

Development and Validation of a Liquid Chromatography Tandem Mass Spectrometry Method for Simultaneous Determination of Four Anthocyanins in Human Plasma after Black Currant Anthocyanins Ingestion

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Black currant anthocyanins consist of delphinidin-3-glucoside (**1**), delphinidin-3-rutinoside (**2**), cyanidin-3-glucoside (**3**), and cyanidin-3-rutinoside (**4**). A liquid chromatography tandem mass spectrometry method for simultaneous determination of four anthocyanins in human plasma was developed and validated. Samples were prepared using solid phase extraction, followed by chromatographic separation with a reverse phase C₁₈ column with gradient elution using mobile phases containing water, acetonitrile, and formic acid. The quantification of four anthocyanins was determined by multiple reaction monitoring using electrospray ionization. The method showed good selectivity, sensitivity (limits of quantification for four anthocyanins were 0.2 nmol/L), linearity (0.2–20 nmol/L; $r > 0.999$), intra- and interday precision, accuracy (<14%), and recovery (62.5–85.7%). Analyte stability was investigated in detail. This method was successfully applied to the determination of delphinidin-3-glucoside (**1**), delphinidin-3-rutinoside (**2**), cyanidin-3-glucoside (**3**), and cyanidin-3-rutinoside (**4**) concentrations in human plasma after ingestion of a single dose of black currant anthocyanins (87.9 μ mol (58.8 mg) total anthocyanins).

KEYWORDS: Black currant; anthocyanins; delphinidin-3-glucoside; delphinidin-3-rutinoside; cyanidin-3-glucoside; cyanidin-3-rutinoside; LC/MS/MS; human

INTRODUCTION

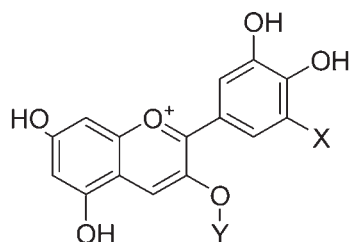
Anthocyanins are a group of naturally occurring phenolic compounds responsible for the color of many flowers, fruits (particularly berries), and vegetables. Because of their widespread distribution in fruits and vegetables, the daily intake of anthocyanins in humans in the United States has been estimated to be 200 mg/day (*1*). Dietary anthocyanins have attracted considerable interest because of their health-promoting benefits, such as reducing the risk of coronary heart disease and preventing several chronic diseases (*2, 3*). In previous studies, we found that oral intake of 50 mg black currant anthocyanins had significant beneficial effects on visual function (*4*). Anthocyanins have been reported to have physiological activities and possible health effects (*5–8*).

In view of the beneficial physiological effects, the bioavailability of anthocyanins is an important issue. Determining the bioavailability requires the development of sensitive and validated analytical methods to quantify anthocyanins in biological fluids. Despite the high sensitivity and selectivity that can be achieved by liquid chromatography tandem mass spectrometry (LC/MS/MS) techniques in quantitative analysis, the most recent reports described quantitative analysis of anthocyanins in plasma

and urine samples based on high performance liquid chromatography (HPLC) with UV/vis detection (HPLC–DAD), using mass spectrometric techniques for compound identification but not for quantification (*9–15*). Recently, the absorption of blood orange juice anthocyanins (delphinidin-3-glucoside, cyanidin-3-glucoside, and cyanidin-3-(6-malonylglucoside)) was studied in humans using LC/MS/MS techniques for quantitative analysis (*16, 17*). In this study, we validated quantification for the rutinosides of anthocyanins using mass spectrometric techniques.

Black currant (*Ribes nigrum* L.) with a high content of anthocyanins is primarily grown for industrial use in making juices, jams, and liquors in many countries of the world. Black currant contains four major anthocyanins (**Figure 1**), delphinidin-3-glucoside (**1**) (13%), delphinidin-3-rutinoside (**2**) (47%), cyanidin-3-glucoside (**3**) (5%), and cyanidin-3-rutinoside (**4**) (35%). Black currant extracts have been used to investigate anthocyanin absorption in humans and experimental animals. A number of studies examined absorption and metabolism of black currant anthocyanins in experimental animals (*9, 13, 18–22*), while few reports are available on humans (*20–22*). In our previous study, subjects were fed >200 mg (300 μ mol) of black currant anthocyanins prepared from black currant juice and four anthocyanins in plasma were quantified by HPLC–DAD (*22*). However, these four anthocyanins could not be quantified in plasma after a single 50 mg dose of black currant anthocyanins because of the lack of sensitivity of analytical method

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No.	compound	X	Y
1	Delphinidin-3-glucoside	OH	glucose
2	Delphinidin-3-rutinoside	OH	rutinoside
3	Cyanidin-3-glucoside	H	glucose
4	Cyanidin-3-rutinoside	H	rutinoside

Figure 1. Structure of the four anthocyanins in black currant.

using HPLC–DAD. At this lower 50 mg level of dosing, there are significant and beneficial effects on visual function.

The aim of the present investigation was to develop a method to determine black currant anthocyanins, delphinidin-3-glucoside (1), delphinidin-3-rutinoside (2), cyanidin-3-glucoside (3), and cyanidin-3-rutinoside (4) in human plasma using LC/MS/MS, and analyze their time course (pharmacokinetics) after a lower, more realistic anthocyanins dose (58.8 mg (87.9 μ mol)).

MATERIALS AND METHODS

Chemicals and Materials. Delphinidin-3-glucoside (1), delphinidin-3-rutinoside (2), cyanidin-3-glucoside (3), and cyanidin-3-rutinoside (4) were extracted and purified from commercial black currant juice according to the methods described previously (23). The flavylium chloride purity was greater than 99.5%, and the structure of each was confirmed by UV, FAB-MS, and NMR spectra analyses (23). Black currant extracts, Currantex20S were purchased from Just the Berries Ltd. (Palmerston North, New Zealand), and contain 23.1% anthocyanins, consisting of 3.03% delphinidin-3-glucoside (1), 9.42% delphinidin-3-rutinoside (2), 1.41% cyanidin-3-glucoside (3), and 9.24% cyanidin-3-rutinoside (4).

Acetone, acetonitrile, formic acid, and hydrochloric acid (HCl) were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). All aqueous solutions, including the HPLC mobile phase, were prepared using water purified with a Milli-Q system (Millipore, Billerica, MA). Oasis HLB 1 cm^3 (10 mg) extraction cartridges for solid phase extraction (SPE) were purchased from Waters (Milford, MA).

Instrumental Conditions. LC/MS/MS analysis was performed using a Quattro premier XE (Waters) via an Acquity Ultra Performance LC (UPLC, Waters). Data were acquired using MassLynx Software version 4.1 (Waters) and data processing used the TargetLynx application manager (Waters). The UPLC system consisted of an Acquity UPLC Binary Solvent Manager and Acquity UPLC Sample Manager. Separation of anthocyanins was achieved using a 50 mm \times 2.1 mm i.d., 1.7 μ m, Acquity UPLC BEH C18 Column (Waters) with the column heater set to 45 $^{\circ}$ C with a 0.6 mL/min flow rate. Gradient elution used two solvents: A, 5% formic acid in water, and B, acetonitrile. The gradient profile was as follows: from 2 to 15% B linear gradient from 0 to 3 min, 80% B isocratic between 3 and 4 min, and then 2% B isocratic between 4 and 5 min. Mass spectrometric analyses were performed using electrospray ionization (ESI) in positive ion mode and multiple reaction monitoring (MRM) mode. The following conditions were used: desolvation temperature = 400 $^{\circ}$ C; desolvation gas flow = 849 L/h; capillary voltage = 1000 V; and source temperature = 120 $^{\circ}$ C. The mass spectrometric parameters for each analyte are described in Table S1 (see the Supporting Information).

Stock Solutions. A mixed standard stock solution of four anthocyanins (1–4) at final concentrations of 100 μ g/mL (193–273 μ mol/L for each anthocyanin) was prepared in 10% aqueous formic acid and stored at 4 $^{\circ}$ C until use. Standard working solutions at 0.5 ng/mL (0.08–0.13 nmol/L for

each anthocyanin) were obtained by further diluting with 10% formic acid. All solutions were stored in the dark to prevent photodegradation.

Preparation of Calibration Curves and Quality Control (QC). Calibration standards of four anthocyanins (1–4) were prepared by adding 0.1 mL of the standard solution and 0.1 mL of 2.4 N HCl to a blank matrix (1 mL) in order to obtain concentrations ranging from 20 to 0.1 nmol/L. Blank plasma was collected and pooled from five healthy volunteers that followed a diet free of anthocyanins for 24 h. QC samples were used in order to establish the precision and the accuracy of the assay and to evaluate the stability of samples under various conditions. QC samples were prepared by spiking blank plasma (1 mL) with standards (0.1 mL) to obtain low (0.44–0.69 nmol/L for each anthocyanin), mid (1.75–2.73 nmol/L), and high (17.52–27.30 nmol/L) levels of concentrations in the range of calibration and were acidified with 0.1 mL of 2.4 N HCl.

Extraction of Anthocyanins from Plasma. Anthocyanins were extracted from plasma samples using a solid phase extraction (SPE) technique, as previously described (12). Before loading on cartridges, the samples were vortexed and centrifuged (14010g, 10 min, 4 $^{\circ}$ C) to remove particulates. Oasis HLB 1 cm^3 (10 mg) extraction cartridges were conditioned sequentially with 1 mL of 10% formic acid in acetone and 1 mL of 10% formic acid in water. Analytical samples (1.2 mL) were loaded, and cartridges were washed with 1 mL of 1% aqueous formic acid, twice. Anthocyanins were eluted sequentially with 0.2 and then 0.1 mL of 10% formic acid in acetone. At all stages of the SPE procedure, a centrifugal evaporator (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) was used to draw solvents through the cartridges until the residue was completely dry. The residue was reconstituted in 200 μ L of 10% formic acid in water. Samples were then filtered through a 0.2 μ m Ultrafree-MC filter (Millipore) and centrifuged at 14010g at 4 $^{\circ}$ C for 10 min prior to analysis by LC/MS/MS.

Validation. Validation assays were established on calibration standards and QC samples of four anthocyanins in blank plasma, prepared as previously described. The selectivity of the method was tested by screening five different batches of blank human plasma. Each blank sample was tested for interferences using the reported extraction procedure and LC/MS/MS conditions. Results were compared with those obtained for a solution of the analytes in 10% aqueous formic acid at a concentration approaching the limit of quantification (LOQ).

Linearity and Accuracy. A calibration curve consisted of a blank sample and eight test samples in the range of 20–0.1 nmol/L. The samples were extracted and analyzed in order to determine the assay linearity and accuracy. Lower concentrations were also prepared for the determination of limit of detection (LOD). Peak integration of the extracted ion chromatograms (m/z values: 465/303 for delphinidin-3-glucoside (1), 611/303 for delphinidin-3-rutinoside (2), 449/287 for cyanidin-3-glucoside (3), and 595/287 for cyanidin-3-rutinoside (4)), calculations of concentrations and regression parameters were performed using TargetLynx application manager.

After back calculations of the concentrations from the regression curve, the accuracy was calculated as [(calculated concentration – nominal concentration)/nominal concentration] \times 100. Accuracy should be accepted within 15%, except at LOQ where it should deviate by more than 20%. LOQ was considered the lowest concentration of each analyte that could be determined with an accuracy of \leq 20%, while the LOD was considered the lowest concentration with a signal intensity at least three times greater than background level.

Intra- and Interday Assays. To determine intraday precision and accuracy of the assays, five replicates of each QC sample at three concentration levels were extracted and analyzed. Interday precision and accuracy were evaluated on three different days, analyzing three QC samples (five or three replicates for each level of concentration) each day. After back calculations of the concentrations from the regression curve, precision was expressed as the relative standard deviation percentage (RSD%) of the recalculated concentrations, and the accuracy was calculated as previously described. Accuracy and precision should be accepted within 15%.

Extraction Recovery. Extraction recovery of analytes was determined by comparing the mean peak area of QC samples (three replicates for two concentration levels, low and high), with the mean peak area of the

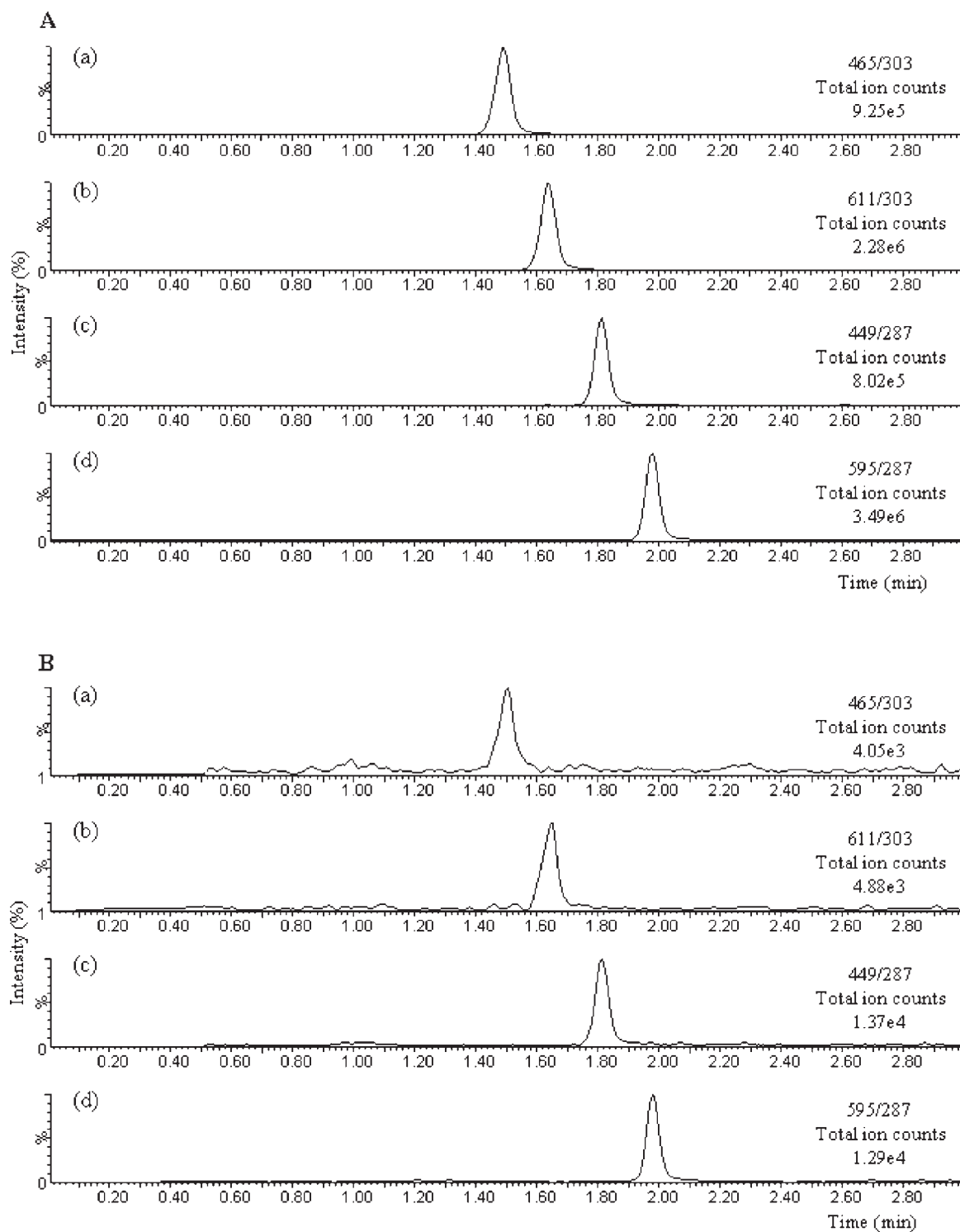


Figure 2. LC/MS/MS analysis of (A) black currant extracts and (B) human plasma spiked with the four black currant anthocyanins (low level concentration). Detection of the respective m/z value of precursor and product ions: (a) delphinidin-3-glucoside (1); (b) delphinidin-3-rutinoside (2); (c) cyanidin-3-glucoside (3); and (d) cyanidin-3-rutinoside (4).

pure standard at the same concentrations. Analyte extracts and standards were dissolved in 10% aqueous formic acid. The reproducibility of the extraction procedure was determined as RSD%.

Stability. All stability assays were conducted on five or three replicates of QC samples at two different concentrations. The analyte stability was expressed as recovery of the measured concentration compared with the analyte concentration of samples immediately extracted and analyzed after spiking.

Short-term stability was evaluated on QC samples kept on ice before sample preparation (4 h). QC samples were extracted and analyzed after

freezing ($-20\text{ }^{\circ}\text{C}$, for 1 day or 12 days) and thawing (on ice). The auto-sampler stability was determined by reanalyzing the processed QC samples after 10 and 20 h at $5\text{ }^{\circ}\text{C}$.

Application of the Method to Human Subjects. The applicability of the proposed analytical method was tested by measuring the concentrations of delphinidin-3-glucoside (1), delphinidin-3-rutinoside (2), cyanidin-3-glucoside (3), and cyanidin-3-rutinoside (4) in human plasma obtained from four healthy volunteers (a man and three women), who were recruited at our laboratory. The study protocol was performed according to the Helsinki Declaration and was approved by the local Ethical Committee,

and the volunteers agreed to participate in the study by written informed consent. Volunteers did not consume any polyphenol-rich products (vegetable, fruits, tea, etc.) or anthocyanin-rich products (red grapes, red wine, eggplant, red onion, red cabbage, etc.) during the 24 h before the experiment or during the experimental period. After an overnight fast, subjects ingested 253 mg of black currant extracts, Currantex20S, 87.9 μmol (58.8 mg) total anthocyanins of delphinidin-3-glucoside (1), 14.1 μmol (7.7 mg), delphinidin-3-rutinoside (2), 33.4 μmol (24.0 mg), cyanidin-3-glucoside (3), 6.8 μmol (3.6 mg) and cyanidin-3-rutinoside (4), 33.6 μmol (23.5 mg) with 100 mL of water. At 2 h postingestion of black currant extracts the subjects were served an anthocyanin-free lunch, consisting of only a rice ball with salt. Water was the only beverage consumed during the experiment.

Seven milliliters of venous blood was collected in heparinized tubes from all volunteers 0, 0.5, 1, 2, 3, 4, and 6 h postingestion of the black currant extracts, and plasma was obtained by centrifugation (1870g, 10 min, 4 °C). A 1 mL portion of plasma was acidified with 0.1 mL of 2.4 N HCl and 0.1 mL of 10% aqueous formic acid, and was extracted and analyzed within 2 h postacidification.

Analysis of the time series concentration data in plasma was performed by noncompartmental model analysis using WinNonlin Professional version 5.2.1 (Pharsight Co., Mountain View, CA).

RESULTS AND DISCUSSION

Method Development. Optimization of mass spectrometric conditions was carried out in a three-step process, referring to Giordano et al. (16). First, we detected molecular ions: m/z 465, 611, 449, and 595 for delphinidin-3-glucoside (1), delphinidin-3-rutinoside (2), cyanidin-3-glucoside (3), and cyanidin-3-rutinoside (4) in positive scan mode, respectively. Among the product ions, the most abundant ions were at m/z 303, 303, 287, and 287 for delphinidin-3-glucoside (1), delphinidin-3-rutinoside (2), cyanidin-3-glucoside (3), and cyanidin-3-rutinoside (4), respectively, corresponding to the loss of sugar moieties. Consequently, the transitions 465/303 for delphinidin-3-glucoside (1), 611/303 for delphinidin-3-rutinoside (2), 449/287 for cyanidin-3-glucoside (3), and 595/287 for cyanidin-3-rutinoside (4) were chosen for MRM analytical mode. Finally, LC/MS/MS analysis by MRM mode combined with LC separation was performed with a C_{18} column using the water–acetonitrile mobile phase containing formic acid, developed with a linear gradient. Analysis of an aqueous solution of black currant anthocyanins using the LC/MS/MS method employed here was capable of resolving four anthocyanins within a run time of 5 min, and identities of these anthocyanins were confirmed employing specific MRM transitions (Figure 2A).

Figure 2B shows a representative chromatogram of spiked human plasma containing four anthocyanins at a concentration of 0.5 nmol/L. Retention times for delphinidin-3-glucoside (1), delphinidin-3-rutinoside (2), cyanidin-3-glucoside (3), and cyanidin-3-rutinoside (4) in plasma were 1.5, 1.6, 1.8, and 2.0 min, respectively. High assay selectivity was achieved using LC coupled to MS/MS. Blank human plasma did not contain any endogenous compounds that could interfere with the assay (data not shown).

Linearity and Accuracy. The LC/MS/MS method was linear over a range of 0.2–20 nmol/L for four anthocyanins (1–4) in plasma, except for 0.1 nmol/L because the values of accuracy for all four analytes exceeded 20% (see the Supporting Information Table S2). The values of the correlation coefficient for calibration curves for all four analytes were < 0.999. The LOQ value was 0.2 nmol/L for four analytes and the LOD value was 0.1 nmol/L for four analytes. These data suggest an increased sensitivity when compared to previously published methods for the analysis of anthocyanins in plasma measured after food ingestion (17, 20, 22, 24), and the LOQ and LOD are as good as the previous LC/MS/MS method (16).

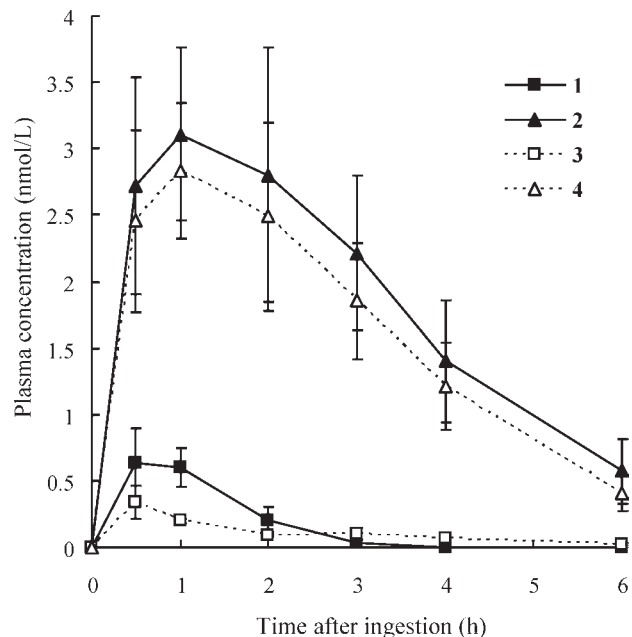


Figure 3. Human plasma concentration profiles of delphinidin-3-glucoside (1), delphinidin-3-rutinoside (2), cyanidin-3-glucoside (3), and cyanidin-3-rutinoside (4) after ingestion of black currant extracts. Values are means \pm SE, $n = 4$.

Intra- and Interday Assays. The intra- and interday variability values are shown in Table S3 (see the Supporting Information). For all samples, precision and accuracy were below 15%. Thus the procedure of extraction and analysis offers excellent repeatability and reproducibility for analysis of four anthocyanins in plasma.

Extraction Recovery. Extraction recoveries for four analytes varied from 62.5% for delphinidin-3-glucoside (1) to 85.7% for cyanidin-3-rutinoside (4) (see the Supporting Information Table S4). Recoveries for delphinidin glycosides were lower compared to cyanidins, so we speculated that this resulted from a lack of stability of delphinidin glycosides as compared with cyanidins (25). Although these recoveries are similar to previous data (12, 20, 22, 26), this method is considerably more rapid, and the required sample volume is smaller than the previous SPE method (22).

Stability. Stability assays were performed in order to cover all possible conditions that would be encountered during sample processing. Considering that biological samples are often stored under varying conditions and processed after different time periods, the lack of data on stability could introduce some discrepancies in the reliability, and this needs to be addressed. Table S5 (see the Supporting Information) summarized the data from stability tests for four analytes. Four anthocyanins were stable in plasma if kept on ice for 4 h, though there was a decrease in recovery only for the low concentration sample (0.5 nmol/L) for delphinidin-3-rutinoside (2). Four anthocyanins were stable in plasma when stored at -20 °C for one day or 12 days and thawed on ice. These tests indicated that storage of plasma samples containing four anthocyanins at -20 °C was adequate when maintained for 12 days and that after thawing on ice, the samples should be extracted immediately. The post-preparative stability of QC samples showed that four analytes were stable in the sample extract when resuspended in 10% aqueous formic acid and kept at 5 °C in autosampler for 20 h. This test indicated that it is reasonable to analyze within 20 h at 5 °C after extraction.

Table 1. Pharmacokinetic Parameters of Four Anthocyanins in Human Plasma after the Ingestion of Black Currant Extracts^a

analyte	C_{\max}^b (nmol/L)	T_{\max}^c (h)	$AUC_{0-\infty}^d$ (nmol·h/L)	$t_{1/2}^e$ (h)
delphinidin-3-glucoside (1)	0.73 ± 0.24	0.75 ± 0.14	1.89 ± 0.17	1.80 ± 0.75
delphinidin-3-rutinoside (2)	3.44 ± 0.99	1.25 ± 0.25	12.78 ± 4.43	1.48 ± 0.20
cyanidin-3-glucoside (3)	0.37 ± 0.12	0.75 ± 0.14	0.71 ± 0.19	1.36 ± 0.26
cyanidin-3-rutinoside (4)	2.99 ± 0.68	1.25 ± 0.25	10.97 ± 2.90	1.49 ± 0.43

^a Values are mean ± SE, $n = 4$. ^b C_{\max} , maximal plasma concentration. ^c T_{\max} , time to reach C_{\max} . ^d AUC, area under the plasma concentration time curve. ^e $t_{1/2}$, elimination half-life.

Method Application. The LC/MS/MS method described above was successfully tested for analysis of plasma samples obtained from four healthy volunteers that received a single dose of 253 mg of black currant extracts, Currantex20S, 87.9 μmol (58.8 mg) total anthocyanins with 100 mL of water. The plasma concentration profiles of four anthocyanins after the ingestion of black currant extracts are shown in **Figure 3**. Plasma samples collected before the ingestion of black currant extracts contained no detectable anthocyanins (data not shown). The concentration reached a maxima of 3.11 ± 0.65 (mean ± SE) nmol/L and 2.84 ± 0.51 nmol/L at 1 h postingestion for delphinidin-3-rutinoside (2) and cyanidin-3-rutinoside (4), respectively, and a maxima of 0.63 ± 0.26 nmol/L and 0.34 ± 0.12 nmol/L at 0.5 h for delphinidin-3-glucoside (1) and cyanidin-3-glucoside (3), respectively, which then gradually decreased over time (**Figure 3**).

The data on plasma concentrations of four anthocyanins were analyzed using a noncompartmental model, and the pharmacokinetic parameters of four anthocyanins are summarized in **Table 1**. T_{\max} (time to reach maximal plasma concentration) values were equal for delphinidin-3-rutinoside (2) and cyanidin-3-rutinoside (4) (1.25 h), which were greater than the values of delphinidin-3-glucoside (1) and cyanidin-3-glucoside (3) (0.75 h), showing an order similar to our previous results, though the values in this study are shorter than those suggested previously (22). These suggest the possibility that absorption is influenced by the difference of the glycoside moiety, as many of the bioavailability studies have suggested (13, 20, 21, 27–29). The C_{\max} (maximal plasma concentration) values and the $AUC_{0-\infty}$ (area under the plasma concentration time curve) values varied in the order delphinidin-3-rutinoside (2) > cyanidin-3-rutinoside (4) > delphinidin-3-glucoside (1) > cyanidin-3-glucoside (3), which was the same order as the quantity of four components in black currant anthocyanins ingested. In our previous report, there was great variability among $t_{1/2}$ (elimination half-time), because of the lack of sensitivity for the HPLC–DAD method and differences among individual absorption. In this study, since we improved the selectivity and sensitivity by using an LC/MS/MS method, we could accurately measure lower concentration in the process of elimination from the blood and so reduce variability among $t_{1/2}$.

Conclusion. The method to determine black currant anthocyanins, delphinidin-3-glucoside (1), delphinidin-3-rutinoside (2), cyanidin-3-glucoside (3), and cyanidin-3-rutinoside (4) concentrations in human plasma using the LC/MS/MS developed in this study had a lower LOQ (0.2 nmol/L) than our previous HPLC–DAD method (4 nmol/L) (22) and used a smaller sample volume (1 mL versus 4 mL) with a rapid SPE preparation method capable of simultaneously analyzing a high number of samples. The reliability as assessed by inter- and intraday precision for QC samples was < 10%. Rapid chromatography and mass-selective detection imparted greater selectivity and sensitivity than the UV detection. As a result, we could analyze the time course (pharmacokinetics) after a lower, more realistic anthocyanins dose (87.9 μmol (58.8 mg)), one-fourth the dosage of a previous human study (22), and precisely determine the pharmacokinetic parameters, especially $t_{1/2}$.

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Supporting Information Available: Optimized mass spectrometric parameters for each analyte (Table S1), linearity of four anthocyanins after extraction from spiked plasma (Table S2), intra- and interday assay precision and accuracy for determination of four anthocyanins in plasma (Table S3), extraction recovery for four anthocyanins from human plasma at two different QC levels (Table S4), and stability of four anthocyanins in human plasma and in prepared sample from human plasma (Table S5). This material is available free of charge via the Internet at <http://pubs.acs.org>.

LITERATURE CITED

- (1) Kuhnu, J. The flavonoids. A class of semi-essential food components: Their role in human nutrition. *World Rev. Nutr. Diet* **1976**, *24*, 117–191.
- (2) Ghosh, D. Anthocyanins and anthocyanin-rich extracts in biology and medicine: biochemical, cellular, and medicinal properties. *Curr. Top. Nutraceutical Res.* **2005**, *3*, 113–124.
- (3) Renaud, S.; de Lorgeril, M. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* **1992**, *339*, 1523–1526.
- (4) Nakaishi, H.; Matsumoto, H.; Tominaga, S.; Hirayama, M. Effects of black current anthocyanoside intake on dark adaptation and VDT work-induced transient refractive alteration in healthy humans. *Altern. Med. Rev.* **2000**, *5*, 553–562.
- (5) Galvano, F.; La Fauci, L.; Vitaglione, P.; Fogliano, V.; Vanella, L.; Felgines, C. Bioavailability, antioxidant and biological properties of the natural free-radical scavengers cyanidin and related glycosides. *Ann. Ist. Super. Sanita* **2007**, *43*, 382–393.
- (6) Prior, R. L.; Wu, X. Anthocyanins: structural characteristics that result in unique metabolic patterns and biological activities. *Free Radic. Res.* **2006**, *40*, 1014–1028.
- (7) Tsuda, T.; Horio, F.; Uchida, K.; Aoki, H.; Osawa, T. Dietary cyanidin 3-*O*-beta-D-glucoside-rich purple corn color prevents obesity and ameliorates hyperglycemia in mice. *J. Nutr.* **2003**, *133*, 2125–2130.
- (8) Sasaki, R.; Nishimura, N.; Hoshino, H.; Isa, Y.; Kadowaki, M.; Ichi, T.; Tanaka, A.; Nishiumi, S.; Fukuda, I.; Ashida, H.; Horio, F.; Tsuda, T. Cyanidin 3-glucoside ameliorates hyperglycemia and insulin sensitivity due to downregulation of retinol binding protein 4 expression in diabetic mice. *Biochem. Pharmacol.* **2007**, *74*, 1619–1627.
- (9) Walton, M. C.; Hendriks, W. H.; Broomfield, A. M.; McGhie, T. K. Viscous food matrix influences absorption and excretion but not metabolism of blackcurrant anthocyanins in rats. *J. Food Sci.* **2009**, *74*, H22–H29.
- (10) Mullen, W.; Edwards, C. A.; Serafini, M.; Crozier, A. Bioavailability of pelargonidin-3-*O*-glucoside and its metabolites in humans following the ingestion of strawberries with and without cream. *J. Agric. Food Chem.* **2008**, *56*, 713–719.
- (11) Tian, Q.; Giusti, M. M.; Stoner, G. D.; Schwartz, S. J. Urinary excretion of black raspberry (*Rubus occidentalis*) anthocyanins and their metabolites. *J. Agric. Food Chem.* **2006**, *54*, 1467–1472.
- (12) Cooke, D. N.; Thomasset, S.; Boocock, D. J.; Schwarz, M.; Winterhalter, P.; Steward, W. P.; Gescher, A. J.; Marczylo, T. H.

- Development of analyses by high-performance liquid chromatography and liquid chromatography/tandem mass spectrometry of bilberry (*Vaccinium myrtillus*) anthocyanins in human plasma and urine. *J. Agric. Food Chem.* **2006**, *54*, 7009–7013.
- (13) Wu, X.; Pittman, H. E.3rd; McKay, S.; Prior, R. L. Aglycones and sugar moieties alter anthocyanin absorption and metabolism after berry consumption in weanling pigs. *J. Nutr.* **2005**, *135*, 2417–2424.
- (14) Talavera, S.; Felgines, C.; Texier, O.; Besson, C.; Gil-Izquierdo, A.; Lamaison, J. L.; Remesy, C. Anthocyanin metabolism in rats and their distribution to digestive area, kidney, and brain. *J. Agric. Food Chem.* **2005**, *53*, 3902–3908.
- (15) Felgines, C.; Talavera, S.; Texier, O.; Gil-Izquierdo, A.; Lamaison, J. L.; Remesy, C. Blackberry anthocyanins are mainly recovered from urine as methylated and glucuronidated conjugates in humans. *J. Agric. Food Chem.* **2005**, *53*, 7721–7717.
- (16) Giordano, L.; Coletta, W.; Rapisarda, P.; Donati, M. B.; Rotilio, D. Development and validation of an LC/MS/MS analysis for simultaneous determination of delphinidin-3-glucoside, cyanidin-3-glucoside, and cyanidin-3-(6-malonylglucoside) in human plasma and urine after blood orange juice administration. *J. Sep. Sci.* **2007**, *30*, 3127–3136.
- (17) Vitaglione, P.; Donnarumma, G.; Napolitano, A.; Galvano, F.; Gallo, A.; Scalfi, L.; Fogliano, V. Protocatechuic acid is the major human metabolite of cyanidin-glucosides. *J. Nutr.* **2007**, *137*, 2043–2048.
- (18) Walton, M. C.; Lentle, R. G.; Reynolds, G. W.; Kruger, M. C.; McGhie, T. K. Anthocyanin absorption and antioxidant status in pigs. *J. Agric. Food Chem.* **2006**, *54*, 7940–7946.
- (19) Matsumoto, H.; Nakamura, Y.; Iida, H.; Ito, K.; Ohguro, H. Comparative assessment of distribution of blackcurrant anthocyanins in rabbit and rat ocular tissues. *Exp. Eye Res.* **2006**, *83*, 348–356.
- (20) Nielsen, I. L.; Dragsted, L. O.; Ravn-Haren, G.; Freese, R.; Rasmussen, S. E. Absorption and excretion of black currant anthocyanins in humans and watanabe heritable hyperlipidemic rabbits. *J. Agric. Food Chem.* **2003**, *51*, 2813–2820.
- (21) McGhie, T. K.; Ainge, G. D.; Barnett, L. E.; Cooney, J. M.; Jensen, D. J. Anthocyanin glycosides from berry fruit are absorbed and excreted unmetabolized by both humans and rats. *J. Agric. Food Chem.* **2003**, *51*, 4539–4548.
- (22) Matsumoto, H.; Inaba, H.; Kishi, M.; Tominaga, S.; Hirayama, M.; Tsuda, T. Orally administered delphinidin 3-rutinoside and cyanidin 3-rutinoside are directly absorbed in rats and humans and appear in the blood as the intact forms. *J. Agric. Food Chem.* **2001**, *49*, 1546–1551.
- (23) Matsumoto, H.; Hanamura, S.; Kawakami, T.; Sato, Y.; Hirayama, M. Preparative-scale isolation of four anthocyanin components of black currant (*Ribes nigrum* L.) fruits. *J. Agric. Food Chem.* **2001**, *49*, 1541–1545.
- (24) Wu, X.; Cao, G.; Prior, R. L. Absorption and metabolism of anthocyanins in elderly women after consumption of elderberry or blueberry. *J. Nutr.* **2002**, *132*, 1865–1871.
- (25) Rubinskiene, M.; Jasutiene, I.; Venskutonis, P. R.; Viskelis, P. HPLC determination of the composition and stability of blackcurrant anthocyanins. *J. Chromatogr. Sci.* **2005**, *43*, 478–482.
- (26) Mazza, G.; Kay, C. D.; Cottrell, T.; Holub, B. J. Absorption of anthocyanins from blueberries and serum antioxidant status in human subjects. *J. Agric. Food Chem.* **2002**, *50*, 7731–7737.
- (27) McGhie, T. K.; Walton, M. C. The bioavailability and absorption of anthocyanins: Towards a better understanding. *Mol. Nutr. Food Res.* **2007**, *51*, 702–713.
- (28) Ichiyanagi, T.; Shida, Y.; Rahman, M. M.; Hatano, Y.; Konishi, T. Bioavailability and tissue distribution of anthocyanins in bilberry (*Vaccinium myrtillus* L.) extract in rats. *J. Agric. Food Chem.* **2006**, *54*, 6578–6587.
- (29) Wu, X.; Pittman, H. E.3rd; Prior, R. L. Pelargonidin is absorbed and metabolized differently than cyanidin after marionberry consumption in pigs. *J. Nutr.* **2004**, *134*, 2603–2610.

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