Research Article

Urinary excretion of strawberry anthocyanins is dose dependent for physiological oral doses of fresh fruit

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There is considerable interest in coloured fruits and berries as sources of biologically active anthocyanins. To examine the relationship between the oral dose and the amount excreted for anthocyanins from a food source across a physiological range of doses, volunteers were fed, in random order, four portions (100 – 400 g) of fresh strawberries as part of a standard breakfast. Urine was collected at 2 h intervals up to 8 h, and for the period 8 – 24 h. Fresh strawberries contained pelargonidin-3-glucoside as the major anthocyanin with smaller amounts of cyanidin-3-glucoside and pelargonidin-3-rutinoside. Anthocyanins were detected in the urine of all volunteers for all doses, predominantly as pelargonidin glucuronide and sulphate metabolites. There was a strong, linear relationship between oral dose and anthocyanin excretion (Pearson's product moment correlation coefficient = 0.692, $p < 0.001$, $n = 40$) which indicated that on an average, every additional unit of dose caused 0.0166 units of excretion. Within individuals, dose – excretion data fitted a linear regression model (median $R^2 = 0.93$). We conclude that strawberry anthocyanins are partially bioavailable in humans with a linear relationship between oral dose and urinary excretion for doses up to 400 g fresh fruit.

Keywords: Bioavailability / Dietary antioxidants / Flavonoids / Human metabolism / Polyphenols

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1 Introduction

Anthocyanins are one of the six classes of flavonoids and are widespread in the plant kingdom where they are responsible for most of the purple, red and blue colours of many fruits, vegetables, leaves and flowers. Anthocyanins are conjugates of anthocyanidins where the anthocyanidin core (aglycone) is *O*-conjugated to give glycosylated or acylglycosylated anthocyanins. The anthocyanidin is a 2-phenylbenzopyrylium or flavylium structure, and the majority of anthocyanins are formed through conjugation with sugars or acyl sugars in the 3- and/or 5-positions, although there are also examples of conjugation through 7-, 3′- and 5′-hydroxy groups (Fig. 1). There is widespread interest in anthocyanins as potentially health-promoting constituents of fruits and vegetables that may contribute to the protection afforded against age-related diseases by diets rich in fruits and vegetables. Anthocyanin containing plant parts are consumed widely, both as intact fruits (*e.g.* strawberries, blackberries, blackcurrants, red/black grapes, blueberries), and vegetables (red cabbage, black carrots) and as processed products such as fruit juices and concentrates, wines, jams and sauces. An estimate of the mean daily intake of anthocyanins in the Unites States (180 – 215 mg/day; [1]) is higher than that estimated for other flavonoid classes (*e.g.* flavonol plus flavone intakes in The Netherlands were estimated to be 23 mg/day; [2]). This is due to their widespread occurrence and typically high concentrations in plant-based foods and beverages. For example, reported levels of anthocyanins in strawberries, raspberries, lowbush and highbush blueberries were 155, 840, 2670 and 4350 μmol/(100 g FW), respectively (67 – 1885 mg/(100 g FW)) [3].

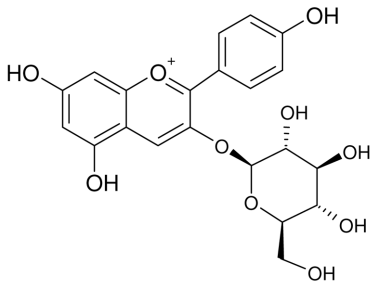

Figure 1. Structures of strawberry anthocyanins.

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Anthocyanins are biologically active, and have been shown, *in vitro*, to be powerful antioxidants [4, 5], to possess antiinflammatory [6], antiplatelet [7] and vasomodulatory [8 – 10] activities, and antitumour activities including inhibition of pro-inflammatory enzymes, inhibition of tumour and tumour cell growth and induction of apoptosis [5, 11 – 13]. However, the data available from a number of reported human intervention trials focused on anthocyanins are difficult to interpret because the foods/extracts used contained a mixture of flavonoids and other phytochemicals. For example, red wine is a source of anthocyanins, and some studies have suggested that clinically relevant responses observed *in vitro* with red wine anthocyanins indicate that they may be the bioactive component of red wines [14], but red wine is also a rich source of flavan-3-ols, flavonols, the stilbene resveratrol, and ethyl alcohol, all of which are biologically active and could explain many of the observed effects.

The potential for any food/beverage phytochemical to influence cell/tissue function depends on their ability to reach the relevant tissues. In order to influence tissues and cells beyond the gastrointestinal tract, anthocyanins must be absorbed and reach the circulatory system. A number of studies concerned with the absorption of anthocyanins have reported that they are rather poorly bioavailable, with only a tiny fraction, often less than 0.1%, of the ingested doses recovered in urine, and plasma peak plasma concentrations reaching only low nanomolar levels [15 – 18]. The majority of dietary flavonoids undergo deglycosylation during absorption and are subsequently modified during first pass metabolism such that the forms in plasma and urine are typically glucuronide and sulphate conjugates of the aglycone and methylated derivatives. In contrast, many anthocyanins appear to escape first pass metabolism, and the ingested forms appear in plasma and urine unaltered [17]. This is almost unique for flavonoids since other classes are efficiently deglycosylated and conjugated with sulphate, glucuronide and methyl groups during absorption [18].

Human systemic exposure to anthocyanins depends on the levels in foods or beverages, the amounts ingested, and the bioavailability of the particular anthocyanin, and may depend on the food matrix. Data concerning the levels in foods and beverages are becoming more complete [19], estimates of average intakes in certain populations are available [19], and relative absorption rate data for some anthocyanins have been reported [20]. Further, routes to increase anthocyanin contents of fruits have been described [10]. Currently, however, it is not known how the absorption of flavonoids varies with the oral dose. The aim of this study was to investigate the bioavailability of strawberry anthocyanins as a function of the oral dose. Accordingly, we measured the urinary excretion of strawberry anthocyanins from volunteers who consumed four different portions of fresh strawberries that were representative of the range of habitual intake. This study allowed us to investigate (i) the variation in the anthocyanin content of strawberries purchased from a local supermarket over a 5-month period, (ii) whether the relationship between individual anthocyanin dose and urinary excretion was linear and (iii) the upper limit of anthocyanin 'exposure' for currently available commercial strawberry fruits.

2 Materials and methods

2.1 Chemicals and reagents

Methanol (HPLC grade) was purchased from Fisher Scientific (Loughborough, Leicestershire, UK). Acetonitrile (HPLC grade) and trifluoroacetic acid (TFA) were purchased from Sigma – Aldrich (Poole, Dorset, UK). All anthocyanins used as standards were obtained from Extrasynthése.

2.2 Strawberries

For the study, fresh strawberries were purchased from a local supermarket, stored in a fridge (2 – 8°C), and used within 24 h. Immediately prior to consumption strawberries were washed, quartered and portioned appropriately. A subsample (50 g) was obtained for immediate analysis. To quantify the strawberry anthocyanins, the 50 g sample was blended (food mixer) into a puree in the presence of 1% HCl. Samples of puree (1 g) were extracted in triplicate with 1% HCl in methanol, filtered (0.45 μm) into HPLC vials and analysed by HPLC. Quantification of strawberry anthocyanins was based on external calibration curves of pelargonidin-3-glucoside over the range of 0.1 – 100 μg/mL (10 μL *per* injection) that were fitted to a linear regression ($R^2 > 0.99$). Intraday variance (SD per cent of mean) for the quantification of anthocyanins in strawberries was < 10%.

2.3 Subjects and study design

Ten apparently healthy volunteers (three men and seven women) aged 20 – 65 years were recruited to participate in this study. All study participants were assessed for eligibility on the basis of a health questionnaire and the results of clinical laboratory tests. The following exclusion criteria applied: smokers; long term medical conditions such as asthma (unless untreated within the past 2 years) heart disease, gastrointestinal disease, diabetes, cancer; regular prescribed medication (except HRT and oral contraceptive); dietary supplements (unless judged not to affect study outcome); BMI < 18.5 or > 35;. Subject characteristics were (mean ± SD): weight 64.9 ± 7.7 kg (range 54 – 76.8 kg), BMI 24 ± 2.6 kg/m^2 (range 21 – 27.1 kg/m^2) and age 40 ± 11 year (range 23 – 56 years). The study was explained to participants and written informed consent subsequently obtained. The study protocol was approved by the Human Research Governance Committee of the Institute of Food Research and the Norwich Research Ethics Committee.

The study was a randomized four phase crossover design investigating the effect of dose on the bioavailability of phytochemicals from fresh strawberries. Each test phase comprised a 3 day period of intervention separated by a washout period of at least 2 days. During each period of intervention subjects followed a low-polyphenol diet. To aid compliance a list of authorized and prohibited foods was provided and discussed during a face-to-face interview prior to participation. On day 2 of the intervention subjects arrived at the Human Nutrition Unit following an overnight fast and a baseline urine sample was obtained. Subjects were given a standard breakfast consisting of two slices of white toast (72 g) with a fat-based spread (10 g) followed by either 100, 200, 300 or 400 g portion of fresh strawberries. Subjects refrained from drinking and eating for 1.5 and 4 h, respectively to limit any variation in food/drink volume consumed. Urine was collected between 0–2, 2–4, 4–6, 6–8 and 8–24 h after strawberry consumption, the amount of urine in each fraction was measured and sub-samples were acidified with HCl to prevent anthocyanin degradation. All urine samples were stored at 4°C and analysed on the day of collection.

2.4 Extraction and analysis of anthocyanins from urine

Prior to the extraction of anthocyanins 50 μL of delphinidin-3-glucoside (0.1 mg/mL) was added to 20 mL of acidified urine and used as an internal standard. Anthocyanins in urine were extracted using a solid phase extraction cartridge (Varian Bond Elut C_{18}) conditioned with 5 mL methanol followed by 10 mL 1% HCl (aq). Following application of urine the cartridge was washed with 10 mL 1% HCl (aq) and anthocyanins eluted directly into autosampler vials with 0.5 mL 1% HCl in methanol. The SPE extract was analysed by HPLC (Agilent HP1100) using a Gemini C18 column (150 × 2.00 mm) with a 5 μm particle size (Phenomenex, Macclesfield, UK). The mobile phase (A, 0.1% TFA in water; B, 0.1% TFA in acetonitrile) was pumped through the column at a flow rate of 0.3 mL/min and samples eluted with a gradient of increasing acetonitrile in 0.1% TFA. Postcolumn, the eluent passed through a UV-diode array detector monitoring over the range of 200–700 nm, and subsequently an electrospray ionisation-mass spectrometer (ESI-MS; Agilent Technologies, Waldbronn, Germany). The mass spectrometer was operated in positive ionisation mode (cone voltage 22 V, source block temp 120°C, desolvation temperature 300°C) with selected ion monitoring. Quantification of strawberry anthocyanins in plasma, urine and food/beverage samples was based on standard curves ($A_{520\,nm}$, range 0.1–100 μg/mL) for pelargonidin-3-glucoside that were run alongside samples with correction for recovery of the internal standard. The assay was linear with regression coefficients for all standard curves >0.99. Recoveries of the internal standard for the urine analyses were 93.1 ± 4.3% (mean ± SD). The repeatability of the assay was evaluated by analysis of replicate samples of a pooled postintervention urine sample; the repeatability (relative standard deviation per cent) was 5.1, 23.1 and 23.8% for total pelargonidin glucuronides, pelargonidin sulphate and pelargonidin aglycone, respectively. Samples were also subjected to mass spectrometry with multiple reaction monitoring using a Micromass Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, UK) coupled to a Jasco PU-1585 triple pump HPLC equipped with an AS-1559 cooled autoinjector, CO-1560 column oven and UV-1575 UV detector (Jasco, Great Dunmow, UK). The HPLC column temperature was maintained at 25°C and the autoinjector at 4°C. The 1 mL/min mobile phase flow exiting the HPLC column was split using an ASI 600 fixed ratio splitter valve (Presearch, Hitchin, UK) so that approximately 200 μL/min entered the mass spectrometer; the remainder of the flow was diverted to the UV detector. The flow split was monitored using a Humonics Optiflow 1000 flowmeter (Sigma–Aldrich) coupled to the outflow of the UV cell. HPLC elution was as described above. Mass spectra were obtained in both positive and negative ion electrospray mode using a Micromass Z-spray™ ion source. The electrospray probe was operated at 25 V, the source and desolvation temperatures were 140 and 350°C, respectively. The nitrogen nebulising and drying gas flow rate were optimized at 15 and 500 L/h, respectively. Spectra were recorded (in centroid mode) between *m*/*z* 50 and 1500 with a scan duration of 1.5 s/scan and an interscan time of 0.1 s. MS1 was set to unit mass resolution or better (LM and HM resolution parameters both set to 15.0). Spectra were processed using MassLynx™ 3.4 software (Micromass).

2.5 Data analysis

Statistical analyses were performed using the R data analysis software [21]. Standard Linear Regression and ANOVA models were employed to analyse this data. When Linear Regression was used, the explanatory variable was always the quantity of dose given and the response variable was either the fraction of the dose excreted or the total amount excreted. The ANOVA models were constructed in a similar way but Subject ID was also included as a fixed effect. For all models, regression diagnostics were checked to determine if data transformations, outlier omissions or alternative nonparametric models were required. All results from the models were considered significant if $p < 0.05$.

3 Results

3.1 Anthocyanin content and composition of strawberries

Anthocyanin content and composition was determined for a subsample of fresh strawberries that was purchased on each

Table 1. Strawberry anthocyanin content and composition

Sample #	Purchasedate	Country of origin	Total anthocyanin (mg/(100 g FW))	Pel-3-Glc (%)[a)]
1	10 April 2006	Spain	80.0	89.2
2	26 April 2006	Spain	64.3	87.9
3	02 May 2006	Spain	47.5	83.7
4	08 May 2006	Spain	84.5	87.6
5	11 May 2006	USA	73.0	83.6
6	17 May 2006	USA	34.5	85.5
7	06 June 2006	USA	28.3	83.5
8	12 June 2006	UK	46.5	95.7
9	19 June 2006	UK	56.0	98.2
10	21 june 2006	UK	52.7	100
11	26 June 2006	Scotland	50.5	99.0
12	03 July 2006	England	112.3	96.1
13	05 July 2006	USA	49.0	87.8
14	10 July 2006	UK	54.0	96.3
15	12 July 2006	UK	43.5	100
16	19 July 2006	UK	47.3	100
17	26 July 2006	UK	54.0	98.1
18	09 August 2006	Scotland	56.7	97.6
19	16 August 2006	Scotland	74.0	94.6
20	30 August 2006	UK	33.5	94.8
Mean			57.1	93
SD			19.8	6.1
Variance (%)			34.7	6.6

a) The contribution of pelargonidin-3-glucoside as a percentage of total anthocyanin in the strawberry sample.

Table 2. Characterisation of urinary anthocyanins and metabolites

	Retention time (min)	Co-elution with standard	*m/z* 447, 271	*m/z* 433, 271	*m/z* 351, 271	*m/z* 271, 121	Compound structure
M1	14.1	None	+	–	–	+	Pelagonidin monglucuronide
M2	16.5	Pelargonidin-3-glucoside	–	+	–	+	Pelargonidin-3-glucoside
M3	16.7	None	+	–	–	+	Pelagonidin monglucuronide
M4	17.8	None	+	–	–	+	Pelagonidin monglucuronide
M5	29.9	None	–	–	+	+	Pelargonidin monosulphate
M6	30.4	Pelargonidin	–	–	–	+	Pelargonidin

Samples of urine were extracted and analysed using HPLC with online diode array and mass spectrometry detection as described in Section 2. Molecular ions as follows: *m/z* 447 = pelargonidin monoglucuronide; *m/z* 433 = pelargonidin-3-glucoside; *m/z* 351 = pelargonidin monosulphates; *m/z* 271 = pelargonidin. Multiple reaction monitoring of pelargonidin also generates an *m/z* = 121. Authentic standards were available for pelargonidin-3-glucoside and pelargonidin (aglycone) but not for any of the putative glucuronide and sulphate metabolites.

study day ($n = 20$). The strawberries were obtained from a single local supermarket over a period of 5 months (April to August 2005) (Table 1). During this period, the strawberries comprising different varieties originated from the UK (Scotland and England), US and Spain. For all samples, up to three anthocyanins were identified and quantified by HPLC separation with online UV diode-array and mass spectrometric detection. Pelargonidin-3-glucoside (see Fig. 1) was the major anthocyanin in all the strawberry samples (93.0 ± 6.1% of total anthocyanins; mean ± SD) with relatively lower amounts of cyanidin-3-glucoside and pelargonidin-3-rutinoside also present. The mean total anthocyanin content of the strawberries was 57.1 ± 19.8 μmol/(100 g FW) (mean ± SD) (Table 1). The total anthocyanin content varied from 28.3 to 112.5 μmol/(100 g FW), indicating that there can be substantial variation in anthocyanin contents over time for strawberries obtained from commercial outlets.

3.2 Identification of anthocyanins in urine

Following consumption of strawberries, six peaks with measurable absorbance at 520 nm and with pelargonidin-like spectra were detected in urine samples (M1 – M6) (Table 2). M2 and M6 co-eluted with authentic standards of pelargonidin-3-glucoside and pelargonidin aglycone, and their identity was confirmed by comparison of UV and MS spectra (*m/z* = 433 and 271, respectively). The main metab-

olite (M3) was identified as a pelargonidin-monoglucuronide based on mass spectrometry ($m/z = 447$, fragment 271). M1 and M4 also gave clear ion currents on the mass spectrometer in selected ion monitoring mode for $m/z = 447$, and parent/daughter ions (m/z 447, 271) when subjected to multiple reaction monitoring, and are probably pelargonidin-monoglucuronides. Since standards were not available, we could not identify the conjugation position for each putative monoglucuronide. However, since pelargonidin has four hydroxyl groups (positions 3, 5, 7 and 4′), and since the 5-position is rarely conjugated, it is probable that M1, M3 and M4 correspond to the 3-, 7- and 4′-glucuronides. M5 appears to be a pelargonidin-sulphate; this peak runs later than the more polar glucuronides and generated the expected parent and daughter ion masses ($m/z = 351$, 271). All the putative pelargonidin conjugates were quantified as pelargonidin-3-glucoside equivalents. For some subjects, peaks M1 and M2 were not fully resolved and so the areas of the peaks corresponding to M1 and M2 were combined. The mean contribution of the individual metabolites to the totals were: M1 + M2, 8.0% (range 3.4 – 25.9%); M3, 63.5% (50.5 – 76.5); M4, 3.0% (1.9 – 4.6); M5, 16.4% (8.3 – 25.4); M6, 9.1% (5.8 – 13.7).

3.3 Urinary excretion and relationship with dose

Each volunteer consumed four different doses of fresh strawberries on four different occasions, with an appropriate washout period between each study day. We were not able to detect anthocyanins in any of the 40 baseline samples, indicating excellent compliance with the dietary restrictions that were designed to essentially eliminate anthocyanin consumption and limit polyphenol intakes to a low level.

When assessed for each individual, the dose excretion curves were linear (Fig. 2). However, for one individual (volunteer 8) at one dose, the amount of anthocyanin excreted was very high compared to the oral dose. On checking the regression diagnostics, this data point was considered an outlier and it was removed from this individual's regression analysis. The mean and median R^2 values for the linear regressions calculated for each individual dose-response curve were 0.86 and 0.93, indicating a good linear fit of the data (Table 3). There was no evidence of nonlinearity of the dose – excretion curves at the lowest or highest doses tested (*e.g.* mean% excretion values were 1.84, 1.77, 1.67 and 1.76% for the 100, 200, 300 and 400 g doses, respectively). Taken together, these data indicate that there is a strong intra-individual linear relationship between the oral dose and urinary excretion of strawberry anthocyanins.

Mean urinary excretion of strawberry anthocyanins for the entire study was 1.71 ± 0.17% (mean ± SEM, $n = 40$) with a range of 0.79 – 2.35%. Least squares linear regression of the entire dataset indicated a linear dose – excretion relationship (adjusted $R^2 = 0.681$, $p < 0.001$, n = 40). Having demonstrated that individual dose – response relationships are strongly linear, and noting that the repeatability of urinary anthocyanin measurements is good (coefficient of variance < 10%), the within individual variation in urinary anthocyanin yields is a good indicator of interday individual variation. Urinary yield variances (SD per cent of mean) were 26.1 ± 15.1 (mean ± SD).

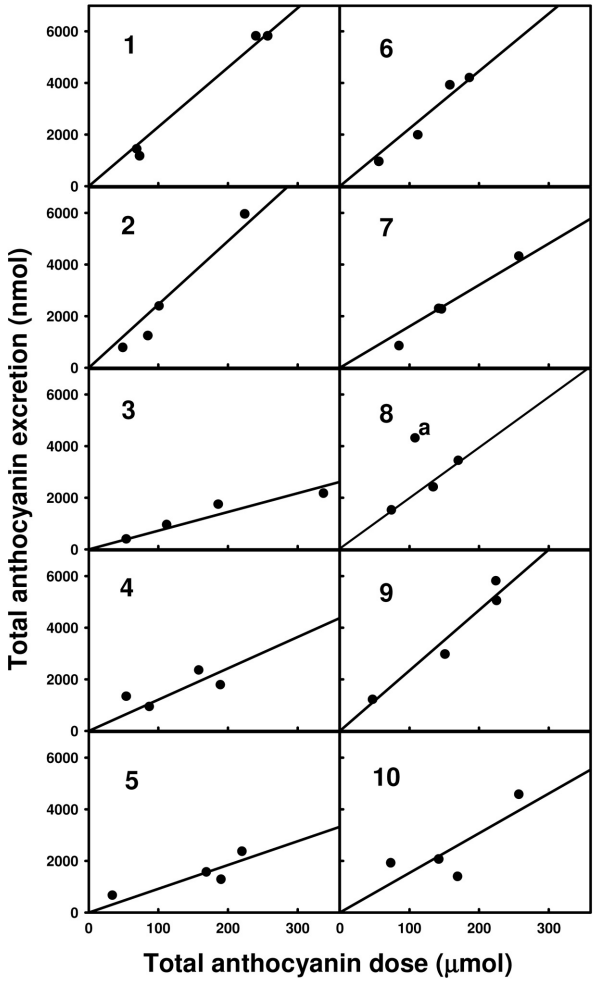

Figure 2. Dose – response for excretion of total pelargonidin metabolites for the study population. Examination of the data with a simple linear regression model suggested that one of the data points (marked 'a') was an outlier. However, it is appropriate to treat the data as repeated measures and treat 'subject' as a fixed effect, which improves the model (ANOVA). However, the potential outlier (a) is not significant in this model and so all the data were included in subsequent analyses. After fitting a linear model to the data, an adjusted R^2 value of 0.681 and a Pearson's product-moment correlation coefficient of 0.692 (95% confidence interval 0.485 – 0.826, $p <$ 0.001) were obtained. The linear model was adequate to fit the data and there was no indication of nonlinearity.

Table 3. Individual dose – excretion data

Volunteer #	Dose[a)]	Excretion (% of dose)	Excretion (mean, %)	SD	Variance (%)	R^2 (linear regression)[b)]
1	73 (100 g)	1.61				
	69 (200 g)	2.09	2.10	0.35	16.9	0.99
	240 (300 g)	2.43				
	257 (400 g)	2.27				
2	49 (100 g)	1.60				
	101 (200 g)	2.37	2.02	0.58	28.9	0.98
	85 (300 g)	1.46				
	224 (400 g)	2.66				
3	54 (100 g)	0.74				
	112 (200 g)	0.85	0.79	0.13	16.2	0.91
	337 (300 g)	0.65				
	186 (400 g)	0.94				
4	54 (100 g)	2.47				
	87 (200 g)	1.08	1.50	0.69	46.1	0.50
	158 (300 g)	1.49				
	189 (400 g)	0.94				
5	34 (100 g)	1.95				
	169 (200 g)	0.93	1.16	0.56	48.0	0.75
	220 (300 g)	1.08				
	190 (400 g)	0.68				
6	112 (100 g)	1.78				
	56 (200 g)	1.71	2.06	0.38	18.3	0.96
	158 (300 g)	2.48				
	186 (400 g)	2.26				
7	85 (100 g)	1.00				
	146 (200 g)	1.56	1.47	0.31	21.3	0.99
	142 (300 g)	1.62				
	257 (400 g)	1.68				
8[a)]	74 (100 g)	2.06				
	108 (200 g)	4.00	2.47	0.14	7.10	0.20
	170 (300 g)	2.03				
	134 (400 g)	1.80				
9	47 (100 g)	2.59				
	225 (200 g)	2.25	2.35	0.30	12.9	0.95
	151 (300 g)	1.97				
	224 (400 g)	2.60				
10	73 (100 g)	2.63				
	169 (200 g)	0.83	1.67	0.75	44.9	0.59
	142 (300 g)	1.45				
	257 (400 g)	1.78				
Mean (obs)[c)]	–	1.71	–	0.42	26.1	0.78
Median (obs)	–	–	–	–	–	0.93
SD (obs)	–	0.54	–	–	–	0.18
Adjusted R^2 [d)]	–	–	–	–	–	0.68

a) Data represent the ingested amount of total pelargonidin in mmoles, with the fresh weight (g) of strawberries in parentheses.
b) R^2 values for linear regression of individual data.
c) (obs) indicates values were calculated from the subject direct measurements.
d) Calculated from the complete study data using a simple ANOVA model with 'volunteer' as a fixed effect.

4 Discussion

There is considerable interest in dietary flavonoids as components of human diets that may protect against age-related diseases such as cancer, cardiovascular disease, cataracts, and neurodegenerative diseases including Alzheimer's disease. Previous studies have investigated the absorption of dietary flavonoids in a number of models (*e.g.* cultured cells, animal models) and these have shown that flavonoids are partially bioavailable and they have been measured in plasma and urine of human subjects [17]. In addition, a number of studies have investigated the form of the flavonoids in plasma and urine, and these have provided data to show that flavonoids are usually transformed during absorption, and the common metabolites in plasma and urine are glucuronidated and sulphated derivatives of the parent flavonoid or methylated derivatives [18]. In this report, we have, for the first time, shown that the urinary

excretion of strawberry anthocyanins varies linearly with oral doses representing commonly consumed portions of fresh strawberries. Using strawberries as a source of anthocyanins, we have demonstrated that at the individual level, there appears to be a linear relationship between the oral dose and the total excretion of pelargonidin metabolites in urine (Table 3, Fig. 2). Even though there was three-fold inter-subject variation in urinary yield *per* unit dose (observe the variation in the slopes of the individual regression lines in Fig. 2), the relationship between oral dose and per cent pelargonidin excretion for the study population was highly significant (Table 3). The coefficients of the model indicate that for each additional unit of dose, the urinary excretion increases by 0.0166 units.

Considering the importance of dose–response relationships in pharmacology, and the burgeoning literature concerned with the bioavailability of flavonoids, it is surprising that the data on dose–response relationships for orally dosed flavonoids are so scarce. Almost exclusively, flavonoid bioavailability studies have reported bioavailability from single doses, which provide some information regarding the approximate range of concentrations that might be achieved in plasma and the approximate fractional excretion. However, single dose studies cannot identify saturation effects or absorption thresholds (*i. e.* where a minimum dose is required before absorption is observed; this may occur, for example, if there are efficient but saturateable active processes for luminal efflux from the enterocytes). It is particularly important to establish if absorption can be saturated at higher doses when efforts are underway to increase the concentration of flavonoids in a variety of foods [10, 22] and flavonoid-rich extracts are marketed as dietary supplements (*e. g.* ‚Flavay', http://www.flavonoid.com/; Pycnogenol, http://www.pycnogenol.com/flash/). Karr *et al*. [23] have shown that urinary isoflavone excretion varies linearly with oral dose of soy protein for three doses covering low to moderate intakes. Urinary isoflavone excretion has also been reported as dose dependent in a study concerned with the effects of oral red clover isoflavones on lipoprotein profiles in postmenopausal women [24] and in an animal model of postmenopausal bone loss (three doses [25]). A report concerned with the absorption and excretion of tea catechins in human subjects provided some evidence of saturation for the highest dose, indicated by no significant increase in plasma C_{max} between the intermediate and highest doses (3.0 and 4.5 g tea solids, respectively) [26]. Manach *et al*. [27] reported the excretion of flavanones from two doses of orange juice, 0.5 and 1.0 L. The reported plasma concentrations achieved were more than two times higher for the 1.0 L dose compared to the 0.5 L dose, but the urinary yields were similar. However, it is not possible to determine whether the relationship is linear from this limited data.

Anthocyanins are less bioavailable than the other classes of flavonoids (see [17]). Nevertheless, strawberry anthocyanins represent the most bioavailable anthocyanins reported to date, with mean urinary yields in the region of 2% (this report and ref. [28]). Strawberry anthocyanins are absorbed rapidly from an oral dose, and this may in part be due to rapid and efficient absorption from the stomach, as reported by Talavéra *et al*. [29] who found substantial (19–37%) absorption of anthocyanins within 30 min using an *in situ* gastric administration rat model, and Passamonti *et al*. [30] who measured anthocyanins in rat portal and systemic plasma within 6 min of gastric dosing..

Recently, Borges *et al*. [31] reported the recovery of 1–2% of orally dosed raspberry anthocyanins in rats. The absorption of anthocyanins is strongly influenced by structural features within the molecule. Specifically, it has been demonstrated that (i) anthocyanins containing a single glucose substituent appear more rapidly in plasma during perfusion of the rat small intestine than those containing other sugar substitutions such as anthocyanin rhamnoglucosides (rutinosides) and arabinosides, and (ii) the structure of the anthocyanidin also has an influence [20]. The most bioavailable anthocyanins reported to date are those from red wine (1.5–5.1% [32]) and from strawberries (mean 1.8% [28]), although more recent studies indicating that the total anthocyanin excretion from red wine was <0.20% and was not higher than from red grape juice casts some doubt on the earlier report [33, 34]. Since the predominant anthocyanin in strawberries is pelargonidin-3-glucoside, it is possible that the presence of a single glucose substitution enhances absorption compared to, for example, rutinoside derivatives, for which the reported absorption and excretion yields are at least an order of magnitude lower [35].

Since we and others [28] have shown that pelargonidin is excreted in urine predominantly as phase 2 metabolites (glucuronides and sulphates), deglycosylation of the fruit anthocyanin has occurred during or following absorption. In studies conducted at our laboratory, we have demonstrated the importance of human lactase (lactase-phlorizin hydrolase, LPH) in the small-intestinal deglycosylation of dietary flavonoids. For example, In a Caco-2 cellular model of intestinal absorption, we demonstrated that flavonoid deglycosylation and trans-membrane transport were supported in cells with detectable lactase activity but not in those that lacked lactase [36]. We also determined the specificity of isolated human lactase for a range of flavonoid glycosides, which indicated that human lactase hydrolysed a range of flavonoids, including anthocyanidin-monoglucosides, with apparent affinities (K_m) in the 10s to 100s of micromolar range, but that only monoglucosides and monogalactosides were substrates. We have also reported that another human β-glucosidase present in enterocytes, the broad specificity cytosolic β-glucosidase, was also capable of hydrolysing flavonoid monoglucosides [36, 37], but cyanidin-3-glucoside was apparently not a substrate for this enzyme. We propose that deglycosylation of pelargonidin-3-glucoside most likely occurs through the action of

human lactase, or by an as yet unknown mechanism, during passage across the intestine wall.

In conclusion, we have shown that urinary excretion of strawberry anthocyanins is significantly correlated with oral dose from fresh strawberry fruits across a physiological range of doses, and that the predominant forms of pelargonidin in urine are phase 2 conjugates, indicting that deglycosylation is an important step in the process of absorption and metabolism.

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