



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

## 1     **Introduction**

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

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

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

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

22

## 23 **Experimental**

24 **Materials and reagents**

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

15

## 16 Metabolite modeling

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

23

## 24 HPLC-MS/MS conditions

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

8

#### 9 SPE conditions

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

16

#### 17 Method Validation

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

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

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

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

14

## 15 **Results**

### 16 **HPLC-MS/MS**

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

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

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

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

21

## 22 Extraction

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

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

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

14

15 Validation using human samples.

16 Thirty six of the 45 putative metabolites were detected in the human samples; 26 within  
17 urine, 25 within plasma and 24 with feces, with the minimum concentrations identified  
18 ranging from 0.4 nM for vanillic acid (VA)-sulfate to 127,899 nM for hippuric acid in urine,  
19 2 nM for methyl-3,4-dihydroxybenzoate to 5,771 nM for hippuric acid in plasma and 0.3 nM  
20 for BA-4-glucuronide to 6,974 nM for 2,3-dihydroxyBA in feces (**Table 3**). The maximum  
21 concentrations identified within participant samples ranged from 3,103,601 nM in urine to  
22 10,106 nM in plasma for hippuric acid and 211,194 nM for ferulic acid in feces.

23

## 24 **Discussion**

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

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

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

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

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

1 and liquid-liquid extraction <sup>27,28</sup> are also often commonly used techniques in the analysis of  
2 polyphenols <sup>23-25</sup>. These methods were also initially and exhaustively explored, however, due  
3 to issues with poor recovery, extremely high variability (inter- and intra-extraction) and  
4 insufficient chromatographic resolution of some analytes (data not shown), they were  
5 abandoned in favor of SPE. Thus, SPE was the optimal method for the extraction of  
6 anthocyanin metabolites, resulting in mean extraction efficiencies of  $88.3 \pm 17.8\%$  for urine,  
7  $86.5 \pm 11.1\%$  for serum and  $80.6 \pm 20.9\%$  for feces for the 45 modeled metabolites. In  
8 addition, the methods provided acceptable reproducibility for the established extraction  
9 efficiencies (Table 2). For urine extraction, the DSC-18 and Strata-X<sup>TM</sup> SPE cartridges  
10 yielded similar recoveries for C3G, PGA and the internal standard (taxifolin), however the  
11 binding characteristics of the polymeric divinylbenzene Strata-X<sup>TM</sup> sorbent allowed large  
12 amounts of polar compounds in the matrix to remain bound to the column after the aqueous  
13 wash, resulting in poor resolution of PCA and PCA-4-glucuronide from other analytes when  
14 using UVvis detection. Therefore, DSC-18 SPE cartridges, were selected as they gave  
15 superior recoveries for compounds from urine. However, when using MRM as a single  
16 detection method, co-elution of background analytes may be of limited significance, thus  
17 permitting the use of Strata-X<sup>TM</sup>. Within serum, the more selective Strata-X<sup>TM</sup> cartridges  
18 were optimal for the extraction of target analytes, as they afforded higher extraction  
19 efficiencies and improved repeatability under the explored conditions. The SPE recoveries of  
20 the 45 analytes of interest ranged from  $10.2 \pm 4.6\%$  to  $121 \pm 41.5\%$  with a high mean  
21 recovery of  $80.6 \pm 20.9\%$ , demonstrating that despite the complexity of the fecal matrix, the  
22 presented method is suitable for the recovery of the target analytes.

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

1 sample and standards are more similar compared to using mobile phase alone. The methods  
2 described herein were successful in identifying 36 metabolites, 26 analytes within urine, 25  
3 within serum, and 24 within feces; 19 of which have now been confirmed as anthocyanin  
4 metabolites in a recent <sup>13</sup>C-labelled anthocyanin study <sup>4</sup>. The lowest concentrations of the  
5 metabolites identified in the urine samples ranged from 0.4 nM for VA-4-sulfate to 127,899  
6 nM for hippuric acid, while concentrations in the plasma ranged from 2 nM for methyl-3,4-  
7 dihydroxybenzoate to 5,771 nM for hippuric acid and concentrations in the feces ranged from  
8 0.3 nM for BA-4-glucuronide to 6,974 nM for 2,3-dihydroxyBA (Table 3). The identification  
9 of these metabolites within clinical samples demonstrates that the methods are suitable and  
10 have acceptable LODs for the detection of anthocyanin metabolites in clinically relevant  
11 samples.

12

### 13 **Conclusion**

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

17

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24 management at St Andrews, and also Mark Philo for his assistance with method  
25 development.

## 1 **Supporting Information Available**

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

6

## 7 **ABBREVIATIONS**

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

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

- 1 Coumaric acid, <sup>32</sup>Phloroglucinaldehyde, <sup>33</sup>Ferulic acid, <sup>34</sup>2-HydroxyBA, <sup>35</sup>Sinapic acid,
- 2 <sup>36</sup>Taxifolin, <sup>37</sup>4-Methoxysalicylic acid, <sup>38</sup>6-Methoxysalicylic acid, <sup>39</sup>Phloridzin. (B) <sup>13</sup>-
- 3 Methylgallic acid, <sup>2</sup>Isovanillic acid, <sup>3</sup>Cyanidin-3-glucoside, <sup>4</sup>Syringic acid, <sup>5</sup>2-MethoxyBA,
- 4 <sup>6</sup>Pelargonidin-3-glucoside, <sup>7</sup>Methyl vanillate, <sup>8</sup>Methyl-3,4-dimethoxybenzoate.

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

| Analyte                      | Ionization | Slope (a) <sup>a</sup> | CV   | R <sup>2</sup> | LOD (nM) |       |       |
|------------------------------|------------|------------------------|------|----------------|----------|-------|-------|
|                              | Mode       | Mean ± SD              | (%)  |                | 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-Methoxysalicylic 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   |
| <i>p</i> -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    |

- 1 <sup>a</sup>Linear regression analysis with a regression equation of  $y = ax + b$ , where  $x$  is the concentration in
- 2  $\mu\text{M}$ ,  $b$  is equal to 0 and  $y$  is the peak area; LOD, the limit of detection (Signal/Noise = 3);  $R^2$ ,
- 3 correlation coefficient of regression equations.

1 Table 2. SPE extraction efficiencies of analytes in urine, serum and faecal matrices as  
 2 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-Methoxysalicylic 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  |
| <i>p</i> -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 <sup>a</sup> | 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 <sup>b</sup>    | 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  |

1 Extraction efficiencies are expressed as mean % ± SD using DSC-18, Strata-X and  
2 Bond Elute C18 SPE cartridges for the extraction of urine, serum and fecal matrices  
3 respectively; VC, volume control; <sup>a</sup>NQ, not quantified due to high background  
4 concentrations in urine; <sup>b</sup>Scopoletin was used as a volume control standard and was  
5 therefore added post extraction only.  
6

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

| Analyte                      | Urine (nM)       |                  | Serum (nM)       |                  | Faeces (nM)      |                  |
|------------------------------|------------------|------------------|------------------|------------------|------------------|------------------|
|                              | Min <sup>a</sup> | Max <sup>b</sup> | Min <sup>a</sup> | Max <sup>b</sup> | Min <sup>c</sup> | Max <sup>d</sup> |
| 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               |
| <i>p</i> -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  |

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