The stomach as a site for anthocyanins absorption from food[1]

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Abstract The ability of anthocyanins to permeate the gastric mucosa can be suggested as a possible explanation of the fast kinetics of plasma appearance of anthocyanins in rats and humans. This paper presents an in vivo experiment aimed to prove the involvement of the stomach in the absorption of grape anthocyanins in rats. The required analytical selectivity and sensitivity was achieved by high-performance liquid chromatography, diode array detection and mass spectrometry. Malvidin 3-glucoside appeared in both portal and systemic plasma after only 6 min. The average concentrations measured in portal and systemic plasma were 0.650 ± 0.162 μM and 0.234 ± 0.083 μM (mean ± S.E.M.), respectively.

Key words: Anthocyanin; Gastric absorption; Digestive tract; HPLC; Mass spectrometry; *Vitis vinifera*

1. Introduction

Anthocyanins are plant secondary metabolites providing pigmentation to flowers, fruits, seeds and leaves [1]. They are particularly abundant in red berries and fruits as well as in red wines; thus their presence in the diet can be noteworthy, estimated to be about 0.2 g/day [2]. They are phenolic compounds, belonging to the vast class of flavonoids, and occur mainly as glycosides. Because of their phenolic structure they are efficient antioxidants [3] and are likely to protect cells against oxygen radical-related damage at the basis of various pathologies, such as, for instance, atherosclerosis [4], some cancers [5], diabetes [6]. Thus, the concept evolves that the human health condition could be partly controlled through the dietary intake of plant polyphenols [7]. It therefore becomes urgent to improve our knowledge of polyphenol availability from the diet.

Numerous independent studies have reported on the detection of anthocyanin glycosides in the blood after oral consumption [8–15]. Anthocyanin glycosides are both bulky and polar compounds and, therefore, carrier-mediated mechanisms of absorption from the gastro-intestinal tract are likely to be involved.

Bilitranslocase is a plasma membrane organic anion carrier (TC #2.A.65.1.1, http://tcdb.ucsd.edu/tcdb/background.php) expressed both in the basolateral domain of the liver plasma membrane [16] and in the gastric epithelium [17]. It is inhibited competitively by a number of anthocyanins, including both the mono- and di-glycosylated derivatives, with apparent inhibition constants in the low-μM range, leading to the hypothesis that it could be involved in the bioavailability of anthocyanins [18], particularly in the absorption at the gastric level.

A prerequisite to postulate this role for bilitranslocase in the stomach is to verify the possibility that absorption of anthocyanins can indeed take place at this level. This paper reports on data obtained from in vivo experiments in rats that confirm this hypothesis.

2. Materials and methods

2.1. Standardised mixture of grape anthocyanins

A standardised mixture containing 15 anthocyanins was isolated from *Vitis vinifera* (cv. Cabernet Sauvignon) grape with the procedure described in [18]. It had the following composition, measured as percentage HPLC (high-performance liquid chromatography) area at 520 nm: delphinidin 3-glucoside 11.89%; cyanidin 3-glucoside 1.94%; petunidin 3-glucoside 13.83%; peonidin 3-glucoside 9.26%; malvidin 3-glucoside 47.18%; delphinidin 3-(6-*O*-acetyl)-glucoside 2.15%; cyanidin 3-(6-*O*-acetyl)-glucoside 0.24%; petunidin 3-(6-*O*-acetyl)-glucoside 2.21%; peonidin 3-(6-*O*-acetyl)-glucoside 1.24%; malvidin 3-(6-*O*-acetyl)-glucoside 7.49%; delphinidin 3-(6-*O*-*p*-coumaroyl)-glucoside 0.12%; cyanidin 3-(6-*O*-*p*-coumaroyl)-glucoside 0.03%; petunidin 3-(6-*O*-*p*-coumaroyl)-glucoside 0.40%; peonidin 3-(6-*O*-*p*-coumaroyl)-glucoside 0.17%; malvidin 3-(6-*O*-*p*-coumaroyl)-glucoside 1.86%.

2.2. In vivo gastric absorption

The method employed for the study of the in vivo gastric absorption was that suggested by [19]. It was compliant with rules on animal experimentation at the University of Trieste. This method employed anaesthetised rats, thus allowing us to open their abdominal cavity, to reach their stomach, to fill it with an anthocyanin solution and, after time intervals, to sample blood from both the portal vein and the heart. Under the conditions of deep anaesthesia that lasted for the whole experiment, body temperature, blood flow and gastro-intestinal motility are obviously decreased with respect to the untreated animal and could affect the rate of absorption of test compounds from the stomach to an extent whose determination fell beyond the scope of this work. Male, Wistar rats (250 g), fasted for 24 h, were anaesthetised by intra-peritoneal injection of 2.5 ml of a 2.5% (wt/v) solution of 2,2,2-tribromoethanol (Sigma-Aldrich, Steinheim, Germany) in ethanol:0.15 M NaCl (1:9 v/v). Induction occurred within a few minutes; if required, one additional aliquot of 0.5 ml was administered intra-muscularly. The abdominal wall was opened longitudinally and the stomach was located. A ligature was tightened around the cardias, in order to prevent reflux into the duodenum. A plastic tubing (outer

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diameter 4 mm) was introduced into the duodenum through an opening and gently forced through the pylorus into the stomach cavity. To ensure tightness of the system and to avoid blood reflux into the stomach, two ligatures were fastened upstream and downstream of the pyloric sphincter. The tube was connected to a syringe to introduce various solutions into the gastric lumen. This was washed repeatedly with 4 ml aliquots of an acidified saline solution (10 mM HCl/0.15 M NaCl) until the effluent was clear. The stomach was finally filled with the standardised grape anthocyanin mixture (2 mg = 3.8 μmol in 4 ml 10 mM HCl/0.15 M NaCl). In order to obtain an even distribution of the test compounds inside the organ, the solution was introduced and withdrawn repeatedly and regularly by means of a syringe. At the end of the incubation times (6, 12, 24 and 36 min), 0.1 ml (500 IU) sodium heparin (Clarisco; Schwarz Pharma, Milan, Italy) was injected into the inferior cava vein. Immediately thereafter, 1 ml blood was sampled from the portal vein. Then, the thorax was opened and 3–4 ml systemic blood was obtained by cardiac puncture. The rat died in less than 1 min.

2.3. Sample preparation

2.3.1. Step 0: preparation of plasma extracts. Blood samples were put in ice and centrifuged for 1 min at $12\,000\times g$. The supernatant consisting of plasma was added to 15 ml plastic tubes containing 4.5 ml (for portal blood samples) or 13.5 ml (for general blood samples) of methanol (HPLC grade; Carlo Erba, Milan, Italy) saturated with nitrogen and kept at −20°C. The tubes were screw-capped, flushed with nitrogen again, vortexed and centrifuged at $3640\times g$ for 10 min at 4°C. Supernatants were decanted in glass tubes under a stream of nitrogen and screw-capped.

2.3.2. Step 1: storage of plasma extracts. Plasma extracts were stored at −20°C until sent to the analytical laboratory for further steps.

2.3.3. Step 2: clean-up of plasma extracts. Methanol was removed from the sample under reduced pressure on a rotavapor (Büchi, Germany) at 38°C to about the initial volume of the plasma (one-tenth). The sample was immediately re-diluted in 10 ml 5 mM H_2SO_4. The SPE cartridge (Sep Pak C18, 0.35 g; Waters, Milford, MA, USA) was previously conditioned with 3 ml methanol followed by 5 ml 5 mM H_2SO_4, and then the sample was quantitatively loaded. The cartridge was then washed with 5 ml 5 mM H_2SO_4. Before elution the cartridge was dried with nitrogen in order to remove the remaining traces of water. Anthocyanins were eluted with 4 ml of methanol. The methanol fraction was evaporated to dryness under reduced pressure, at 38°C. The residue was immediately redissolved in 0.5 ml of a mixture suitable for HPLC analysis (50% methanol, 50% of 5% formic acid (HPLC grade; Carlo Erba) in H_2O). The sample was filtered through a 0.22 μm polyvinylidene difluoride filter (Millipore, Bedford, MA, USA) into a HPLC vial, and then analysed by HPLC. A scheme summarising the steps of cyanidin 3-glucoside extraction from plasma and the relative recoveries is given in Fig. 1.

2.4. HPLC analysis

Analyses were carried out on a Waters 2690 HPLC system equipped with Waters 996 diode array detector (DAD) (Waters), Micromass ZQ electrospray ionisation-mass spectrometry (ESI-MS) system (Micromass, Manchester, UK), and MassLynx Software version 3.5 (Micromass). Separation was performed using a column ODS Hypersil 200×2.1 mm (5 μm) with guard ODS Hypersil 20×2.1 mm (5 μm) (Agilent, Palo Alto, CA, USA). The mobile phases consisted of 5% formic acid in H_2O (A) and 5% formic acid in methanol (B). Separation was carried out at 40°C in 30 min, under the following conditions: linear gradients starting at 16% B, to 36% B in 10 min, to 45.2% B in 7 min, to 51.2% B in 4 min, to 64% B in 5 min, to 90% B in 4 min. The column was equilibrated for 4 min prior to each analysis. The flow rate was 0.4 ml/min and the injection volume 40 μl. The UV–VIS (visible) spectra were recorded from 230 to 600 nm, with detection at 520 nm. The MS detector operated at a capillary voltage of 3000 V, an extractor voltage of 6 V, a source temperature of 105°C, a desolvation temperature of 200°C, a cone gas flow (N_2) of 30 l/h, a desolvation gas flow (N_2) of 450 l/h. The outlet of the HPLC system was split (9:1) to the ESI interface of the mass analyser. ESI-mass spectra ranging from *m/z* 200 to 700 were taken in positive mode with a dwell time of 0.1 s. The cone voltage was set in scan mode at the value of 65 V for the identification based on the aglycon peak, and at 30 V for the identification based on both the fragment aglycon and molecular ion. The following single ions (*m/z*) were monitored for the quantification: 287.2 (50 V) for cyanidin derivatives, 301.2 (50 V) for peonidin derivatives, 303.2 (50 V) for petunidin derivatives, 317.2 (50 V) for delphinidin derivatives and 331.3 (65 V) for malvidin derivatives. The calibration curves for standard malvidin 3-glucoside, petunidin 3-glucoside, delphinidin 3-glucoside, peonidin 3-glucoside and cyanidin 3-glucoside were linear in the range 140–4800 nM, with coefficient of correlation ≥0.9983 for MS detection and ≥0.9995 for VIS detection. Samples were quantified by single ion monitoring in MS, with the external standard method. An example of the chromatograms of the anthocyanins identified in a sample of rat portal plasma is given in Fig. 2.

3. Results and discussion

3.1. Method validation

To unambiguously detect trace levels of anthocyanins in plasma, a HPLC method with double detection, consisting of ESI-MS and DAD detection, was developed. The single ion monitoring by MS was the first choice for the quantification, being both more sensitive and more selective than the DAD detection at 520 nm. The limit of detection (LOD) of the five anthocyanins was in the range 19–54 nM (Table 1) with signal-to-noise ratio (S/N) ≥3. The limit of quantification ($S/N \geq 10$) was in the range 63–182 nM. This sensitivity was considered adequate for detecting anthocyanins in plasma, also taking into account that sample preparation resulted in ca 0.8-fold and three-fold concentration of portal and systemic plasma samples, respectively. The MS signal in scan mode allowed us to confirm the identity of the peaks by comparing their spectra with those of pure standards. The DAD signal provided a second way for the quantification of the peaks above the limit of quantification (LOQ) (Table 1), and gave additional information on the identity of the compounds, by comparing their UV–VIS spectra with that of pure compounds.

The distribution of the test results under repeatability conditions [20] was estimated for the retention times and for the determination of the concentration of the main anthocyanins in a diluted stock solution ($N = 11$, Table 1). The coefficient of variation (CV) for the retention times was in the range 0.20–0.50% for MS signal, and 0.22–0.30% for DAD, while the CV

Table 1
Instrumental LOD, LOQ and repeatability for MS single ion monitoring and DAD of anthocyanins

Anthocyanin	Detection by MS, single ion			Diode array detection, 520 nm		
	LOD (nM)	LOQ (nM)	Repeatability[a] (CV, %)	LOD (nM)	LOQ (nM)	Repeatability[a] (CV, %)
Malvidin 3-glucoside	28.74	95.69	8.90	62.03	206.70	5.94
Petunidin 3-glucoside	54.59	181.63	11.76	71.29	237.58	3.38
Delphinidin 3-glucoside	52.92	176.74	11.34	54.52	181.53	2.87
Peonidin 3-glucoside	18.85	62.95	8.51	48.32	161.00	2.94
Cyanidin 3-glucoside	25.37	84.79	11.76	57.14	190.62	3.23

[a]Standard solution containing 300 ng/ml of each compound, $N = 11$.

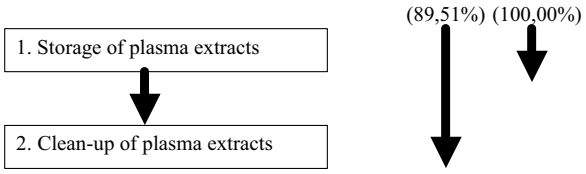

Fig. 1. Flow sheet of the sample preparation steps with indication of the recovery in parentheses.

of the concentration ranged between 8.51% and 11.76% for MS, and between 2.87% and 5.94% for DAD detection.

The experimental recoveries of cyanidin 3-glucoside, malvidin 3-glucoside, peonidin 3-glucoside, petunidin 3-glucoside and malvidin 3-glucoside acetic ester from blood spiked with the pure anthocyanin mixture were, respectively, 57.6% (CV = 20,8%), 41.9% (CV = 19.7%), 42.6% (CV = 17.6%), 54.4% (CV = 17.8%), 48.4% (CV = 19.5%) ($N = 9$). Cyanidin 3-glucoside was used in further tests aimed to assess both the stability during storage and the efficiency of extraction from plasma. The results of these tests are summarised in Fig. 1. Diluted stock solutions of cyanidin 3-glucoside in methanol or in 0.5% acetic acid in methanol were proved to be stable up to 7 days at −20°C, +4°C and room temperature (ca 20°C) (step 1, Fig. 1). Methanol was preferred for the storage of diluted plasma samples, since the acid solution is known to exert hydrolytic activity on the acetic esters of anthocyanins [21]. The recovery of cyanidin 3-glucoside from a methanolic solution during the sample clean-up by C18 cartridge ($N = 4$) was complete (98.69%), and repeatable (CV = 3.87%). The experimental recovery from rat plasma (steps 1+2), spiked with cyanidin 3-glucoside, was estimated to be 89.51% with CV = 4.76% ($N = 6$). In conclusion, these data show that a substantial fraction (about 50%) of the mixture components was associated with red blood cells and that the recovery from plasma was quite satisfactory.

3.2. Absorption of anthocyanins from the stomach

To test whether the stomach is a site of absorption of anthocyanins, a surgical procedure was implemented on anaesthetised rats [19], allowing us to introduce solutions of an anthocyanin mixture isolated from *V. vinifera* (cv. Cabernet Sauvignon, Fig. 2A) grape into the stomach, ligated at both the cardias and the pylorus levels. Rats were grouped according to the duration of the absorption experiment and blood samples were ultimately taken from both their portal vein and their heart. Analysis of anthocyanins in plasma (Fig. 2B) revealed that of all components of the mixture, malvidin 3-glucoside was detected at the highest concentration, which was expected from its being the most abundant single anthocyanin

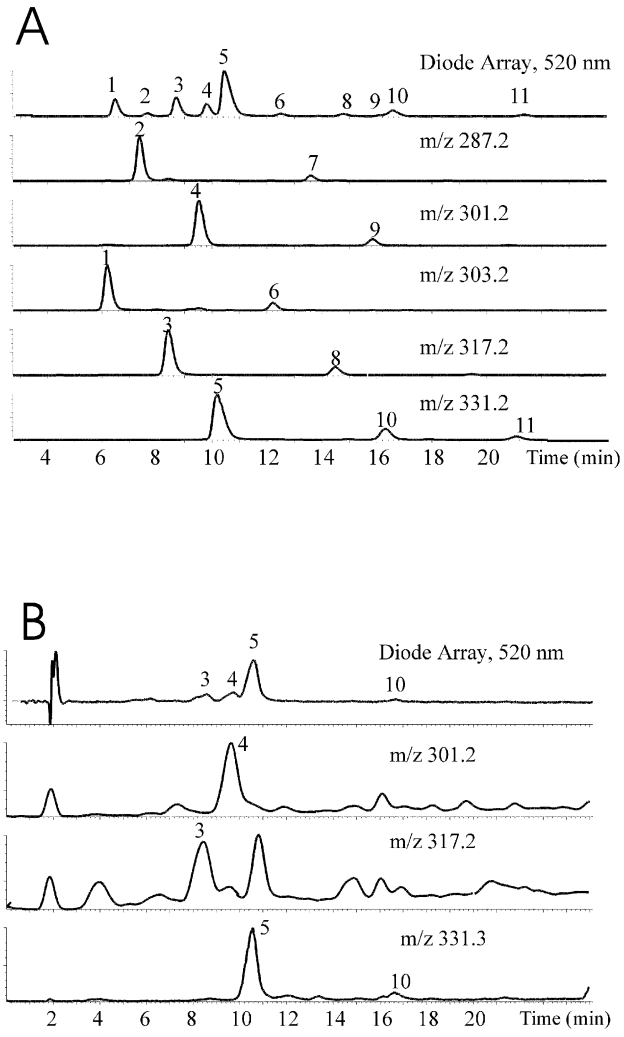

Fig. 2. HPLC-DAD-MS analysis of anthocyanin standard mixture (A) and a sample of rat portal plasma (B). The diode array signal at 520 nm (upper trace) and the MS single ion signals (lower traces) at *m/z* 287.2, 301.2, 303.2, 317.2 and 331.3 are shown. The vertical axis is normalised. 1 = delphinidin 3-glucoside; 2 = cyanidin 3-glucoside; 3 = petunidin 3-glucoside; 4 = peonidin 3-glucoside; 5 = malvidin 3-glucoside; 6 = delphinidin-3-(6-*O*-acetyl)-glucoside; 7 = cyanidin 3-(6-*O*-acetyl)-glucoside, 8 = petunidin 3-(6-*O*-acetyl)-glucoside, 9 = peonidin 3-(6-*O*-acetyl)-glucoside, 10 = malvidin 3-(6-*O*-acetyl)-glucoside, 11 = malvidin 3-(6-*O*-coumaroyl)-glucoside.

in the standardised grape extract. Some chromatograms also revealed the presence of peonidin 3-glucoside, petunidin 3-glucoside and minute amounts of the malvidin 3-glucoside acetic ester (Fig. 2B). Interestingly, neither anthocyanin agly-

Table 2
Malvidin 3-monoglucoside concentration in plasma sampled from either the portal vein or the heart

Time (min)	[Malvidin 3-glucoside] in plasma (μM)									
	Portal plasma					Systemic plasma				
	Means	*N*	Std error	Min	Max	Means	*N*	Std error	Min	Max
6	0.789	5	0.491	0.043	2.554	0.098	5	0.078	0.009	0.410
12	0.468	5	0.228	0.062	1.203	0.300	5	0.239	0.013	1.254
24	0.470	4	0.285	0.021	1.303	0.307	4	0.273	0.006	1.126
36	0.838	5	0.275	0.157	1.341	0.263	6	0.103	0.023	0.664
All groups	0.650	19	0.162	0.021	2.554	0.234	20	0.083	0.006	1.254

cones nor conjugated derivatives could be detected in the blood extracts. Table 2 shows malvidin 3-glucoside concentration in either portal or systemic plasma from rats that received the anthocyanin mixture for the times indicated. High animal variability accounted for the relatively large values of standard errors, presumably due in large part to the deep anaesthesia, affecting differently animals already worn out by 24 h fasting.

Malvidin 3-glucoside appeared in both portal and systemic plasma after only 6 min, and by this time an apparent steady state was reached. This can be estimated by averaging all the data of either the portal or the systemic plasma samples. The values are 0.650 ± 0.162 and 0.234 ± 0.083 μM (mean ± S.E.M.), respectively. The concentrations found in the portal blood are always higher than those in the systemic circulation, obviously due to the proximity of the portal blood district to the site of absorption. The lower concentration values of the systemic plasma may reflect: (a) specific hepatic uptake, (b) dilution into a larger vascular compartment, and (c) distribution in peripheral tissues. The data in systemic plasma are of the same order of magnitude as those reported in studies with human subjects receiving comparable amounts/kg body weight [8,9], confirming that anthocyanin bioavailability in mammals is limited [22]. Nevertheless, malvidin 3-monoglucoside concentrations in systemic plasma are still in a range compatible with effective antioxidant activity [23] and, in addition, the concentrations found in the portal blood are close to the half-saturation of bilitranslocase [18], which could thus play a relevant role in facilitating their uptake into the liver, where they have been shown to protect against oxidative stress [24].

The ability of anthocyanins to permeate the gastric mucosa, and possibly through a bilitranslocase-mediated mechanism, could be at the basis of the fast kinetics of plasma appearance of anthocyanins in rats [15], as well as in humans [9,10,12]. Although absorption is likely to occur also in the upper intestine, the data presented above raise the question of the relative contribution of the stomach in the process of absorption of anthocyanins, whose fate in lower segments of the digestive tract is complicated by degradation, dependent on both pH and microbial metabolism.

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