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RESEARCH ARTICLE

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# HPLC-MS/MS analysis of anthocyanins in human plasma and urine using protein precipitation and dilute-and-shoot sample preparation methods, respectively

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## Abstract

A high-performance liquid chromatography tandem–mass spectrometry (HPLC-MS/MS) method has been developed to analyze anthocyanins in urine and plasma to further understand their absorption, distribution, metabolism and excretion. The method employed a Synergi RP-Max column (250 × 4.6 mm, 4 µm) and an API 4000 mass spectrometer. A gradient elution system consisted of mobile phase A (water–1% formic acid) and mobile phase B (acetonitrile) with a flow rate of 0.60 mL/min. The gradient was initiated at 5% B, increased to 21% B at 20 min, and then increased to 40% B at 35 min. The analysis of anthocyanins presents a challenge because of the poor stability of anthocyanins during sample preparation, especially during solvent evaporation. In this method, the degradation of anthocyanins was minimized using protein precipitation and dilute-and-shoot and sample preparation methods for plasma and urine, respectively. No interferences were observed from endogenous compounds. The method has been used to analyze anthocyanin concentrations in urine and plasma samples from volunteers administered saskatoon berries. Cyanidin-3-galactoside, cyanidin-3-glucoside, cyanidin-3-arabinoside, cyanidin-3-xyloside and quercetin-3-galactoside, the five major flavonoid components in saskatoon berries, were identified in plasma and urine samples.

**KEYWORDS**

anthocyanin, flavonoid, HPLC, mass spectrometer, plasma, urine

## 1 | INTRODUCTION

Anthocyanins constitute the largest group of water-soluble pigments in plants and are responsible for the blue, purple and red colors of many fruits, flowers and leaves. Types and concentrations of anthocyanins vary widely in food (Bhagwat, Haytowitz, Wasswa-Kintu, & Holden, 2013). Many population-based investigations have associated the intake of individual flavonoids with a reduced incidence of diseases such as cardiovascular disease, diabetes mellitus and cancer (Del Rio et al., 2013). Food intervention studies have shown that long-term administration of fruits rich in anthocyanins can improve clinical and biomedical indexes in patients with various health conditions (Del Rio et al., 2013).

The saskatoon (*Amelanchier alnifolia* Nutt., Rosaceae) is a fruit-producing shrub or small tree native to the North American Great Plains (Mazza, 1986). Its berry is also referred to as the juneberry or serviceberry and was a traditional food used by Aboriginal people. Saskatoon berries have been grown on the Canadian prairies commercially since the mid-1960s. Although saskatoon berry and blueberry (*Vaccinium* spp.) belong to different genera and species, they share many characteristics that are important to consumers, including fruit color, shape, texture and nutrition (Mazza, 1982). Both saskatoon berries and blueberries are rich in anthocyanins (Bakowska-Barczak & Kolodziejczyk, 2008; Lavola, Karjalainen, & Julkunen-Tiitto, 2012; Ozga, Saeed, Wismer, & Reinecke, 2007).

**Abbreviations:** Cy-3-ara, cyanidin-3-arabinoside; Cy-3-gal, cyanidin-3-galactoside; Cy-3-glu, cyanidin-3-glucoside; Cy-3-rut, cyanidin-3-rutinoside; De-3-glu, delphinidin-3-glucoside; De-3-rut, delphinidin-3-rutinoside; Ma-3-gal, malvidin-3-galactoside; Ma-3-glu, malvidin-3-glucoside; MRM, multiple reaction monitoring; Pg-3-glu, pelargonidin-3-glucoside; Pn-3-glu, peonidin-3-glucoside; Qu-3-gal, quercetin-3-galactoside; TFA, trifluroacetic acid.

  

Appropriate analytical methods are critical in understanding the absorption, distribution, metabolism and excretion of anthocyanins. The absorption anthocyanins follows a unique pattern rather different from those of other flavonoids (Fang, 2014a; Fernandes, Faria, Calhau, de Freitas, & Mateus, 2014; Rodriguez-Mateos et al., 2014). Anthocyanins such as cyanidin-3-glucoside (Cy-3-glu) and pelargonidin-3-glucoside (Pg-3-glu) could be absorbed in their intact form into the gastric and intestinal wall (He, Wallace, Keatley, Failla, & Giusti, 2009; Talavera et al., 2005). They can then undergo extensive first-pass metabolism and enter the systemic circulation as metabolites (Fang, 2014b). Some anthocyanins can reach the large intestine intact in large quantities where they are decomposed by gut bacteria.

Although many LC-MS/MS methods have been developed for the analysis of anthocyanins, most of them involve time-consuming sample preparation procedures. These sample preparation procedures are generally employed to remove salts, and to purify and concentrate analytes for better sensitivity and reproducibility. Solid-phase extraction is the most commonly used sample preparation method for the isolation of anthocyanins from plasma and urine samples (Cao, Muccitelli, Sanchez-Moreno, & Prior, 2001; Cooke et al., 2006; de Ferrars et al., 2014; Felgines et al., 2005; Giordano, Coletta, Rapisarda, Donati, & Rotilio, 2007; Ichiyanagi et al., 2005; Kay, Mazza, Holub, & Wang, 2004; Miyazawa, Nakagawa, Kudo, Muraishi, & Someya, 1999; Talavera et al., 2005; Tian, Giusti, Stoner, & Schwartz, 2006; Urpi-Sarda et al., 2009). However, the analysis requires relatively large quantities of plasma or serum samples. Further, anthocyanins are unstable during sample preparation procedures, especially during the evaporation step, which is often required for solid-phase extraction (Woodward, Kroon, Cassidy, & Kay, 2009).

This paper reports on a LC-MS/MS method for the analysis of anthocyanins and flavonols in human plasma and urine using a simple protein precipitation and a dilute-and-shoot sample preparation method, respectively. These sample preparation methods improve the stability of anthocyanins, require small volumes of blood samples and can potentially improve the reproducibility of the analysis of flavonoids. The analytical method was successfully applied to the analysis of anthocyanins in plasma and urine samples from volunteers who had ingested saskatoon berries.

## 2 | EXPERIMENTAL

### 2.1 | Materials and reagents

Delphinidin-3-glucoside (De-3-glu), delphinidin-3-rutinoside (De-3-rut), cyanidin-3-galactoside (Cy-3-gal), cyanidin-3-glucoside (Cy-3-glu), cyanidin-3-rutinoside (Cy-3-rut), cyanidin-3-arabinoside (Cy-3-ara), malvidin-3-galactoside (Ma-3-gal), malvidin-3-glucoside (Ma-3-glu), peonidin-3-glucoside (Pn-3-glu) and quercetin-3-galactoside (Qu-3-gal) were purchased from Extrasynthese (Genay, France). Mass spectrometry-grade water and acetonitrile were used in the preparation of the mobile phase (Fisher Scientific, New Jersey, USA). All chemicals purchased were of the highest grade commercially available.

### 2.2 | HPLC-MS/MS conditions

The high-performance liquid chromatography (HPLC) was conducted on an Agilent 1200 Series (Mississauga, Ont, Canada) system consisting of an online degasser, a quaternary pump, a thermostatted column compartment and an autosampler with a thermostat. Separation of flavonoids was accomplished using a Synergi RP-Max column (250 × 4.6 mm, 4 μm; Phenomenex, Torrance, CA, USA) with a matching guard column, maintained at 40°C. Sample aliquots of 30 μL were injected onto the column and eluted using a gradient flow of 0.60 mL/min. The mobile phase A consisted of water with formic acid (1%) and mobile phase B was acetonitrile. The gradient was initiated at 5% B, increased linearly to 21% B at 20 min, and then increased linearly to 40% B at 35 min.

Quantification was achieved with multiple reaction monitoring (MRM) in positive ion mode electrospray ionization for the analytes using an AB Sciex 4000 hybrid triple quadropole linear ion trap mass spectrometer (Concord, ON, Canada) equipped with a Turboionspray™ interface. Curtain gas, nebulizer gas and heater gas parameters were 40, 50 and 40, respectively. Nitrogen was used for all gases. The ionspray voltage was set at 5500 V and source temperature was 550°C. Entrance potential and collision cell exit potential were set at 10 V. The MRM parameters of each analyte are presented in Table 1. The dwell time for each transition was 300 ms using unit resolution. To protect the mass spectrometer from the electrolyte and other hydrophilic components of urine and plasma, an actuator (model EHMA, Vaco Instruments Co. Inc, Ontario, Canada) was installed between the HPLC column and the mass spectrometer to divert HPLC solvent from the mass spectrometer for the first 8 min of the gradient run. Applied Biosystems/MDS Sciex Analyst software (version 1.6.0) was used to control the LC-MS/MS system and process the data. Peak heights of analytes were used for quantification because this parameter may be less sensitive to variations in the baselines.

### 2.3 | Sample preparations

#### 2.3.1 | Plasma and urine

Plasma or urine samples (100 μL) were mixed with trifluroacetic acid (TFA) aqueous solution (50 μL, 20% v/v) and then centrifuged at 12,000 ***g*** for 15 min to remove protein and solid particles. The supernatants were injected (30 μL) directly for HPLC-MS/MS analysis.

#### 2.3.2 | Extraction of anthocyanins from saskatoon berries

Anthocyanins of saskatoon berry slurry (100 mg, containing 50 mg frozen whole saskatoon berries) were extracted (vortexed for 15 min) with 1 mL HPLC-grade methanol–0.1% hydrochloric acid four times (Bakowska-Barczak, Marianchuk, & Kolodziejczyk, 2007). The extracts were combined and diluted 100 times with 0.1% hydrochloric acid and subjected to HPLC-MS/MS analysis (30 μL).

**TABLE 1** Optimized SRM conditions for analyzing the anthocyanins by HPLC-MS/MS

| Flavonoids | RT (min) | SRM transition (*m/z*) $[M + H]^+$ | Declustering potential (V) | Collision energy (V) | Biological samples |
|---|---|---|---|---|---|
| Delphinidin-3-glucoside | 15.0 | 465.1 → 303.0 | 55 | 30 | ND |
| Delphinidin-3-rutinoside | 15.4 | 611.3 → 303.2 | 57 | 37 | ND |
| Cyanidin-3-galactoside | 15.8 | 449.2 → 287.1 | 55 | 27 | U, P |
| Cyanidin-3-glucoside | 16.3 | 449.2 → 287.1 | 55 | 27 | U, P |
| Cyanidin-3-rutinoside | 16.6 | 595.2 → 287.1 | 64 | 40 | ND |
| Cyanidin-3-arabinoside | 16.9 | 419.1 → 287.0 | 50 | 24 | U, P |
| Malvidin-3-galactoside | 18.0 | 493.2 → 331.1 | 45 | 28 | ND |
| Peonidin-3-glucoside | 18.1 | 463.2 → 301.1 | 45 | 28 | U, P |
| Malvidin-3-glucoside | 18.4 | 493.2 → 331.1 | 45 | 28 | ND |
| Quercetin-3-galactoside | 23.8 | 465.1 → 303.0 | 30 | 20 | U, P |

U, Urine; P, plasma; ND, not detected.

## 2.4 | Method validation

### 2.4.1 | Linearity and lower limit of quantification

The linearity of the method was assessed by preparing five different concentrations of standards with blank plasma or urine ranging from 0.3 to 100 ng/mL. Blank plasma and urine samples were obtained from volunteers who followed a low anthocyanin diet for 2 days and fasted overnight. A calibration curve was created by using a weighted least squares linear regression. A new calibration curve was constructed for each analysis run. The lower limit of quantification (LLOQ) of the method was defined as the lowest concentration of standards giving a signal-to-noise ratio of 5:1.

### 2.4.2 | Recoveries following plasma protein precipitation

The recoveries of each analyte were determined for plasma following protein precipitation at three concentrations (1, 10 and 100 ng/mL). Blank human plasma was pre-spiked with the above concentration of standards and prepared as described above (Section 2.3). For comparison, blank human plasma was subjected to protein precipitation procedures as described above. The supernatant was post-spiked with reference standards at equivalent concentrations. The recoveries were determined by comparing the peak height of pre-spiked and post-spiked samples.

## 2.5 | Human study

Saskatoon berries (Martin variety) were purchased from a local farm and frozen at −20°C until use. Saskatoon berry supplement was prepared by homogenizing saskatoon berries with water (1:1, w/v) in a kitchen blender. The slurries were consumed within 3 h of preparation.

The study protocol was approved by the University of Saskatchewan's Biomedical Research Ethics Board. The three subjects gave written informed consent. Two days before the study, the participants were asked to avoid antioxidant-rich food such as fruits and vegetables and their derived products. Following overnight fasting, the volunteers ate the saskatoon berry slurries (containing 100 g saskatoon berries) in the morning. Blood samples were taken before and at 0.5, 1.5, and 4 h after supplementation. Urine samples were collected before and 0–0.5, 0.5–1.5 and 1.5–4 h after supplementation.

# 3 | RESULTS

## 3.1 | Method validation

### 3.1.1 | Selectivity

MRM transitions were tested for each analyte and the optimal ion pairs are presented in Table 1. The MRM parameters of each analyte (declustering potentials and collision energy) were optimized and are presented in Table 1.

Figure 1 (Reference standard) is a chromatogram of a mixture of reference standards of De-3-glu, De-3-rut, Cy-3-gal, Cy-3-glu, Cy-3-rut, Cy-3-ara, Ma-3-gal, Ma-3-glu, and Qu-3-gal. Figure 1 (plasma) is a chromatogram of plasma from a volunteer 1.5 h after ingestion of the saskatoon berry supplement. Figure 1 (urine) is a chromatogram of urine 1.5 h after saskatoon berries. The plasma and urine samples after overnight fasting were found to be devoid of any peaks interfering with the above-mentioned analytes.

### 3.1.2 | Lower limit of quantification and linearity

The lower limits of quantitation were 0.15 or 0.5 ng/mL for the analytes (Table 2). Standard calibration curves, ranging from 0.3 to 100 ng/mL, were constructed. The data from the standard curves were analyzed using regression analysis to obtain the correlation coefficients ($r^2$), which were >0.99 for all analytes (Table 2).

### 3.1.3 | Reproducibility

Intraday reproducibility was determined by injecting the same reference standard samples four times on the same day. Data taken from samples prepared on four different days were used to determine interday reproducibility (Table 3).

### 3.1.4 | Percentage recovery

Different concentrations of TFA (5, 10, 20% v/v) were evaluated for the clarity of the resultant supernatant from plasma. It was found that 20% TFA produced the clearest supernatant. The percentage recovery for the anthocyanins and quercetin-3-galactoside was determined by comparing the amounts of each compound in the extracted plasma samples spiked with reference standards with those obtained from plasma supernatant post-spiked with reference standards. The average

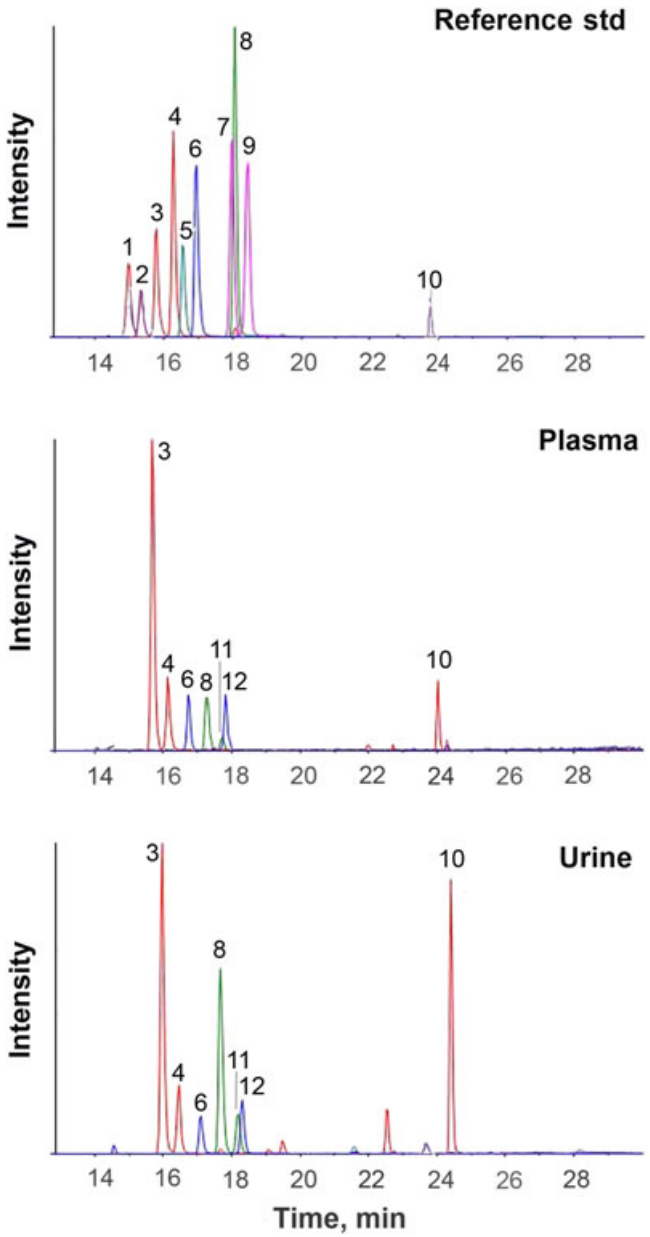


**FIGURE 1** HPLC chromatograms of flavonoids reference standards, a plasma sample from a volunteer 1.5 h after saskatoon berries, and a urine sample from a volunteer 1.5 h after saskatoon berry ingestion. 'Reference std' is a chromatogram of a mixture of reference standards; 'Plasma' is a chromatogram of plasma from a volunteer 1.5 h after ingestion of the saskatoon berry supplement. 'Urine' is a chromatogram of urine 1.5 h after saskatoon berries. Peak identities: 1: De-3-glc; 2: De-3-rut; 3: Cy-3-gal; 4: Cy-3-glc; 5: Cy-3-rut; 6: Cy-3-ara; 7: Ma-3-gal; 8: Pn-3-gal; 9: Ma-3-glc; 10: Qu-3-gal; 11: Pn-3-glc; 12: Cy-3-xyl.

percentage recovery was calculated for each compound (see Table 3). The protein precipitation process appeared to cause some loss of analytes, possibly owing to binding of analytes to the proteins. However, these losses seem to be consistent considering the excellent linearity and reproducibility of the assay. The recovery of quercetin-3-galactoside could be improved to 56.4 and 63.6% at concentrations of 5 and 50 ng/mL, respectively, by adding organic solvent to the protein precipitation medium (10% acetonitrile, 20% TFA). The 'dilute-and-shoot' sample preparation method seem to result in no loss of analytes in urine.

**TABLE 2** Linearity and lower limit of quantification (LLOQ)

| Compound | Matrix | LLOQ (ng/mL)[a] | $r^2$ Values[b] |
|---|---|---|---|
| Delphinidin-3-glucoside | Plasma<br>Urine | 0.15<br>0.5 | 0.9976<br>0.9961 |
| Delphinidin-3-rutinoside | Plasma<br>Urine | 0.15<br>0.5 | 0.9986<br>0.9983 |
| Cyanidin-3-galactoside | Plasma<br>Urine | 0.15<br>0.15 | 0.9994<br>0.9986 |
| Cyanidin-3-glucoside | Plasma<br>Urine | 0.15<br>0.15 | 0.9985<br>0.9987 |
| Cyanidin-3-rutinoside | Plasma<br>Urine | 0.15<br>0.15 | 0.9988<br>0.9974 |
| Cyanidin-3-arabinoside | Plasma<br>Urine | 0.15<br>0.15 | 0.9985<br>0.9968 |
| Malvidin-3-galactoside | Plasma<br>Urine | 0.15<br>0.15 | 0.9994<br>0.9978 |
| Peonidin-3-glucoside | Plasma<br>Urine | 0.15<br>0.15 | 0.9992<br>0.9980 |
| Malvidin-3-glucoside | Plasma<br>Urine | 0.15<br>0.15 | 0.9990<br>0.9972 |
| Quercetin-3-galactoside | Plasma<br>Urine | 0.15<br>0.15 | 0.9976<br>0.9957 |

[a]Limits of quantification are presented as concentrations in plasma or urine. LLOQ is defined as the lowest concentration measured where the peak heights are at least 5 times higher than baseline.

[b]$r^2$ Values of the calibration curves (arithmetic means of five findings).

### 3.2 | Human volunteer study

Figure 2 presents the plasma and urine concentration vs time curves for saskatoon berry flavonoids from three volunteers (Table 4) administered saskatoon berries. This demonstrated the applicability of the HPLC-MS/MS method for analysis of plasma and urine concentrations of flavonoids in biological samples. The flavonoid contents of saskatoon berries are presented in Table 5.

## 4 | DISCUSSION

This report describes an HPLC-MS/MS method with a protein precipitation and a dilute-and-shoot sample preparation method for the determination of common anthocyanins and their metabolites in human plasma and urine, respectively. The compounds were well resolved, confirming the selectivity of the HPLC-MS/MS method (Figure 1). The analytical method is also sensitive enough for the analysis of flavonoids in plasma and urine samples. The LLOQ limit can be further decreased using scheduled MRM where each analyte is monitored only around the time of their retention time. Fewer MRMs per unit time results in longer dwell time and improved sensitivity when large numbers of analytes are monitored.

The recoveries of anthocyanins from protein precipitation were between 55 and 89%, which is fairly high. The recoveries of quercetin-3-galactoside were lower at 29–33%. This recovery could be improved by adding organic solvent to the protein precipitation medium. In the literature, recoveries following solid-phase extraction could reach >90% (Cao et al., 2001). No recovery data has been found in literature about the protein precipitation method (Felgines et al., 2002).

**TABLE 3** Reproducibility and recovery for the HPLC-MS/MS analysis of flavonoids

| Compound | Matrix | Concentration (ng/mL) | Recovery (%)[a] | Intraday variabilities[b] (SD, %) | Interday variabilities[c] (SD, %) |
|---|---|---|---|---|---|
| Delphinidin-3-glucoside | Plasma | 1 | 72.8 | 0.7 | 15.3 |
| | | 10 | 67.6 | 8.8 | 12.2 |
| | | 100 | 70.2 | 4.2 | 1.5 |
| | Urine | 1 | – | 10.5 | 23.4 |
| | | 10 | – | 1.9 | 10.0 |
| | | 100 | – | 6.6 | 0.6 |
| Delphinidin-3-rutinoside | Plasma | 1 | 89.3 | 1.8 | 17.8 |
| | | 10 | 77.5 | 7.1 | 11.1 |
| | | 100 | 82.5 | 3.5 | 1.3 |
| | Urine | 1 | – | 4.1 | 20.5 |
| | | 10 | – | 0.2 | 7.3 |
| | | 100 | – | 11.5 | 0.1 |
| Cyanidin-3-galactoside | Plasma | 1 | 59.5 | 2.0 | 12.2 |
| | | 10 | 62.0 | 16.9 | 7.9 |
| | | 100 | 59.9 | 6.1 | 1.0 |
| | Urine | 1 | – | 3.0 | 13.2 |
| | | 10 | – | 8.9 | 7.5 |
| | | 100 | – | 6.6 | 0.6 |
| Cyanidin-3-glucoside | Plasma | 1 | 67.0 | 2.7 | 14.8 |
| | | 10 | 63.2 | 5.5 | 6.4 |
| | | 100 | 68.1 | 9.6 | 1.0 |
| | Urine | 1 | – | 2.1 | 13.7 |
| | | 10 | – | 10.1 | 7.4 |
| | | 100 | – | 3.9 | 0.5 |
| Cyanidin-3-rutinoside | Plasma | 1 | 72.3 | 0.7 | 11.9 |
| | | 10 | 65.6 | 4.2 | 6.2 |
| | | 100 | 76.3 | 11.6 | 0.9 |
| | Urine | 1 | – | 3.6 | 16.4 |
| | | 10 | – | 2.6 | 9.9 |
| | | 100 | – | 2.9 | 0.5 |
| Cyanidin-3-arabinoside | Plasma | 1 | 63.3 | 1.3 | 10.1 |
| | | 10 | 54.8 | 8.4 | 8.8 |
| | | 100 | 70.4 | 10.0 | 1.1 |
| | Urine | 1 | – | 3.1 | 6.7 |
| | | 10 | – | 11.2 | 3.2 |
| | | 100 | – | 3.9 | 1.1 |
| Malvidin-3-galactoside | Plasma | 1 | 68.4 | 6.1 | 13.3 |
| | | 10 | 72.5 | 11.7 | 10.0 |
| | | 100 | 72.7 | 3.6 | 0.7 |
| | Urine | 1 | – | 3.5 | 11.8 |
| | | 10 | – | 8.9 | 15.0 |
| | | 100 | – | 8.0 | 1.8 |
| Malvidin-3-glucoside | Plasma | 1 | 77.9 | 3.0 | 17.3 |
| | | 10 | 62.6 | 6.5 | 15.9 |
| | | 100 | 75.4 | 5.9 | 0.7 |
| | Urine | 1 | – | 7.9 | 12.9 |
| | | 10 | – | 7.9 | 5.7 |
| | | 100 | – | 6.4 | 1.1 |
| Quercetin-3-galactoside | Plasma | 1 | 33.1 | 4.1 | 22.4 |
| | | 10 | 29.5 | 6.8 | 10.2 |
| | | 100 | 32.7 | 4.4 | 1.1 |
| | Urine | 1 | – | 2.4 | 17.2 |
| | | 10 | – | 3.7 | 7.3 |
| | | 100 | – | 4.1 | 1.0 |
| Peonidin-3-glucoside | Plasma | 1 | 65.9 | 2.7 | 19.4 |
| | | 10 | 62.1 | 8.4 | 10.1 |
| | | 100 | 71.5 | 4.3 | 1.3 |
| | Urine | 1 | – | 2.7 | 9.9 |
| | | 10 | – | 7.2 | 11.1 |
| | | 100 | – | 8.8 | 0.7 |

[a]Arithmetic means of a triplicate study. The study was repeated with similar results.

[b]Arithmetic means of four findings.

[c]Arithmetic means of four different days.

Most existing LC-MS/MS methods involve time-consuming sample preparation procedures. Although adding acid to the biological samples can stabilize anthocyanins (Cao & Prior, 1999; He et al., 2006), solvent evaporation procedures were found to be mainly responsible for the degradation of anthocyanins during their analysis (Woodward et al., 2009). Off-line solid-phase extraction extraction techniques are the most commonly used sample preparation procedures for anthocyanins (Bitsch, Netzel, Frank, Strass, & Bitsch, 2004;

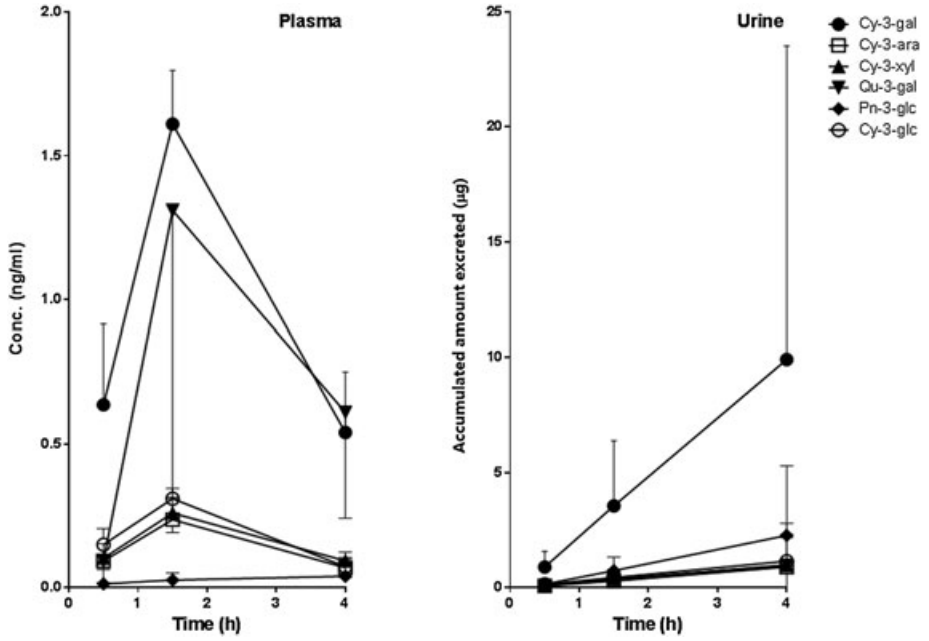


**FIGURE 2** Plasma concentration and urine excretion of anthocyanins of three volunteers following administration of saskatoon berries. Blood samples were taken at 0.5, 1.5 and 4 h after administration of 100 g saskatoon berries. Urine samples were collected at 0–0.5, 0.5–1.5 and 1.5–4 h after supplementation. Urine data are presented as accumulated amount excreted (i.e. entire amount excreted prior to the time point)

**TABLE 4** Volunteer characteristics

| Gender | Age | Ethnic origin | Body mass index |
|---|---|---|---|
| F | 24 | Mixed | 29.3 |
| M | 51 | Asian | 24.3 |
| M | 24 | Caucasian | 21 |

**TABLE 5** Flavonoids contents of saskatoon berries used in the present study

| | Saskatoon berries (mg/100 g) |
|---|---|
| Cyanidin-3-galactoside | 123.5 ± 10.2 |
| Cyanidin-3-glucoside | 29.7 ± 2.5 |
| Cyanidin-3-arabinoside | 13.0 ± 1.7 |
| Cyanidin-3-xyloside[a] | 10.1 ± 1.7 |
| Quercetin-3-galactoside | 47.3 ± 7.0 |

*n* = 3.

[a]Quantified as Cy-3-ara equivalent.

de Ferrars et al., 2014). Most liquid–liquid and solid-phase extraction procedures require an evaporation step, which has been shown to decompose anthocyanins (Woodward et al., 2009). Our sample preparation approaches are particularly helpful for identifying and measuring new metabolites whose extraction efficiencies are unknown with conventional extraction methods. Possible metabolites that can be formed from anthocyanins include their glucuronide, sulfate conjugates and phenolic acid breakdown products (de Ferrars et al., 2014). Since the recovery for quercetin-3-galactoside was relatively low, it is recommended to add organic solvent to the plasma protein precipitation medium to further improve the recovery. Our simple sample preparation procedures did not involve steps which lead to large variations, such as solvent transfer and solvent evaporation. This maximizes the reproducibility of the method and therefore an external standard method was employed. The simple sample preparation methods were made possible with the sensitivity and selectivity offered by the 4000 QTRAP mass spectrometer.

Our HPLC-MS/MS method requires as little as 30 µL plasma volume for the analysis of flavonoids. For studies on the absorption of fruit flavonoids, blood samples are usually obtained by venipuncture procedures performed by licensed personnel in dedicated facilities. In a preliminary study, our method was successfully used for the analysis of flavonoids in capillary blood samples from a volunteer administered saskatoon berries (unpublished data).

For most reported analytical methods, relatively large quantities of plasma or serum samples are required in the analysis of anthocyanins. The smallest volume of plasma (350 µL) required for the analysis of anthocyanins was reported in a method using microelution SPE (µSPE) plates to isolate flavonoids in biological samples (Marti et al., 2010). This method also eliminated the solvent evaporation and reconstitution steps, which reduced the extraction time and degradation of the flavonoids. HPLC solvents were diverted from the mass spectrometer for the first 8 min of the gradient run in our method. This was designed to elute the electrolyte and other polar components of urine that would cause deterioration of instrument performance.

To the best of our knowledge, this is the first report on the application of these simple sample preparation methods for anthocyanins in human plasma and urine samples. Protein precipitation is a simple, high-throughput pretreatment strategy for plasma samples. This method was used to analyze urine and plasma samples from rats fed with very high doses of anthocyanins (Felgines et al., 2002). HPLC-UV method was used to analyze the supernatants. Our study demonstrated that protein precipitation can be used to analyze plasma samples from humans ingested low doses of anthocyanins. Protein

precipitation as the only sample preparation procedure has been successfully used to analyze isoflavonoids (Zhao, Zhao, Liu, Han, & Yu, 2012), flavanone (Liu, Xu, Zhang, Song, & Tian, 2008) and puerarin (Prasain et al., 2007) in rat plasma or serum with HPLC-MS/MS. For the analysis of small molecules, plasma protein precipitation was commonly facilitated by adding an acid. Direct injection has been reported for LC-MS/MS analysis of isoflavones and their conjugates in urine samples or diluted urine samples from rats administered soy (Fang, Yu, & Badger, 2002).

The LC-MS/MS method was successfully applied to measure plasma and urine concentrations of flavonoids from volunteers administered saskatoon berries (Figure 2). It can be seen that maximum concentrations for the concerned flavonoids are achieved at 1.5 h in plasma. In plasma, Cy-3-gal and quercetin-3-galactoside were the most prominent flavonoid found, which is consistent with their high content in saskatoon berries (Table 5). In urine, Cy-3-gal is the most prominent anthocyanin found.

The Synergi RP-Max column has been employed successfully to fingerprint a wide array of flavonoid contents in different fruits owing to excellent peak resolutions (Borges, Degeneve, Mullen, & Crozier, 2010). It is therefore possible that the method could be applied for the analysis of flavonoids in other fruits consumed in human volunteer studies.

In summary, our method improved the LC-MS/MS analysis of anthocyanins using protein precipitation and dilute-and-shoot sample preparation methods for plasma and urine, respectively. Our 4000 QTrap mass spectrometer demonstrated excellent selectivity and sensitivity for the analysis of these compounds in urine and plasma samples. The method was successfully applied to the analysis of anthocyanins and quercetin-3-galactoside in plasma and urine samples from volunteers administered saskatoon berries.

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