UHPLC-MS/MS Triple Quadrupole Analysis of Anthocyanin Metabolites in Human Plasma Using Protein Precipitation and Solid Phase Extraction For Determination of Uptake from Food

Application Note

Food

Authors

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Abstract

Matrix effects in biological samples have been shown to be a source of variability and inaccuracy in liquid chromatography mass spectrometry (LC/MS). In this study, we developed a method for the analysis of anthocyanin metabolites in human plasma. In addition, we compared two sample preparation techniques, Protein Precipitation (PPT) and Solid Phase Extraction (SPE), for the extraction of five different anthocyanin metabolites from human plasma. Blood samples were collected from healthy female volunteers (age 20–35 years) after consumption of a strawberry beverage (40 g freeze dried strawberry powder) at 0, 2, and 3 hours. For PPT, acidified acetonitrile (1.5 mL) was added to plasma (500 µL), and samples were vortexed and centrifuged. The supernatant was collected and dried under nitrogen before triple quadrupole LC/MS analysis. The SPE technique involved loading the diluted plasma sample on SPE tubes (Agilent Bond Elut Plexa, 3 mL), followed by washing with water, and finally collecting the eluent with acidified methanol. The collected eluent was dried under nitrogen before analyzing on a triple quadrupole LC/MS. Anthocyanins/metabolites (cyanidin-3-glucoside, C3G; cyanidin-3-rutinoside, C3R; pelargonidin-3-glucoside, P3G; pelargonidin-3-rutinoside, P3R and pelargonidin glucuronide, PG) were identified and quantified using triple quadrupole LC/MS.

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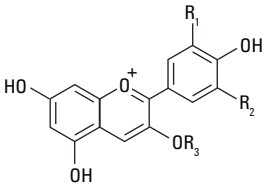
Excellent linearity and detection limits were obtained for these metabolites using matrix-matched standards added after the sample preparation procedures. Peak anthocyanin metabolite content was observed at 2 hours post-consumption. P3G and PG content was significantly higher ($p < 0.05$) in the SPE samples compared to the PPT samples. However, no differences were observed in C3G, C3R, and P3R content between the two techniques. Recovery (%) was in the range of 4.2–18.4% with PPT and 60.8–121.1% with SPE depending upon the compound. Our results indicate that sample preparation using SPE can concentrate the anthocyanin metabolites with better recovery.

Introduction

Anthocyanins belong to the flavonoid category of polyphenols. They are abundant in red, blue, or purple colored fruits and vegetables such as berries, apples, cabbage, corn, grapes, potatoes, and so forth. They not only impart bright red/purple color to fruits and vegetables, but also play an important role in seed dispersal and pollination. The basic structure of anthocyanins is composed of flavylium cation (C6-C3-C6), which could be linked to different sugars, hydroxyl, or methyl groups resulting in different anthocyanins. In addition, the sugar residues could be acylated by aromatic/aliphatic acids [1]. The aglycones (without sugar) of anthocyanins are called anthocyanidins, and the six major ones are cyanidin, peonidin, delphinidin, pelargonidin, malvidin, and petunidin [2]. Consumption of diets rich in anthocyanins has been linked with health benefits and prevention of diseases such as hypertension, cardiovascular diseases, diabetes, obesity, and age-related neurodegenerative disorders [3].

Among berries, strawberries are a good source of anthocyanins. The total anthocyanin content in strawberries varies from 20–60 mg/100 g fresh weight depending upon the variety [4]. Pelargonidin-3-*O*-glucoside is the major anthocyanin in strawberries (83%), followed by pelargonidin-3-*O*-rutinoside (8%) and cyandin-3-*O*-glucoside (7%) [4]. Structures of anthocyanins occurring in strawberry are shown in Figure 1. Anthocyanins can be absorbed as glycosides, however, the bioavailability of anthocyanins is very low. Clinical trials are being conducted to study the health benefits imparted by anthocyanins from various berries. The major challenge faced by researchers for method development is the presence of these compounds in very low levels in blood, and their sensitivity to pH, being more stable under acidic conditions. Therefore, efficient extraction techniques are required to extract anthocyanin metabolites from plasma. Protein precipitation (PPT) is most commonly used for the preparation of biological samples since it is inexpensive and easy, but the results are not often accurate and reproducible. Solid Phase Extraction (SPE) is gaining importance for sample cleanup before HPLC analysis because of better recovery and reproducibility. It also helps to concentrate the metabolites and limit the interferences from unwanted compounds in the plasma matrix.

This application note describes the methodology for analysis of these metabolites including a comparison of PPT and SPE for the extraction of anthocyanin/metabolites from human plasma. For sample cleanup, Agilent Bond Elut Plexa SPE tubes were compared with the traditional protein precipitation method.



Names	R1	R2	R3
Pelargonidin	H	H	H
Cyanidin	OH	H	H
Pelargonidin-3-*O*-glucoside	H	H	Glucose
Cyanidin-3-*O*-glucoside	OH	H	Glucose
Pelargonidin-3-*O*-rutinoside	H	H	Rutinose
Cyanidin-3-*O*-rutinoside	OH	H	Rutinose

Figure 1. Structure of the monomeric anthocyanins occurring in strawberries and their corresponding anthocyanidins.

Experimental

Study design

Plasma samples were collected from three female volunteers (20 to 35 years). The volunteers followed a low polyphenol diet for two days prior to the study and during the treatment day. A strawberry drink (made from 40 g freeze dried strawberry powder) was provided after the baseline blood draw (0 hours). Table 1 shows the anthocyanin composition of the strawberry drink. Blood was collected from the antecubital vein into prelabeled vacutainer (3 × 4 mL EDTA) tubes at 0, 2, and 3 hours. Plasma was transferred to microcentrifuge tubes in aliquots of 500 µL, and stored at –80 °C until analysis.

Table 1. Anthocyanin Content (mg) in Strawberry Drink (40 g Strawberry Powder)

Anthocyanins	Strawberry drink (mg/40 g strawberry powder)
Cyanidin-3-*O*-glucoside	17.60 ± 0.40
Cyanidin-3-*O*-rutinoside	0.74 ± 0.01
Pelargonidin-3-*O*-glucoside	160.12 ± 1.60
Pelargonidin-3-*O*-rutinoside	28.66 ± 0.24

Data are mean ± standard deviation for three replicates

Sample preparation: Extraction of anthocyanins/metabolites from plasma

PPT

The plasma samples were thawed on ice, and 1.5 mL of acidified acetonitrile (1% formic acid) was added to 500 µL of sample. The samples were kept in the refrigerator (4 °C) for 1 hour, and vortexed every 30 minutes. The samples were centrifuged at 1,789 rcf for 10 minutes at 4 °C. The supernatant was collected and dried under nitrogen. The dried sample was dissolved in acetonitrile (5% containing 1% formic acid), centrifuged, and analyzed on HPLC to a final volume of 125 µL.

SPE

Plasma was thawed on ice, and 500 µL of sample was diluted with 1.5 mL of acidified water (1% formic acid). Conditioning of SPE cartridges (Agilent Bond Elut Plexa, 200 mg 3 mL, p/n 1219610) was done with 2 mL of acidified methanol (1% formic acid) followed by 2 mL of acidified water (1% formic acid). Samples were loaded onto the conditioned cartridges under gravity. The SPE cartridges were then washed with 1.5 mL of acidified water (1% formic acid). Elution of metabolites was done with 1.5 mL of acidified methanol (1% formic acid). The collected elute was dried under nitrogen. The dried sample was redissolved in 125 µL of the initial UHPLC mobile phase, centrifuged, and analyzed on HPLC.

Standard preparation

Standards of the four glycosylated anthocyanins were added to blank plasma after sample preparation at the concentrations used to generate the calibration curve through the linear range investigated. Limits of detection (LODs) and limits of quantification (LOQs) were estimated from the response obtained from the lowest standard using only the SPE sample preparation method. These provide method LODs and LOQs, and accurate representation of the concentrations measured in the test subject plasma samples. The glucuronide metabolite concentrations were estimated using the response obtained from the pelargonidin-3-*O*-glucoside standard because an authentic standard of this metabolite could not be obtained.

Instrumentation

The extracted anthocyanins/metabolites were analyzed using an Agilent 1290 Infinity UHPLC system with an Agilent 6460 Triple Quadrupole Mass Spectrometer. The UHPLC consisted of:

- Agilent 1290 Infinity Binary Pump (G4220A)
- Agilent 1290 Infinity Autosampler (G4226A) with an Agilent 1290 Infinity Thermostat (G1330B)
- Agilent 1200 Series Thermostatted Column Compartment (G1316B)

LC/MS/MS analysis was performed using the conditions shown in Table 2, and the MRM target transitions used for quantification are shown in Table 3. One MRM time segment was used with a dwell time of 100 ms for each transition.

Table 2. Agilent 6460 Triple Quadrupole LC/MS Run Conditions

LC run conditions	
Column	Agilent Poroshell 120 Stablebond C18, 2.1 mm × 150 mm, 2.7 µm (p/n 683775-902)
Column temperature	30 °C
Injection volume	5 µL
Mobile phase	A) 1% Formic acid in ddH_2O B) Acetonitrile
Run time	22 minutes
Flow rate	0.3 mL/min
Gradient	Initial 5% B; 5 to 15% B from 0 to 10 minutes; 20% B at 15 minutes; 30% B at 18 minutes; 90% B at 20 minutes and back to 5% B at 22 minutes
Triple quadrupole MS conditions	
Ionization mode	Positive electrospray with Agilent Jet stream technology
Sheath gas	300 °C, 11 L/min
Drying gas flow	9 L/min
Nebulizer pressure	35 psi
Capillary voltage	4,500 V

Standards of C3G, C3R, P3G, and P3R were optimized for collision energies and MRM transitions using an Agilent MassHunter Optimizer. The MRM transition for PG was based on Q-TOF analysis conducted in our previous study [5]. Linearity, LOD, and LOQ are shown in Table 4. The recovery was also studied for both techniques by spiking the blank plasma at known concentrations of standards (that is, low spike 37 ng/mL and high spike 74 ng/mL), and comparing the response to standards prepared in solvent.

Table 3. Target Transitions for Anthocyanins/Metabolites Identified and Quantified by Agilent 6460 Triple Quadrupole LC/MS

Compound	Retention time (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (V)	Fragmentor (V)
Cyanidin-3-*O*-glucoside	9.80	449.1	287.1	22	82
Cyanidin-3-*O*-rutinoside	10.37	595.2	287.1	38	89
Pelargonidin-3-*O*-glucoside	11.26	433.1	271.0	18	87
Pelargonidin glucuronide	11.44	447.1	271.1	22	89
Pelargonidin-3-*O*-rutinoside	11.99	579.2	271.1	30	89

Table 4. Linearity, LOD, and LOQ of Anthocyanins

Compound	Linearity (ng/mL)	R^2	LOD (S/N = 3) (ng/mL)	LOQ (S/N = 10) (ng/mL)
Cyanidin-3-*O*-glucoside	6.31–202.00	0.9999	0.30	0.50
Cyanidin-3-*O*-rutinoside	7.72–247.00	0.9999	0.50	1.50
Pelargonidin-3-*O*-glucoside	6.47–207.00	0.9999	0.03	0.10
Pelargonidin-3-*O*-rutinoside	6.56–210.00	0.9999	0.30	1.25

Results and Discussion

The plasma samples extracted with SPE were visually cleaner than those extracted with PPT (Figure 2). A comparison of metabolites extracted by PPT and SPE techniques is shown in Table 5. There was a significant ($p < 0.05$) increase in the content of P3G and PG in SPE samples compared to PPT in 2-hour plasma samples. At the 3-hour time point, a significant increase in all plasma metabolites was observed in SPE samples, except for P3R. Strawberry anthocyanins/metabolites were not observed in baseline plasma (t = 0 hours). PG was the only metabolite found in plasma, while the remaining four compounds were the anthocyanins present in the strawberries that were absorbed without any transformation, and appeared in plasma. The peak plasma concentration of strawberry anthocyanins/metabolites was achieved around 2 hours followed by a decrease at 3 hours. The concentration of different anthocyanins/metabolites appeared to be very low after strawberry drink consumption, indicating low absorption and bioavailability of these compounds in humans.

The results obtained with SPE are more reliable since with the PPT technique there is possibility of binding/coprecipitating of compounds with proteins. The recovery of anthocyanins using SPE and PPT is shown in Table 6. Recovery (%) was in the range of 4.2–18.4% with PPT, and 60.8–121.1% with SPE depending upon the compound. For sample preparation in complex matrices such as plasma, SPE is superior to PPT. Our results indicate that SPE helps in sample cleanup, recovery, and concentration of anthocyanin/metabolites.

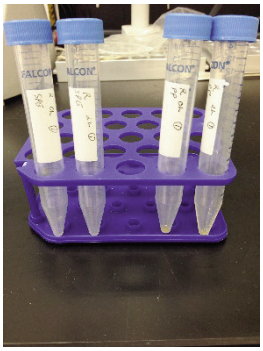


Figure 2. Visual appearance of SPE and PPT tubes after centrifugation.

Table 5. Comparison of Anthocyanins/Metabolites Extracted from Plasma by PPT and SPE Techniques in ng/mL of Plasma

	Technique					
	Protein Precipitation (PPT) Time (hours)			Solid Phase Extraction (SPE) Time (hours)		
Anthocyanins/Metabolites (ng/mL)	0	2	3	0	2	3
Cyanidin-3-*O*-glucoside	0.07 ± 0.00	0.25 ± 0.06	0.21 ± 0.01	0.07 ± 0.00	0.23 ± 0.15	0.24 ± 0.06
Cyanidin-3-*O*-rutinoside	0.41 ± 0.00	0.83 ± 0.00	0.78 ± 0.00	0.41 ± 0.00	0.83 ± 0.01	0.79 ± 0.01
Pelargonidin-3-*O*-glucoside	0.00 ± 0.00	1.34 ± 0.86	0.59 ± 0.34	0.00 ± 0.00	1.92 ± 0.66	1.20 ± 0.69
Pelargonidin-3-*O*-rutinoside	0.00 ± 0.00	0.15 ± 0.15	0.05 ± 0.06	0.00 ± 0.00	0.30 ± 0.59	0.08 ± 0.11
Pelargonidin glucuronide	0.00 ± 0.00	1.27 ± 1.13	0.77 ± 1.21	0.00 ± 0.00	3.32 ± 2.02	1.97 ± 1.36

Data are mean ± standard error for three replicates

Table 6. Recovery of Anthocyanins by PPT and SPE

Technique	PPT		SPE	
Metabolites	Recovery (%) Low spike	Recovery (%) High spike	Recovery (%) Low spike	Recovery (%) High spike
Cyanidin-3-*O*-glucoside	16.12 ± 1.55	10.33 ± 0.06	84.55 ± 1.21	75.16 ± 0.15
Cyanidin-3-*O*-rutinoside	18.35 ± 1.57	12.04 ± 2.71	103.82 ± 3.19	97.3 ± 6.26
Pelargonidin-3-*O*-glucoside	11.21 ± 1.79	6.79 ± 2.14	68.7 ± 1.42	60.84 ± 2.37
Pelargonidin-3-*O*-rutinoside	8.60 ± 3.16	4.18 ± 1.67	121.05 ± 13.76	97.8 ± 4.87

Data are mean ± standard deviation for four replicates.

Figure 3 shows the chromatographic results of the analysis of the four parent compounds and one metabolite in one subject at two hours. Good separation is achieved for all analytes except P3G and PG. However, the difference in the two precursor ions is more than sufficient to separate the signals for the two compounds. It is interesting to note that two other compounds well-separated chromatographically from the analytes have the same precursor/product transitions as C3R and PG. They may be related compounds, but this could only be assessed with full spectral MS/MS data.

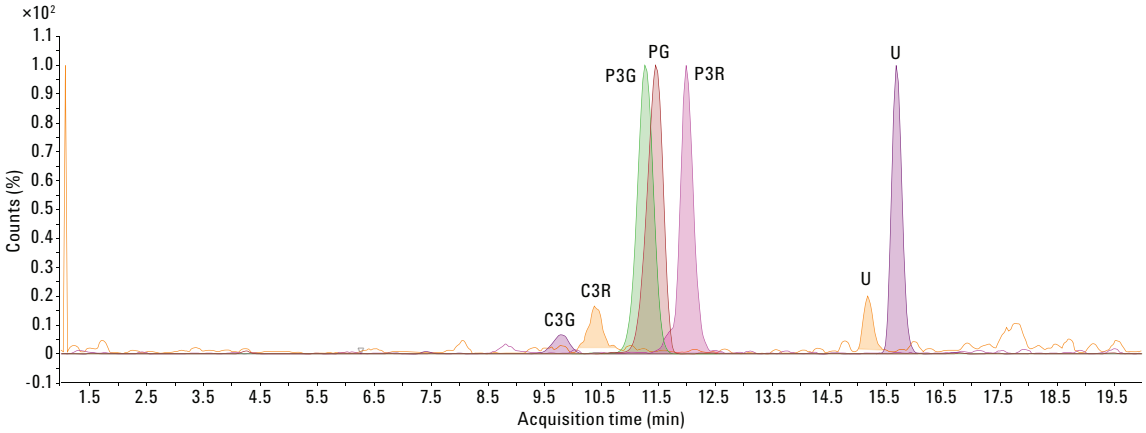


Figure 3. MRM chromatogram of ion transitions of anthocyanins/metabolites (overlaid) in plasma, two hours after consumption of strawberry drink; C3G (Cyanidin-3-O-glucoside), C3R (Cyanidin-3-O-rutinoside), P3G (Pelargonidin-3-O-glucoside), PG (Pelargonidin glucuronide) and P3R (Pelargonidin-3-O-rutinoside); U (unknown peaks).

Conclusions

Using LC/MS/MS with a triple quadrupole, uptake of glycosylated anthocyanins and their metabolites can accurately be measured in human plasma samples to very low levels. The use of SPE improves the recovery, accuracy, and precision of this method. The high selectivity of MRM provides both the sensitivity and selectivity needed. These studies provide insight into the use of these potentially health promoting compounds.

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