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Investigating the Transport Dynamics of Anthocyanins from Unprocessed Fruit and Processed Fruit Juice from Sour Cherry (*Prunus cerasus* L.) across Intestinal Epithelial Cells

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ABSTRACT: Anthocyanins can contribute to human health through preventing a variety of diseases. The uptake of these compounds from food and the parameters determining uptake efficiency within the human body are still poorly understood. Here we have employed a Caco-2 cell based system to investigate the transport of key antioxidant food components from sour cherry (*Prunus cerasus* L.) across the intestinal epithelial barrier. Anthocyanins and (−)-epicatechin were supplied in three contrasting matrices: fruit, processed fruit cherry juice, and polyphenolic fractions obtained by solid-phase extraction. Results show that both compound types behave differently. Fruit or juice matrices display comparable transport across the epithelial cell layer. The juice supplements sucrose and citric acid, which are regularly added to processed foods, have a positive effect on stability and transport. Polyphenolic fractions display a lower transport efficiency, relative to that of the fruit or juice, indicating the importance of food matrix components for intestinal absorption of polyphenols.

KEYWORDS: *sour cherry, Prunus cerasus L., nectar processing, anthocyanins, in vitro, bioavailability, Caco-2 cell model*

INTRODUCTION

Anthocyanins, one of the major subgroups of the flavonoids, are naturally occurring plant secondary metabolites that are present in many foods such as red fruits, vegetables, and beverages. Recent studies have linked the consumption of these compounds to the prevention of a variety of human diseases, such as cancer,[1,2] cardiovascular disease,[3] obesity, and diabetes.[4] Usually, these plant metabolites are consumed in processed form, for instance, in wine or fruit juice.[5,6] In general, people have a high intake of anthocyanins compared to other flavonoid groups, particularly, for example, through the consumption of fruit juices.[5,7] However, while flavonoids such as quercetin glycosides in foods are absorbed well by the human gastrointestinal tract and can be detected in plasma and urine,[7,8] the absorption of anthocyanins is notoriously poor.[9] In addition, anthocyanins are sensitive to oxygen, enzymatic activity, light, temperature, and particularly pH.[10] The varying pH conditions occurring during food processing, storage, and on passing through the gastrointestinal (GI) tract may change the chemical form of anthocyanins from red flavylium cation forms (which occur at pH < 2) to quinonoidal bases, hemiketals, and chalcones, which occur at neutral pH values. These different anthocyanin forms will differ not only in stability but also in bioactivity.[9] Thus, how much of the anthocyanins originally present in fruits and their products is actually available to exert bioactivity in humans depends on many parameters.

Fruit processing may have strong effects on the concentration of anthocyanins in the final product. For example, the preparation of grape juice from red grapes leads to a loss of the vast majority of anthocyanins during the process.[11] Sour cherry is commonly consumed as a nectar, which is produced from a fruit juice concentrate by the addition of water, sucrose syrup, and citric acid.[12] In contrast to grape, the production of sour cherry nectar is not associated with large losses of anthocyanins or with large changes in the content of other dominant phenolic compounds (e.g., flavan-3-ols, such as (−)-epicatechin), as was recently demonstrated in detail by our group.[12] Therefore, comparing the fate of anthocyanins between sour cherry fruit and nectar samples represents a valuable opportunity to study the effect of traditional industrial processing strategies on bioavailability.

One of the aspects that can influence the bioavailability of anthocyanins is their transport across the gut epithelium into the blood. The small intestine is primarily responsible for the uptake of food components. Intestinal functions can be influenced by such components, and responses can be reproduced using *in vitro* cell lines to analyze food−intestine interactions at the molecular level.[13] The Caco-2 cell line, a

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human intestinal epithelial cell model derived from a colon carcinoma, is known to be able to demonstrate many *in vivo* intestinal functions and has regularly been used to study the epithelial transport of many purified plant compounds.[14,15] Good correlations have been reported between *in vitro* Caco-2 cell permeability and *in vivo* absorption rates in rats[16] and humans.[17] The bioavailability of various phytochemicals, such as quercetin from red onions[18] and the tea flavonoid (−)-epicatechin,[15] as well as the antioxidant efficiency of bioactives, such as anthocyanins from bilberry extracts,[19] has also been investigated using the *in vitro* Caco-2 cell model system. However, this system has rarely been used to study the transport of such phenolic compounds when delivered in a fruit matrix or in processed products. Nevertheless, such studies are highly relevant since phenolic compounds can interact with each other and with other components in the food matrix,[20] which in turn may significantly influence their overall effect on human physiology.

In the work described here, we have aimed to assess the transport of sour cherry anthocyanins across the gut epithelium, using the human intestinal Caco-2 cell system as a model. The main objective was to evaluate the influence of processing sour cherry fruit into nectar on anthocyanin stability and bioavailability by exposing the cells to samples of sour cherry fruit and sour cherry nectar. In addition, we have investigated the effect of several processing treatments on the epithelial transport of anthocyanins, including the addition of sucrose and/or citric acid, as well as solid-phase extraction effects.

MATERIALS AND METHODS

Sour Cherry Fruit and Nectar Samples. Sour cherry fruit and the industrial-scale processed product, called nectar, were directly obtained from a Turkish fruit juice factory (Aroma Bursa Fruit Juice and Food Industry Inc., Turkey). Processing of fruit to juice (nectar) has been described in detail before.[12] During nectar production, raw sour cherry fruit material was first processed to the concentrated juice. Most importantly, production of concentrate included mash heating (80 °C, 90 s) and mash pressing (110 bar) steps. Press cake, a waste material, was subsequently subjected to three further extraction steps with water, in order to increase the juice yield from 73% to 85%. Collected press fractions were pooled and further pasteurized (95 °C, 90 s), enzyme treated (50 °C, 2 h), clarified (50 °C, 1 h), filtered and evaporated to concentrated juice (65°Bx). The concentrated juice was then processed to the nectar by addition of water, sucrose syrup and citric acid, and subsequent pasteurization (95 °C, 45 s).

Fruit and nectar samples were snap-frozen in liquid nitrogen, ground to a fine powder, and then stored at −80 °C until used.

Solid-Phase Extract Preparation. The phenolic compounds, including anthocyanins and procyanidins, in fruit and nectar samples were purified using a solid-phase extraction (SPE) procedure. OASIS HLB cartridges (Waters OASIS HLB 3 cc column, Waters Corp., Milford, MA) were first conditioned by rinsing with 6 mL 100% methanol followed by rinsing with 4 mL MQ water. Sour cherry fruit and nectar samples were prepared by dissolving 0.5 g fresh weight powder in 1.6 mL 5% methanol in MQ water (v:v), followed by 10 min of sonication and 10 min of centrifugation at 2500 rpm. To obtain the solid-phase extracted fruit (fruit-SPE) and the solid-phase extracted nectar (nectar-SPE) samples, these extracts were loaded onto the activated HLB cartridges that were subsequently washed with 4 mL MQ water. All cartridges were then eluted with 1 mL 75% methanol in MQ water (v:v) and 0.4 mL 100% methanol. The solvent was removed from the eluates using a speed vacuum concentrator (Christ RVC 2-18, Germany) until totally dry pellets of fruit-SPE and nectar-SPE samples were obtained. These were stored at −20 °C until further analysis.

Cell Culture. Media used for incubations with cells included Hank's Balanced Salt Solution (HBSS) (Invitrogen, 14170; no calcium, no magnesium, no phenol red), HBSS buffered with 100 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Sigma, H3375), Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, 42430-082; with 4.5 g/L glucose, no pyruvate, 4 mM L-glutamine, and 25 mM HEPES), with or without a supplement of 9.1% HyClone Fetal Bovine Serum (FBS, Fisher Scientific CH 30160.03) that had been heat-inactivated at 56 °C for 45 min. The Caco-2 human colon cancer cell line was obtained from the American-Type Culture Collection (ATCC HTB-37TM; USA). The cells were routinely grown in 75 cm^2 tissue culture flasks using DMEM + 9.1% FBS. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. Medium was changed three times per week, and cells were subcultured every 7 days (at 80−90% confluence). For subculturing, the cells were detached from the bottom of the flask using a 1-mL 0.25% trypsin-EDTA wash for 5−10 min at 37 °C and at 5% CO_2 and subcultured into new flasks with a dilution ratio varying from 1:2 to 1:6.

For transport experiments, Caco-2 cells, having a passage number between 40 and 51, were seeded in 6-well tissue culture plate inserts (Greiner bio-one 657640; translucent, 0.4 μm pores, 1×10^8 pores/cm, 452.4 mm^2 surface area for cell growth) at a concentration of approximately 6.0×10^5 cells/mL per well. Cells were allowed to grow and differentiate to confluent monolayers for 23−24 days post seeding. Culture medium was changed three times a week. To ensure that the monolayers exhibited the properties of a tight biological barrier, trans-epithelial electrical resistance (TEER) was monitored using a MilliCell-ERS voltohmmeter (Millipore Co., United States). Monolayers with TEER values exceeding 200 Ω cm^2 were used exclusively for transport experiments since TEER values above this value are generally considered as acceptable.[21] TEER values were also determined after completion of the exposure experiments to check for maintenance of integrity.

Selection of Cell Culture Medium. Four media, including HBSS, HBSS + 100 mM HEPES, DMEM, and DMEM + 9.1% FBS, were tested for their effect on the stability of sour cherry phenolic compounds. Both fruit and nectar samples were dissolved in these different media at a concentration of 16.7 mg dry-weight/mL medium and incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C for 6 h. Samples were taken before and after a 6-h incubation period. Samples were stabilized in 1:1 (v:v) methanol (adjusted to pH 2 with formic acid), and analyzed using HPLC for concentrations of cyanidin-3-(2^G-glucosylrutinoside) (referred to as C3GR) and (−)-epicatechin (using the procedure described below).

The effects of these four different media on the TEER value of a Caco-2 cell layer were analyzed. Cells were grown for 3 weeks in inserts using the standard DMEM + 9.1% FBS medium, and the TEER value was recorded. The standard medium was then replaced by the different media as used above for the stability analysis and preincubated in these media for 1 h. After 1 h (t = 0) and 7 h (t = 6) the TEER value was recorded.

Transport Experiments. Transport experiments were carried out using fruit and nectar samples, as well as their solid-phase extracted forms (fruit-SPE and nectar-SPE), dissolved in DMEM (without FBS), which was selected as transport medium. In order to use sample concentrations that do not negatively influence TEER values of Caco-2 cells, preliminary studies were performed in which varying sample concentrations were analyzed during several hours of exposures to cells (data not shown). TEER analyses indicated that concentrations lower than 50 mg dry-weight/mL medium provided TEER values that were greater than 80% of the initial TEER value. This led to the choice of using fruit and nectar samples at concentrations of 23 ± 2 and 48 ± 5 mg dry-weight/mL, respectively, for the transport experiments. The higher amount of nectar was chosen to compensate for the presence of sucrose in the dry matter of nectar (>50%). In each sample, these concentrations correspond to equal amounts (55 ± 5 μM) of cyanidin-3-(2^G-glucosylrutinoside) (C3GR, the major anthocyanin compound in sour cherry). Epicatechin concentrations in the same fruit and nectar samples were 12.7 and 36.2 μM, respectively. In order to make these calculations, all samples were analyzed with HPLC for their

Table 1. Changes (%) in Cyanidin-3-(2^G-glucosylrutinoside) and Epicatechin Concentrations in Fruit and Nectar During 6 h of Incubation in Selected Test Media (without Cell Monolayers)

	fruit		nectar	
medium	C3GR[a]	epicatechin[a]	C3GR[a]	epicatechin[a]
HBSS	101 ± 2 a	99 ± 3 a	98.4 ± 0.4 a	101 ± 2 a
HBSS + HEPES	53 ± 3 b	70 ± 5 b	84 ± 2 b	96 ± 1 a
DMEM	37.6 ± 0.5 c	24 ± 2 c	71.1 ± 0.1 c	77 ± 4 b
DMEM + FBS	56 ± 2 b	77 ± 2 b	47 ± 4 d	71 ± 3 b

[a]Values represent the percentage of initial concentrations measured at 0 h (measurements performed in triplicates). Different letters in the columns represent statistically significant differences ($p < 0.05$).

anthocyanin and procyanidin levels, using the procedure described below. Furthermore, the possible effects of sucrose and/or citric acid, which are routinely added to the nectar during processing and differentiated nectar from fruit, on transport through Caco-2 cells were assessed. Citric acid was added to the fruit sample as 2.4 μL citric acid (50% w/v)/mL fruit in DMEM to provide the same pH value (pH 4) as obtained for the nectar sample. The added sucrose constituted approximately 50% of the dry matter of the fruit sample in DMEM (21 mg sucrose added to 23 ± 2 mg dry-weight fruit). In the third sample, both citric acid and sucrose were added to the fruit sample. This led to a total of five samples: fruit (F); fruit + citric acid (F+CA); fruit + sucrose (F+SUC); fruit + citric acid + sucrose (F+CA+SUC); and nectar (N).

Additionally, fruit-SPE and nectar-SPE pellets were diluted in transport medium in order to obtain the anthocyanin concentrations equal to the concentrations used for fruit and nectar samples, which was regulated on the basis of the HPLC measurements. Since both SPE samples gave a pH value of pH 8 in transport medium, also a set of SPE samples was analyzed where citric acid was added to adjust the pH to 4, similar to the situation with untreated nectar.

The Caco-2 cells used in transport experiments were seeded, grown, and analyzed for TEER values as detailed above. First, the transport medium was changed with the culture medium, and 1 h of preincubation time was applied. Then, the samples in transport medium were loaded into the apical compartment of the culture wells in a volume of 2 mL. Transport medium alone (2.5. mL) was loaded into the basolateral compartment. The Caco-2 cells were incubated with fruit/nectar materials for 6 h ($t = 6$) at 37 °C and 5% CO_2. Subsequently, apical and basolateral sides were sampled (≥3 replicates), stabilized in 1:1 (v:v) methanol (adjusted to pH 2 with formic acid), and stored at −20 °C until HPLC analysis. The analysis of anthocyanins and flavan-3-ols was carried out using methanolic extracts of both apical and basolateral medium using RP-HPLC as described below. The HPLC results obtained for apical side samples were used to evaluate the recovery of anthocyanins from the cell layer. The results obtained for samples from basolateral compartments were used to assess the passage of anthocyanins through cell monolayer during the 6-h incubation.

HPLC Analysis. HPLC analysis was carried out to detect sour cherry anthocyanins and flavan-3-ols. Methanolic extracts were stored at −20 °C till the analysis and were sonicated for 10 min, centrifuged for 5 min at 13200 rpm, and filtered through 0.45 μm filters (Minisart SRP4, Biotech GmbH, Germany) into glass vials before the analysis. A W600 Waters HPLC system was used with a reversed phase (RP) Luna C18 column (150 mm × 4.6 mm, 3 μm; Phenomenex, Torrance, CA, USA) heated to 40 °C. A gradient from 95% to 25% MQ and 5% to 75% acetonitrile, both in 0.1% trifluoroacetic acid (1 mL min^{-1} flow rate) was applied for separation over a period of 50 min. Eluting compounds were monitored continuously between 240 and 600 nm using a PDA detector, after which anthocyanins were quantified at 512 nm. The detection of procyanidins was performed using the same HPLC system coupled to a Waters 2475 Fluorescence Detector with excitation at 275 nm and emission at 310 nm.[22] Absorbance spectra and retention times of eluting peaks were compared with those of available standards (Extrasynthese, France; Sigma, St. Louis, USA). For quantification, dose−response curves of cyanidin-3-glucoside and (−)-epicatechin (0−50 μg/mL) were used as references.

Statistical Analysis. Data were subjected to statistical analysis using Minitab software (version 16.1.0) for the analysis of variance (ANOVA). Pairwise comparisons between the treatments were performed using the Tukey test with a 95% confidence level.

RESULTS

Cell Culture Medium. The protocol applied to select the optimum culture medium considered both the stability of sour cherry phenolics and the cell monolayer integrity. The stability analysis revealed that the highest recoveries, for both anthocyanins and procyanidins, were observed when the samples were incubated in HBSS, in which recovery after incubation was close to 100% (Table 1). The lowest recovery of fruit compounds was observed in DMEM medium (37.6% for C3GR and 24.5% for epicatechin), whereas compounds in nectar material showed the lowest recovery in DMEM + 9.1% FBS (47.0% for C3GR and 71.2% for epicatechin) (Table 1). Since large effects of pH on stability of anthocyanins have been reported, the pH of the media with samples was recorded. In HBSS, both fruit and nectar samples led to a pH of 4, while in the other three media the pH was 7. This could indicate that the reduced levels found after incubation are due to a lower stability of the compounds as an effect of the high pH but also that FBS (containing proteins) might cause reduced recovery.

Although these different media have been used in published studies, we aimed to analyze whether these cell culture media, when incubated with Caco-2 cells, lead to a loss of cell layer integrity that would result in diffusion of metabolites, rather than transport. The TEER value measurements performed to investigate the layer integrity indicated that both DMEM media displayed a maintenance of the TEER value at 80−90% of the initial values at $t = 0$ and $t = 6$ (Table 2). On the other hand,

Table 2. TEER (Ω cm^2) Measurements Performed in Cell Culture Medium and During Incubation in Four Different Cell Media

medium	initial TEER value in culture medium	TEER at $t = 0$	TEER at $t = 6$
HBSS	277 ± 21	157.5 ± 0.7 (57%)[a]	110 ± 6 (40%)
HBSS with 100 mM HEPES	287.5 ± 0.7	157 ± 27 (55%)	120 ± 13 (42%)
DMEM with 9.1% FBS	273 ± 7	248 ± 3 (91%)	226 ± 17 (83%)
DMEM (no FBS)	285 ± 9	244 ± 6 (86%)	232 ± 5 (81%)

[a]Numbers in brackets represent the TEER values relative to the initial TEER value in percentages.

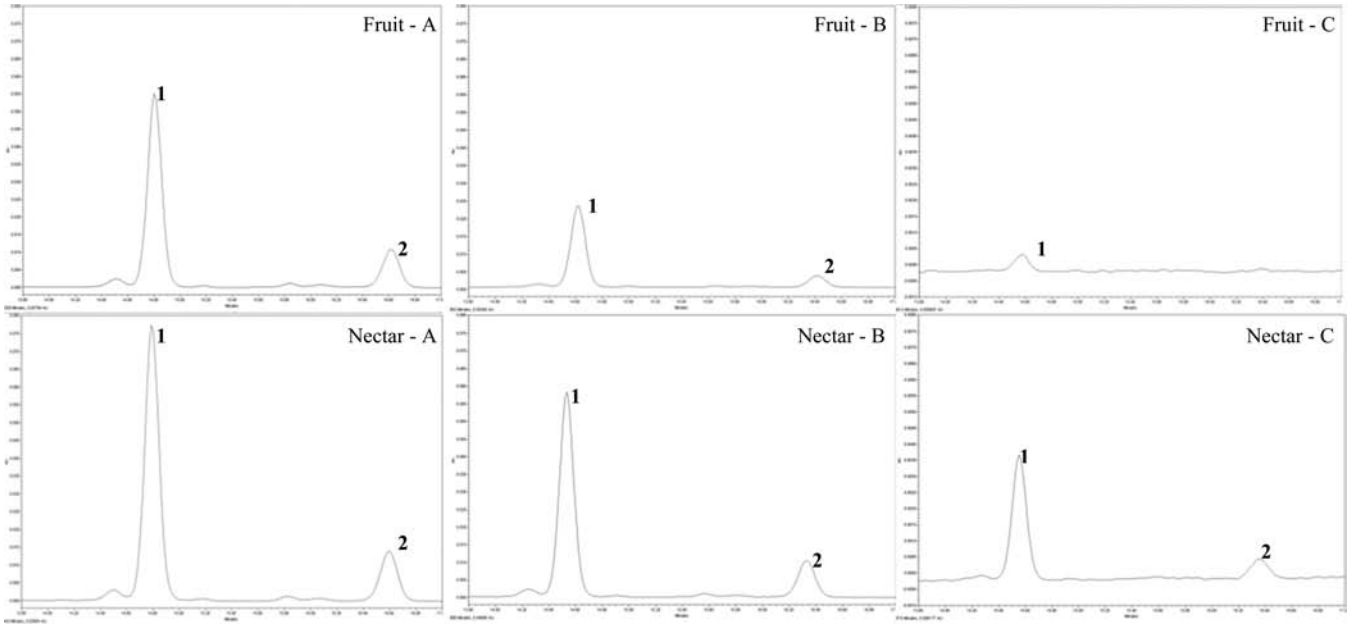

Figure 1. Representative HPLC chromatograms of sour cherry fruit (upper panel) and nectar (lower panel) anthocyanins in the apical side extracts at 0 h (A); in the apical side extracts after 6 h incubation (B); and in the basolateral side extracts after 6 h incubation (C). Numbers refer to the anthocyanins peaks: (1) cyanidin-3-(2^G-glucosylrutinoside), (2) cyanidin-3-rutinoside. Please note the scale differences among the A, B and C figures. The *x*, *y* scales for A figures: *x*, 13.8−17.0 min; *y*, −0.002−0.080. B figures: *x*, 13.8−17.0 min; *y*, −0.002−0.080. C figures: *x*, 13.8−17.0 min; *y*, −0.001−0.008.

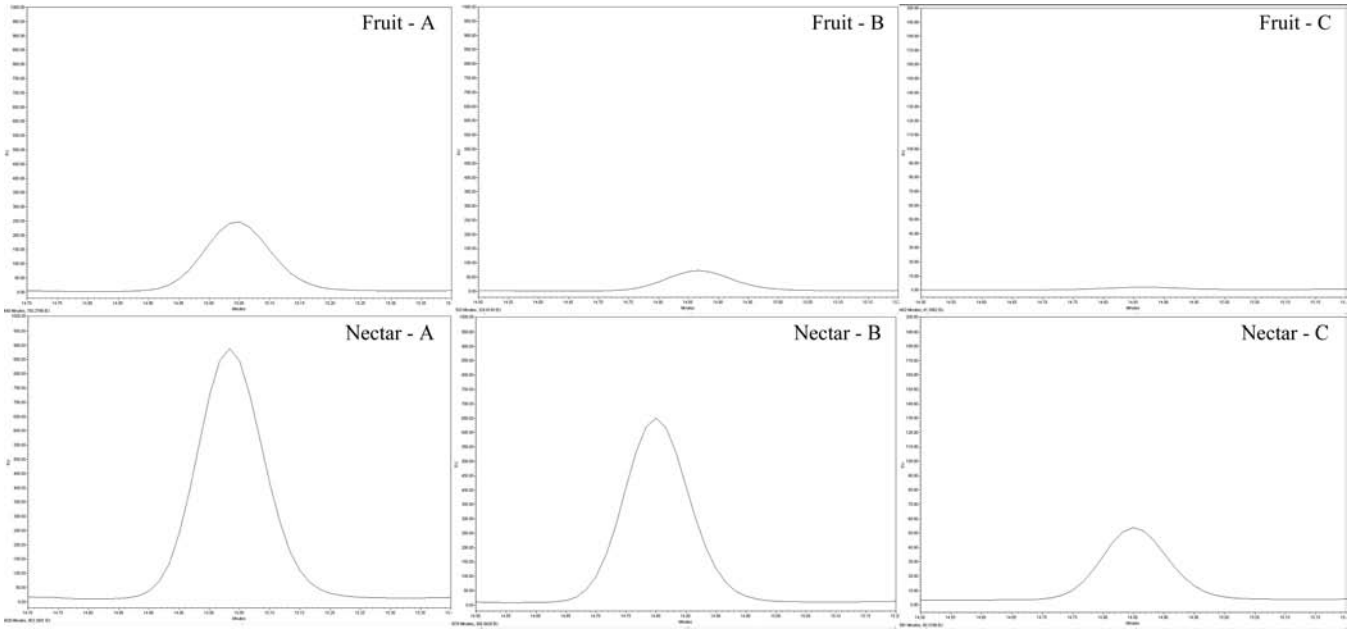

Figure 2. Representative HPLC chromatograms of the epicatechin peak in sour cherry fruit (upper panel) and nectar (lower panel) samples; in the apical side extracts at 0 h (A); in the apical side extracts after 6 h incubation (B); and in the basolateral side extracts after 6 h incubation (C). Please note the scale differences between the A, B, and C figures. The *x*, *y* scales for A figures: *x*, 14.7−15.4 min; *y*, −20−1000. B figures: *x*, 14.5−15.2 min; *y*, −20−1000. C figures: *x*, 14.5−15.2 min; *y*, −5−200.

both HBSS media led to a rapid decline in TEER values at $t = 0$ of incubation (below 200 Ω cm^2), and after prolonged incubation (at $t = 6$) TEER values had dropped even further, indicating a loss of integrity of the cell layer.

On the basis of these observations it was decided that HBSS media could not be used since they led to a high loss of cell layer integrity. Moreover, the low pH of HBSS, which likely explains the high stability of anthocyanins and procyanidins, does not reflect the natural conditions in the gut, which is generally maintained at pH = 6−7.[23] Inclusion of FBS in the medium could potentially lead to interactions of proteins with sour cherry phenolic compounds.[24,25] Therefore, incubations for transport assays were performed in DMEM medium without FBS, but in view of the reduced stability of the compounds in this medium, the stability (recovery in the apical side of cell layer) was monitored in each transport experiment.

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Table 3. Basolateral and Apical Side Recoveries and Transport Efficiencies of C3GR and Epicatechin for Fruit and Nectar Samples

		C3GR			epicatechin		
sample[a]	pH	basolateral side recovery (%)[b]	apical side recovery (%)[c]	transport efficiency[d]	basolateral side recovery (%)[b]	apical side recovery (%)[c]	transport efficiency[d]
F	6	0.52 ± 0.08 c	45 ± 2 c	0.011 ± 0.001 d	1.1 ± 0.4 c	33 ± 1 c	0.03 ± 0.01 a
F+CA	4	2.3 ± 0.9 b	78.4 ± 0.1 ab	0.03 ± 0.01 c	2.2 ± 1.1 bc	60 ± 6 b	0.03 ± 0.01 a
F+SUC	6	2.43 ± 0.09 ab	45.1 ± 0.2 c	0.054 ± 0.002 a	1.8 ± 0.1 bc	35 ± 2 c	0.053 ± 0.005 a
F+CA+SUC	4	3.9 ± 0.5 a	82 ± 2 a	0.048 ± 0.006 ab	3.8 ± 0.6 a	71 ± 1 a	0.054 ± 0.008 a
N	4	2.6 ± 0.4 ab	77 ± 2 b	0.034 ± 0.005 bc	3.3 ± 0.4 ab	76 ± 2 a	0.043 ± 0.005 a

[a]Abbreviations used for the sample names: F, fruit; N, nectar; CA, citric acid; SUC, sucrose. [b]Basolateral side recovery percentages were calculated as (C3GR or epicatechin concentrations at the basolateral side after 6 h of incubation)/(C3GR or epicatechin concentrations at the apical side at 0 h of incubation) × 100. Values represent the averages of triplicates ± standard deviation. Samples not sharing a letter are significantly different ($p < 0.05$). [c]Apical side recovery percentages were calculated as (C3GR or epicatechin concentrations at the apical side after 6 h of incubation)/(C3GR or epicatechin concentrations at the apical side at 0 h of incubation) × 100. Values represent the averages of triplicates ± standard deviation. Samples not sharing a letter are significantly different ($p < 0.05$). [d]Transport efficiency was calculated as (basolateral side recovery (%))/(apical side recovery (%)). Values represent the averages of triplicates ± standard deviation. Samples not sharing a letter are significantly different ($p < 0.05$).

Table 4. Recovery (%) on Apical Side and Transport Efficiency (%) to the Basolateral Side of C3GR and Epicatechin for SPE Samples

		C3GR			epicatechin		
sample[a]	pH	basolateral side recovery (%)[b]	apical side recovery (%)[c]	transport efficiency[d]	basolateral side recovery (%)[b]	apical side recovery (%)[c]	transport efficiency[d]
fruit-SPE	8	0.30 ± 0.06 b	52 ± 3 c	0.006 ± 0.001 a	0.00 ± 0.00 b	59 ± 4 b	0.000 ± 0.000 b
fruit-SPE + CA	4	0.57 ± 0.07 ab	117 ± 22 a	0.005 ± 0.002 a	0.4 ± 0.2 a	95 ± 2 a	0.005 ± 0.003 a
nectar-SPE	8	0.5 ± 0.1 ab	81 ± 10 bc	0.006 ± 0.002 a	0.33 ± 0.08 ab	86 ± 17 a	0.004 ± 0.002 ab
nectar-SPE + CA	4	0.58 ± 0.03 a	101 ± 1 ab	0.006 ± 0.000 a	0.3 ± 0.1 ab	98 ± 1 a	0.003 ± 0.001 ab

[a]Abbreviations used for the sample names: fruit-SPE, solid-phase extracted fruit; nectar-SPE, solid-phase extracted nectar; CA, citric acid. [b]Basolateral side recovery percentages were calculated as (C3GR or epicatechin concentrations at the basolateral side after 6 h of incubation)/(C3GR or epicatechin concentrations at the apical side at 0 h of incubation) × 100. Values represent the averages of triplicates ± standard deviation. Samples not sharing a letter are significantly different ($p < 0.05$). [c]Apical side recovery percentages were calculated as (C3GR or epicatechin concentrations at the apical side after 6 h of incubation)/(C3GR or epicatechin concentrations at the apical side at 0 h of incubation) × 100. Values represent the averages of triplicates ± standard deviation. Samples not sharing a letter are significantly different ($p < 0.05$). [d]Transport efficiency was calculated as (basolateral side recovery (%))/(apical side recovery (%)). Values represent the averages of triplicates ± standard deviation. Samples not sharing a letter are significantly different ($p < 0.05$).

Transport Experiments with Fruit and Nectar Samples. The fruit and nectar samples, including F, F+CA, F+SUC, F+CA+SUC, and N, were added to the apical (top) side of the Caco-2 cells growing in transwells, and samples were collected from the apical compartment after 0 and 6 h of incubation and from the basolateral (bottom) compartment after 6 h. After 6 h of exposure, TEER values were also measured and compared to their respective media controls, which were DMEM (control medium for samples not including citric acid and/or sucrose) or DMEM with citric acid and/or sucrose added (control medium for samples including citric acid and/or sucrose). The values were between 90.5% and 114.5% of the values recorded for their controls (data not shown). In addition, all average TEER values, before/after treatment, were above 250 Ω cm^2, which is considerably above the recommended minimum level (200 Ω cm^2)[21] needed for monolayer integrity. Therefore, samples were considered to be nontoxic to the cells and did not affect the paracellular transport, which could influence bioavailability calculations. At the end of 6 h of exposure, recoveries on the apical side and the basolateral side were quantified, for C3GR and epicatechin, as a percentage of the amount of anthocyanin/epicatechin loaded to the apical side at 0 h, using HPLC (Figures 1 and 2; Table 3). Both from fruit and from nectar, C3GR and (−)-epicatechin could be detected at the basolateral side (Figures 1 and 2; Table 3).

The anthocyanin recovery at the basolateral side was ±5 times higher for the nectar sample (N) (2.59%) compared to the fruit sample (F) (0.52%) (Table 3). When the fruit material was supplemented with nectar ingredients, either sucrose (F+SUC) or citric acid (F+CA), the recovery at the basolateral side increased to similar levels as in the nectar (2.43% and 2.34%, respectively) (Table 3). One explanation for the higher recovery at the basolateral side could be the differences in anthocyanin stability at different pH values, depending strongly on the presence of citric acid (Table 3). Therefore, the recovery of anthocyanin at the apical side (indicating stability) was also taken into account and found to be ±2-fold higher at pH = 4 (N, F+CA, F+SUC+CA) than at pH = 6 (F, F+SUC). The basolateral side recovery (indicating transport) was corrected for the apical side recovery (indicating stability), and this value is referred as "transport efficiency" in Table 3. The transport efficiency of C3GR was still significantly higher ($p < 0.05$) for nectar (approximately 3-fold higher) than was obtained for fruit (0.011 for fruit and 0.034 for nectar).

The basolateral side recovery of epicatechin was 3-fold higher when supplied in the nectar matrix (3.29%) compared to the fruit matrix (1.14%) (Table 3). Again, transport efficiency was calculated considering the differences in apical side recovery in different samples. However, transport efficiency of epicatechin from nectar was found to be not significantly different ($p < 0.05$) (0.035 for fruit and 0.043 for nectar) (Table 3).

Effect of Solid-Phase Extraction on Transport. To investigate the effect of the matrix on transport of C3GR and epicatechin, solid-phase extraction (SPE) was performed on both fruit (F) and nectar (N) samples. This treatment will remove most polar matrix compounds including carbohydrates, organic acids, salts, and proteins. When mixing solid-phase extracts with cell culture medium, we noted that the pH was 8. Therefore, here also a set of samples was analyzed where citric acid was added to adjust the pH to 4, similar to the situation with untreated nectar. While the recovery at the apical side was high, particularly when citric acid was added (Table 4), the recovery percentage at the basolateral side was ±5-fold (for C3GR) to 10-fold (for epicatechin) lower than the values obtained with the set of samples those were not treated with SPE (Table 3).

DISCUSSION

In this study we aimed to investigate the effect of food processing on absorption of phenolic compounds in the human digestive system using a recognized cell-based assay. A number of studies have been carried out into the effect of food processing on the bioavailability of micronutrients (e.g., Hotz and Gibson[26]) and also on the bioavailability of carotenoids.[27] However, the effects of processing on uptake of phenolic compounds from food matrices are largely unknown. Here we have used the Caco-2 cell system as a model to study the effect of food processing on transport of sour cherry flavonoids across the gut epithelium. Sour cherry fruit is rich in anthocyanins (cyanidin-3-(2^G-glucosylrutinoside) (C3GR) as the major anthocyanin component), and flavan-3-ols (represented mainly by (−)-epicatechin). These compounds have been reported to be well-recovered into the nectar fraction during processing.[12] Therefore, in the work described here, we have focused on the predominant anthocyanin and flavan-3-ol in sour cherry.

The major conclusion from this work is that transport of the predominant sour cherry anthocyanin and flavan-3-ol is not negatively affected by processing. Indeed, a positive effect on transport was observed for anthocyanin, which was transported three times more efficiently and significantly higher ($p < 0.05$) from processed nectar than from fruit (with transport efficiency values of 0.034 for nectar and 0.011 for fruit; Table 3). This positive effect appears to correlate with the added presence of sucrose and citric acid, since these constitute the major differences between fruit and nectar.[12] A strong reduction of transport of both anthocyanins and flavan-3-ols was observed when the fruit and nectar samples were presubjected to a SPE procedure that was included to remove most of the polar compounds in the food matrix including carbohydrates, organic acids, salts, and proteins.

Although both anthocyanins and flavan-3-ols are major phytochemicals in fruit and vegetables, their bioavailability is known to be relatively low compared to that of other flavonoids.[9,28−30] The trans-epithelial transport of anthocyanins and flavan-3-ols has been studied before using Caco-2 cells where, for example, Yi et al.[31] followed the transport of anthocyanin monoglycosides extracted from blueberries across Caco-2 cell monolayers and reported transport efficiencies of 3−4%. This is in the same range as observed here for C3GR present in sour cherry nectar or supplemented fruit (Table 3). Faria et al.[32] tested grape skin extracts, mainly containing malvidin-glucoside, which was also transported at similar levels. Trans-epithelial transport of (−)-epicatechin was studied by Vaidyanathan and Walle,[15] who reported no transport of epicatechin to the basolateral side of Caco-2 cell layers. Notably, we also could not observe any transport of epicatechin when supplied in SPE-purified form from the fruit sample. Since the transport of this group of flavonoids generally appears to be relatively poor, it may be of significance to study other food-related factors that could enhance the transport of these flavonoids across the gut epithelium. As has been demonstrated in this study, the effect of fruit processing, sucrose, citric acid, and the fruit matrix on transport of C3GR and epicatechin across Caco-2 cell monolayers can be influential in this process.

Effect of Sucrose. In our study, sucrose was found to enhance the trans-epithelial transport of both C3GR and epicatechin from sour cherry fruit by ca. 5-fold (from 0.52% to 2.43%) and 1.6-fold (from 1.14% to 1.85%), respectively (Table 3). While this was a significant increase for the anthocyanin ($p < 0.05$), it was not for epicatechin ($p > 0.05$). Apparently there is a difference between the effect of sucrose on the transport of the anthocyanin C3GR and the flavan-3-ol epicatechin. The role of sucrose in the bioavailability of anthocyanins has been addressed previously in several *in vivo* experiments, testing differences between contrasting processing products including grape juice and red wine.[33,34] Other studies, focusing, for instance, on malvidin-glucoside in grape juice and wine, indicated that sucrose did not affect the total quantity of absorbed malvidin-glucoside in grape juice, relative to red wine, but that large interindividual differences might exist, in particular for plasma levels of anthocyanins absorbed from sucrose-supplemented grape juice.[33] In the study of Frank et al.,[34] it was observed that the relative bioavailability of most anthocyanins (including cyanidin-3-glucoside) was 1.5−2 times higher in sucrose-containing juice than in wine. This observation is in line with our observation that sucrose enhances transfer of C3GR to the basolateral side of Caco-2 cells. On the other hand, Mulleder et al.[35] investigated and compared the urinary excretion of cyanidin-3-glycosides in humans after consumption of elderberry anthocyanins with and without simultaneous ingestion of sucrose. In contrast, they observed that the ingestion of sucrose led to a reduced excretion of cyanidin-3-glucoside. However, here, without information on uptake/excretion dynamics, it is difficult to make any conclusions on bioefficacy and to compare with our results. Other studies, using the related flavonoid quercetin-glucoside, reported inhibition of flavonoid uptake in Caco-2 cells by glucose.[36] Quercetin glycosides have been shown to be absorbed in the small intestine by interaction with the intestinal glucose transporter (SGLT-1, sodium dependent glucose transporter 1).[37] Though some studies[38] suggest glucose transporters as the candidates for anthocyanin absorption/transport, others[39] could not find any relation between the presence of glucose and the absorption of cyanidin-3-glucoside by jejunum tissue.[40]

Effect of Citric Acid. From the results in Table 3, it appears that citric acid also enhances transport across Caco-2 cells (∼5-fold higher recovery for C3GR and 2-fold higher recovery for (−)-epicatechin). Part of this effect can most likely be linked to a higher stability at lower pH values, specifically for anthocyanins.[10] In this study we tried to choose a culture medium such that there was no measurable effect on pH. Nevertheless, a reduced pH was still observed on the apical side of the samples when citric acid was present (Table 3). When the transport efficiency was calculated, an improvement in the transport of C3GR was still observed (∼3 times higher and statistically significant ($p < 0.05$)), while there was no

equivalent effect for epicatechin ($p > 0.05$). Therefore, it appears that the effect of citric acid on C3GR transport is not wholly the consequence of increased apical side recovery but points to an enhancing effect on transport. Yi et al.[31] suggested that the effect of pH on transport observed in Caco-2 cells may have some physiological relevance. Even though the cellular interstices and blood have a pH of around pH 7.4, the pH in the upper gastrointestinal tract under fasting conditions ranges from 5.0 to 6.5. In addition, the pH of the acidic microclimate just above the epithelial cell layer has been reported to be between 5.8 and 6.3.[23]

Effect of the Fruit Matrix. Solid-phase extraction appeared to have a major impact on the transport of anthocyanins (C3GR) and flavan-3-ols ((−)-epicatechin) and reduced the transport efficiency by 5- to 10-fold (Tables 3 and 4). Several components of the SPE protocol could be responsible for this effect. McDougall et al.[41] also suggested a positive effect of food matrix on anthocyanin bioavailability, based on a study on raspberry anthocyanins, in the form of extracts, using an *in vitro* digestion system. Felgines et al.[42] investigated the bioavailability of strawberry anthocyanins, consumed in the form of whole fruits, and suggested that anthocyanin absorption was likely affected by the food matrix. The matrix components and/or contaminants involved have so far hardly been explored but clearly deserve further investigation.

Implications for Processing. What has become evident from this research is that the uptake and transportation of important potentially bioactive food components such as common polyphenols is a complex process that is under the influence of multiple physicochemical and biochemical factors. Particularly important from a food processing perspective is that standard food supplements such as sucrose and citric acid appear to have a positive effect on trans-epithelial transportation of these compounds, and furthermore, as also been shown previously, the natural food matrix itself has a strong influence on bioavailability. Both observations entail that these aspects need to be taken into proper account when food processors wish to design new and improved product development strategies. Just ensuring the presence of key compounds in the basic ingredients is no guarantee that these will be taken up and transported across the gut into the bloodstream, from which they only then will have the potential to exert their physiological effect.

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Notes

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