Human intervention study to investigate the intestinal accessibility and bioavailability of anthocyanins from bilberries

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Abstract

We investigated the importance of the large intestine on the bioavailability of anthocyanins from bilberries in humans with/without a colon. Low bioavailability of anthocyanins in plasma and urine was observed in the frame of this study. Anthocyanins reached the circulation mainly as glucuronides. Analysis of ileal effluents (at end of small intestine) demonstrated that 30% of ingested anthocyanins were stable during 8 h passage through the upper intestine. Only 20% degradants were formed and mostly intact anthocyanins were absorbed from the small intestine. Higher amounts of degradants than anthocyanins reached the circulation after bilberry extract consumption in both groups of subjects. Comparison of the bioavailability of anthocyanins in healthy subjects versus ileostomists revealed substantially higher amounts of anthocyanins and degradants in the plasma/urine of subjects with an intact gut. The results suggested that the colon is a significant site for absorption of bioactive components such as anthocyanins and their degradation products.

Introduction

Most previous studies investigating the bioavailability of anthocyanins focused on their respective parent structure and phase II metabolites (such as methylated, glucuronated and sulfated forms) (Felgines et al., 2003; Kay, Mazza, Holub, & Wang, 2004). Despite the known limited stability of anthocyanins, only a few studies have measured the different degradation products and their respective metabolites potentially formed in vivo and attempted to estimate the total bioavailability of anthocyanins (Czank et al., 2013; Ferrars et al., 2014). In vitro fermentation studies have demonstrated a degradation pathway of anthocyanins to phloroglucinol aldehyde (from the A-ring) and phenolic acids (from the B-ring) (Keppler & Humpf, 2005) depending on the pattern of substitution of the parent anthocyanin. Recent studies have revealed the complexity of the degradation in vivo (Czank et al., 2013; Ferrars et al., 2014). Czank et al. reported that after ingestion of single 13C-labeled cyanidin-3-glycoside, up to 49 metabolites were identified. In the present study, we aimed to investigate the intestinal accessibility and bioavailability of anthocyanins from bilberries in humans with/without a colon.
and degradation products could be detected *in vivo* associated with the ingested parent anthocyanin due to the stable isotope labeling. The relative bioavailability of the metabolites and degradants was estimated as 12% (Czank et al., 2013).

Fig. 1 shows the possible metabolism and degradation pathways of anthocyanins under physiological conditions. Beside phenolic acids and phenylaldehydes, other degradants, such as phenylacoholes, phenylacetic acids and phenylpropenoic acids, have been identified (Fernandes, Faria, Calhau, de Freitas, & Mateus, 2014; Ferrars, Cassidy, Curtis, & Kay, 2014). Depending on the constitution and polarity of these compounds, they may also be present systemically as metabolites.

Phenolic acids have mainly been detected as glucuronides, sulfates or methylated forms (Felgines et al., 2003; Kay et al., 2004). The glycine conjugated hippuric acid was shown to be the major metabolite in urine over several days (Czank et al., 2013; Ferrars et al., 2014). To date, few studies have investigated intermolecular interactions of anthocyanins with themselves and other molecules (e.g., polyphenols, phenolic acids, proteins, etc.) or their complex-forming properties *in vivo* (Cavalcanti, Santos, & Meireles, 2011; Eiro & Heinonen, 2002; He, Xu, Zeng, Qin, & Chen, 2016; Ribnicky et al., 2014; Soares, Mateus, & De Freitas, 2007; Tang, Zuo, & Shu, 2014). Thus, the role of these *in vitro* confirmed characteristics on the human bioavailability and biological effectiveness is still unclear. Thus, prospective investigations are essential to identify the importance of dietary anthocyanin intake and their effects and distribution in humans (Czank et al., 2012; Rodriguez-Mateos, Feliciano, Cifuentes-Gomez, & Spencer, 2016; Williamson & Clifford, 2010) especially given their previously underestimated bioavailability. More studies on the bioavailability, site of absorption and metabolism of anthocyanins in humans are useful to clarify previous results (Fernandes, Faria, de Freitas, Calhau, & Mateus, 2015; Kay, Pereira-Caro, Ludwik, Clifford, & Crozier, 2017).

Bilberries (*Vaccinium myrtillus* L.) contain 15 anthocyanins which differ in functional groups of the aglyca and the binding sugar. So, bilberry extract represents a broad spectrum of anthocyanins compared to other fruits and extracts (Müller, Schantz, & Richling, 2012). For this reason our pilot interventions were designed to elucidate the amounts of bilberry anthocyanins reaching the human intestine and circulation. Thus, the studies were performed on volunteers with or without a colon. Ileostomists (with a terminated artificial small bowel outlet) represent an adequate model to demonstrate how anthocyanins reach the small intestine. Information obtained from analyzing ileostomy effluents at the end of the small intestine can be used to identify compounds available to the colon, and therefore possible candidates for colonic absorption in healthy subjects with an intact colon. Following anthocyanin intake, their effluents were collected in bags at the end of the small intestine and analyzed after simple preparation to determine the amounts of anthocyanins and degradation products present. This non-invasive method allows direct comparison of data for healthy subjects and ileostomists to investigate the bioavailability of anthocyanins and formation of degradation products. We employed two groups of volunteers using the same intervention design. Each subject was administered one bolus of 10 g anthocyanin rich bilberry extract (BE) and blood and urine samples were collected within 8 and 24 h and ileostomy fluid within 8 h. The samples were analyzed for their content of intact anthocyanins and glucuronides and specific degradation products (e.g., phenolic acids, phenylaldehydes) to identify any differences or similarities.
in their accessibility and bioavailability. However, not all anthocyanin degradation products were recorded owing to the complex mixture of anthocyanins in the BE (15 different compounds ingested). The spectrum of metabolites generated from each anthocyanin may be multifarious, as reported by Czank et al. (2013). Thus, only a few specific metabolites were selected to investigate the role that the small intestine and colon play in the absorption kinetics of consumed bilberry anthocyanins.

2. Materials and methods

2.1. Study design

Details of this human pilot study were first described in Kropat et al. relating to an investigation of physiological effects in the blood cells of subjects (Kropat et al., 2013). Specific details relating to the pharmacokinetic analyses are given below. After approval from the ethics commission of the University of Wuerzburg (No. 32/10), the study was carried out with two groups of subjects. Group A were ileostomists (N = 5 females, BMI = 27 ± 4, age = 41 ± 8). Owing to Crohn’s disease, these volunteers had been fitted with an artificial gut outlet at the end of their small intestine (stoma of the terminal ileum). The volunteers of group B were healthy subjects with an intact gut (N = 5 females, BMI = 23 ± 3, age = 33 ± 7). The time axis of the study is shown in Fig. 2. Prior to ingestion of the bolus of anthocyanin rich BE, a wash out phase was employed of 48 h on an anthocyanin- and polyphenol-free diet, whereby foods such as red fruits, vegetables, chocolate and coffee were omitted. Samples of urine (−24–0 h), blood (0 h, immediately before extract consumption), and additionally for group A, ileostomy fluid (−12–0 h), were collected to check for background and compliance. After the wash out period, each subject consumed a single portion of 10 g BE stirred into 150 g of low fat yogurt (3.4% cow milk protein, 1.5% fat, 5.0% carbohydrates), corresponding to 4.95 mmol anthocyanins (2.4 g). The following samples were collected for both groups (A and B) after extract consumption: total volume of urine at four time intervals (0–2 h, 2–4 h, 4–8 h, 8–24 h) and about 27 ml of blood drawn from vena brachialis at four time points (1 h, 2 h, 4 h, 8 h). In addition, the total volume of ileostomy fluid collected from participants of group A was measured at five time intervals (0–1 h, 1–2 h, 2–4 h, 4–6 h, 6–8 h). For further details, see the section entitled “Sample preparation” below. During the 24 h of intervention (after BE consumption), all subjects received the same diet. After 1 h, a breakfast was provided of white bread and artificial honey. At lunch time (after 5 h), the volunteers received white bread with cheese and ham, and after 9 h, they were provided with wheat pasta with carbonara sauce. It is known that the stability and absorption of anthocyanins can be influenced by food ingredients (e.g. whey proteins, sugar) (He et al., 2016; Schantz et al., 2014; Williamson, 2013). Besides these effects, the volunteers got the bolus of anthocyanins in combination with yogurt (without extra sugar) to make the intake of astringent BE as pleasant as possible.

2.2. Chemicals, standards and bilberry extract

The BE (Symrise GmbH & Co. KG, Holzminden, Germany, pr. No. 399916; batch 29) was produced from bilberries (Vaccinium myrtillus L.) pomace via ethanolic and subsequent concentration of polyphenols by column chromatography. The BE consisted of 24 ± 1% of 15 different anthocyanins based on the five aglyca delphinidin (del), cyanidin (cy), petunidin (pet), malvidin (mal) and peonidin (peo) and the three sugar residues glucose (glc), galactose (gal), and arabinose (ara) in the 3–0 position. The anthocyanin profile is shown in Fig. 5A. Further components, such as quercetin glycosides, phenolic acids, tannins, carbohydrates, fibers and traces of free anthocyanidins, may also be present (Juadjur & Winterhalter, 2012). The degradation products under this study (GA, PCA, HBA, HBAL, MGA, PGA, SA, VA) were 0.4% in the ingested BE. All standards had at least a purity of 95%. Anthocyanin standards were obtained from three sources: cy-3-glc, delphinidin-3,5-diglucopyranoside (del-3,5-diglc, internal standard, IS) and cy were purchased from Extrasynthese (Genay Cedex, France); cy-3-gal, cy-3-ara, peo-3-glc, peo-3-gal, peo-3-ara, del-3-glc, pet-3-glc, and mal-3-glc were purchased from Polyphenols (Sandes, Norway); del-3-gal, del-3-ara, pet-3-gal, pet-3-ara, del-3-glc, and mal-3-glc were kindly provided by Peter Schreier and Michael Kraus (University of Wuerzburg, Germany). The possible degradation products 4-hydroxybenzoic acid (HBA), gallic acid (3,4,5-trihydroxybenzoic acid, GA) and syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid, SA) were obtained from VWR International (Radnor, Pennsylvania, USA); protocatechuic acid (3,4-dihydroxybenzoic acid, PCA), vanillic acid (4-hydroxy-3-methoxybenzoic acid, VA) and phloroglucinol aldehyde (2,4,6-trihydroxybenzaldehyde, PGA) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA); 3-0-methylgallic acid (3,4-dihydroxy-5-methoxybenzoic acid, MGA) was purchased from Abcr GmbH & Co. KG (Karlsruhe, Germany); and the internal standard isorhamnetic acid-d$_3$ (3-hydroxy-4-methoxyisocinnamic acid-d$_3$,...
iso-FA-d₃) was from Toronto Research Chemicals Inc. (Toronto, Canada). HPLC solvents were of analytical grade and purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and VWR International (Radnor, Pennsylvania, USA).

2.3. Sample storage

The blood samples were centrifuged at 4000 rcf and 4 °C for 10 min. Afterwards, the plasma was separated and the pH adjusted to pH 3 with formic acid (50% (v/v)). To 1 ml of plasma, 24 µl of formic acid was added. Immediately after, the samples were frozen with liquid nitrogen and stored at −80 °C until analysis. Before the urine sampling started, the urine containers were prepared with 2–10 ml of hydrochloric acid (1 M) depending on the collection interval to ensure rapid acidic stabilization of the anthocyanins. After collection, all urine samples were adjusted to pH 2.5, then dispensed into aliquots and stored at −80 °C prior to analysis. The ileostomy effluents of group A were collected in standardized bags, immediately frozen with liquid nitrogen and stored at −20 °C.

2.4. Sample preparation

Ileostomy fluid was lyophilized to dryness (90 h, −54 °C, 0.47 mbar) and then homogenized. For anthocyanin analysis, the ileostomy samples were extracted with solvent A (87% acetonitrile/3% water/10% formic acid (v/v/v)) using an ultraturrax (60 s, 20,000 rcf) and centrifuged (20 min, 4500 g, 25 °C). For further analyses, the supernatants were used. For degradant analysis, ileostomy fluid samples were extracted with 90% water/10% formic acid (v/v) for 2 h with a thermomix (23 °C, 1400 rpm). The suspensions obtained were centrifuged (1 h, 4 °C, 20,000 rcf) and the supernatants were diluted 1:1 with ice-cold ethanol, mixed, and cooled at −80 °C at least for 1 h to precipitate the proteins. This was followed by a second centrifugation, supernatants were

Fig. 4. Kinetics of anthocyanins and degradation products in ileostomy fluid (A, B), plasma (C, D) and urine (E, F) of ileostomists and healthy subjects after BE consumption.
evaporated and the residues were redissolved in 90% water/10% formic acid prior to analysis. Urine was purified and concentrated via solid phase extraction (SPE method 1) using a reversed phase Strata C18 U (500 mg/3 ml) from Phenomenex (Aschaffenburg, Germany). First the solid phase was conditioned with 4 ml methanol (100%), equilibrated with 4 ml of water/formic acid (95%/5%, v/v) and then loaded with urine (6 ml for the samples at 0–12 h, 0–2, 2–4, 4–8 h and 9 ml for the samples at 8–24 h). The following elution step was carried out with 4 ml water/formic acid (95%/5%, v/v). Each eluate was dried in a vacuum centrifuge and the pellet redissolved in 300 μl 87% acetonitrile/3% water/10% formic acid (v/v/v).

This solid phase extraction method without a washing step (see SPE method 1) was insufficient for the plasma samples. Plasma

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**Fig. 5.** Profiles of anthocyanins and glucuronides in BE, ileostomy fluid and plasma samples of healthy subjects (A), profiles of degradation products in ileostomy fluid, plasma and urine of healthy subjects (B).
contains a lot of proteins that can influence the analysis. Consequently, it was necessary to develop an alternative extraction method to provide adequate recoveries and sufficient purification. Using a washing step during SPE method 1, the recovery of the low molecular weight phenolic acids and aldehydes was low. The interaction between the polar solvent (water) and the phenolic acids/ aldehydes seemed to be stronger than the interaction with the solid phase. Hence, we developed a method employing two combined sorbent materials to ensure adequate recovery of anthocyanins and phenolic acids/aldehydes in the same step. We overlaid two different sorbent materials. The lower sorbent was the Strata X material (60 mg), a reversed phase of styrol divinylbenzol chains with γ-lactam moieties. The upper sorbent was the Strata C18 U sorbent (200 mg), which consists of C18 hydrocarbon chains. The first step of SPE method 2 was conditioning using 4 ml methanol (100%, v/v) followed by equilibration with 4 ml water/formic acid (95%/5%, v/v). The next step was loading with 4 ml of the plasma sample (diluted 1:1 with water/formic acid (95%/5%, v/v)) followed by a washing step with 2 ml water/formic acid (95%/5%, v/v). The sample loading step (with 4 ml plasma/water) and washing step (2 ml water/formic acid) were then repeated. The samples were eluted with 3 ml methanol/formic acid (95%/5%, v/v). This protocol was carried out in parallel to the preparation of urine samples (see above).

The sample preparation of urine and ileostomy fluid was performed in triplicate, whereas single plasma samples were analyzed owing to limited collection volumes. Prior to HPLC-analysis internal standards were added to all samples: 10 μg/ml del-3,5-diglc for HPLC-DAD and 0.1 μg/ml del-3,5-diglc for HPLC-MS/MS, and 0.2 μg/ml iso-FA-d3 for HPLC-MS/MS analysis, respectively. The recovery from ileostomy fluid was 95 ± 7% for anthocyanins and 95 ± 10% for degradants. The recovery using SPE method 1 was 102 ± 6% from water (pH 2.5) and 89 ± 25% from an anthocyanin free urine matrix for anthocyanins, and 102 ± 4% from water (pH 2.5), and 106 ± 5% from the urine matrix for degradation products. SPE method 2 yielded an anthocyanin recovery values of 95 ± 7% from water (pH 2.5) and 91 ± 15% from plasma, and degradation values of 95 ± 10% from water (pH 2.5), and 84 ± 15% from plasma.

2.5. Qualitative and quantitative analysis of anthocyanins and degradation products

Standard references were used for optimization of the HPLC instrument (Agilent 1200), which was coupled with a tandem mass spectrometer system equipped with an ESI interface (API 3200, ABSciex). The identification of analytes in plasma, urine and ileal fluids was based on comparison of the chromatographic retention times and MS² fragmentation with ≥3 parent-daughter ion-fragmentation transitions to the reference data. Glucuronides and sulfates were verified by neutral loss scan (cleavage of a glucuronyl group with a loss of 176 amu and cleavage of a sulfate group with a loss of 80 amu) and MS² fragmentation compared with published data (Ferrars et al., 2014). The detected analytes in the different types of matrix, i.e., plasma, urine and ileal fluid, are shown in Fig. 3.

Anthocyanins in BE, ileal fluids, and urine samples were quantified by a previously described HPLC-DAD method (Kropat et al., 2013; Müller et al., 2012). For their quantification in plasma, a HPLC-ESI-MS/MS instrument operating in the multi reaction monitoring mode (MRM) was used. HPLC chromatographic separation was carried out with a Luna C18 (u) column (Phenomenex, Aschaffenburg, Germany) at 23 °C, with a flow rate of 0.5 ml/min and an injection volume of 20 μl. The mobile phase consisted of water/acetoniitri/formic acid (92%/3%/5%; v/v/v) solvent A and water/acetoniitri/formic acid (45%/50%/5%; v/v/v) solvent B. The gradient comprised 0–20 min, 2–14% B; 20–40 min, 14% B; 40–50 min, 14–15% B; 50–55 min, 15–19% B; 55–65 min, 19–20% B; linear step to 99% B for 5 min; and return to initial condition of 2% B for 10 min. The ESI-MS instrument-parameters were carried out in positive mode: curtain gas, 30 psi; ion spray, 5000 V; temperature, 450 °C; gas 1, 50 psi; gas 2, 40 psi. The substance-specific parameters of individual compounds and transitions are listed in Table 15 – see supplementary data. Semi-quantification was performed with external calibration of cy-3-glc in polyphenol-free plasma (every 25 measurements) and the internal standard del-3,5-diglc. Calibration curves ranged from 0.002 to 2 μmol/L with linearity coefficient of 0.999 R² and a limit of quantification (LOQ) of 0.002 μmol/L.

Degradation products in plasma, urine, and ileal fluids were quantified after extraction using HPLC-ESI-MS/MS in the negative MRM mode. Separations of phenolic acids and aldehydes were performed using the same column as reported before. The mobile phase was pumped at a flow rate of 0.5 ml/min and contained 0.5% aqueous acetic acid (A) and 100% acetoniitri (B). The injection volume was 20 μl and the following gradient protocol was used: 0 min, 10% B; 15 min, 10% B; 25 min, 15% B; 35 min, 15% B; 40 min, 35% B; 52 min, 35% B; 52.1 min, 90% B; 55 min, 90% B; 55.1 min, 10% B; and 60 min, 10% B. To enhance the selectivity of the individual mass traces, the method was divided into three separate time periods: the instrument-specific parameters during Period 1 (0–26 min) were set to 30 psi curtain gas, −4500 V ion spray, 700 °C temperature, 60 psi gas 1 and 40 psi gas 2, whereas during Period 2 (26–44 min) and Period 3 (44–59 min), they were changed to 25 psi curtain gas, −4000 ion spray, 50 psi gas 1 and 70 psi gas 2. The substance-specific parameters are listed in Table 15 – see supplementary data. The references and internal standard (iso-FA-d3) were added to samples of polyphenol-free ileal fluid, urine and plasma to generate individual calibration curves and compensate for suppression of ionization by the matrix. Calibration curves ranged between 0.003 μmol/L to 6 μmol/L (R² > 0.99) and the LOQ was 0.003–0.190 μmol/L depending on the analytes.

2.6. Determination of intestinal passage time

To determine how fast food components (anthocyanins) were excreted, the volunteers under study drank 330 ml of conventional bilberry juice. For the group of ileostomists, the individual intestinal passage time was defined as the time of coloring of ileostomy fluid, whereas for the subjects with intact gastrointestinal passage, the coloring of feces was monitored and documented.

2.7. Statistical analysis and calculations

Due to the low number of subjects in this study, no significant testing was meaningful. This study is a pilot intervention and gives hints and motivation to design more powerful intervention studies. Pharmacokinetic parameters (see Table 1), such as the area under the curve (AUC) of plasma analytes, were determined by integration of the concentration curves between 0 to 8 h using the linear trapezoidal rule. The time point where maximum of plasma concentration (Cmax) was reached was defined as Tmax. The plasma concentrations of anthocyanins and degradants were expressed as a Bateman function, which is representative of oral ingestion. Elimination started at Tmax and followed first order kinetics for an approximate one compartment system. For calculation of the half life, T1/2, we plotted the natural logarithm (ln) of the concentration against time and fitted the data to a linear equation to calculate the elimination rate constant from the slope: T1/2 was then calculated from the slope using a quotient of ln(2). All parameters must be considered with appropriate insecurities corresponding to the low number of subjects.
3. Results

3.1. Anthocyanins and degradation products in ileostomy fluid

We investigated the time it took for food components to reach the small intestine and their subsequent elimination. The intestinal transit times of ileostomists were estimated to be between 15–60 min after juice intake. The maximal coloring of feces of subjects with an intact gut was observed by themselves within 24 h.

Fig. 4 shows the amounts of total anthocyanins (Fig. 4A) and total degradation products per ileostomy bag (Fig. 4B) at selected times.

The majority of ingested anthocyanins were excreted between 0 and 2 h, i.e., 0 to 1 h: 748.3 ± 353.3 nmol anthocyanins; 1–2 h: 566.8 ± 69.7 nmol anthocyanins per ileostomy bag, corresponding to 15.1 ± 7.1% and 11.5 ± 1.5% of consumed anthocyanins from BE, respectively. Between 2–4 h and 4–6 h, only 1.9 ± 1.6% (92.6 ± 78.3 μmol) and 1.1 ± 0.9% (54.3 ± 46.5 μmol), respectively, of ingested anthocyanins were excreted via ileostomy fluid. After 8 h, no excretion of anthocyanins was detected for most subjects except two participants, for which 1–4 μmol anthocyanins were detected in the effluents. On the contrary, the kinetics of anthocyanins in the plasma of subjects with a colon followed a different trend. After 1 h, the amount of plasma anthocyanins was 75.6 ± 19.1 nmol/L and the maximum concentration was observed at 1 h after ingestion of the BE and corresponded to 67.4 ± 5.8 nmol/L. At later time points, the anthocyanin concentration decreased continuously to 51.9 ± 38.4 nmol/L after 2 h, 20.8 ± 7.5 nmol/L after 4 h and 9.6 ± 13.4 nmol/L after 8 h. In contrast, the kinetics of anthocyanins in the plasma of subjects with a colon followed a different trend. After 1 h, the amount of plasma anthocyanins was 512.0 ± 128.0 nmol/L and the maximum concentration was obtained after 2 h of 91.2 ± 41.8 nmol/L anthocyanins. After 4 h and 8 h, the concentration was only 54.0 ± 52.5 and 9.1 ± 8.5 nmol/L, respectively. Fig. 4D shows base line amounts of degradants were present at the first time point (0 h), just prior the BE bolus intake. The amounts reached 143.3 ± 195.9 nmol/L for ileostomists and 132.2 ± 154.9 nmol/L for the subjects with a colon. After 1 h, the concentration of degradation products increased to a maximum of 1077.1 ± 229.4 nmol/L for ileostomists and 1494.8 ± 275.7 nmol/L for ‘healthy’ subjects. With increasing time, the amount of degradation products in plasma then declined: after 2, 4 and 8 h, the concentrations were 527.9 ± 385.7 nmol/L, 179.6 ± 96.3 nmol/L and 77.3 ± 26.2 nmol/L in the plasma of ileostomists and 1061.6 ± 241.7 nmol/L, 395.0 ± 180.1 nmol/L and 150.5 ± 86.8 nmol/L in the plasma of healthy subjects, respectively.

The rate of occurrence of intestinal degradation products was similar to the kinetics of anthocyanins, although the inter-individual concentrations varied significantly. Again, maximum concentrations were achieved at 0–1 h (137.5 ± 89.1 μmol per bag) and 1–2 h (117.8 ± 24.4 μmol per bag), but the amounts were 20% lower compared to those of anthocyanins. After 2–4 h and 4–6 h, the intestinal amount of degradation products decreased to 24.4 ± 13.7 μmol and 20.4 ± 10.3 μmol, respectively, around 3 to 4 times lower than the anthocyanin concentration at the same times. At around 8 h after BE consumption, the intestinal concentration of degradation products was as low as 1.5 ± 1.9 μmol. The total amount of degradation products in the small intestine quantified over 8 h of excretion was 301.6 ± 79.1 μmol, corresponding to only a fifth of the analyzed total anthocyanins in this efflux compartment.

3.2. Concentrations of anthocyanins and their degradation products in the blood circulation

Fig. 4C and D show the time-dependent concentration of consumed anthocyanins reaching the human blood circulation in their intact and conjugated form (mainly glucuronides, see Fig. 5A) and as degraded products, namely GA, PCA, MGA, HBA, VA, SA, HBAL, PGAL.

The maximum concentration of anthocyanins in the plasma of ileostomists was reached 1 h after ingestion of the BE and corresponded to 67.4 ± 5.8 nmol/L. At later time points, the anthocyanin concentration decreased continuously to 51.9 ± 38.4 nmol/L after 2 h, 20.8 ± 7.5 nmol/L after 4 h and 9.6 ± 13.4 nmol/L after 8 h. In contrast, the kinetics of anthocyanins in the plasma of subjects with a colon followed a different trend. After 1 h, the amount of plasma anthocyanins was 75.6 ± 19.1 nmol/L and the maximum concentration was obtained after 2 h of 91.2 ± 41.8 nmol/L anthocyanins. After 4 h and 8 h, the concentration was only 54.0 ± 52.5 and 9.1 ± 8.5 nmol/L, respectively. Fig. 4D shows base line amounts of degradants were present at the first time point (0 h), just prior the BE bolus intake. The amounts reached 143.3 ± 195.9 nmol/L for ileostomists and 132.2 ± 154.9 nmol/L for the subjects with a colon. After 1 h, the concentration of degradation products increased to a maximum of 1077.1 ± 229.4 nmol/L for ileostomists and 1494.8 ± 275.7 nmol/L for ‘healthy’ subjects. With increasing time, the amount of degradation products in plasma then declined: after 2, 4 and 8 h, the concentrations were 527.9 ± 385.7 nmol/L, 179.6 ± 96.3 nmol/L and 77.3 ± 26.2 nmol/L in the plasma of ileostomists and 1061.6 ± 241.7 nmol/L, 395.0 ± 180.1 nmol/L and 150.5 ± 86.8 nmol/L in the plasma of healthy subjects, respectively.

3.3. Elimination of anthocyanins and degradation products via urine

Fig. 4E and F present the urinary elimination kinetics of anthocyanins and degradation products. In the urine samples of ileostomists the concentration of degradation products was as low as 1.5 ± 1.9 μmol. The total amount of degradation products in the small intestine quantified over 8 h of excretion was 301.6 ± 79.1 μmol, corresponding to only a fifth of the analyzed total anthocyanins in this efflux compartment.

Table 1
Pharmacokinetic parameters (AUC, Cmax, Tmax, T1/2) of most abundant anthocyanins and specific degradants in plasma of ileostomists (−colon) and healthy subjects (+colon) after intake of bilberry extract.

<table>
<thead>
<tr>
<th>Anthocyanins</th>
<th>AUC0-8h [(nmol/L)*h]</th>
<th>Cmax [nmol/L]</th>
<th>Tmax [h]</th>
<th>T1/2 [h]</th>
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<tbody>
<tr>
<td>Plasma (− colon)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Mal-gluc</td>
<td>49 ± 28</td>
<td>13 ± 3</td>
<td>1.4 ± 0.5</td>
<td>2.2 ± 0.9</td>
</tr>
<tr>
<td>Peo-gluc</td>
<td>100 ± 66</td>
<td>31 ± 13</td>
<td>1.4 ± 0.5</td>
<td>2.2 ± 2.0</td>
</tr>
<tr>
<td>Degradants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>36 ± 20</td>
<td>13 ± 4</td>
<td>1.2 ± 0.5</td>
<td>2.3 ± 1.0</td>
</tr>
<tr>
<td>MGA</td>
<td>76 ± 35</td>
<td>40 ± 10</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>PCA</td>
<td>91 ± 32</td>
<td>40 ± 9</td>
<td>1.0 ± 0.0</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>PGAL</td>
<td>128 ± 73</td>
<td>35 ± 16</td>
<td>1.8 ± 1.3</td>
<td>3.8 ± 2.7</td>
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<tr>
<td>SA</td>
<td>1057 ± 376</td>
<td>541 ± 101</td>
<td>1.0 ± 0.0</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>VA</td>
<td>498 ± 132</td>
<td>252 ± 39</td>
<td>1.0 ± 0.0</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>Plasma (+ colon)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Mal-gluc</td>
<td>109 ± 55</td>
<td>27 ± 11</td>
<td>1.4 ± 0.5</td>
<td>2.0 ± 0.4</td>
</tr>
<tr>
<td>Peo-gluc</td>
<td>194 ± 120</td>
<td>43 ± 21</td>
<td>2.2 ± 1.1</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>Degradants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>81 ± 47</td>
<td>25 ± 12</td>
<td>1.4 ± 0.5</td>
<td>4.0 ± 4.8</td>
</tr>
<tr>
<td>MGA</td>
<td>194 ± 39</td>
<td>79 ± 25</td>
<td>1.0 ± 0.0</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>PCA</td>
<td>206 ± 93</td>
<td>81 ± 32</td>
<td>1.0 ± 0.0</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>PGAL</td>
<td>144 ± 52</td>
<td>29 ± 8</td>
<td>2.4 ± 0.9</td>
<td>6.1 ± 2.3</td>
</tr>
<tr>
<td>SA</td>
<td>2008 ± 522</td>
<td>777 ± 182</td>
<td>1.0 ± 0.0</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>VA</td>
<td>1182 ± 387</td>
<td>379 ± 111</td>
<td>1.2 ± 0.5</td>
<td>1.8 ± 0.3</td>
</tr>
</tbody>
</table>
mists, the main excretion of anthocyanins occurred between 0–2 h (2.2 ± 1.7 μmol/g creatinine) and 2–4 h (2.6 ± 1.8 μmol/g creatinine). Between 4–8 h after BE consumption, the mean concentration was 0.9 ± 0.6 μmol/g creatinine, compared to 0.1 ± 0.1 μmol/g creatinine between 8–24 h. For 0–2 h, the concentration of anthocyanins in the urine of healthy subjects was 1.9 ± 1.4 μmol/g creatinine. After 2–4 h, a maximum was reached of 3.6 ± 3.0 μmol anthocyanins per g creatinine. However, high inter-individual differences were observed. After 4–8 h, the anthocyanin concentration in urine decreased to 1.1 ± 0.6 μmol/g creatinine, and after 8–24 h, to 0.2 ± 0.2 μmol/g creatinine.

Similar to the plasma findings, the results of the degradation products in urine showed a baseline concentration of anthocyanins prior BE intake. During the polyphenol-free diet prior to BE consumption (between –24–0 h), the subjects with and without colon excreted 5.0 ± 1.7 μmol/g creatinine and 2.2 ± 1.9 μmol/g creatinine, respectively. After intake at 0–2 h and 2–4 h, the concentration of anthocyanins in urine of ileostomists increased to 23.3 ± 19.1 μmol/g creatinine and 26.4 ± 17.7 μmol/g creatinine, respectively. After 4–8 h, the urine contained 4.2 ± 3.4 μmol/g creatinine, and after 8–24 h, only 2.4 ± 2.0 μmol/g creatinine were excreted per g creatinine.

The urinary contents of degradation products between 8–24 h, to 0.2 ± 0.2 μmol/g creatinine. After 0–2 h and 2–4 h, the concentration in urine decreased to 1.1 ± 0.6 μmol/g creatinine, and after intake at 0–2 h and 2–4 h, the concentration of anthocyanins in urine of ileostomists increased to 23.3 ± 19.1 μmol/g creatinine and 26.4 ± 17.7 μmol/g creatinine, respectively. After 4–8 h, the urine contained 4.2 ± 3.4 μmol/g creatinine, and after 8–24 h, only 2.4 ± 2.0 μmol/g creatinine were excreted per g creatinine. After 0–2 h and 2–4 h, the concentration of anthocyanins in urine of ileostomists increased to 23.3 ± 19.1 μmol/g creatinine and 26.4 ± 17.7 μmol/g creatinine, respectively. After 4–8 h, the urine contained 4.2 ± 3.4 μmol/g creatinine, and after 8–24 h, only 2.4 ± 2.0 μmol/g creatinine were excreted per g creatinine.

The plasma profiles of anthocyanins, metabolites and degradation products demonstrate the variety of analytes reaching the human circulation after anthocyanin exposure to BE. Table 1 summarizes the kinetic characteristics (AUC, Tmax, T1/2) of the main anthocyanins (such as Mal-gluc and Peo-gluc) and degradation products (such as glucuronides and sulfates) were identified by LC-MS/MS neutral loss detection (mass loss of 176 and 80 amu). These results and previous data from the literature (Ferrars et al., 2014) confirm the presence of VA-glucuronides and PCA-glucuronides in plasma and urine. However, the glucuronides could not be quantified in the present study owing to a lack of suitable standard references. Few standards were offered for purchase but most had to be synthesized by ourselves in chemical or enzymatic ways (Heleno, Martins, Queiroz, & Ferreira, 2015; Stachulski & Meng, 2013). In addition, the urine samples were analyzed for their occurrence of ferulic acid, coumaric acid, and hippuric acid. MS data was not quantified but showed high peak areas in urine after anthocyanin exposure as well in baseline urine without any intake of polyphenols.

The plasma profiles of anthocyanins, metabolites and degradation products demonstrate the variety of analytes reaching the human circulation after anthocyanin exposure to BE. Table 1 summarizes the kinetic characteristics (AUC, Cmax, Tmax, T1/2) of the main anthocyanins (such as Mal-gluc and Peo-gluc) and degradation products (such as GA, MGA, PCA, PGAL, SA and VA) in plasma. In this table, data from the plasma of ileostomists (without colon, Table 1, top) are compared with those of healthy subjects (with colon, Table 1, bottom). As shown in Fig. 4, higher amounts of anthocyanins and their degradation products were obtained in the presence of a healthy gut, a trend also reflected in the individual values of AUC and Cmax in Table 1. The results of Tmax were similar for both groups of subjects. The largest difference was for PCA-gluc, which showed Tmax of 2.2 ± 1.1 h for subjects with a colon compared to 1.4 ± 0.5 h for subjects without a colon. High inter-individual variation was observed for Tmax of PGAL, i.e., 2.4 ± 0.9 h for subjects with a colon and 1.8 ± 1.3 h for subjects without a colon. The half-life T1/2 indicates the time required for the concentration to drop to 50% of Cmax and allows conclusions regarding the elimination rate to be drawn. The value of T1/2 for anthocyanin glucuronides was 2.2 h and 1.8–2.0 h in plasma for subjects without a colon and with a colon, respectively. MGA, PCA, SA and VA were more rapidly eliminated via plasma with a range of T1/2 from 1.0–1.4 h (without colon) and 1.2–1.8 h (with colon). Both GA and PGAL showed relatively long residence times in plasma with high inter-individual differences: T1/2 of GA in plasma was 2.3 h (without colon) and 4.0 h (with colon), and the highest T1/2 values were calculated for PGAL of 3.8 h (without colon) and 6.1 h (with colon).
4. Discussion

4.1. Availability of consumed anthocyanins and formation of degradation products in the small intestine

Owing to the small number of subjects (n = 5) in each group and high inter-individual differences, significance tests were not particularly meaningful. Hence, this study represents a pilot study and the inter-individual differences could be improved by recruiting a larger number of volunteers. The results suggested that anthocyanins might reach the small intestine within 1 h and were at least partially stable at intestinal pH. Our results and others have shown, that the amount of anthocyanins along the intestinal gut passage corresponds to on average 30–50% of the ingested anthocyanins (Kraus et al., 2010; Wu, Pittman, & Prior, 2006), but the availability in plasma is very low. This raises the question – what happened to the missing 50–70% after intake? Under an acidic pH, anthocyanins have been shown to be very stable inside the stomach (Schantz et al., 2014). By entering the intestine, there is an abrupt change in pH, which might adversely affect the stability of anthocyanins. Fig. 1 shows the reported diversity of structural modifications of anthocyanins under physiological conditions (Czank et al., 2013; Ferrars et al., 2014; Ferrars et al., 2014; Kalt, Liu, McDonald, Vinqvist-Tymchalk, & Fillmore, 2014; Kepper & Humpf, 2005). In the past, phenolic acids and phloroglucinol aldehyde were principally identified as intestinal degradation products (Kepper & Humpf, 2005). To check the whereabouts of the anthocyanins, we investigated for the first time the presence of phenolic acids and aldehydes in ileostomy fluid after BE consumption. After reaching the blood circulation, Phase II metabolites were expected to reenter the intestine via enterohepatic circulation into the ileostomy fluid. In our ileal samples, glucuronated or sulfated forms of anthocyanins or degradants were not detected, in good agreement with other studies (Kahle et al., 2006). The kinetics of degradation products in ileostomy fluid were comparable to the kinetics of anthocyanins. The total amounts of degradants over 8 h corresponded to only one fifth of the anthocyanins. This fact corresponding with the low bioavailability indicates that anthocyanins must exist in other structures along the gastrointestinal passage. The extent to which aglyca and chalcones exist in the intestine has not been described to date. The detection of unstable aglyca and chalcones poses a considerable analytical challenge (Kalt et al., 2014). The manner, by which these structures react with other molecules in vivo and the effects of co-pigmentation or association with other molecules of the chyme, matrix or tissue remain unknown. Anthocyanins and their aglyca may form co-pigments (Eiro & Heinonen, 2002) and complexes (Cavalcanti et al., 2011) in aqueous solution, but whether this holds significance in vivo has so far not been examined. Further associations of anthocyanins or chalcones with other molecules are conceivable. Initial evidence suggests that binding is possible between anthocyanins and plasma proteins. For instance, measurement of the intrinsic fluorescence intensity of protein tryptophan residues demonstrated the binding affinity of Mal-glc to bovine serum albumin (Soares et al., 2007). However, the nature of binding has not yet been investigated.

4.2. Bioavailability of anthocyanin associated structures in the human circulation after BE consumption and its urinary elimination in the presence of an intact gut

Anthocyanins are absorbed rapidly after ingestion (maximum after 2 h). The bioavailability of anthocyanins in plasma (over 8 h after BE intake) was low, corresponding to only 0.02% (calculated for a total plasma volume of 2–3 L) of ingested anthocyanins. In urine samples, 0.03 ± 0.02% of ingested anthocyanins over the entire 24 h were found. The major anthocyanins (see Fig. 5A) in the plasma and urine samples were the glucuronated forms of malvidin (28%) and peonidin (46%). The minor compounds were 0.2–4% of the profile and the glucuronated forms of delphinidin and petunidin could not detected. Although delphinidin, cyanidin and petunidin were in higher amounts in the initial extract, they showed minor concentrations in plasma. A reason of this could be effects of dehydroxylation and methylation in the organism. This trend is in good agreement with previous studies (Cooke et al., 2006; Fernandes et al., 2014; Pojer, Mattivi, Johnson, & Stockley, 2013)

Current research has shown that the formation of degradation products and metabolites occurs in significant quantities in blood and urine after anthocyanin intake (Czank et al., 2013; Ferrars et al., 2014). This fact is of great importance for the total bioavailability of anthocyanins and their benefits in humans, especially phenolic acids, which are thought responsible for the main effects (Lu, Nie, Belton, Tang, & Zhao, 2006; Nile & Park, 2014; Peiffer et al., 2014; Russell & Duthie, 2011). Our data demonstrate that the formation of degradants is very fast. Rapid appearance in the circulation (within 0–1 h) was accompanied by rapid urinary elimination (within 0–4 h). The major degradants in plasma, i.e., SA and VA (originating from the major anthocyanins in plasma, malvidin and peonidin), were also identified in previous studies after bilberry consumption (Nurmi et al., 2009). The kinetic characteristics of PGAL were different from those of the phenolic acids because of the extended long half-life (1/2, 6.1 h). It was also the third most abundant metabolite in the small intestine after BE consumption (Fig. 5B) and can theoretically result from any anthocyanin, irrespective of their B-ring. PGAL was not observed as a major metabolite in urine or plasma samples. It is conceivable that other structures result from the A-ring or PGAL continues to react in vivo. First tests show certain binding properties between these degradation products with bovine serum albumin (data not shown). Further effects with endogenous structures or different ways of degradation are possible. The complex mixture of anthocyanin metabolites generated may be the main reason for the potentially beneficial effects in humans. The formation of many different metabolites with different functionalities may cause synergistic effects in vivo. For this case further investigations are needed. Our results revealed that the plasma level of degradation products was up to 20 times higher than that of anthocyanins. In urine, the ratio of degradants to anthocyanins was as high as 38:1. This is consistent with other results (Czank et al., 2013). However, our study did not consider all the possible metabolites formed. A further limitation of our study was the timeframe. Even 8 h after BE consumption, there was still a plasma level of degradation products. This was consistent with the urine results: several degradants were still present after 24 h and elimination seemed not complete at this time point. We found a baseline plasma level of HBA and HBAL prior to BE intake and comparable results for urine baseline, with high amounts of HBA and moderate concentrations of HBAL and VA. Additionally, hippuric acid was identified between –24–0 h in urine but was not further quantified because of its non-specificity. Sources for these metabolites could be other polyphenols or grain products, which contain, e.g., ferulic acid and p-coumaric acid (Mattila, Pihlava, & Hellström, 2005). Endogenous formation of vanillic acid has also been described in the literature (Rösen & Goodall, 1962). In the literature, there is already evidence that anthocyanin associated metabolites remain up to 48 h in urine. Czank and coworkers found that after 13Cy-3-glc ingestion, high concentrations of the glycine conjugated hippuric acid were present in the urine of subjects after 48 h (Czank et al., 2013). Almost all phenolic compounds can be metabolized to hippuric acid. Thus, hippuric acid can only be used as a metabolite specific for anthocyanin exposure if, and only if, the parent anthocyanin was labeled.
prior to ingestion. Human studies with many initial anthocyanins (BE contains 15 several anthocyanins) may implicate multiple metabolites. Carrying out these studies with fruit, juices or extracts as a source of anthocyanins (unlabeled) may complicate unambiguous assignment to the parent compounds. Nevertheless, such investigations mimic the actual route of exposure through the daily diet and include potential synergy effects with other polyphenols or matrix constituents.

4.3. Role of the small and large intestine in the resorption of consumed anthocyanins

The bowel plays an important role in the bioavailability of anthocyanins. This was demonstrated by the results for subjects with an intact healthy gut compared to ileostomists with an interrupted intestinal passage lacking a colon. The amounts of anthocyanins in the plasma and urine of healthy subjects was 79% and 44% higher, respectively, than in the plasma and urine of ileostomists. This indicates considerably higher bioavailability in the presence of an intact upper gut. Previous studies have assumed that anthocyanins could reach the circulation via the stomach (Passamonti, Vrhovsek, Vanzo, & Mattivi, 2003), despite the fact that at pH 1–2, they exist as polar flavylium cation, which impedes their passive diffusion through the mucosa. These studies suggested that anthocyanins may interact with bilitranslocase to transport these compounds from the lumen into the epithelial layers of the gastric mucosa (Passamonti, Vrhovsek, & Mattivi, 2002). Fernandes and coworkers demonstrated transport of anthocyanins through adenocarcinoma stomach cells (MKN-28), which could cause their absorption by facilitated diffusion (Fernandes, de Freitas, Reis, & Mateus, 2012). More investigations are useful because the mechanism and the identity of the transporter involved in the absorption of anthocyanins at the gastric level are still unknown (Fernandes et al., 2012).

As the ileostomists in the present study were found to absorb some anthocyanins, the stomach could still be a potential site of absorption. However, much more important for uptake seems to be the upper small intestine, mainly the jejunum as the main site of absorption (Matuschek, Hendriks, McGhie, & Reynolds, 2006; McGhie & Walton, 2007; Talavéra et al., 2004). The small intestine was present in both the ileostomists and healthy subjects, but the anthocyanins collected in the ileostomy bags would not be available for absorption in the intestine of ileostomists. This might explain the higher absorption in the healthy subjects. Furthermore, the lower gut in subjects with a colon may serve as another site of uptake. The rapid attainment of plasma anthocyanins (maximum 2 h) implicates the small intestine as the main site of absorption via active transporters or passive diffusion following deglycosylation (Kay, 2006).

Even more striking are the results of the degradation products. 100% more degradants reached the plasma of subjects with healthy gut and 75% more degradants were excreted via urine. Firstly, more degradation products might reach the circulation via the intact gut. Secondly, absorbed anthocyanins in the plasma may directly form degradation products in the circulation at pH 7.4. However, the importance of the enterohepatic circulation or influence of transit time was not investigated here.

In conclusion, after oral intake of BE, anthocyanins were absorbed rapidly and the human bioavailability of initial anthocyanins was very low (0.02% plasma/0.03% urine). Anthocyanins, which form structures based on the flavylium cation, reached the circulation mainly as glucuronides, especially of the malvidin and peonidin type. In contrast, anthocyanin-associated mononcylic degradation products were formed to a greater extent after BE consumption. Our study revealed that the plasma level of several selected degradants was up to 20 times higher in plasma and 38 times higher in urine compared to the parent anthocyanins. Other results have also demonstrated that the estimated total bioavailability is increased substantially when degradation products are considered in the calculation (Czank et al., 2013; Ferrars et al., 2014).

The microbiota in the small intestine may be different in healthy and ileostomy subjects. Nevertheless, ileostomists represent a simple, noninvasive model to demonstrate how food ingredients reach the small intestine and are therefore available for colonic absorption in healthy subjects. We detected about 30% of ingested anthocyanins in ileostomy fluid and, these concentrations remained relatively stable over 8 h during upper intestinal passage. The fate of the remaining 70% is difficult to identify, but some indication is provided by the analysis of formed degradation products reaching the gut. At first time, we investigated the amounts of specific degradants present in ileostomy fluid. GA, PCA and PGAL were the main degradants in the small intestine. However, in the circulation, SA and VA were predominant. PGAL had the highest half live in plasma, and therefore remained longest in the human circulation. The intestinal concentration of degradation products was just a fifth of that of the anthocyanins. While these results provide useful insights, further studies are essential to understand the complexity and whereabouts of anthocyanins during human gut passage.

The innovative aspect of the study reported here was the direct comparison of healthy subjects and ileostomists regarding the bioavailability of anthocyanins and influence of the small intestine. We found higher bioavailability of anthocyanins in the presence of an intact gut which confirms the gut as a potential site of action.

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Appendix A. Supplementary data

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References


