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Research Article

Uptake of grape anthocyanins into the rat kidney and the involvement of bilitranslocase

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Anthocyanins are among the most common flavonoids in the human diet. In spite of their very low bioavailability, anthocyanins are indicated as active in preventing the progress of cardiovascular and neurodegenerative diseases, obesity, inflammation, and cancer. Any piece of knowledge concerning absorption, tissue distribution, metabolism, and excretion of dietary anthocyanins is expected to help understanding the apparent paradox between their low concentrations in cells and their bioactivity. The aim of this work was to investigate the renal uptake of dietary anthocyanins and the underlying molecular mechanism. A solution containing anthocyanins extracted from grape (*Vitis vinifera*) was introduced into the isolated stomach of anesthetized rats; after both 10 and 30 min, plasma, liver, and kidney were analyzed for their anthocyanin contents. While anthocyanins in the liver were at apparent equilibrium with plasma both after 10 and 30 min, kidney anthocyanins were 3- and 2.3-fold higher than in plasma, after 10 and 30 min, respectively. Since the transport activity of the bilitranslocase in kidney basolateral membrane vesicles was competitively inhibited by malvidin 3-glucoside (K_i = 4.8 ± 0.2 μM), the anthocyanin uptake from blood into kidney tubular cells is likely to be mediated by the kidney isoform of this organic anion membrane transporter.

Keywords: Anthocyanins / Bilitranslocase / Grape (*Vitis vinifera*) / Kidney / Rat

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1 Introduction

Many fruits and vegetables in the human diet are rich in anthocyanins. Therefore these flavonoid-based pigments [1] are among the most common dietary polyphenols [2]. In recent years, numerous studies have indicated that anthocyanins might be positively implicated in the human health [3]. It has been reported that anthocyanins reduce inflammation [4–6], reduce the risk of coronary heart disease [7–9], exert vasoprotective effects [7, 10, 11], and inhibit the cellular growth of cancer cells *in vitro* [12–14]. Anthocyanins are reported to exert beneficial antidiabetic effects [15] and even to prevent adipocyte dysfunction which leads to obesity [16, 17]. In addition, anthocyanins have been reported to be effective in reversing age-related neurodegenerative diseases [18] and these findings are supported by the identification of anthocyanins in the brain as intact glycosides [19–21]. Anthocyanins, though absorbed rapidly from the stomach [22, 23] and the intestine [24], are poorly bioavailable, reaching a plasma concentration of 10–50 nM after oral administration in humans [25, 26]. They are efficiently eliminated in the bile and in the urine within 24 h after administration [24, 27–30].

The aim of this work was to investigate the renal uptake of dietary anthocyanins and the underlying molecular mechanism. A solution containing anthocyanins extracted from grape (*Vitis vinifera*) was introduced into the stomach, ligated both proximally and distally, of anesthetized rats, where anthocyanins are chemically stable [23] and absorption takes place much faster than metabolism, thus enabling to reach an appreciable plasma concentration (0.5–1.5 μM) of intact compounds [22, 23]. Plasma, liver, and kidney were analyzed for their anthocyanin contents after both

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Abbreviation: BSP, bromosulfophthalein

10 min ($n = 8$) and 30 min ($n = 8$) of exposure, in order to explore the so far poorly investigated early phase of tissue distribution and metabolism of anthocyanins. This part of the study was expected to enable the comparison of the respective efficiencies of anthocyanin uptake in the two main excretory organs. The second part of the study investigated if bilitranslocase is involved in anthocyanin uptake into the kidney. Bilitranslocase is a hepatic membrane transporter with a high-affinity for dietary anthocyanins [31, 32], of which an isoform also occurs in the kidney [33].

2 Materials and methods

2.1 Materials

2.1.1 Chemicals

Methanol (HPLC grade) was obtained from Merck (Darmstadt, Germany). Perchloric acid was obtained from Carlo Erba (Milan, Italy). Aqueous solutions were made with Milli-Q water (Millipore, Bedford, USA). PBS solution: 6.03 mM Na_2HPO_4, 3.91 mM NaH_2PO_4, and 139 mM NaCl (Carlo Erba) were dissolved in Milli-Q water; pH was adjusted to 7.4 with 1 or 0.1% HCl. Anesthetic: 2.5% w/v solution of 2,2,2-tribromoethanol (Sigma–Aldrich, Steinheim, Germany) in ethanol: 0.15 M NaCl (1:9 v/v). Heparin was from Schwarz Pharma (Milan, Italy). Cyanidin 3-glucoside, peonidin 3-glucoside, petunidin 3-glucoside, delphinidin 3-glucoside, and malvidin 3-glucoside were from Polyphenol Laboratories AS (Sandnes, Norway). Tris, HEPES, bromosulfophthalein (BSP), valinomycin, and Percoll were from Sigma–Aldrich. Standard grape anthocyanin mixture administered into the stomach of rats was isolated from *V. vinifera* (cv. Cabernet Sauvignon) as previously described [32]. It consisted of 15 anthocyanidin-glucosides. The composition was the following (measured as percentage HPLC area at 520 nm): delphinidin 3-*O*-glucoside, 11.89%; cyanidin 3-*O*-glucoside, 1.94%; petunidin 3-*O*-glucoside, 13.83%; peonidin 3-*O*-glucoside, 9.26%; malvidin 3-*O*-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%.

2.1.2 Animals and diets

Male Wistar rats (250 g) were used for this study. They were fed standard laboratory chow (Harlan Teklad 2018) and tap water *ad libitum*; they were housed in temperature-controlled rooms at 22–24°C and 50–60% humidity. All animals were maintained and handled at the Animal House of the University of Trieste according to the provisions of the European Community Council Directive (n.86/609/CEE) and to the provisions of Italy (D.L.vo. 116/92). The experimental protocol was approved by the University of Trieste Committee for animal studies. Rats ($n = 16$) were fasted for 24 h before the absorption experiment. They were randomly divided into two groups: A and B. A solution containing grape anthocyanins was kept into the isolated stomach for either 10 min (group A, $n = 8$) or 30 min (group B, $n = 8$), respectively. For the preparation of kidney basolateral plasma membrane vesicles, six fed rats from the same cage were used.

2.2 Methods

2.2.1 Anthocyanin administration into the stomach of rats

The absorption of grape anthocyanins from isolated stomach was investigated *in vivo*. The surgical procedure for administering anthocyanins into the stomach of anesthetized rats was the same as previously described [22]. The stomach, ligated both proximally and distally, was rinsed with acidified saline solution (10 mM HCl/0.15 M NaCl) until the effluent was clear. Then the stomach was filled with the standard grape anthocyanin mixture (2 mg in 4 mL of 10 mM HCl/0.15 M NaCl). Considering that rats weighted 250 g, the administered dose corresponded to *ca.* 8 mg/kg of body weight. For comparison, serving 1 glass (100 mL) of young red wine can provide up to 30 mg grape anthocyanins [34]. The human daily intake of anthocyanins should be 0–2.5 mg/kg of body weight, as suggested by the World Health Organization. In order to obtain an even distribution of the test compounds inside the organ, the solution was introduced and withdrawn from the stomach repeatedly and regularly by means of a syringe for 10 min (group A) and for 30 min (group B). At the end, 0.1 mL (500 IU) sodium heparin was injected into the inferior cava vein. Immediately, 1 mL of blood was sampled from the portal vein whereas systemic blood was drained from the inferior cava vein. Then, a PBS (about 20 mL) was injected into the cava vein; this procedure lasted less than 1 min and resulted in washing out of the blood from the right kidney and most of the liver. The rat was euthanized by decapitation. Immediately thereafter, the right kidney and one lobe of the liver were excised.

2.2.2 Preparation of tissue samples

Plasma was obtained by centrifugation of blood samples and anthocyanins were extracted from plasma with nine volumes of ice-cold methanol, as described [22]. Both the liver and the kidneys were homogenized in four volumes of PBS w/w. Anthocyanins were extracted by adding 1 mL homogenates to 9 mL of ice-cold methanol, previously saturated with nitrogen. The tubes were screw-capped, flushed with nitrogen, 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. Samples in methanol were stored at −20°C. Clean-up of tissue extracts in methanol was performed using a SPE as described in [22]. Methanol was evaporated; the residue was diluted in acidified Milli-Q water and loaded on the solid phase cartridge (Sep-Pak, 0.35 g; Waters, Milford, MA). Anthocyanins were eluted with methanol, evaporated until dryness, and reconstituted in solution corresponding to the initial HPLC conditions. The sample was filtered through a 0.22 μm PVDF filter (Millipore) into an HPLC vial and immediately analyzed by HPLC.

2.2.3 HPLC analysis

An Agilent 1100 HPLC with DAD, coupled to an Agilent NDS ChemStation (Agilent Technologies, Palo Alto, USA), was used for anthocyanin detection and quantification. Separation was performed using a C18 column (250×4.6 mm^2, 5 μm; Purospher, Merck) with a C18 guard column (4×4 mm^2, 5 μm; Purospher). The mobile phase consisted of solvent A (0.3% perchloric acid in Milli-Q water) and solvent B (100% methanol). Separation was carried out at 35°C for 50 min. The gradient was linear (from 28.5% B, to 51% B in 42 min, to 68.5% B in 3 min, to 100% B in 2 min, 100% B for 3 min). The column was equilibrated for 6 min prior to each analysis. The flow rate was 0.55 mL/min and the injection volume was 70 μL. The UV–Vis spectra were recorded from 200 to 700 nm, with the detection at 520 nm. The conditions of this method enable the detection of anthocyanin structures in the extracts that regenerate to the red flavylium cation upon sample preparation.

2.2.4 Preparation of kidney basolateral plasma membrane vesicles

The preparation of basolateral plasma membrane vesicles was done following the method described by Jensen and Berndt [35] with some modifications [36, 37]. Approximately 4 g of kidney cortex was placed in a Dounce homogenizer containing 50 mL of 250 mM sucrose, 5 mM Tris–HEPES pH 7.40 (buffer A). After four gentle strokes with the loosely fitting pestle, the suspension was homogenized further with a motor-driven Teflon pestle (600 rpm/5 strokes) and centrifuged at $1200 \times g$ for 15 min. The supernatant was aspirated and centrifuged at $22\,000 \times g$ for 15 min. The fluffy, beige upper layer of the resulting pellet (crude plasma membranes) was resuspended in about 1 mL of supernatant and added to 19 mL buffer A. The membrane suspension was homogenized gently through a 16-gauge gavage needle, followed by the addition of 3.7 mL of 100% Percoll. The Percoll-membranes mixture was centrifuged at $39\,250 \times g$ for 30 min. The top, clear layer was discarded and the top darkest band was collected. This layer was composed primarily of basolateral membranes as established by marker enzymes analysis. Vesicles were suspended in 85 mM KCl, 83 mM sucrose, 2 mM Tris–HEPES pH 7.40 (buffer B) at a ratio of 8 mL/g original cortex wet weight. Then, vesicles were pelleted at $30\,000 \times g$ for 30 min and washed three times with buffer B. Finally, vesicles were resuspended in 0.3 mL of 250 mM sucrose, 10 mM Tris–HEPES, pH 7.40. Aliquots of the membranes were stored at −70°C. Each preparation represented cortical tissue from six animals.

2.2.5 Preparation of bilitranslocase antibody

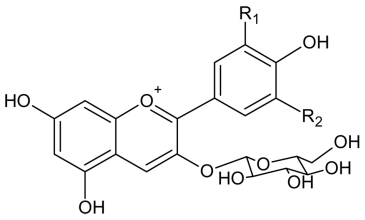
An antisequence bilitranslocase polyclonal antibody was used. The pure antibody was obtained by affinity purification of rabbit (*Oryctolagus cuniculus*, white New Zealand strain) sera; rabbits were immunized at the Animal House of the University of Trieste with the peptide EFTYQLTSSPTC, corresponding to the segment 235–246 of the primary structure of bilitranslocase. The production and biological properties of this antibody, named antibody B, have been previously described [31, 38].

2.2.6 Assay of electrogenic BSP uptake into kidney basolateral plasma membrane vesicles

Bilitranslocase transport activity was assayed in membrane vesicles, using BSP as its standard transport substrate, as previously described in detail [32]. BSP concentration in the assay was recorded in real time at the wavelength pair 580–514 nm by a double-wavelength spectrophotometer (Sigma-ZWS II). Briefly, 4 μL (12 μg protein) of kidney basolateral plasma membrane vesicles was added to a stirred spectrophotometric polystyrene cuvette, containing 2 mL of assay medium (0.1 M potassium phosphate, pH 8.0), with different BSP concentrations (in the range of 5–80 μM), at room temperature. This addition caused an instantaneous drop in absorbance. After the attainment of a steady-state (4 s), a second drop in absorbance was brought about by valinomycin-induced K^+ diffusion potential by adding 2 mg valinomycin in 2 μL methanol. Such a K^+ diffusion drove the substrate into the vesicles. The slope of the linear phase of this absorbance drop, lasting about 1 s, is referred to as electrogenic BSP uptake and is related to bilitranslocase transport activity [39]. The pH in the assay medium was constant throughout the duration of the test, as previously shown with an analogous preparation from rat liver [40].

2.2.7 Electrogenic BSP uptake inhibition by malvidin 3-glucoside

For transport inhibition assays, cuvettes, containing the assay medium with different BSP concentrations as described above, were supplemented with 2 μL malvidin 3-glucoside, prepared at the concentrations of either 2 or 5 mM in DMSO; the addition was done 5 s before the addition of the vesicles, in order to minimize tautomerization of malvidin 3-glucoside. To check if malvidin 3-glucoside interfered with the valinomycin-induced change of absorb-



R_1	R_2	
H	OH	cyanidin 3-glucoside
H	OCH_3	peonidin 3-glucoside
OH	OH	delphinidin 3-glucoside
OH	OCH_3	petunidin 3-glucoside
OCH_3	OCH_3	malvidin 3-glucoside

Figure 1. Molecular structure of grape anthocyanins. The glucosyl moiety can be esterified with either acetic acid or *p*-coumaric acid at the 6″ position.

ance at the wavelength pair 580 – 514 nm, a control experiment was carried out by adding just malvidin 3-glucoside to the assay medium, in the absence of BSP. Under these conditions, absorbance remained constant on addition of valinomycin to the vesicle suspension, thus confirming that the inhibitor did not interfere with the assay.

2.2.8 Electrogenic BSP uptake inhibition by bilitranslocase antibodies

The kinetics of bilitranslocase transport activity inhibition by antibodies were examined by preincubating 30 μL (=117 μg protein) kidney basolateral plasma membrane vesicles at 37°C with 12 μL antibody B at the final concentrations of 2.1, 4.2, and 8.4 μg IgG/mL. Controls were carried out by using equivalent amounts of IgG purified from preimmune rabbit sera [38]. Aliquots (6 μL) of the preincubation mixtures were withdrawn and added to the transport medium containing 60 μM BSP for the assay of electrogenic BSP uptake activity. Under these conditions, all components of the preincubation mixture were diluted 3.3×10^2 times, so that they did not interfere with the activity of bilitranslocase.

2.2.9 Data analyses

Data were analyzed by means of SigmaPlot 2001 (SPSS Science Software, Erkrath, Germany). Comparison of the results of tissue anthocyanins after 10 and 30 min of administration was done by the Student's *t*-test (ANOVA); differences with $p < 0.05$ were considered significant. Data for the characterization of the kinetics of electrogenic BSP uptake in kidney basolateral membrane vesicles fitted the Michaelis – Menten equation and the apparent K_M and V_{max} values were derived with their standard errors. The competitive K_i values were derived from the equations $K_{Mi} = K_M (1 + [I]/K_i)$, where i stands for inhibitor. Two K_i values were obtained from the two apparent K_{Mi} obtained at the two concentrations of malvidin 3-glucoside tested. The mean K_i and its standard error reported in the text were obtained by averaging the two K_i values obtained. Data of the time course of inactivation of electrogenic BSP uptake by the antisequence bilitranslocase antibody fitted the single exponential decay equation, as specified in the legend to the figures, thus enabling the characterization of the kinetics of inactivation.

3 Results and discussion

3.1 Detection and identification of grape anthocyanins in rat tissues

Grape anthocyanins (Fig. 1) in both the standard mixture and tissue extracts were separated by HPLC (Fig. 2). Anthocyanin identification was based on their retention times and UV/Vis spectra; these properties coincided with those of pure standards. Two of them (malvidin-3-acetyl glucoside and malvidin 3-*p*-coumaroyl glucoside, corresponding to peaks 10 and 15, respectively) are not commercially available but have been isolated by us as previously reported [32]. The standard mixture and samples of plasma, liver, and kidney extracts were also analyzed by HPLC-DAD-MS, as already described [19], in order to confirm the identity and purity of each anthocyanin on the basis of their base fragment, corresponding to the aglycon, and molecular ion. Anthocyanins were quantified by optical absorption at $\lambda = 520$ nm and expressed as malvidin 3-glucoside equivalents. A nine-point standard calibration curve was linear in the range of 0.005 to 2.5 mg/L ($r^2 = 0.99962$). Repeatability tests were carried out by sequentially injecting malvidin 3-glucoside (0.25 mg/L; $n = 11$) into the HPLC column. CV of concentration and retention time were 2.545% or 0.350%, respectively. The LOD of malvidin 3-glucoside (S/N ≥ 3) was 0.0053 mg/L; the limit of quantification (S/N ≥ 10) was 0.018 mg/L. Anthocyanins were not detected in plasma, liver, and kidney of control animals. In order to assess recoveries of anthocyanins from tissues, known amounts of grape anthocyanin mixture (2.84 μg) were spiked into tubes containing blood (1.4 mL), kidney or liver homogenates (1 mL), prepared as described above (Section 2.2.2) from control animals. After extraction (Section 2.2.2), the respective recoveries (mean ± SEM) of grape anthocyanins were 46 ± 3% ($n = 9$), 63 ± 3% ($n = 10$), and 53 ± 3% ($n = 5$). Data presented in the tables have been corrected for recovery.

3.2 Detection of anthocyanins in tissues after 10 min of gastric absorption of a standard grape anthocyanin mixture

Grape anthocyanins were administered into the ligated stomach of anesthetized rats as described in Section 2. After

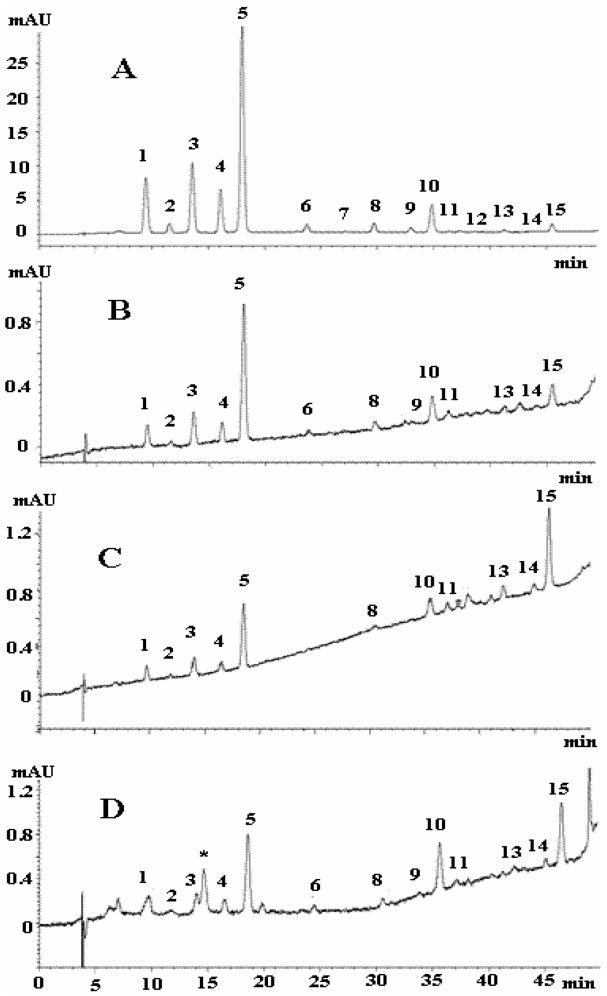


Figure 2. HPLC chromatograms at 520 nm of a standard grape anthocyanin mixture (a) and of anthocyanins detected in systemic plasma (b), liver (c), and kidneys (d) after 30 min of its administration into the ligated stomach of an anesthetized rat. The peak eluting close to petunidin 3-glucoside in the kidney (d) and labeled with * is unidentified; its UV – Vis spectrum did not correspond to an anthocyanin-like spectrum. Peaks are numbered as follows: anthocyanidin 3-*o*-glucosides (1 – 5): 1, delphinidin; 2, cyanidin; 3, petunidin; 4, peonidin; 5, malvidin; anthocyanidin 3-(6-*o*-acetyl)-glucosides (6 – 10): 6, delphinidin; 7, cyanidin; 8, petunidin; 9, peonidin; 10, malvidin; anthocyanidin 3-(6-*o*-*p*-coumaroyl)-glucosides (11 – 15): 11, delphinidin; 12, cyanidin; 13, petunidin; 14, peonidin; 15, malvidin. The sharp rise of the optical signal at 50 min in the chromatogram shown in panel (d) is not an elution peak, but an optical artifact due to the change of solvents at 45 min.

10 min, plasma, liver, and kidney were collected and analyzed for their anthocyanin concentrations (Table 1).

3.2.1 Plasma

Six out of 15 anthocyanins of the standard anthocyanin mixture were quantitatively detected in plasma obtained from both portal and systemic blood samples, in agreement with previous observations [19]. The total anthocyanin concentration (mean ± SEM) in portal plasma was 688 ± 246 ng/mL, *i.e.*, 2.8-fold higher than in systemic plasma (247 ± 72 ng/mL), as previously observed [22]. The anthocyanins that could not be quantified in plasma samples were the least abundant components of the mixture (each <2.2%), with the exception of malvidin 3-(6-*O*-*p*-coumaroyl)-glucoside (1.86% of the mixture). Its high occurrence in plasma (both portal and systemic) points to its highly efficient absorption from the stomach.

3.2.2 Liver

Five out of six anthocyanins found in plasma (both portal and systemic) were also quantitatively detected in the liver. The missing compound was delphinidin 3-glucoside. The total anthocyanin concentration in the liver was 188 ± 44 ng/g, *i.e.*, 3.7- and 1.3-fold lower than in either portal or systemic plasma, respectively. These data show that anthocyanin concentrations in liver and systemic plasma are roughly at equilibrium, as previously observed [30, 31] and consistent with the conclusion that no significant first-pass metabolism of anthocyanins takes place there [29].

3.2.3 Kidney

Five out of six anthocyanins found in plasma (both portal and systemic) were also detected in the kidney. Nevertheless, from a quantitative point of view, the situation in the kidney appeared quite different from both systemic plasma and liver. The total anthocyanin concentration in the kidney was three-fold higher than in systemic plasma (726 ± 174 ng/g *vs.* 247 ± 72 ng/mL, respectively), in agreement with previous observations [30]. The most striking difference concerned petunidin 3-glucoside, whose concentration was 12-fold higher in the kidney than in the systemic plasma. This might be the result of either a more efficient rate of its transport into the kidney or of a high rate of *O*-methylation of its precursor, delphinidin 3-glucoside, in this organ. Though it has been reported that, in the liver, the latter was methylated only at the level of the 4′ position [41], we cannot exclude that, in the kidney, methylation occurred also at the level of the 3′ position. Since our chromatographic conditions are similar to those used for the separation of peonidin 3-glucoside from isopeonidin 3-glucoside [42], our method might enable an at least partial separation of the two putative petunidin 3-glucoside isomers. Moreover, genetic polymorphism of catechol-*O*-methyl transferase giving rise to variations in the enzyme catalytic activities has been documented also in rat strains [43], suggesting that some apparently conflicting findings with respect to the methylation products of anthocyanins might have a biological rather than a methodological basis. Should part of petunidin 3-glucoside (or its isomer) in the kidney arise from methylation of delphinidin 3-glucoside, this would demonstrate that the latter was indeed bioavailable in the kidney, but it escaped detection because it was efficiently metabolized. Similar results were obtained with

Table 1. Occurrence of grape anthocyanins in rat tissues after 10 min of administration into the ligated stomach (group A, $n = 8$)

	Delphinidin 3-glucoside	Petunidin 3-glucoside	Peonidin 3-glucoside	Malvidin 3-glucoside	Malvidin 3-(6-*O*-acetyl)-glucoside	Malvidin 3-(6-*O*-*p*-coumaroyl)-glucoside	Sum	%[a]
Portal plasma								
N positive[b]	4	4	4	8	1	8	8	
Mean ± SEM (ng/mL)	35 ± 17	91 ± 43	54 ± 26	376 ± 145	14 ± 14	111.8 ± 8.0	688 ± 246	
Systemic plasma								
N positive	2	5	5	8	2	7	8	
Mean ± SEM (ng/mL)	10 ± 7	32 ± 12	22 ± 8	143 ± 43	6 ± 4	30 ± 5	247 ± 72	
Amount (ng)	88 ± 62	282 ± 106	94 ± 70	1258 ± 378	53 ± 35	264 ± 44	2174 ± 634	0.11 ± 0.03
Liver								
N positive	0	8	5	5	4	2	8	
Mean ± SEM (ng/g)	0	45 ± 7	19 ± 7	89 ± 32	11 ± 5	24 ± 22	188 ± 44	
Amount (ng)	0	315 ± 49	133 ± 49	623 ± 224	77 ± 35	189 ± 154	1316 ± 308	0.07 ± 0.02
Kidney								
N positive	0	8	6	8	7	3	8	
Mean ± SEM (ng/g)	0	372 ± 62	45 ± 30	240 ± 11	50 ± 8	11 ± 7	726 ± 174	
Amount (ng)	0	653 ± 149	108 ± 72	576 ± 26	120 ± 19	26 ± 17	1742 ± 418	0.09 ± 0.02
Standard grape anthocyanin mixture								
Concentration (mg/mL)	0.06	0.07	0.05	0.24	0.04	0.01		
Amount (μg)	240	280	180	940	140	40	2000	100

Only compounds above the limit of quantification in tissues are listed. Data are expressed as malvidin 3-glucoside equivalents and corrected for the recovery factors reported in Section 3.1. Amounts of individual compounds in all tissues, with the exception of portal blood, were calculated considering that rat plasma volume is 8.8 mL (http://www.ratbehavior.org/Stats.htm) and that liver and kidney masses were 7 and 2.4 g, respectively.

a) Percent amount of administered standard grape anthocyanin mixture detected in tissues.

b) Number of rats in which individual anthocyanins were detected.

a bilberry mixture, which contained delphinidin 3-glucoside: despite its detection in plasma, it was absent not only in both the liver and the kidney, but also in bile and urine [27]. This might serve as a good example of underestimation of anthocyanin bioavailability. Another distinctive feature of the kidney *versus* both the systemic plasma and the liver was the ten-fold lower concentration of malvidin 3-(6-*O*-*p*-coumaroyl)-glucoside. Such situation could result from its less efficient uptake into the kidney than into the liver, or from a higher rate of its excretion *via* the kidney, or even from the hydrolysis of its ester bond.

3.3 Detection of anthocyanins in tissues after 30 min of gastric absorption of a standard grape anthocyanin mixture

After 30 min, plasma, liver, and kidney were collected and analyzed for their anthocyanin concentrations (Table 2).

3.3.1 Plasma

The total anthocyanin concentration in both portal and systemic plasma were slightly lower after 30 min than after 10 min (451 ± 240 ng/mL *vs.* 688 ± 246 ng/mL, respectively), though not to a statistical significance. This shows that transfer of anthocyanins from the stomach into the blood can reach a steady-state level by 10 min and perhaps earlier [22].

3.3.2 Liver

The total anthocyanin concentration in the liver was not statistically different at either 10 or 30 min of absorption, showing that the anthocyanin concentration in the liver was maintained at equilibrium with systemic plasma also for longer times. The concentrations of the individual compounds did not change, except for the noticeable case of petunidin 3-glucoside, which dropped by about one order of magnitude (from 24 to 3 ng/g) in a 20 min span, being detected in just one out of eight rats (*vs*. in all eight rats at 10 min, Table 1). A similar finding was also obtained in the kidney and it is discussed below.

3.3.3 Kidney

The total anthocyanin concentration in the kidney was lower at 30 min that at 10 min, though not to a statistical significance. As in the liver, also in the kidney did the concentration of petunidin 3-glucoside drop significantly. In the kidney, the drop was as high as 27-fold (from 372 to 14 ng/g), being detected in three out of eight rats (*vs*. in all

Table 2. Occurrence of grape anthocyanins in rat tissues after 30 min of administration into the ligated stomach (group B, $n = 8$)

	Delphinidin 3-glucoside	Petunidin 3-glucoside	Peonidin 3-glucoside	Malvidin 3-glucoside	Malvidin 3-(6-*O*-acetyl)-glucoside	Malvidin 3-(6-*O*-*p*-coumaroyl)-glucoside	Sum	%[a]
Portal plasma								
N positive[b]	2	2	4	7	1	8	8	
Mean ± SEM (ng/mL)	33 ± 23	62 ± 47	43 ± 24	234 ± 123	17 ± 17	61 ± 3	451 ± 240	
Systemic plasma								
N positive	4	6	6	7	1	5	7	
Mean ± SEM (ng/mL)	18 ± 10	36 ± 18	20 ± 9	97 ± 54	5 ± 5	11 ± 3	189 ± 98	
Amount (ng)	158 ± 88	317 ± 158	176 ± 79	854 ± 475	44 ± 44	97 ± 26	1663 ± 862	0.08 ± 0.04
Liver								
N positive	1	1	2	6	2	3	6	
Mean ± SEM (ng/g)	3 ± 3	6 ± 6	21 ± 20	129 ± 76	12 ± 9	53 ± 30	223 ± 112	
Amount (ng)	21 ± 21	42 ± 42	147 ± 140	903 ± 532	84 ± 63	371 ± 210	1561 ± 784	0.08 ± 0.04
Kidney								
N positive	2	3	5	8	2	2	8	
Mean ± SEM (ng/g)	21 ± 14	14 ± 7	53 ± 30	297 ± 135	21 ± 14	33 ± 22	438 ± 201	
Amount (ng)	50 ± 34	34 ± 17	127 ± 72	713 ± 324	50 ± 34	79 ± 53	1051 ± 482	0.05 ± 0.02
Standard grape anthocyanin mixture								
Concentration (mg/mL)	0.06	0.07	0.05	0.24	0.04	0.01		
Amount (mg)	240	280	180	940	140	40	2000	100

Only compounds above the limit of quantification in tissues are listed. Data are expressed as malvidin 3-glucoside equivalents and corrected for the recovery factors reported in Section 3.1. Amounts of individual compounds in all tissues, with the exception of portal blood, were calculated considering that rat plasma volume is 8.8 mL (http://www.ratbehavior.org/Stats.htm) and that liver and kidney masses were 7 and 2.4 g, respectively.

a) Percent amount of administered standard grape anthocyanin mixture detected in tissues.

b) Number of rats in which individual anthocyanins were detected.

eight rats at 10 min, Table 1). It should be noticed that its systemic plasma concentration did not change appreciably over time. This observation does not support the hypothesis of efficient uptake of petunidin 3-glucoside as the mechanism of its accumulation in the kidney. Rather, it possibly shows the contrary, *i.e.*, efflux of petunidin 3-glucoside from the kidney to the blood. However, a number of other different biochemical events might account for the drop of kidney petunidin 3-glucoside concentration: (i) efficient excretion *via* the urine, as already documented [27, 44]; (ii) further methylation to malvidin 3-glucoside by catechol-*O*-methyl transferase [45]; and/or (iii) decreased methylation of delphinidin 3-glucoside, due to consumption of *S*-adenosyl methionine [46], *i.e.*, the methyl group donor of the methylation reaction; the concurrent increase of the coproduct of the methylation reaction, *i.e.*, *S*-adenosyl homocysteine, might have accumulated and inhibited catechol-*O*-methyl transferase [43], thus further decreasing the overall rate of methylation. The latter hypothesis might be supported by the fact that, in the kidney, delphinidin 3-glucoside, while undetectable at 10 min, could be detected at 30 min, though only in two rats out of eight. As a consequence of the drop in petunidin 3-glucoside contents in the kidney, at 30 min the prevailing anthocyanin in the kidney is malvidin 3-glucoside.

No other anthocyanin-related metabolite was detected at 520 nm to any significant extent in the investigated tissues (Figs. 2B – D), after either 10 or 30 min of administration. Such results show that, shortly after anthocyanin absorption, the prevailing metabolic reaction involving grape anthocyanins appears to be *O*-methylation. These data are in agreement with the finding that no anthocyanin metabolites (aglycones, sulfo/glucurono-conjugates) were detected in urine, following ingestion of delphinidin 3-rutinoside. In the same study, only the intact compound and its 4′-*O*-methyl derivative was detected in the urine [28]. Nevertheless, some metabolic products, *i.e.*, glucuronyl derivatives and even aglycones, have indeed been detected either in plasma or urine, though hours after oral administration [47]. Presumably, the absence of detectable amount of metabolites in the examined tissues might be due to the relatively short time of exposure of organs to plasma anthocyanins and the restricted site of absorption, *i.e.*, the stomach, where no anthocyanin metabolites are formed in 30 min, as seen both by another group [23] and us. In particular, no cathecol *O*-methyl transferase activity takes place

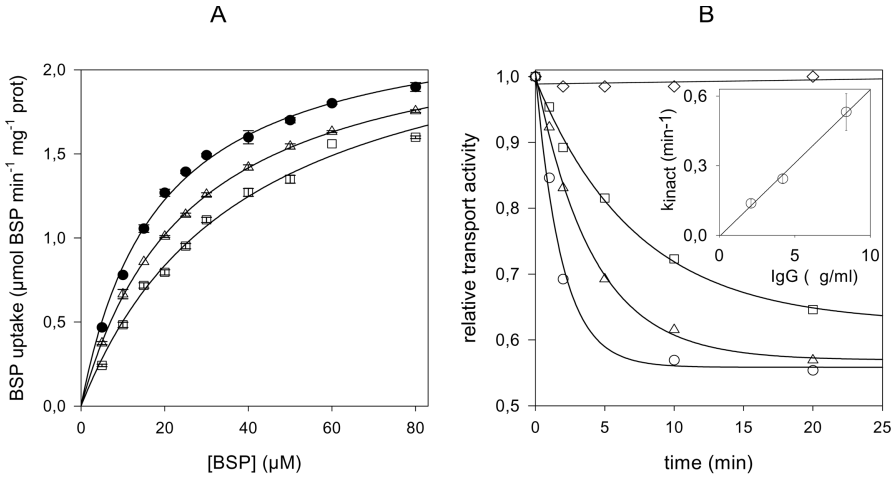


Figure 3. Uptake of BSP into kidney basolateral plasma membrane vesicles (A and B). (A) Dependence of uptake on [BSP] and inhibition by malvidin 3-glucoside. Uptake was assayed as described in Section 2 both in the absence (●) and in the presence of either 2 (△) or 5 (▫) µM malvidin 3-glucoside. Data were analyzed as described in Section 2.2.9; fitting data to the Michaelis–Menten equation yielded the following parameters: V_{max} = 2.35 ± 0.05 (●), 2.33 ± 0.015 (△), 2.45 ± 0.012 (▫) µmol min^{-1}mg^{-1} protein; apparent K_M = 18.37 ± 1.03 (●), 25.66 ± 0.39 (△), 38.57 ± 3.83 (▫) µM BSP. (B) Inactivation of BSP uptake by an antisequence bilitranslocase antibody. Vesicles were preincubated either in the absence (◇) or in the presence of antibody B at the following concentrations: 2.1 (▫), 4.2 (△), or 8.4 (○) µg IgG/mL; at the times indicated, electrogenic BSP uptake activity was assayed as described in Section 2. Transport activity data were fitted to the following equation: $y = y_0 + a\ e^{-kt}$, where y = relative transport activity, y_0 = relative uptake rate at the inhibition steady-state, $a = 1 - y_0$, e = 2.7183, t = time, and k = first order rate constant of transport inhibition. The following parameters were obtained: y_0 = 0.62 ± 0.02, a = 0.37 ± 0.02, k_1 = 0.14 ± 0.02 min^{-1} (△); y_0 = 0.57 ± 0.01, a = 0.43 ± 0.01, $k_2$2 = 0.24 ± 0.02 min^{-1} (△); y_0 = 0.56 ± 0.02, a = 0.45 ± 0.03, k_3 = 0.53 ± 0.08 min^{-1} (○). The inset shows the relationship between k and the antibody concentration ([IgG]) in the preincubation. Data were fitted to a straight line by linear regression. The parameters were: intercept at the y-axis = −0.006 ± 0.002; slope = 0.063 ± 0.004 min^{-1} µg^{-1} IgG mL; r^2 = 0.995.

in the gastric lumen, as observed with typical substrates of this enzyme, *i.e.*, chlorogenic [48] and caftaric acid [49], respectively. In this context, it should be added that collection of either bile or urine was unfeasible, due to their small volumes produced during the experiments.

Data presented in Tables 1 and 2 show that about 0.1% of the administered dose of anthocyanins appears in plasma 10 or 30 min after its introduction into the stomach. Such value is in line with what observed in most studies, where plasma anthocyanins are analyzed following oral administration [26].

The possibility that absorption might have occurred due to unspecific damage to the gastric mucosa should be ruled out, since this did not occur, as ascertained by macroscopic examination at the end of each experiment; should microscopic injuries have occurred, the physiologic reaction would be vasoconstriction [50], which would limit rather than favor trans-epithelial transport, due to loss of the available absorbing surface. In our experimental model, the amounts of anthocyanins in the liver and in the kidneys were both about 0.1% of the administered dose (Tables 1 and 2). The amounts of anthocyanins that were found in plasma, liver, and kidneys after 30 min of intragastric administration were not significantly different than those detected at 10 min (*t*-test, $p < 0.05$). However, due to the lower mass of the kidney than that of the liver, the concentration of anthocyanins in the kidney was two to four-fold higher than in the liver, showing that, in the short term, the kidney is more efficient than the liver at taking up anthocyanins.

The data presented above show that anthocyanins, shortly after their appearance in plasma, are taken up more efficiently into the kidney than into the liver. Preferential urinary over bile excretion has also been observed by other studies testing pure anthocyanins [28, 30, 51]. Because of the high urinary excretion rate reported in these studies, anthocyanins are considered to be eliminated from the body by both glomerular filtration and renal tubular secretion [30]. The sequential steps of anthocyanin uptake into cells, methylation and tubular secretion into the urine might act as an overall mechanism for anthocyanin nephrotropism.

3.4 Involvement of bilitranslocase in uptake of malvidin 3-glucoside in kidney tubular cells

While glomerular filtration occurs passively, tubular secretion requires two sequential steps of membrane transport, the first one being influx from the peri-tubular capillaries into tubular cells and the second one efflux from there into the tubule (Fig. 4). Due to the polar nature of anthocyanins, their membrane transport is expectedly mediated by specific membrane carriers.

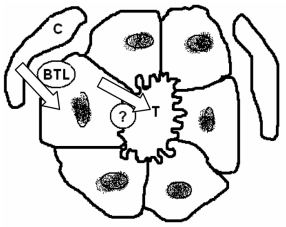


Figure 4. Scheme of the membrane transport steps of renal tubular secretion of organic anions. C, peri-tubular capillary; T, tubular lumen; BTL, bilitranslocase, set at the basolateral domain of the cell plasma membrane and involved in organic anion uptake from the blood into tubular cells;?, unknown membrane transporter(s) at the apical domain of the cell plasma membrane and involved in organic anion efflux into the tubular lumen.

So far, carrier-mediated membrane transport of anthocyanins has been described only in liver cells, where it has been shown to be strongly dependent on the organic anion carrier named bilitranslocase [31]. That result was predictable, since malvidin 3-glucoside is a strong competitive inhibitor of bilitranslocase transport in rat liver plasma membrane vesicles [32].

Bilitranslocase is also expressed in the kidney at the level of the basolateral membrane domain, but not at the apical one [33] (Fig. 4). In order to directly check if the kidney isoform of bilitranslocase can interact with malvidin 3-glucoside, basolateral plasma membrane vesicles were prepared from kidney cortex. The transport activity of bilitranslocase was assayed as valinomycin-induced BSP uptake into vesicles, according to standard procedures [32, 38]. Figure 3A shows that such BSP transport activity displays saturation with respect of the transported substrate. The calculated Michaelis–Menten constant ($K_M = 18.4 \pm 0.6$ μM) was similar to that reported earlier [33] and higher than found in the liver ($K_M = 5.36 \pm 0.63$ μM [52]). From these data, it can be speculated that the transport site of the kidney isoform of bilitranslocase is structurally different than the corresponding site of the liver isoform. Malvidin 3-glucoside behaved as a pure competitive inhibitor, as previously observed in the liver [32]. The calculated inhibition constant ($K_i = 4.8 \pm 0.2$ μM) was however again higher than in the liver ($K_i = 1.42 \pm 0.13$ μM). The ratio K_M kidney/K_M liver = 3.47 is essentially identical to the ratio K_i kidney/K_i liver = 3.43, showing that the loss of function with respect to either electrogenic BSP transport or inhibition by malvidin 3-glucoside had the same extent. Though this might be a sheer coincidence, these observations could also be interpreted as experimental evidence that both BSP and the anthocyanin engage at the same site of the carrier through a common set of chemical interactions, supported by a set of hydrogen bonds certainly involving also the B ring, as deduced by neural network-assisted quantitative structure–activity relationship [53]. Figure 3B shows that valinomycin-induced BSP uptake into vesicles was inhibited by an antisequence bilitranslocase antibody. This is meaningful, since the same antibody (antibody B) was also shown to inhibit malvidin 3-glucoside uptake into cultured liver cells [31].

4 Concluding remarks

Glomerular filtration of anthocyanins might be a mechanism of anthocyanin excretion into the urine and it is the most plausible mechanism for the elimination of intact anthocyanins with relative molecular mass close to 1000, as for instance acylated anthocyanins [54]. However, the finding that bilitranslocase is selectively expressed at the basolateral domain of the kidney tubular cell plasma membrane (Fig. 4) suggests that anthocyanins that escaped glomerular filtration might be efficiently taken up into tubular cells and then secreted into the urine. This conclusion is also supported by the evidence shown above of active anthocyanin metabolism in kidney tubular cells. The highly likely involvement of catechol-*O*-methyl transferase in methylating anthocyanins seems to be the required step to both accumulate methylated anthocyanins in the kidney and to direct them into the tubular lumen by transport across the apical domain of the kidney tubular cell plasma membrane. The same paradigm of urinary excretory pathway has also been claimed for *trans*-caftaric acid, another grape polyphenol [49]. It can be speculated that the last membrane transport step (*i.e.*, secretion from tubular cells into urine, Fig. 4), yet to be characterized, has to be quite efficient, since methylated anthocyanins might be transported back from the cells to the blood and this back-transport might be catalyzed again by bilitranslocase, which is a bi-directional carrier (Terdoslavich and Passamonti, unpublished data) and, in its liver isoform, has higher affinity for anthocyanosides with methoxyl rather than hydroxyl groups on the B ring [32]. Further investigation on the interaction of anthocyanins with bilitranslocase and other transporters expressed at both sides (basolateral and apical) of kidney tubular cells might provide important clues to understand the kidney transport and metabolism of these flavonoids.

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