Bioavailability of strawberry antioxidants in human subjects

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Strawberries contain many antioxidant phytochemicals such as vitamin C, carotenoids and phenolic compounds including anthocyanins (ACN). In the present study, antioxidant composition of fresh strawberries (FS) and stored strawberries (SS) and the bioavailability of the main strawberry bioactive compounds were determined in human subjects. Thirteen healthy volunteers consumed 300 g of FS and SS on two separate occasions. Blood, before and at different time points from meal consumption, as well as 24 h urine, was collected, and parent compounds and metabolites of the different compounds were determined by HPLC or LC/MS/MS. A reduction in α-carotene plasma concentrations *v.* baseline values was recorded after the consumption of FS, although the amount of this carotenoid was higher in the SS. On the contrary, a significant increase of plasma vitamin C after 2, 3 and 5 h ($P < 0.05$) of FS and SS consumption was recorded. No quercetin and ACN were found in plasma, while coumaric acid, 4-hydroxybenzoic acid (4HBA, 56 and 54 % of pelargonidin-3-glucoside (Pel-glc) ingested with FS and SS, respectively) and protocatechuic acid (59 and 34 % of cyanidin-3-glucoside ingested with FS and SS, respectively) over 8 h from strawberry consumption were retrieved in the plasma. Pelargonidin glucuronide, pelargonidin glucoside and pelargonidin aglycone peaked in urine within 2 h of strawberry consumption, and the 24 h amount excreted was always approximately 0·9 % of the Pel-glc dose ingested. The data indicated that the content of phytochemicals in strawberries may influence the bioavailability of individual compounds. Furthermore, in the present study, the metabolism of Pel-glc was elucidated, and, for the first time, 4HBA was suggested to be a major human metabolite of Pel-glc.

Anthocyanins: Bioavailability: Metabolism: Phenolic acids: Strawberries

Strawberries (*Fragaria* × *ananassa* Duch.) are consumed worldwide, and they represent by far the most common dietary source of anthocyanins (ACN) among red berries.

Strawberries contain many antioxidant phytochemicals such as vitamin C, carotenoids and phenolic compounds including ACN, mainly pelargonidin-3-glucoside (Pel-glc)[1–3]. ACN are water-soluble polyphenolic compounds; they are responsible for the blue, purple and red colours of many plant tissues, and they are found principally in fruits and juices[4,5].

ACN are associated with a wide variety of health benefits including decreased risk of CHD and CVD[6–8], reduced risk of cancer[7,9,10], improved neurofunction[11–13] and protection of brain tissue against hypoxic ischaemic injury[14,15]. Improved vision[16] and memory[17], as well as inhibition of weight gain[18], have also been attributed to ACN.

Health benefits may be due to the high antioxidant activity of ACN demonstrated in various *in vitro*[19–22] and *in vivo* studies[23–25]. However, the bioactivity of all dietary compounds is mediated by their appearance in blood and tissue; thus, bioavailability represents a fundamental issue.

Recent bioavailability studies have demonstrated that ACN are quickly absorbed from the stomach[26,27] and in the small intestine[28], and that they appear in plasma and urine in their parental form or as methylated, glucuronidated or sulphated compounds[29–32]. Previously, low bioavailability has been reported for ACN, and their metabolism is still not fully understood[29,33–37].

Pel-glc has been reported to be the most bioavailable since it can be measured in urine after the ingestion of only low doses of the compound (1·8 % dose of Pel-glc ingested *v.* 0·1 % dose of other ACN)[30,38,39]. Few human studies have dealt specifically with Pel-glc bioavailability, and little conclusive data are available. Strawberries represent an excellent food to study the bioavailability of Pel-glc as they contain – almost exclusively – this ACN.

In the present study, antioxidant composition of fresh and stored strawberries (SS) and the bioavailability of the main

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Abbreviations: 4HBA, 4-hydroxybenzoic acid; ACN, anthocyanins; FS, fresh strawberries; SS, stored strawberries; PCA, protocatechuic acid; Pel-glc, pelargonidin-3-glucoside; Pel-glu, pelargonidin glucuronide; Pel-sulph, pelargonidin sulphated derivatives.

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strawberry bioactive compounds, including Pel-glc, were determined in human volunteers.

Materials and methods

Subjects

Thirteen volunteers (nine males and four females) recruited among the students and personnel of the local universities and research institutes were selected and enrolled into the study. The following criteria were considered: absence of acute or chronic diseases or metabolic disorders, smoking habits (<10 g tobacco/d), moderate alcohol consumption (<30 g/d for men and <20 g/d for women), and taking no drug or vitamin or mineral supplements 2 weeks before the experiments.

The volunteers were aged between 26 and 37 years (26 (SEM 7) years), and they had a mean BMI of 22·6 (SEM 2·6) kg/m^2.

Test meals

Strawberries (cv Favetta) of the same strain and harvested under the same conditions were delivered to the laboratory by a local agricultural producer (Latina, Italy). The strawberries were washed and portioned for immediate consumption (fresh strawberries, FS) or stored in a plastic box at +4°C for 4 d by covering with a cotton cloth (thus allowing fruit respiration and oxygen exchange) (SS). On the test mornings, FS and SS were served in 300 g portions, and they were consumed by the volunteers.

The identity and amount of bioactive compounds ingested by the subjects were characterised using the methods described below.

Study design

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the University of Rome 'La Sapienza' Ethical Committee. Written informed consent was obtained from all the participants before enrolment into the study.

Volunteers followed a low antioxidant diet, excluding some fruits and vegetables and beverages 3 d before each test meal.

On the test mornings, the volunteers presented at the laboratory fasted for at least 12 h, and consumed an allocated portion of either FS or SS. Before and at specific time points after the test meal consumption, blood and urine samples were collected from each subject. In particular, blood samples were drawn at baseline and 0·5, 1, 2, 3, 5, and 8 h after the test meal consumption. Urine was collected at 0–2, 2–4, 4–6, 6–8, 8–12 and 12–24 h after the ingestion of the test meals, and the volume at each interval was recorded. The same protocol was followed on each occasion for each volunteer with an interval of 4 d between the consumption of FS and SS.

Sample treatment

Blood samples were collected in EDTA-containing tubes. After centrifugation at 3000 rpm for 10 min at 4°C, plasma was collected and stored at −80°C for analyses to determine vitamins A, E and C, some carotenoids, anthocyanins and phenolic acids.

Sodium azide (1 g/l) and ascorbic acid (1 g/l) were added to each of the urine samples, which were analysed subsequently for ACN concentration and related metabolites (described below).

Chemicals

All the solvents and reagents were of HPLC or Optima grade; common reagents and standards were purchased from Sigma–Aldrich Srl (Milan, Italy), Extrasynthese (Genay, France), Carlo Erba (Milan, Italy) and BDH Laboratory Supplies (Poole, UK), and were of the highest grade available. Double-distilled water (Millipore, Milan, Italy) was used throughout the study.

Test meal antioxidant characterisation

Carotenoids. Carotenoids were extracted using the method described by Sharpless *et al.*[40]. The determination of carotenoid concentrations was carried out by HPLC as described previously by Maiani *et al.*[41].

Phenolics and total ascorbic acid. Phenolics and total ascorbic acid were extracted from strawberries using the methods described previously by Hertog *et al.*[42] and Margolis *et al.*[43], respectively. The quantitative analyses were performed using an HPLC system equipped with a coulometric detector (ESA model 580; Chelmsford, MA, USA), and data processing was done using a reversed phase with gradient elution. The chromatographic separation was done by applying the methods described by Serafini *et al.*[44].

Anthocyanins. ACN were extracted using a method adapted from Kay *et al.*[45]. The extracts were analysed using a HPLC/MS/MS system, API 3000 triple quadrupole mass spectrometer (Applied Biosystem Sciex, Concord, Ontario, Canada), with a Turboionspray interface, coupled with HPLC binary micropumps (Perkin Elmer, Boston, MA, USA; model Series 200), using the analytical conditions described previously by Vitaglione *et al.*[46].

Biological analyses

Carotenoids (such as lutein, zeaxanthin, cryptoxanthin, lycopene, α-carotene, and β-carotene), vitamin A and vitamin E as well as total vitamin C plasma concentrations were measured using the methods reported previously for the test meal analysis[41,43].

Plasma phenolic compounds (such as protocatechuic acid (PCA), hydroxybenzoic acid, coumaric acid, quercetin and kaempferol) were determined in their free and glycosylated forms, after enzymatic and acidic hydrolysis as described by Serafini *et al.*[44]. Briefly, the enzymatic hydrolysis was performed by incubating plasma with a mixture containing sulphatase and β-glucuronidase (type HP1 from Helix pomatia, Sigma–Aldrich Srl). After acidification and precipitation of the proteins, extraction of phenolic compounds was performed using ethyl acetate, and the quantitative analysis was carried out by HPLC (temperature 30°C; flow rate of 0·8 ml/min; solvent A: 0·02 mol $NaH_2PO_4.H_2O$ adjusted to a pH of 2·8 with 85 % orthophosphoric acid; solvent B: methanol). The linear gradient that was used consisted of 10 % solvent B, increasing to 30 % over 7 min before being held for 19 min, increasing to 33 % over 4 min, and reaching

Table 1. The amount of bioactive compounds ingested by the subjects in the two experimental treatments (300 g)

(Mean values and standard deviations for triplicates)

Bioactive compounds	Fresh strawberries		Stored strawberries		Statistics*
α-Carotene (μg)	20·58	5·48	37·53	7·73	$P<0·05$
Vitamin C (mg)	119·7	23·2	127·3	30·2	NS
Phenolic compounds (mg)					
Coumaric acid	4·44	0·93	4·38	1·52	NS
Quercetin	10·41	4·57	19·79	11·46	$P<0·05$
Kaempferol	5·57	1·71	9·44	3·63	$P<0·05$
Anthocyanins (mg)					
Cyanidin-3-glucoside	0·20	0·12	0·20	0·05	NS
Pelargonidin-3-glucoside	8·93	1·20	6·66	0·97	$P<0·05$
Pelargonidin malonyl glucoside	0·28	0·11	0·22	0·12	NS
Pelargonidin acetyl glucoside	0·16	0·06	0·11	0·03	NS

* Repeated measures $P<0·05$.

100 % over 15 min before being held for 5 min at 100 % and returning to 10 % solvent B over 5 min, where it was maintained for a further 5 min. The setting potentials were 60, 120, 200, 340, 480, 620, 760 and 900 mV.

ACN and their metabolites were extracted from the biological fluids using C18 column solid phase (Supelclean ENVI-18, 6 ml, 500 mg; Sigma) according to the method described by Kay *et al.*[45], and the extracts were analysed using HPLC/MS/MS[46]. The quantification of parent ACN and pelargonidin metabolites was done using a calibration curve constructed using pure Pel-glc.

Statistical analysis

All data were checked for normal distribution using the Shapiro−Wilk test. Student's t test for dependent samples was applied for food and dietary intake. ANOVA for repeated measures and Bonferroni's two-tailed t test for matched pairs were applied, assuming the baseline values as reference category. $P<0·05$ was considered significant. Plasma and urine C_{max} and the time of peak concentration (t_{max}) were recorded. The area under the curves for all the compounds and plasma and urine concentration−time (0−8 h for plasma and 0−24 h for urine) curves using the linear trapezoidal rule were estimated.

Results

Strawberries contain vitamin C, α-carotene, some phenolics and ACN as reported in Table 1. Data showed that the content of α-carotene, quercetin and kaempferol in SS was significantly higher than that of those in FS, while the concentration of Pel-glc was lower. No significant effect of vitamin C concentration was found. These findings are in line with the literature: storage does not necessarily cause a reduction in bioactive content, but may, in fact, increase the total antioxidant content[1,47–49].

Analysis of plasma showed no difference between FS and SS in terms of vitamins A and E (data not shown).

In addition, no traces of circulating quercetin and kaempferol, present in strawberries, were found.

The plasma concentration of lutein plus zeaxanthin, cryptoxanthin, lycopene and β-carotene was not significantly different after acute ingestion of FS *v.* SS (data not shown).

As shown in Fig. 1, mean α-carotene plasma concentration was significantly higher when the subjects consumed FS than when they consumed SS. The concentrations over the 8 h post FS consumption were never significantly different from baseline, and a trend of reduction in α-carotene plasma concentrations, significantly different from baseline only 8 h after SS consumption ($P<0·05$), was found. These data were not consistent with the higher amount of α-carotene ingested with SS compared with that ingested with FS (37·53 (SEM 7·73) *v.* 20·58 (SEM 5·48) μg/300 g test meal, respectively).

As shown in Fig. 2, plasma vitamin C was higher after the consumption of SS than after the consumption of FS (area under the curves being 0·13 (SEM 0·02) mmol × h/l and 0·08 (SEM 0·01) mmol × h/l, respectively; $P>0·05$), although the concentration of vitamin C in the strawberries was the same (127·3 (SEM 30·2) mg in SS *v.* 119·7 (SEM 23·2) mg in FS; $P=$NS).
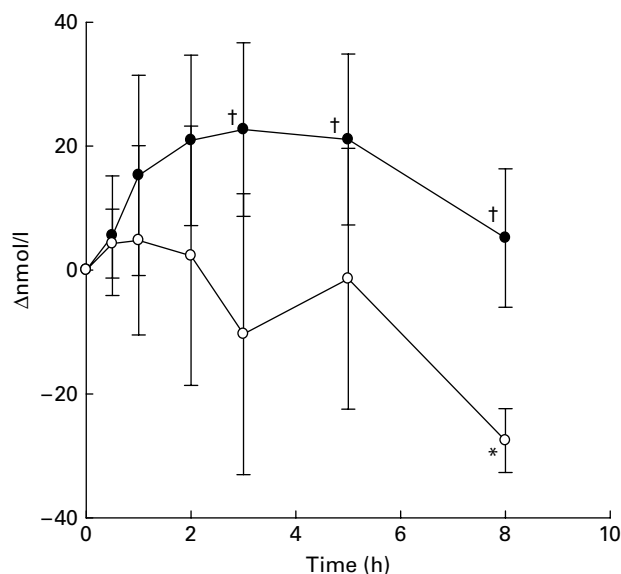

Fig. 1. Variations from baseline of α-carotene plasma concentrations (nmol/l) upon consumption of 300 g fresh strawberries (●) or stored strawberries (○). Values are means with their standard errors (*n* 13). * Mean value was significantly different when compared with baseline ($P<0·05$); † Mean value was significantly different from that for stored strawberries ($P<0·05$).
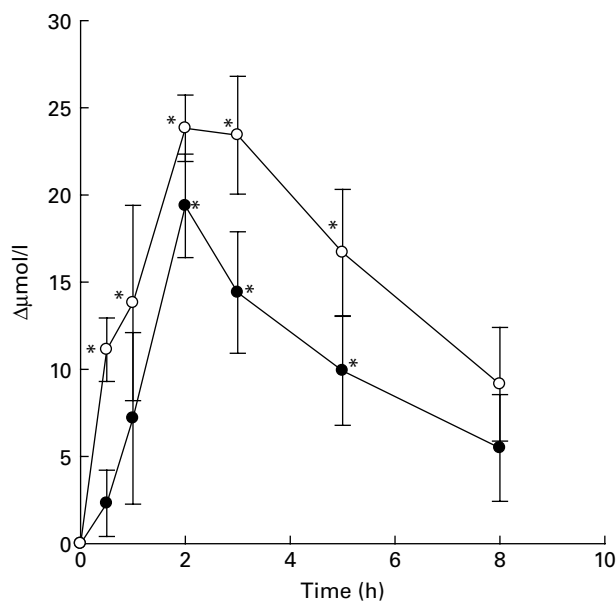
Fig. 2. Variations from baseline of ascorbic acid plasma concentrations (mmol/l) upon consumption of 300 g fresh strawberries (●) or stored strawberries (○). Values are means with their standard errors (*n* 13). * Mean value was significantly different when compared with baseline (*P* < 0.05).

No traces of parent ACN or their conjugated metabolites, namely glucuronidated, methylated and sulphated, were found in the plasma samples. The LC/MS/MS had a lowest limit of detection of 10 μmol/l. In comparison, plasma phenolic acids (namely coumaric acid; PCA, 4-hydroxybenzoic acid, 4HBA) were detected in the plasma collected at baseline, and their concentrations increased after the consumption of both the strawberry meals.

Table 2 and Fig. 3 show that 4HBA was, by far, the main phenolic acid, and that it peaked between 2 and 3 h after strawberry consumption. The concentrations of phenolic acids that were measured in the plasma after the consumption of SS were always lower than those found after the consumption of FS. In particular, a decreased area under the curves of phenolic acid concentration–time curves by 29, 26 and 42 % for coumaric acid, 4HBA and PCA, respectively, for the plasma samples obtained after the consumption of SS compared with those obtained after the consumption of FS was recorded. These data are in line with the decrease of ACN in the SS.

The urine samples were analysed for the presence of phenolic acids and flavonols, but neither traces of them nor free or acylated forms were found.

The 24 h urine samples showed the presence of Pel-glc, the derived aglycone (Pel), and pelargonidin glucuronide (Pel-glu). The mean amounts at each time point are reported in Table 3. For all compounds, the maximum amount was excreted within 2 h of meal consumption, with Pel-glc and Pel-glu being significantly higher after the consumption of FS than after the consumption of SS (30·1 (SEM 4·5) *v.* 15·0 (SEM 1·3) nmol and 312·6 (SEM 34·5) *v.* 233·1 (SEM 16·8) nmol, respectively). These results are consistent with the higher amount of Pel-glc present in the FS than in the SS.

Discussion

The formation of 4HBA from Pel-glc has been demonstrated in some *in vitro* studies and in animals, but not in human subjects, upon consumption of a Pel-glc-rich food. Taking the mean plasma 4HBA recoveries of 23 and 17 mmol (corresponding to the percentages of 54 and 56% of the Pel-glc ingested) after FS and SS consumption, respectively, calculated from areas under the curve of 4HBA between baseline and 8 h, into consideration and by considering a mean volume of 6 litres of blood in the body, it was demonstrated that the formation of the corresponding phenolic acid represents the major metabolic pathway for pelargonidin-3-glucoside metabolism as demonstrated previously in human subjects by Vitaglione *et al.*[46] for cyanidin-3-glucoside upon consumption of blood orange juice. In this case, the authors found a serum recovery of PCA corresponding to 44 % of cyanidin-3-glucosides ingested.

Accordingly, in the present study, PCA was measured at a level corresponding to 59 and 34 % of cyanidin 3-glucoside ingested with FS and SS, respectively. The differences, despite the same intake, are probably due to the influence of other compounds present in the strawberries on cyanidin 3-glucoside metabolism.

In the plasma samples, coumaric acid was found in an amount that was higher than the dose ingested with the strawberries, suggesting that this hydroxycinnamic acid might also derive from the metabolism of other strawberry components.

To the best of our knowledge, only four studies that had dealt with the bioavailability of strawberry ACN in human subjects[30,51−53] have been published previously. Three of them reported only the urinary excretion of ACN and their

Table 2. Data of plasma concentration of phenolic acids*

(Mean values with their standard errors)

	4-Hydroxybenzoic acid				Protocatechuic acid				Coumaric acid			
	Fresh		Stored		Fresh		Stored		Fresh		Stored	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
C_{max} (μmol/l)	2·5	0·7	2·0	0·4	0·17	0·03	0·15	0·03	0·37	0·06	0·29	0·06
AUC (μmol × h/l)	7·6	0·2	5·6	0·2	0·18	0·01	0·10	0·01	0·62	0·02	0·45	0·02
t_{max} (h)	1·9	0·3	1·6	0·2	3·8	0·8	2·9	0·5	1·3	0·4	1·4	0·4

C_{max}, Maximum plasma concentration; AUC, area under the curve from zero to the last sampling time; t_{max}, time to reach the maximum plasma concentration.
* No differences were observed between plasma phenolic acid levels for fresh strawberries *v.* stored strawberries.
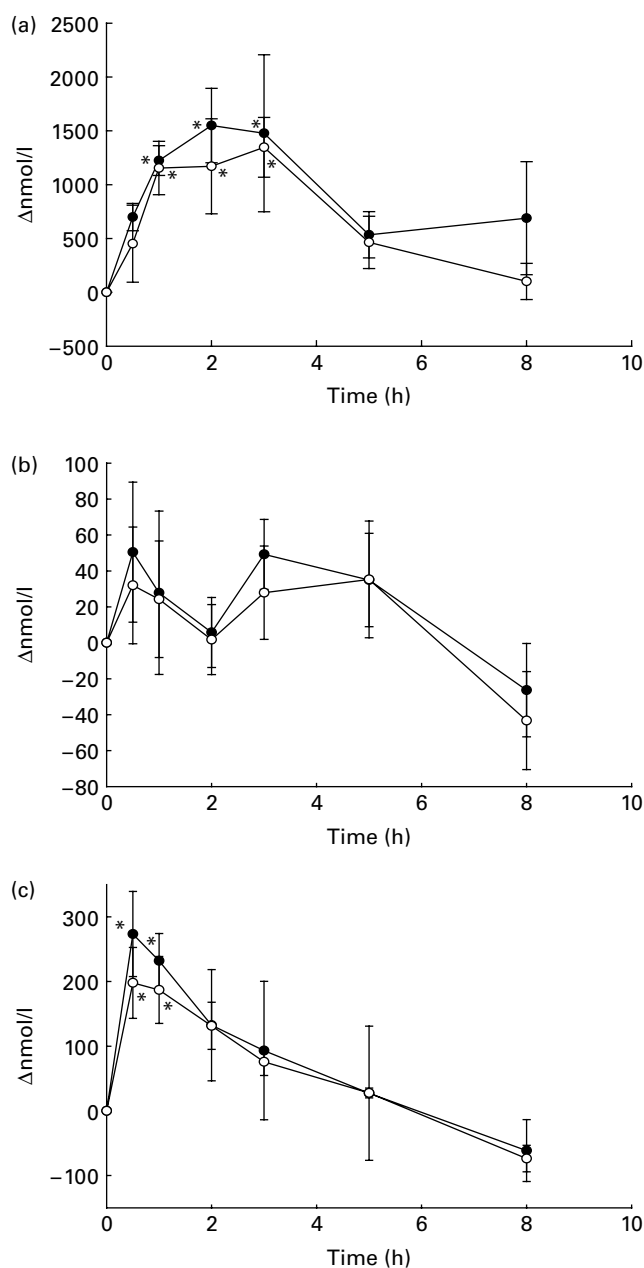
Fig. 3. Plasma phenolic acid concentrations at each monitored time point subtracted from the relative baseline value after the consumption of 300 g fresh strawberries (●) or stored strawberries (○). Values are means with their standard errors. *Mean value was significantly different when compared with baseline (P < 0.05). (a) 4-Hydroxybenzoic acid; (b) protocatechuic acid; and (c) coumaric aid.

metabolites[30,51,52], while in the work by Mullen et al.[53], the plasma concentrations were reported. A comparison between the findings of these studies and the present study is summarised in Table 4.

The data confirmed the findings of all the previous studies in which experimental meal was constituted solely by strawberries (Mullen et al.[53], arm without cream) or by strawberries with a non-energetic sweetener[51]. A delay of maximum urinary excretion up to 4 h was found when strawberries were ingested together with 100 ml of cream[53], or were included in a standard breakfast[30].

Among the urinary metabolites, Pel-glu represented 91 and 95 % of the total compounds after FS and SS consumption, respectively. This result is in accordance with the previous studies reporting that Pel-glu was the most abundant urinary compound in human subjects, ranging from 90 up to 97 % of the total compounds excreted in 24 h independently from the dose of Pel-glc ingested and from the type of the experimental meal[30,51,53]. In the present study and in that by Carkeet et al.[51], the absence of sulphated derivatives (Pel-sulph) might be due to very low metabolite concentrations (under the limit of detection). Anyway, by comparing these studies with the other three human studies, it was deduced that the absence of Pel-sulph reflected the low dose of Pel-glc ingested, which ranged between 13 and 54 μmol in both the studies, compared with the Pel-glc doses which were always higher than 100 μmol in the studies done by Felgines et al.[30], Hollands et al.[52] and Mullen et al.[53]. Thus, it may be hypothesised that at high doses, saturation of Pel-glucuronidation pathway and the initiation of Pel-sulphation pathway may occur. This feature is also consistent with the absence of Pel-sulph compared with the considerable amount of Pel-glu in the 24 h urine collected from subjects after the ingestion of ACN-rich foods in which Pel-glc represented a minor ACN[46].

A general consensus exists on the fact that Pel-glc is more bioavailable than other ACN, as demonstrated by the amount of parent pelargonidin compounds and metabolites excreted v. the Pel-glc dose ingested. In particular, after strawberry consumption, pelargonidin urinary levels have been claimed to range from 0.75 to 2.4 % of the Pel-glc ingested[30,51,52].

In the present study, the pelargonidin recoveries of 0.9 and 0.8 % of Pel-glc dose from FS and SS were slightly higher than those obtained in the study done by Mullen et al.[53], but they were lower than those obtained in studies done by Carkeet et al.[51], Hollands et al.[52] and Felgines et al.[30]. Data suggested that consumption of sweetened strawberries alone or by inclusion in a complete meal (typical breakfast) may increase the bioavailability of Pel-glc.

The slower excretion observed in the study done by Mullen et al.[53] may be explained by co-ingestion of paracetamol and lactulose with strawberries. In fact, paracetamol metabolism comprises a rapid hepatic detoxifying step through glucuronidation by UDP glucuronosyl transferase. Thus, competition between the two substrates (paracetamol and Pel-glc) may delay the glucuronidation of Pel-glc.

The influence of food matrix on the absorption of pelargonidin from strawberries, catechins from cocoa and flavanones from orange juice has been described recently by Mullen et al.[53–55]. In all these studies, the authors concluded that co-ingestion of polyphenols with a source of proteins or fats (cream, milk or full-fat yogurt, respectively) delayed the urinary excretion of the compounds, without a significant modification in the total amount excreted over 24 h or alteration of plasma pharmacokinetics.

Unfortunately, in the studies done by Hollands[52] and Felgines[30], who supplemented the subjects with Pel-glc doses similar to those used by Mullen et al.[53] without paracetamol, blood concentration of Pel-glc and its derived compounds was not reported. The absence of these compounds in the present study may be due to the much lower Pel-glc doses ingested compared with those used in the study done by Mullen et al.[53].

Table 3. Twenty-four-hour urinary excretion of parent anthocyanins and their metabolites from the subjects after the consumption of fresh strawberries (FS) and stored strawberries (SS)

(Mean values with their standard errors (nmol), *n* 13)

| | Pelargonidin | | | | Pelargonidin glucoside | | | | Pelargonidin glucuronide | | | |
| | FS | | SS | | FS | | SS | | FS | | SS | |
Time (h)	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	0		0		0		0		0		0	
0–2	7·9	1·4	8·4	1·7	19·2	3·6	8·2*	1·2	145·9	23·5	102·5*	13·3
2–4	0		0		8·2	0·8	4·0*	0·3	83·1	7·8	64·4*	5·5
4–6	0		0		3·1	0·5	2·8	0·1	50·4	5·4	33·7*	3·3
6–8	0		0		2·2	0·4	0		24·2	7·8	12·9*	1·1
8–24	0		0		0		0		36·3	9·0	19·6*	2·2
Total	7·9	1·4	8·4	1·7	30·1	4·5	15·0*	1·3	312·6	34·5	233·1*	16·8

* Mean value was significantly different from that for SS ($P < 0.05$).

From the results of the present study and from a review of the literature, the main pathways of absorption and metabolism of Pel-glc are proposed in Fig. 4. Briefly, it shows that while a fraction of Pel-glc may be rapidly absorbed through the stomach and may pass through the portal vein into the liver, the other fraction may pass into the small intestine. Hepatic and intestinal pelargonidin glucuronidation and sulphation by UDP glucose dehydrogenase and

Table 4. Comparison of the main characteristics of the present study with those of the other human studies in the literature dealing with pelargonidin glucoside bioavailability upon consumption of a strawberry-based meal

| Subject number | Pel-glc source/dose/exp meal composition (μmol Pel-glc) | Results | | Reference |
		Urine (nmol in 24 h)	Plasma (nmol in 8 h)	
13	FS: Pel-glc 137 nmol/g 300 g FS → 41 μmol Stored (SS): Pel-glc 103 nmol/g 300 g SS → 31 μmol	Pel-glu: 312·6; 233·0 Pel-glc: 30·0; 15·0 Pel: 7·9; 8·4 Total: 350·6; 256·4 (0·9 % dose by FS) (0·8 % dose by SS)	Pel-glu: not found Pel: not found 4HBA: 22 900; 16 800 (56 % of dose by FS) (54 % of dose by SS) PCA: 530; 310 CA: 1850; 1330	The present study
12	Strawberry purée Pel-glc 133·4 nmol/g (1) 100 g → 13·4 μmol (2) 200 g → 26·8 μmol (3) 400 g → 53·6 μmol + one packet of non-energetic sweetener	Pel-glu: 298·1; 646·7; 1125·4 Pel-glc: 22·3; 29·4; 37·4 Total: 320·4; 676·1; 1162·8 (2·4 % dose by 1) (2·4 % dose by 2) (2·1 % dose by 3)	NE	Carkeet *et al.*[51]
10	FS Pel-glc 1224 nmol/g (1) 100 g → 122 μmol (2) 200 g → 244 μmol (3) 300 g → 366 μmol (4) 400 g → 488 μmol + two slices of white toast with spread	Pel-glu + Pel-sulph 1·84 % dose by (1) 1·77 % dose by (2) 1·67 % dose by (3) 1·76 % dose by (4)	NE	Hollands *et al.*[52]
6	Strawberries Pel-glc 895 nmol/g 200 g → 179 μmol +15 g sugar, 60 g bread and 10 g butter	Pel-glu: 2895 Pel-sulph: 134 Pel-glc: 118 Pel: 79 Total: 3226 (1·8 % dose)	NE	Felgines *et al.*[30]
6	Strawberries Pel-glc 1110 nmol/g 200 g → 222 μmol (1) without 100 g cream (2) with 100 g cream +1 g paracetamol and 5 g lactulose	Pel-glu: 1611; 2098 Pel-glc: 31; 55 Pel-sulph: 3; 3 Pel: 26; 68 Total: 1672; 2217 (0·75 % dose by 1) (1 % dose by 2)	Pel-glu: approximately 2568; 2685* (1·1 % dose by 1) (1·2 % dose by 2) Pel: trace amounts	Mullen *et al.*[53,54]

Pel-glc, pelargonidin-3-glucoside; FS, fresh strawberries; Pel-glu, pelargonidin glucuronide; Pel, pelargonidin; 4HBA, 4-hydroxybenzoic acid; PCA, protocatechuic acid; CA, coumaric acid; NE, not evaluated; Pel-sulph, pelargonidin sulphated derivatives.
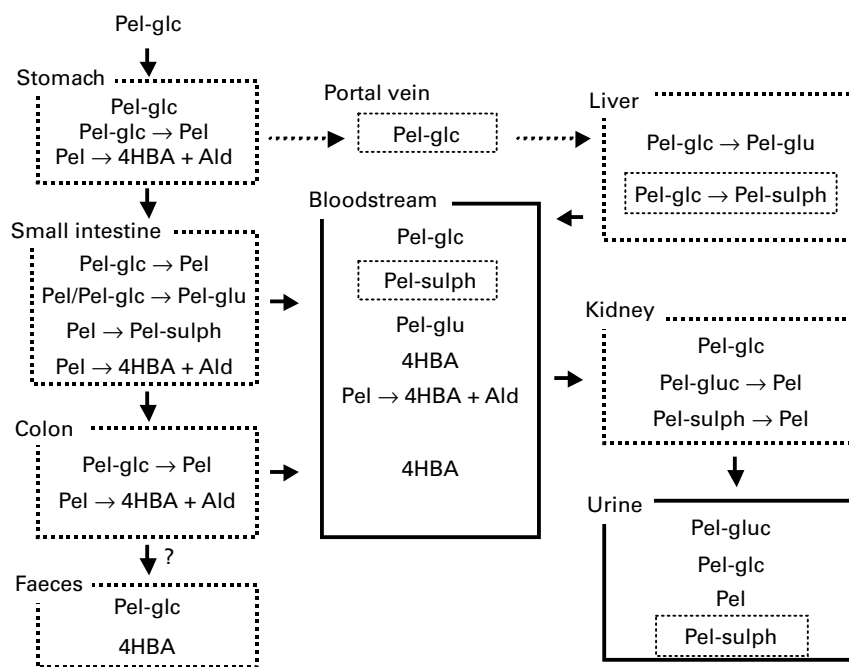* Calculated from AUC (0–8) considering a 3 litres mean amount of plasma.

Fig. 4. Proposed pathways for the absorption and metabolism of pelargonidin-3-glucoside (Pel-glc) in human subjects as indicated by the results of the present study and literature data. 4HBA, 4-hydroxybenzoic acid; Pel-glu, pelargonidin glucuronide; Pel-sulph, pelargonidin sulphated derivatives Ald, aldehyde.

sulphotransferases, via a pathway that requires deglycosylation of the ACN or that directly depends on the Pel-glc, may occur[56,57]. Pel-glu and Pel-sulph formed in the liver and in the small intestine pass into the bloodstream. Pel aglycone in the stomach, in the intestine or, after absorption, in the plasma may be rapidly degraded to 4HBA because of its chemical instability[50]. This figure showing the formation from Pel-glc is consistent with the time course of 4HBA concentration in plasma (Fig. 3). 4HBA plasma concentration is already high at 30 min, peaking at 2 h after strawberry consumption, and a role of gut microflora in this time window can be ruled out. Many studies indicated that 4HBA can also be formed by the degradation of quercetin as well as of catechin and procyanidins upon incubation *in vitro* with human faecal microflora[58–60]. Quercetin and procyanidins are present in strawberries[61]; however, using the time–concentration curves of plasma phenolic acids (see Fig. 3), no significant variation from the baseline concentrations was reported for 4HBA, PCA and coumaric acid 5 and 8 h after food consumption, which is the mean time approximately taken by the bolus to arrive to the colon[62]. Thus, it can be argued that the high concentration of 4HBA found in serum up to 5 h derives almost exclusively from Pel-glc through its chemical degradation along the gastrointestinal tract. At the same time, the presence of 4HBA in the plasma and of other phenolic acids in the blood samples collected 8 h after strawberry consumption could also be related to the other flavonoids present in the fruit.

The presence of 4HBA and Pel-glc in faeces has been hypothesised on the basis of animal studies[39,50,63], but this has not been demonstrated in human subjects yet. Further metabolism of Pel-glu and Pel-sulph due to the activity of glucuronidases and sulphatases present in the kidney and urine may explain the presence of Pel aglycone in urine[64,65].

In conclusion, data obtained in the present study show that storage of strawberries modifies relative content of some bioactive compounds. A significant increase in α-carotene, quercetin and kaempferol contents over strawberry storage is accompanied by a significant decrease in Pel-glc content.

The altered composition of FS and SS influenced the human absorption and metabolism of strawberry bioactive compounds. The present results contribute to the understanding of the absorption, metabolism and excretion of pelargonidin in human subjects. In the present study, a correlation between Pel-glc content in fruits and pelargonidin urinary excretion as parent compounds or metabolites was found. 4HBA was demonstrated to be the major human metabolite of Pel-glc at least over 8 h of strawberry consumption, with it being present in the plasma at mean levels of approximately 55 % of the Pel-glc dose ingested; this result confirms the fundamental role of the phenolic acids in the human metabolism of ACN.

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