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Encapsulation of anthocyanins from bilberries – Effects on bioavailability and intestinal accessibility in humans

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Protocatechuic acid (PubChem CID: 72)
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ABSTRACT

Anthocyanins are flavonoids that have been suggested to provide beneficial health effects. The biological activity of anthocyanins is influenced by their pharmacokinetic properties, but anthocyanins are associated with limited bioavailability in humans. In the presented study, we investigated how the encapsulation of bilberry extract (BE), a source of anthocyanins, with either whey protein or citrus pectin influences the bioavailability and intestinal accessibility of anthocyanins in humans. We performed an intervention study that analyzed anthocyanins and their degradation products in the urine, plasma, and ileal effluent of healthy volunteers and ileostomists (subjects without an intact colon). We were able to show, that whey protein encapsulation modulated short-term bioavailability and that citrus pectin encapsulation increased intestinal accessibility during passage through the small intestine and modulated the formation of the degradation product phloroglucinol aldehyde (PGAL) in human plasma.

1. Introduction

Bilberries (*Vaccinium myrtillus* L.) are an important source of anthocyanins in the diet, and are also rich in other phytochemicals such as procyanidins, phenolic acids and flavonoids (Clifford, 2000; Juadjur & Winterhalter, 2012). Various studies have presented strong evidence for the preventive health potential of anthocyanins, which are red/blue pigments found in fruits and flowers (Schantz, Baum, & Richling, 2011; Triebel, Trieu, & Richling, 2012; Wallace, Slavin, & Frankenfeld, 2016). The pharmacokinetic properties of these flavonoids (bioavailability, metabolism, excretion) affect their biological activity. However, intact anthocyanins are associated with limited bioavailability (Fang, 2014; Gonzalez-Barrio, Borges, Mullen, & Crozier, 2010; Kraus et al., 2010). The high polarity of anthocyanin structures may underlie their low *in vivo* absorption efficiency. The low bioavailability may also stem from the instability caused by pH changes and microbial degradation during gastrointestinal (GIT) passage (Aura et al., 2005; Keppler & Humpf, 2005; Pina, Oliveira, & de Freitas, 2015). Therefore, it would be important to study how the stability and physical properties of anthocyanins are affected by GIT passage, and how these changes influence the bioaccessibility and bioavailability of this group of antioxidants.

Encapsulation can be a viable approach for stabilizing active food ingredients (Fang & Bhandari, 2010; Gibbs, Kermasha, Alli, & Mulligan, 1999; Madene, Jacquot, Scher, & Desobry, 2006). Several studies on the *in vitro* effects of anthocyanin encapsulation are available (Fang & Bhandari, 2010; Flores et al., 2015; Munin & Edwards-Lévy, 2011;

Abbreviations: Ara, arabinoside; BE, bilberry extract; CPC, citrus pectin encapsulated BE; Cy, cyanidin; Del, delphinidin; GA, gallic acid; Gal, galactose; Glc, glucose; Gluc, glucuronide; HBA, 4-hydroxybenzoic acid; HBAL, 4-hydroxybenzaldehyde; IS, internal standard; Mal, malvidin; MGA, 3-*O*-methylgallic acid; PCA, protocatechuic acid; PGAL, phloroglucinol aldehyde; Peo, peonidin; Pet, petunidin; SA, syringic acid; VA, vanillic acid; WPC, whey protein encapsulated BE

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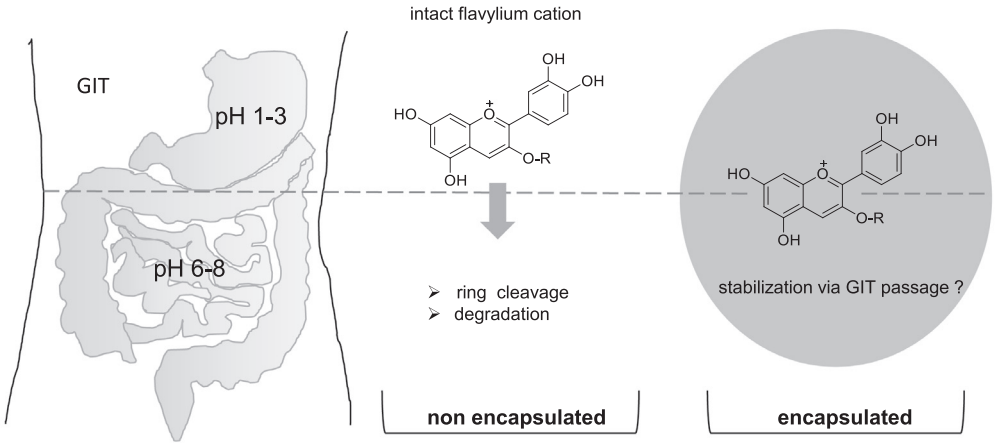

Fig. 1. Overview of which anthocyanins are present at physiological GIT pH in the non-encapsulated and encapsulated forms of bilberry extract.

Oehme, Valotis, Krammer, Zimmermann, & Schreier, 2011; Schantz et al., 2014). Furthermore, our previous research has already demonstrated successful stabilization of anthocyanins with conserved biological activity (Baum et al., 2014; Schantz et al., 2011, 2014). However, as of yet, no studies have assessed how anthocyanin encapsulation affects chemical stability, intestinal accessibility, and bioavailability in humans. The current intervention study evaluated whether encapsulated and non-encapsulated anthocyanins differ in terms of *in vivo* pharmacokinetics (Fig. 1).

The degradation of anthocyanins in humans follows different pathways (Mueller et al., 2017). Recent studies on the bioavailability and biological effectiveness of anthocyanins have demonstrated the importance of their metabolites and degradation products (Czank et al., 2013; Faria, Fernandes, Mateus, & Calhau, 2013; Fernandes, Faria, de Freitas, Calhau, & Mateus, 2015; Ferrars et al., 2014; Kalt, Liu, McDonald, Vinqvist-Tymchuk, & Fillmore, 2014; Kay, Pereira-Caro, Ludwig, Clifford, & Crozier, 2017). Our previous study indicated that the plasma concentration of degradation products can be up to 20 times higher than the corresponding concentration of anthocyanins (Mueller et al., 2017). Thus, the *in vivo*-formed metabolites and degradation products have to be considered when the 'real bioavailability' of anthocyanins is determined.

In light of this, the current study also aims to address what effect the encapsulation of anthocyanins has on their *in vivo* degradation. The presented human intervention study was integrated into a previously published study by Mueller et al. (2017). In the previous study, which represents part one, volunteers received non-encapsulated anthocyanin-rich bilberry extract (BE). The research reported in this article represents part two, during which volunteers ingested bilberry extract that was encapsulated with either citrus pectin or whey protein. The intestinal accessibility and bioavailability were then measured and compared with data from volunteers who had ingested non-encapsulated bilberry extract (Mueller et al., 2017) (see Fig. 2). These studies included both ileostomists (group A, without a colon) and volunteers with a colon (group B) to provide detailed information about the intestinal absorption of anthocyanins when the results for encapsulated and non-encapsulated forms were compared. Part I of the study included a single bolus of BE (corresponding to 4.95 mmol anthocyanins). During part II of the study, each subject received equimolar concentrations of anthocyanins encapsulated with either whey protein (WCP) or citrus pectin (CPC).

The contents of intact anthocyanins, selected glucuronides and degradation products (e.g. phenolic acids and phenylaldehydes) in intestinal effluent, plasma and urine samples were then determined to demonstrate how encapsulation affects the pharmacokinetics of anthocyanins.

2. Material and methods

2.1. Study design

Details of this human pilot study are described in detail by Mueller et al. (2017). There were two groups of subjects. Group A included ileostomists (N = 5 females, BMI = 27 ± 4, age = 41 ± 8) and group B included healthy subjects who had an intact gut (N = 5 females, BMI = 23 ± 3, age = 33 ± 7). The intervention consisted of two parts. The initiation of each part was preceded by a wash-out period of 48 h with a polyphenol-free diet. The two parts were also separated by a period of at least two weeks. The subjects received equimolar amounts of anthocyanins (a total of 10 g), either in the form of a single bolus of non-encapsulated BE (10 g) (for details, see Mueller et al. (2017)), a single bolus of BE encapsulated with whey protein (WPC, 144 g), or a single bolus of BE encapsulated with citrus pectin (CPC, 30 g) (see Fig. 2). All of the study participants received an identical diet, described in detail by Mueller et al. (2017), throughout the study period. The sample collection of ileostomy fluids, blood and urine was analogous to what was described in Mueller et al. (2017). At the end of the study period, group A, parts I & II, included five subjects and group B, parts I & part II with CPC intake, included five subjects while part II with WPC intake included four subjects. Unfortunately, one volunteer quit the study due to illness. The results of study part I and study part II are comparable because they were performed with the identical study design. Sample preparation was also done using the analogue methods.

2.2. Chemicals, bilberry extract and stabilization systems (capsules)

The qualities of standards used for quantification and chemicals used in experiments were analogous to those reported in Mueller et al. (2017). The BE (Symrise GmbH & Co. KG, Holzminden, Germany, pr. no. 399916, batch 29) was produced from bilberries (*Vaccinium myrtillus* L.) and included 24 ± 1% of 15 different anthocyanins (for details and anthocyanin profile, see Mueller et al. (2017)).

2.2.1. Encapsulation with whey proteins (WPC)

The whey protein capsules (WPC) were produced by the group of Prof. Dr. U. Kulozik (Technische Universitaet Muenchen, Germany). A 30% whey protein solution was prepared from WPI BiPro® Protein Isolate (94% (w/w)) (Davisco Foods International Inc., Le Seur, MN, USA) and distilled water, after which BE powder was slowly added and allowed to dissolve. The pH was adjusted to 1.5 with aqueous hydrochloric acid (Merck, Darmstadt, Germany), and the final concentrations of whey protein and BE in the final solution were 20% and 10%,

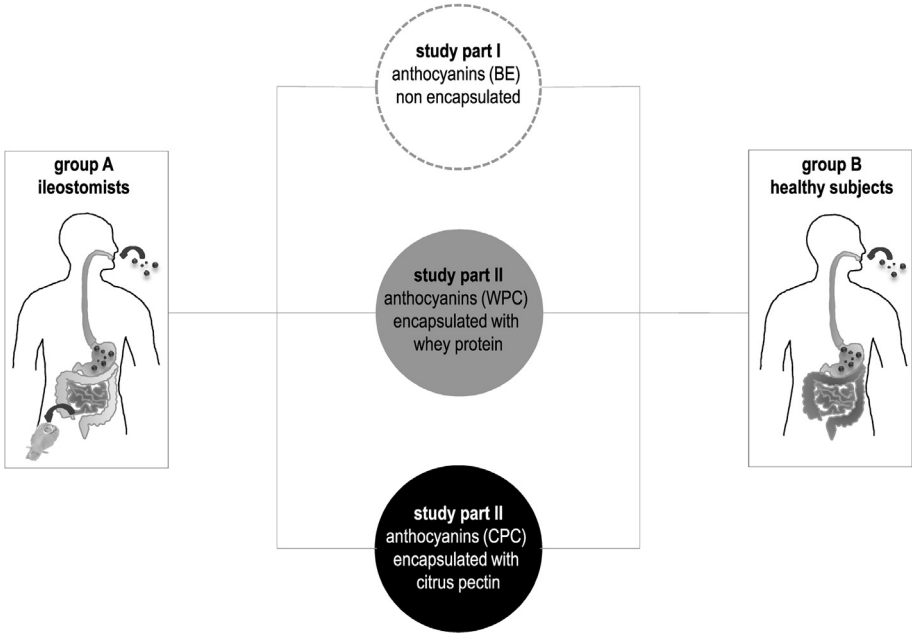

Fig. 2. The intervention study was performed three times with a group of ileostomists (A) and healthy subjects with an intact gut (B) who were given either non-encapsulated anthocyanins (study part I), anthocyanins encapsulated with whey protein or anthocyanins encapsulated with citrus pectin (study part II).

respectively. The solution was then centrifuged (5000*g*, 2 min) to remove any insoluble components. Microencapsulation was performed by emulsification and thermal gelation. An emulsion of BE/whey protein solution and sunflower oil was prepared by heating the solution to 50 °C while slowly stirring. Within six minutes, the emulsion was heated to 80 °C and maintained at this temperature for 10 min to denature proteins and form gel-like capsules. The suspension was cooled to 20 °C and oil residues were separated by centrifugation (1000*g*, 2 min). The mean capsule diameter was 200 µm (Oidtmann et al., 2012). An HPLC analysis of the whey protein encapsulated BE, described in Section 2.4, revealed an anthocyanin content of 7%.

2.2.2. Encapsulation with citrus pectin (CPC)

The citrus pectin capsules (CPC) were produced by the group of Prof. Dr. K. Schwarz (University of Kiel, Germany) (Berg, Bretz, Hubbermann, & Schwarz, 2012). BE (40%) was mixed with trehalose (42%), highly esterified citrus pectin, with 71% degree of esterification (5%, Herbstreith & Fox KG, Neuenbuerg, Germany) and citric acid (13%, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and dissolved in water. The solution was spray-dried, and the resulting microcapsules were granulated with ethanolic shellac solution (10%, shellac: SSB57 Pharma, Stoever GmbH & Co. KG, Bremen, Germany). The granules were then coated in a fluidized bed process, with an application quantity of 25 g of shellac per 100 g of granulate. Following drying, the coated product was classified as having a target particle size of 250–500 µm. An HPLC analysis (see Section 2.4) of the citrus pectin encapsulated BE revealed an anthocyanin content of 34%.

2.3. Sample storage

The storage conditions of ileostomy fluid, plasma, and urine samples were analogous to those described by Mueller et al. (2017).

2.4. Sample preparation and analysis

The sample preparation procedure is described in detail by Mueller et al. (2017). Ileostomy fluids were lyophilized, after which samples were extracted for analysis. Urine and plasma samples were extracted with a solid phase extraction method (see Mueller et al. (2017)). The WPC and CPC capsules were extracted to determine the anthocyanin content in the two encapsulated forms of BE: Whey protein encapsulated BE (WPC) was extracted with solvent A (87% acetonitrile/3% water/10% formic acid (v/v/v)) using an ultra turrax (60 s, 20,000 rcf), and was then centrifuged (20 min, 4500*g*, 25 °C). Citrus pectin encapsulated BE (CPC) was extracted with solvent A using a stirrer (20 h, 25 °C, light protected), and was then centrifuged (20 min, 4500*g*, 25 °C). The quantification of anthocyanins, along with their degradation products and glucuronides of interest, in the extracted samples was performed using the HPLC-DAD and HPLC-MS/MS methods described by Mueller et al. (2017).

2.5. Statistical analysis and calculations

The results were not analyzed for statistical significance because of the low number of subjects in this intervention study. The area under the curve (AUC) of plasma analytes was determined by integration of the concentration curves between 0 and 8 h using the linear trapezoidal rule (Kaplan, Jack, Cotler, & Alexander, 1973).

3. Results

This section presents the results from parts I and II of the intervention study, which included a non-encapsulated form and two encapsulated forms of BE, respectively. The anthocyanins identified in the ileostomy fluid, urine, and plasma following the ingestion of non-encapsulated BE are shown in Fig. 3A (Mueller et al., 2017). Fig. 3B shows the degradation products that were identified in ileostomy fluid, urine, and plasma of volunteers who had received non-encapsulated BE. Within eight hours of BE consumption, 1547 ± 309 µmol of anthocyanins and 302 ± 79 µmol of degradation products were found in ileostomy fluids (N = 5). The concentrations of anthocyanins and degradation products in ileal effluent (N = 5) within the same time period (0–8 h) after the consumption of anthocyanins stabilized with whey protein (WPC) were 1390 ± 586 µmol and 361 ± 154 µmol, respectively. Eight hours after the intake of BE encapsulated with citrus pectin (CPC), 1916 ± 210 µmol of anthocyanins and 342 ± 33 µmol of degradation products were recovered from ileal fluids (N = 5). The total amounts of anthocyanins and degradation products that were excreted in the urine of healthy subjects (with intact colon) and ileostomists (without colon) within 24 h are shown in Fig. 3. After the intake of non-encapsulated BE, 0.9 ± 0.5 µmol of anthocyanins and 11.6 ± 5.6 µmol of degradation products were excreted via urine in

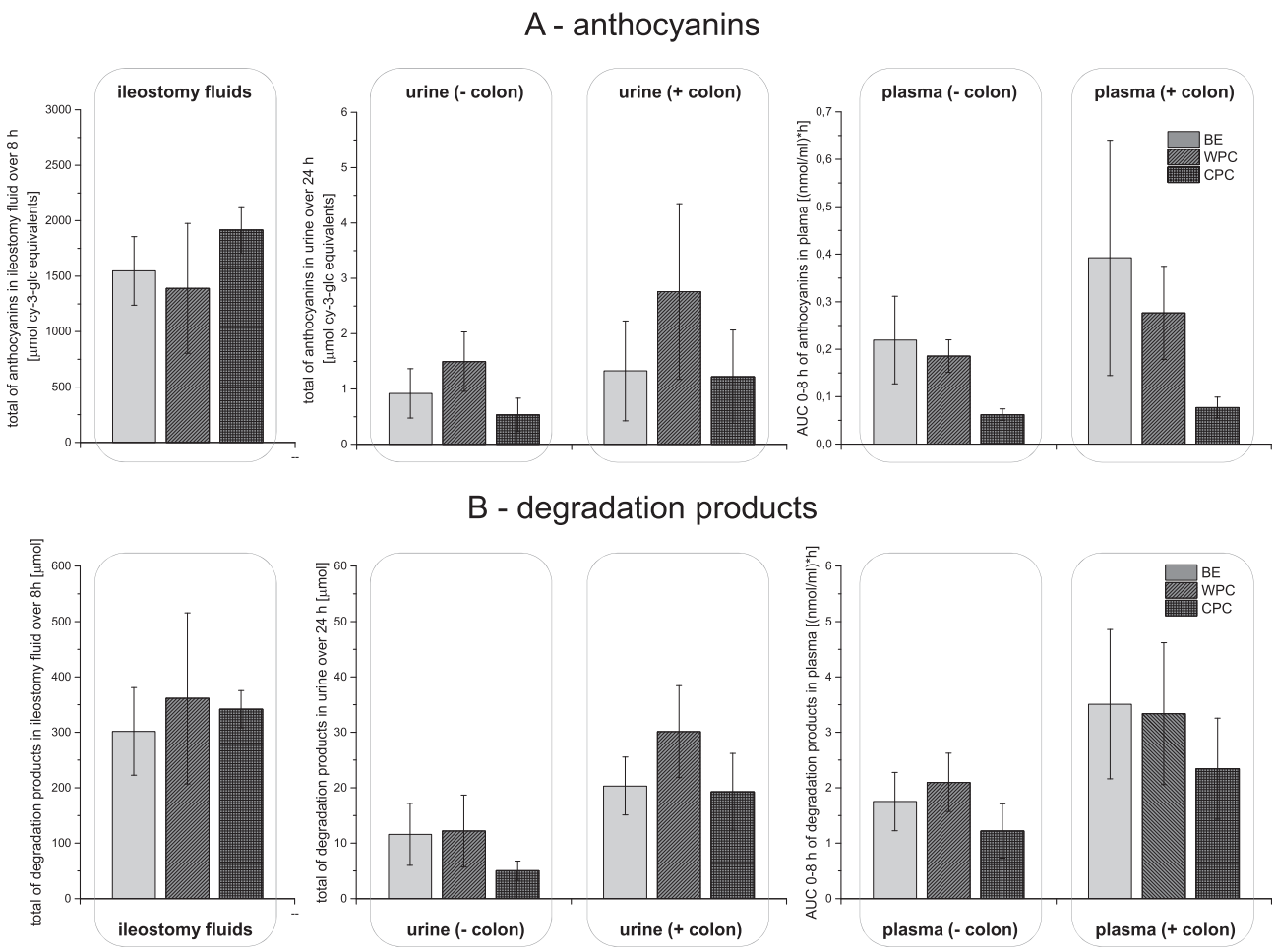

Fig. 3. The anthocyanins (A) and degradation products (B) that were identified during study parts I (BE) and II (N = 4–5) from ileostomy effluents (over 8 h), urine samples (over 24 h), and plasma samples (shown as AUC from 0 to 8 h).

ileostomists (N = 5), and 1.3 ± 0.9 µmol of anthocyanins and 20.3 ± 5.2 µmol of degradation products were excreted via urine in healthy volunteers (N = 5). When BE was encapsulated with WPC, the urine samples of ileostomists (N = 5) showed 1.5 ± 0.5 µmol and 12.2 ± 6.5 µmol of anthocyanins and degradations products, respectively, while the urine samples of health volunteers (N = 4) showed 2.8 ± 2.0 µmol and 30.1 ± 8.3 µmol of anthocyanins and degradation products, respectively. Following the ingestion of BE encapsulated with CPC, the urine samples of ileostomists (N = 5) showed 0.5 ± 0.3 µmol of anthocyanins and 5.0 ± 1.7 µmol of degradation products while the urine samples of healthy patients (N = 5) showed 1.2 ± 0.8 µmol of anthocyanins and 19.3 ± 6.9 µmol of degradation products. The AUC (between 0 and 8 h) indicates the concentration of anthocyanins and specific degradation products in the plasma. Following non-encapsulated BE intake, the AUCs for anthocyanins and degradation products were 0.22 ± 0.09 nmol/ml∗h and 1.8 ± 0.5 nmol/ml∗h, respectively, in ileostomists (N = 5), while the corresponding AUCs for healthy subjects (N = 5) were 0.39 ± 0.25 nmol/ml∗h and 3.5 ± 1.3 nmol/ml∗h, respectively. After the ingestion of BE encapsulated with WPC, the AUCs for anthocyanins and degradation products were 0.18 ± 0.03 nmol/ml∗h and 2.1 ± 0.5 nmol/ml∗h, respectively, in ileostomists (N = 5), while the corresponding AUCs for healthy subjects (N = 4) were 0.28 ± 0.10 nmol/ml∗h and 3.3 ± 1.3 nmol/ml∗h, respectively. The AUCs for anthocyanins and degradation products following the ingestion of BE encapsulated with CPC were 0.06 ± 0.01 nmol/ml∗h and 1.2 ± 0.5 nmol/ml∗h, respectively, in ileostomists (N = 5), and 0.08 ± 0.02 nmol/ml∗h and 2.3 ± 0.9 nmol/ml∗h, respectively, in healthy subjects (N = 5).

In Fig. 4, the results from study part II are overlaid onto the data reported by Mueller et al. (2017). This figure shows how the amounts of anthocyanins and degradation products in the ileal effluent (Fig. 4A and B), plasma (Fig. 4C and D), and urine (Fig. 4E and F) of ileostomists and healthy subjects change over time. The maximum anthocyanin concentration in ileostomy fluids was detected 0–1 h after non-encapsulated BE intake, both 0–1 h and 1–2 h after intake of BE encapsulated with WPC and 1–2 h after intake of BE encapsulated with CPC (Fig. 4A). The amount of degradation products in the three samples showed a similar pattern. The maximum amount of degradation products was observed 0–1 h after non-encapsulated BE intake, both 0–1 h and 1–2 h after intake of BE encapsulated with WPC, and 1–2 h after intake of BE encapsulated with CPC (Fig. 4B). The maximum concentration of anthocyanins in plasma was reached 2 h after ingestion of non-encapsulated BE and BE encapsulated with WPC, but already 1 h after intake of BE encapsulated with CPC, after which the concentration stayed constant until 2 h (Fig. 4C). For all three forms of BE, the maximum plasma level of degradation products was observed after 1 h (Fig. 4D), whereas the maximum concentrations of anthocyanins (Fig. 4E) and degradation products in urinary excretions occurred 2–4 h after ingestion (Fig. 4E and F).

Fig. 5 illustrates which metabolites were identified in plasma samples after the ingestion of encapsulated and non-encapsulated anthocyanins in relation to findings by Mueller et al. (2017). The figure presents patterns of anthocyanin-glucuronides such as Mal-gluc and Peo-gluc, phenolic acids such as GA, PCA, MGA, SA, VA, and phenylaldehydes such as PGAL. It can be noted that the profiles of Mal-gluc and Peo-gluc changed with different forms of BE. The ingestion of pure

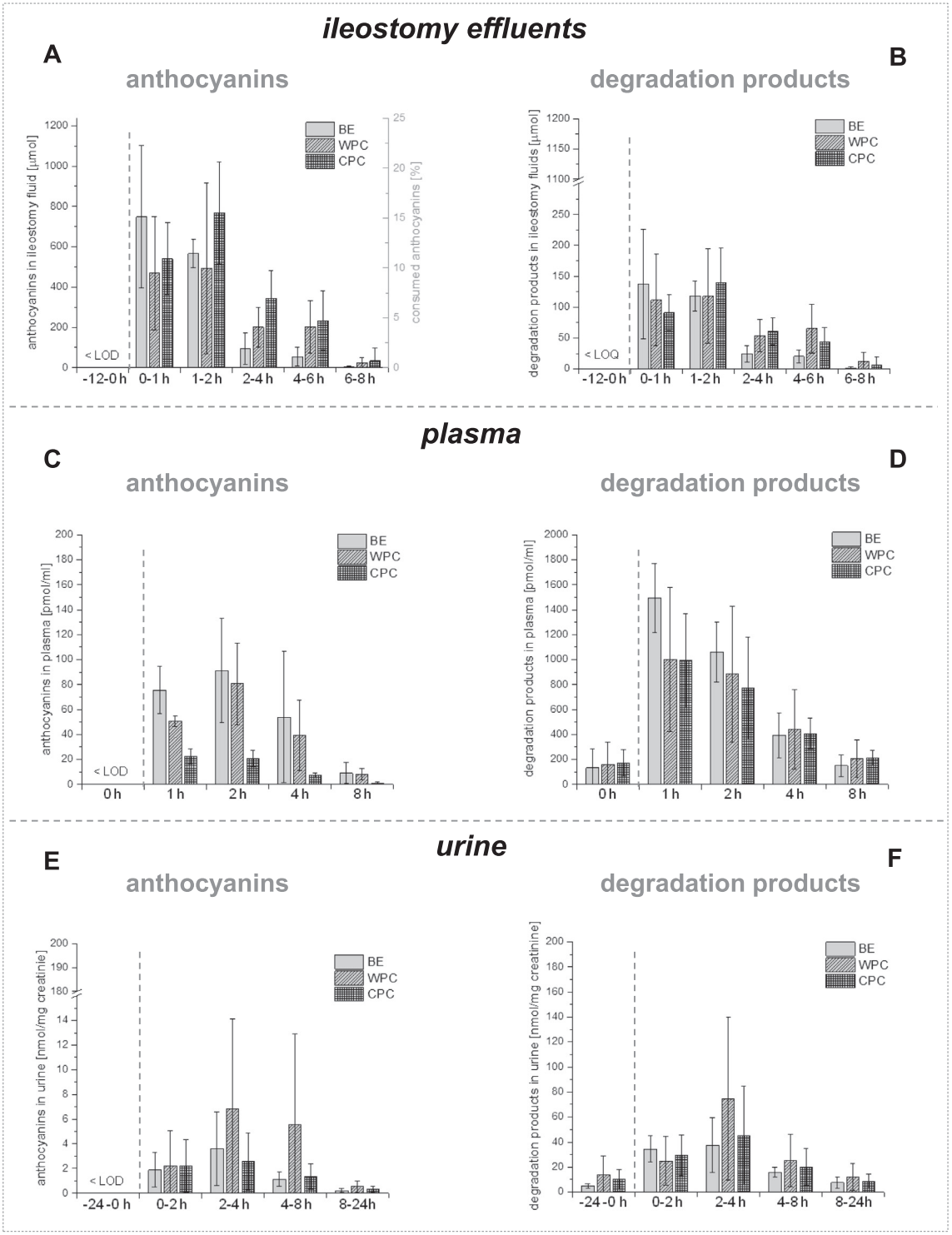

Fig. 4. The average amounts of total anthocyanins and degradation products in ileostomy fluid (A, B) of ileostomists (N = 5) and plasma (C, D) and urine (E, F) of healthy subjects (N = 4–5) after the consumption of non-encapsulated (BE: part I) and encapsulated anthocyanins (WPC, CPC: part II).

BE showed the highest amounts of Mal-gluc and Peo-gluc in plasma samples. The intake of BE encapsulated with whey protein (WPC) showed similar profiles for Mal-gluc and Peo-gluc, but with lower concentrations. In contrast, the intake of BE encapsulated with CPC was followed by very low Mal-gluc concentrations in the plasma samples. The most prominent glucuronide in the plasma sample was Peo-gluc, but the concentration after ingestion of BE encapsulated with CPC was far lower than what was observed after the ingestion of non-capsulated BE or BE encapsulated with WPC. The plasma profiles of phenolic acids had a similar trend for each form of BE. The concentrations decreased more or less in the following rank: SA > VA > PCA = MGA > GA. The BE forms differed based on the temporal occurrences of certain metabolites in the plasma samples. For example, MGA and SA concentrations were lower after the ingestion of BE encapsulated with CPC than after the ingestion of non-encapsulated BE or BE encapsulated with WPC. The highest PGAL concentration was observed after intake of BE encapsulated with CPC.

4. Discussion

The study addressed two main topics: how does anthocyanin encapsulation modulate absorption and bioavailability in comparison to the ingestion of pure berry extract; and how does passage through the gastrointestinal tract modulate the biological effect exerted by anthocyanins? By including ileostomists (subjects without an intact colon) and collecting ileal effluents, we created a model that can adequately demonstrate how ingested compounds – in our case anthocyanins (encapsulated versus non-encapsulated) – reach the large intestine (Mueller et al., 2017). The results of intervention study part I, during which volunteers consumed pure BE, have been published in detail (Mueller et al., 2017). The findings presented in this article demonstrate how two encapsulated forms of BE (encapsulation with whey protein or citrus pectin) differ from pure BE in terms of anthocyanin bioavailability and metabolism. Generally, the absorption and elimination of

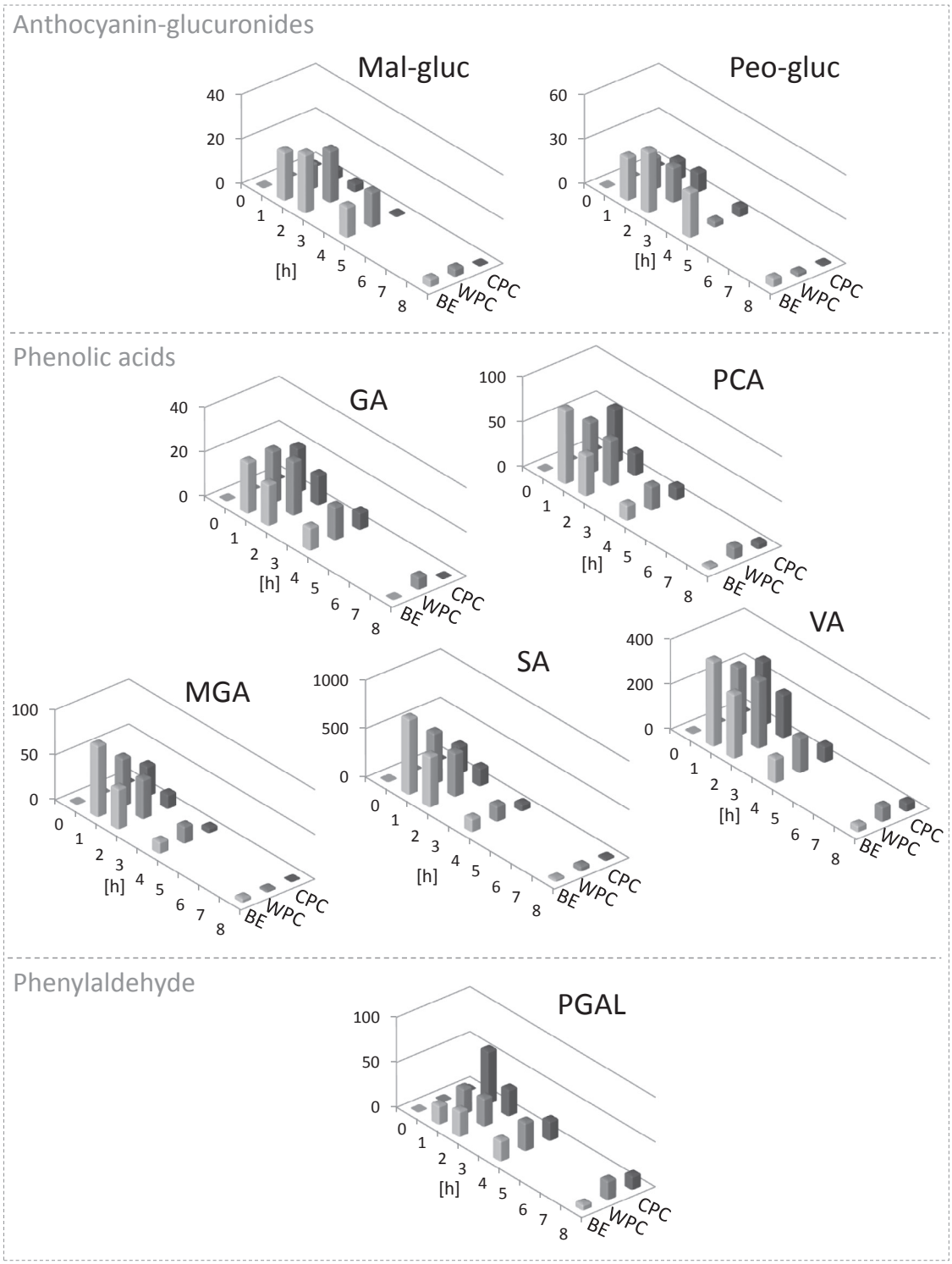

Fig. 5. Kinetic profiles of anthocyanin-glucuronides (Mal-gluc, Peo-gluc), phenolic acids (GA, PCA, MGA, SA, VA) and phloroglucinol aldehyde (PGAL) in the plasma samples of healthy subjects with an intact gut during study parts I (N = 5) and II (N = 4–5) in nmol/L (BE: part I; WPC, CPC: part II).

both anthocyanins and degradation products was higher in the group of healthy subjects than in the ileostomists, regardless of the formulation (BE, WPC, or CPC). Passage through the small and large intestine strongly influences the bioavailability of anthocyanins independent of whether or not they are encapsulated. Various studies have presented details of how the intestine is a site of anthocyanin absorption (Matuschek, Hendriks, McGhie, & Reynolds, 2006; McGhie & Walton, 2007; Mueller et al., 2017; Talavéra et al., 2004).

4.1. Pharmacokinetic effects of whey protein encapsulation (WPC) on anthocyanins and their degradation products

Whey protein stabilization decreased the total anthocyanin content (over 8 h) in the ileal effluents by 10% when compared to pure BE (Fig. 3A). The two formulations varied greatly in terms of degradation products that were identified in the ileal effluent. The degradation product content in the ileal fluid samples was about 20% higher after subjects ingested BE encapsulated with WPC rather than pure, non-encapsulated BE (Fig. 3B). Although there were high inter-individual differences, the results demonstrate that whey protein encapsulation had almost no effect (only by trend) on the stabilization of anthocyanins during intestinal passage, and increased the rate of degradation when compared to what was observed for non-encapsulated BE.

The concentration of a compound in the plasma and urine is a good representation of how much of the compound is being absorbed and what amount will reach systemic circulation. After the ingestion of anthocyanin encapsulated with WPC, 28% less anthocyanins and 6% less degradation products were detected in the plasma of healthy subjects. The profile of the identified metabolites is shown in Fig. 5. On the other hand, WPC encapsulation led to the elimination of 108% more anthocyanins and 48% more degradation products via urine than had been observed after pure BE intake. Each of the recovered degradation products showed a higher concentration in samples from subjects who ingested BE capsulated with WPC rather than non-encapsulated BE. A specific trend was not apparent (data not shown). The differences between the results from plasma and urine samples could stem from different sampling periods. The plasma concentrations only demonstrate concentrations at certain time points (0 h, 1 h, 2 h, 4 h, 8 h). The calculation of the area under curve (AUC) shows a certain trend, but concentrations at specific times between two sampling points remain unknown. In contrast, a certain volume of urine was collected from the subjects during each sampling period (− 24 to 0 h, 0–2 h, 2–4 h, 4–8 h, 8–24 h). Therefore, the urine samples demonstrate the total amount of a compound over a certain time period (see Fig. 3).

In summary, these results show that whey protein encapsulation, when compared with non-encapsulated anthocyanins, leads to higher concentrations of anthocyanins and their degradation products in the urine. Whey protein encapsulation might increase systemic concentrations and short-term bioavailability. Several studies have provided evidence that whey proteins exert a stabilizing effect on anthocyanins (Schantz et al., 2014; Viljanen, Kylli, Hubbermann, Schwarz, & Heinonen, 2005). However, our results showed that whey protein capsules did not stabilize anthocyanins at the bowel milieu, and absorption via the stomach could be a reason for the high urinary levels of anthocyanins and their degradation products. Several studies have demonstrated that whey proteins prolong the duration of stomach passage (Buraczewski, Porter, Rolls, & Zebrowska, 1971; Stanstrup, Schou, Holmer-Jensen, Hermansen, & Dragsted, 2014). It is possible that the release of anthocyanins from the capsules may have been delayed by the presence of whey proteins, a mechanism that has been reported before (Schantz et al., 2014). The temporal shift in the maximum concentrations of anthocyanins and their degradation products supports this theory. Absorption increases with the time that anthocyanins are stable and available at a specific compartment for absorption.

4.2. Pharmacokinetic effects of citrus pectin encapsulation (CPC) on anthocyanins and their degradation products

The investigation of ileal effluents revealed that citrus pectin encapsulation seems to stabilize anthocyanins during intestinal passage. About 24% more anthocyanins were recovered in the ileostomy effluents when subjects ingested CPC-encapsulated rather than pure BE (Fig. 3A). The shellac coating of these capsules may have well delayed the release of anthocyanins. Shellac is resistant against the low pH environment of the stomach (Farag & Leopold, 2011). This allows anthocyanins encapsulated with CPC to enter the intestine unaffected. The concentrations of degradation products were in line with this theory and on average, 13% higher when subjects ingested BE encapsulated with CPC rather than pure BE (Fig. 3B). This could be due to the stabilization of anthocyanin in the stomach, which is followed by intestinal transit and increased degradation. The time-dependent collection of ileal effluent following ingestion of BE encapsulated with citrus pectin demonstrated substantial temporal shifts in the maximum concentrations of anthocyanins and their degradation products when compared to the concentrations observed after ingestion of pure BE (Fig. 4A and B). The levels of anthocyanins and their degradation products were lower in the plasma samples of subjects who had ingested anthocyanins encapsulated with CPC than those who had ingested anthocyanin in the non-encapsulated form. The concentrations in the urine differ slightly. The concentrations of anthocyanins in the plasma and urine samples were 80% and 8% lower, respectively, in healthy subjects who had ingested BE encapsulated with CPC rather than pure BE. The amounts of degradation products were also lower in the plasma and urine samples of healthy subjects (34% and 5%, respectively) when BE encapsulated with CPC was ingested instead of pure BE. Hence, citrus pectin encapsulation might increase intestinal concentrations of anthocyanins, but will not trigger the absorption and therefore, not improve bioavailability. The results regarding PGAL concentrations revealed an interesting exception (Fig. 5). Every analyzed metabolite (anthocyanin-glucuronides, phenolic acids) was present at lower concentrations in all three tested samples (ileal effluent, plasma, urine) when the ingestion of BE encapsulated with CPC was compared to the ingestion of non-encapsulated BE. The only exception was PGAL, which showed a higher plasma level in subjects with an intact colon who had consumed BE encapsulated with CPC rather than non-encapsulated BE. This trend was reversed in the group of ileostomists (data not shown). This may be explained by the absence of a large intestine, which would otherwise provide microbiota that cleave anthocyanins to anthocyanidins and further degradation products (Keppler & Humpf, 2005). Further prospective studies are necessary to verify these results. The metabolite profile of the urine sample from healthy subjects did show the same trend as that of plasma. In the urine samples, the concentrations of most metabolites were lower when BE encapsulated with CPC was ingested than when pure BE was ingested. The subjects who had ingested CPC-encapsulated BE and non-capsulated BE did not differ in urinary PGAL concentrations. The formulations did differ in terms of SA concentrations, as the ingestion of CPC-encapsulated BE resulted in slight higher SA concentrations than the ingestion of pure BE.

4.3. Conclusion

This study is limited by the low number of subjects and the incompleteness of the detected metabolites and degradation products. Previous research has reported that many different metabolites, namely cy-3-glu, form after anthocyanin intake (Czank et al., 2013; Kay et al., 2017). Even though encapsulation did not strongly influence the total bioavailability of anthocyanins, a closer examination reveals certain modulatory effects. The large dose of anthocyanin ingested by the subjects (2.4 g) may be one reason that the effects were not more pronounced, as this could have saturated the enzymatic and absorption systems.

The presented results raise the question of whether it is important to increase the bioavailability of intact anthocyanins, or if it would be more useful to focus on degradation products, which demonstrated higher absorption rates and could therefore be responsible for the physiological effects of anthocyanins. These aspects do not yet have answers. Intervention studies have shown that the intake of anthocyanins induces the antioxidative defense mechanism via Nrf2 and protects DNA from oxidative damage (Kropat et al., 2013). We recently showed that the consumption of anthocyanin-rich fruit juice over eight weeks reduces DNA damage in the white blood cells of volunteers within the first hours of intervention as well as throughout the whole eight-week study period (unpublished data). Hence, the intake of anthocyanin-rich foods exerts protective effects, but the identities of the active antioxidative compounds remain at the speculative stage.

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References

Aura, A. M., Martin-Lopez, P., O'Leary, K. A., Williamson, G., Oksman-Caldentey, K. M., Poutanen, K., & Santos-Buelga, C. (2005). In vitro metabolism of anthocyanins by human gut microflora. *European Journal of Nutrition, 44*(3), 133–142.

Baum, M., Schantz, M., Leick, S., Berg, S., Betz, M., Frank, K., ... Schuchmann, H. (2014). Is the antioxidative effectiveness of a bilberry extract influenced by encapsulation? *Journal of the Science of Food and Agriculture, 94*(11), 2301–2307.

Berg, S., Bretz, M., Hubbermann, E. M., & Schwarz, K. (2012). Influence of different pectins on powder characteristics of microencapsulated anthocyanins and their impact on drug retention of shellac coated granulate. *Journal of Food Engineering, 108*(1), 158–165.

Buraczewski, S., Porter, J., Rolls, B., & Zebrowska, T. (1971). The course of digestion of different food proteins in the rat. *British Journal of Nutrition, 25*(2), 299–306.

Clifford, M. N. (2000). Anthocyanins – Nature, occurrence and dietary burden. *Journal of the Science of Food and Agriculture, 80*(7), 1063–1072.

Czank, C., Cassidy, A., Zhang, Q., Morrison, D. J., Preston, T., Kroon, P. A., ... Kay, C. D. (2013). Human metabolism and elimination of the anthocyanin, cyanidin-3-glucoside: A 13C-tracer study. *American Journal of Clinical Nutrition, 97*(5), 995–1003.

Fang, J. (2014). Bioavailability of anthocyanins. *Drug Metabolism Reviews, 46*(4), 508–520.

Fang, Z., & Bhandari, B. (2010). Encapsulation of polyphenols – A review. *Trends in Food Science & Technology, 21*(10), 510–523.

Farag, Y., & Leopold, C. S. (2011). Development of shellac-coated sustained release pellet formulations. *European Journal of Pharmaceutical Sciences, 42*(4), 400–405.

Faria, A., Fernandes, I., Mateus, N., & Calhau, C. (2013). Bioavailability of anthocyanins. *Natural products*(pp. 2465–2487). Springer.

Fernandes, I., Faria, A., de Freitas, V., Calhau, C., & Mateus, N. (2015). Multiple-approach studies to assess anthocyanin bioavailability. *Phytochemistry Reviews, 14*(6), 899–919.

Ferrars, R., Czank, C., Zhang, Q., Botting, N., Kroon, P., Cassidy, A., & Kay, C. (2014). The pharmacokinetics of anthocyanins and their metabolites in humans. *British Journal of Pharmacology, 171*(13), 3268–3282.

Flores, G., del Castillo, M. L. R., Costabile, A., Klee, A., Guergoletto, K. B., & Gibson, G. R. (2015). In vitro fermentation of anthocyanins encapsulated with cyclodextrins: Release, metabolism and influence on gut microbiota growth. *Journal of Functional Foods, 16*, 50–57.

Gibbs, B. F., Kermasha, S., Alli, I., & Mulligan, C. N. (1999). Encapsulation in the food industry: A review. *International Journal of Food Sciences and Nutrition, 50*(3), 213–224.

Gonzalez-Barrio, R., Borges, G., Mullen, W., & Crozier, A. (2010). Bioavailability of anthocyanins and ellagitannins following consumption of raspberries by healthy humans and subjects with an ileostomy. *Journal of Agricultural and Food Chemistry, 58*(7), 3933–3939.

Juadjur, A., & Winterhalter, P. (2012). Development of a novel adsorptive membrane chromatographic method for the fractionation of polyphenols from bilberry. *Journal of Agricultural and Food Chemistry, 60*(10), 2427–2433.

Kalt, W., Liu, Y., McDonald, J. E., Vinqvist-Tymchuk, M. R., & Fillmore, S. A. (2014). Anthocyanin metabolites are abundant and persistent in human urine. *Journal of Agricultural and Food Chemistry, 62*(18), 3926–3934.

Kaplan, S., Jack, M., Cotler, S., & Alexander, K. (1973). Utilization of area under the curve to elucidate the disposition of an extensively biotransformed drug. *Journal of Pharmacokinetics and Biopharmaceutics, 1*(3), 201–216.

Kay, C. D., Pereira-Caro, G., Ludwig, I. A., Clifford, M. N., & Crozier, A. (2017). Anthocyanins and flavanones are more bioavailable than previously perceived: A review of recent evidence. *Annual Review of Food Science and Technology, 8*, 155–180.

Keppler, K., & Humpf, H.-U. (2005). Metabolism of anthocyanins and their phenolic degradation products by the intestinal microflora. *Bioorganic & Medicinal Chemistry, 13*(17), 5195–5205.

Kraus, M., Kahle, K., Ridder, F., Schantz, M., Scheppach, W., Schreier, P., & Richling, E. (2010). Colonic availability of bilberry anthocyanins in humans. *ACS Symposium Series: Flavor and Health Benefits of Small Fruits, American Chemical Society, 1035*(10), 159–176.

Kropat, C., Mueller, D., Boettler, U., Zimmermann, K., Heiss, E. H., Dirsch, V. M., ... Marko, D. (2013). Modulation of Nrf2-dependent gene transcription by bilberry anthocyanins in vivo. *Molecular Nutrition & Food Research, 57*(3), 545–550.

Madene, A., Jacquot, M., Scher, J., & Desobry, S. (2006). Flavour encapsulation and controlled release – A review. *International Journal of Food Science & Technology, 41*(1), 1–21.

Matuschek, M. C., Hendriks, W. H., McGhie, T. K., & Reynolds, G. W. (2006). The jejunum is the main site of absorption for anthocyanins in mice. *Journal of Nutritional Biochemistry, 17*(1), 31–36.

McGhie, T. K., & Walton, M. C. (2007). The bioavailability and absorption of anthocyanins: Towards a better understanding. *Molecular Nutrition and Food Research, 51*(6), 702–713.

Mueller, D., Jung, K., Winter, M., Rogoll, D., Melcher, R., & Richling, E. (2017). Human intervention study to investigate the intestinal accessibility and bioavailability of anthocyanins from bilberries. *Food Chemistry, 231*, 275–286.

Munin, A., & Edwards-Lévy, F. (2011). Encapsulation of natural polyphenolic compounds: A review. *Pharmaceutics, 3*(4), 793–829.

Oehme, A., Valotis, A., Krammer, G., Zimmermann, I., & Schreier, P. (2011). Preparation and characterization of shellac-coated anthocyanin pectin beads as dietary colonic delivery system. *Molecular Nutrition and Food Research, 55*, 1–11.

Oidtmann, J., Schantz, M., Mader, K., Baum, M., Berg, S., Betz, M., ... Richling, E. (2012). Preparation and comparative release characteristics of three anthocyanin encapsulation systems. *Journal of Agricultural and Food Chemistry, 60*(3), 844–851.

Pina, F., Oliveira, J., & de Freitas, V. (2015). Anthocyanins and derivatives are more than flavylium cations. *Tetrahedron, 71*(20), 3107–3114.

Schantz, M., Baum, M., & Richling, E. (2011). Antioxidative efficiency of an anthocyanin rich bilberry extract in vitro. *Journal of Berry Research, 383* 92–92.

Schantz, M., Berg, S., Betz, M., Kulozik, U., Leick, S., Rehage, H., …, Richling, E. (2014). Triggered gastrointestinal release of anthocyanins from bilberries (*Vaccinium myrtillus* L.). *Acta Horticulturae* (*ISSN 0567-7572*) peer reviewed series, Proceedings *International Society for Horticultural Science*, pp. 381–385.

Stanstrup, J., Schou, S. S., Holmer-Jensen, J., Hermansen, K., & Dragsted, L. O. (2014). Whey protein delays gastric emptying and suppresses plasma fatty acids and their metabolites compared to casein, gluten, and fish protein. *Journal of Proteome Research, 13*(5), 2396–2408.

Talavéra, S., Felgines, C., Texier, O., Besson, C., Manach, C., Lamaison, J.-L., & Rémésy, C. (2004). Anthocyanins are efficiently absorbed from the small intestine in rats. *Journal of Nutrition, 134*(9), 2275–2279.

Triebel, S., Trieu, H. L., & Richling, E. (2012). Modulation of inflammatory gene expression by a bilberry (*Vaccinium myrtillus* L.) extract and single anthocyanins considering their limited stability under cell culture conditions. *Journal of Agricultural and Food Chemistry, 60*(36), 8902–8910.

Viljanen, K., Kylli, P., Hubbermann, E.-M., Schwarz, K., & Heinonen, M. (2005). Anthocyanin antioxidant activity and partition behavior in whey protein emulsion. *Journal of Agricultural and Food Chemistry, 53*(6), 2022–2027.

Wallace, T. C., Slavin, M., & Frankenfeld, C. L. (2016). Systematic review of anthocyanins and markers of cardiovascular disease. *Nutrients, 8*(1), 32.