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Rapid method for quantification of anthocyanidins and anthocyanins in human biological samples

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Abstract

Using simple solvent extraction, and enzymatic hydrolysis, a rapid LC-MS/MS method for quantification of free and conjugated forms of anthocyanidins and anthocyanins in plasma and urine samples was developed and validated. A mixed enzymatic treatment containing β-glucuronidase (100 U mL^{-1}) and sulfatase (2.5 U mL^{-1}) for 5 min (37 °C; pH 6) was optimal condition for deconjugation of anthocyanidins and anthocyanins in urine and plasma samples. The LC-MS/MS allowed quantifying thirteen different anthocyanidins and anthocyanins simultaneously. The developed LC-MS/MS method was precise and accurate over multiple days and nominal concentrations. The stability assessment study confirmed that the long-term storage and/or periodic use of plasma and urine samples might have a considerable impact on the stability of some anthocyanidins. The method was successfully applied to measure anthocyanidins and anthocyanins in plasma and urine samples following consumption of acute blueberry test meals.

Keywords: anthocyanidin, anthocyanin, acute blueberry consumption, plasma, urine

1. Introduction

Anthocyanins are water-soluble vacuolar plant pigments, which undergo structural re-arrangements in response to changes in pH, and form different colours from red to violet (Li, Ma, Huang, Li, & Yao, 2013). They are responsible for the colours of several fruits and flowers (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009a; Ignat, Volf, & Popa, 2011). Chemically, anthocyanins consist of an aglycone (anthocyanidin), which is bound to one or more sugar moieties. Structurally, anthocyanidins are polyhydroxy or polymethoxy derivatives of 2-phenylbenzopyrylium: two benzoyl rings (A and B) separated by a heterocyclic (C) ring (Mazza & Miniati, 1993). Around 17 anthocyanidins have been identified, whereas, only six of them are common and widely distributed; cyanidin (Cy), delphinidin (Dp), petunidin (Pt), peonidin (Pn), pelargonidin (Pg) and malvidin (Mv) (Fang, 2014). The sugar moieties commonly attached to anthocyanidins are glucose, galactose, rhamnose, and arabinose, which form different mono-, di- or tri-saccharide containing anthocyanins (Jaakola, 2013).

Anthocyanins are key components of the human diet due to their high abundance in fruits and vegetables such as berries, black carrot, red cabbage, and purple potato (Khoo, Azlan, Tang, & Lim, 2017). Recent studies attributed numerous health-promoting effects to anthocyanins such as antioxidant, anti-inflammatory, anti-diabetic and anti-cancer activities (Lala, Malik, Zhao, He, Kwon, Giusti, et al., 2006; Sancho & Pastore, 2012; Seeram, Momin, Nair, & Bourquin, 2001). These beneficial effects of anthocyanidins and anthocyanins depend on their absorption efficiency (Hribar & Ulrih, 2014). Upon absorption, they metabolised in the small intestine, liver, and kidney. In blood, anthocyanins occur in their native form or as methyl, sulfate and glucuronyl conjugates. Finally, they may be transformed into their corresponding phenolic acid and aldehyde metabolites in the gut (Fang, 2014; Hribar & Ulrih, 2014). Several factors such as dose, chemical structure, food or beverage matrix, processing, age and gender of the

individuals reported to have a major impact on the bioavailability and metabolism of anthocyanins (Fernandes, Faria, Calhau, de Freitas, & Mateus, 2014; Novotny, Clevidence, & Kurilich, 2012; Yang, Koo, Song, & Chun, 2011).

Under physiological conditions, anthocyanins and anthocyanins undergo degradation and structural rearrangements, which makes them difficult to quantify (Brouillard & Delaporte, 1977; Fernandes, Faria, Calhau, de Freitas, & Mateus, 2014; Fernandes, Faria, de Freitas, Calhau, & Mateus, 2015; Hribar & Ulrih, 2014). High-performance liquid chromatography (HPLC) is commonly used technique to quantify anthocyanidins and/or anthocyanins in biological samples including plasma and urine (Felgines, Talavéra, Gonthier, Texier, Scalbert, Lamaison, et al., 2003; Ling, Ren, Mallery, Ugalde, Pei, Saradhi, et al., 2009; G. Mazza, Kay, Cottrell, & Holub, 2002; Nakamura, Matsumoto, Morifuji, Iida, & Takeuchi, 2010). The use of liquid chromatography-mass spectrometry (LC-MS) has been growing due to the high sensitivity and selectivity of this technology. Thus far, only a few LC-MS methods have been reported for quantification of anthocyanidins and/or anthocyanins in biological samples (Giordano, Coletta, Rapisarda, Donati, & Rotilio, 2007; Kalt, Blumberg, McDonald, Vinqvist-Tymchuk, Fillmore, Graf, et al., 2008; Ling, et al., 2009). However, most of these methods quantify only anthocyanidins or anthocyanins and have long chromatographic run-times. Moreover, little is known about the applicability of these methods to both urine and plasma samples.

Solid phase extraction (SPE) is commonly used method for isolation of anthocyanidins and anthocyanins from biological matrices including plasma and urine. Although an efficient technique, the overall procedure is cumbersome and often results in significant losses. Also, this method is not considered as a suitable method for preparation of a large number of clinical samples, which contain unstable analytes such as anthocyanidins and anthocyanins (Van Eeckhaut, Lanckmans, Sarre, Smolders, & Michotte, 2009). Additonally, detection and

qualification of various forms of sulfo- and glucuro- anthocyanidins and anthocyanins conjugates is difficult in biological samples, due to lack of their respective analytical standards, and little is known about their exact site of conjugation. Therefore, enzymatically deconjugation is the preferred approach for quantitative estimation of conjugated forms of anthocyanidins and anthocyanins in biological samples. However, there is no exact information about the optimal enzymatic treatment for hydrolysis of conjugated forms of anthocyanidins and anthocyanins in plasma and urine samples.

Considering the above difficulties, a simple isolation and rapid LC-MS/MS method was developed for quantification of the free and conjugated forms of the major anthocyanidins and anthocyanins in plasma and urine.

2. Materials and Methods

2.1. Materials and reagents

The anthocyanidin and anthocyanin standards: Cy, Dp, Pt, Pn, Pg, Mv, cyanidin 3-*O*-glucoside (Cy3Glu), cyanidin-3-*O*-galactoside (Cy3Gal), cyanidin-3-*O*-arabinoside (Cy3Ara), delphinidin-3-*O*-galactoside (Dp3Gal), malvidin-3-*O*-galactoside (Mv3Gal), pelargonidin-3-*O*-glucoside (Pg3Glu), petunidin-3-*O*-glucoside (Pt3Glu) were purchased from Phytolab GmbH & Co. KG, Labor Addipharma, Hamburg, Germany (structure are shown in Supplementary Fig. S1). The internal standard (IS) 2-amino-3,4,7,8-tetramethyl-3H-imidazo[4,5-F] quinoxaline was obtained for Toronto Research Chemicals, Ontario, Canada. HPLC-grade methanol and acetonitrile, analytical grade acetic acid and hexane, β-glucuronidase and sulfatase were procured from Sigma-Aldrich, UK. Water used was of Milli-Q grade.

2.2. Standard preparation

The influence of several solvents (0.1% formic acid in methanol, 0.1% acetic acid in water,

0.1% acetic acid in water:acetonitrile (50:50, v/v); data is not shown) on the signal intensity was investigated. Amongst these, 0.1% formic acid in methanol was found to provide maximum signal intensity and good separation of anthocyanidins and anthocyanins. Hence, stock solutions (1 mg mL^{-1}) of each of the anthocyanidins and anthocyanins were prepared in 0.1% formic acid in methanol (v/v). The working standard (WS) solution was prepared by combining stock solutions of anthocyanidins and anthocyanins and adjusting their concentration by dilution with 10 mM acetic acid in methanol. Similarly, the working internal standard (IS) solution was prepared by dissolving and diluting accurately weighed 2-Amino-3,4,7,8-tetramethyl-3H-imidazo[4,5-F] quinoxaline in 10 mM acetic acid in methanol.

2.3. Human intervention studies, samples collection and preparation

The fresh blueberries (variety-Duke and Altair) were purchased from the local supermarket of Aberdeen, UK. The anthocyanidin and anthocyanin content of this blueberries sample is shown in Supplementary Table S1. Healthy human donors (n=2) were recruited for the collection of plasma and urine samples. The volunteers were asked to avoid the consumption of foods rich in anthocyanidins and anthocyanins before sample collections. Two test meals were provided on separate days. The first meal comprised 375 g of whole fresh blueberries and in the second each volunteer received 460 g of a blueberry smoothie (prepared in skimmed yogurt), which was equivalent to nearly 350 g of fresh blueberries. Both meals had to be consumed within 30 min. For each intervention, the blood (10 mL) was collected directly into heparinised tubes before the intervention and at three-time intervals (30, 60 and 180 min) following consumption. The samples were immediately placed ice and centrifuged (1500 x g; 15 min; 4 °C) to separate the plasma. Urine samples were collected before the intervention and at two-time intervals (60 and 180 min) after consumption and the weight and volume recorded. The harvested plasma and the urine were aliquoted and stored at -70 °C until analysis. the human study was approved by the Rowett Research Institute Human Studies Management Committee.

2.4. LC-MS/MS instrumentation

LC-MS/MS analysis was conducted using a liquid chromatography system coupled to a triple quadrupole mass spectrometer (Agilent 6490, USA). The separation of samples was achieved on Phenomenex Synergi Polar-RP C18 column (250×4.6 mm). The LC conditions were optimized as follows: solvent A was 2% formic acid, and solvent B was acetonitrile-methanol (85:15, v/v)(Buchert, Koponen, Suutarinen, Mustranta, Lille, Törrönen, et al., 2005). The gradient program for pump B was as follows: 0.00–25 min, 5-25%; 25-40 min, 25-80%; 40-40.2 min, and 40.2-45 min, 5%. The flow rate was set to 0.4 mL min^{-1}, and the column temperature was set at 40 °C. The mass spectra were acquired at positive mode using Agilent Jet Stream electrospray ionization, and quantification of all analytes was performed in multiple reaction monitoring (MRM) mode. The other operating parameters were as follows: nebulizer gas pressure, 60 psi; nebulizer gas flow, 19 L min^{-1}; sheath gas flow, 10 L min^{-1}; nebulizer gas temperature, 230 °C; sheath gas heater temperature, 400 °C. Mass hunter (Agilent) software was used to control the instruments and for data acquisition and processing.

2.5. Calibration curves

For quantification of anthocyanidins and anthocyanins from plasma and urine samples, a matrix-matched calibration curve was prepared at five calibration points by serial increasing the addition of the WS solution (starting from 1 to 50 μL) to 100 μL blank plasma or urine. The constant volume of the working IS solution (50 μL) was added, and the reaction mixture was then diluted to 500 μL with 10 mM acetic acid in methanol. The calibration samples were centrifuged (12500×g; 5 min; 4 ºC) to remove precipitated proteins. The samples further purified by adding n-hexane (500 μL). After centrifugation (12500×g for 5 min at 4 ºC), the lower phase was collected in brown-coloured vials. Calibration standards were freshly prepared for each analysis.

2.6. The extraction efficiency of anthocyanins and anthocyanidins

In plasma and urine, the extraction efficiency of anthocyanins and anthocyanidins were studied in different acidified methanolic conditions such as 10 mM formic acid in methanol, 10 mM acetic acid in methanol, 1 and 10 mM hydrochloric acid in methanol. Individual recoveries of each anthocyanidin and anthocyanin were analysed by spiking blank plasma/urine with a known amount of the WS (50 ng mL^{-1}), and the amounts of respective anthocyanin were estimated by measuring the peak area ratios and fitting these values to the straight-line equation of the calibration curve.

2.7. Extraction protocol for free anthocyanidins and anthocyanins

Combined protein precipitation and solvent extraction method was used to liberate the free anthocyanidins and anthocyanins in plasma and urine samples. Frozen samples (plasma and urine) were thawed to room temperature, and 100 µL of each sample was transferred into 1.5 mL Eppendorf tubes. The IS (50 µL) was added, and all tubes diluted to 500 µL with 10 mM acetic acid in methanol. Following centrifugation (12500×g; 5 min; 4 ˚C), the supernatant was transferred, and n-hexane (500 µL) added. The samples were mixed well (vortex), centrifuged (12500 × g; 5 min; 4 ˚C) and the lower phase transferred to brown-coloured vials for LC-MS/MS analysis (Fig. 1).

2.8. Optimization of enzymatic hydrolysis of anthocyanidin and anthocyanin conjugates

Treatment with β-glucuronidase and sulfatase enzymes was optimized to hydrolyse the sulfo- and glucuro-conjugated forms of anthocyanidins and anthocyanins in the plasma and urine samples. Preliminary studies on the stability of anthocyanidins and anthocyanins in the presence 25000 U mL^{-1} of β-glucuronidase at different pH (4, 5 and 6) and incubation times (5 and 30 min at 37 °C) was performed (data is not shown). Maximum stability was obtained at pH 6, with a 5 min incubation at 37 °C. Therefore, all further work regarding optimization of concentrations of β-glucuronidase and sulfatase for hydrolysis of conjugated anthocyanidins and anthocyanins in plasma and urine were at the above conditions. Initially, plasma samples

were subjected to enzymatic hydrolysis with different concentrations of individual and combined treatments of β-glucuronidase and sulfatase. Based on results of this study, the combined treatment of β-glucuronidase and sulfatase were optimized for urine samples by studying their three different combinations: 1) β-glucuronidase (100 U mL^{-1}) and sulfatase (5 U mL^{-1}), 2) β-glucuronidase (100 U mL^{-1}) and sulfatase (2.5 U mL^{-1}), 3) β-glucuronidase (50 U mL^{-1}) and sulfatase (2.5 U mL^{-1}).

2.9. Extraction of de-conjugated anthocyanidins and anthocyanins

For extraction of de-conjugated anthocyanidins and anthocyanins, the standard addition method was adapted to compensate for their enzymatic degradation. Briefly, 100 µL of the reaction mixture containing citrate phosphate buffer (0.3 M; pH 6), β-glucuronidase (100 U mL^{-1}) and sulphatase (2.5 U mL^{-1}) was mixed with the working standard pre-added plasma/urine sample (110 µL) and immediately incubated for 5 min at 37 ˚C. 10 mM acetic acid in methanol (240 µL) and IS (50 µL) were added to the reaction mixture to stop the enzymatic reaction and to perform protein precipitation. After centrifugation (12500 × g; 5 min; 4 ˚C), the supernatant was transferred to clean Eppendorf tubes, and n-hexane (500 µL) added. The samples tubes were mixed well (vortex), centrifuged (12500 × g; 5 min; 4 ˚C) and the lower aqueous layer transferred to brown-coloured vials for LC-MS/MS analysis. The overall experimental workflow for the de-conjugation and extraction of anthocyanidins and anthocyanins is shown in Fig. 1.

2.10. Method validation

Validation experiments were performed as per guidance issued by the U.S. Food and Drug Administration (Health & Services, 2001) and the European Medicines Agency (Smith, 2012). The method was validated for linearity, precision, and accuracy. Moreover, matrix effect and stability of analytes in biological matrices were also studied. Matrix-matched calibration curves were established, and the peak area ratios of each analyte to the IS versus their respective

analyte concentration were used to calculate linear regression (r^2). The lowest concentration of each analyte on the calibration curve was recognized as the lower limits of quantification (LLOQ). Limits of detection (LOD) and limits of quantification (LOQ) were extrapolated from the slope and standard deviation of the response of linear calibration curves (Shrivastava & Gupta, 2011). The inter-assay variabilities were determined on three consecutive days. The accuracy and precision of the method were assessed by analysing plasma and urine samples at low, medium, and high concentrations of each analyte on the calibration curve. For evaluation of the matrix effect, blank human plasma and urine samples spiked to the medium concentration of each anthocyanidin and anthocyanin analyte and one concentration of the internal standard (100 ng mL^{-1}). The matrix effect was calculated by comparing the average area count for the spiked analyte in the extracted matrix after the extraction procedure with the average area count for the same concentration of the analyte in neat solution (Chambers, Wagrowski-Diehl, Lu, & Mazzeo, 2007; Matuszewski, Constanzer, & Chavez-Eng, 2003). To assess the stability of anthocyanidins and anthocyanins in plasma and urine, samples were spiked with a known concentration (40 ng mL^{-1}) of each analyte and stored at -70 °C for 30 days. Similarly, to understand freeze and thaw stability, plasma and urine samples containing known concentrations of WS were frozen at -70 °C for 24 hours and allowed to thaw completely to room temperature. After three freeze-thaw cycles (as described) the samples were analysed and compared.

3. Results and discussion

3.1. Optimization of LC-MS/MS conditions

Initially, manual detection of mass spectrum scanning mode, dwell time, compensation voltage (CV), as well as a precursor and product ion for each anthocyanidin, anthocyanin, and IS was performed. These parameters are essential to confirm and quantify each of the individual analytes. Optimized reaction monitoring conditions for each anthocyanidin, anthocyanin and

IS are summarized in Supplementary Table S2. All anthocyanidin and anthocyanin standards showed intense precursor and product ion peaks at positive scanning mode. Reversed-phase C18 columns are commonly used for the separation of anthocyanins (da Costa, Horton, & Margolis, 2000). Hence, the separation of anthocyanidin and anthocyanin mixture was initially assessed using Synergi 4µ Fusion-RP 80A, Jupiter 5µ C18 300A, Phenomenex Synergi Polar-RP C18, Luna 5µ C18 100A, and Kinetex 5µ C18 100A columns. Amongst these, a Phenomenex Synergi Polar-RP C18 column was selected further for analysis, based on best resolution and separation abilities. In this study, an MRM-based quantification approach was adapted for concurrent quantification of 13 different anthocyanidins and anthocyanins in human urine and plasma samples. MRM is a sensitive and rapid method for removal of interfering co-eluting endogenous compounds of biological matrices including lipids and sterols. Supplementary Fig. S2 shows a representative MRM chromatogram of anthocyanidin and anthocyanin analytes in a spiked human plasma sample. Short LC run-times is a prerequisite condition for analysis of a large number of samples as commonly generated in clinical studies. Several solvent systems were tried to achieve good separation, resolution and short run time. The solvent system [Solvent A: 2% formic acid in water and Solvent B: acetonitrile: methanol (85:15 v/v)] originally described by Buchert et al. (Buchert, et al., 2005) was most feasible. Slight solvent and gradient modification in above method lead to a significant reduction in run-time from 67 to 45 min. In addition, the developed method facilitates separation and accurate quantification of anthocyanin isomers like Cy3Glu and Cy3Gal.

3.2. Extraction efficiency of anthocyanidins and anthocyanins

Herein, a simple protein precipitation-solvent extraction and enzymatic hydrolysis protein precipitation-solvent extraction procedures were developed for rapid isolation of free and de-conjugated forms of anthocyanidins and anthocyanins, respectively from both urine and plasma

samples. It has been demonstrated that both anthocyanidins and anthocyanins have better stability in methanol, and acidification of methanol further provides added stability (Aqil, Vadhanam, Jeyabalan, Cai, Singh, & Gupta, 2014; Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009b; da Costa, Horton, & Margolis, 2000). However, the extraction efficiency of anthocyanidins and anthocyanins in acidified methanol from biological matrices was variable, and also depends on the type of acid used (Aqil, Vadhanam, Jeyabalan, Cai, Singh, & Gupta, 2014). In earlier studies, methanol that contains small amounts of hydrochloric or formic acid was found to ideal for extraction of anthocyanidins and anthocyanins from biological samples (Aqil, Vadhanam, Jeyabalan, Cai, Singh, & Gupta, 2014; Felgines, Talavera, Texier, Gil-Izquierdo, Lamaison, & Remesy, 2005). In this study, extraction efficiencies of 13 anthocyanidins and anthocyanins from human plasma and urine samples in different acidified methanolic (HCI, formic acid and acetic acid in methanol) solvents were studied. Results of recoveries of spiked anthocyanidins and anthocyanins from plasma samples extracted into methanol under different acidic conditions are shown in Supplementary Table S3 and S4. Only two anthocyanidins Mv and Pn, were detectable in plasma samples following extraction with 1 mM HCl in methanol. In both plasma and urine, extraction with 10 mM HCl and acetic acid in methanol showed similar recoveries (>70%) for almost all anthocyanidins/anthocyanins except Dp and Cy, which had low recoveries (60% and 30%, respectively from urine). In some cases, recoveries of some anthocyanidins and anthocyanins were found to be skewed, which may be due to the matrix effect or acidic hydrolysis of the anthocyanins. In the previous study, it has been found that anthocyanins and anthocyanidins had a comparatively good recovery from plasma with HCI compared with phosphoric and trifluoroacetic acid (Aqil, Vadhanam, Jeyabalan, Cai, Singh, & Gupta, 2014). This study has shown that all of the studied anthocyanin and anthocyanidin compounds had much better recoveries in acetic acid compared with hydrochloric acid from

both plasma and urine. Based on the maximum recoveries of a wide range of spiked anthocyanidins and anthocyanins, 10 mM acetic acid in methanol was found to be the most suitable for extracting solvent for both urine and plasma samples.

3.3. Optimal enzymatic condition for deconjugation of anthocyanidins and anthocyanins

Currently, there is only one reported method for de-conjugation of glucuronidated forms of anthocyanidins and anthocyanins from human urine samples (Felgines, Talavera, Texier, Gil-Izquierdo, Lamaison, & Remesy, 2005). In this study, it has been observed that β-glucuronidase and sulfatase acted very quickly, and longer treatments (up to 30 min) with these enzymes led to the degradation of the majority of studied anthocyanidins and anthocyanins. Moreover, glucuronidase (10^6 U L^{-1}) treatment to urine samples for 5 min at 37 °C was effective to de-conjugate glucuronidated anthocyanidins and anthocyanins (Felgines, et al., 2003; Felgines, Talavera, Texier, Gil-Izquierdo, Lamaison, & Remesy, 2005). Similarly, we also found that incubation of biological samples with β-glucuronidase and sulphatase mixture for 5 min at 37 °C was an optimal treatment for de-conjugation of both sulfated and glucuronidated anthocyanidins and anthocyanins in both plasma and urine.

The summary of optimization of β-glucuronidase and sulfatase activities for deconjugation of anthocyanidins and anthocyanins in plasma samples after blueberry-rich meals consumption by human volunteers are shown in Supplementary Fig. S3. Activities of β-glucuronidase and sulfatase were optimized for hydrolysis of sulfo- and glucuro-conjugated anthocyanidins and anthocyanins in plasma and urine at previously established conditions of pH 6, for 5 minutes at 37 °C. The enzymatic treatment containing 100 U mL^{-1} of β-glucuronidase and 2.5 U mL^{-1} of sulfatase was found to provide optimal stability and hydrolysis of conjugated anthocyanidins and anthocyanins in plasma samples. Similarly, Analogous to plasma, the treatment of β-glucuronidase and sulfatase at concentration 100 U $mL^{-1}$and 2.5 U mL^{-1}, respectively were also

observed to be optimal for de-conjugation of sulfo- and glucuro- conjugates of anthocyanidins and anthocyanins in urine samples (Supplementary Fig. S4).

3.4. Method validation

Method validation is essential to demonstrate that methods are reliable, reproducible and fit for purpose (Health & Services, 2001). The coefficient of determination (r^2) for anthocyanidin and anthocyanin was evaluated for linearity analysis by plotting the peak area ratio versus the concentration in triplicate. The LC-MS/MS method was linear for all analytes over the concentration range (10-500 ng mL^{-1} for Cy, Dp, Pg, Pt; and 2-100 ng mL^{-1} for Mv, Pn, Cy3Glu, Cy3Gal, Cy3Ara, Dp3Gal, Mv3Gal, Pg3Glu, Pt3Glu) studied in both plasma and urine. The resultant r^2 values for all anthocyanidins and anthocyanins were close to 1 (Table 1), which indicated good linearity within the considered concentration ranges (Bosco, Daeseleire, Van Pamel, Scariot, & Leus, 2014). The LOD and LOQ values of all anthocyanidin and anthocyanin analytes were calculated in blank plasma and urine to determine the sensitivity of the method. LOD and LOQ in plasma were within the range 0.05-3.66 ng mL^{-1} and 0.18-12.20 ng mL^{-1}, respectively. In urine, LOD and LOQ were 0.09-6.66 ng mL^{-1} and 0.31-22.19 ng mL^{-1}, correspondingly (Table 1). Blank plasma and urine samples were spiked with low, medium, and high nominal concentrations to determine the accuracy, inter-day precision, and matrix effect. The results of all precision, accuracy and matrix effect studies for all anthocyanidins and anthocyanins in plasma are summarized in Table 2. Similarly, Table S5 shows summary of results of precision, accuracy and matrix effect studies for anthocyanidins and anthocyanins in urine. The percent coefficient of variation (% CV) values of all anthocyanidins and anthocyanins analytes studied were within the acceptable limits (<15%) for both plasma and urine at respective their nominal concentrations. Likewise, the results of the percent recovery (% RE) of all anthocyanidin and anthocyanin analytes were also within an acceptable range

(80–120%) for both plasma and urine (Table 2 and S5). These results altogether confirmed that the developed method was precise and accurate.

3.5. Matrix Effects

Matrix effect is a direct or indirect alteration or interference in response due to the presence of unintended analytes (for analysis) or other interfering substances in the sample (Health & Services, 2001) and it is important that this effect is established. Usually, the matrix effect is high for biological samples. Therefore, to compensate for this and to improve detection, matrix-matched calibration curves were considered for further quantification of anthocyanidins and anthocyanins in plasma and urine samples (Stuber & Reemtsma, 2004).

Matrix effect is expressed as the % SSE (percent signal enhancement or suppression) (King, Bonfiglio, Fernandez-Metzler, Miller-Stein, & Olah, 2000). The values of % SSE for all anthocyanidin and anthocyanin analytes in plasma and urine are shown in Table 2 and Table S5, respectively. A negative % SSE value indicates ion suppression, and a positive % SSE indicates enhancement of the analyte signal (Chambers, Wagrowski-Diehl, Lu, & Mazzeo, 2007). Except Cy, all anthocyanidin and anthocyanin analytes were showing enhanced ionization in plasma. In urine, Cy, Dp, and Cy3Gal showed suppressed ionization whereas, all other analytes showed enhancement in the ionization process.

3.6. Stability assessment

Stability of the analytes during the whole analytical procedure is a prerequisite for reliable quantification (Peters, 2007). In this study, longer-term storage (30 days), and freeze-thaw stability assessments were performed to understand the stability of anthocyanidins and anthocyanins analytes in a given matrix (plasma and urine) under storage and experimental conditions. The stability assessment is summarised in Table 3. Results indicated that almost all of the anthocyanidins and anthocyanins analytes were degraded after three freeze and thaw cycles and during long term storage at -70 ^{o}C (around 30 days) in both plasma and urine.

However, this observed degradation was not found to be significant, since %CV values of all anthocyanidins and anthocyanins analytes were within 15%. Anthocyanidin, Dp was found to be completely degraded during 30 days storage and with freeze and thaw cycles in both plasma and urine. Besides having considerable long-term stability, Cy was also found to be completely degraded after three freeze and thaw cycles in urine.

3.7. Quantification of anthocyanidin and anthocyanin in human biological samples

To demonstrate the efficiency of the method, we quantified anthocyanidins and anthocyanins in plasma and urine samples from two healthy volunteers after consuming fresh blueberries (375 g), and results of this are presented in Fig. 2. In plasma, only Mv3Gal was observed in its native form in low amounts (< 2 ng mL^{-1}). This was also the most abundant unconjugated anthocyanin in urine (> 10 ng mL^{-1}), present along with lesser amounts of other free anthocyanins. The majority of the anthocyanins and anthocyanidins in both plasma and urine were found to be sulfo- and glucuro- conjugates.

Anthocyanidin and anthocyanin contents were also assessed in plasma and urine samples following consumption of crushed blueberries in the form of a yogurt-based smoothie (Fig. 3). As for the fresh blueberries, it was also observed that most of the anthocyanins were in their free or native forms, whereas the anthocyanidins were in their conjugated forms in plasma and urine. For both test meals, Mv3Gal was the predominant anthocyanin in its native form in both plasma and urine.

4. Conclusions

This study provides a simple method for extraction and LC-MS/MS quantification of both free and conjugated forms of anthocyanidins and anthocyanins in urine and plasma samples. The developed method was rapid and reliable, which allows the simultaneous quantification of thirteen different anthocyanidins and anthocyanins in human plasma and urine samples. The

method presented in this study can be used to understand bio-availability, metabolism, and excretion of different plant sourced anthocyanidins and anthocyanins in human, which is critical to informing on the biological activity of this important group of plant metabolites.

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Appendix A. Supplementary data

The following are the Supplementary data to this article:

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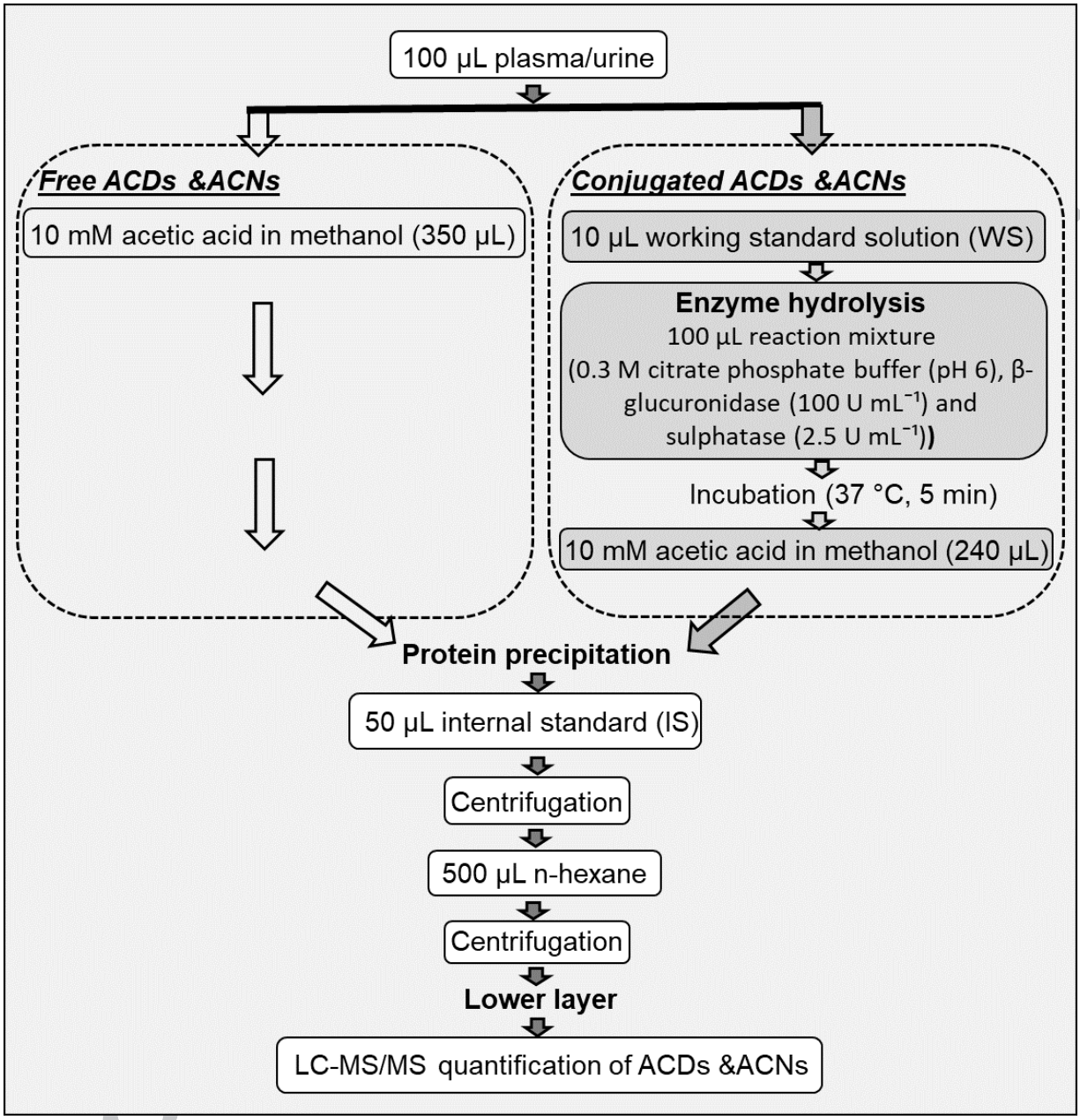

Fig. 1. Experimental workflow for the extraction of anthocyanidins (ACDs) and anthocyanins (ACNs) from plasma and urine samples.

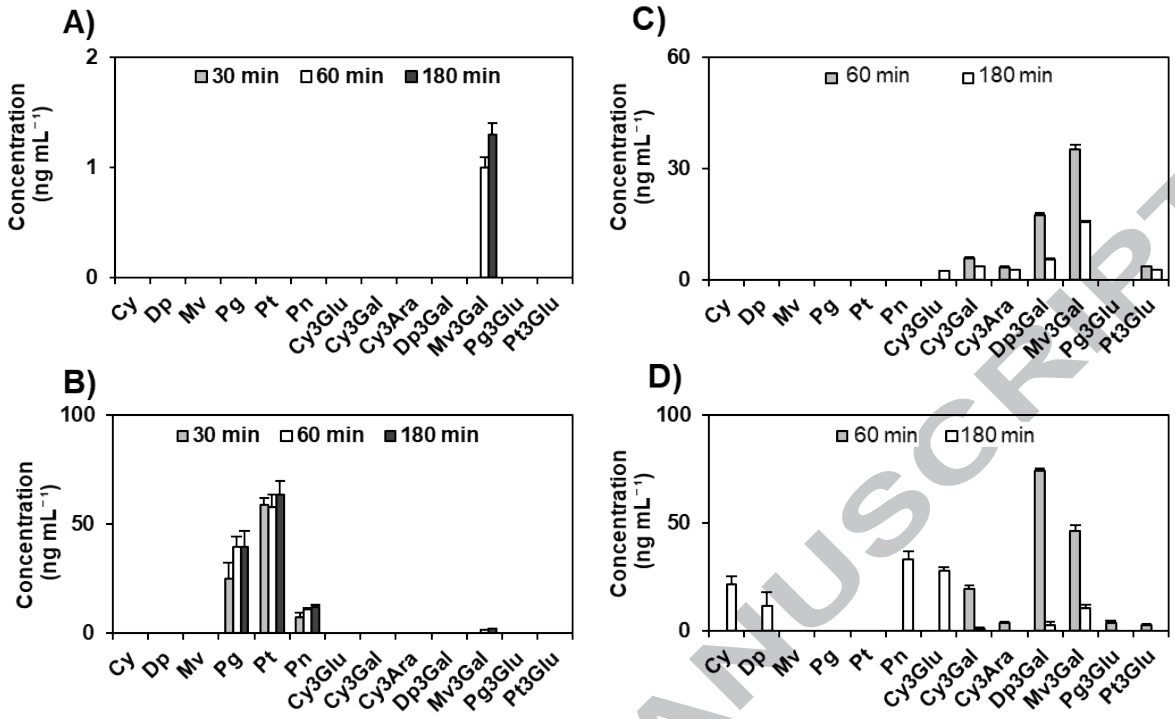

Fig. 2. Anthocyanidins and/or anthocyanins content present as free, and sulfo- and glucuro-conjugated forms in human plasma and urine samples from two human volunteers consumed the whole blueberry [A - free forms in plasma; B - conjugated forms in plasma; C - free forms in urine; D - conjugated forms in urine]. Results are mean of six technical replicates ± SE. (Cy – cyanidin, Dp – delphinidin, Mv – malvidin, Pg – pelargonidin, Pt – petunidin, Pn – peonidin, Cy3Glu - cyanidin 3-glucoside, Cy3Gal - cyanidin 3-galactoside, Cy3Ara - cyanidin 3-arabinoside, Dp3Gal - delphinidin 3-galactoside, Mv3Gal - malvidin 3-glactoside, Pg3Glu - pelargonidin 3-glucoside, Pt3Glu - petunidin 3-glucoside).

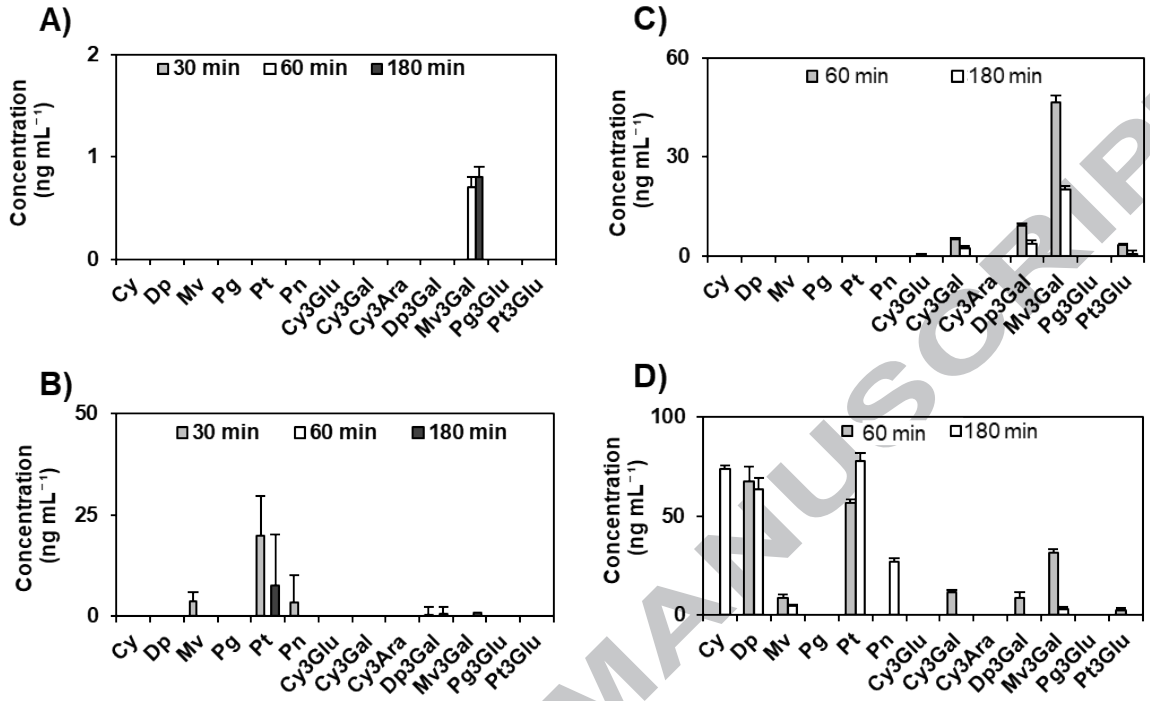

Fig. 3. Anthocyanidins and/or anthocyanins content present as free, and sulfo- and glucuro-conjugated forms in human plasma and urine samples from two human volunteers after the consumption of blueberry smoothie [A - free forms in plasma; B - conjugated forms in plasma; C - free forms in urine; D - conjugated forms in urine]. Values represents mean of six technical replicates ± SE. (Cy – cyanidin, Dp – delphinidin, Mv – malvidin, Pg – pelargonidin, Pt – petunidin, Pn – peonidin, Cy3Glu - cyanidin 3-glucoside, Cy3Gal - cyanidin 3-galactoside, Cy3Ara - cyanidin 3-arabinoside, Dp3Gal - delphinidin 3-galactoside, Mv3Gal - malvidin 3-glactoside, Pg3Glu - pelargonidin 3-glucoside, Pt3Glu - petunidin 3-glucoside).

Table 1. Coefficient of determination (r^2), limits of detection (LOD) and limits of quantification (LOQ) values for anthocyanidins and anthocyanins in human plasma and urine.

Sr. No.	Anthocyanidin/ Anthocyanin	Plasma			Urine		
		r^2	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	r^2	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
1.	Cyanidin	0.9963	0.87	2.59	0.9999	1.15	3.82
2.	Delphinidin	0.9959	0.76	2.52	0.9992	0.72	2.40
3.	Malvidin	0.9983	1.38	4.63	0.9997	2.25	7.52
4.	Pelargonidin	0.9998	0.51	1.68	0.9985	2.20	7.33
5.	Petunidin	0.9989	0.81	2.69	0.9992	1.36	4.55
6.	Peonidin	0.9995	3.66	12.20	0.9996	6.66	22.19
7.	Cyanidin 3-glucoside	0.9992	0.23	0.78	0.9988	0.09	0.31
8.	Cyanidin 3-galactoside	0.9983	0.18	0.61	0.9985	0.19	0.62
9.	Cyanidin 3-arabinoside	0.9992	0.07	0.22	0.9993	0.34	1.13
10.	Delphinidin 3-galactoside	0.9983	0.20	0.68	0.9997	0.15	0.51
11.	Malvidin 3-glactoside	0.9999	0.10	0.34	0.9995	0.43	1.44
12.	Pelargonidin 3-glucoside	0.9998	0.05	0.18	0.9988	0.43	0.42
13.	Petunidin 3-glucoside	0.9996	0.10	0.34	0.9991	0.21	0.69

Table 2. Summary of precision (% CV), accuracy (%RE) and matrix effect (%SSE) studies for anthocyanidins and anthocyanins in human plasma (n=3).

Sr. No.	Anthocyanidin/ Anthocyanin	Nominal conc. (ng mL^{-1})			Precision (% CV) at conc. (ng mL^{-1})			Accuracy (%RE) at conc. (ng mL^{-1})			%SSE at medium nominal conc. (ng mL^{-1})
		Low	Medium	High	Low	Medium	High	Low	Medium	High	
1.	Cyanidin	10	100	500	4.97	3.36	3.07	109.9	95.2	103.0	-3.51
2.	Delphinidin	10	100	500	3.99	2.02	3.49	107.0	96.2	102.7	4.81
3.	Malvidin	2	20	100	11.87	1.35	0.47	95.0	99.93	100.4	232.9
4.	Pelargonidin	10	100	500	6.65	1.77	1.57	101.0	97.10	100.5	36.5
5.	Petunidin	10	100	500	5.13	3.67	0.91	109.1	94.2	102.0	79.0
6.	Peonidin	10	100	500	4.20	4.66	0.51	98.28	101.0	100.3	40.1
7.	Cyanidin 3-glucoside	2	20	100	1.26	3.92	0.96	104.8	97.6	101.8	17.2
8.	Cyanidin 3-galactoside	2	20	100	5.65	3.33	0.65	101.9	98.2	102.1	2.90
9.	Cyanidin 3-arabinoside	2	20	100	2.73	2.19	1.54	107.8	97.1	102.8	12.9
10.	Delphinidin 3-galactoside	2	20	100	5.37	1.38	1.82	103.2	97.9	101.7	23.9
11.	Malvidin 3-glactoside	2	20	100	3.46	5.44	1.15	106.3	95.1	101.2	22.8
12.	Pelargonidin 3-glucoside	2	20	100	1.61	0.63	1.98	111.2	95.4	101.8	15.9
13.	Petunidin 3-glucoside	2	20	100	0.70	2.54	1.06	105.3	97.06	101.9	22.7

Table 3. Stability of anthocyanidins and anthocyanins in human plasma and urine samples (n = 3) [%RE = percent recovery; %CV= percent coefficient of variation].

Sr. No.	Anthocyanidins/anthocyanins	Freeze and thaw stability (3 cycles, −70 °C)					Long-term stability (30 days, −70 °C)				
		Nominal conc. (ng mL^{-1})	Plasma		Urine		Nominal conc. (ng mL^{-1})	Plasma		Urine	
			%RE	%CV	%RE	%CV		%RE	%CV	%RE	%CV
1.	Cyanidin	100	14.6	9.24	n.d.	n.d.	40	76.7	5.68	75.9	8.72
2.	Delphinidin	100	n.d.	n.d.	n.d.	n.d.	40	n.d.	n.d.	n.d.	n.d.
3.	Malvidin	20	12.1	2.42	31.7	10.3	40	62.8	9.45	44.3	9.90
4.	Pelargonidin	100	28.3	2.43	35.6	7.55	40	192.4	2.86	85.2	9.27
5.	Petunidin	100	8.9	8.60	23.1	1.72	40	56.9	1.77	70.2	6.73
6.	Peonidin	20	25.3	9.01	25.6	1.41	40	110.0	5.55	45.2	5.48
7.	Cyanidin 3-glucoside	20	83.2	10.96	107.2	5.03	40	78.5	2.94	62.3	7.19
8.	Cyanidin 3-galactoside	20	104.1	6.32	107.4	3.37	40	83.5	2.16	65.6	6.28
9.	Cyanidin 3-arabinoside	20	101.2	4.30	106.5	1.02	40	86.4	2.46	57.9	4.94
10.	Delphinidin 3-galactoside	20	92.8	7.50	98.9	4.07	40	83.2	0.77	70.6	10.4
11.	Malvidin 3-glactoside	20	75.1	5.90	71.5	4.27	40	62.3	3.87	42.3	10.1
12.	Pelargonidin 3-glucoside	20	91.9	5.02	82.4	3.47	40	92.1	2.60	58.6	6.54
13.	Petunidin 3-glucoside	20	84.0	4.75	96.7	3.53	40	75.5	4.64	66.4	8.19

Highlights

- Rapid method to measure anthocyanidins and anthocyanins in biological samples.

- Extracts both free and conjugated forms from blood and plasma
- Thirteen major compounds quantified by LC-MS

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: