Absorption and metabolism of red orange juice anthocyanins in rats

Catherine Felgines[1]*, Séverine Talavéra[1], Odile Texier[1], Catherine Besson[2], Vincenzo Fogliano[3], Jean-Louis Lamaison[1], Luca la Fauci[4], Giacomo Galvano[5], Christian Rémésy[2] and Fabio Galvano[4]

[1]*Laboratoire de Pharmacognosie, Faculté de Pharmacie, Clermont-Ferrand, France*
[2]*Laboratoire des Maladies Métaboliques et des Micronutriments, Institut National de la Recherche Agronomique de Clermont-Ferrand/Theix, France*
[3]*Department of Food Science, University of Naples 'Federico II', Naples, Italy*
[4]*Department of Agro-forestry and Environmental Science, Mediterranean University of Reggio Calabria, Italy*
[5]*Department of Agronomical, Agrochemical and Animal Production Science, University of Catania, Italy*

Anthocyanins are natural pigments that could be involved in various health effects. Red oranges are an important dietary source of anthocyanins, including cyanidin 3-glucoside (Cy 3-glc) and an acylated derivative, cyanidin 3-(6″-malonyl)-glucoside (Cy 3-malglc). The aim of this study was to evaluate the absorption and metabolism of red orange anthocyanins in rats fed an anthocyanin-enriched diet for 12 d (approximately 2·8 μmol anthocyanins/d). Furthermore, the absorption of these anthocyanins was studied in both the stomach and intestine using *in situ* models in rats. Anthocyanin metabolites were identified and quantified by HPLC–electrospray ionization tandem MS and HPLC–diode array detection, respectively. The red orange anthocyanins, Cy 3-glc and Cy 3-malglc, as well as their respective methylated derivatives, were recovered in urine after red orange juice intake. The 24 h urinary excretion of total anthocyanins was low (0·081 (SEM 0·009) % of the ingested amount). However, a high proportion (about 20 %) of red orange anthocyanins was absorbed from the stomach. More Cy 3-malglc than Cy 3-glc was absorbed in the intestine. This study thus indicated that red orange juice anthocyanins were rapidly absorbed from both stomach and small intestine, and then excreted in the urine as intact and methylated forms. Moreover, the absorption and metabolism of acylated anthocyanins and non-acylated anthocyanins were similar.

Red orange anthocyanins: Rat: Absorption: Metabolism: Acylation

Anthocyanins (from the Greek *antos*, flower, and *kyanos*, blue) are natural pigments widely distributed in fruits. They are important in nutrition because their daily intake, which is estimated at around 200 mg in the USA, is higher than that of other flavonoids (Kühnau, 1976). Anthocyanins play a role in a wide range of biological activities. They may reduce the risk of coronary heart disease (Renaud & de Lorgeril, 1992), reduce inflammatory insult (Youdim *et al.* 2002), modulate the immune response (Wang & Mazza, 2002) and exert anticarcinogenic activities *in vitro* (Fimognari *et al.* 2004). These actions might be mediated by their antioxidant activity (Tsuda *et al.* 1998; Mazza *et al.* 2002) owing to their particular chemical structure, which is characterized by an electron deficiency making them particularly reactive to reactive oxygen species (Galvano *et al.* 2004).

Although numerous studies have evaluated the absorption and metabolism of anthocyanin glycosides (Miyazawa *et al.* 1999; Tsuda *et al.* 1999; Bub *et al.* 2001; Cao *et al.* 2001; Matsumoto *et al.* 2001; Felgines *et al.* 2002; Wu *et al.* 2002; Frank *et al.* 2003; McGhie *et al.* 2003; Nielsen *et al.* 2003; Bitsch *et al.* 2004; Cooney *et al.* 2004; Ichiyanagi *et al.* 2004, 2005; Kay *et al.* 2004; Talavéra *et al.* 2006), only a few reports are available on the absorption and metabolism of acylated anthocyanins (Suda *et al.* 2002; Harada *et al.* 2004). However, these acylated compounds are present in a number of edible plants, including red oranges, red cabbage and purple sweet potatoes (Giusti & Wrolstad, 2003; Galvano *et al.* 2004). Pigmented oranges (*Citrus sinensis* varieties: Moro, Tarocco and Sanguinello), also known as 'red' or 'blood' oranges and typically grown in the Etna volcano region of Sicily (Italy) as well as in Florida (USA), are an important dietary source of anthocyanins such as cyanidin 3-glucoside (Cy 3-glc) and cyanidin 3-(6″-malonyl)-glucoside (Cy 3-malglc), responsible for their brilliant red colour. However, no data are available on the bioavailability of red orange juice anthocyanins. Thus, the aim of this study was to evaluate the absorption and metabolism of red orange juice anthocyanins in rats fed for 12 d with an anthocyanin-enriched diet ('adapted rats'). In addition, the absorption of these anthocyanins was studied using *in situ* models in rats (Talavéra *et al.* 2003, 2004).

Materials and methods

Chemicals

Cy 3-glc and cyanidin 3,5-diglucoside were purchased from Extrasynthèse (Genay, France). Red orange juice lyophilisate was supplied by Liofil S.r.l. (Ribera, Sicily, Italy).

Animals and diets

'Adapted rat' study. Twelve male Wistar rats (Iffa-Credo, L'Arbresle, France) weighing approximately 200 g were housed two per cage in temperature-controlled rooms (22°C), with the a controlled dark period from 08.00 to 20.00 h and access to food from 08.00 to 16.00 h. They were fed a semi-purified control diet (755 g/kg wheat starch, 150 g/kg casein, 50 g/kg peanut oil, 35 g/kg AIN-93M mineral mixture, 10 g/kg AIN-76A vitamin mixture) for 6 d (Felgines *et al.* 2002). They were then individually housed in metabolic cages fitted with urine–faeces separators and received the control diet supplemented with red orange juice lyophilisate (200 g/kg diet) for 12 d. The rats ate approximately 16·5 g/d diet, i.e. approximately 3·3 g/d red orange juice lyophilisate. To quantify dietary anthocyanin content, red orange juice lyophilisate (1 g) was treated for 30 min under agitation with 45 ml methanol–water (50:50 v/v). After filtration, the volume of solution was adjusted to 50 ml, and this solution was diluted twice with 0·12 M-HCl before HPLC analysis (20 μl) as described below.

In situ experiments. Twelve male Wistar rats (Iffa-Credo) weighing approximately 200 g were housed two per cage in temperature-controlled rooms (22°C), with a controlled dark period from 20.00 to 08.00 h and access to food from 16.00 to 08.00 h. They were fed the semi-purified control diet for 2 weeks.

All animals were maintained and handled according to the recommendations of the Institutional Ethic Committee (INRA), in accordance with French decree No 87–848.

Sampling procedure during 'adapted rat' study

Rats were killed 3 h or 6 h after the beginning of the last experimental meal (i.e. at 11.00 h and 14.00 h, respectively) after being anaesthetised with sodium pentobarbital (40 mg/kg body weight). Blood was withdrawn from the abdominal aorta into heparinised tubes. Plasma was acidified with 240 mM-HCl. Urine present in the bladder was collected and acidified with 240 mM-HCl. The day before the rats were killed, urine was collected over 24 h in tubes containing 1 ml 1·8 M-HCl. Collecting urine on HCl allowed a regeneration of the coloured structure of anthocyanins and thus increased their stability. We previously ensured that Cy 3-malglc was stable in these acidic conditions. Caecal contents were drained by finger pressure into microfuge tubes. All samples were rapidly frozen and stored at −20°C until analysis.

Anthocyanin administration during in situ experiments

Rats fasted for 24 h were anaesthetised with sodium pentobarbital (40 mg/kg body weight, intraperitoneal injection) and kept alive under anaesthesia throughout the experiments.

In situ gastric administration. After cannulation of the bile duct, the pylorus was ligated and a physiological buffer was injected into the stomach across the cardia. This sphincter was ligated to prevent any gastro-oesophageal reflux. The stomach was filled *in situ* with 5 ml of a buffer specially developed to mimic the osmotic and pH conditions found in the stomach during a meal. This buffer (pH 3) contained KH_2PO_4 (7·5 mM), NaCl (50 mM), KCl (50 mM), $CaCl_2$ (2 mM), acetic acid (25 mM), lactic acid (25 mM), $MgSO_4$ (1 mM) and polyethylene glycol 6000 (5 g/l) and was maintained at 37°C (Talavéra *et al.* 2003). It was supplemented with approximately 22 μmol/l of red orange juice anthocyanins. The amounts of anthocyanins infused into the stomach (about 110 nmol) were consistent with those previously used in similar models (Crespy *et al.* 2002; Passamonti *et al.* 2003; Talavéra *et al.* 2003). Red orange anthocyanin extract was obtained as described later. At 30 min after administration, the stomach contents were collected and blood samples were withdrawn from the abdominal aorta into heparinised tubes. Bile was collected throughout the 30 min of the experiment. Plasma and bile samples were acidified with 240 mM-HCl, whereas the stomach contents were acidified with 40 mM-HCl. All samples were stored at −20°C until analysis.

In situ intestinal perfusion. After cannulation of the bile duct, a perfusion of the jejunoileal segment of the intestine (from 5 cm distal to the duodenojejunal flexure up to the ileocaecal valve) was prepared by installing cannulas at each extremity. This segment was continuously perfused *in situ* for 45 min at a flow rate of 0·75 ml/min with a buffer containing KH_2PO_4 (5 mM), K_2HPO_4 (2·5 mM), $NaHCO_3$ (5 mM), NaCl (50 mM), KCl (40 mM), $CaCl_2$ (2 mM), $MgSO_4$ (1 mM), $K_3C_6H_5O_7$ (10 mM), glucose (12 mM), glutamine (2 mM) and taurocholic acid (1 mM), pH 6·6, at 37°C (Talavéra *et al.* 2004). The buffer was supplemented with approximately 22 μmol/l of red orange anthocyanins. Red orange anthocyanin extract was obtained as described later. The intestine was washed of its contents during the first 25 min. Effluents were directly collected at the exit of the ileum during the last 5 min of perfusion. Effluent volume was estimated by weighing. Bile was collected throughout the 45 min of the experiment. At the end of the experiment, blood samples were withdrawn from the abdominal aorta into heparinised tubes. Urine present in the bladder was also collected. Perfused solution, effluent, bile, plasma and urine samples were rapidly acidified with 240 mM-HCl and stored at −20°C until analysis.

To determine the stability of anthocyanins throughout the *in situ* perfusion experiment (at 37°C, pH 6·6), an aliquot of the perfused buffer maintained at 37°C was collected at the beginning (t = 0), at t = 25 min and at the end (t = 45 min) of the perfusion period, and the anthocyanins were analysed by HPLC after acidification with 240 mM-HCl, as described later. The overall percentage of degradation was calculated by the decrease in anthocyanin concentrations between 0 and 45 min. Moreover, anthocyanin degradation was a linear function of time. Thus, the amounts of anthocyanin perfused were determined from the mean of the anthocyanin concentrations in the perfused buffer at t = 0 and t = 45 min.

Red orange anthocyanin extract was prepared from red orange juice lyophilisate. It was obtained from 40 g powder treated for 30 min under agitation with 400 ml methanol and then filtered. The filtrate was evaporated to dryness using a rotary evaporator at 35°C, and then dissolved in

20 ml 0·12 M-HCl and briefly sonicated. The solution was centrifuged for 5 min at 12 000 **g** at room temperature, and the supernatant was later diluted 20-fold in the gastric or intestinal buffer to obtain a final concentration of approximately 22 μmol/l Cy 3-glc equivalents.

Sample preparation

Anthocyanins present in the urine samples were extracted using a solid phase extraction cartridge (Sep-Pak C$_{18}$ Plus; Waters, Milford, MA, USA), as follows. The cartridge was washed with 10 ml methanol and equilibrated with 10 ml 12 mM-aqueous HCl before use. Twenty-four-hour urine samples spiked with 3 nmol cyanidin 3,5-diglucoside as an internal standard were loaded onto the cartridge. The cartridge was then washed with 10 ml 12 mM-aqueous HCl, and anthocyanins were eluted with 3 ml 12 mM-HCl in methanol. The methanolic extract was evaporated to dryness under reduced pressure using a rotary evaporator at 35°C. The dried extract was dissolved with 300 μl 0·12 M-aqueous HCl. After centrifugation for 5 min at 12 000 **g**, the supernatant (60 μl) was analysed by HPLC as described below.

Caecal contents (0·2 g) were extracted with 1·8 ml of water–acetone (1:1 v/v) containing 500 mM-HCl, and then briefly sonicated and centrifuged for 5 min at 12 000 **g** at room temperature. Supernatants were evaporated under a stream of N$_2$ gas to half their initial volume to eliminate the acetone. Finally, aqueous extracts (60 μl) were analysed for anthocyanin content as described later.

Stomach contents were centrifuged for 5 min at room temperature, and then filtered on fritted glass and analysed (20 μl) by HPLC. Absorption through the gastric wall was estimated by the difference between the amount of anthocyanins administered into the stomach and the amount recovered at the end of incubation.

After centrifugation for 5 min at 12 000 **g**, the supernatants of intestinal effluents were analysed (20 μl) by HPLC as described later. All the concentrations measured in the effluent samples were corrected by taking into account the intestinal absorption of water. Water absorption was estimated by calculating the difference between effluent flow and perfusion flow (0·75 ml/min). Absorption through the intestinal barrier was estimated by calculating the difference between the amount of anthocyanins administered through the intestinal segment and the amount recovered at the end of the ileal segment. These amounts were determined for the last 5 min of perfusion. Anthocyanin stability was also taken into account in evaluating intestinal absorption.

HPLC analysis

Quantification of anthocyanins was performed by HPLC using a photodiode array detector (DAD 200; Perkin Elmer, Courtabœuf, France) and a UV-visible detector (785A; Perkin Elmer) at 524 nm. Samples were loaded onto a 150 mm × 4·6 mm Hypersil C18-5 μm column protected by a 10 × 4 mm Hypersil C18-5 μm guard column (Interchim, Montluçon, France) and analysed as previously described (Felgines *et al.* 2002). Anthocyanin quantification was expressed as Cy 3-glc equivalents.

Red orange anthocyanins and anthocyanin metabolites were identified by HPLC–electrospray ionization tandem MS

analysis. These analyses were performed on a Hewlett-Packard HPLC system equipped with MS–MS detection (API 2000; Applied Biosystems, Les Ulis, France) as previously described (Felgines *et al.* 2003). The MS data were collected in multiple reaction monitoring mode by monitoring the transition of parent and product ions specific for each compound, using a dwell time of 0·5 s. Anthocyanins were detected according to the respective *m/z* values of their parent and product ions: Cy 3-glc, 449/287; Cy 3-malglc, 535/287; methyl Cy 3-glc, 463/301; methyl Cy 3-malglc, 549/301.

Polyethylene glycol measurements

Polyethylene glycol, a compound that is not absorbed by the stomach, was added to the gastric buffer. Its concentration in the gastric buffer was determined by the method of Powell and Malawer (1968). The ratio between the initial concentration and that measured at the end of the experiment reflected the intensity of gastric secretion (gastric volume at the end of the experiment: approximately 5·8 ml). This parameter has to be taken into account in order to obtain the correct concentration of anthocyanins at the end of the experiment.

Data analysis

Values are given as means with their standard errors. When appropriate, significance of differences between values was determined by unpaired *t* test (GraphPad; Instat, San Diego, CA, USA). Values of $P < 0.05$ were considered significant.

Results

'Adapted rat' study

The HPLC profile of red orange juice lyophilisate showed two main peaks as well as other smaller ones (Fig. 1(a)). Peaks 1
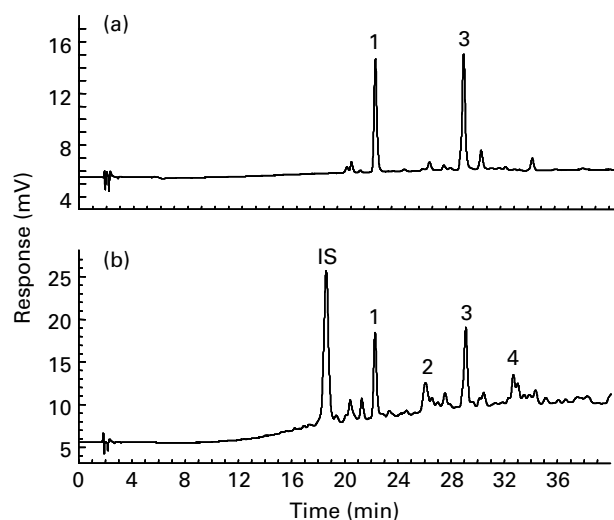

Fig. 1. HPLC chromatogram of red orange juice lyophilisate (a) and 24 h urine from rats fed red orange juice (b). Detection was performed at 524 nm. IS, internal standard (cyanidin 3,5-diglucoside). Peaks are as follows: (1) cyanidin 3-glucoside, (2) methyl cyanidin 3-glucoside, (3) cyanidin 3-malonylglucoside, (4) methyl cyanidin 3-malonylglucoside.

and 3 were identified as Cy 3-glc and Cy 3-malglc, respectively. Concentrations of Cy 3-glc and Cy 3-malglc in the anthocyanin-supplemented diet were $74.4\ \mu mol/kg$ and $90.4\ \mu mol/kg$, respectively. Therefore, rats ate approximately $1.23\ \mu mol/24\,h$ and $1.50\ \mu mol/24\,h$ of Cy 3-glc and Cy 3-malglc, respectively.

Several peaks were detected in the urine from red orange anthocyanin-fed rats (Fig. 1(b)). Urine collected in the bladder and 24 h urine both presented the same HPLC profile. The urinary HPLC profile showed red orange juice anthocyanins as well as methylated derivatives. The presence of red orange anthocyanins, Cy 3-glc (peak 1) and Cy 3-malglc (peak 3) was confirmed by HPLC–electrospray ionization tandem MS analysis by detection of their specific parent and product ions (m/z values 449/287 and 535/287, respectively; Fig. 2(c), (e)). Peak 2 was identified as methyl Cy 3-glc according to its parent and product ion pair (463/301; Fig. 2(d)). Peak 4 displayed a parent and product ion pair at m/z 549/301, suggesting the presence of methyl Cy 3-malglc (Fig. 2(f)). The mean urinary excretion of anthocyanins over a 24 h period was estimated taking account of these four major peaks (Table 1). The data demonstrated that only a negligible amount of the anthocyanins (below 0.1 %) was recovered in the urine.

The caecal contents contained Cy 3-glc and Cy 3-malglc as well as the other red orange juice anthocyanins (Fig. 3). A low amount of cyanidin was also recovered. Total caecal anthocyanins and cyanidin contents were 12.5 (SEM 2.0) nmol and 15.9 (SEM 2.2) nmol (n 6) at 3 and 6 h after the beginning of the last meal, respectively. Caecal Cy 3-glc content was significantly higher at 6 h than at 3 h ($P<0.05$).

In situ *gastric administration*

We first ensured that red orange anthocyanins were stable under the experimental conditions, i.e. incubated for 30 min at 37°C in the gastric buffer (pH 3).

About 20 % of the anthocyanins were absorbed from the gastric lumen (Table 2). Absorption of Cy 3-glc at the gastric level was not significantly different from that of Cy 3-malglc. No metabolites of anthocyanins were observed in the stomach contents after 30 min of incubation. HPLC analysis of urine revealed the presence of native red orange juice anthocyanins (Cy 3-glc, Cy 3-malglc) as well as their methylated derivatives. The low doses infused did not enable the detection of metabolites in either bile or plasma.

In situ *intestinal perfusion*

The overall percentage of anthocyanin degradation during the 45 min of perfusion was less than 2 %.

HPLC profiles were similar between the effluents and the red orange anthocyanin extract perfused. Absorption from the intestinal lumen was about 10 % (Table 3), and there was a higher intestinal absorption of Cy 3-malglc than Cy 3-glc ($P<0.05$). Red orange anthocyanins as well as their methylated derivatives were detected in urine collected after *in situ* intestinal perfusion. No anthocyanin was detected in plasma or bile samples owing to the low amount of anthocyanins perfused.
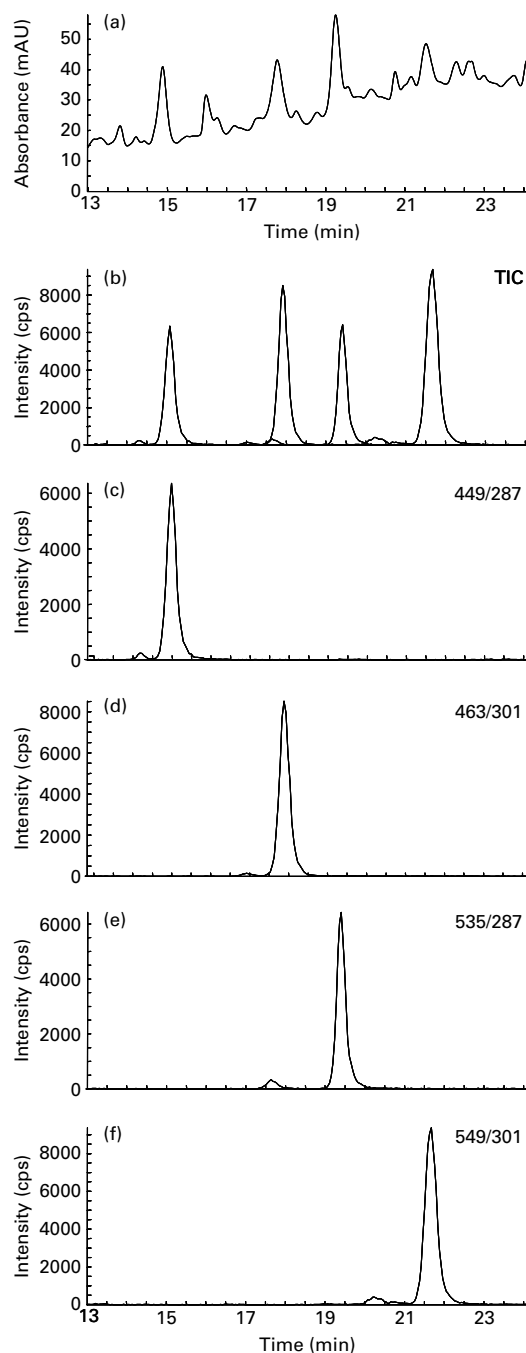

Fig. 2. HPLC–electrospray ionization tandem MS analysis of anthocyanin metabolites in urine from rats fed red orange juice. (a) Detection at 524 nm. Detection of the respective m/z values of the parent and product ions: (b) total ionic current (TIC), (c) cyanidin 3-glucoside, (d) methyl cyanidin 3-glucoside, (e) cyanidin 3-malonylglucoside, (f) methyl cyanidin 3-malonylglucoside.

Discussion

The aim of this study was to evaluate the bioavailability and sites of absorption of anthocyanins contained in red oranges. A preliminary step involved determining the anthocyanin content of the red orange juice lyophilisate used in these experiments. The red orange juice contained two major anthocyanins, Cy 3-glc and Cy 3-malglc, as previously reported

Table 1. Urinary anthocyanin excretion following ingestion of red orange anthocyanins

(Means and their standard errors, *n* 9)

Compound	Anthocyanin ingestion (μmol/24 h)		Urinary anthocyanin excretion			
			(nmol/24 h)		(% of amount ingested)	
	Mean	SEM	Mean	SEM	Mean	SEM
Cy 3-glc	1·23	0·05	0·71	0·14	0·057	0·011
Methyl Cy 3-glc			0·47	0·05	0·038†	0·003
Cy 3-malglc	1·50	0·06	0·68	0·12	0·045	0·008
Methyl Cy 3-malglc			0·38	0·08	0·025‡	0·004
Total anthocyanins	2·73	0·11	2·24	0·29	0·081	0·009

Cy 3-glc, cyanidin 3-glucoside; Cy 3-malglc, cyanidin 3-(6″-malonyl)-glucoside.
† Related to Cy 3-glc ingestion.
‡ Related to Cy 3-malglc ingestion.

(Lee, 2002; Dugo *et al.* 2003; Hillebrand *et al.* 2004). These two pigments accounted for more than 82 % of the total anthocyanins.

Although numerous studies have focused on anthocyanin glycoside bioavailability (Miyazawa *et al.* 1999; Tsuda *et al.* 1999; Bub *et al.* 2001; Cao *et al.* 2001; Matsumoto *et al.* 2001; Felgines *et al.* 2002; Wu *et al.* 2002; Frank *et al.* 2003; McGhie *et al.* 2003; Nielsen *et al.* 2003; Bitsch *et al.* 2004; Cooney *et al.* 2004; Ichiyanagi *et al.* 2004, 2005; Kay *et al.* 2004; Talavéra *et al.* 2006), there have been very few attempts to evaluate the absorption and metabolism of acylated anthocyanins. Previous studies have investigated only the absorption of complex acylated anthocyanins from purple sweet potato in rats receiving a single bolus of anthocyanins by gavage (Suda *et al.* 2002; Harada *et al.* 2004). Our study reports the first information on the urinary excretion of acylated anthocyanins after the adaptation of rats to an anthocyanin-enriched diet for 12 d. This model mimics a regular intake of anthocyanins. Expressed as dry weight, rats eat approximately 20 g/d diet whereas humans eat in the region of 400 g/d food. Therefore the consumption of approximately 3·3 g/d red orange juice lyophilisate by rats would correspond to about 66 g/d in human subjects, i.e. about
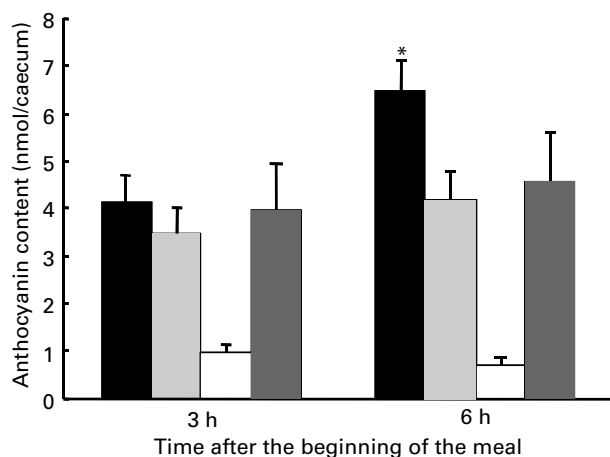

Fig. 3. Caecal anthocyanin contents of rats fed red orange juice anthocyanins. Values are means with their standard errors, shown by vertical bars (*n* 6). *P< 0·05. ■, Cyanidin 3-glucoside; ▨, Cyanidin 3-(6″ malonyl)-glucoside; □, Cyanidin; ▨, Other anthocyanins .

Table 2. Anthocyanin absorption after a 30 min administration of red orange anthocyanins into the stomach of rats

(Means and their standard errors, *n* 5)

Compound	Anthocyanins injected into the gastric lumen (nmol)		Anthocyanins absorbed from the gastric lumen (nmol)		Anthocyanin absorption from the gastric lumen (% of the injected dose)	
	Mean	SEM	Mean	SEM	Mean	SEM
Cy 3-glc	52·8	0·5	11·1	1·5	20·9	2·7
Cy 3-malglc	55·3	0·3	9·85	1·21	17·8	2·2

Cy 3-glc, cyanidin 3-glucoside; Cy 3-malglc, cyanidin 3-(6″-malonyl)-glucoside.

660 ml red orange juice since the dry content of this juice is approximately 100 g/l. Such an amount can easily be consumed daily by humans. Red orange juice anthocyanins were recovered in the urine and were accompanied by methylated derivatives of both Cy 3-glc and Cy 3-malglc. These methylated conjugates could result from hepatic methylation at the 3′ hydroxyl moiety position of cyanidin derivatives by catechol *O*-methyltransferase, as previously described (Felgines *et al.* 2002; Ichiyanagi *et al.* 2005; Talavéra *et al.* 2005).

The percentage of red orange anthocyanins and their methylated derivatives recovered in the urine (calculated as the ratio of anthocyanins excreted to anthocyanins ingested) was low (about 0·08 %). Given that the methyl Cy 3-glc and methyl Cy 3-malglc found in the urine resulted from the methylation of Cy 3-glc and Cy 3-malglc, respectively, their excretion could be linked to the ingestion of Cy 3-glc and Cy 3-malglc. We can thus consider that the urinary recovery of Cy 3-glc and Cy 3-malglc as either the intact or the methylated forms was in the region of 0·095 % and 0·070 % of the ingested amounts, respectively. Although there are scarce quantitative data on the urinary excretion of anthocyanins in rats, the urinary recovery of red orange anthocyanins was of the same order of magnitude as previously reported (Felgines *et al.* 2002; Harada *et al.* 2004; Talavéra *et al.* 2006). Moreover, these results clearly show that acylation did not affect the absorption, metabolism or excretion of anthocyanins. In the present study, we failed to detect

Table 3. Anthocyanin absorption after perfusion of red orange anthocyanins through the intestinal lumen of rats

(Means and their standard errors, *n* 5)

Compound	Anthocyanins perfused through the intestinal lumen† (nmol)		Anthocyanins absorbed from the intestinal lumen† (nmol)		Anthocyanin absorption from the intestinal lumen (% of the perfused dose)	
	Mean	SEM	Mean	SEM	Mean	SEM
Cy 3-glc	65·2	11·0	4·31	0·52	6·79	0·37
Cy 3-malglc	69·9	11·7	9·40*	1·85	13·3*	0·6

Cy 3-glc, cyanidin 3-glucoside; Cy 3-malglc, cyanidin 3-(6″-malonyl)-glucoside.
Mean value was significantly different from that for Cy 3-glc: *P < 0·05.
† These amounts correspond to the last 5 min of perfusion.

glucurono-conjugates of anthocyanins in the urine. This was probably due to the low amounts of anthocyanins present in the diet. Indeed, we have previously identified cyanidin and peonidin monoglucuronides in urine from rats that had ingested 80-fold higher anthocyanin contents than in the present study, but these metabolites were present only in very small proportions (Talavéra *et al.* 2005).

Analysis of anthocyanins in the caecal contents revealed the presence of red orange anthocyanins as well as cyanidin. We have previously recovered glycosides as well as their corresponding aglycones in caecal contents from blackberry or bilberry anthocyanin-fed rats (Felgines *et al.* 2002; Talavéra *et al.* 2006). Cyanidin probably resulted from the hydrolysis of anthocyanins by caecal microflora glycosidases (Griffiths & Smith, 1972) as red orange juice did not contain this aglycone. The instability of anthocyanidins at a physiological pH (near neutrality) was probably responsible for the low cyanidin content in the caecum, and cyanidin was probably rapidly transformed to non-coloured and/or degradation products, as recently suggested (Felgines *et al.* 2002; Fleschhut *et al.* 2006; Talavéra *et al.* 2006). Furthermore, the rats were adapted to the anthocyanin diet for 12 d. The recovery of low amounts of red orange anthocyanins could result from adaptation of the microflora to anthocyanin degradation, as previously shown in rats adapted to flavanone-enriched diets (Felgines *et al.* 2000). The proportion of anthocyanin glucosides in the caecum was slightly different from that of the red orange juice. The Cy 3-glc to Cy 3-malglc ratio in red orange juice was 45:55. Six hours after the beginning of the last meal, this caecal content ratio was 61:39. Acylated Cy 3-glc could be less stable at caecal pH or more sensitive to enzymatic degradation by microflora than Cy 3-glc. Moreover, the degradation of Cy 3-malglc by microflora could lead to the formation of Cy 3-glc. Indeed, Fleschhut *et al.* (2006) have shown that human faecal microflora were able to hydrolyse acylated groups from acylated anthocyanins.

To gain further insight into the behaviour of acylated anthocyanins, we investigated red orange anthocyanin absorption in the stomach and intestine using low, physiological amounts of anthocyanin. Gastric absorption was evaluated after direct administration of red orange anthocyanins (about 110 nmol) into the rat stomach using an *in situ* gastric administration model (Talavéra *et al.* 2003). Intestinal anthocyanin absorption was investigated using *in situ* intestinal (jejunoileal) perfusion as previously described (Talavéra *et al.* 2004). Our results indicated that a high proportion (about 20 %) of red orange anthocyanins was rapidly absorbed from the stomach. Moreover, absorption levels were close to those of previous reports for various anthocyanin monoglucosides under the same conditions (Talavéra *et al.* 2003). The acylation of Cy 3-glc did not modify its gastric absorption. We have previously shown that anthocyanins are absorbed across the stomach as intact forms (Talavéra *et al.* 2003, 2005). The mechanism of anthocyanin permeation in this organ remains unknown. Passamonti *et al.* (2002) suggested that bilitranslocase, which is an organic anion carrier expressed in the gastric epithelium, could be involved in the gastric absorption of anthocyanins. These authors have reported that the acylation of anthocyanin glucosides weakened the interaction between the anthocyanins and the bilitranslocase transport site. However, our results did not reveal differences between Cy 3-glc and Cy 3-malglc gastric

absorption. The absorption and further metabolism of Cy 3-glc and Cy 3-malglc occurred very quickly, since native as well as methylated anthocyanins were recovered in the urine only 30 min after the beginning of the experiment.

On the other hand, our results indicated that red orange juice anthocyanins were also absorbed from the small intestine. Two observations can be drawn from the *in situ* intestinal perfusion results. First, the intestinal absorption of Cy 3-malglc was almost double that of Cy 3-glc, suggesting that acylation could favour intestinal absorption. Second, the absorption of Cy 3-glc from red orange juice was lower than the absorption of purified Cy 3-glc or Cy 3-glc from blackberry or bilberry anthocyanin extract, which has been found to be about 20 % using the same model (Talavéra *et al.* 2004). We have previously hypothesized (Talavéra *et al.* 2004) that anthocyanin glucosides, which have a basic flavonoid structure, could interact with the intestinal sodium-dependent glucose transporter 1 (SGLT1), as has been reported for quercetin glucosides (Walgren *et al.* 2000; Wolffram *et al.* 2002). The red orange juice extract used in these *in situ* experimentations contained a high amount of sugars. We have determined that the perfused solution contained 71 mM-glucose (compared with 12-mM in the intestinal buffer, as previously used during the perfusion of purified molecules (Talavéra *et al.* 2004)). We thus suggest that this high amount of glucose could lead to a competition-induced decrease in Cy 3-glc absorption *via* the SGLT1 carrier. Indeed, previous studies have shown that anthocyanin glucoside absorption was delayed and even decreased with a concomitant administration of sugar, and the authors have thus hypothesized that this pattern could result from a competitive action of glucose and anthocyanin glucosides on SGLT1 (Bub *et al.* 2001; Mulleder *et al.* 2002). However, this hypothesis requires further investigation to confirm the role of SGLT1 in anthocyanin glucoside absorption.

In conclusion, this study showed that red orange juice anthocyanins were rapidly absorbed from both stomach and small intestine and then excreted in the urine as intact and methylated forms. Moreover, absorption and metabolism were very similar between acylated anthocyanins and non-acylated anthocyanins. Future research will be aimed at investigating acylated anthocyanin distribution to various tissues and evaluating their potent biological activities.

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