
IsoVA-3-glucuronide	89.6 ± 5.0	86.7 ± 12.0	76.6 ± 8.2
IsoVA-3-sulfate	96.2 ± 6.3	92.6 ± 9.3	84.2 ± 13.0
Syringic acid	104.5 ± 8.3	87.7 ± 2.6	82.7 ± 4.7
3-Methylgallic acid	79.6 ± 17.4	95.3 ± 3.7	75.2 ± 3.8
Benzoic acid-4-glucuronide	85.8 ± 1.9	103.5 ± 5.6	42.0 ± 11.6
Benzoic acid-4-sulfate	77.3 ± 7.1	94.2 ± 3.3	89.6 ± 15.3
Methyl-3,4-dihydroxybenzoate	87.2 ± 9.4	82.6 ± 17.8	79.2 ± 6.1
Methyl-3,4-dimethoxybenzoate	96.8 ± 3.9	73.3 ± 3.2	73.0 ± 7.2
Methyl vanillate	101.5 ± 11.8	83.6 ± 2.1	80.9 ± 13.6
Methyl gallate	87.7 ± 13.0	64.9 ± 5.3	101.4 ± 23.0
4-Hydroxybenzyl alcohol	72.9 ± 8.7	80.0 ± 3.9	92.7 ± 12.2
p-Coumaric acid	93.5 ± 10.2	88.1 ± 6.0	87.6 ± 9.4
Caffeic acid	78.6 ± 9.1	75.7 ± 6.0	98.6 ± 12.8

Ferulic acid	105.3 ± 2.1	100.0 ± 4.4	74.3 ± 6.8
Sinapic acid	110.1 ± 5.6	88.7 ± 0.5	79.6 ± 9.8
Phloroglucinaldehyde	64.5 ± 5.9	79.7 ± 8.4	66.4 ± 7.0
4-Hydroxybenzaldehyde	85.8 ± 8.7	73.7 ± 6.1	97.0 ± 13.4
3,4-Dihydroxybenzaldehyde	77.8 ± 7.9	83.8 ± 9.2	109.4 ± 26.8
4-Hydroxyphenylacetic acid	87.2 ± 2.8	87.6 ± 7.9	82.1 ± 19.9
HomoPCA	60.0 ± 11.6	72.0 ± 3.2	88.2 ± 11.8
HomoVA	81.9 ± 9.7	107.3 ± 1.1	63.4 ± 7.3
Hippuric acid	NQ[a]	98.9 ± 2.4	87.3 ± 11.5
3-Methylhippuric acid	76.6 ± 9.6	95.4 ± 0.4	85.4 ± 10.5
4-Methylhippuric acid	92.5 ± 1.7	90.3 ± 6.2	77.1 ± 6.2
	Internal standards		
Phloridzin	81.6 ± 6.4	77.2 ± 15.0	75.5 ± 7.4
Scopoletin[b]	VC	VC	VC
Taxifolin	96.6 ± 0.9	88.6 ± 4.4	76.6 ± 11.0
7,8-Dihydroxycourmarin	88.8 ± 0.8	87.5 ± 5.4	98.0 ± 12.5

Extraction efficiencies are expressed as mean % ± SD using DSC-18, Strata-X and Bond Elute C18 SPE cartridges for the extraction of urine, serum and fecal matrices respectively; VC, volume control; [a]NQ, not quantified due to high background concentrations in urine; [b]Scopoletin was used as a volume control standard and was therefore added post extraction only.

Table 3. Minimum and maximum concentrations of analytes detected in spot urine, serum and fecal samples post consumption of 500 mg anthocyanins.

Analyte	Urine (nM)		Serum (nM)		Faeces (nM)	
	Min[a]	Max[b]	Min[a]	Max[b]	Min[c]	Max[d]
Cyanidin-3-glucoside	2	6348	4	7	2017	2017
2-Hydroxybenzoic acid	37	2919	173	211	ND	ND
3-Hydroxybenzoic acid	60	21,024	21	36	ND	ND
4-Hydroxybenzoic acid	656	9892	30	39	34	3,026
2,3-Dihydroxybenzoic acid	12	12,360	ND	ND	6974	21,044
Protocatechuic acid (PCA)	31	8117	11	24	8	33,081
PCA-3-glucuronide	2	8161	3	15	20	713
PCA-4-glucuronide	7	2771	4	14	9	1,127
PCA-3 and 4-sulfate	14	29,403	734	358	2	872
3,5-Dihydroxybenzoic acid	134	21,328	18	50	ND	ND
4-Methoxysalicylic acid	ND	ND	0.3	2	29	11,420
Vanillic acid (VA)	66	18,076	6	62	282	16,663
VA-4-glucuronide	74	18,929	16	120	29	285
VA-4-sulfate	0.4	75,259	23	161	0.3	1968
IsoVA	ND	ND	ND	ND	177	230
IsoVA-3-glucuronide	4	15,680	10	24	18	241
IsoVA-3-sulfate	0.4	75,259	23	161	1	4993
Syringic acid	ND	ND	5	22	ND	ND
Benzoic acid-4-glucuronide	3	623	7	10	0.3	1477
Benzoic acid-4-sulfate	ND	ND	66	196	ND	ND
Methyl-3,4-dihydroxybenzoate	ND	ND	2	6	58	7425
Methyl gallate	ND	ND	3	5	ND	ND
Methyl vanillate	ND	ND	ND	ND	2726	2726
4-Hydroxybenzyl alcohol	228	17,663	ND	ND	ND	ND
p-Coumaric acid	13	3040	ND	ND	ND	ND
Caffeic acid	ND	ND	ND	ND	4168	9285
Ferulic acid	15	9908	7.7	28	131	211,194
Sinapic acid	5	8842	ND	ND	ND	ND
Phloroglucinaldehyde	7	3477	4	103	10	11,216
4-Hydroxybenzaldehyde	ND	ND	97	182	9	105
3,4-Dihydroxybenzaldehyde	ND	ND	17	23	11	724
HomoPCA	309	22,045	ND	ND	8	1879
HomoVA	1493	289,697	ND	ND	ND	ND

Hippuric acid	127,899	3,102,601	5771	10,106	13	748
3-Methylhippuric acid	29	5831	ND	ND	ND	ND
4-Methylhippuric acid	16	2963	ND	ND	ND	ND

ND, not detected; [a]Absolute minimum and [b]maximum analyte concentration detected within fasting pre bolus and 1, 2 and 3 hours post bolus spot urine and serum samples of 15 participants fed 500 mg elderberry anthocyanins [5,30]; [c]Absolute minimum and [d]maximum analyte concentration detected within 8 participants fed 500 mg ^{13}C-labelled cyanidin-3-glucoside, where samples were collected for 48 hours post bolus [4].

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