Human anthocyanin bioavailability: effect of intake duration and dosing

Article *in* Food & Function · November 2017

DOI: 10.1039/C7FO01074E

5 authors, including:

Jane E. Mcdonald
Agriculture and Agri-Food Canada
24 PUBLICATIONS **1,698** CITATIONS

SEE PROFILE

Melinda Vinqvist-Tymchuk
Agriculture and Agri-Food Canada
15 PUBLICATIONS **916** CITATIONS

SEE PROFILE

Sherry A E Fillmore
Agriculture and Agri-Food Canada
119 PUBLICATIONS **1,487** CITATIONS

SEE PROFILE

Food & Function

PAPER

View Article Online
View Journal | View Issue

Cite this: *Food Funct.*, 2017, **8**, 4563

Human anthocyanin bioavailability: effect of intake duration and dosing

Wilhelmina Kalt, *[a] Jane E. McDonald,[a] Melinda R. Vinqvist-Tymchuk,[a] Yan Liu[b] and Sherry A. E. Fillmore[a]

Received 18th July 2017,
Accepted 10th October 2017

DOI: 10.1039/c7fo01074e

rsc.li/food-function

While *in vitro* and animal evidence supports a role for anthocyanins in human health, future opportunities in berry health benefits will rest upon evidence from clinical intervention trials. Because little is known about the behaviour of anthocyanins during long term intake in humans, several clinical design factors were examined. Urine from volunteers (n = 17) who consumed blueberry juice daily was analysed using LC-MS/MS for predicted flavonoid-based products of anthocyanins in relation to a 5-day anthocyanin-free run-in, 28 days of blueberry juice intake, a 7-day washout and two dosing regimens. Total and parent anthocyanin content in urine varied 10-fold among the 17 participants. A high 24–0 h total anthocyanin excretion was associated with high anthocyanin retention (*i.e.* 0 h, before blueberry juice intake). Total anthocyanin excretion was not different before and after up to 7 days of washout indicative of a slow release of anthocyanins. Urinary excretion of anthocyanins declined during the 36-day study. The 24–0 h excretion was greater for total anthocyanins but not for parent anthocyanins when daily blueberry juice was taken all at once rather than as $\frac{1}{3}$ doses taken thrice daily. However parent anthocyanins were retained better (higher 0 h) with 1× dosing. These findings could aid in the design of clinical research on anthocyanins and health.

1. Introduction

Anthocyanin (Anc) pigments are associated with human health benefits based on a growing body of evidence (for review see ref. 1). Anc compounds are most abundant in berries that have red, purple or blue coloration. Blueberries (*Vaccinium* sp.) are one of the richest common food sources of Anc.[2] Compelling evidence of Anc health benefits comes from the epidemiological association between Anc intake and protection against diabetes[3] and in the promotion of cardiovascular[4–6] and neurological health.[7] These associations are observed in highly diverse, well-nourished western populations where Anc containing foods are widely available.

Although epidemiological evidence supports Anc health benefits, it has been difficult to integrate evidence obtained from *in vitro*, *in vivo* and clinical research due in part to the poor *in vivo* bioavailability of food forms of Anc *i.e.* parent Anc. Parent Anc levels *in vivo* are substantially lower than levels used *in vitro*.[1] The low level of parent Anc *in vivo* is due to fission of the C ring of the Anc flavonoid structure. Further metabolism after ring fission leads to an abundance of phenolic acids and aldehydes that are thought to contribute to the health benefits of ingested Anc.[8–10]

In a recent clinical study that tracked flavonoid-based metabolites of Anc during long term blueberry juice intake a pool of >350 parent and Anc metabolites was detected with MS/MS.[11,12] Of this total, approximately 50 Anc-based compounds made up about 80% of the total daily nanomoles excreted in urine.[12] The study showed that flavonoid-based products are well-retained *in vivo* and remain abundant in urine due to their enterohepatic circulation.[11] Enterohepatic circulation is characterized by modified pharmacokinetics involving delayed clearance, multiple peaks in excretion, a longer half-life and increased plasma exposure.[13]

Anc metabolites were present in a 20-fold excess compared to parent Anc and when counted, raised bioavailability estimates to about 1.0% of the dose. In contrast ^{13}C-labelled phenolic moieties arising from ^{13}C cyanidin 3-glucoside were in about a 50-fold excess to cyanidin 3-glucoside in urine.[14]

One report from the same study characterized urinary Anc on the first day of the study[11] while a second report characterized the pool of 371 Anc products for all collection dates combined.[12] The present report describes the behaviour of urinary Anc with respect to design factors, namely dosing regimen, intake duration as well as Anc-free run-in and washout, and inter-individual variation over the 36 day study. The objective of the study was to characterize the impact of study design factors in human research using berry Anc.

[a]*Agriculture and Agri-Food Canada, Kentville Research and Development Centre, 32 Main St, Kentville, NS, B4N 1J5, Canada. E-mail: kalt.willy@gmail.com; Fax: +1 (902) 365-8455; Tel: +1 (902) 365-8561*
[b]*Institute of Special Economic Animal and Plant Sciences, Chinese AAS. No.4899 Juye Street, Changchun, 130112 China*

2. Materials and methods

2.1. Recruitment and dietary compliance

The study complied with all institutional and national guidelines, as per the Canadian Research Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2 2014). The protocol was approved by Agriculture and Agri-Food Canada Human Research Ethics Committee and the study was registered at clinicaltrials.gov (NCT01789359). After obtaining written informed consent, seventeen healthy volunteers (age 24–60, 13 women and 4 men) were enrolled in the randomized study. Using self-reporting questionnaires throughout the study, volunteers were encouraged to report their intake of non-prescribed Acn-foods or their deviations from the prescribed blueberry juice (BJ) intake.

2.2. Study design

No Acn-containing foods were consumed by the 17 volunteers for 5-days prior to the start of the study. Then for 28 days, 250 mL commercially-available single-strength wild blueberry juice (Van Dyks Health Juice Products Limited, NS Canada) containing 216 mg (448 μmol) cyanidin 3-glucoside equivalents was consumed daily. Wild blueberries (*Vaccinium angustifolium* Aiton) contain a particularly complex profile of ±30 parent Anc, however they lack pelargonidin-based Anc.[15]

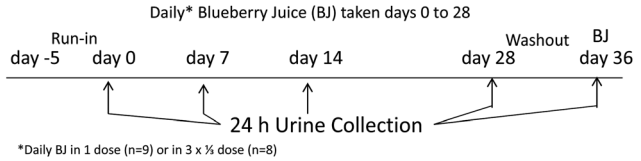

Fig. 1 Schematic outline of anthocyanin bioavailability clinical trial with 17 volunteers.

Assignment of volunteers to the two dosing regimens was balanced based on their reported intake of Anc in a pre-trial questionnaire.[11] In one group nine individuals took the 250 mL volume of BJ at one time (denoted 1×), before 10:00 in the morning. In the second group eight individuals took $\frac{1}{3}$ of this dose (83 mL) thrice daily (denoted $3^{*}\frac{1}{3}\times$). The last 83 mL dose was taken no later than 17:00 in the afternoon. A 7-day washout took place between day 29 and 35 and on the 36th day a final BJ dose was taken (Fig. 1).

Volunteers carried out 24 h urine collections on days 0, 7, 14, 28 and on day 36, after washout. This protocol yielded 662 individual urine samples with an average of eight voids per day per person. Methods employed for urine sample collection and storage have been described by Kalt *et al.*[11]

2.3. Urine sample preparation and HPLC-ESI-MS/MS analysis

Solid phase extraction of urine was carried out using Oasis HLB (30 μm) 2 mg cartridges (Waters, Ltd) exactly as described by Kalt *et al.*[11] HPLC-ESI-MS/MS analysis of urine samples after solid phase extraction was carried exactly as described by Kalt *et al.*[11] using multiple reaction monitoring to detect 18 parent Anc and 41 predicted Anc metabolites (Table 1). A sample chromatogram from this study is shown in ref. 12. The 18 parent Anc were identified in urine by their MS/MS and a retention time (RT) that matched the pure Anc standard. The 41 MS/MS transitions for predicted flavonoid-based C6–C3–C6 metabolites from parent Anc were detected by their parent mass, their major aglycone mass and their RT. Except for the 18 parent Anc each MS/MS × RT was designated as an Anc metabolite (Table 1).

2.4. Statistical analysis

MS/MS signal calibration and handling of calibrated data was as described by Kalt *et al.*[11] MS/MS signals were integrated using MultiQuant software (AB-SCIEX, Toronto, ON) and

Table 1 Summary of mass transitions (MRM) scanned. Among the 60 MRM were 18 parent anthocyanins (in bold) and 41 predicted anthocyanin metabolites. A single MRM for phospholipid was also monitored. Glucuronide, gluc

Parent	Fragment	Cyanidin Total/Fragment *m*/*z*	Delphinidin	Malvidin	Pelargonidin	Peonidin	Petunidin
Pure standard	**Glu**	**448.9/287.1**	**465.0/303.1**	**493.3/331.2**	**433.1/271.0**	**463.2/301.1**	**479.0/317.1**
Pure standard	**Gal**	**449.0/287.0**	**464.5/303.3**	**493.0/330.9**		**463.2/301.0**	**478.9/317.1**
Pure standard	**Ara**	**419.0/287.1**	**435.0/303.0**	**463.2/331.0**		**433.0/301.1**	**448.8/317.1**
Pure standard	**Di-glu**	**611.1/449.1**		**655.2/331.1**			
Gluc	Glu/Gal	625.0/287.0	641.0/303.0	669.0/331.0	609.0/271.0	639.1/301.1	655.1/317.1
Methyl gluc	Glu/Gal	639.0/301.0	655.0/317.0	683.0/345.0	623.0/285.0	653.0/315.0	669.1/331.1
Methyl	Glu/Gal	463.0/301.0	479.0/317.0	507.0/345.0	447.0/285.0	447.0/315.0	493.1/331.1
Sulphate	Glu/Gal	529.0/286.0	545.0/302.0	573.0/330.0	513.0/270.0	543.0/300.0	559.1/316.1
Methyl sulphate	Glu/Gal						
	Aglycone	287.0/137.0	301.0/283.0	331.0/204.0	271.0/271.0	301.0/284.0	317.1/317.1
Gluc	Aglycone	463.0/287.0	479.0/303.0	507.0/331.0	447.0/271.0	447.1/301.1	493.1/317.1
Methyl gluc	Aglycone	477.0/301.0	493.0/317.0	521.0/345.0	461.0/285.0	491.0/315.0	507.1/331.1
Methyl	Aglycone						
Sulphate	Aglycone						
Methyl sulphate	Aglycone						
Di-glucur	Aglycone						
Chalcone							
Phospholipid	184/184						

manually reviewed for consistency. Integrated signals with S/N > 10 were further analysed using Genstat (VSN International).[16] Among 662 urine samples 371 MS/MS × RT were detected, including 17 of 18 parent Anc. The 371 MS/MS × RT were sought in all urine samples and if not present (S/N > 10) a 0 value was recorded. The data set of mean nanomoles for 371 Anc across 82 combinations of 17 volunteers in one of two dosing regimens for five 24 h collection dates yielded a data set of over 35 000 lines. Data for each Anc was fitted to an exponential curve to predict the mean nanomoles excreted. The 0 h value was the Anc content in the single void preceding BJ intake. The 24–0 h value was the cumulative nanomoles of Anc excreted in all voids for the 24 h after BJ intake.

Box plots (Fig. 2) and scatter plots (Fig. 3) were prepared using calibrated data before ANOVA. ANOVA was used to examine effects of duration (Fig. 4) and dosing (Fig. 5 and Table 2). Repeated measures analysis was used to examine responses related to intake duration (days 7, 14 and 28) and dosing (1× *versus* 3*$\frac{1}{3}$× daily) (Fig. 5).

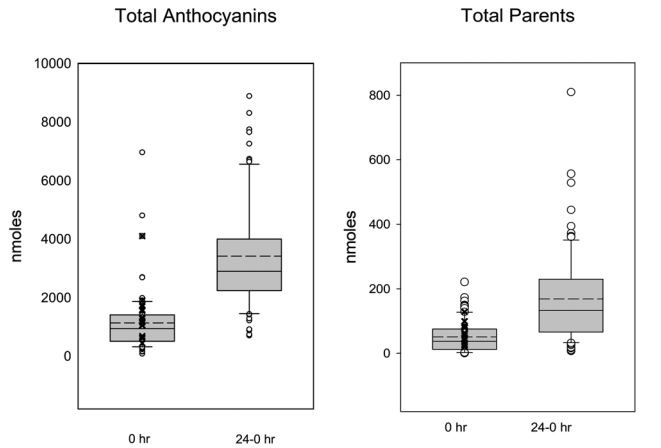

Fig. 2 Box plots with 82 values each for 17 volunteers and five 24-hour urine collections and three missing values. The dashed line in the box shows the mean; the solid line shows the median. In 0 h boxplots, the day 0 points (*i.e.* after run-in, before blueberry juice) are shown with an ×.

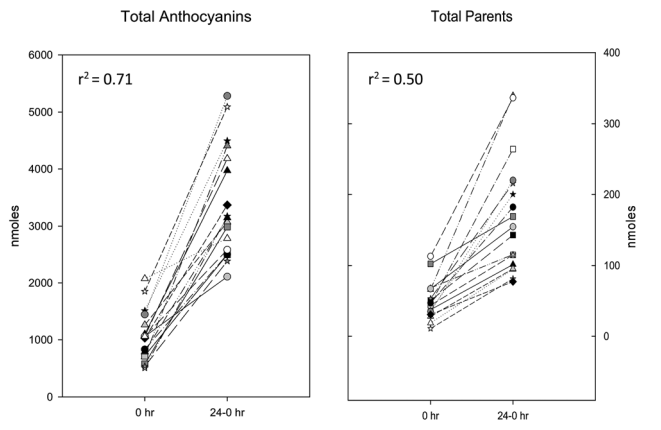

Fig. 3 Excretion of total and parent anthocyanins at 0 h and 24–0 h based on the mean of five 24 h urine collections among 17 volunteers. The 0 h and 24–0 mean is connected by a line for each of the 17 to illustrate inter-individual variation.

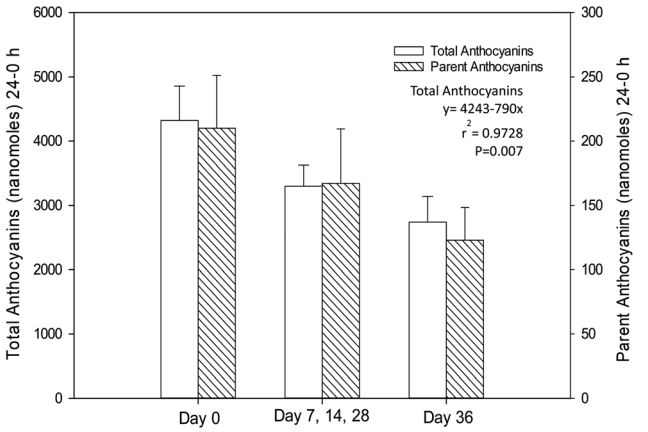

Fig. 4 ANOVA results showing the decline in total 24–0 h anthocyanins over the 36-day study. Shown are day 0 and 36 and the mean of days 7, 14 and 28. Open bars, total anthocyanins; hatched bars, parent anthocyanins.

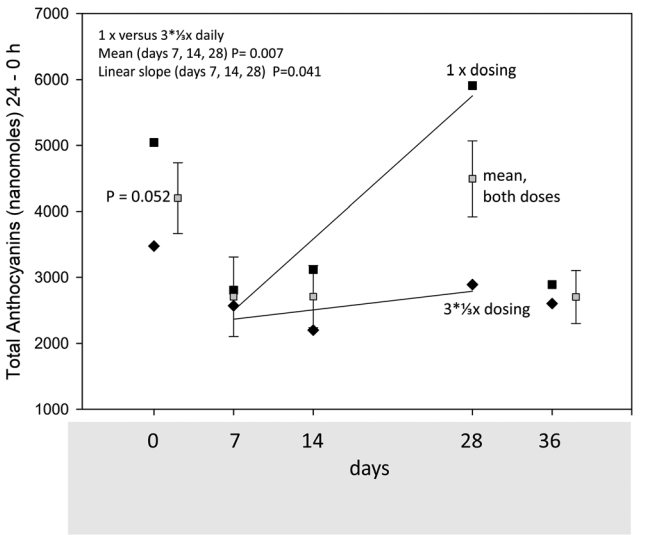

Fig. 5 ANOVA results showing the 24–0 h mean and SEM ▩ for anthocyanin excretion at five collection dates (day 0, 7, 14, 28, 36) and for both dosing regimens combined. Fitted lines illustrate the response to dosing regimens (■ 1× daily and ◆ 3*$\frac{1}{3}$× daily) including linear response line means over day 7, 14 and 28 determined by repeated measures analysis and ANOVA.

3. Results

3.1. Variation among volunteers

All volunteers completed the five 24 h urine collections, except three people did not take part in the final 24 h collection on day 36 (Fig. 1). Total nanomole Anc excreted during 24–0 h

Table 2 Effects of blueberry juice dosing regimen (1× *vs.* 3*$\frac{1}{3}$× dose daily) on total and parent anthocyanins and other selected categories of urinary anthocyanin intermediates in urine. Statistics are described for the void at 0 h and cumulative excretion for 24–0 h

			Days 7, 14, 28 mean		Day 0		Day 28		Day 36	
Anthocyanin	%	Dosing	24–0 h	0 h	24–0 h	0 h	24–0 h	0 h	24–0 h	0 h
			nmol							
Total aglycones	91.9	1×	3666	917	4727	1415	5491	1245	2801	1165
		3*$\frac{1}{3}$×	2319	1018	3122	1104	2585	778.8	2244	1093
		SEM	297.3	330.2	522.7	281.5	560.1	199.8	385.8	211.3
		F Prob	0.005	ns	0.041	ns	0.002	ns	ns	ns
Aglycone glucuronides	65.3	1×	2687	726.1	3370	1047	4181	993.5	2106	886.2
		3*$\frac{1}{3}$×	1556	781.7	2088	782.3	1841	552.2	1582	785
		SEM	214.4	289.6	471.4	207.2	495.8	177.2	338.1	168.5
		F Prob	0.002	ns	0.066	ns	0.004	0.090	ns	ns
Simple aglycones	26.6	1×	978.7	190.9	1357	368.4	1310	251.3	684.8	279.1
		3*$\frac{1}{3}$×	763.4	235.9	1033	321.3	743.5	226.5	662.5	307.8
		SEM	115.6	49.62	174.1	86.07	129.2	49.67	103.9	6.08
		F Prob	ns	ns	ns	ns	0.006	ns	ns	ns
Total glycosides	8.19	1×	291.1	94.12	327.3	121.9	423	154.1	158.4	76.61
		3*$\frac{1}{3}$×	244.8	59.84	371.9	66.59	311.1	78.47	249.5	46.86
		SEM	55.72	15.83	51.33	15.77	57.18	25.53	34.16	8.086
		F Prob	ns	ns	ns	0.022	ns	0.034	ns	.020
Parent anthocyanins	5.07	1×	180.2	66.11	190.1	80.29	240.7	113.2	85.4	48.93
		3*$\frac{1}{3}$×	152.7	35.9	232.2	35.98	179.9	45.2	165.2	24.37
		SEM	42.11	13.0	41.17	7.667	42.02	18.37	25.47	7.038
		F Prob	ns	ns	ns	<0.001	ns	0.017	0.042	0.026

across the five collection dates for the 17 volunteers (82 values) spanned approximately a 10-fold range (Fig. 2). In all cases the mean Anc (dashed line) was greater than the median Anc (solid line) which was due to some high values in the upper 50% of the observations while the bottom 50% of values were similar and low (Fig. 2). The 24–0 h total Anc mean and median were 3415 and 2892 nmol respectively, whereas the 75th percentile was 4000 nmol (Fig. 2). Parent Anc which was about 5% of the total Anc also ranged approximately 10-fold at 24–0 h with only one of the 82 values beyond this range (Fig. 2). The 24–0 h parent Anc mean and median were 169 and 132 nmol respectively, whereas the 75th percentile was 229 nmol (Fig. 2).

At 0 h the mean value also exceeded the median for both total Anc and parent Anc. In Fig. 2 points denoted with an × indicate the 17 values for the 0 h void on day 0, to illustrate that Anc were abundant in urine even after a 5-day Anc-free run-in and before BJ (*i.e.* 0 h).

3.2. Relation between 0 h and 24–0 h Anc excretion

To further illustrate inter-individual variation in Anc excretion scatter plots are shown for 0 h and 24–0 h as the mean of five collection dates for each of the 17 volunteers (Fig. 3). Lines connecting 0 h and 24–0 h values shows that in general volunteers who had a high 0 h total nanomoles Anc also had high nanomoles Anc for 24–0 h. Conversely, volunteers with low 0 h Anc also had relatively low 24–0 h Anc excretion (r^2 = 0.71). Parent Anc showed this pattern of inter-individual variation but less strongly (r^2 = 0.50) (Fig. 3).

3.4. Decline in total Anc excretion during long-term intake

Based on ANOVA total mean Anc excretion for dosing regimens declined (P = 0.007) over the five collection dates during the 36-day trial (Fig. 4). The decline is depicted by comparing day 0 after the 5-day run-in with excretion during chronic intake (*i.e.* days 7, 14 and 28) and after washout on day 36. While individual means increase between day 7, 14 and 28 (Fig. 5) this effect was mainly due to dosing as described below. A day 36 after washout, Anc excretion was significantly less than on day 0. PAnc also declined over the 36-day trial, but not significantly (Fig. 4). This overall decline in Anc excretion (Fig. 4) occurred during daily intake of equivalent to about 200 g of wild blueberry fruit in BJ, and the decline persisted even after a 7-d Anc-free washout (days 29–36) (Fig. 1).

3.5. Effects of BJ dosing regimen

At day 0 after the 5-day Anc-free run-in, Anc excretion was greater among those who consumed BJ 1× daily compared to those who took BJ 3*$\frac{1}{3}$× daily (P = 0.052) (Fig. 5). Between day 0 and day 7 excretion of Anc declined in both dosing regimens and the two groups were not different at day 7 and 14. However between day 14 and day 28 Anc excretion increased among participants who took BJ 1× daily while there was no change in the BJ 3*$\frac{1}{3}$× group during the same period (Fig. 5).

To further examine these responses repeated measures analysis was conducted within ANOVA on the three mid-study collection dates (days 7, 14 and 28). These three dates were examined separately because they were preceded by at least 7 days of daily BJ intake (Fig. 1). The two dosing regimens differed based on mean total Anc (P = 0.007) and its linear trend (P = 0.041) over the 7, 14 and 28 collection dates (Fig. 5). Greater nanomole Anc excretion occurred when BJ was taken 1× daily (Fig. 5) as compared to $3*\frac{1}{3}\times$ daily. At day 28 the total nanomoles Anc excreted when BJ was taken $3*\frac{1}{3}\times$ daily was only 65% compared to when BJ was taken 1× daily. However on day 36 following the 7-day washout there was no longer an effect of dosing on total 24–0 h total Anc excretion (Fig. 5).

The same 24–0 h dosing effect (*i.e.* $1\times > 3*\frac{1}{3}\times$ BJ daily) that was seen for total Anc (Fig. 5) was also seen for abundant groups of metabolites including the total Anc aglycones (P = 0.005) and Anc aglycone glucuronides (P = 0.002) (Table 2). Marginally significant dosing effects were seen on day 0 only for total Anc (P = 0.052) (Fig. 5), total Anc aglycones (P = 0.041) and aglycone glucuronides (P = 0.066) (Table 2) but effects were much more pronounced at day 28 where F probabilities were: total Anc, P = 0.002 (Fig. 5); total aglycones, P = 0.002 and aglycone glucuronides, P = 0.004 (Table 2). On day 36 after 7 days of washout there was no dosing effect for total Anc (Fig. 5) or for the abundant Anc metabolite groups (Table 2). At 0 h there was no effect of dosing on total Anc (not shown) or any abundant Anc metabolite group on any collection date (Table 2).

No 24–0 h dosing differences were found for total glycosides or parent Anc on any collection date except for parent Anc on day 28 (Table 2). However glycosidic forms of Anc (8.2% of total Anc) and parent Anc (5.1% of total Anc) were affected by dosing at 0 h (Table 2). At 0 h Anc glycosides were more abundant when BJ was taken 1× daily on all dates *i.e.* (1) after the Anc-free run-in on day 0 (P = 0.022), (2) after long-term intake (day 28) (P = 0.034) and (3) after 7-days of washout (day 36) (P = 0.020). Similar (*i.e.* $1\times > 3*\frac{1}{3}\times$) but even greater dosing effects were seen for parent Anc at the same three time points (P = <0.001, 0.017, 0.026 respectively) (Table 2).

4. Discussion

This report describes only the urinary metabolites possessing a flavonoid backbone that are predicted to arise from all six anthocyanidins (Table 1). Not all predicted metabolites could be scanned therefore quantities reported are underestimated.[12] Non-flavonoid products arising from C ring fission were not tracked in this study. Non-flavonoid products arising from Anc intake have been relatively well characterized and are known to be abundant.[1,14]

The two earlier reports[11,12] from this study document the relative abundance and nature of the total pool of urinary C6–C3–C6 products for all dates and two dosing regimens combined[12] and provide an early look into the retention of Anc *in vivo*.[11] The present report documents the effects of intake duration, dosing regimen, run-in, washout, and inter-individual variation (Fig. 1). Results are discussed in relation to optimizing the body's exposure to Anc to achieve health outcomes.

4.1. Human phenotypic variation

The complex pool of Anc-derived products[12] reflects the physical and chemical conditions *in situ* plus the collective effect of human xenobiotic processes and, to a limited extent, gastrointestinal microbiota. The pool of Anc metabolites[12] reflects human phenotypic variation in the xenobiotic enzymatic and transporter machinery responsible for modification, conjugation, transport and excretion of Anc. These aspects have been examined for selected flavonoids (for example see ref. 17 and 18) and reviewed.[19–21] The 10-fold range in total Anc and parent Anc among the 17 participants (Fig. 2) was small compared to the 35- to 87-fold range documented *in vitro* for human β-glucosidase (n = 10) with various glycoside substrates.[22] When the human wild-type efflux transporters BCRP and MDR1 were tested with sixteen different Anc *in vitro* substantial variation was seen.[23] When the metabolic fate of five classes of flavonoids was examined using intestinal perfusion and cannulation techniques a great deal of structural specificity was documented.[24]

4.2. Capacity to excrete and retain Anc

Scatter plots of Anc excretion (*i.e.* 24–0 h) and retention (*i.e.* 0 h) (Fig. 3) showed that volunteers who had a high capacity for 24–0 h Anc excretion generally had high Anc content in their 0 h void (Fig. 3) which was novel. If capacity for Anc metabolism, excretion and retention was found to correlate within an individual's capacity for other physiological responses to parent Anc, then a randomization of volunteers based on their baseline urinary Anc should be considered.

4.3. Anc persistence

Persistence *in vivo* was apparent by the high urinary Anc concentration at 0 h (*i.e.* before BJ) after both a 5-day Anc-free run-in (Fig. 2) and a 7-day washout (Table 2). The long *in vivo* residence time of Anc is attributed to their uptake into bile and circulation between the small intestine and liver *via* enterohepatic circulation. Enterohepatic circulation appears to be common for flavonoids[25] but not for phenolic acids and esters.[26] Parent Anc are found in bile within 5 min after gastric delivery[27] and Anc are readily soluble in bile phospholipids[11] behaving similarly to other flavonoids in this way.[17,18,20] Conjugation and de-conjugation events during enterohepatic circulation leads to a complex pool of metabolites. Solutes remain in enterohepatic circulation until they are distributed to peripheral sites (tissues, organs) or excreted in feces or urine.[13]

Other studies document the persistence of Anc *in vivo*, for example when radiolabeled cyanidin 3-glucoside was administered to humans[14] and mice[28] label was rapidly distributed into numerous products including fluids[14,28] and tissues.[28] At 48 h after administration, radio-label in feces was still increasing and only 43% of the label had been accounted for.[14] Dose–

dependent tissue disposition of Anc has been reported in pigs.[29,30] Quercetin persistence in the gastrointestinal tract has been reported in humans.[31] The notion of the Anc metabolite persistence is also supported by reports of significant stability of Anc *in vivo*.[32,33]

The persistence *in vivo* of Anc complicates a comparison of animal and human Anc studies. Whereas human volunteers will have a significant amount of urinary Anc at baseline, well-controlled animal studies can mitigate or eliminate this complication. The lack of previous dietary exposure to parent Anc in laboratory animals may also contribute to a greater capacity for a response by an animal to parent Anc intervention.

4.4. Decline in Anc excretion over 36 days

The decline in urinary Anc during long-term BJ intake (Fig. 4) may be due to a reduction in Anc uptake by the small intestine and increased Anc partitioning into feces which has not been previously reported. Amounts of metabolites in urine are roughly correlated with maximum plasma concentration[21] and therefore a decline in urinary Anc over time means a reduction in systemic exposure to Anc. A decline in excretion of both total Anc and parent Anc was still apparent even after 7 days of washout suggesting that Anc partitioning between feces and urine is slow to change (Fig. 4 and 5). Saturation or inhibition effects of Anc on xenobiotic machinery may contribute to the urinary Anc decline during prolonged intake.[21,34] Non-specific Anc effects including adsorption to biomolecules like polysaccharide, protein, fat including intestinal mucin[35] which could obstruct continued Anc uptake, cannot be ruled out.

4.5. Effects of dosing

Because Anc are rapidly broken down in the lumen of the gastrointestinal tract, but well-retained after intestinal absorption[33] (Fig. 2) then affecting intestinal uptake could be a means to increase systemic Anc exposure. Greater 24–0 h excretion of both total Anc and groups of abundant Anc occurred when BJ was taken 1× daily compared to $3*\frac{1}{3}\times$ daily (Fig. 5 and Table 2) which may have been due to greater coverage of the stomach and small intestine by the larger 1× BJ volume (250 mL) compared to intestinal coverage by 83 mL of BJ (*i.e.* $3*\frac{1}{3}\times$). Coverage of a greater portion of the stomach and small intestine at one time may be a means to increase intestinal absorption of Anc. Also the 250 mL BJ dose would be more likely than an 83 mL dose to reach the jejunum for uptake into enterohepatic circulation, before the loss of the flavonoid structure.

The greater Anc excretion observed in the 1× dosing group was due to high Anc excretion at day 28. At day 28 many of the same Anc observed at earlier dates were present but at higher levels. The high level of Anc excretion at 28 days could not be readily associated with the identification of new Anc forms.

Parent Anc at 0 h was greater when BJ was taken 1× daily (Table 2). This is interesting because parent Anc taken 1× daily will have resided in the gastrointestinal tract at least seven hours longer than the parent Anc ingested in the last 83 m BJ ($\frac{1}{3}$ dose) which was taken as late as 17:00. It appears that when parent Anc had access to more surface area of the gastrointestinal tract, due to the greater BJ volume a greater amount of parent Anc was retained.

In light of the complex human pharmacokinetics of Anc due to extensive xenobiotic metabolism and enterohepatic circulation it is particularly valuable to understand the impact of study design factors. The design of clinical trials involving Anc may be guided by results reported here. The retention of Anc shown in the present study means that human base-line levels of Anc far exceed those of laboratory animals that have had no exposure to dietary Anc. Anc decline during long-term BJ intake highlights the importance of designing research using readily achievable dietary Anc doses. The effect of dosing regimen (1× daily *vs.* $3*\frac{1}{3}\times$ daily) illustrates the extensive and dynamic first pass metabolism of Anc along the gastrointestinal tract. Results related to dosing regimen suggest that a specified PAnc dose taken in a large volume would lead to greater Anc absorption than the same PAnc dose taken in a smaller volume.

Abbreviations

Anc	Anthocyanin(s)
BJ	Blueberry juice
RT	Retention time(s)

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

This research was financially supported by the Wild Blueberry Association of North America and Agriculture and Agri-Food Canada.

References

1 E. Pojer, F. Mattivi, D. Johnson and C. S. Stockley, *Compr. Rev. Food Sci. Food Saf.*, 2013, **12**, 483–508.
2 http://www.ars.usda.gov/nutrientdata, 2013.
3 N. M. Wedick, A. Pan, A. Cassidy, E. B. Rimm, L. Sampson, B. Rosner, W. Willett, F. B. Hu, Q. Sun and R. M. van Dam, *Am. J. Clin. Nutr.*, 2012, **95**, 925–933.
4 A. Cassidy, K. J. Mukamal, L. Liu, M. Franz, A. H. Eliassen and E. B. Rimm, *Circulation*, 2013, **127**, 188–196.
5 A. Jennings, A. A. Welch, S. J. Fairweather-Tait, C. Kay, A.-M. Minihane, P. Chowienczyk, B. Jiang, M. Cecelja, T. Spector, A. Macgregor and A. Cassidy, *Am. J. Clin. Nutr.*, 2012, **96**, 781–788.
6 A. Cassidy, E. J. O'Reilly, C. Kay, L. Sampson, M. Franz, J. P. Forman, G. Curhan and E. B. Rimm, *Am. J. Clin. Nutr.*, 2011, **93**, 338–347.

7 E. E. Devore, J. H. Kang, M. M. Breteler and F. Grodstein, *Ann. Neurol.*, 2012, **72**, 135–143.
8 C. D. Kay, P. A. Kroon and A. Cassidy, *Mol. Nutr. Food Res.*, 2009, **53**, S92–S101.
9 S. C. Forester and A. L. Waterhouse, *J. Nutr.*, 2009, **138**, 1824S–1831S.
10 G. Williamson and M. N. Clifford, *Br. J. Nutr.*, 2010, **104**, S48–S66.
11 W. Kalt, Y. Liu, J. E. McDonald, M. R. Vinqvist-Tymchuk and S. A. E. Fillmore, *J. Agric. Food Chem.*, 2014, **62**, 3926–3934.
12 W. Kalt, J. E. McDonald, Y. Liu and S. A. E. Fillmore, *J. Agric. Food Chem.*, 2017, **65**, 1582–1591.
13 M. S. Roberts, B. M. Magnusson, F. J. Burczynski and M. Weiss, *Clin. Pharm.*, 2002, **41**, 751–790.
14 C. Czank, A. Cassidy, Q. Zhang, D. J. Morrison, T. Preston, P. A. Kroon, N. P. Botting and C. D. Kay, *Am. J. Clin. Nutr.*, 2013, **97**, 995–1003.
15 W. Kalt, J. E. McDonald, R. D. Ricker and X. Lu, *Can. J. Plant Sci.*, 1999, **79**, 617–623.
16 *Genstat for Windows*, VSN International, Hemel Hempstead UK, 2013.
17 B. Xia, Q. Zhou, Z. Zheng, L. Ye, M. Hu and Z. Liu, *Mol. Pharm.*, 2012, **9**, 3246–3258.
18 X. Jia, J. Chen, H. Lin and M. Hu, *J. Pharmacol. Exp. Ther.*, 2004, **310**, 1103–1113.
19 B. Wu, K. Kulkarni, S. Basu, S. Zhang and M. Hu, *J. Pharm. Sci.*, 2011, **100**, 3655–3681.
20 L. Zhang, Z. Zuo and G. Lin, *Mol. Pharmaceutics*, 2007, **4**, 833–845.
21 Z. Liu and M. Hu, *Expert Opin. Drug Metab. Toxicol.*, 2007, **3**, 389–406.
22 K. Németh, G. W. Plumb, J.-G. Berrin, N. Juge, R. Jacob, H. Y. Naim, G. Williamson, D. M. Swallow and P. A. Kroon, *Eur. J. Nutr.*, 2003, **42**, 29–42.
23 A. Dreiseitel, B. Oosterhuis, K. V. Vukman, P. Schreier, A. Oehme, S. Locher, G. Hajak and P. G. Sand, *Br. J. Pharmacol.*, 2009, **158**, 1942–1950.
24 V. Crespy, C. Morand, C. Besson, N. Cotelle, H. Vezin, C. Demigne and C. Remesy, *Am. J. Physiol.:Gastrointest. Liver Physiol.*, 2003, **284**, G980–G988.
25 M. R. Dobrinska, *J. Clin. Pharm.*, 1989, **29**, 577–580.
26 S. Lafay, A. Gil-Izquierdo, C. Manach, C. Morand, C. Besson and A. Scalbert, *J. Nutr.*, 2006, **136**, 1192–1197.
27 S. Talavera, C. Felgines, O. Texier, C. Besson, J.-L. Lamaison and C. Remesy, *J. Nutr.*, 2003, **133**, 4178–4182.
28 C. Felgines, S. Krisa, A. Mauray, C. Besson, J.-L. Lamaison, A. Scalbert, J.-M. Merillon and O. Texier, *Br. J. Nutr.*, 2010, **103**, 1738–1745.
29 W. Kalt, A. Hanneken, P. Milbury and F. Tremblay, *J. Agric. Food Chem.*, 2010, **58**, 4001–4007.
30 W. Kalt, J. B. Blumberg, J. E. McDonald, M. R. Vinqvist-Tymchuk, S. A. E. Fillmore, B. A. Graf, J. M. O'Leary and P. E. Milbury, *J. Agric. Food Chem.*, 2008, **56**, 705–712.
31 J. F. Young, S. E. Nielsen, J. Haraldsdottir, B. Daneshva, S. T. Lauridsen, P. Knuthsen, A. Crozier, B. Sandstrom and L. O. Dragsted, *Am. J. Clin. Nutr.*, 1999, **69**, 87–94.
32 J. Fleschhut, F. Kratzer, G. Rechkemmer and S. E. Kulling, *Eur. J. Nutr.*, 2006, **45**, 7–18.
33 J. Fang, *J. Agric. Food Chem.*, 2014, **62**, 3904–3911.
34 M. Zeng, R. Sun, S. Basu, Y. Ma, S. Ge, T. Yin, S. Gao, J. Zhang and M. Hu, *Mol. Nutr. Food Res.*, 2016, **60**, 1006–1019.
35 G. B. Gonzales, G. Smagghe, C. Grootaert, M. Zotti, K. Raes and J. V. Camp, *Drug Metab. Rev.*, 2015, **47**, 175–190.