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ORIGINAL CONTRIBUTION

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Abstract
Purpose This study investigated the absorption mechanism of the phytochemicals indicaxanthin and betanin and the influence of their food matrix (cactus pear and red beet) on the intestinal transport.
Methods Trans-epithelial transport of dietary-consistent amounts of indicaxanthin and betanin in Caco-2 cell monolayers seeded on Transwell^{R} inserts was measured in apical to basolateral (AP-BL) and basolateral to apical (BL-AP) direction, under an inwardly directed pH gradient (pH 6.0/7.4, AP/BL) mimicking luminal and serosal sides of human intestinal epithelium. The effect of inhibitors of membrane transporters on the absorption was also evaluated. Contribution of the paracellular route was investigated after EDTA treatment of the cell monolayer. In vitro digestion of betalainic food was performed to provide a post-intestinal fraction containing bioaccessible pigments.
Results Apparent permeability coefficients (P_{app}) in the absorptive direction were $(4.4 \pm 0.4) \times 10^{-6}$ and $(3.2 \pm 0.3) \times 10^{-6}$ cm s^{-1} for indicaxanthin and betanin, respectively. Transport of indicaxanthin was non-polarized, linear as a function of time and concentration, and unaffected by inhibitors of membrane transporters. Betanin exhibited significantly different bidirectional P_{app} values and non-linear efflux kinetics. The concentration-dependent betanin efflux was described by a kinetic model including one non-saturable ($K_d = 0.042$ μL cm^{-2} min^{-1}) and one saturable component identified as the apical multidrug resistance–associated protein 2 (MRP2; $K_m = 275$ μM; $J_{max} = 42$ pmol min^{-1} cm^{-2}). Permeation of both betalains increased remarkably after EDTA treatment of the cell monolayer. Neither indicaxanthin nor betanin underwent metabolic transformation. Food matrix did not affect trans-epithelial transfer of indicaxanthin, but reduced the absorption rate of betanin, red beet more than cactus pear.
Conclusions Dietary indicaxanthin and betanin can substantially be absorbed through paracellular junctions of intestinal epithelial cells. Additional trans-membrane permeation can be considered for betanin, whose absorption is limited by a MRP2-mediated efflux and negatively affected by its food matrix. Present findings are consistent with the quite higher bioavailability of indicaxanthin over betanin established in humans.

Keywords Betalains · Intestinal absorption · Caco-2 cells · Betalainic food · Indicaxanthin · Betanin

Introduction

Health benefits of dietary phytochemicals have been suggested in recent years. Among thousands of different compounds, phenolics, terpenoids, and sulfur-containing compounds have been considered because of reducing power and potential to affect redox-modulated cellular processes [1–3]. Betalains, which occur in a number of vegetables of the Cariophyllalae order, with cactus pear (*Opuntia* genus) fruits and red beet (*Beta vulgaris*) as the more representative dietary sources, are nitrogen-containing compounds, the structure of which comes from a tyrosine derivative known as betalamic acid. Depending on the components bonded to the main structure, violet-red

L. Tesoriere · C. Gentile · F. Angileri · A. Attanzio · M. Tutone · M. Allegra · M. A. Livrea (✉)
Dipartimento di Scienze e Tecnologie Molecolari e Biomolecolari (STEMBIO), Università di Palermo, Via Michele Cipolla 74, 90123 Palermo, Italy
e-mail: maria.livrea@unipa.it

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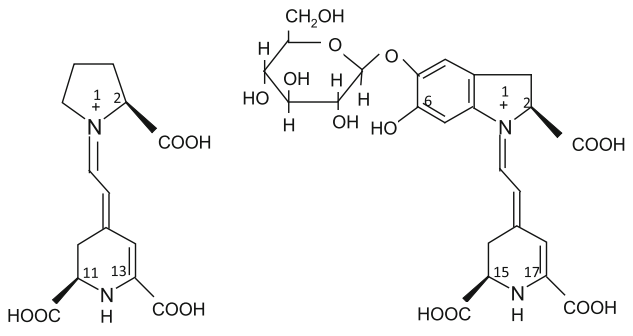
betacyanins or orange-yellow betaxanthins arise, the former when the component is 3,4-dihydroxy-phenylalanine (DOPA), which may be or may be not glycosylated, and the latter if the conjugation partners are amino acids or derived amines [4, 5]. The potential use of this class of molecules as natural dyes has widely been explored in the past, and experimental evidence has been provided about safety and factors affecting their stability under a variety of conditions [6, 7]. More recently, a number of bioactivities have emerged from in vitro studies in solution and in cell cultures. In particular, indicaxanthin and betanin (Fig. 1), the adducts of betalamic acid with proline and cyclo-DOPA 5-*O*-β glucoside, respectively, exhibited antioxidant activity in biological lipid environments from human low-density lipoproteins to membranes and whole cells [8–12], were able to modulate redox-mediated signal transduction pathways involved in inflammation in cultured endothelium [13], and showed antiproliferative effects in human tumor cell lines [14–16].

The health benefits of dietary phytochemicals cannot easily be deduced from in vitro studies, one main reason being that phytochemicals are processed as xenobiotics, that is, they may be or may be not absorbed to a suitable amount, transported in blood and distributed to tissues, metabolized and excreted. Human studies after the ingestion of dietary amounts of cactus pear fruit [17] or red beet juice [18, 19] indicated that indicaxanthin and betanin reach plasma concentrations of a micromolar order, which is quite higher than other phytochemicals such as polyphenols [20, 21]. In other studies [22], by a simulated gastro-intestinal digestion, we were able to demonstrate that digestive stability and additional factors relevant to the solubilization from food matrix and style of food processing influence the fraction of soluble betalains in the post-intestinal digesta potentially available for trans-epithelial transfer. Other factors that may concur to betalain bioavailability, including intestinal metabolism and mechanism of trans-epithelial transport, as well as interference of the food matrix on the absorptive process, are still unknown. In this study, we investigate intestinal permeation of indicaxanthin and betanin using Caco-2 cell monolayers seeded on Transwell® insert, a well-established model of the intestinal barrier. Originating from a human colorectal carcinoma, these cells spontaneously differentiate into polarized monolayers that exhibit morphological and functional characteristics of the intestinal absorptive epithelium, including intercellular tight junctions and apical microvilli, carriers for nutrient as well as efflux proteins, and a number of phase-2 enzymes required to xenobiotic disposition [23, 24]. In addition, since food matrix can affect absorption, the trans-epithelial permeation of betalains from various betalainic food submitted to a simulated gastro-intestinal digestion was compared with that of the pure compounds.



Indicaxanthin Betanin

Fig. 1 Molecular structure of indicaxanthin and betanin

Materials and methods

Unless stated otherwise, all reagents and materials were from Sigma Chemical Co. (St Louis, MO, USA) and solvents were of the highest purity or HPLC grade.

Betalain pigments

Betanin and indicaxanthin were isolated from cactus pear fruits as previously reported [25] and then purified according to Stintzing et al. [26]. The betalains were spectrophotometrically quantified using molar absorbance of 65,000 at 536 nm and 42,800 at 482 nm for betanin and indicaxanthin, respectively.

Physicochemical properties

Molecular descriptors of the betalains such as ClogP, ClogD were computed by Qikprop 3.1 predict program (Schrodinger, LLC, New York, NY, USA). The non-polar surface area (NPSA) was obtained as the difference between the molecular surface area (MSA) and polar surface area (PSA), calculated by CODESSA PRO software [27]. pK_a values of indicaxanthin were obtained by two different approaches, that is, the semi-empirical partial charge related and the Hammet and Taft linear free-energy relationships. Semi-empirical calculations were made by means of Marvin Sketch 5.0.6.1 prediction program (ChemAxon, Budapest, Hungary), based on the calculation of Mulliken partial charge of atoms in the molecule. The Hammet and Taft linear free-energy relationships were calculated by Epik 1.6 software (Schrödinger, LLC, New York, NY, USA), which adopts the combination of Hammet and Taf methods in conjunction with ionization and tautomerization tools. The pK_a values of betanin were obtained from the literature [28].

Cell culture

Caco-2 cells, obtained from the American Type Culture Collection (Rockville, MD, USA), were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco Life Technologies, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (Gibco Life Technologies), 1 % non-essential amino acids, 10 mM HEPES, 50 units/mL penicillin, 50 μg/mL streptomycin, and 100 μg/mL gentamicin and were maintained at 37 °C in 5 % CO_2 and 95 % humidity. Medium was changed 2–3 times per week. Caco-2 cells were used between passages 27 and 31. The cells were monitored by phase contrast microscopy, and fluorescent staining of DNA by Hoechst 33,258 (Thermo Fisher Scientific, Rockford, IL, USA) was used once a month to rule out mycoplasma contamination.

In vitro simulated digestion and preparation of the bioaccessible fraction from betalainic food

Fresh cactus pear fruits (*O. ficus indica* L. Mill.) from yellow and red Sicilian cultivars and red beet roots (*B.* V*ulgaris* L. ssp V*ulgaris*) from Tuscany (Italy) cultivations were obtained from a local market. Red beet juice (Biotta AG, CH-8274, Tagewiler, Switzerland) was purchased in a healthy food store. Cactus pear fruit juice was prepared after brief homogenization of the pulp in a kitchen-type blender and filtration through a colander (0.2 mm mesh size). Aliquots (20 g of each food preparation) was chewed 10 times by a single investigator and subsequently expelled in a tared beaker. The post-oral material was briefly homogenized in Hanks' balanced salt solution, pH 7.4 (HBSS, 1:2, w:v) for 2 min in a laboratory blender (Waring, New Hartford, CT), acidified at pH 2.0 with HCl, and 8 mg/mL porcine pepsin (3,200–4,500 units/mg) was added. After incubation in a shaking (100 rpm) water bath (type M 428-BD, Instruments s.r.l., Bernareggio, Mi, Italy) at 37 °C, for 2 h to simulate the gastric phase, the pH of the sample was immediately increased to 7.4 with 0.5 M $NaHCO_3$. The small intestinal phase of digestion was started after the addition of 2.4 mg/mL porcine bile extract and 0.4 mg/mL pancreatin. After incubation in the shaking water bath as above, for 2 h at 37 °C, the post-intestinal digest was centrifuged at 167,000*g*, for 35 min at 4 °C in a Beckman Optima TLX ultracentrifuge, equipped with an MLA-55 rotor (Beckman Instruments, Inc., Palo Alto, CA, USA), to separate the aqueous bioaccessible fraction (BF) from particulate material. Digestive enzymes were removed by ultracentrifugation through YM-10 membranes, and the betalain content of BF was measured by HPLC. Food BF was stored at −80 °C until use.

Cytotoxicity of the BFs on Caco-2 cells was checked in pilot studies. Caco-2 cells were seeded at 5×10^5 cells well in a six-well flat-bottom plate, and the medium was changed three times a week. After 15 days from confluence, differentiated monolayers were washed three times with HBSS, and then 2 mL of food BF was added. The BFs were filtered through a Millex HV 0.2 μm filter (Millipore, Billerica, MA, USA) immediately before the use. After a 90-min incubation, cells were washed and 50 μL HBSS containing 5 mg/mL MTT was added. The medium was discarded after a 4-h incubation at 37 °C, and formazan blue formed in the cells was dissolved in DMSO. The absorbance at 540 nm of MTT formazan of untreated cells was taken as 100 % viability. In addition, viability of the cells after the treatment was determined by trypan blue exclusion and microscopy examination. Neither treatment caused cell toxicity.

Trans-epithelial transport

Transport experiments were carried out using Transwell^R inserts (polycarbonate membrane, 0.4 μm pore size, 24 mm diameter, Corning Inc., Corning, NY, USA). Inserts were placed in 6-well plates. Caco-2 cells were seeded at 5×10^4 cells per cm^2 on the membrane insert with 1.5 mL of medium in the apical/luminal side (AP) and 2.5 mL of medium in the basolateral/serosal side (BL). Cells reached confluence within 5 days post-seeding. Culture medium was changed three times a week. On day 15 after reaching confluence, the DMEM was removed and the cells were rinsed three times with HBSS. The integrity of Caco-2 cell monolayers was evaluated by measuring the trans-epithelial electric resistance (TEER), according to Hidalgo et al. [29]. TEER values across the cell monolayers were measured using a Millicell-ERS voltohmeter (Millipore Corp., Bedford, MA). Only monolayers with TEER > 300 Ω/cm^2 were utilized. After washing of the cells with HBSS as reported above, 100 μM indicaxanthin, betanin or food BF in HBSS was added to the donor compartment and HBSS to the acceptor one. HBSS in the apical compartment was buffered at pH 6.0 with 20 mM 2-(*N*-morpholine) ethanesulfonic acid (MES). When necessary, HBSS of various either inhibitors (pravastatin, 5 mM; verapamil, 5 mM; indomethacin, 10 mM) or substrates (ferulic acid, 10 mM; acetic acid, 5 mM; valproic acid, 10 mM; glucose 10 mM) of membrane transporters was added in the AP side. Concentration-dependent trans-epithelial transport of either indicaxanthin or betanin was measured by varying the betalain concentrations between 100 μM and 2 mM under all other conditions unaltered. Cultures were incubated (37 °C, 5 % CO_2) and the acceptor medium was taken at 15-min time-intervals between 0 and 90 min and replaced with fresh HBSS. The acceptor medium was centrifuged at 1,000 *g* for 10 min at 4 °C and submitted to HPLC analysis of betalains. In parallel experiments, the flux of marker

compounds, phenolsulfonaphtalein (phenol red, 5 mM) and testosterone (100 μM), was evaluated by spectrophotometric and HPLC analysis, respectively [30]. Thermal stability of betalains (100 μM to 2 mM) under the conditions of the experiments was checked in the absence of cells. No significant loss of both pigments was observed after 90-min incubation at 37 °C.

The effect of either purified betalains or food BF on the barrier integrity of Caco-2 cell monolayers was assessed by checking the TEER values at the end of each transport experiment. In addition, the transfer of phenol red from the AP-to-BL compartment was also measured. Under the conditions applied, treatment with either betalains or BF did not significantly modify the monolayer resistance or barrier integrity.

The apparent permeability coefficients (P_{app}) were calculated according to the equation

$$P_{app} = \frac{V}{AC_o}\frac{dC}{dt}\ \text{cm s}^{-1} \quad (1)$$

where V = the volume of solution in the receiving compartment, A = the membrane surface area (4.71 cm^2), C_o = the initial concentration in the donor compartment, and dC/dt = the steady-state flux across the monolayer calculated as the slope of the curve betalain concentration in the receiving compartment versus time [31].

The mass balance, calculated as the percentage of the original pigment mass accounted for at the end of the transepithelial transport experiments, was assessed by evaluating the compound recovered in the donor and receiving chambers, and associated with cells. To this end, the cell monolayer was washed three times with 2 mL of HBSS containing 5 mmol/L sodium taurocholate, to remove the pigment adhered to cell surface. Rinsed cells were scraped in methanol, and each well was washed three times with the same solvent. Cells with washing solvent were immediately extracted, sonicated in ice bath for 5 min, and centrifuged at 2,000 *g* for 5 min. Methanol supernatant was collected, and the cells were re-suspended in methanol and extracted again as above. The methanol extracts were gathered and reconstituted in suitable solvent for HPLC analysis of betalains.

Kinetic parameters for the efflux of betanin were obtained by fitting the data to a model incorporating saturable and non-saturable components, according to Eq. 2 (Gepasi software package, 3.30):

$$J = \frac{J_{max}C}{K_m + C} + K_dC\ \text{pmol min}^{-1}\ \text{cm}^{-2} \quad (2)$$

where J is the flux normalized to unit surface area, J_{max} is the maximum efflux rate, K_m is the kinetic constant for saturable transport, K_d is the kinetic constant for non-saturable transport, and C is the betanin concentration.

Transport after alteration in the tight junction barrier

A 10 μM EDTA solution was prepared using PBS solution without Ca^{2+}/Mg^{2+} and applied to the apical and basolateral sides of Caco-2 cell monolayers for 5 min at 37 °C [32]. After the solution was removed, cells were washed three times with the PBS without Ca^{2+}/Mg^{2+}, before adding betalains (100 μM), phenol red (5 mM) or testosterone (100 μM), at the apical compartment, under pH gradient conditions (pH 6.0/7.4; AP/BL).

HPLC analysis of betalains

HPLC measurements of betalain pigments were performed as reported [25] using a RP-18e Performance column (100 × 4.6 mm; Merck, Darmstadt, Germany), equipped with RP-18e Chromolith guard cartridge (5 × 4.6 mm, Merck), eluted with a 20-min linear gradient elution from solvent A (1 % acetic acid in water) to 20 % solvent B (1 % acetic acid in acetonitrile), at a flow rate of 1 mL/min. Spectrophotometric detection of indicaxanthin (Rt 9.3 min) or betanin (Rt 12.5 min) was at 482 nm or 536 nm, respectively. Quantitation was by reference to curves constructed with 1–100 ng of the purified compounds, and by relating the amount of compound under analysis to the peak area.

Statistical analysis of data

All data are expressed as mean ± SD. Three independent observations were made for each experiment. All experiments have been replicated two to three times, to have $6 < n < 9$. Statistical difference was calculated using unpaired t test. Significance was accepted if the null hypothesis was rejected at the $p < 0.05$ level. Calculations and graphs were obtained by Instat-3 statistical software (GraphPad Software Inc., San Diego, CA).

Results

Physicochemical parameters

Physicochemical parameters and ionization constants calculated for indicaxanthin and betanin are reported in Table 1. Indicaxanthin and betanin are cationized molecules, with a positive charge localized in proximity of the N1 nitrogen (Fig. 1). Both molecules possess a number of ionizable carboxyl groups, with pK_a between 2.0 and 5.4 from our calculations and literature data. In addition, betanin bears a phenol hydroxyl at the cyclo-Dopa, the pK_a of which has been reported more acidic than expected [28]. In accordance with the measurements, indicaxanthin

Table 1 Physicochemical parameters of indicaxanthin and betanin

	MW	logP[a]	logD[b]	PSA (Å^2)	NPSA (Å^2)	pK_a	
						(COOH)[c]	(phenol-OH)[c]
Indicaxanthin	309	0.362	−7.25	126.92	161.11	$5.0_{(2)};3.7_{(11)};2.6_{(13)}$[d]	
Betanin	551	−1.767	−15.63	246.53	229.26	$<3.4_{(2)};3.4_{(15)};3.4_{(17)}$[e]	$<7.4_{(6)}$[e]

The molecular surface is described by the polar surface area (PSA) and the non-polar surface area (NPSA)

[a] Octanol/water partition coefficient

[b] Octanol/buffer pH 6.0 partition coefficient

[c] C atom in bracket

[d] Theoretically calculated pK_a values (see "Materials and methods")

[e] pK_a values from Ref [28]

mainly exists as a bis-anion at both pH 6.0 and 7.4, whereas betanin must shift toward a tris-anion around pH 7.0 [18, 28].

Trans-epithelial transport of pure betalains

The bidirectional trans-epithelial transport of indicaxanthin and betanin across Caco-2 cell monolayers was investigated under a pH gradient (6.0/7.4; AP/BL). Functional characteristics of the monolayer were preliminarily checked using phenol red and testosterone as markers for paracellular and trans-cellular permeation, respectively. The values of $P_{app\ (AP\text{-}to\text{-}BL)}$ calculated for these compounds were $0.28 \pm 0.01 \times 10^{-6}$ cm s^{-1} and $30.1 \pm 0.6 \times 10^{-6}$cm s^{-1}, respectively. Unless specified, transport experiments were done with 100 μM of either betalains and the amount of pigment in the receiving compartment was monitored at 15-min time-intervals for 90 min, under initial rate conditions.

The P_{app} measured for indicaxanthin across the Caco-2 cell monolayer in the absorptive AP-to-BL direction ($4.2 \pm 0.4 \times 10^{-6}$ cm s^{-1}) was comparable with that measured in the secretive BL-to-AP direction ($4.4 \pm 0.4 \times 10^{-6}$ cm s^{-1}, Fig. 2), indicating a non-polarized transport. In contrast, the calculated P_{app} of betanin was significantly higher in the absorptive AP-to-BL direction ($3.2 \pm 0.33 \times 10^{-6}$ cm s^{-1}) than in the efflux direction ($2.5 \pm 0.23 \times 10^{-6}$ cm s^{-1}, Fig. 2). Due to the –OH phenol group, the pK_a value of which is near to the pH in the donor BL compartment, variation in the ratio between bis-anion and tris-anion species in favor of the latter could limit the efflux of betanin, resulting in an asymmetric flux of the phytochemical.

HPLC measurements of the medium in the apical and in basolateral chambers were done to evaluate the mass balance of either indicaxanthin or betanin at the end of each experiment. Peaks relevant to the parent compounds provided evidence of a complete mass balance of both betalains, on the basis of the sum of the cumulative amounts recovered in the receiving chamber and the residual compound in the donor one, expressed as percent of the original pigment added. Only traces of the molecules were found in cells. These data indicate that no significant metabolism of betalains had occurred in intestinal cells.

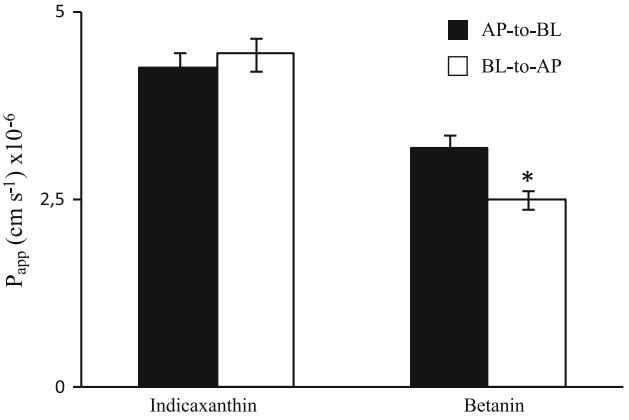


Fig. 2 Bidirectional apparent permeability coefficient (P_{app}) of betalains across Caco-2 cell monolayers under inwardly directed pH gradient (AP pH 6.0/BL pH 7.4). Trans-epithelial transport AP-to-BL (*black bars*) and BL-to-AP (*white bars*) and P_{app} measurements were as reported in Methods. Values are the mean ±SD of three separate experiments carried out in triplicate. *Statistically significant vs the relevant $P_{app\ (AP\text{-}to\text{-}BL)}$ direction with $p < 0.001$ (Student's t test)

The cumulative amounts of either indicaxanthin or betanin transported into the receiving chamber as a function of time are shown in Fig. 3. The amount of indicaxanthin crossing the monolayer increased linearly with time within 90 min, in both the absorptive and efflux direction, with comparable transport rates (Fig. 3a). For betanin, the relationship was linear in the absorptive direction, whereas a quite different relationship was observed in the efflux transport (Fig. 3b).

In the presence of the proton gradient applied, H^+-dependent influx carriers were considered. Then, the AP-to-BL trans-epithelial transport of betanin was measured in the presence of substrates for the monocarboxylate

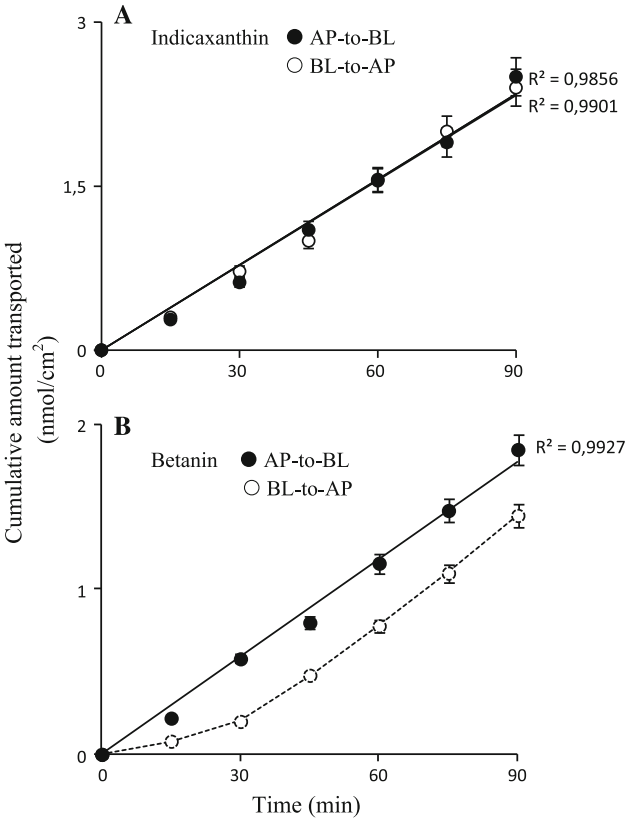


Fig. 3 Kinetics of the bidirectional trans-epithelial transport of indicaxanthin (**a**) and betanin (**b**). Measurement conditions were as reported in Methods. Data are the mean ± SD of three separate experiments carried out in triplicate. Trend lines (*full line*) from linear regression analysis fitted through zero showing good linearity ($R^2 > 0.95$) are reported

transporter (MCT1) and the organic anion–transporting polypeptide (OATP2B1), as potential competitors. Permeation of betanin was unaffected by either ferulic acid or acetate, both substrates of MCT1 [33, 34], and by pravastatin, a substrate for OATP2B1, whereas valproate, a substrate for both transporters [34], caused an unexpected increase of P_{app} (Table 2). The AP-to-BL trans-epithelial transport of betanin was also investigated in the presence of either verapamil or indomethacin as specific inhibitors of the efflux proteins P-glycoprotein and multidrug resistance–associated protein 2 (MRP2), respectively [35]. Indomethacin, but not verapamil, caused a significant increase in betanin permeation (Table 2), indicating that absorption of betanin across the cell monolayer was negatively affected by an MRP2-mediated efflux. An inhibition of permeation of around 35 % can be calculated at the applied betanin concentration. As a corollary, these data provided an explanation to the increase of P_{app} evaluated for betanin in the presence of valproate, since valproate has been shown to be a substrate of MRP2 [36]. Finally, the absorption of glycosylated phytochemicals could involve the Na^+-dependent glucose transporter of the brush border

Table 2 Influence of various compounds on the transport rate of betanin across Caco-2 cells monolayers

Compound	Concentration (mM)	P_{app} (AP-BL) ($\times 10^{-6}$cms^{-1})	n
Control		3.21 ± 0.30	4
Ferulic acid	10	3.17 ± 0.29	2
Acetic acid	5	3.25 ± 0.31	2
Pravastatin	5	3.11 ± 0.35	2
Valproic acid	10	4.51 ± 0.38*	3
Verapamil	5	3.09 ± 0.31	2
Indomethacin	10	4.93 ± 0.33*	3
Glucose	10	3.15 ± 0.28	2

Betanin (100 μM) was added to the apical (AP) compartment of monolayers in the absence (control) or in the presence of each compound at the indicated concentration. Trans-epithelial transport experiments were carried out under gradient pH conditions (pH 6.0/7.4; AP/BL), at 37 °C, for 60 min as described in Methods. Data are the mean ± SD of n separate experiments carried out in triplicate. * Statistically significant vs control with $p < 0.005$ (Student's t test)

membrane [37]. When 100 μM betanin was co-incubated in the apical side with excess glucose, the permeation rate was not significantly modified (Table 2).

In parallel experiments, the AP-to-BL permeability of indicaxanthin was measured in the presence of the mentioned competitors for H^+-dependent carriers and efflux transporters, to rule out that the apparent absence of a carrier-mediated transport actually resulted from a false negative. None of the compounds caused variations in the calculated P_{app} of indicaxanthin (not shown), ruling out the effects of the pigment on the transporters considered.

To further characterize the transfer of betalains, the flux of indicaxanthin and betanin in both directions was evaluated as a function of concentration. The observed relationship was linear for indicaxanthin in both the absorptive and efflux directions (Fig. 4a), in accordance with a simple diffusion process. For betanin, the relationship was linear in the absorptive direction only, which suggested a non-saturable process, whereas approached saturation was in the BL-to-AP direction (Fig. 4b). The experimental efflux data were then analyzed according to Eq. 2 describing a model consisting of one saturable and one non-saturable term (Fig. 4b, *inset*). The J_{max} and K_m estimated for the betanin efflux were 42 ± 5.0 pmol min^{-1} cm^{-2} and 275 ± 12 μM, respectively. The estimated coefficient of non-saturable transport K_d was 42 ± 2 nL min^{-1} cm^{-2}. When comparing K_d with the saturable transport clearance (J_{max}/K_m, 152 μL min^{-1} cm^{-2}), it appears that 73 % of the overall betanin efflux occurs via a saturable process, for concentrations $\ll K_m$. A comparison of the slopes from the curves representing the non-saturable AP-to-BL and BL-to-AP transports (Fig. 4b and *inset*) indicates that under the

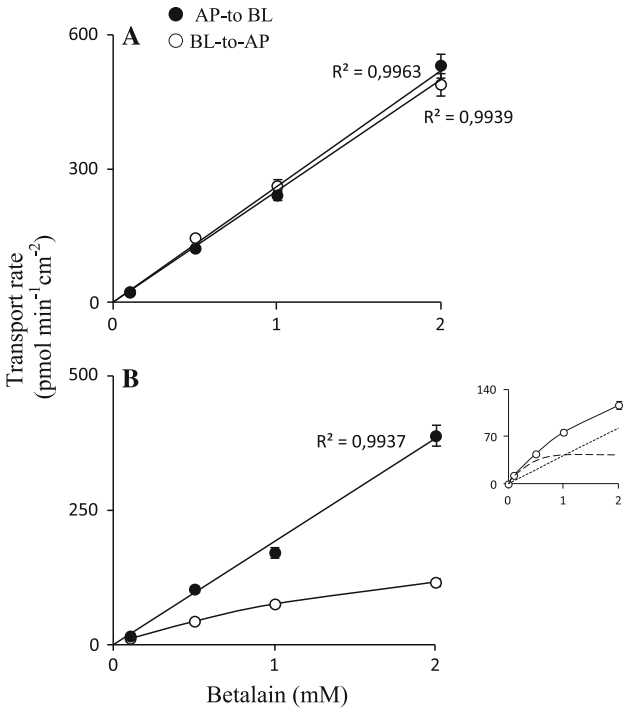


Fig. 4 Concentration-dependent transport of indicaxanthin (**a**) and betanin (**b**) across Caco-2 cell monolayers. Measurement conditions are reported in Methods. Data are the mean ± SD of three separate experiments carried out in triplicate. Trend lines (*full line*) from linear regression analysis fitted through zero showing good linearity ($R^2 > 0.95$) are reported. *Inset* B: the *solid*, *dashed*, and *dotted lines* represent the best fit to the efflux data, saturable and non-saturable components of the transport, respectively, according to Eq. 2

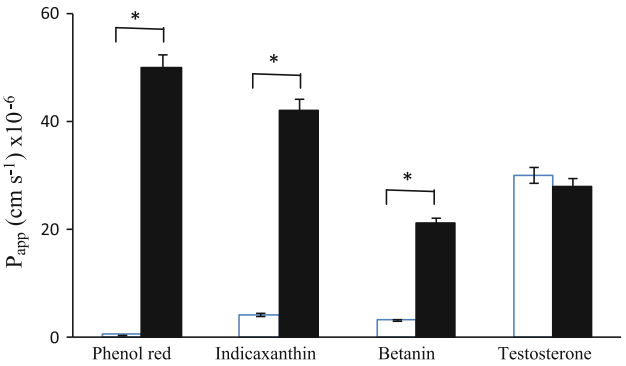


Fig. 5 Apparent permeability coefficient (P_{app}) for betalains across Caco-2 cell monolayer with intact tight junctions (*open bars*) and after opening of tight junctions with 5 mM EDTA (*black bars*), in comparison with phenol red and testosterone. Data are the mean ± SD of two separate experiments carried out in triplicate. *Statistically significant with $p < 0.001$ (Student's t test)

Table 3 Apparent permeation coefficient (P_{app}, AP-BL) across Caco-2 cell monolayers of betalains from the bioaccessible fraction (BF) of betalainic foods

Betalain	BF source	Concentration (μM)	P_{app} (10^{-6} cms^{-1})
Indicaxanthin	Cactus yellow fruit	30.5 ± 2.3	4.32 ± 0.35
	Cactus yellow fruit juice	29.8 ± 3.2	4.15 ± 0.41
Betanin	Raw red beet	62.5 ± 5.3	1.98 ± 0.21*
	Red beet juice	11.7 ± 1.1	1.82 ± 0.20*
	Cactus red fruit	12.2 ± 1.5	2.78 ± 0.34**
	Cactus red fruit juice	13.1 ± 1.2	2.61 ± 0.31**

Preparation of BF from foods and incubation conditions of Caco-2 monolayers were as reported in methods

Values are the mean ± SD of three determinations carried out on two different food samples. Significantly different from P_{app} of the relevant pure compound, * $p < 0.001$, ** $p < 0.05$ (Student's t test)

experimental conditions applied, betanin diffusion is fourfold higher in the influx than in the efflux direction.

The P_{app} of the AP-to-BL transfer of indicaxanthin and betanin across Caco-2 cell monolayers was evaluated after treatment of the monolayer with EDTA, which affects paracellular permeability via loosening of the tight junctions [31], in comparison with markers for paracellular and trans-cellular pathways. The P_{app} value of indicaxanthin increased around 11-fold, and that of betanin sevenfold (Fig. 5). As expected, an effective transfer of phenol red across the epithelial cell layer was observed only after loosening of the TJs, whereas the permeability of testosterone, the transfer of which occurs via a passive trans-cellular pathway, was almost unaffected by the EDTA treatment of the cells (Fig. 5). It was concluded that the transport of both betalains substantially occurred through paracellular permeation.

Transport of betalains from food digesta

The absorption of betalains from digested betalainic food was evaluated. Cactus pear fresh fruits and juice as a source of indicaxanthin and betanin, as well as raw red beet and red beet juice, as a source of betanin, were processed through a simulated in vitro digestion, and the BFs so obtained were placed at the apical side of Caco-2 cells layered on a Transwell insert, after measuring their betalain content. Permeation of indicaxanthin and betanin is reported in Table 3. Whereas permeation of indicaxanthin from cactus pear either fruit or juice was quite comparable with that of the pure pigment, the food matrix reduced the transport rate of betanin, red beet more than cactus pear (Table 3).

Discussion

Bi-directional transfer of indicaxanthin and betanin through Caco-2 cell monolayers

The intestinal absorption of two bioavailable dietary betalains, betanin and indicaxanthin, has been investigated

using Caco-2 cell monolayers grown on permeable polyester membranes as a model for the small intestinal mucosal epithelium, and the influence of the betalainic food matrix on the absorption process has been evaluated. Different tracts of the gastro-intestinal lumen are characterized by different pH values, and further pH changes are measured during digestion [38]. The pH in the upper gastro-intestinal tract under fasting conditions ranges from 5.0 to 6.5, and it has been reported to be 5.8 to 6.3 just above the absorbing epithelial cell layer [39]. We then measured the flux of betalains across the Caco-2 cell monolayer under conditions of an inwardly directed pH gradient (pH 6.0/7.4) approaching the pH microclimate at the luminal and serosal sides of human intestinal cells, with maintaining first-order conditions to mimic physiological absorption circumstances at the small intestine. In addition, the bulk of the experiments were carried out using a 100-μM betalain concentration, which approached the amount in the intestinal digesta from dietary amounts of either cactus pear fruit pulp (200 g, 24 mg indicaxanthin) or raw red beet (100 g, 71 mg betanin) [22], when considering an intestinal volume of 600 mL [40].

Like for xenobiotics, the intestinal absorption of phytochemicals may occur passively, through trans-cellular permeation or paracellular route in accordance with molecular mass and physicochemical characteristics, and could involve either influx or efflux membrane transporters. Our computational analysis provided solubility parameters and the polar and NPSA of indicaxanthin and betanin, as well as dissociation constants of the indicaxanthin carboxyl groups. According to our calculations and literature data [18, 28], both compounds mainly occur as bis-anions at pH 6.0. The octanol/water partition coefficients Clog*P* and Clog*D* (pH 6.0) indicated that indicaxanthin is moderately less polar than betanin. Finally, the calculated NPSA provided evidence that both betalains have a quite large non-polar surface, accounting for around 50 % of surface area, which substantiated previous observations on the ability of these molecules to interact with lipid environments from membranes [9, 19, 41, 42] to low-density lipoproteins [8, 11, 17].

Generally, unless utilizing transporters in the epithelial membrane, charged solutes of a suitable molecular mass should diffuse through the paracellular route and be transported passively by solvent drag [43–46]. Quite consistent with the physicochemical features of indicaxanthin and betanin, our study shows that both compounds may cross the epithelial cell layer by passive diffusion via the paracellular pathway, without any metabolic transformation. Some findings, however, suggest a mixture of paracellular and trans-cellular transport for betanin.

The effects of perturbation on the permeation of solutes are used as criteria to determine the preferred route of intestinal transport [32, 47]. If the permeation is significantly affected by perturbation of cellular tight junctions (TJs), then the permeation is considered to occur predominantly by the paracellular route. We observed that an opening of TJs with EDTA induced a remarkable increase in the indicaxanthin and betanin influx, consistent with a paracellular transport of both compounds across the Caco-2 cell monolayer. Indeed, the trans-epithelial transfer of indicaxanthin did not appear to be polarized, did not show saturation kinetics, nor the absorption was varied by inhibiting membrane transporters, in accordance with the simple diffusion-solubility criteria governing paracellular transports. On the other hand, the absorption of the glycosylated betanin appeared polarized, and the cumulative transfer in the efflux direction was not linear with time nor with concentration. Kinetic modeling of the efflux data revealed parallel contribution of one non-saturable and one saturable component, identified as the apical efflux transporter MRP2. Quantitative analysis of the data showed that the betanin efflux mainly occurs through the saturable pathway (73 %) at betanin concentrations far below K_m (i.e. $\ll$275 μM), whereas the betanin diffusion rate is fourfold higher in the influx than in the efflux direction. Then, in spite of the apical efflux, under the present experimental set-up mimicking physiological pH and betalain concentration conditions, the permeation rate of betanin was higher in the absorptive than in the secretive direction, which should assure that absorption at the intestine will prevail. It may be worthwhile to mention that our experiments have been done with amounts of betalains consistent with the intestinal content after a reasonable dietary intake, which may be critical when dealing with carrier-mediated processes.

The involvement of MRP2 in the transport discloses interesting features of the betanin ability to migrate through the Caco-2 cell monolayer. While indicating that the bioavailability of this phytochemical is limited by an absorption barrier, our findings suggest that trans-cellular transfer of betanin could occur in parallel with the paracellular one. Partition of various ionized species into chemical bilayers has been determined [48], and trans-cellular transport of ionized species across Caco-2 cell monolayers has been suggested in other studies [49, 50]. Moreover, previously performed chemico-physical partition studies in our laboratory provided evidence that betanin locates at the phospholipid core of the bilayer in an aqueous phosphatidylcholine liposomal system [41]. Then, it seems reasonable to suppose betanin permeation of the cell membrane at the apical side, with access to the apical membrane efflux transporter, and possibly lateral diffusion to the basal side of the cell and release to the basolateral compartment.

With solutes of like charge, paracellular permeability is a function of the molecular mass and decreases with the

increase in molecular weight [43, 44, 46]. Since indicaxanthin and betanin have a comparable ionic charge at pH 6.0, the molecular mass should favor the paracellular permeation of indicaxanthin. Noteworthy that in the presence of indomethacin, that is, blocking the MRP2 efflux transporter, the P_{app} for the absorption of betanin has appeared as high as that of indicaxanthin may be an indirect evidence that additional trans-membrane transport of betanin does occur.

The intestinal absorption of xenobiotics is considered negligible if the $P_{app} < 0.1 \times 10^{-6}$ cm s^{-1} and essentially complete if the trans-epithelial $P_{app} > 5.0 \times 10^{-6}$ cm s^{-1} [51, 52]. With the observed permeability coefficients, the trans-epithelial gradient of indicaxanthin and betanin at the intestinal lumen after ingestion, and their continuous removal by the bloodstream at the serosal side, could account for a significant intestinal transport. Present data indeed are consistent with and appear to validate the high fraction of dietary indicaxanthin absorbed in vivo [17]. Bioavailability of betanin in humans has been shown much lower than that of indicaxanthin [17–19]. According to previous studies [22], around 50 % of betanin is lost during the digestive process; however, its recovery in human urine was found no more than 3 % of the compound ingested with various foods [17–19]. Present findings on the intestinal processing and the calculated P_{app} do not fully consist with the bioavailability measurements. The complexity of the in vivo system could involve other significant losses, possibly hydrolytic processes by glycosidases of the intestinal microflora, and/or some oxidation of the pigment in the body [12]. Other factors, including the food matrix, could play additional roles.

Influence of betalainic food matrix on the intestinal permeation of the pigments

The absorption efficacy of phytochemicals can be affected by the mixture of their food matrix [53]. With respect to the pure compounds, the trans-epithelial transport rate of betanin from the soluble fraction of betalainic food digesta was strongly reduced, whereas that of indicaxanthin was not. Phenolic groups of phytochemicals may be involved in hydrogen bonding with protein moiety [54], and then complexes between betanin and soluble protein fragments in the post-intestinal digesta could prevent a fraction of the pigment to be absorbed. Eventual competition by other betalainic food components for the paracellular pathway could also be hypothesized. Present observations that betanin from cactus pear appeared more readily absorbed than from red beet are quite in accordance with previous studies in humans, showing higher bioavailability of betanin from dietary cactus pear fruit than from red beet juice [17–19]. This may deserve consideration for nutritional purposes.

Conclusive remarks

Dietary bioactive phytochemicals are now considered potential nutraceuticals/pharmacological molecules [55], and then analyzing mechanisms and factors affecting intestinal transfer could help to predict their eventual effects in the body. Definite evidence has been provided that the very high bioavailability of dietary indicaxanthin in humans [17] results not only from relatively high stability of the molecule to the digestive process [22], but also from favorable intestinal absorption through paracellular route by solvent drag, and easy release from food. Betanin bioavailability, instead, appears to be limited by digestive instability [22] and by an intestinal efflux mechanism reducing the absorption by around 35 % for betanin concentrations consistent with a nutritional intake. Furthermore, its intestinal permeation may negatively be influenced by its food matrix. Importantly, indicaxanthin and betanin do not need metabolic transformations to be released in plasma and circulate as unconjugated molecules. Taking all these facts into consideration, beneficial effects of dietary betalain pigments, as well as the impact of betalainic foods on human health [56], may be considered and results from appropriately planned in vitro studies may be interpreted to suggest real effects in vivo. Present data may provide a basis for research on the potential health effects of these substances, eventually to be orally administered as purified compounds.

Conflict of interest The authors declare that there are no conflicts of interest.

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