Pharmacokinetic Characterization and Bioavailability of Strawberry Anthocyanins Relative to Meal Intake

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ABSTRACT: Plasma strawberry anthocyanins were characterized in overweight (BMI: 26 ± 2 kg/m^2) adults ($n = 14$) on the basis of meal timing. At each visit, subjects ingested three study drinks: two control and one strawberry drink. A strawberry drink was given at either 2 h before the breakfast meal (BM), with the meal (WM), or 2 h after the meal (AM), and control drinks were given at the alternative time points. Plasma anthocyanins and their metabolic conjugates were assessed hourly for 10 h using a triple-quadrupole liquid chromatography mass spectrometer. Maximum concentrations (C_{max}), area under the curve (AUC), and bioavailability of pelargonidin-based anthocyanins determined from the main conjugated metabolite (pelargonidin glucuronide) were greater when a strawberry drink was consumed 2 h before the meal (BM) compared to consumption WM or AM ($p < 0.05$). Our results indicate that the timing of strawberry consumption relative to a meal impacts anthocyanin pharmacokinetic variables.

KEYWORDS: anthocyanins, bioavailability, glucuronide, strawberries, HPLC

■ INTRODUCTION

Fresh strawberries represent the largest retail market by volume compared to other berries in the United States.[1] One factor that has contributed to the tremendous growth of the strawberry market is the recognition that they are good source of essential nutrients[2] and bioactive compounds that may promote human health and protect against various chronic diseases. Data from various epidemiological studies indicate an inverse correlation between berry consumption and the incidence of chronic diseases, such as type 2 diabetes and cardiovascular diseases.[3−5] In addition, various clinical trials show beneficial changes in chronic-disease risk factors after strawberry consumption acutely[6,7] and chronically.[8−10]

Strawberries contain flavonols, flavanols, ellagitannins, and condensed tannins and, most notably, anthocyanins, which account for greater than 75% of strawberry polyphenols.[11] Anthocyanins are water-soluble pigments and belong to the flavonoid group of polyphenols. The basic structure of anthocyanins is composed of flavylium cations (C6-C3-C6), which could be linked to different sugars or hydroxyl or methyl groups resulting in different anthocyanins. In addition, the sugar residues could be acylated by aromatic or aliphatic acids. The structure, solubility, and reactivity of anthocyanins is affected by the sugar and hydroxylation pattern. There are six main anthocyanidins (aglycone, without sugar) found in the diet: cyanidin, peonidin, delphinidin, pelargonidin, malvidin, and petunidin. Strawberries contain principally cyanidin and pelargonidin glycosides. The total anthocyanin content in strawberries varies from 20 to 60 mg per 100 g of fresh weight, depending upon variety. Pelargonidin-3-O-glucoside is the most abundant anthocyanin in strawberries (77−90%), followed by pelargonidin-3-O-rutinoside (6−11%) and cyanidin-3-O-glucoside (3−10%).[12] Structures of anthocyanins found in strawberry and their corresponding anthocyanidins are shown in Figure 1.
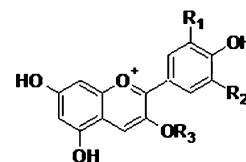
Names	R$_1$	R$_2$	R$_3$
Pelargonidin	H	H	H
Cyanidin	OH	H	H
Pelargonidin-3-O-glucoside	H	H	Glucose
Cyanidin-3-O-glucoside	OH	H	Glucose
Pelargonidin-3-O-rutinoside	H	H	Rutinose
Cyanidin-3-O-rutinoside	OH	H	Rutinose

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

Previous studies conducted by our research group showed that anthocyanins delivered in 10 g of freeze-dried strawberries (FDS) (~110 g of fresh weight) significantly attenuated postprandial oxidative stress and inflammation induced by a high-carbohydrate, high-fat meal, with corresponding improvements in the insulin response.[6,13] More recently, our group tested different doses of strawberry in individuals with insulin resistance and found an inverse correlation of pelargonidin metabolites in plasma to insulin and glucose responses

compared to the control.[14] Pharmacokinetic parameters of anthocyanins and their metabolic conjugates revealed that with the increase in the dose, there was an increase in the maximum concentrations (C_{max}) and area under the curve (AUC), which may have influenced the biological activity. However, if the health promoting action of anthocyanins is dependent upon the bioavailability or achieving a certain blood concentration to impart biological activity, then understanding the factors that influence these variables is critical. Thus, the objective of the present study was to determine whether the timing of strawberry consumption relative to meal intake would impact the kinetic profile and bioavailability of strawberry anthocyanins and their metabolic conjugates.

■ MATERIALS AND METHODS

Chemicals and Materials. High-performance liquid chromatography (HPLC) grade acetonitrile and methanol and reagent-grade formic acid were purchased from Fisher Scientific (Houston, TX). Cyanidin-3-O-glucoside (C3G), cyanidin-3-O-rutinoside (C3R), pelargonidin-3-O-glucoside (P3G), pelargonidin-3-O-rutinoside (P3R), and malvidin-3-O-glucoside (M3G) were purchased from Extrasynthese (Genay, France). Bond Elut Plexa (3 mL) C18 cartridges for solid-phase extraction (SPE) were obtained from (Agilent Technologies, Santa Clara, CA).

Study Design. The study was conducted at the Clinical Nutrition Research Center (CNRC) at the Illinois Institute of Technology (IIT) (Chicago, Illinois) with the approval of study procedures by IIT's Institutional Review Board (IRB). The study is registered on ClinicalTrial.gov (registration number NCT01856153). All subjects provided written informed consent via the IRB-approved consent form prior to the initiation of any study-related procedures. The study was designed as a within-subjects crossover design, delivering one strawberry drink and two control drinks in a computer-generated randomized sequence based on time on three separate occasions to healthy adult men and women. Strawberry drinks were provided either at 2 h before the standard breakfast meal (BM), with the meal (WM), or 2 h after the breakfast meal (AM), and control drinks were provided at the alternative times, such that three drinks were consumed per study day. For example, when the strawberry drink was provided 2 h before the meal (BM sequence), control drinks were provided with the meal and 2 h after the meal. The "WM" treatment sequence provided the strawberry drink with the meal and control drinks 2 h before and 2 h after the meal. The "AM" treatment sequence provided the strawberry drink 2 h after the meal and control drinks 2 h before and with the meal (See the study schema in Figure 2). A separate demographically matched group (n = 10) drank only the control drinks at all time points and served as the reference group. The reference group was a single-blinded, one-arm design to compare the influence of strawberry consumption on metabolic and inflammation markers.[15]
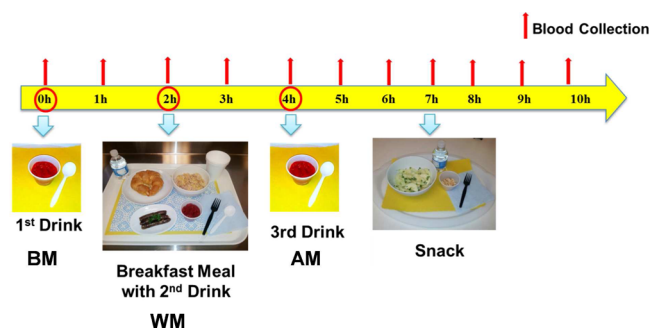

Figure 2. Postprandial study schema for 10 h visit. BM: before meal, WM: with meal, and AM: after meal.

Study Test Drinks and Meals. The "strawberry-containing" drink (34.7 ± 0.5 mg anthocyanins per drink) was prepared from 12 g of FDS powder (California Strawberry Commission, Watsonville, CA), which is equivalent to ~132 g of fresh strawberries. The control drink was prepared using a nonstrawberry containing powder matched closely with the energy, sugar, and micronutrient content of the strawberry powder but did not contain anthocyanin or other polyphenol components (control powder provided by California Strawberry Commission, Watsonville, CA). Strawberry or control powders were blended with 60 g of ice and 5 g of cold water to serve as a slushy drink (77 mL) to study participants at protocol-specified time points (sequence BM, WM, or AM). Participants were given 230 mL of water on the side to drink with the slushy.

The standard breakfast meal was a high-fat and high-carbohydrate (HFHC) meal consisting of a croissant with butter and apple jelly, frosted-flake cereals, whole milk, and breakfast sausage links. The breakfast meal provided 838 kilocalories with 44% energy from fat and 46% energy from carbohydrates. A snack was provided at 7 h to ease subjects' hunger during the 10 h visit. The snack contained 254 kilocalories (21 g of fat and 11 g of carbohydrates) and consisted of dry-roasted peanuts and fresh peeled cucumber with ranch dressing.

Study Participants. Overweight healthy men and premenopausal women were recruited for the study. Men and women who were between 18 and 45 years old with body mass index (BMI) values between 25 to 29.9 kg/m^2 (as an exception for the Asian population, the BMI ranged from 23 to 27.4 kg/m^2), nonsmoker, and in relatively good health with no previous history or current clinical evidence of cardiovascular, metabolic, respiratory, renal, gastrointestinal, or hepatic diseases were eligible to participate. Subjects taking medications (e.g., gastrointestinal medications, antibiotics, and diuretics) or dietary supplements (e.g., fiber supplements, probiotics and prebiotics, and antioxidants) that would interfere with outcomes of the study were not eligible to participate. Women who were pregnant or lactating or anyone reporting allergies or sensitivity to berry products or any foods offered during the study were not eligible to participate in the study. A total of 42 subjects were screened for the strawberry group, of which 13 were not eligible due to BMI outside the specified range (n = 12) and smoking (n = 1). Out of the remaining 29 screened subjects, 14 subjects participated in the study.

Study-Day Visit Procedures. Qualified participants prepared for each study day by strictly limiting dietary anthocyanin intake 3 days prior to each study visit and as much as possible on other days while participating in the study. A prestudy period was conducted to assess subject's usual dietary intake patterns using a 3 day scale weighed food diaries. Study dietitians used these records to guide subjects while on study to avoid anthocyanin-containing foods choosing alternative foods. Food diaries were kept throughout the study for review, checking for consistency in dietary patterns and avoidance of anthocyanin foods. Subjects were rescheduled if noncompliant. A standardized dinner meal was provided the day before the study visit to help control variance in food intake the night before each study visit. On each study visit, subjects arrived to the CNRC fasted and hydrated. After the standard admission procedures were completed (e.g., a review of compliance with dietary requirements of 3 days prior to study day, dinner meal intake, and usual sleep patterns), a catheter was placed by a registered nurse in subjects' nondominant arm, and a fasting (baseline) blood sample was collected (0 h). Thereafter, subjects were provided a study drink according to their assigned sequence of BM, WM, or AM. Blood samples were collected for anthocyanin analysis at 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 h after the first drink. The HFHC meal plus the sequence-assigned second drink was provided after the 2 h blood draw, and subjects had 20 min to finish the entire meal and drink. After the 4 h blood draw, the third study drink was given according to the sequence. The snack was provided after the 7 h blood draw. Blood sampling continued throughout until 10 h.

Sample Preparation and HPLC Analysis of Anthocyanins and Their Metabolic Conjugates. Whole-blood samples were collected in tubes containing EDTA as an anticoagulant. Blood samples were centrifuged at 453g for 15 min at 4 °C. Plasma was

separated from blood samples after centrifugation and aliquots of plasma were stored at −80 °C for subsequent analysis. SPE was used for the extraction of anthocyanins and their metabolic conjugates from the plasma. Briefly, plasma was thawed on ice, and 500 μL of the sample was diluted with 1.5 mL of acidified water (1% formic acid). Samples were loaded on the preconditioned cartridges under gravity. The SPE cartridges were washed with 1.5 mL of acidified water (1% formic acid). The elution of metabolites was done with 1.5 mL of acidified methanol (1% formic acid). The collected elute was dried under nitrogen at 25 °C. The dried sample was dissolved in acetonitrile (5% containing 1% formic acid) and centrifuged at 18514g for 10 min at 4 °C. Samples were transferred to amber HPLC vials and were analyzed using an Agilent 1290 Infinity ultrahigh-performance liquid chromatography (UHPLC) system with an Agilent 6460 Triple Quadrupole Mass Spectrometer (Agilent Technologies, Santa Clara, CA). The system was equipped with a binary pump with an integrated vacuum degasser, an autosampler with a thermostat, and a column compartment with a thermostat. Separation of the compounds was done using poroshell 120 stablebond C18 column (2.1 mm × 150 mm, 2.7 μm, Agilent Technologies) at a constant temperature of 30 °C. The mobile phase used for the separation of compounds consisted of acidified water (1% formic acid) and acetonitrile. The injection volume was 5 μL. The flow rate was maintained at 0.3 mL/min, and the gradient for the separation of compounds was as follows: 5 to 15% B from 0 to 10 min; 20% B at 15 min; 30% B at 18 min; 90% B at 20 min, and back to 5% B at 22 min. The column was re-equilibrated to the initial mobile-phase conditions for 5 min before starting the next run to ensure consistent chromatography. The initial analysis was conducted by using a full MS scan and MS^2 product-ion scan followed by multiple-reaction monitoring (MRM) transitions. Spectra were recorded in positive-ion mode with capillary voltage of 4500 V and drying gas flow rate of 9 L/min at 200 °C. The sheath gas temperature and flow rate were 300 °C and 11 L/min, respectively.

Standards of C3G, C3R, P3G, P3R, and M3G were optimized for collision energies, fragmentor voltages, and MRM transitions using Mass Hunter Optimizer. A single transition per compound was used using a dwell time of 75 ms. The MRM transition for pelargonidin glucuronide (PG), a phase II conjugate, was based on an UHPLC accurate mass quadrupole time-of-flight mass spectrometry with electrospray ionization analysis conducted in our previous study.[16] Standards were prepared in blank plasma (charcoal-stripped human plasma obtained from BioreclamationIVT) for matrix match (6.25−200 ng/mL). The limit of quantification for all the anthocyanins was determined in blank plasma by signal-to-noise ratio (C3G, C3R, and P3R, 0.08 ng/mL; P3G, 0.002 ng/mL). Malvidin-3-O-glucoside was used as an internal standard to account for any extraction losses before HPLC analysis. PG was quantified using P3G due to unavailability of PG standard.

Calculations and Statistical Analysis. Anthocyanin analysis focused on measuring parent compounds and conjugated parent metabolites maintaining the base C6-C3-C6 structure because these have been shown previously[16] to be the main compounds increasing in plasma within the first few hours of consumption and likely influenced by meal timing or physiological state (i.e., fasting or fed). C_{max} (nmol/L) is defined as the maximum concentration of anthocyanins and their metabolic conjugates in plasma from time 0−10 h after strawberry-drink consumption. T_{max} is the time (h) when C_{max} was achieved. Area under the plasma concentration time curve (AUC$_{0-10h}$, nmol × h/L) was calculated by the linear trapezoidal method[17] using Microsoft Excel 2013 version 15. Relative bioavailability of anthocyanins was based on parent compounds and metabolites maintaining the parent C6-C3-C6 flavonoid structure. It was calculated based on compounds and their metabolic conjugates AUC$_{0-10h}$ corrected for individual plasma volume based on sex and body weight (kg) according to the methods described in the technical manual of American Association of Blood Banks,[18] where plasma volume is estimated to be 36 mL/kg for women and 40 mL/kg for men. This method to calculate total plasma volume is also used by other researchers.[19,20] The nmol of each compound in plasma was calculated by multiplying the sum of area

under curve of that compound with the estimated total plasma volume in the body. The percent bioavailability was calculated by dividing the nmol of compound in plasma to the nmol of anthocyanin or its metabolic conjugate per drink and multiplying by 100. The collected data was analyzed by repeated measures analysis of variance (RM-ANOVA) using the MIXED procedure of SAS 9.3 (SAS Institute Inc., Cary, NC) to test the main effects of the strawberry treatment (BM, WM, and AM), time (h), and time by treatment interaction. Sequence, period, and treatment by period interaction did not significantly affect responses observed and, therefore, were not included in the final statistical models. The final models contained time, treatment, and treatment by time interaction. The Kenward−Roger correction was used to compute the denominator degrees of freedom for the tests of fixed effects, and the method of restricted maximum likelihood were used in all Mixed Models.[21−23] The main effects of treatment and time were assessed with the subject as the blocking variable. All outcome variables (pharmacokinetic parameters, including bioavailability) were first examined for normality by Shapiro−Wilk's tests,[24] and data not conforming to normal distribution patterns were log-transformed prior to analysis. Anthocyanin pharmacokinetic parameters (C_{max}, AUC, and percent bioavailability) were analyzed using one-way ANOVA followed by a Tukey−Kramer adjusted t test for the pairwise multiple comparisons using the MIXED procedure of SAS 9.3 (SAS Institute Inc.). T_{max} was analyzed by paired t tests after adjusting for strawberry-drink timing of intake. The results of the statistical analysis are presented as least-squares means of treatment effect ± standard error of means (SEMs) unless indicated otherwise, and the level used to determine statistical significance was $p < 0.05$.

RESULTS

Subject Demographics. A total of 14 (male, $n = 9$ and premenopausal female, $n = 5$) subjects were enrolled in the study. A separate demographically matched group was enrolled as a reference group ($n = 10$). All subjects completed the study. The mean age and BMI for the strawberry group was 25 ± 4 years and 26 ± 2 kg/m^2, respectively, and for the reference group it was 27 ± 4 years and 27 ± 2 kg/m^2, respectively. All subjects were healthy and free of chronic diseases and were not on any prescription medications on a regular basis. Subject demographic characteristics are listed in Table 1.

Table 1. Subject Demographic Characteristics

variable	strawberry group ($n = 14$)[a]	reference group ($n = 10$)[a]
age (year)	25 ± 4	27 ± 4
weight (kg)	79 ± 8	79 ± 7
BMI (kg/m^2)	26 ± 2	27 ± 2
waist (cm, mid point)	87 ± 7	87 ± 6
male/female	9:5	7:3
ethnicity (Asian/Hispanic/ Caucasian/African American)	4:2:6:2	4:1:3:2

[a]Values are mean ± SEMs.

Anthocyanin Content of Test Drinks. A total of four anthocyanins were identified and quantified in the strawberry drink, i.e., C3G, C3R, P3G, and P3R, which were detected previously in strawberries by other researchers.[25−27] The content of individual anthocyanins and total anthocyanins in strawberry and control drink is shown in Table 2. P3G was the major anthocyanin in the strawberry drink comprising about 76.8% of total strawberry anthocyanins followed by P3R (12.7%), C3G (10.2%), and C3R (0.3%). Similar content of anthocyanins was reported in five strawberry cultivars.[12] The concentration of total anthocyanins was 76.6 ± 1.2 μmol (34.7 ± 0.5 mg) in 77 mL of strawberry drink.

Table 2. Anthocyanin Content of Test Drinks

anthocyanins	strawberry drink (μmol/drink)[a]	control drink (μmol/drink)[a]
cyanidin-3-O-glucoside	7.8 ± 0.1	0.0 ± 0.0
cyanidin-3-O-rutinoside	0.3 ± 0.0	0.0 ± 0.0
pelargonidin-3-O-glucoside	58.8 ± 1.0	0.1 ± 0.0
pelargonidin-3-O-rutinoside	9.7 ± 0.0	0.0 ± 0.0
total anthocyanins	76.6 ± 1.2	0.1 ± 0.0

[a]Data are mean ± standard deviation for three replicates.

Kinetic Profile and Pharmacokinetic Parameters. A total of five compounds were detected in plasma including four parent anthocyanins (C3G, C3R, P3G, and P3G) and one conjugated metabolite PG, which was the major anthocyanin metabolite maintaining the C6-C3-C6 parent anthocyanin structure (Figure 3). C3R was present only in trace amounts and could not be quantified in most of the plasma samples. Anthocyanins and their metabolic conjugates were not detected in the baseline samples ($t = 0$ h). The peak plasma concentration of PG and P3G was achieved between ~1−3 h after consumption of the strawberry drinks, depending on when strawberry drinks were consumed relative to the meal (Figure 4). Consuming the strawberry drink with the meal delayed the appearance of pelargonidin-based anthocyanins in the blood by ~1−1.5 h compared to results from consuming the strawberry drink on an empty stomach (BM condition, $p < 0.05$) or 2 h post-meal (AM condition, $p < 0.05$ for PG and P3G only). After the peak concentrations were achieved, the content of anthocyanins and their metabolic conjugates in plasma decreased with time; however, they were still detectable at the final time point (10 h). The highest C_{max} values were obtained for PG while the lowest values were observed for C3G and P3R. Significant differences among treatment sequences ($p < 0.05$) were observed in the C_{max} of PG: consuming the strawberry drink before (BM, 38.0 ± 6.7 nmol/L) or after (AM, 34.5 ± 7.4 nmol/L) the meal resulted in significantly higher C_{max} compared to results when the strawberry drink was consumed with the meal (WM, 12.8 ± 2.1 nmol/L). However, C_{max} values for P3G, P3R, and C3G were not significantly different after strawberry drink consumption relative to meal timing (Table 3). The T_{max} (values corrected to time of strawberry consumption) of pelargonidin-based anthocyanins and their metabolic conjugates was significantly longer than ~3 h when strawberry drink was consumed with the meal (WM) compared to before (BM) or after the meal (AM) (Table 3).

AUC provides information on relative exposure. There was a significant increase in the AUC_{0-10h} of PG when the strawberry drink was consumed before (BM: $p = 0.0002$) and after the meal (AM: $p = 0.0025$) compared to with the meal (WM). However, AUC_{0-10h} was greatest for P3G when the strawberry drink was consumed with the meal (WM versus AM and BM, <0.05). Similarly, the AUC of P3R was greater in the WM condition compared to the BM condition ($p < 0.05$) but not compared to the AM condition. No effect was observed in the
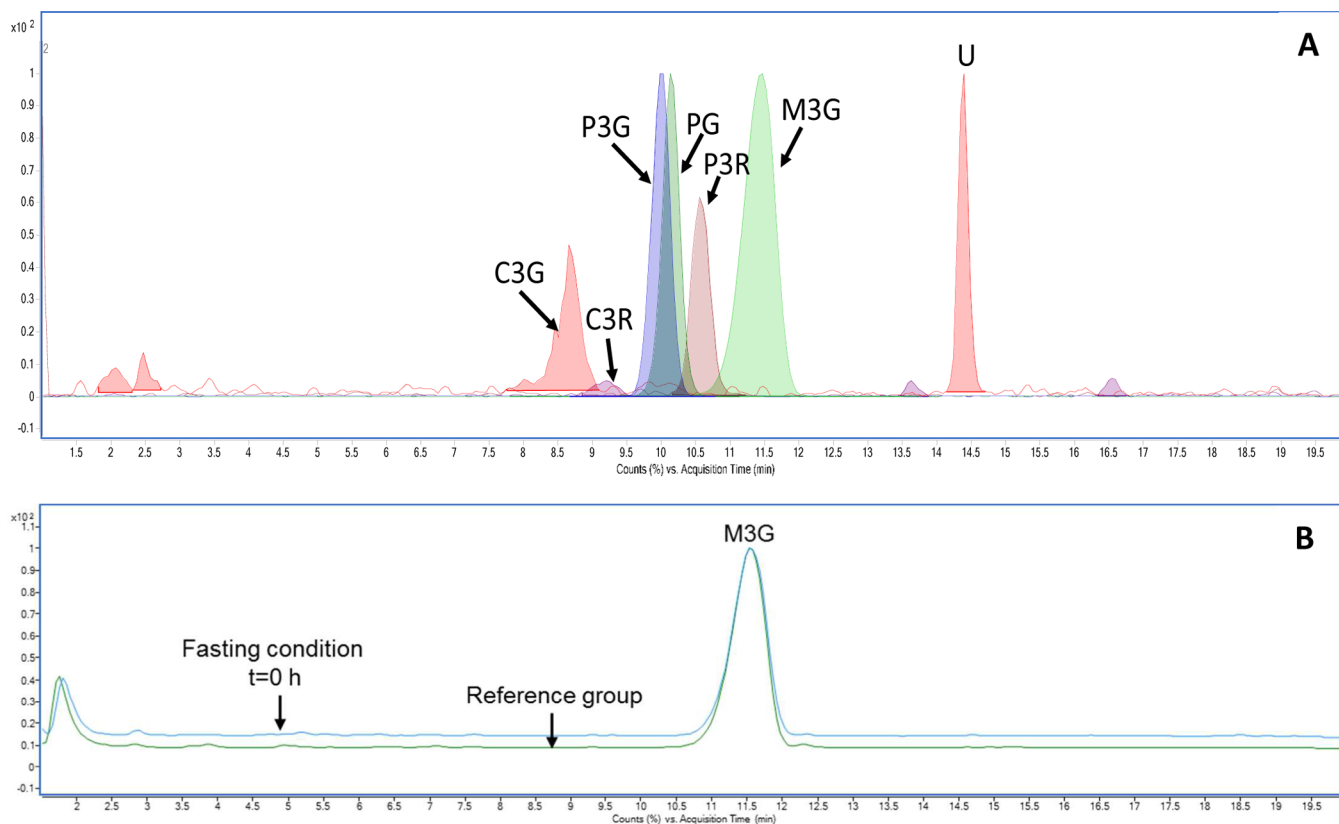

Figure 3. (A) MRM chromatogram of ion transitions of anthocyanins and their metabolic conjugates (overlaid) in plasma 2 h after the consumption of strawberry drink before the meal (BM); C3G (cyanidin-3-O-glucoside), C3R (cyanidin-3-O-rutinoside), P3G (pelargonidin-3-O-glucoside), PG (pelargonidin glucuronide), P3R (pelargonidin-3-O-rutinoside), M3G (malvidin-3-O-glucoside; internal standard), and U (unknown peaks). (B) Total-ion chromatogram of subject in fasting condition ($t = 0$ h) and a reference-group subject ($t = 2$ h); M3G (malvidin-3-O-glucoside; internal standard).
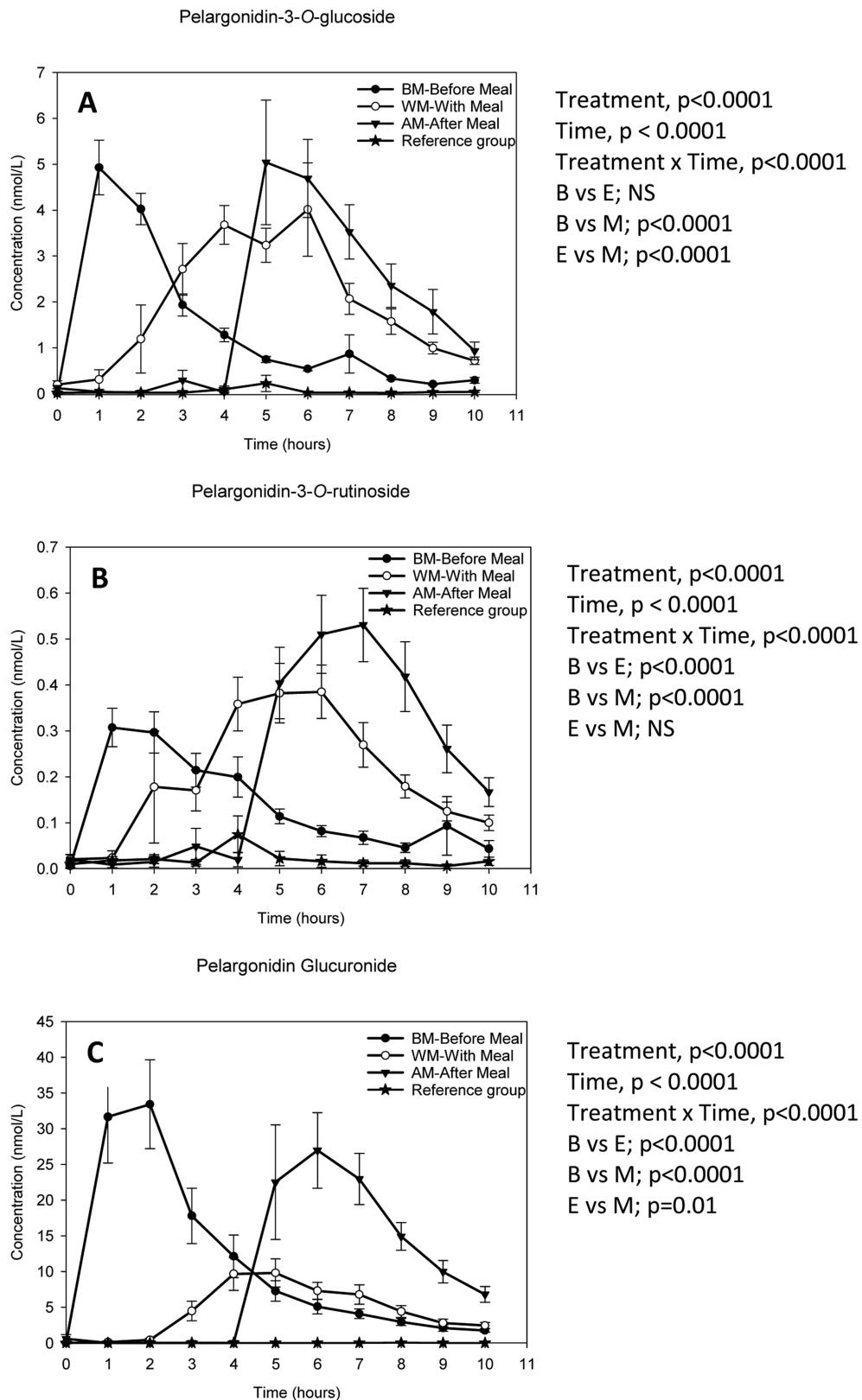
Pelargonidin-3-O-glucoside

Treatment, p<0.0001
Time, p < 0.0001
Treatment x Time, p<0.0001
B vs E; NS
B vs M; p<0.0001
E vs M; p<0.0001

Pelargonidin-3-O-rutinoside

Treatment, p<0.0001
Time, p < 0.0001
Treatment x Time, p<0.0001
B vs E; p<0.0001
B vs M; p<0.0001
E vs M; NS

Pelargonidin Glucuronide

Treatment, p<0.0001
Time, p < 0.0001
Treatment x Time, p<0.0001
B vs E; p<0.0001
B vs M; p<0.0001
E vs M; p=0.01

Figure 4. Plasma concentration vs time profiles of (A) pelargonidin-3-O-glucoside, (B) pelargonidin-3-O-rutinoside, and (C) pelargonidin glucuronide 0−10 h after ingestion of 77 mL (12 g of freeze-dried strawberry powder) of strawberry drink; $n = 14$ and reference group $n = 10$. RM-ANOVA using the MIXED procedure of SAS 9.3 to test the main effects of the strawberry treatment (BM, WM, and AM), time (h), and time by treatment interaction is shown on the right-hand side of each figure. NS: Not significant.

AUC_{0-10h} of C3G ($p = 0.5520$) regardless of meal timing (Table 3).

Bioavailability (Percent Anthocyanins Maintaining C6-C3-C6 Structure Relative to Intake of Anthocyanins).

Table 3. Pharmacokinetic Parameters of Anthocyanins and Their Metabolic Conjugates in the Plasma of Healthy Volunteers

anthocyanins and their metabolic conjugates	C_{max} (nmol/L)[a]			T_{max} (h)[a]			AUC (nmol × h/L)[a]		
	BM[b]	AM	WM	BM	WM	AM	BM	WM	AM
pelargonidin glucuronide	38.0 ± 6.6, a	12.8 ± 2.1, b	34.5 ± 7.3, a	1.7 ± 0.3, b	2.9 ± 0.30, a	1.9 ± 0.2, b	123.8 ± 26.5, a	47.3 ± 6, b	100.0 ± 19.1, a
pelargonidin-3-O-glucoside	5.2 ± 0.6	5.4 ± 0.9	6.4 ± 1.2	1.3 ± 0.3, b	2.8 ± 0.3, a	1.7 ± 0.2, b	15.0 ± 1.3, b	42.6 ± 20.5, a	18.1 ± 3.2, b
pelargonidin-3-O-rutinoside	0.4 ± 0.0	0.5 ± 0.1	0.6 ± 0.1	1.9 ± 0.4, b	2.9 ± 0.4, a	2.3 ± 0.2, ab	1.4 ± 0.2, b	2.5 ± 0.4, a	2.3 ± 0.4, ab
cyanidin-3-O-glucoside	0.6 ± 0.3	0.3 ± 0.0	0.4 ± 0.1	2.1 ± 0.4	2.9 ± 0.4	2.3 ± 0.5	1.7 ± 0.6	1.3 ± 0.2	1.5 ± 0.6

[a]Values are mean ± SEMs (n = 14). [b]BM: Drink before meal (t = 0 h); AM: drink after meal (t = 4 h); WM: drink with meal (t = 2 h); C_{max}: maximum content in time period of 0–10 h; T_{max}: time point when C_{max} was achieved, values corrected to time when strawberry drink was consumed; AUC: area under curve derived by trapezoidal method. Different letters in each column indicate significant differences in numbers (p < 0.05).

PG was the most abundant metabolite circulating in the blood of subjects when the strawberry drink was consumed regardless of meal timing. The percent relative bioavailability of pelargonidin anthocyanins as measured by PG was significantly higher when the strawberry drink was consumed before (BM, 0.6 ± 0.0%; p = 0.0004) and after the meal (AM, 0.5 ± 0.0%; p = 0.0039) compared to with the meal (WM, 0.2 ± 0.0%). However, percent relative bioavailability was significantly higher for P3G (0.2 ± 0.0%; p = 0.0341) and P3R (0.1 ± 0.0%; p = 0.0265) when the strawberry drink was consumed with the meal (WM) (Table 4). No effect of meal timing was observed

Table 4. Percent Relative Bioavailability of Anthocyanins and Their Metabolic Conjugates in Plasma of Healthy Volunteers

anthocyanins and their metabolic conjugates	percent bioavailability		
	BM[ab]	WM[a]	AM[a]
pelargonidin glucuronide	0.6 ± 0.0, a	0.2 ± 0.0, b	0.5 ± 0.0, a
pelargonidin-3-O-glucoside	0.1 ± 0.0, b	0.2 ± 0.0, a	0.1 ± 0.0, ab
pelargonidin-3-O-rutinoside	0.0 ± 0.0, b	0.1 ± 0.0, a	0.1 ± 0.0, ab
cyanidin-3-O-glucoside	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
total anthocyanins	0.8 ± 0.0	0.6 ± 0.0	0.8 ± 0.0

[a]Values are mean ± SEMs (n = 14). [b]BM: Drink before meal (t = 0 h); WM: drink with meal (t = 2 h); AM: drink after meal (t = 4 h). Different letters in each column indicate significant differences in numbers (p < 0.05).

on the percent relative bioavailability of C3G. The percent relative bioavailability for total anthocyanins quantified was 0.8 ± 0.0%, 0.6 ± 0.0%, and 0.8 ± 0.0% for BM, WM, and AM sequence interventions, respectively (Table 4). Overall, percent relative bioavailability of total anthocyanins was not affected relative to meal timing, although there was a trend for enhanced bioavailability (~26%) when the strawberry drink was consumed before the meal (BM) compared to results from consuming with the meal (WM, p = 0.0698).

■ DISCUSSION

The bioavailability of parent anthocyanins and their conjugated metabolites (glucuronide, sulfate, and methyl conjugates of the precursor anthocyanins) has been reported to be relatively low compared to other flavonoids (flavonols and flavanals). Several factors affect bioavailability including food matrix, structural variation of anthocyanins, dose, inter-individual variations, complex interactions with other compounds during absorption and digestion, and instrumental and methodological challenges that result in low values.[28] Most of the clinical studies investigating the bioavailability of anthocyanins administered single doses (varying from 150 mg to 2 g) of total anthocyanins, generally in the form of whole berries,[29] juice,[30] berry extracts,[31] or concentrates[32] in the fasted state. Because foods are consumed at different times of the day under varying metabolic conditions, it is unclear how bioavailability and ascribed biological effects may vary when dietary anthocyanins are consumed under fasting conditions, with a meal, or for a snack between meals. To our knowledge, this is the first study reporting the effect of timing of strawberry consumption relative to a meal on the pharmacokinetic parameters, including the bioavailability of strawberry anthocyanins.

Unlike most other polyphenols, anthocyanins can be absorbed in their intact glycated form without structural modification.[33] After absorption, anthocyanins can exist as glycosides, aglycones, and their chalcones, all of which possess C6-C3-C6 flavonoid-like structure that remains intact even after phase II metabolism.[34] Consistent with previous reports, the data obtained in the current study show that after strawberry drink consumption by human subjects, PG is the major pelargonidin metabolite found in plasma. However, only one isomer was detected and quantified in this study as compared to previous studies.[16,35] We observed higher concentrations of PG in blood plasma ($C_{max} \approx 66\%$ increase) when the strawberry drink was consumed in a fasted state compared to the fed state (i. e., WM) (Table 3). Mullen et al.,[29] studied the influence of consuming cream with strawberries on the absorption of pelargonidin from strawberries in humans. In the strawberry-only condition, the C_{max} of PG was reported at 274 ± 24 nmol/L after 1.1 ± 0.4 h in eight volunteers (six male and two female). The reported C_{max} was 86% higher than the C_{max} of PG in the present study under similar biological conditions (fasting). The difference in C_{max} between the two studies could be attributed to the differences in the form and dose of strawberries consumed (Mullen et al.:[29] 200 g of frozen defrosted strawberries with 222 μmol of P3G versus the present study: 12 g of freeze-dried strawberries equivalent to 132 g fresh weight containing 58.8 μmol P3G) as well as variation in subject population. A dose-response increase in C_{max} has been observed with strawberry intake in previous studies conducted by our research group.[14,16]

Anthocyanins may be rapidly absorbed by the stomach, passing to the liver via the portal vein, and other fractions may be absorbed from the small intestine, which is the primary absorption site of flavonoids.[36] Data suggest that anthocyanins could be absorbed from the stomach via bilitranslocase transporter[37,38] and from the small intestine via transporters such as sodium glucose linked transporter 1 (SGLT1) and glucose transporter 2 (GLUT2).[39,40] P3G could be metabolized to glucuronide, either directly by the action of UDP−glucose dehydrogenase and UDP−glucuronosyltranferases (UGT) or after being deglycosylated by β-glucosidases or lactase phlorizen hydrolase in the intestinal wall,[36] or undergo glucuronidation in the liver by UDP−glucose dehydrogenase and UGT.[41] The plasma data from the present study showed that the C_{max} and T_{max} of PG was ~63−66% higher and occurred ~1 h sooner (Table 3), respectively, when the strawberry drink was consumed 2 h before or after the meal compared to with the meal, suggesting potential interference by meal constituents impacting pelargonidin metabolism and PG kinetics. This could occur either directly via competition for transporters or above-mentioned enzymes or indirectly via meal-associated physiochemical properties and effects on gastrointestinal function. Alternatively, anthocyanins are more stable under acidic conditions,[42] so it is also possible that the lower gastric pH during the fasting and postabsorptive conditions (pH 1.5−3.0) had a stabilizing effect on anthocyanins for enhanced gastric absorption as opposed to the meal condition in which gastric pH increases above the pK_a of anthocyanins (pK_a = 3.7).

In contrast, P3R and P3G showed enhanced absorption with the meal (expressed by AUC) compared to the fasting state (Table 3). This may explain, at least in part why PG was much lower during the "meal" sequence because P3G and P3R were absorbed intact bypassing metabolism to the glucuronide conjugate. The explanation for this observation is uncertain

from the present data, however; a review of the time-by-concentration curves suggested a slower absorption time of P3G and P3R with the meal. This may have been due to the transient formation of anthocyanin complexes with other meal components (i.e., carbohydrates, proteins, and organic acids)[43] and the slower release of anthocyanins for uptake into enterocytes influencing concentration-dependent kinetics of phase II enzymes for glucuronidation.[44,45] The food matrix can have an effect on the absorption profile, with varied outcomes on bioavailability of polyphenols.[43] For example, food components such as proteins, fats, and carbohydrates can complex with anthocyanins, and bioavailability could be enhanced, lowered, or unaltered by such processes.[46] The delayed absorption of P3R is most likely due to the effect of sugar moiety, i.e., rutinoside,[31] which is somewhat evident in comparing the P3G and P3R T_{max} values before the meal (p = 0.01) and 2 h after the meal (p = 0.01) but not with the meal (p = 0.77). Similar effects of rutinoside have been observed with quercetin absorption.[47]

Bioavailability was assessed for parent anthocyanin compounds absorbed in native form as well as relative to conjugated form. Overall, the bioavailability of total strawberry anthocyanins was not affected by the timing of strawberry consumption relative to the meal (Table 4). However, differences in bioavailability according to meal intake were detected and indicated according to individual anthocyanins and their metabolic conjugates. PG was the most abundant anthocyanin metabolite detected in plasma, and when assessed according to the dose of pelargonidin-based anthocyanins consumed, PG values indicated that the bioavailability of pelargonidin-based anthocyanins were highest when strawberry intake was 2 h before or 2 h after the meal. Further, PG values suggested that pelargonidin anthocyanins are more bioavailable than cyanidin anthocyanins, which has been reported previously.[41]

More than 99% of the anthocyanins were not recovered in the plasma. Recent evidence on the bioavailability of anthocyanins suggest that they undergo extensive presystemic metabolism rather than poor absorption.[48] The bioavailability of anthocyanins is underestimated because the metabolites formed during the course of digestion by colonic microflora are not reported in most of the human studies on anthocyanins.[29,35,49] A study conducted using isotopically labeled C3G showed a relative bioavailability of $12.3 \pm 1.3\%$ in humans,[50] and less than 50% of the ingested dose of labeled carbon (^{13}C) was recovered in urine, breath, and feces, with the fate of the remaining ^{13}C unknown in the study. These labeled metabolites remained in circulation for more than 48 h after ingestion because the C_{max} of ^{13}C metabolites in feces was not achieved by this time. It was speculated that a considerable portion of unrecovered ^{13}C remained in feces. Therefore, a significant amount of anthocyanins can reach the colon, where they are broken down into smaller molecules by bacteria. Absorption of these colonic-derived metabolites of anthocyanins would be considered another source of the original anthocyanins contributing to their overall bioavailability and possibly bioactivity.

Our results indicate that pelargonidin glucuronide was the major anthocyanin metabolite detected after strawberry drink consumption. The peak plasma concentration of anthocyanins and their metabolic conjugates was reached within 1−3 h after strawberry consumption with or without a meal. The C_{max}, AUC, and percent relative bioavailability of PG was significantly higher when strawberry drink was consumed 2 h before and

after the meal, compared with results for consumption with the meal. In contrast, parent pelargonidin-based compounds were highest in the plasma when consumed with the meal. Hence, the overall bioavailability of total anthocyanins quantified in this study was not affected by the timing of strawberry drink consumption relative to the meal. However, the biological relevance of the pharmacokinetic differences observed in this study requires further investigation. Understanding the timing of intake, matrix, and dose and amount of strawberries or other fruits delivering bioactive components should be considered in the development of dietary guidance to maximize the delivery of health benefits. This study is the first to report on the effects of meal timing and the pharmacokinetics of strawberry anthocyanins.

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