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Chapter 12

COMPARATIVE HUMAN *IN VITRO* AND *IN VIVO* BIOAVAILABILITY INVESTIGATION OF BILBERRY ANTHOCYANINS IN DIFFERENT COMPLEX LIGANDS WITH DIFFERENT COPIGMENTATION STATUS

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ABSTRACT

The colorful anthocyanins are well recognized members of the bioflavonoid phytochemicals. Anthocyanins have gained much attention as the food ingredients with health-promoting functions for recent years. Bilberries have been particularly known as one of the richest sources of anthocyanins.

The physiological effects of anthocyanins in humans are dependent on the absorption after ingestion. Clinical studies have demonstrated that the bioavailability of anthocyanins is very low and highly variable because of their instability in physiological absorption conditions. The targets for the development of new anthocyanin products are improved absorption and reduced absorption variability. One way to achieve these targets is an increase of the stability of anthocyanins under physiological conditions.

Amongst others, copigmentation of anthocyanins is a natural occurring mechanism to stabilize anthocyanins. For this reason, anthocyanin extracted from bilberry fruits have been investigated for their copigmentation effects in solution.

Bilberry Anthocyanin Extracts (BAEs) exerting strong copigmentation effects were tested by both, an *in vitro* CaCo-2 cell culture system and an *in vivo* human clinical trial. It could be shown convincingly that copigmentation effects of BAEs were correlated with an increased absorption as well as with reduced absorption variability.

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Keyword: BAE 2 (MyrtArgos), *vaccinium myrtillus* anothocyanins (VMA), copigmentation, bioavailability, CaCo-2 cell, randomized human clinical trial (RTC)

ABBREVIATIONS

Dp-Gal : delphinidin 3-*O*-galactoside; Dp-Glu:delphinidin 3-*O*-glucoside; Dp-Ara:delphinidin 3-*O*-arabinoside;

Cy-Gal : cyanidin 3-*O*-galactoside;Cy-Glu:cyanidin 3-*O*-glucoside; Cy-Ara:cyanidin 3-*O*-arabinoside;

Pt-Gal : petunidin 3-*O*-galactoside; Pt-Glu: petunidin 3-*O*-glucoside; Pt-Ara: petunidin 3-*O*-arabinoside;

Pe-Gal: peonidin 3-*O*-galactoside; Pe-Glu: peonidin 3-*O*-glucoside; Pe-Ara: peonidin 3-*O*-arabinoside;

Mv-Gal: malvidin 3-*O*-galactoside; Mv-Glu: malvidin 3-*O*-glucoside; Mv-Ara: malvidin 3-*O*-arabinoside.

1. INTRODUCTION

Anthocyanins belong to the flavonoid group of polyphenolic phytochemicals, generally occurring in fruits, vegetables, nuts, plant oils, cocoa and cereals. Flavonoids are comprised of a huge group (> 10.000) of diverse aromatic plant compounds with multiple substitution patterns. Among the flavonoid family, anthocyanidins are of special interest because their intake from the daily human diet is estimated to be more than 100 mg/day [1-3]. However, the bioavailability of anthocyanins in humans has been regarded very low and in studies investigating the urinary excretion the bioavailability was actually ranging only from 0.018% to 0.37% of the ingested does [4].

2. GENERAL

Based on increasing consumer awareness for natural instead of synthetic food ingredients, anthocyanins have been introduced into the food industries as coloring food ingredients and natural food colorants. More recently anthocyanins were recognized as functional food ingredients due to a number of physiological effects attributed to a daily ingestion of anthocyanins [5-8].

The increasing awareness of anthocyanins has promoted research into the stability and coloring properties, the structural elucidation and the distribution of anthocyanins in functional foods. Anthocyanin extracts prepared from edible berry fruits have gained much attention in many functional foods applications [9-13]. Among berry fruits, bilberries, *Vaccinium Myrtillus* L., (Photo 1) have been used with special interest for health-promoting foods because of their high natural contents of different types of anthocyanins [14-16]. *Vaccinium myrtillus* anthocyanins (VMA) consist of at least 15 anthocyanins which are

composed of 5 anthocyanidin skeletons, each occurring as 3-O-glycoside with glucose (Glu), galactose (Gal) or arabinose (Ara) (Figure 1) [17, 18].

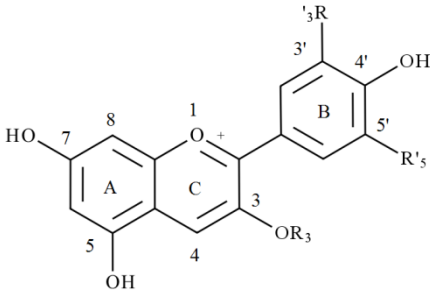
2.1. *In Vivo* Studies with Berry Anthocyanins

Pharmacokinetic studies in humans and animals demonstrated that edible berry anthocyanins were absorbed intact into the blood. However, the bioavailability is poor most likely because of the high instability of anthocyanins under physiological absorption conditions. Anthocyanins are quickly degradaded before absorption. Therefore, the low bioavailability of anthocyanins can be improved simply by improvement of absorption [19-24].

Poor bioavailability of nutrients is of no major concern unless the absorption is highly variable. Several published studies strongly indicated that the absorption of anthocyanins is highly variable. High variability of poorly absorbed compounds causes variable physiological effects and finally makes prediction for a dose-depending cause-and-effect relationship difficult, if not impossible.

2.2. Stability of Anthocyanins Depending on pH

Development of novel functional food products rich in anthocyanins must overcome the instability of anthocyanins under different physiological conditions. The stability of anthocyanins in solution and the color appearance are strongly correlated [25-31]. Depending on the pH value, the color of anthocyanins changes substantially (Figure 2). The changes observed depend on a structural change of the anthocyanidin skeleton (Figure 3). The change substantially influences the maximum absorption wavelength (λ_{max}) and the extinction value (E) at λ_{max} (Figure 4). In the acidic region, the color is stable over time indicating stability of the corresponding anthocyanidin structure. However, at physiologically relevant weakly acidic and neutral pH, the color of anthocyanins is unstable over time indicating degradation the corresponding anthocyanin structures. Determination of color stability over time using visual spectrum or color measurement (CIE-Lab) could be used to estimate the stability of anthocyanins [32-35]. To obtain quantitative information on the degradation of anthocyanins, high performance liquid chromatography (HPLC) analyses are used [36, 37].



Compound	R'3	R'5
cyanidin (Cy)	OH	H
delphinidin (Dp)	OH	OH
petunidin (Pt)	OH	OCH_3
peonidin (Pe)	OCH_3	H
malvidin (Pt)	OCH_3	OCH_3

Figure 1. Structures of 5 anthocyanidins occurring in bilberry.

Photo 1. Bilberry fruits in harvest time. Photographed by Hannu Huttsu, Aug 2006.

In their natural environment, anthocyanins appear to be stable under conditions resembling the physiological absorption conditions. Several, naturally occurring mechanisms for anthocyanin stabilization in plants have been identified [38]. In addition to the stabilization of anthocyanin structures, those mechanisms cause usually significant changes in the color appearances which explains the enormous color variation of anthocyanins in nature. Color changes could be explained by both, a bathochromic (λ_{max} drifts to higher wavelengths) and a hyperchromic (Є at λ_{max} increases) shift.

Figure 2. pH-dependent shift of the color in aqueous solutions of VMA extracts (BAEs) (McIlvaine buffer system, numbers correspond to pHs).

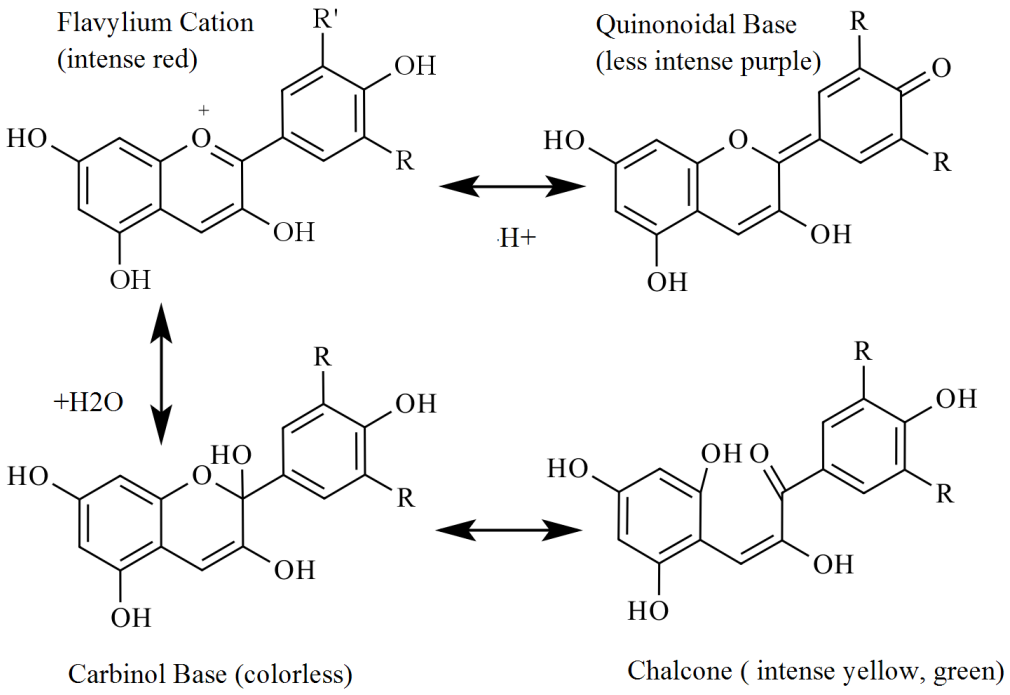


Figure 3. pH-dependent change of the anthocyanidin skeleton structure.

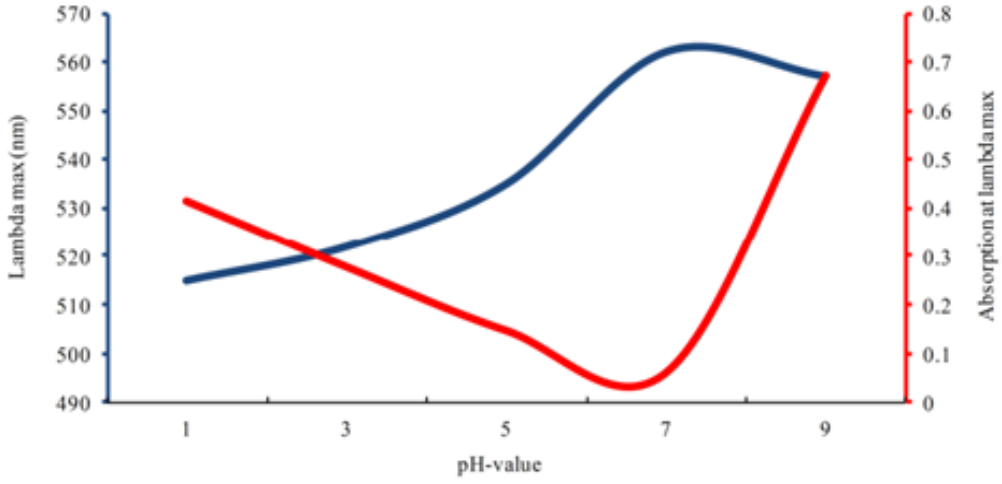


Figure 4. pH-dependent shift of λ_{max} [blue line] and Є at λ_{max} [red line] (10^{-3} M aqueous solution Cy-Glu in McIlvaine buffer system).

2.3. Formation of Anthocyanin-Complex with Metal Ions

Anthocyanins can form stable complexes in presence of metal ions such as Al^{3+} (to a lesser extent with Mg^{2+}, Fe^{2+} and other metal ions) as shown in Figure 5 [39].

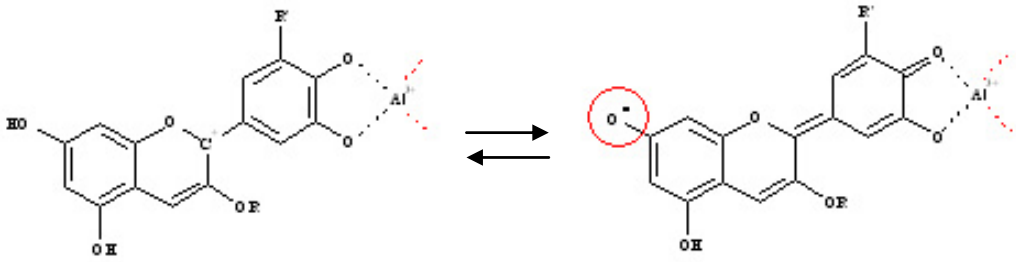


Figure 5. Complex of anthocyanin with Al^{3+}. Left: pH around 3-4; Right: pH around 4-6 (red parts indicate anchor point for other anthocyanin molecules).

Anthocyanins with acyl groups bound to the sugars may form complexes with other flavonoids around a central metal ion. Such complexes are known as metalloanthocyanins possessing interesting properties in terms of color, stability and solubility [40]. Examples are the metalloanthocyanins occuring in *Centaurea cyanus* L. (protocyanin) and of *Commelina communis* L. (commelinin) [41, 42].

2.4 Anthocyanin – Copigmentation

Copigmentation comprises of several mechanisms for anthocyanin stabilization (Figure 6) and is an important phenomenon strongly contributing to the stability and color variation of anthocyanins. Also, copigmentation effects can be observed as batho- and hyperchromic shifts in solutions. The magnitude of both batho- and hyperchromic shifts depend on the copigmentation mechanism, the content of the copigmentation formation, the ratio of anthocyanins to copigments, and the pH of the solution. Although there is no strict correlation, larger batho- and hyperchromic shifts indicate higher copigmentation strength. Copigmentation effects are also described for the anthocyanins occurring in bilberries [43-47].

Current production techniques of commercial anthocyanin extracts used in functional food ingredients are focused on optimum anthocyanin extraction yield and neglect to investigate whether copigmentation effects are retained in the final products. Innovative products with a strong copigmentation effect should lead to higher anthocyanin stability under physiological conditions and provide a possibility to increase the absorption of anthocyanins.

For this reason, it was attempted to develop novel anthocyanin extracts from bilberries with a special focus on copigmentation effects observed under physiological conditions. These candidate extracts were tested using both *in vitro* and *in vivo* to confirm whether copigmentation could increase the absorption or reduce variability of the absorption of anthocyanins.

This chapter presents the results of both *in vitro* and *in vivo* experiments to investigate the absorption of VMA from extracts with different copigmentation strengths.

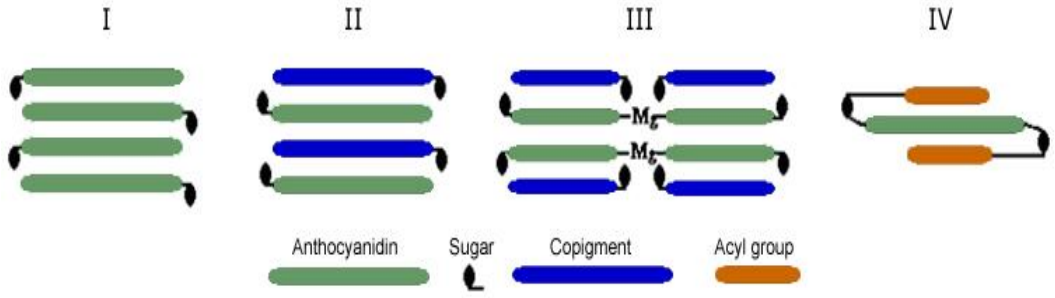


Figure 6. Overview of copigmentation mechanisms.
I : Intermolecular self-association of anthocyanins.
II : Intermolecular association of anthocyanins and copigments (flavone or flavonol).
III: Intermolecular association of anthocyanins and copigments augmented by metal ions.
IV: Intramolecular association of acylated anthocyanins (acyl group is the copigment).

3. MATERIALS

Solvents (HPLC-grade), standard chemicals including *p*-coumaric acid (p.a.-grade or better) and stationary phase material (Sephadex LH20, Amberlite XAD-7 and poly(vinylpolypyrrolidone 110 µm mesh) were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA) or VWR International, LLC (Radnor, USA). Anthocyanin reference compounds were obtained from Extrasynthese (Lyon, France). Bilberries (crop 2006) were harvested from Savukoski area Finland (Photo 1). BAE 1 (Standard Bilberry extract) and BAE 2 (MyrtArgos) were kindly supplied by Omnica Co., Ltd. in Tokyo, Japan.

4. METHODS

4.1. Copigmentation Test

Single anthocyanins, VMA extracts and copigments were dissolved in McIlvaine citrate-phosphate buffer (pH 4.5 or 7.5). Copigmentation of single anthocyanins with copigments was determined at a molar ratio of 1:20. BAEs were dissolved directly in buffer and cleared by filtration. For comparative purposes, tentative copigments present in BAEs were removed by purification over poly(vinylpolypyrrolidone) at the conditions described below.

Visible spectrum analyses and color measurements were performed with a Hunter Lab ColorQuest XE. Incubations were performed at 37 °C for up to 360 min with samples taken every 10-15 minutes. For HPLC conditions see 4.4. All tested samples were adjusted for the same content of anthocyanins.

4.2. Preparation of Bilberry Extracts

An almost pure anthocyanin concentrate lacking copigments (PBAC) was prepared as a control following previously published techniques [48-51]. Briefly, 3 kg bilberries collected in Finland and provided by Omnica Co., Ltd., were crushed and extracted 3 times with ethanol/formic acid=9/1 (v/v). The combined extracts (9 L) were evaporated by vacuum distillation. The dry residue was dissolved in 100 mL deionized water/formic acid=99.95/0.05 (v/v). Aliquots of 5 mL were loaded on a semi-preparative column (Amberlite XAD-7, 40 x 3.5 mL bed volume) equilibrated with water/formic acid=99.95/0.05 (v/v). The column was washed with deionized water/formic acid=99.95/0.05 (v/v), the anthocyanin fraction was then eluted with ethanol/formic acid=9/1 (v/v). The combined anthocyanin fractions were evaporated by vacuum distillation. After dissolution in 50 mL water/formic acid=99.95:0.05 (v/v) aliquots of 5 mL were loaded onto on a semi-preparative column (Sephadex LH-20, 40 x 3.5 mL bed volume) equilibrated with deionized water/formic acid=99.95/0.05 (v/v). After washing of the column with deionized water/formic acid=99.95/0.05 (v/v), elution of anthocyanins was accomplished by a stepwise gradient from 5 to 70 % ethanol in deionized water/formic acid=99.95/0.05 (v/v). The combined anthocyanin fractions were evaporated by vacuum distillation. After dissolution in 50 mL deionized water/trifluoroacetic acid=99/1 (v/v) aliquots of 5 mL were loaded on a semi-preparative column (poly(vinylpolypyrrolidone) 110 µm mesh, 40 x 3.5 mL bed volume) equilibrated with deionized water/trifluoroacetic acid=99/1 (v/v). After washing of the column with deionized water/trifluoroacetic acid=99/1 (v/v), elution of anthocyanins was accomplished with ethanol/trifluoroacetic acid=95/5 (v/v). The combined fractions were evaporated by vacuum distillation and dissolved in 10 mL trifluoroacetic acid followed by drop-wise addition to 1 Liter ice-cold water free diethylether for crystallization of anthocyanins. The overall extraction yield was 7.2 g PBAC.

In cooperation with Omnica Co., Ltd. (Tokyo, Japan) BAEs were produced and characterized for copigmentation effects. For series I of BAEs, bilberries were grinded and extracted in an ethanol/water/phosphoric acid solution. After concentration by vacuum distillation, the anthocyanins were further concentrated by preparative resin absorption. Afterwards they were fractionated and eluted in ethanol/water mixtures. The anthocyanin containing fractions were concentrated by vacuum distillation and finally spray dried. Variations included the ethanol/water ratio in the extraction solvent and the addition of antioxidant (*L-cysteine*) to prevent oxidation processes during manufacturing.

For series II of BAEs, bilberries were squeezed to obtain juice. The press residue from juice preparation was grinded and extracted in an ethanol/water/phosphoric acid solution. The juice was further processed as described above. Further on, the juice and the extract were combined in varying ratio and processed as described above. Again, variations included the ethanol/water ratio in the extraction solvent and addition of antioxidants.

For comparative purposes, potential copigments were removed from the BAEs produced by purification over poly(vinylpolypyrrolidone) chromatography as described above.

4.3. CaCo-2 Cell Test System

The CaCo-2 cell culture test system was chosen to estimate the rate and extent of absorption *in vitro* [52-54]. CaCo-2 cells were cultured in Dulbeccos's Modified Eagle

Medium containing 20% fetal bovine serum, 1.2 % non-essential amino acids, 0.83 mM L-glutamine, 1.2 % penicillin-streptomycin and 0.1 % mercaptoethanol in an atmosphere of 5% CO_2 and 95% air at 37°C. Cells were grown in 75 cm^2 culture-flasks (T75) and sub-cultured after one week. Prior to experiments, cells were seeded in wells at a density of $3x10^5$ cells/well (standard 6-well cell culture plate) and grown in an atmosphere of 5% CO_2 and 95% air at 37°C until confluence was reached. For the experiments, BAEs were dissolved in incubation medium (adjusted to 1 mg anthocyanins/ml medium) and transferred into the wells. The cells were incubated for 30, 60 and 90 minutes at 37 °C. At each time point, the cells from 5 wells were removed for further processing (removal of medium, washing of cells with buffer, harvest of cells, and disruption of cells in acidified methanol, centrifugation and storage of the clear supernatant until analysis by HPLC/UV. Each experiment was performed in three replicates providing 15 samples/experiment/time-point. The individual anthocyanin concentrations observed in each well were summed up. Results given are mean values of the summarized anthocyanin concentration observed per well and time point (Figure 13, Table 3).

4.4. HPLC Analysis

Separation of anthocyanins was achieved on a Knauer, Hypersil ODS (250×4.6 mm) column with a flow rate of 1.5 mL/min using a linear gradient from 100 % deionized water/formic acid =90/10 (v/v) to 30 % methanol/acetonitrile/formic acid/water=20/20/10/50 (v/v) in 40 minutes. The column was kept at 45 °C. Detection was performed with UV/VIS (520 nm) coupled to positive mode ESI/MS [55, 56]. Quantification was performed against external standardization (Cy-Glu, Dp-Glu, Mv-glu, Pe-Glu) using single ion monitoring selective for anthocyanins and anthocyanidins occurring in bilberries. The qualitative elucidation of anthocyanin structures was performed using total ion current settings.

During the copigmentation and the *in vitro* experiments the samples collected were submitted to HPLC without further treatment. Samples obtained during the clinical study were subjected to solid phase extraction before analysis [57, 58].

4.5. Pharmacokinetic Study in Healthy Human

For pharmacokinetic investigations in humans, a randomized crossover design with multiple treatments in 12 subjects of either gender was chosen (Figure 12). The study design followed GCP guidelines, the Declaration of Helsinki, and fulfilled the guideline for bioequivalence testing [59-61]. The design of the study was approved by the Responsible Ethics Committee.

Primary aim of the study was to investigate the pharmacokinetic profile of VMA after single and multiple intakes of two selected BAEs (BAE 1 or BAE 2) in order to compare the rate and extent of absorption of the anthocyanins. During each period, the participants received once daily treatments for 7 consecutive days with 185 mg anthocyanins from either BAE 1 or BAE 2. Between the 2 periods a wash-out period of 7 days was introduced. During each period blood samples were drawn for pharmacokinetic assessments on the corresponding treatment days 1 (single dose) and 7 (repeated dose) immediately before and 0.5, 1.5, 2.5 and 4 hours after administration (Figure 13). Blood samples drawn were immediately cooled to

4 °C and centrifuged at 4 °C to obtain plasma. Plasma samples were acidified with formic acid (1800 µL blood + 200 µL formic acid), vortexed for 5 minutes and centrifuged again at 13.000 rpm for 5 minutes. The supernatant was collected, transferred into an appropriately labeled vial and stored at -80 °C until analysis. From the plasma concentrations observed, the pharmacokinetic parameters C_{max} (maximum concentration) (Table 4, Figure 13) and $AUC_{0\text{-}inf}$ (area under the concentration time curve extrapolated from 0 to infinity) (Tables 6, 7) were calculated using non-compartmental methods. Bioequivalence assessment was performed according to published methods [62, 63]. Bioequivalence assessment consisted of the calculation of the intra-individual ratios of C_{max} and $AUC_{0\text{-}inf}$ values BAE 2/BAE 1, the calculation of the corresponding mean ratio (comparative bioavailability) and the confidence interval (C.I.). If the mean ratio, together with the confidence interval, lies within the margins of 80-125 % the two products tested are bioequivalent; otherwise the products are not bioequivalent (Tables 4,5,6,7).

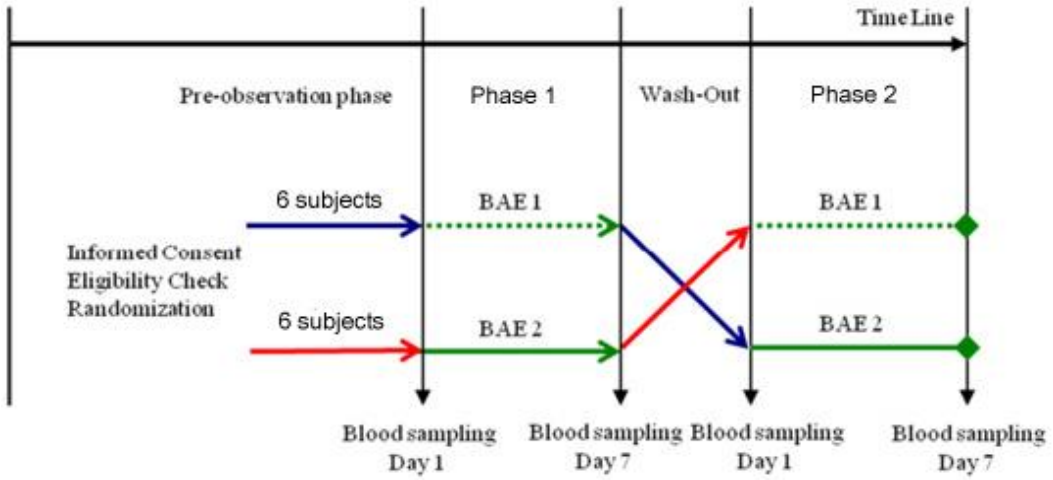


Figure 12. Flowchart of clinical study interventions.
Total (n=12) average age 33 y; male (n=3) average age 34 y; female (n=9) average age 38 y.

5. RESULTS

5.1. Effects of Copigmentation

Exemplifying for the intermolecular association of anthocyanins and copigments, the batho- and hyperchromic shift obtained for copigmentation of Cy-Glu with *p*-coumaric acid at pH 4.5 are shown in Figure 7. In Figure 8, the stabilization observed for copigmentation of Cy-Glu with coumaric acid at pH=7.5 and 37 °C is presented. The results obtained indicate that copigmented anthocyanins are substantially more stable under the physiologically relevant conditions.

5.2. Development of Copigmented Bilberry Anthocyanin Extracts (BAEs)

The primary target was to elucidate the influence of different manufacturing processes on copigmentation effects of BAEs.

A reference PBAC completely devoid of copigments was prepared. The anthocyanin content in PBAC was determined to be >85 %. All 15 anthocyanins occurring in bilberry were retrieved by HPLC in their approximate relative distribution. This indicated that the applied purification technique yielded an apparently pure anthocyanin fraction with the naturally occurring anthocyanin pattern. The visible spectrum and color characteristics of PBAC obtained were interpreted as typical for VMA without copigmentation effects.

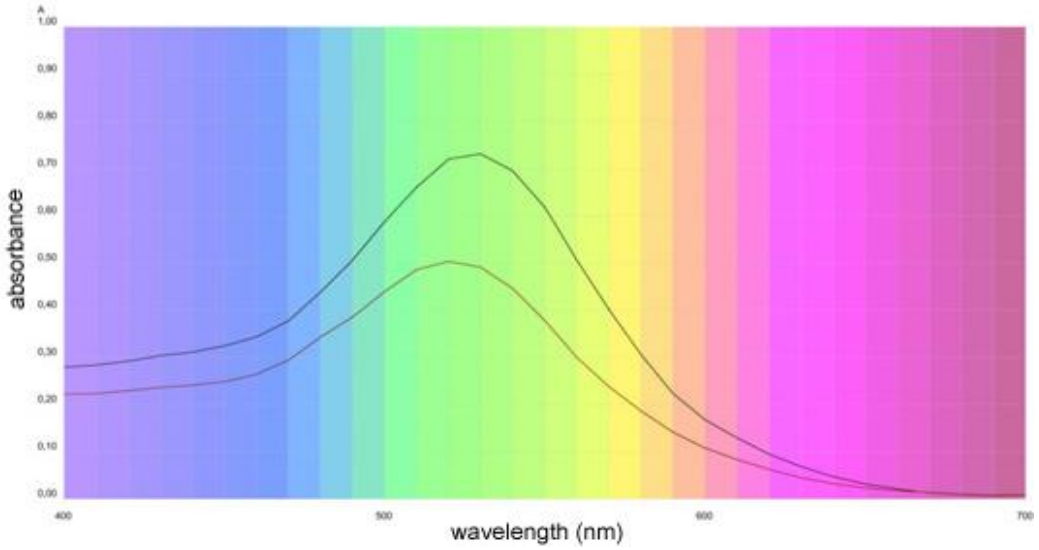


Figure 7. Visible absorption spectrum of pure (lower trace: red) and Cy-Glu copigmented with coumaric acid (upper trace: black) at pH 4.5 (bathochromic shift: +15 nm; hyperchromic shift: +50 %).

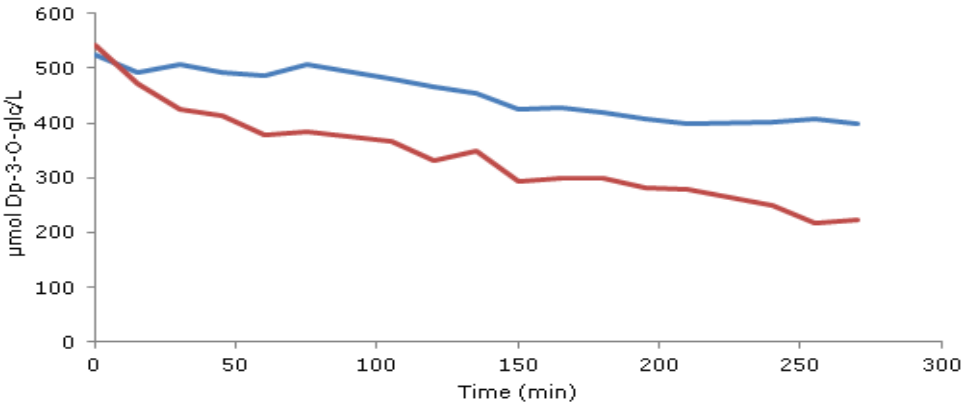


Figure 8. Degradation of Cy-Glu copigmented *p*-coumaric acid at pH 7.5 and 37 °C.

The BAEs prepared were compared with PBAC for copigmentation effects. Based on the results obtained, two extracts denominated as BAE 1 (low copigmentation effect) and BAE 2 (high copigmentation effect, MyrtArgos) were selected for further experimental works. The visible spectrum and color characteristics as well as the stability (aqueous buffer pH=7.5, 37 °C, up to 6 hours) of BAE 1 and BAE 2 are shown in Tables 1, 2, and Figures 9, 10. BAE 1 was obtained from series I with 80 % ethanol in the extraction solvent and antioxidants included. The anthocyanin content was 34.5 %. BAE 2 was obtained from series II with supplementary antioxidants and combining the whole press residue extract (extracted with 80 % ethanol) with the juice. The anthocyanin content was 38.3 %.

The results strongly indicate that BAE 1 and BAE 2 show spectroscopic properties of copigmentation. That copigmentation reactions are the reason for the spectroscopic changes could be derived from the comparison of BAEs prior to and after removal of copigments. In a general agreement, the magnitude of the copigmentation effect was taken as an estimate of the copigmentation strength. The results of color measurement indicated that the color stability correlated with the copigmentation strength. The results of the stability revealed that the stability of anthocyanins under physiological conditions correlates with the copigmentation strength observed. The removal of copigments diminished the stabilization effect.

Table 1. Summary of visible spectra characteristics of three VMA extracts (aqueous buffer pH 4.5) PBAC, BAE 1 and BAE 2

Sample characteristic	Bathochromic shift (difference in λ_{max}, nm)	Hyperchromic shift (difference in E_{max}, %)
PBAC	Reference=520 nm	Reference=100 %
BAE 1	+10	123
BAE 1 (after removal of copigments)	+2	104
BAE 2	+25	167
BAE 2 (after removal of copigments)	+5	107

Table 2. Color characteristics (CIE-lab) of three VMA extracts in aqueous solution (pH=4.5) obtained immediately after dissolution, and after 24 hours at room temperature

Sample characteristic	L*	a*	b*	dL*	da*	db*	dE*
PBAC	83.37	11.87	-2.65	-12.42	11.92	-2.53	12.03
PBAC after 24 hours	89.67	5.28	0.23	-6.12	5.33	0.35	5.16
BAE 1	77.90	12.40	-0.02	-17.89	12.45	0.10	12.27
BAE 1 after 24 hours	81.79	10.22	-0.07	-14.00	10.27	0.05	10.09
BAE 2	83.47	10.87	1.30	-12.33	10.93	1.42	10.82
BAE 2 after 24 hours	82.56	12.55	-0.55	-13.24	12.60	-0.43	12.43

L*:lightness; a*:color strength between purple-red and blue-green; b*:color strength between yellow and blue; dL*, da*, db*: L*, a*, b* corrected for standard; dE*:total color difference.

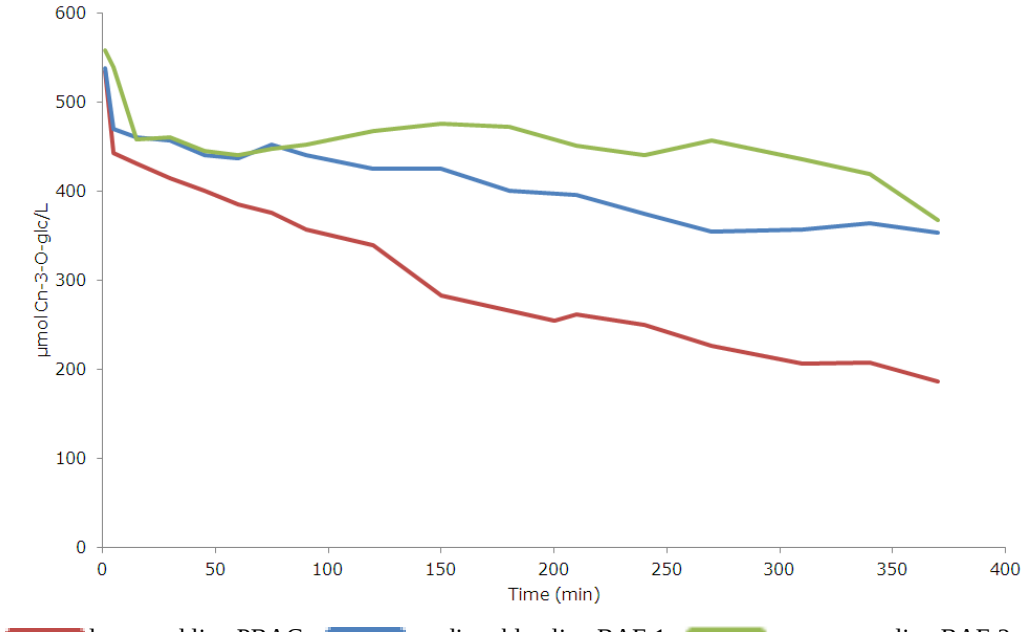


Figure 9. Comparative stability of 3 VMA extracts (aqueous buffer pH=7.5, 37 °C, HPLC/UV with external standardization).

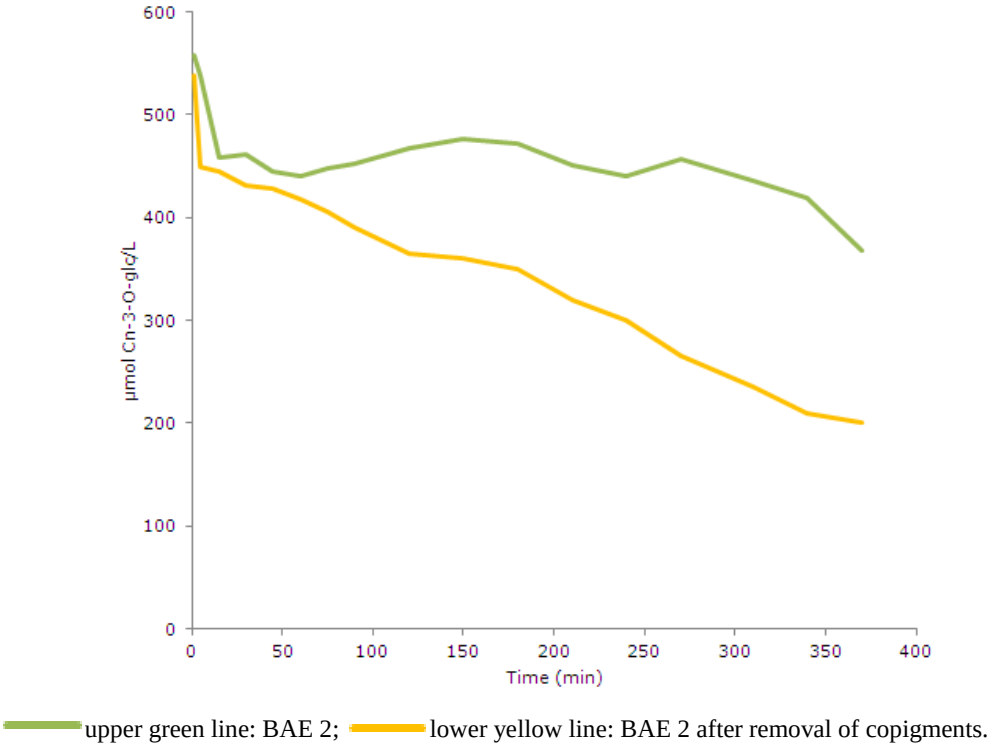


Figure 10. Stability of VMA extracts in relation to copigmentation (aqueous buffer pH=7.5, 37 °C, HPLC/UV).

5.3. *In Vitro* Investigations with CaCo-2 Cell

The absorption of anthocyanins from PBAC, BAE 1 and BAE 2 were investigated *in vitro* using the CaCo-2 cells culture system. Figure 11 shows the amount of anthocyanins recovered in the cells at the indicated time points, whereas Table 3 contains the amount of 15 anthocyanins determined after 60 minutes of incubation. These results strongly indicate that the absorption of anthocyanins into CaCo-2 Cell correlates with the copigmentation strength. An estimate of the increase could be derived from the concentrations of 15 anthocyanins observed after 60 minutes of incubation. Strong copigmentation doubled the amount of anthocyanins absorbed when compared to PBAC lacking copigmentation. It should be noted that the amount of all 15 anthocyanins was increased suggesting that copigmentation could protect all anthocyanins present in bilberries.

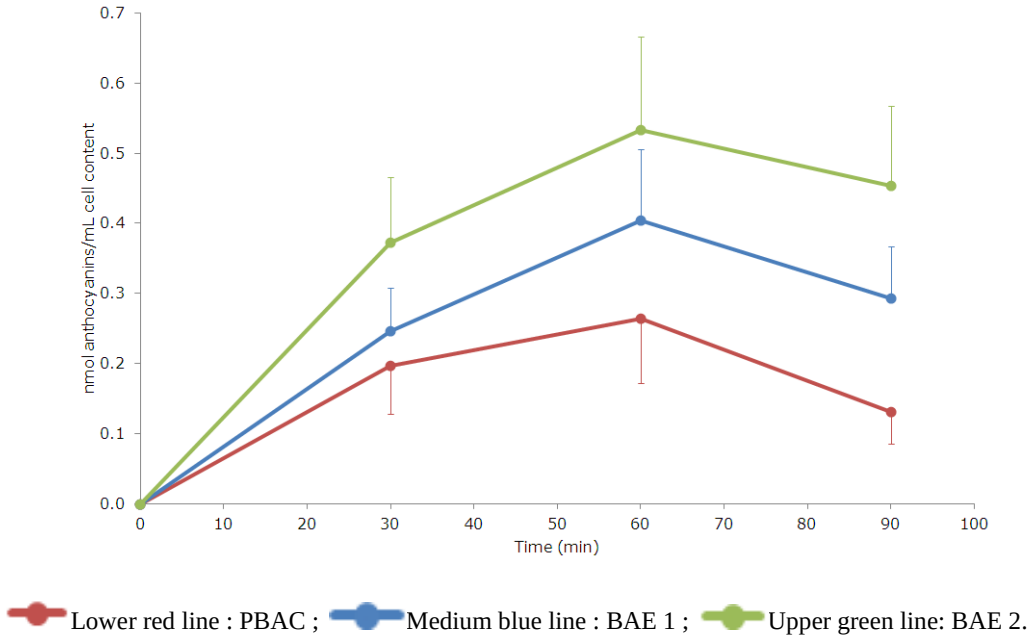


Figure 11. Mean values of anthocyanins ± s.d. (n=15) absorbed into CaCo-2 cells from 3 bilberry extracts.

5.4. Pharmacokinetic Study in Humans

The absorption of anthocyanins from BAE 1 and BAE 2 was investigated *in vivo* in a randomized, cross-over pharmacokinetic study in 12 subjects with single and repeated oral administration.

Figure 13 shows the mean values of anthocyanidin-related structures observed in plasma after single and repeated administration of either BAE 1 or BAE 2. The values shown refer to the sum of all individual structures bearing the corresponding anthocyanidin. Table 4 gives the maximum plasma concentrations observed for 15 anthocyanins after treatment with BAE 1 or BAE 2. Table 5 shows the scattering of C_{max} values observed. The results of the bioequivalence assessment are shown in Tables 6 and 7.

Table 3. Mean values of 15 anthocyanins (nmol/mL cell content) absorbed into CaCo-2 cell from three bilberry extracts after 60 minutes incubation (n=15) at 37C

Compound	PBAC	BAE 1	BAE 2
Dp-Gal	0.0303	0.0450	0.0601
Dp-Glc	0.0306	0.0443	0.0629
Dp-Ara	0.0268	0.0367	0.0460
Cy-Gal	0.0365	0.0557	0.0669
Cy-Glu	0.0287	0.0454	0.0620
Cy-Ara	0.0110	0.0132	0.0211
Pt-Gal	0.0284	0.0319	0.0511
Pt-Glu	0.0174	0.0270	0.0318
Pt-Ara	n.a.	0.0124	0.0169
Pe-Gal	n.a.	0.0114	0.0130
Pe-Glu	0.0166	0.0240	0.0258
Pe-Ara	n.a.	n.a	n.a
Mv-Gal	0.0170	0.0251	0.0240
Mv-Glc	0.0201	0.0329	0.0358
Mv-Ara	n.a.	n.a	0.0194
Sum	0.264	0.405	0.537
(%)	(100 %)	(153 %)†	(203 %)†

n.a.:not applicable (below limit of detection); †:relative to sum of PBAC.

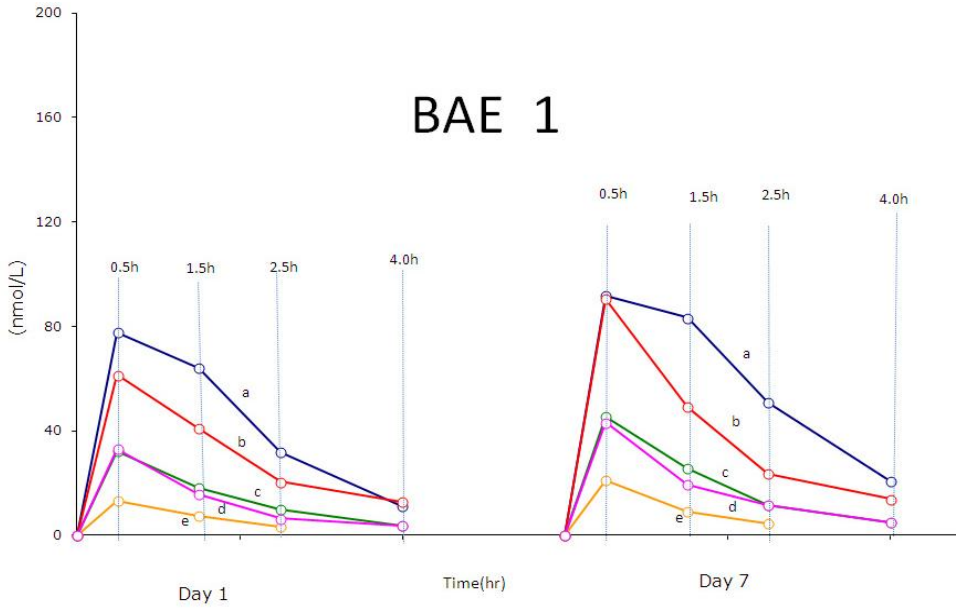


Figure 13. (A) Mean concentrations of anthocyanidin-relates compounds (nmol/L) after single (day 1) and repeated (day 7) oral administrations of BAE 1 (n=12). Each plasma level/time curve covers 0-4 hours (at 0.5h/1.5h/2.5h/4.0h, respectively) after treatment; blue line a: cyanidin; red line b: delphinidin; green line c: petunidin; orange line d: peonidin; magenta line e: malvidin.

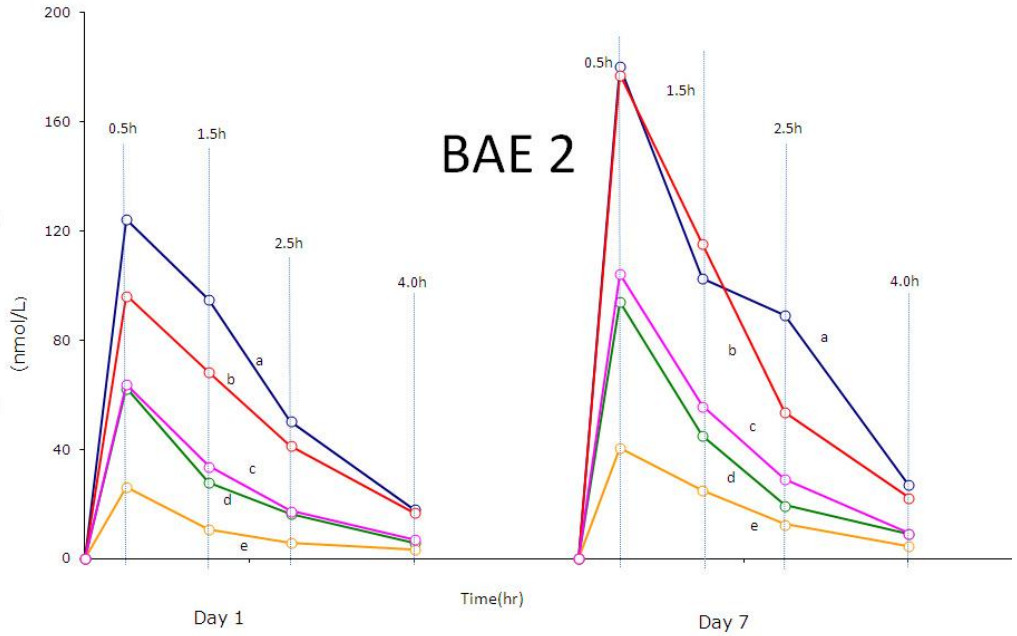


Figure 13. (B) Mean concentrations of anthocyanidin-relates compounds (nmol/L) after single (day 1) and repeated (day 7) oral administrations of BAE 2 (n=12). Each plasma level/time curve covers 0-4 hours (at 0.5h/1.5h/2.5h/4.0h, respectively) after treatment; blue line a: cyanidin; red line b: delphinidin; green line c: petunidin; orange line d: peonidin; magenta line e: malvidin.

Table 4. Mean maximum concentrations (nmol/L) of VMA after single and repeated administration of BAE 1 and BAE 2 (n=12, respectively)

Compound	C_{max}, single administration		C_{max}, repeated administration	
	BAE 1	BAE 2	BAE 1	BAE 2
Cy-Glu	21.4	42.5	31.0	64.8
Cy-Glu	11.6	17.1	16.5	33.9
Cy-Ara	8.35	15.5	12.9	26.3
Dp-Glu	11.6	23.4	19.6	37.0
Dp-Gal	9.03	16.3	13.1	30.3
Dp-Ara	9.20	16.8	14.3	25.7
Pt-Glc	11.9	20.0	16.7	35.3
Pt-Gal	4.18	7.10	5.64	12.7
Pt-Ara	2.96	7.74	4.33	12.8
Pe-Glc	6.26	13.8	10.4	23.8
Pe-Gal	1.99	4.75	3.02	8.86
Pe-Ara	2.54	4.39	2.77	8.31
Mv-Glc	12.2	24.9	17.0	44.0
Mv-Gal	4.06	8.32	6.29	12.8
Mv-Ara	3.46	7.56	5.18	12.5

Table 5. Scattering of C_{max} values (Sum of anthocyanidin-related structures) after single (day 1) and repeated (day 7) treatments with BAE 1 and BAE 2, coefficient of variation (%) (n=12, respectively)

Compound	Single treatment		Repeated treatment	
	BAE 1	BAE 2	BAE 1	BAE 2
Cyanidin-relates	26.6	21.2	24.0	20.5
Delphinidin-relates	29.4	23.3	27.8	24.9
Petunidin-relates	30.2	26.1	29.8	25.4
Peonidin-relates	27.8	24.6	29.5	25.0
Malvidin-relates	24.3	19.7	23.9	20.1

The results strongly indicate that the mean anthocyanin plasma levels were considerably higher after treatment with BAE 2 when compared to BAE 1. Likewise the values for C_{max} and $AUC_{0\text{-}inf}$, indicative for the rate and extent of absorption, of BAE 2 were higher when compared to that of BAE 1. The effects observed are more significant after repeated treatments when compared to single treatment. Interestingly, the individual values for C_{max} after treatment with BAE 1 indicated higher variability when compared to BAE 2 (Table 7). Whether this points to reduced variability in the absorption due to higher copigmentation strength cannot be concluded at this stage. However, the finding could indicate that copigmented anthocyanins were stabilized irrespectively of the individual physiological conditions at the absorption site whereas the stability of anthocyanins devoid of copigmentation effects was more dependent on individual conditions.

The results of the bioequivalence assessment demonstrated a significantly increased relative bioavailability (supra-bioavailability) of BAE 2 when compared to BAE 1 after single (day 1) and repeated treatment (day 7).

Table 6. Results for the bioequivalence test of pharmacokinetic parameters after single treatments with BAE 1 and BAE 2

Compound	Comparative bioavailability BAE 2/BAE 1 (%)		Confidence interval BAE 2/BAE 1 (%)	
	C_{max}	$AUC_{0\text{-}inf}$	C_{max}	$AUC_{0\text{-}inf}$
Cyanidin-relates	162.0[1)]	161.1[1)]	145.5-180.5[1)]	150.6-172.2[1)]
Delphinidin-relates	158.4[1)]	126.4[1)]	142.9-175.6[1)]	89.2-178.2[1)]
Petunidin-relates	182.0[1)]	n.a.	160.9-205.8[1)]	n.a.
Peonidin-relates	215.1[1)]	n.a.	185.0-250.2[1)]	n.a.
Malvidin-relates	197.6[1)]	n.a.	173.1-225.5[1)]	n.a.

1) statistically significant better bioavailability of BAE 2; n.a. : not applicable (AUC could not be determined).

Table 7. Results for the bioequivalence test of pharmacokinetic parameters d after repeated (day 7) treatment with BAE 1 and BAE 2

Compound	Comparative bioavailability BAE 2/BAE 1 (%)		Confidence interval BAE 2/BAE 1 (%)	
	C_{max}	$AUC_{0\text{-}inf}$	C_{max}	$AUC_{0\text{-}inf}$
Cyanidin-relates	196.3[1)]	187.1[1)]	181.3-212.7[1)]	163.4-202.2[1)]
Delphinidin-relates	188.5[1)]	206.4[1)]	163.3-217.7[1)]	165.3-257.8[1)]
Petunidin-relates	201.2[1)]	n.a.	177.6-228.0[1)]	n.a.
Peonidin-relates	202.6[1)]	n.a.	183.9-223.2[1)]	n.a.
Malvidin-relates	237.9[1)]	n.a.	208.8-271.1[1)]	n.a.

1) statistically significant better bioavailability of BAE 2; n.a.: not applicable (AUC could not be determined).

6. DISCUSSION

VMA have gained much attention as functional food ingredients. However, the bioavailability in humans is poor because VMA are instable under physiological conditions, especially under the pH conditions at the absorption site. Moreover, published studies indicate that absorption of anthocyanins is highly variable.

The current knowledge of anthocyanin copigmentation effects could provide a way to investigate whether copigmentation can overcome the instability of anthocyanins under physiological conditions leading to better absorption and reduced variability of absorption.

The investigations at hand showed that the conditions of the manufacturing process for bilberry anthocyanin extracts influence the copigmentation strength of the resulting product. As described in the experimental setups, the direct extraction of bilberries yields a low copigmentation effects only. Strong copigmentation effects were only achieved by obtaining a berry juice, extraction of the press residue and combining both, the juice and the press residue extract. Moreover, a positive effect of antioxidants on the resulting copigmentation strength was observed. The investigations showed that different manufacturing processes can yield extracts with substantially higher copigmentation strength regardless of their content of anthocyanins. To provide conclusive results, further investigations into the detailed phytochemical composition of differently prepared VMA extracts following up published procedures should be performed [64-66]. Comparison of the phytochemical composition in the extracts with special emphasis laid on copigment structures and the copigmentation strength should provide the conclusive results for the phenomena observed.

In this study, the stability of VMA from different bilberry extracts under physiological conditions correlated with the copigmentation strength. Moreover it could be demonstrated that the removal of the copigment fraction diminished these effects. It is therefore reasonable to conclude that the copigmentation phenomena observed in the natural environment can be retained in VMA extracts if a special manufacturing process is chosen.

Following the hypothesis, it was expected that the stabilization of anthocyanins under physiological conditions could provide an opportunity for improved absorption. Two BAEs,

selected on the basis of their copigmentation strengths (low for BAE 1 and high for BAE 2, respectively), were further submitted to *in vitro* and *in vivo* absorption studies.

The *in vitro* tests for the absorption of anthocyanins into CaCo-2 cells provided strong evidence that the copigmentation strength observed in the BAEs tested correlated with improved absorption of anthocyanins The detailed mechanism of absorption of anthocyanins is unknown; hence, the improved absorption observed cannot be explained conclusively yet. The increased stability of anthocyanins resulting in higher concentrations of intact anthocyanins over time might be just one explanation. It might also be speculated that copigmented anthocyanins are better absorbed than single anthocyanins or that copigmented anthocyanins follow a different absorption route than single anthocyanins. In both cases, this might be due to changes in the physiochemical properties in response to copigmentation. Moreover it cannot be completely excluded that the correlation of copigmentation strength and improved absorption pretends a true, yet unknown, mechanism for improving absorption.

The *in vivo* test investigating the pharmacokinetics in humans confirmed the *in vitro* test results observed. Based on the C_{max} and $AUC_{0\text{-}inf}$ values calculated from the anthocyanin plasma concentrations, a substantially improved absorption correlating with the copigmentation strength can be derived. Bioequivalence assessment demonstrated that VMA from an extract with strong copigmentation effects are significantly better bioavailable than from an extract with low copigmentation effects.

The results observed in our *in vitro* test system and in our *in vivo* pharmacokinetic study in humans could support the copigmentation hypothesis discussed above. Based on the C_{max} and $AUC_{0\text{-}inf}$ values calculated from the anthocyanin plasma concentrations observed in the human *in vivo* study, a substantially improved absorption correlating with the copigmentation strength could be derived. Bioequivalence assessment demonstrated that VMA from an extract with strong copigmentation effects were significantly higher bioavailable than that of an extract with low copigmentation effects.

Based on the scattering of the C_{max} values, also a trend to the reduced variability of the absorption could also be observed for the bilberry extract with a strong copigmentation effect. Although statistically not significant, it can be speculated that increased stability based on copigmentation effects at the physiological site of absorption might lead to and maintain a more consistent anthocyanin absorption. Consistency of absorption points to reduced variability in plasma concentrations and would make a dose-effect relationship more predictable.

CONCLUSION

Taken together, the extent of anthocyanins absorbed correlates *in vitro* and *in vivo* with copigmentation effects observed. Bilberry anthocyanin extracts with a strong copigmentation effect -observed as changes in the visible spectrum and increased stability at physiologically relevant conditions- are statistically better absorbed in humans. Further investigations will focus on the detailed composition of bilberry anthocyanin extracts which should provide insights into the exact mechanisms of copigmentation.

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