Tissue bioavailability of anthocyanins from whole tart cherry in healthy rats

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ABSTRACT

Our aim was to confirm and identify the presence of tart cherry anthocyanins in several target tissues of healthy rats. Liquid chromatography–mass spectrometry analysis was employed for detection and characterisation of anthocyanin metabolites. It was shown that four native anthocyanins, namely cyanidin 3-glucosylrutinoside, cyanidin 3-rutinoside, cyanidin 3-rutinoside 5-β-$_D$-glucoside, and peonidin 3-rutinoside were differentially distributed among targeted tissues of rats. Bladder and kidney contained more total anthocyanins than all other tissues analysed. It was also revealed that the bioavailability pattern of these native anthocyanins among tissues is varied. The highest concentration of individual anthocyanin cyanidin 3-glucosylrutinoside (2339 picograms/gram of tissue) was detected in bladder, followed by cyanidin 3-rutinoside 5-β-$_D$-glucoside (916 picograms/gram) in the liver of rats. Although the diverse distribution of tart cherry anthocyanins in different rat tissues still requires further explanation, it may provide an evidentiary link between tissue bioavailability and health-enhancing properties of anthocyanins at target sites.

1. Introduction

Interest in the biological effects of anthocyanin compounds has substantially increased in recent years due to evidence of therapeutic effects in animal models (He & Giusti, 2010; Tsuda, 2012) and in humans with anthocyanin-rich foods. Anthocyanins have antioxidant activity and exert anti-inflammatory effects (Heinonen, 2007; Tsuda, 2012). Although the role of anthocyanins in functional foods is less well established as compared to other flavonoids, recent studies reveal their intracellular and molecular effects (Tsuda, 2012; and references cited therein). For example, anthocyanins can alter the activity of peroxisome proliferator-activated receptors (PPARs) that affect energy substrate metabolism and inflammation (Seymour et al., 2008; Seymour et al., 2011). These effects suggest a possible benefit for the phenotypes of metabolic syndrome (Tsuda, 2008) and its pathologic sequelae. In general, studies suggest that anthocyanins are associated with health benefits against chronic disease including cardiovascular disease (Mink et al., 2007) and cancer (McCune, Kubota, Stendell-Hollis, & Thomson, 2010).

Tart cherry (*Prunus cerasus*) fruits and their relevant processed food products are rich sources of dietary anthocyanins, as reported earlier (Kirakosyan, Seymour, Urcuyo Llanes, Kaufman, & Bolling, 2009; Ou, Bosak, Brickner, Iezzoni, & Seymour, 2012). We previously reported that tart cherry phytochemicals have *in vitro* synergistic effects on antioxidant capacity (Kirakosyan et al., 2010). In various rat models, we reported that tart cherry fruit intake reduces blood lipids and inflammation and alters phenotypes of metabolic syndrome (Seymour et al., 2008; Seymour et al., 2009). Tart cherry also affects activity of PPARs in various tissues and various gene transcripts related to inflammation (Matchynski et al., 2013; Seymour et al., 2009; Zhou, Nair, & Claycombe, 2012). Phytochemicals in tart cherry fruits may be directly cytoprotective by altering gene transcription and translation, thereby improving resistance to oxidative and inflammatory stressors.

The health-enhancing properties of anthocyanins cannot be fully understood without analysis of their tissue distribution. This information affects hypotheses of the molecular mechanisms of bioactive compounds at target sites and how they vary among tissues. We previously showed in humans that intake of individually quick frozen (IQF) whole tart cherries yielded plasma and urine anthocyanins and their related metabolites (Uhley et al., 2009). Because anthocyanins have perceived low tissue bioavailability, limited studies have measured tissue levels following intake of anthocyanin-rich foods (Felgines et al., 2009;

Passamonti, Vrhovsek, Mattivi, & Vanzo, 2005; Talavéra et al., 2005). Furthermore, tissue bioavailability of tart cherry anthocyanins is unknown. This knowledge gap should be filled to bridge what is known of the phenotypic and transcriptional effects of tart cherry-enriched diets in diverse experimental models.

In the present study, our working hypothesis is that intake of tart cherry is associated with anthocyanin appearance in several tissues of healthy rats (heart, kidney, liver, brain, bladder, and adipose tissue), and that sugar conjugates of native anthocyanins and/or their metabolites are differentially distributed among tissues.

2. Materials and methods

2.1. Animals and diets

Wistar rats (Male, N = 18, six weeks old) were acquired from Harlan Corp. (Indianapolis, IN, USA) and were housed two animals per cage in temperature-controlled rooms (22 °C) with a 12 h light:12 h dark photoperiod regime. Animals were fed each day ($\sim$4 pm) a semi-purified control diet AIN-76A (Dyets, Inc.) for seven days, for washout from their previous standard "chow" diet and to acclimate to powder-based feeding. For the experimental diets, AIN-76a was altered by adding 1% or 10% w:w IQF tart cherry powder (harvested in northern Michigan and prepared by VanDrunen Farms, Momence, IL). Tart cherry powder nutrient analysis was conducted by VanDrunen Farms and its subsidiary Futureceuticals (Momence, IL). Further phytochemical analysis was conducted by our group using liquid chromatography–mass spectrometry (LC/MS). Diets were mixed weekly, vacuum-sealed, and stored at 4 °C. This approach guarantees anthocyanin stability, as shown in our previous studies. Nine rats were assigned to 1% tart cherry diet (1-TC) and nine to 10% tart cherry diet (10-TC). Rats were provided 20 g of diet powder per animal per day; this was approximately 10% below *ad libitum* intake to ensure complete consumption and equal food intake among all rats in the study. Water was provided *ad libitum*. This protocol was approved by the University of Michigan's University Committee on the Use and Care of Animals.

2.2. Tissue collection and extraction

After three weeks of feeding, rats were fasted for ⩾18 h and sacrificed by 4% isoflurane inhalation followed by decapitation and exsanguinations (with a rat's body weight of 295 ± 10 g). Tissues were harvested and weighed, including heart (ventricles), brain (cortex), liver, kidneys, bladder, and retroperitoneal fat. Tissues were minced, washed with PBS, and snap-frozen in liquid nitrogen, and stored at −80 °C until further analysis. The time from tissue harvest to freezing was less than 5 min.

2.3. Sample preparation

Both brain and bladder samples were merged from all animals in a group during extraction to have enough tissue for extraction. All other tissues were sampled from individual animals. Frozen tissues were crushed using a tissue homogenizer (Polytron Brinkmann) in methanol containing 5% formic acid (6 ml/g of tissue), and centrifuged 10,000×g for 10 min. Supernatants were collected, and pellets were re-extracted with 5% formic acid in methanol (4 ml/g of tissue). The two methanolic supernatants were combined, and evaporated to dryness using a Labconco rotary evaporator at room temperature under reduced pressure.

To determine anthocyanin recovery following extraction, cyanidin 3-glucoside was used as an internal standard. Dried tissue extracts were dissolved in 500 µl of 5% formic acid aqueous solution. After centrifugation (10,000×g, 10 min), an aliquot (100 µl) was immediately analysed by HPLC for internal standard recovery. The internal standard recovery was 28%, 40%, 33%, 35%, 60% and 34% in the heart, kidney, liver, brain, retroperitoneal fat, and bladder, respectively. The % recovery values were later used for data correction.

Final preparation of extracts involved evaporating the methanolic solution from the extracts, diluting the residue in acidified water (5% formic acid), and extracting anthocyanins by solid-phase adsorption onto a hydrophobic matrix (Sep-Pak C18, 0.35 g, Waters, Milford, MA). Anthocyanins were eluted with 5% formic acid in 80% methanol, then evaporated to dryness. 400 µl of 0.5% FA aqueous solution was added to the sample, vortexed 10 min, and centrifuged at 15,000×g for 20 min. The supernatant (100 µl) was immediately subjected to LC/MS/MS analysis.

2.4. LC–MS/MS analysis

Samples (in triplicate) were injected into a Dionex UHPLC UltiMate 3000 liquid chromatograph interfaced to an amaZon SL ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). A C18 Kinetex column (2.6 micron particle size, 150 mm × 4.6 mm, Phenomenex, CA, USA) or a similar phase Poroshell 120 EC18 column (2.7 micron particle size, 150 mm × 4.6 mm, Agilent, DE, USA.) were used for chromatographic separation. The targeted ions retention times were different for each columns, and the mass spectrometry method was adjusted for MRM, accordingly. The initial mobile phase condition started from 90% A (0.1% formic acid in water) and 10% B (0.1% formic acid in acetonitrile), then changed to 20% B in 5 min. After staying constant for 5 min at 20% B, the gradient was changed linearly to 100% acetonitrile in 4 min and remained there for 3 min. The total run was 18 min with 5 min initial condition equilibration time. The flow rate was maintained at 0.4 ml/min. The mass spectrometer electrospray capillary voltage was maintained at 4.5 kV, and the drying temperature at 250 °C with drying gas flow rate of 10 l/min. Nebulizer pressure was 40 psi. Nitrogen was used as both nebulising and drying gas, and helium was used as collision gas at 60 psi. The mass-to-charge ratio 757, 609 and 595 were selected as precursor ions in enhanced resolution positive-ion MRM mode. Isolation width 4 and fragmentation amplitude 1 were used to quantify the cyanidin-specific fragment ion 287 from transition 757 → 287 and 595 → 287. The fragment ion 301 was used to quantify the peonidin specific residue from transition 609 → 301. The Smart Parameter Setting (SPS) was used to automatically optimise the trap drive level for precursor ions. The instrument was externally calibrated with the ESI TuneMix (Agilent, DE, USA).

2.5. Statistical methods

Results are provided as descriptive statistics, namely, mean ± SD.

3. Results

We previously reported that the major anthocyanin compound in 'Montmorency' cultivar of tart cherry is cyanidin 3-glucosylrutinoside, and that cyanidin species in 'Montmorency' cherries are about 93% of total anthocyanins present (Kirakosyan et al., 2009). In the current study, tart cherry IQF powder was analysed to determine the levels of individual anthocyanins: cyanidin 3-glucoside; cyanidin 3-rutinoside; cyanidin 3-glucosylrutinoside; cyanidin 3-sophoroside; peonidin 3-glucoside; and pelargonidin. The results in Table 1 show that the major anthocyanin compound in IQF tart

Table 1
Individual anthocyanin constituents in 'Montmorency' tart cherry powder.

Anthocyanins	µg/g dry weight	% of individual anthocyanins
Cyanidin 3-sophoroside	2.8 ± 0.5	0.4
Cyanidin 3-glucosylrutinoside	325.9 ± 57.2	42
Cyanidin 3-rutinoside 5-β-D-glucoside	120.2 ± 20.5	15.5
Cyanidin 3-glucoside	3.1 ± 0.7	0.4
Cyanidin 3-rutinoside	274.2 ± 43.2	35.4
Peonidin 3-glucoside	7.4 ± 1.1	0.9
Peonidin 3-rutinoside	40.4 ± 7.7	5.2
Pelargonidin	1.2 ± 0.4	0.1

cherry powder is cyanidin 3-glucosylrutinoside, followed by cyanidin 3-rutinoside.

These results largely agree with our previous data with cherry powder (Kirakosyan et al., 2009). However, we also detected and identified two other anthocyanin compounds, namely, cyanidin 3-rutinoside 5-β-D-glucoside and peonidin 3-rutinoside (Figs. 1 and 2). Based on our quantification, the cherry powder contained approximately 0.775 mg/g dry weight of total anthocyanins. This value was used to calculate total anthocyanins consumed in the daily diet (∼0.155 mg/day for 1% cherry diet, 1.55 mg/day for 10% cherry diet).

Our main goal for the present work was to determine and compare the bioavailability data for tart cherry anthocyanins in targeted tissues of rats. Results in Table 2 show that four different native (parent) anthocyanin species were detected and quantified in several tissues.

Bladder and kidney contained more total anthocyanins than all other tissues analysed. The highest concentration of cyanidin 3-glucosylrutinoside anthocyanin is 2339 picograms per gram tissue in 10-TC rat bladder, which is almost ten times higher than the mean amount in other tissues. The second highest concentration of individual anthocyanin (916 picograms/gram) was cyanidin 3-rutinoside 5-β-D-glucoside in the liver of 10-TC rats. In liver tissue, three native anthocyanins except cyanidin 3-glucosylrutinoside were detected. In heart tissues of both rat groups, all four anthocyanins were detected and quantified. These four anthocyanins were present also in 1-TC bladder tissue. In brain tissue samples, only cyanidin 3-rutinoside 5-β-D-glucoside and peonidin 3-rutinoside, were detected and quantified. No anthocyanins were detected in retroperitoneal fat.

It is noteworthy that the bioavailability pattern of these four anthocyanins among tissues is varied. For example, the occurrence of cyanidin 3-rutinoside is high in organs of excretion/elimination – the kidney and bladder. Cyanidin 3-glucosylrutinoside is high in bladder, whereas cyanidin 3-rutinoside 5-β-D-glucoside and
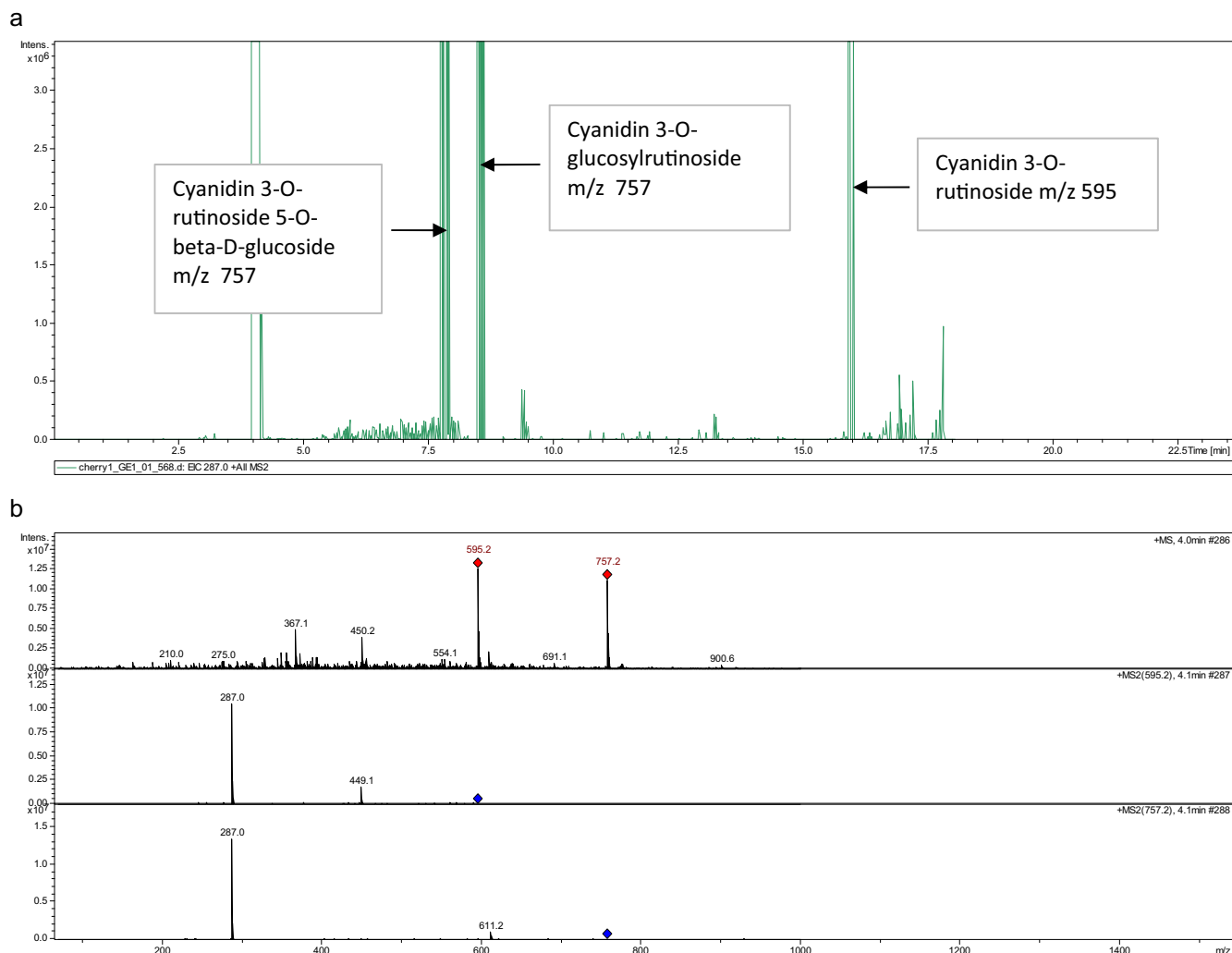

Fig. 1. (a) Extracted ion chromatograms (either in IQF powder or target tissue) of all eluted ions which have characteristic m/z 287, typical for the cyanidin moiety. (b) Fragmentation of cyanidin 3-rutinoside 5-β-D-glucoside.
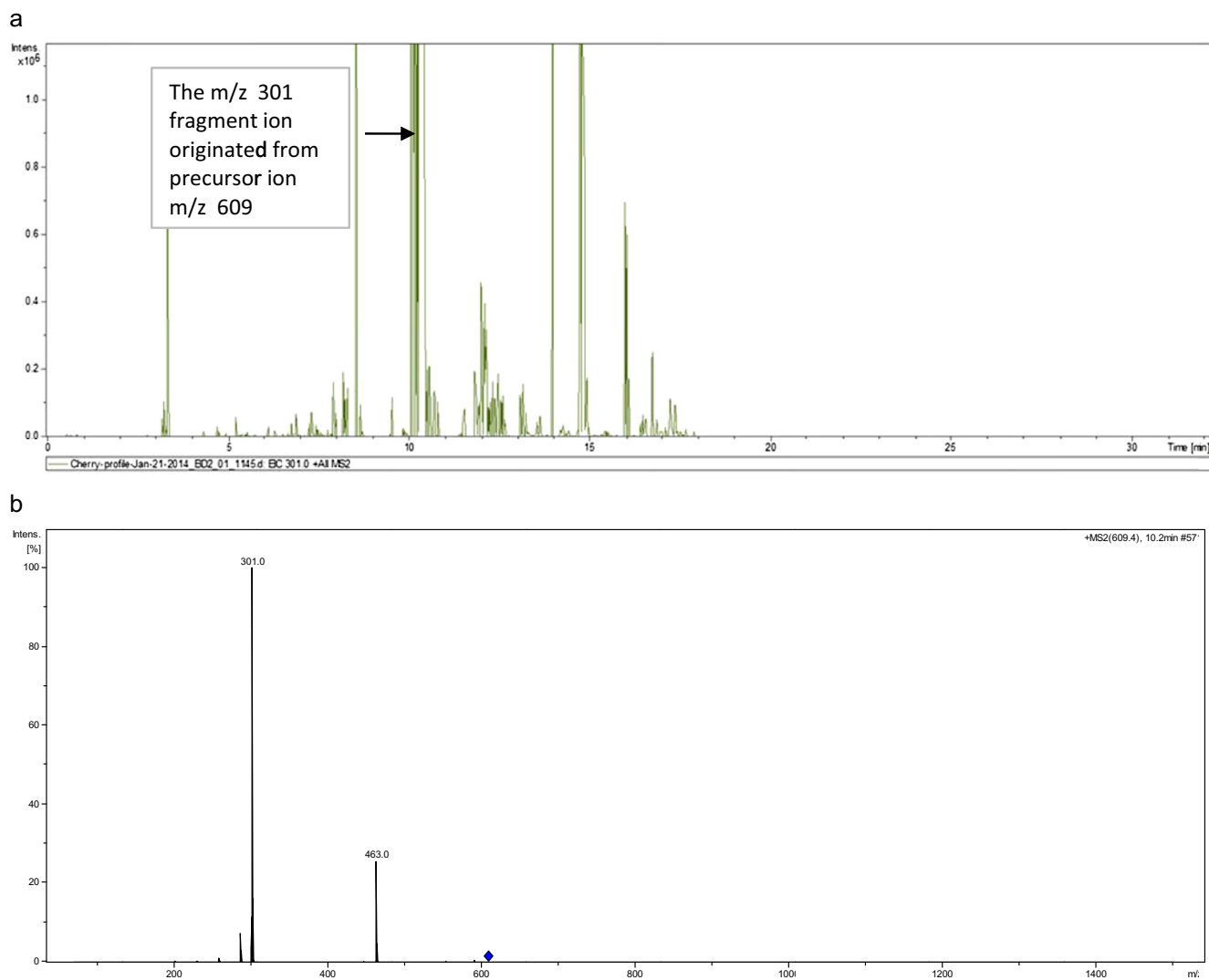
a

b

Fig. 2. (a) Extracted ion chromatograms (either in IQF powder or target tissue) of all eluted ions which have characteristic m/z 301, typical for peonidin moiety. (b) Fragment ions of m/z 609 (supposedly peonidin 3-rutinoside).

Table 2
Anthocyanin content (picograms per gram tissue) in tissues of rats fed with tart cherry-supplemented diets.

Tissue (cherry dose group)	Cyanidin 3-rutinoside	Cyanidin 3-glucosyl rutinoside	Cyanidin 3-rutinoside 5-β-D-glucoside	Peonidin 3-rutinoside	Total anthocyanin content
Bladder					
(1-TC group)	275.8 ± 17.2	430.9 ± 27.2	171 ± 11.8	4.7 ± 0.5	882.4 ± 56.8
(10-TC group)	nd	2339 ± 122.1	343.9 ± 19.2	8.7 ± 0.7	2691.6 ± 142
Kidney					
(1-TC group)	113.2 ± 13.6	nd	733.3 ± 32.3	58.4 ± 4.3	904.9 ± 50.2
(10-TC group)	275 ± 18.4	nd	483.7 ± 21.8	35 ± 4.4	793.7 ± 44.6
Liver					
(1-TC group)	nd	nd	313.1 ± 17.9	20.4 ± 1.2	333.5 ± 19.1
(10-TC group)	146.4 ± 10.7	nd	916.9 ± 46.2	71.4 ± 5.3	1134.7 ± 62.2
Heart					
(1-TC group)	236.4 ± 16.1	47.4 ± 5.9	119 ± 8.2	30.2 ± 4.4	433 ± 34.6
(10-TC group)	99.5 ± 8.4	78.2 ± 5.3	180.5 ± 11.8	9.4 ± 0.9	367.6 ± 26.4
Brain					
(1-TC group)	nd	nd	147.5 ± 9.3	5.1 ± 0.4	152.6 ± 9.7
(10-TC group)	nd	nd	317.5 ± 15.6	20.3 ± 1.8	337.8 ± 17.4
Retroperitoneal fat					
(1-TC group)	nd	nd	nd	nd	–
(10-TC group)	nd	nd	nd	nd	–

peonidin 3-rutinoside were high in liver. Organs not involved in metabolism and elimination (brain, heart, and retroperitoneal fat) showed an expected reduction in tissue anthocyanin content. Interestingly, these native anthocyanins were absent in abdominal retroperitoneal fat, a proposed target of anthocyanin bioactivity (Prior et al., 2010; Seymour et al., 2011; Tsuda, Horio, Uchida, Aoki, & Osawa, 2003) although the presence of other metabolised anthocyanins in this experiment cannot be excluded.

The dose-dependency of the tissue bioavailability revealed both expected and unexpected results. Liver and brain had expectedly higher levels of total anthocyanin (sum of the detected anthocyanins) in 10-TC versus 1-TC rats. In heart tissue, cyanidin 3-rutinoside and peonidin 3-rutinoside were higher in 1-TC than 10-TC rats, as were total anthocyanins. The same situation was observed in kidney tissue; cyanidin 3-rutinoside 5-β-D-glucoside and peonidin 3-rutinoside were higher in 1-TC than 10-TC rats, as were total anthocyanins.

4. Discussion

Bioavailability is defined in various ways. With oral intake of nutrients, bioavailability generally refers to the quantity or fraction of the ingested dose that is absorbed. Specifically, bioavailability is the proportion of the nutrient that is digested, absorbed and metabolised through normal pathways (McGhie & Walton, 2007). Consequently, it is not only important to know how much of a nutrient is present in a food or dietary supplement, but it is also critical to know how much of that nutrient is bioavailable (Azzini et al., 2010). The same principles apply to the non-nutrient phytochemicals found in plant-based foods and beverages.

Anthocyanin tissue uptake may depend on their chemical structure, as influenced by the nature of the attached sugar moiety, and also by the structure of the aglycone. As reported, anthocyanins have low bioavailability; the majority of studies show a recovery of <1% of the ingested anthocyanin dose (He & Giusti, 2010; Manach, Williamson, Morand, Scalbert, & Remesy, 2005; McGhie & Walton, 2007). Recent bioavailability studies have demonstrated that anthocyanins are quickly absorbed from the stomach and in the small intestine, appearing in the bloodstream within few minutes (about 5–20 min) after consumption, and reaching maximum blood levels after half to two hours (Azzini et al., 2010; McGhie & Walton, 2007; Pojer, Mattivi, Johnson, & Stockley, 2013; Vanzo, Vrhovsek, Tramer, Mattivi, & Passamonti, 2011; Vanzo et al., 2013). Anthocyanins are exposed to different pH environments during digestion which affects the stability of many anthocyanin species. Anthocyanins are also subjected to degradation and metabolism by intestinal enzymes such as the glycosidases, esterases, oxidases, and hydrolases (Sousa et al., 2008). They appear in plasma and urine in their parental form or as methylated, glucuronidated or sulphated compounds (McGhie & Walton, 2007). Some ingested anthocyanins are absorbed intact and circulated in the plasma and passed into urine without undergoing metabolic changes (Pojer et al., 2013; and references cited therein).

We previously studied the pharmacokinetics of whole tart cherry in humans (Uhley et al., 2009). Some anthocyanin metabolites may be present in plasma even after fasting the rats for 18 h. However, our clinical study showed that plasma cyanidin 3-glucosylrutinoside and cyanidin 3-rutinoside returned to baseline 12 h after whole tart cherry intake (Uhley et al., 2009). As such, any residual blood in tissue is unlikely to contribute to our results because of fasting, exsanguination, tissue sectioning/mincing, washing, and extraction procedures.

The diverse distribution of tart cherry anthocyanins in different tissues requires some explanation as well. The parent phytochemicals contain functional groups (e.g., a hydroxyl group) that often undergo conjugation reactions with endogenous compounds to yield more polar and water-soluble compounds. The latter are usually ideal substrates for active transport out of the cell and eventual excretion from the body. The sugar conjugates of anthocyanins may also have an effect on the bioavailability of bioactive phytochemicals. In our experiments, we observed exclusively anthocyanins, whereas none of the known anthocyanidins were detected. It is noteworthy that the brain contains no cyanidin 3-rutinoside, but peonidin 3-rutinoside is present. In addition, the more polar and more complex congener of cyanidin 3-rutinoside, namely, cyanidin 3-rutinoside 5-glucoside was detected. Also, the most abundant compound, cyanidin 3-glucosyl rutinoside, was absent in excretory organs (liver, kidney), but was found in the bladder and in the heart. One can interpret these data as a mechanism for elimination due to glomerular filtration (without renal excretion) and adsorption of a largely glycosylated compound on the surface of the bladder epithelium and on the cardiac vessels.

Finally, some of the proposed transporters for anthocyanins may have diverse structural preferences or affinities, and these transporters may vary by tissue type (Schinkel & Jonker, 2003; Vanzo et al., 2008).

Several studies have investigated anthocyanin uptake in the liver. A study with bilberry anthocyanins in mice estimated that ~50% of tissue anthocyanins following intake were located in the liver (Sakakibara et al., 2009). These observations suggest that the liver may be the main target for anthocyanin accumulation. In our study, we observed only high levels of cyanidin 3-rutinoside 5-β-D-glucoside anthocyanin in liver tissue, particularly at the 10-TC dose.

The kidneys are also a target for accumulation of anthocyanins. For example, grape anthocyanins appear in the kidneys of rats 10 min after gastric administration, where the total concentration was almost two times higher than in the systemic circulation (Vanzo et al., 2008). Anthocyanin-rich diets have conferred protection to kidneys from experimental hypertension (Elks et al., 2011) and diabetes (Kang, Lim, Lee, Yeo, & Kang, 2013). In kidneys, anthocyanins may undergo active metabolism depending on the level of native anthocyanins. This may explain the discrepancy of native anthocyanin concentration in kidney, where native anthocyanin concentration in 1-TC rats appears higher than in 10-TC rat kidney.

Another important site of anthocyanin distribution and availability is brain tissue (Andres-Lacueva et al., 2005; Rendeiro, Guerreiro, Williams, & Spencer, 2012). Recently, we showed that intake of 1% tart cherry diet (same powder as employed here) significantly reduced stroke-related phenotypes in rats. Tart cherry intake also reduced brain NFκB activity and related pro-inflammatory transcripts (Seymour et al., 2013). The current results confirm that tart cherry anthocyanins cross the blood–brain barrier. Studies with whole blueberry show brain bioavailability of blueberry anthocyanins and neuroprotection from normal ageing (Andres-Lacueva et al., 2005), Parkinson's disease (Strathearn et al., 2014), and toxic insult (Poulose, Bielinski, Carrihill-Knoll, Rabin, & Shukitt-Hale, 2014).

The detection of native anthocyanins in brain, heart, and other tissues may also correlate with the effects of anthocyanin-rich diets on transcription factors and genes/proteins related to antioxidant and anti-inflammatory defence, as observed in other studies using tart cherry, grape, blueberry and bilberries (Mauray et al., 2012; Mykkänen et al., 2012; Seymour et al., 2009; Seymour et al., 2011; Seymour et al., 2013).

While the current work focuses on anthocyanin tissue bioavailability, results may be impacted by the complex polypharmacy of bioavailable tart cherry phytochemicals and their varied sugar moieties that affect absorption, distribution, metabolism, and excretion. The mechanisms of anthocyanin cellular transport are postulated to include bilitranslocase and the ATP-binding cassette

(ABC) family (Schinkel & Jonker, 2003; Vanzo et al., 2008). Since it is not clear from the current preliminary study how variation in anthocyanin accumulation among tissues is affected by the varied tissue expression of these transporters, this topic deserves further study. Finally, the extent to which some bioavailable metabolites may contribute to beneficial health effects of anthocyanins is largely unknown and needs further investigation.

Acknowledgements

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