Food Chemistry 168 (2015) 233–240

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Flavonoids and phenolic acids from cranberry juice are bioavailable and bioactive in healthy older adults

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ARTICLE INFO

Article history:
Received 21 February 2014
Received in revised form 7 June 2014
Accepted 9 July 2014
Available online 16 July 2014

Chemical compounds studied in this article:
Proanthocyanidin A2 (PubChem CID: 124025)
Protocatechuic acid (PubChem CID: 72)
Quercetin (PubChem CID: 5280343)
Vanillic acid (PubChem CID: 8468)
4-Hydroxyphenylacetic acid (PubChem CID: 127)
Epicatechin (PubChem CID: 72276)
4-Hydroxy-3-methoxyphenylacetic acid (PubChem CID: 1738)
Gentisic acid (PubChem CID: 3469)
3,4-Dihydroxyphenylacetic acid (PubChem CID: 547)

Keywords:
Cranberry
Polyphenols
Flavonoids
Phenolics
Proanthocyanidins
Antioxidant
Bioavailability

ABSTRACT

Cranberries (*Vaccinium macrocarpon*) are a rich source of phenolic phytochemicals, which likely contribute to their putative health benefits. A single-dose pharmacokinetic trial was conducted in 10 healthy adults ⩾50 y to evaluate the acute (24-h) absorption and excretion of flavonoids, phenolic acids and proanthocyanidins (PACs) from a low-calorie cranberry juice cocktail (54% juice). Inter-individual variability was observed in the C_{max} and T_{max} of many of these compounds in both plasma and urine. The sum total concentration of phenolics detected in plasma reached a peak of 34.2 μg/ml between 8 and 10 h, while in urine this peak was 269.8 μg/mg creatinine, and appeared 2–4 h earlier. The presence of PAC-A2 dimers in human urine has not previously been reported. After cranberry juice consumption, plasma total antioxidant capacity assessed using ORAC and TAP assays correlated with individual metabolites. Our results show phenolic compounds in cranberry juice are bioavailable and exert antioxidant actions in healthy older adults.

1. Introduction

Observational studies have consistently shown the consumption of fruits and vegetables is inversely associated with the risk of developing chronic diseases, such as cardiovascular diseases (CVD), certain forms of cancer, and neurodegenerative diseases. The phytochemical constituents of these foods appear to contribute substantially to this benefit (Arts & Hollman, 2005). Phenolic phytochemicals have a wide range of biological actions, including the ability to act as antioxidants, ameliorate inflammation, modulate enzyme activity, and regulate gene expression.

Abbreviations: AUC, area under the curve; BMI, body mass index; BODIPY, 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-/s/-indacene-3-undecanoic acid; BP, blood pressure; CHD, coronary heart disease; CJC, cranberry juice cocktail; C_{max}, maximum concentration; CVD, cardiovascular diseases; FRAP, ferric reducing antioxidant power; HNRCA, Human Nutrition Research Center on Aging; LDL oxidation, susceptibility of LDL to Cu^{2+}-induced lipid oxidation; MeOH, methanol; ORAC, oxygen radical absorbance capacity; PAC, proanthocyanidin; TAP, total antioxidant performance; TE, Trolox equivalents; T_{max}, time to maximum concentration.

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http://dx.doi.org/10.1016/j.foodchem.2014.07.062
0308-8146/

Among the 20 most commonly consumed fruits in the American diet, cranberries appear to have the highest total phenol content (Vinson, Su, Zubik, & Bose, 2001). Cranberries (*Vaccinium macrocarpon*) are a rich source of phenolic compounds, particularly phenolic acids (including benzoic, hydroxycinnamic and ellagic acids) and flavonoids (including flavonols, flavan-3-ols, anthocyanins, and proanthocyanidins [PACs]). These phenolics appear responsible for the putative health benefits of cranberry consumption, such as the prevention of urinary tract infections (Howell et al., 2010) and stomach ulcers (Zhang et al., 2005), as well as improved oral health (Weiss et al., 2004). These actions may result from the ability of cranberry phenolics to interfere with the adhesion of some bacteria to select cell types and surfaces. Cranberry polyphenols may also contribute to reducing the risk of CVD (Ruel et al., 2008) and improving glucoregulation in diabetics at high risk for CVD (Wilson, Meyers, Singh, Limburg, & Vorsa, 2008).

Cranberry products and cranberry phenolics have also been shown to possess antibacterial (Caillet, Cote, Sylvain, & Lacroix, 2012), anti-mutagenic (Vattem, Jang, Levin, & Shetty, 2006), anti-carcinogenic (Vu et al., 2012), anti-angiogenic (Roy et al., 2002), and antioxidant activities (Caillet, Lorenzo, Cote, Sylvain, & Lacroix, 2012); however, most of this evidence is derived from *in vitro* studies and animal models. More information is required on the bioavailability and metabolism of cranberry bioactives in humans to better understand their impact on pertinent risk factors for chronic diseases and to inform the design of future clinical trials (McKay & Blumberg 2007).

Previously, we characterised the pharmacokinetics of anthocyanins from cranberry juice in patients with coronary heart disease (CHD), and found most appeared in plasma at nM concentrations within 1–2 h of consumption (Milbury, Vita, & Blumberg, 2010). We have expanded on this work by examining the bioavailability of a broader array of phenolics following cranberry juice consumption, as well as their bioactivity, in a small human study. Healthy older adults were selected as subjects for this study as we anticipate future trials of the putative benefits of cranberry juice on risk factors for chronic age-related diseases, including CVD, type 2 diabetes, and cancer will be conducted in this age group.

2. Materials and methods

2.1. Participants

Ten healthy, nonsmoking men and postmenopausal women age 50–70 y with a body mass index (BMI) of 18.5–29.9 kg/m^2 were recruited from the Boston area. Postmenopausal status in women was defined as the absence of menses for $\geqslant$1 y. The exclusion criteria used to screen for their eligibility included: presence of cardiovascular, endocrine, gastrointestinal, and renal diseases; use of estrogen, with or without progesterone; use of medications known to affect lipid metabolism; use of medications known or suspected to influence blood pressure (BP); gastrointestinal diseases and conditions or medications influencing gastrointestinal absorption; chronic kidney disease; endocrine disorders including diabetes and untreated thyroid disease; rheumatologic disorders; active treatment for cancer of any type (except basal cell carcinoma) $\geqslant$1 y; regular use of oral steroids; systolic blood pressure >150 mmHg and/or diastolic blood pressure >95 mmHg; regular use of any dietary supplements within previous 30 d; usual daily ethanol intake of $\geqslant$2 drinks; cigarette smoking and/or nicotine replacement use; laboratory blood or urine biochemistries outside of normal ranges; EKG abnormalities. The study design was approved by the Institutional Review Board of Tufts University Health Sciences Campus and Tufts Medical Center. All participants signed a written informed consent agreement before participating. This study was registered with the public registry ClinicalTrials.gov (ID # NCT00740077).

2.2. Study design

A single-dose pharmacokinetic trial was conducted to evaluate the acute (24-h) bioavailability of flavonoids and phenolic acids from 237 ml of a double-strength (54% juice) low-calorie, low-sugar cranberry juice cocktail (CJC). The CJC was specially formulated and produced in a single batch by Ocean Spray. Qualified subjects were asked to consume foods low in phenols and polyphenols for 48 h prior to the CJC intervention. Examples of the restricted foods include certain fruits, berries, vegetables, juices, nuts, tea, herbal tea, coffee, cocoa, chocolate and wine. The purpose of these dietary restrictions was to reduce any residual dietary phenolic compounds from the body (these compounds are typically cleared from blood and urine within 48 h of consumption) and to ensure phenolic compounds present in the blood and urine samples collected during the intervention were derived from the CJC alone. Prior to the day of the CJC intervention, subjects reported to the Jean Mayer USDA Human Nutrition Research Center on Aging (HNRCA) at Tufts University for an overnight stay no later than 7:00 PM in the evening. All subjects consumed the same low phenolics meal prepared in the HNRCA Metabolic Research Unit (MRU) kitchen, and refrained from consuming any food or beverages, except for water, for the next 12 h. Prior to administering the CJC the following morning, fasting blood and urine (first morning void) samples were collected for baseline measurements. A single dose of CJC was then administered orally under close observation by the MRU staff. No other food or beverages were provided at this time. Following administration of the CJC, blood samples were collected via indwelling catheter at 0.25, 0.5, 1–6, and 10 h. Urine was collected every 2 h for the next 10 h. Lunch and dinner meals, prepared by the kitchen, were low in phenols and polyphenols and provided 5 h and 10 h post-administration. At the end of 10 h, subjects were allowed to leave the HNRCA with 24 h urine collection materials. Subjects returned to the HNRCA the following morning after having fasted for 12 h. Fasting blood and urine (excreted since the 10 h intervention) were collected within 24 h of administering the CJC. Vital signs, including blood pressure, temperature, pulse and respiration rate, were monitored regularly following CJC consumption.

2.3. Sample preparation

Collected blood samples were assessed for phenolic acids, flavonoids (including flavonols, flavanols, anthocyanins, PAC), and selected measures of total antioxidant capacity – Oxygen Radical Absorbance Capacity (ORAC) with perchloric acid precipitation, Ferric Reducing Antioxidant Power (FRAP), Total Antioxidant Performance (TAP) – and susceptibility of LDL to Cu^{2+}-induced lipid oxidation (LDL oxidation). Blood samples for the analysis of phenolic acids, flavonoids, FRAP, TAP, and LDL oxidation were collected in EDTA-containing evacuated tubes and centrifuged within 15 min of drawing (1000×*g*, 15 min, 4 °C) with a SUR-Sep cap (Organon Teknika, Durham, NC). Blood samples for the ORAC were collected in heparin tubes, and processed similarly. Plasma samples for anthocyanin analysis were prepared by adding 30 μl of 12 mol/l HCl to 1.5 ml of plasma followed by centrifugation (1000×*g*, 15 min, 4 °C) and aliquoted immediately into 2 ml NUNC tubes (Vanguard Cryotubes, Neptune, NJ). Plasma samples for the analysis of LDL oxidation were prepared by adding 111 μl of 6% sucrose solution to 1 ml plasma, and stored at −80 °C for no longer than 8 wk before analyzing. Urine samples for anthocyanin analysis were prepared by adding 0.2 ml of 12 mol/l HCl to 50 ml aliquots. All samples were stored at −80 °C until analysis. All samples for

each participant were analysed within the same run for every assay performed.

2.4. Biochemical analyses

2.4.1. Anthocyanins

Anthocyanins in 1 ml urine and 1.5 ml plasma samples were determined according to the method of Milbury et al. (2010) with slight modifications for LC–MS/MS analysis using an API 3000 Triple Quadrupole mass spectrometer with Turbo IonSpray ionisation (Applied Biosystems, Carlsbad, CA). Quantification of the anthocyanins was completed via comparison with a standard curve obtained using known concentrations of authentic anthocyanin standards with an adjustment for the internal standard (malvidin-di-glucoside). Since authentic standard for anthocyanidin glucuronides are not commercially available, their values are expressed as cyanidin glucose equivalents.

2.4.2. Flavanols, flavonols, and phenolic acids

Plasma (500 μl), urine (200 μl), and CJC beverage flavonoids (including the flavanols catechin and epicatechin and the flavonols quercetin, myricetin, kaempferol and isorhamnetin) and phenolic acids (including caffeic, ferulic, vanillic and *p*-coumaric acid) were determined according to Chen, Milbury, Lapsley, and Blumberg (2005) for HPLC analysis using a ESA CoulArray System (ESA, Inc. Chelmsford, MA). Analyte separation was achieved using a Zorbax ODS C18 column (4.6 × 150 mm, 3.5 μm). Quantification of phenolic acids and flavonoids in unknown samples were calculated based on standard curves constructed using authentic standards with adjustment for the internal standard (4′-hydroxy-3′-methoxyacetophenone).

2.4.3. Proanthocyanidins

Urine (3 ml) was spiked with 76 μmol (300 ng) daidzein as an internal standard. Daidzein was selected due to its expected absence in samples from subjects on a low polyphenol diet, as well as its absence in cranberry products. After acidification with 40 μl glacial acetic acid, the mixture was incubated for 60 min at 37 °C with 40 μl β-glucuronidase/sulphatase. After the addition of ~2 g NaCl, PAC-A2 was extracted twice with 3 ml ethyl acetate. The combined ethyl acetate fractions were dried under N_2 gas, reconstituted in 1.25 ml water with brief sonication, and then loaded onto a 200 mg Bond Elut-C18 cartridge (Agilent, Santa Clara, CA) which was first activated with 3 ml methanol and 3 ml water. After the cartridge was washed with 10 ml water and 3 ml 10% methanol, PAC-A2 and daidzein were eluted with 6 ml acetonitrile, dried under N_2 gas at ambient temperature under red light, and reconstituted with 250 μl of 5% acetonitrile in 0.1% aqueous formic acid for LC–MS/MS analysis. The elution efficacy of acetonitrile was superior to methanol. PAC-A2 and daidzein in the reconstituted samples was quantified using an Agilent 1200 series LC system, equipped with a switching valve, a Phenomenex Synergi Max-RP 80 Å C12 column (150 × 4.6 mm i.d., 4 μm; Torrance, CA), and an API 3000 Triple Quadrupole mass spectrometer with Turbo IonSpray ionisation (Applied Biosystems, Carlsbad, CA). Compound separation was performed using a linear gradient consisting of mobile phase A (water/formic acid, 99.9:0.1, v/v) and B (acetonitrile/formic acid, 99.9:0.1, v/v) at a flow rate of 400 μl/min. The gradient was set as follows: 0–20 min, 10–100% B; 20–25 min, 100% B; 25–26 min, 100–10% B; and 26–30 min, 10% B. The injection volume was 100 μl. The 10–18 min elute from the column was directed into the MS. The parameters of the MS were set according to Urpi-Sarda et al. (2009) with slight modifications. The collision energy for PAC-A2 and daidzein was optimised at −25 V, respectively. The dwell time was 1000 ms. After testing for signal performance in both positive and negative ionisation modes, negative ionisation mode was selected. The MS data were collected using a multiple reaction monitoring (MRM) mode with 575/289 for PAC-A2 and 253/208 for daidzein. LOD and LOQ for PAC-A2 were 0.625 and 1.25 ng/ml, respectively, and intra-day and inter-day CV was 6.6% and 7.9%.

Plasma PAC-A2 was extracted according to Tomas-Barberan et al. (2007) with slight modifications (Chen et al., 2005). After incubation for 60 min at 37 °C, the mixture was acidified with 80 μl *o*-phosphoric acid and 1 ml of ascorbic acid (0.2 mg/ml) and extracted using preconditioned 500 mg Bond Elut-C18 (Agilent, Santa Clara, CA) and 500 mg Oasis HLB (Waters, Milford, MA) cartridges. PAC-A2 was eluted from the cartridge with 15 ml acetonitrile, dried under N_2 gas at ambient temperature under red light, and reconstituted with 250 μl of 5% acetonitrile in 0.1% aqueous formic acid for LC–MS/MS analysis (see above). The LOQ for plasma PAC-A2 was 2.5 ng/ml.

2.4.4. Antioxidant capacity markers

The ORAC value of perchloric acid treated protein-free plasma was determined according to the method of Huang, Ou, Hampsch-Woodill, Flanagan, and Prior (2002) with a FLUOstar OPTIMA plate reader using a fluorescence filter (excitation wavelength, 485 nm; emission wavelength, 520 nm) and the data expressed as μmol Trolox Equivalents (TE)/L. The FRAP value of whole plasma was determined by the spectrophotometric method of Benzie and Strain (1996) and the data expressed as μmol TE/L. Serum TAP was determined according to Aldini, Yeum, Russell, and Krinsky (2001) to measure total antioxidant capacity in both the hydrophilic and lipophilic compartments of serum, and validated by Beretta et al. (2006). The results are expressed as TAP values, which represent the percentage of inhibition of BODIPY oxidation in human serum with respect to that occurring in a control sample consisting of BODIPY in phosphatidylcholine liposomes.

2.4.5. Resistance of LDL against Cu^{2+}-induced oxidation

The *ex vivo* resistance of LDL to Cu^{2+}-induced oxidation was determined by monitoring the formation of conjugated dienes at 37 °C over 3 h with a Shimadzu UV1601 spectrophotometer at an absorbance of 234 nm according to Chen et al. (2005). The results are expressed as lag time (min).

2.5. Statistical analyses

Statistical analyses were performed using SPSS version 21.0 (SPSS, Inc., Chicago, IL). The maximum plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) were recorded as observed. The area under the curve (AUC) of individual phenolic acids, anthocyanins, flavanols, flavonols, PACs, and their metabolite concentrations in blood and urine vs. time curve (0–24 h) were calculated using the linear trapezoidal integration method (Nielsen et al., 2006), adjusted for baseline concentrations. Pearson correlation coefficients (r) and nominal P values were computed to assess the linear relationship between changes in plasma phenolic acids and flavonoids with markers of antioxidant activity and lag time of LDL oxidation from baseline. Unless otherwise noted, results are expressed as mean ± SD. P values <0.05 were considered statistically significant.

3. Results

3.1. Subject characterisation

Subjects were generally healthy and absent chronic diseases (Supplemental Table 1).

3.2. CJC beverage

The principle phenolics in the test beverage were the anthocyanins peonidin-3-galactoside and -arabinoside, followed by cyanidin-3-arabinoside and -galactoside, and the flavonols hyperoside and quercetin. (Supplemental Table 2). In total, the 237 ml dose of CJC contained 188.5 mg phenolics.

3.3. Flavonoids and phenolic acids in plasma and urine

The profile of compounds analysed includes 13 phenolic acids, 2 flavanols, 4 flavonols, 6 anthocyanins, 4 anthocyanin glucuronides, and 1 PAC using 3 analytic platforms. A bimodal distribution of peak plasma concentrations was observed for all of the flavanols and flavonols and 7 phenolic acids (Table 1). In each case, the respective value for C_{max1} was similar to C_{max2} except quercetin and isorhamnetin, which tended to be smaller. The C_{max1} (T_{max1}) for most phenolic acids, flavonols, and epicatechin appeared within the first 0.5–2.6 h and, with the exception of salicylic and caffeic acids, the C_{max2} (T_{max2}) appeared at 6.1–8.8 h. A trimodal distribution of C_{max} was noted for caffeic acid with the T_{max} appearing at 0.8, 3.7, and 8.2 h. Four phenolic acids, protocatechuic, 4-OH-3-methoxy-phenylacetic, 3, 4-OH-phenylacetic, and gentisic acids displayed a C_{max} between 6.1 and 8.4 h. Six anthocyanins (galactosides, glucosides and arabinosides of cyanidin and peonidin) had a T_{max} between 0.9 and 4.7 h. Amongst these anthocyanins, peonidin-3 glucoside had wide range of T_{max} values because it was not detected in all time points of the 3 subjects and one subject had a T_{max} of 24 h. All 6 anthocyanins were only detected in the plasma of 3 of the 10 study subjects. Catechin, anthocyanidin glucuronides and PAC-A2 were not detected in plasma. In urine, a bimodal distribution was observed for the flavonol isorhamnetin and 3 phenolic acids, 4-OH-benzoic, 4-OH-phenylacetic, and 4-OH-3-methoxy-phenylacetic acids, with a T_{max1} at 4.8–6.6 h and T_{max2} at 10–24 h (Table 2). Among all 25 compounds detected in urine, 6 phenolic acids (4-OH-phenylacetic, protocatechuic, 3,4-OH-phenylacetic, 4-OH-3-methoxy-phenylacetic, 3-OH-benzoic, gentisic acids), catechin, epicatechin, and PAC-A2 displayed a T_{max1} later than 6 h. Five compounds detected in urine, including protocatechuic, 4-OH-phenylacetic, 3,4-OH-phenylacetic, 4-OH-3-methoxy-phenylacetic, and *p*-coumaric acids, had a $C_{max1} \geqslant 1.8$ μg/mg creatinine. Pronounced inter-individual variability in the C_{max} and T_{max} of many compounds detected in both plasma and urine were observed. The sum total of phenolics including phenolic acids, flavonols, and flavanols detected in plasma reached a peak concentration of 34.2 μg/ml at 8–10 h (Fig. 1), while in urine the peak concentration of the sum was 269.8 μg/mg creatinine, and appeared 2–4 h earlier (Fig. 2). In plasma, protocatechuic acid, quercetin, and vanillic acid were the highest contributors to this total. In urine, the highest contributors were protocatechuic acid followed by 4-OH-phenylacetic acid.

Anthocyanins, as well as anthocyanin glucuronides, were detected in urine (Table 2, Fig. 3), and their concentrations varied widely. Peonidin-3-galactoside, a form of cyanidin-galactoside methylated at the 3′ position of the B ring, was the predominant anthocyanin detected in both plasma and urine. Peonidin-3-arabinoside and cyanidin-3-glucoside, the two minor anthocyanins detected in urine, were each observed in only 5 subjects. Cyanidin, peonidin, delphinidin, and petunidin glucuronides were detected in urine with a total of 19 metabolites (Table 2). Only cyanidin (RT = 41.9), delphinidin (RT = 39.9), and peonidin glucuronides (RT = 30.0 and 42.4) were observed in all subjects. The excretion of anthocyanidin glucuronides in urine was slightly later (~1–2 h) than the anthocyanins, particularly some peonidin and petunidin glucuronides, which reached C_{max} after 4 h (Fig. 3). Although specific glucuronides could not be accurately quantified, the C_{max} of the 5 predominant glucuronides was ⩾2-fold larger than the parent anthocyanidins.

The presence of PAC-A2 in urine was observed (Table 2, Fig. 4). The T_{max} at 11 h appeared after all of phenolic acids and flavonoids. Among the 10 subjects, 4 had a T_{max} at 10 h, 2 had a T_{max} between 10 and 24 h, and one had a T_{max} at 2 h.

Table 1
Pharmacokinetic parameters of phenolic acids and flavonoids identified in plasma following CJC consumption.[a]

Compound	C_{max1} μg/ml	T_{max1} h	C_{max2} μg/ml	T_{max2} h	C_{max3} μg/ml	T_{max3} h	AUC_{0-24} μg h/ml
Protocatechuic acid	1.6 ± 1.6	8.8 ± 1.4					14.1 ± 18.5
	ng/ml	h	ng/ml	h	ng/ml	h	ng h/ml
Quercetin	340.2 ± 121.8	1.4 ± 1.3	463.0 ± 183.8	7.8 ± 1.9			2984.9 ± 2368.6
Vanillic acid	1997.9 ± 360.9	0.7 ± 0.3	1854.3 ± 378.2	6.1 ± 2.5			2617.7 ± 1721.6
Salicylic acid	53.5 ± 91.8	1.0 ± 0.8	36.6 ± 55.6	4.6 ± 2.3			635.7 ± 1257.6
4-OH-benzoic acid	197.7 ± 88.1	0.8 ± 0.7	198.6 ± 76.4	7.2 ± 2.3			492.4 ± 723.8
4-OH-phenylacetic acid	98.3 ± 51.2	1.5 ± 1.3	119.2 ± 68	7.8 ± 2.5			349.4 ± 348.6
4-OH-3-methoxy-phenylacetic acid	60.9 ± 3.9	7.1 ± 2.9					228.7 ± 138.2
Epicatechin	23.7 ± 18.0	2.6 ± 2.4	22.9 ± 21.4	8.1 ± 2.4			170.1 ± 312.2
Ferulic acid	56.3 ± 16.8	0.6 ± 0.3	55.3 ± 20.8	8.6 ± 1.6			128.6 ± 265.5
Myricetin	21.6 ± 5.7	1.7 ± 1.3	16.0 ± 4.5	7.8 ± 2.0			101.6 ± 107.7
Kaempferol	20.8 ± 10.7	0.8 ± 0.8	21.1 ± 10.9	7.3 ± 3.0			85.6 ± 152.0
Isorhamnetin	31.3 ± 35.4	0.5 ± 0.6	48.3 ± 39.7	7.3 ± 2.1			84.3 ± 119.8
ρ-Coumaric acid	32.4 ± 5.6	0.6 ± 0.3	30.6 ± 6.1	6.4 ± 2.6			79.8 ± 113.0
3,4-OH-phenylacetic acid	14.0 ± 7.2	8.4 ± 6.1					58.6 ± 68.5
Sinapinic acid	19.6 ± 3.9	0.6 ± 0.5					54.3 ± 71.5
Gentisic acid	7.7 ± 2.3	6.1 ± 2.0					41.5 ± 35.6
Caffeic acid	4.9 ± 2.3	0.8 ± 0.5	4.1 ± 3.0	3.7 ± 1.5	5.1 ± 3.2	8.2 ± 1.5	12.9 ± 16.4
Anthocyanins	pg/ml	h	pg/ml	h	pg/ml	h	pg h/ml
Peonidin-3-galactoside	981.2 ± 1123.2	1.6 ± 2.6					2450.3 ± 3436.6
Cyanidin-3-arabinoside	199.4 ± 189.3	3.3 ± 3.7					1472.9 ± 1523.3
Peonidin-3-arabinoside	411.5 ± 544.6	0.9 ± 0.5					985.6 ± 1454.8
Cyanidin-3-galactoside	327.1 ± 267.1	2.3 ± 3.2					603.4 ± 965.1
Peonidin-3-glucoside	77.2 ± 72.6	4.7 ± 9.5					176.0 ± 322.6
Cyanidin-3-glucoside	84.6 ± 77.0	1.7 ± 0.6					41.7 ± 76.9

[a] Values are means ± SD, $n = 10$.

Table 2
Pharmacokinetic parameters of phenolic acids and flavonoids identified in urine following CJC consumption.[a]

Compound	C_{max1} μg/mg[b]	T_{max1} h	C_{max2} μg/mg	T_{max2} h	AUC_{0-24} μg h/mg
Protocatechuic acid	21.1 ± 28.4	9.3 ± 7.2			95.9 ± 207.1
4-OH-phenylacetic Acid	9.6 ± 7.4	6.6 ± 1.7	6.7 ± 6.8	10.0 ± 0.0	78.1 ± 102.1
ρ-Coumaric acid	1.8 ± 2.0	5.4 ± 1.0			15.9 ± 22.7
3,4-OH-phenylacetic Acid	2.9 ± 4.1	7.0 ± 2.4			15.6 ± 21.0
4-OH-3-methoxy-phenylacetic Acid	1.8 ± 1.3	6.2 ± 2.2	0.74 ± 0.58	10.0 ± 0.0	9.8 ± 7.9
Vanillic acid	1.5 ± 1.1	5.6 ± 2.8			9.3 ± 6.9
4-OH-benzoic Acid	1.4 ± 1.5	5.4 ± 1.7	1.1 ± 1.0	22.6 ± 4.4	7.3 ± 9.8
Catechin	0.60 ± 0.63	8.4 ± 6.2			5.7 ± 6.0
3-OH-benzoic Acid	0.92 ± 1.37	10.4 ± 7.3			5.2 ± 7.9
Salicylic acid	1.5 ± 3.6	7.6 ± 6.0			4.4 ± 9.6
Ferulic acid	0.61 ± 0.59	4.2 ± 2.0			1.7 ± 2.1
Sinapinic acid	0.39 ± 0.40	3.8 ± 2.2			1.6 ± 2.3
Kaempferol	0.29 ± 0.30	5.3 ± 1.4			1.3 ± 1.3
Epicatechin	0.30 ± 0.26	6.5 ± 2.3			0.82 ± 1.07
Isorhamnetin	0.18 ± 0.10	4.8 ± 1.5	0.19 ± 0.14	24.0 ± 0.0	0.80 ± 0.89
Quercetin	0.14 ± 0.09	3.8 ± 2.0			0.51 ± 1.09
Gentisic Acid	0.16 ± 0.19	8.0 ± 2.0			0.50 ± 0.90
Caffeic Acid	0.13 ± 0.20	4.8 ± 1.5			0.14 ± 0.45
Myricetin	0.02 ± 0.02	5.4 ± 1.9			0.08 ± 0.10
	ng/mg	h	ng/mg	h	ng h/mg
Proanthocyanidin A2	24.4 ± 13.8	11.0 ± 6.9			169.4 ± 161.1
Anthocyanins	pg/mg	h	pg/mg	h	pg h/mg
Peonidin-3-galactoside	274.8 ± 536.8	3.4 ± 1.9			936.5 ± 1321.1
Cyanidin-3-galactoside	140.0 ± 201.2	2.8 ± 1.9			507.8 ± 549.8
Cyanidin-3-arabinoside	35.2 ± 58.9	3.4 ± 2.5			151.1 ± 200.6
Peonidin-3-glucoside	28.9 ± 33.6	3.2 ± 1.9			104.9 ± 111.7
Peonidin-3-arabinoside	69.4 ± 46.1	3.6 ± 2.6			69.4 ± 95.6
Cyanidin-3-glucoside	6.4 ± 6.5	3.2 ± 1.8			13.1 ± 24.2
Delphinidin glucuronide (m/z transition 479/303)					
RT = 28.9	185.7 ± 266.2	3.3 ± 2.1			279.2 ± 311.0
RT = 32.1	628.8 ± 991.0	3.3 ± 1.0			1803.8 ± 3456.9
RT = 34.6	144.9 ± 165.5	3.7 ± 2.3			283.5 ± 384.6
RT = 37.1	1767.3 ± 3607.7	3.1 ± 1.1			5498.7 ± 11301.8
RT = 38.3	126.6 ± 166.1	3.8 ± 2.0			323.9 ± 495.1
RT = 39.9	2384.6 ± 2427.6	3.3 ± 2.0			6764.9 ± 6716.2
RT = 41.2	61.9 ± 41.6	4.5 ± 1.8			203.7 ± 191.3
Cyanidin glucuronide (m/z transition 463/287)					
RT = 24.3	513.7 ± 800.4	3.3 ± 1.4			2141.7 ± 4022.8
RT = 30.1	1189.2 ± 2001.2	3.3 ± 1.4			4585.6 ± 9130.8
RT = 37.2	360.5 ± 642.5	3.3 ± 1.6			327.4 ± 572.6
RT = 40.5	140.4 ± 164.5	4.2 ± 2.1			193.5 ± 292.3
RT = 41.9	6393.5 ± 10911.3	4.0 ± 2.0			16271.7 ± 25567.0
Peonidin glucuronide (m/z transition 477/301)					
RT = 20.5	231.7 ± 554.2	9.3 ± 6.9			1102.1 ± 3058.5
RT = 30.0	652.5 ± 434.2	4.4 ± 1.9			2961.3 ± 2663.3
RT = 42.4	1707.3 ± 2569.1	4.0 ± 2.0			3897.6 ± 4894.9
Petunidin glucuronide (m/z transition 493/317)					
RT = 26.5	256.3 ± 208.1	4.3 ± 2.9			366.9 ± 543.5
RT = 30.7	255.5 ± 267.2	2.7 ± 1.0			480.1 ± 864.7
RT = 32.9	87.8 ± 81.2	4.0 ± 1.3			185.4 ± 230.1
RT = 35.7	72.4 ± 37.8	4.9 ± 2.3			234.4 ± 308.4

[a] Values are means ± SD, $n = 10$.
[b] Units per mg creatinine.

3.4. Plasma total antioxidant capacity

The total antioxidant capacity of plasma was assessed using the FRAP, ORAC, and TAP assays. The post-intervention values are expressed as a percentage of the baseline (Supplemental Fig. 1). FRAP measures both the hydrophilic and lipophilic ferric reducing antioxidant power in plasma, ORAC uses a hydrophilic peroxy radical generator, and TAP uses a lipophilic methoxy radical generator. FRAP peaked within 30 min, then again at 6 h; ORAC dipped at 1 h, returned to baseline, and later increased at 6 and 8 h; and TAP values showed a progressive rise over time starting at 30 min with no return to baseline after 24 h. Among these three assays, the mean TAP values increased the most after CJC consumption. The different shape of each curve suggests each assay responds in a discrete manner to the absorbed cranberry phenolics. Correlations were observed between ORAC and protocatechuic acid ($r = 0.440$, $P = 0.00$), quercetin ($r = 0.371$, $P = 0.00$), epicatechin ($r = 0.341$, $P = 0.001$), 4-OH-3-methoxy-phenylacetic acid ($r = 0.235$, $P = 0.018$), gentisic acid ($r = 0.210$, $P = 0.045$), and 3,4-OH-phenylacetic acid ($r = 0.216$, $P = 0.03$), as well as between TAP and gentisic acid ($r = 0.285$, $P = 0.01$) and protocatechuic acid ($r = 0.233$, $P = 0.036$). No correlations were observed between FRAP and the concentration of plasma phenolics.

3.5. Resistance of LDL against Cu^{2+}-oxidation

There was a 17.8% increase in the resistance of LDL to Cu^{2+}-induced oxidation at 8 h compared to baseline. This result

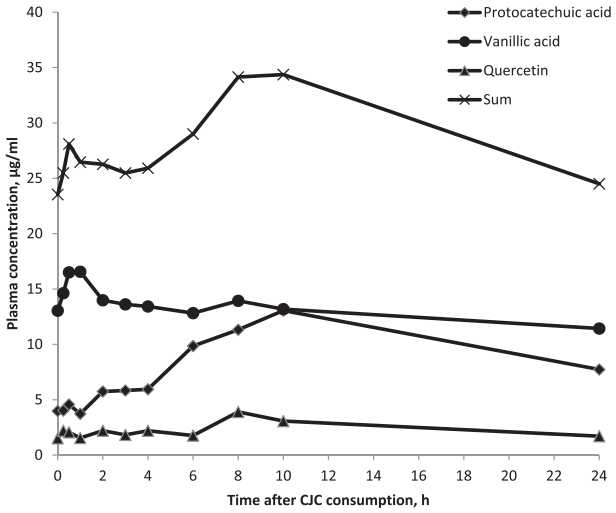

Fig. 1. Plasma concentration of the principle phenolics (vanillic acid, protocatechuic acid, and quercetin), and the sum of all phenolics over time following CJC consumption. Values are means, $n = 10$.

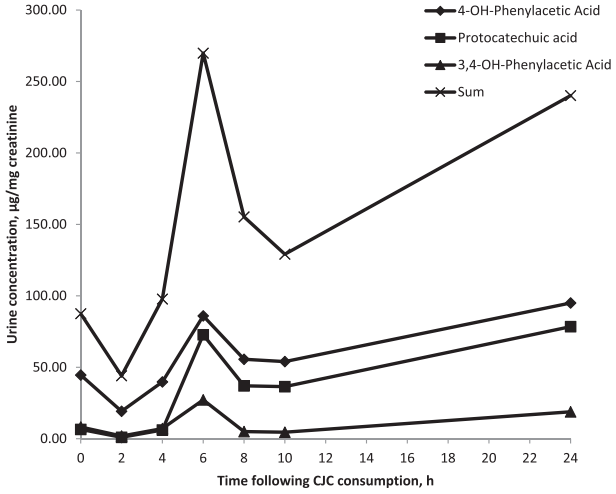

Fig. 2. Urine concentration of the principle phenolic acids (4-OH-phenylacetic acid, protocatechuic acid, and 3,4-OH-phenylacetic acid), and the sum of all phenolics over time following CJC consumption. Values are means, $n = 10$.

appears to parallel the response of the ORAC assay (Supplemental Fig. 2). The resistance of LDL against oxidation tended to be higher at 10 h. LDL resistance was correlated with protocatechuic acid ($r = 0.503$, $P = 0.00$).

4. Discussion

While the bioavailability of phenolic compounds from other fruits has been reported in many studies, only 4 human studies have examined the absorption and excretion of cranberry phenolic acids and flavonoids (Milbury et al., 2010; Ohnishi et al., 2006; Wang, Zuo, Vinson, & Deng, 2012; Zhang & Zuo, 2004). This study is the first to demonstrate that cranberry bioactives, particularly PAC-A2, are bioavailable in healthy older adults over a 24-h period. The marked variation in C_{max} and T_{max} between the different cranberry phenolics suggest they are absorbed and metabolized at different locations within the gastrointestinal (GI) tract. After consumption, flavonoids are subject to extensive metabolism via conjugation mediated by phase II enzymes for rapid excretion in

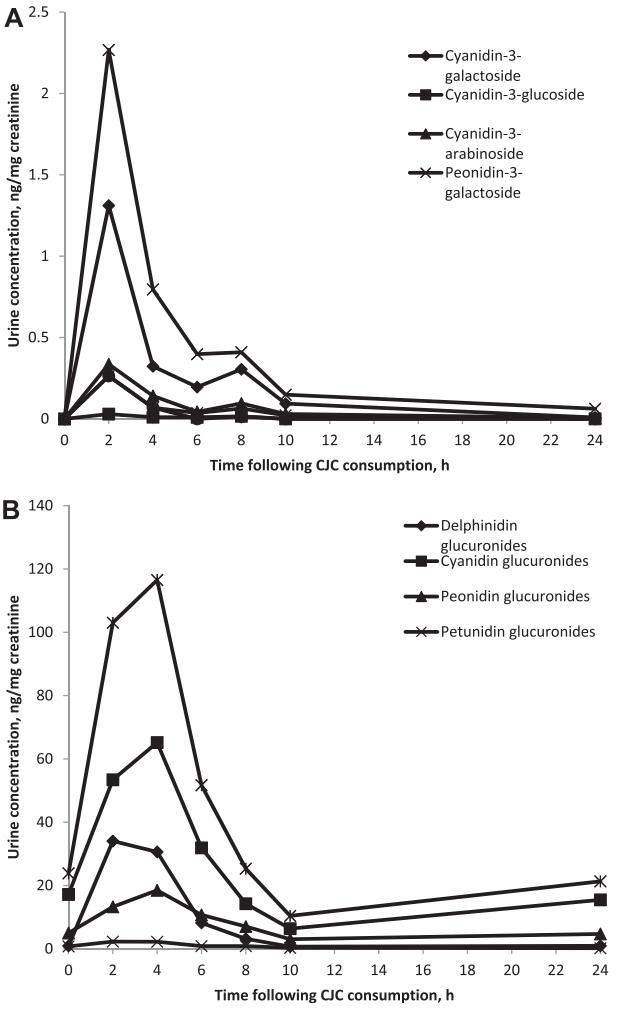

Fig. 3. Urine concentration of the anthocyanins (A), anthocyanin glucosides and their sum (B) over time following CJC consumption. Values are means, $n = 10$.

the small intestine and liver, non-enzymatic degradation in the GI tract, and enzymatic catabolism mediated by gut microflora (Del Rio, Borges, & Crozier, 2010). Using a ^{13}C-tracer cyanidin-3 glucose (500 mg), Czank et al. (2013) found 24 metabolites with concentrations ranging 0.1–42.2 μmol/l in human plasma, reflecting the complexity of flavonoid metabolism.

Consistent with the results of Ohnishi et al. (2006) and Milbury et al. (2010), we detected cranberry anthocyanins in plasma and urine, with peonidin and cyanidin galactosides and arabinosides being the most abundant. Consistent with other reports (Czank et al., 2013; Milbury et al., 2010), we found a marked inter-individual variation in anthocyanin pharmacokinetics. In part, this variation may result from the conversion of cyanidin glycosides to peonidin glycosides via hepatic phase II methylation (Kay, Mazza, & Holub, 2005; Wu, Cao, & Prior, 2002), a reaction that limits the ability to precisely attribute the bioavailability of an individual anthocyanin. The maximum concentration of CJC anthocyanins were typically reached in plasma within 3 h and in urine within 4 h after consumption.

Conjugation of phase II moieties to anthocyanins has been shown in a limited number of studies. Kalt et al. (2008) detected anthocyanidin glucuronides in the brain, eyes, and liver of pigs fed a 2% blueberry diet for 8 wk. Marczylo, Cooke, Brown, Steward, and Gescher (2009) found 19 glucuronidated, sulphated, and methylated cyanidin metabolites in mice gavaged with 500 mg/kg BW cyanidin-3-glucose. Similarly, Kay et al. (2005)

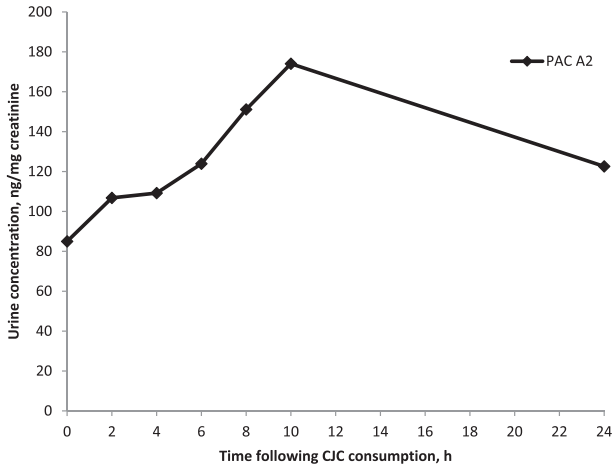

Fig. 4. Urine concentration of proanthocyanidin A2 over time following CJC consumption. Values are means, $n = 10$.

observed cyanidin 3-glycosides in human serum and urine primarily as glucuronide and methyl-glucuronide metabolites after an oral dose of chokeberry extract, though only the glucuronides were found in urine. Czank et al. (2013) detected cyanidin-3-glucoside metabolites in urine, but not in plasma. In contrast, Kay, Mazza, Holub, and Wang et al. (2004) and Wu et al. (2002) found anthocyanidin glucuronides in human plasma but with concentrations significantly lower than anthocyanins. Thus, anthocyanidin glucuronides may be present in plasma following acute intake of CJC but at concentrations lower than the LOQ of our LC–MS/MS method. While we were unable to quantify individual anthocyanidin glucuronides in urine, when expressed as cyanidin-3-glucoside equivalents their concentration was higher than the anthocyanins, suggesting rapid phase II transformation and excretion of anthocyanins after consumption. The pathway for the metabolism of anthocyanidin glucuronides from anthocyanins remains to be fully established. Future studies are warranted to identify other phase II anthocyanin and anthocyanidin metabolites, such as cyanidin and peonidin sulphates.

Cranberries are a rich source of A-type PACs, flavonoids not commonly found in plant foods. Little information is available regarding the pharmacokinetics of A-type PACs though their dimers, trimers, and tetramers have been found to be taken up by Caco-2 cells *in vitro* (Ou, Percival, Zou, Khoo, & Gu, 2012). We provide here the first observation that PAC-A2 can be quantified in the urine of healthy volunteers following an acute dose of CJC. The T_{max} of PAC-A2 at 11 h suggests that most of this compound was absorbed in the lower GI tract after being produced from oligomers and polymers via catabolism by gut microbiota. Thus, urinary PAC-A2 is unlikely reflective of the specific bioavailability of PAC-A2 from CJC intake. While we were able to identify PAC-A2 in plasma, its apparent concentration was below the LOQ of the method. The apparent lack of compliance to the daily intake of CJC in long-term clinical trials has confounded the interpretation of several trials investigating its effect on health outcomes, particularly urinary tract infections. The measurement of PAC-A2 in urine suggests its potential as a biomarker of cranberry intake and compliance. In contrast to the A-type, B-type PACs have been readily detected in human plasma and urine after consumption of grape seed extracts and cocoa (Holt et al., 2002; Sano et al., 2003).

Phenolic acids are abundant in CJC and may contribute to its putative health benefits. Following the consumption of 1.8 L cranberry juice cocktail (27% juice) by one individual, Zhang and Zuo (2004) found benzoic acid to be the most abundant compound in plasma at 45 and 270 min (at 4.40 and 3.11 μg/ml, respectively), but did not analyse urine. Wang et al. (2012) gave 11 healthy young adults 240 ml of cranberry juice containing 2.18 g total phenolics and found benzoic acid in plasma at 30 min (~1.2 μg/ml) and 180 min (~0.98 μg/ml) in the one subject on whom they reported. We found 3 phenolic acids – procatechuic, 4-OH-phenylacetic, and 3,4-OH-phenylacetic acids (in ascending order of the increase from baseline and adjusted for creatinine) – as the most abundant in urine following CJC consumption. However, a direct comparison between the results of Wang et al. (2012) and our study cannot be made due to the differences in methodology for measuring phenolic acids (GC/MS vs. HPLC–ECD), subject age (young vs. old adults), time frame of sample collection (6 vs. 24 h), cranberry juice concentration (100% vs. 54%) and sugar content (25 vs. 0 g), and the reported number of subjects tested for the various analytes (1–11 vs. 10). As marked inter-individual differences are typically found in the pharmacokinetics of flavonoids and phenolic acids, caution is warranted when drawing conclusions about these parameters in a single subject.

Phenolic acids in CJC are not the only source of these compounds in vivo. Phenolic acids are generated in the GI tract via non-specific degradation and microbiota-mediated catabolism. Despite our use of a controlled diet during this acute intervention, a limitation of our study is the absence of a control group where the effect of the meals served on phenolic acid status could be examined. In a human study, Vitaglione et al. (2007) found cyanidin glucoside was degraded to procatechuic acid, whose plasma concentration accounted for 44% of the ingested anthocyanin. Using germ-free rats, Hanske et al. (2013) found human gut microbiota catalysed the deglycosylation of cyanidin glucoside which subsequently led to production of 3,4-dihydroxybenzoic acid and 2,4,6-trihydroxybenzaldehyde via bacterial enzymes and/or spontaneous degradation of cyanidin. The ^{13}C tracer study with cyanidin glucoside by Czank et al. (2013) confirmed the significant contribution of ingested anthocyanins to the phenolic acid pool in systemic circulation and urine, particularly procatechuic acid. Other phenolic acids with a later T_{max}, such as phenylacetic acids and gentisic acid, are likely the products of bacterial degradation of anthocyanins and PACs (Saura-Calixto et al., 2010). These phenolic acids may contribute substantially to the short-term increase in plasma antioxidant capacity observed after the intake of flavonoid-rich foods as well as their putative health benefits (Vitaglione et al., 2007).

The effect of cranberry juices on antioxidant capacity in humans has been previously reported (Duthie et al., 2006; Pedersen et al., (2000); Ruel, Pomerleau, Couture, Lamarche, & Couillard, 2005; Vinson, Bose, Proch, Al Kharrat, & Samman, 2008). In an acute study of CJC, Pedersen et al. (2000) observed an increase in plasma total antioxidant capacity, assessed by the reduction potential of plasma constituents, and attributed the effect to an increase in plasma vitamin C as well as total phenols. Vinson et al. (2008) noted an increase in FRAP following acute consumption of 27% CJC relative to a control beverage. Ruel et al. (2005) reported that 2-wk CJC consumption decreased circulating oxidised LDL and increased plasma antioxidant capacity assessed by the TEAC assay. While our results confirm these findings, this is the first study to correlate changes in individual cranberry metabolites, e.g., protocatechuic acid, with an array of measures of antioxidant capacity over time.

5. Conclusion

In conclusion, we have demonstrated that phenolic acids and flavonoids, in CJC, are bioavailable and increase antioxidant capacity in healthy older adults. We also found that PAC-A2 is detectable in plasma and quantifiable in urine after an acute dose of cranberry juice. The plasma pharmacokinetics of CJC in humans indicate the maximum phenolic concentration, plasma antioxidant activity,

and LDL antioxidant effect occur ~8 h after consumption. The considerable inter-individual variability in the pharmacokinetics of these phytochemicals appears likely due to individual differences in phase II enzyme polymorphisms as well as composition of GI microbiota. This information can help inform the design of future clinical studies on cranberry foods and beverages.

Acknowledgements

Supported by the USDA Agricultural Research Service under Cooperative Agreement No. 58-1950-014 and Ocean Spray. This material is based upon work supported by the U.S. Department of Agriculture, under agreement No. 58-1950-0-014. Any opinions, findings, conclusion, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the U.S. Department of Agriculture.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.07.062.

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