

## Uptake and bioavailability of anthocyanins and phenolic acids from grape/blueberry juice and smoothie *in vitro* and *in vivo*

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### Abstract

The goal of eating five servings of fruits and vegetables a day has not yet been achieved. The intake of polyphenols such as anthocyanins (ACN) could be improved by consuming smoothies and juices that are increasingly popular, especially in children; however, bioavailability data concerning food matrix effects are scarce. Thus, we conducted a randomised, cross-over, bioavailability study ( $n$  10) to determine the bioavailability of ACN and their metabolites from an ACN-rich grape/blueberry juice (841 mg ACN/litre) and smoothie (983 mg ACN/litre) *in vivo*, and the uptake of a corresponding grape/blueberry extract *in vitro*. After the intake of beverage (0.33 litres), plasma and fractionated urine samples were collected and analysed by ultra-performance liquid chromatography coupled to MS. The most abundant ACN found in plasma and urine were malvidin and peonidin as native ACN and as glucuronidated metabolites as well as 3,4-dihydroxybenzoic acid (3,4-DHB); minor ACN (delphinidin, cyanidin and petunidin) were only detected as native glycosides. Plasma pharmacokinetics and recoveries of urinary metabolites of ACN were not different for juice or smoothie intake; however, the phenolic acid 3,4-DHB was significantly better bioavailable from juice in comparison to smoothie. *In vitro* data with absorptive intestinal cells indicated that despite their weak chemical stability, ACN and 3,4-DHB could be detected at the basal side in their native forms. Whether smoothies as well as juices should be recommended to increase the intake of potentially health-promoting ACN and other polyphenols requires the consideration of other ingredients such as their relatively high sugar content.

**Key words:** Bioavailability: Grapes: Blueberries: Anthocyanins: *In vivo* studies: *In vitro* studies

Consumption of fruits and vegetables (FV) has been found to be associated with a decreased risk of diet-related diseases such as obesity, diabetes, CVD, CHD, stroke and some types of cancer<sup>(1–4)</sup>. In spite of this knowledge, guidelines and programmes to promote a healthy lifestyle, the compliance with higher FV intake is less promising<sup>(5)</sup>. In particular, FV intake in children is still far below the recommendations, e.g. less than five FV servings per d<sup>(6,7)</sup>. Therefore, the increase in FV intake in the form of juices or smoothies is very popular, especially in young age groups, and could thus be a source of health-promoting polyphenols with antioxidant and anti-inflammatory activities<sup>(8)</sup>. Polyphenols such as anthocyanins (ACN) are present in FV. High amounts of polyphenols are

found in red and purple coloured fruits and account for 50–80% of the total polyphenol content in berries. Grapes, blueberries, blackberries, cherries or cranberries can reach concentrations of up to 3000 mg/kg fresh weight<sup>(9–11)</sup>. It is estimated that the average total intake of ACN may be approximately 200 mg/d, which is four times higher than that of other polyphenols<sup>(12,13)</sup>. The main glycosylated ACN in fruits are cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin. Despite their high content, the bioavailability of ACN is considered to be relatively low compared with that of other flavonoids<sup>(14)</sup>. Studies investigating the pharmacokinetics of cyanidin or malvidin have shown that cyanidin-3-glucoside (Cya-3-glc) or malvidin-3-glucoside

**Abbreviations:** 3,4-DHB, 3,4-dihydroxybenzoic acid; ACN, anthocyanin;  $C_{\max}$ , maximal concentration; Cya-3-glc, cyanidin-3-glucoside; Del-3-glc, delphinidin-3-glucoside; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; FV, fruits and vegetables; Mal-3-glc, malvidin-3-glucoside; Mal-3-glucuro, malvidin-3-glucuronide; PA, phenolic acid; Peo-3-glc, peonidin-3-glucoside; Peo-3-glucuro, peonidin-3-glucuronide; Pet-3-glc, petunidin-3-glucoside; SPE, solid-phase extraction; TEAC, Trolox equivalent antioxidant capacity;  $t_{\max}$ , time to maximal concentration; UPLC-MS, ultra-performance liquid chromatography MS.

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(Mal-3-glc) reach a maximum plasma level at 30–60 min after ingestion with concomitant enrichment in urine after 2 h. In contrast, cyanidin or malvidin glucuronide or methylated glucuronides were detected in urinary concentrations that were four to six times higher compared with native Cya-3-glc or Mal-3-glc<sup>(15,16)</sup>. Although in most studies glucuronidation or methylation seem to be the predominant phase II metabolisms in the liver, few studies have also identified sulphation of the native forms<sup>(17,18)</sup>. However, in all these studies, the bioavailability is less than 2% with concentrations of native ACN found in the plasma ranging from 1 to 100 nmol/l<sup>(19,20)</sup>. These findings of low concentrations in plasma and urine may be a consequence of the ACN instability at physiological pH and their degradation by the intestinal microflora<sup>(21–23)</sup>. While most studies have focused on absorption, metabolism and excretion of single or mixed ACN with controversial results, little information is available on the effects of the food matrix on their bioavailability<sup>(24)</sup>. Recently, it has been shown that the availability of ACN from berry juice is lower than that from berries<sup>(25,26)</sup>. In contrast, Hagl *et al.*<sup>(27)</sup> demonstrated that polyphenols from an apple smoothie reached the colon, and their availability for the intestinal microbiota could be more effective in the prevention of chronic colon diseases than apple juice or apple cider. In addition, Nurmi *et al.*<sup>(28)</sup> investigated the occurrence of ACN and phenolic acids (PA) in urine after ingestion of fruit puree with and without oat cereals. They observed a delayed maximum urinary excretion of dietary PA after consumption of puree and cereal compared with consumption of puree alone<sup>(28)</sup>. We aimed at investigating whether the *in vivo* intake of ACN-rich beverages such as grape/blueberry juice or smoothie, which are very popular in young age groups, differed with regard to the bioavailability of phenolic compounds. Furthermore, the *in vivo* bioavailability was compared with *in vitro* studies using extracts with a comparable ACN pattern.

## Materials and methods

### Chemicals

All chemicals were of analytical grade. Formic acid and ascorbic acid were from Sigma-Aldrich. Pelargonidin-3-O-rutinoside was purchased from Extrasynthese; 2-chloro-5-nitrobenzoic acid, formic acid and water (HPLC grade) from Fluka; trifluoroacetic acid and methanol from Merck; and Mal-3-glc from Roth.

### *In vitro* and *in vivo* study design

#### *In vitro* studies

**Cell culture.** The human colon adenocarcinoma cell line Caco-2 (ATCC<sup>®</sup>HTB37<sup>™</sup>) was obtained from the American Type Culture Collection (LGC Standards GmbH). Cells were routinely grown in 75 cm<sup>2</sup> culture flasks using Dulbecco's modified Eagle's medium (DMEM) at pH 7.4 with 1% non-essential amino acids and 20% fetal calf serum (FCS). Cells were maintained in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. Stock passages were subcultured every 3–4 d. For incubation studies,

pre-confluent cells were trypsinised with a 0.25% (w/v) trypsin/0.53 mM-EDTA solution, and 1 × 10<sup>4</sup> cells were seeded onto a transwell insert with a polycarbonate membrane (diameter 6.35 mm, pore size 0.4 μm; Becton, Dickinson and Company) and placed in a twenty-four-well plate. Cells were allowed to grow to confluence and to differentiate to absorptive enterocytes within 21 d. The culture medium was changed every 2–3 d at the apical (0.35 ml) and basal side (1.35 ml). Transepithelial electrical resistance was monitored using a Millicell-ERS Volt-Ohm Meter (Millipore). Only transwell filters with transepithelial electrical resistance values above 350 Ωcm<sup>2</sup> were used for absorption experiments (*n* 3 in duplicates).

**Degradation and incubation studies.** For incubation studies, inserts with cells were carefully washed at 37°C with DMEM containing 10% FCS. Apical compartments representing the luminal side of the gut and basal compartments representing the serosal side were filled for equilibration (30 min, 37°C) with 0.5 and 1.5 ml of medium (DMEM, 10% FCS), respectively. Then, grape/blueberry extract dissolved in medium was added to the apical compartment. The concentration of total ACN as the sum of individual ACN was 2613 (SEM 19) μmol/l, respectively 1290 (SEM 9) mg/l (Table 2). ACN-rich extract was dissolved in cell culture medium (DMEM, 10% FCS) at 37°C. The pH was adjusted to 7.4 for incubation studies and to 2.0 or 7.4 for degradation studies. The final concentration of the extract solution was about 50 μmol/l Mal-3-glc equivalents. After 0, 30, 60 and 90 min, medium from the apical and basal compartments was removed, mixed with 1% formic acid to prevent further degradation and stored immediately at –80°C until ACN quantification. Control chambers without cells were run as a control to measure ACN degradation at pH 2.0 and 7.4. At the same time points, aliquots were taken and treated in the same way as the samples that had cell contact. ACN in apical compartments were analysed by HPLC with UV detection (HPLC-UV). Due to lower concentrations, basal compartments were analysed by ultra-performance liquid chromatography coupled to MS (UPLC-MS) after solid-phase extraction (see 'Analysis of *in vitro* and *in vivo* samples' section).

#### *In vivo* studies

**Design and study subjects.** From May to August 2010, we conducted a randomised, double-blind, cross-over study to investigate the bioavailability of ACN from ACN-rich juice and smoothie in healthy young individuals at the Justus-Liebig University Giessen (Germany). The present study was carried out according to the guidelines laid down in the Declaration of Helsinki, and the local ethics committee (registration no. 13/10) approved all procedures involving human subjects. Written informed consent was obtained from all participants. Cluster sample size calculation for 2 × 2 cross-over designs with type 1 and 2 errors of 0.05 and 0.2, respectively, was performed with Stata version 12 (cluster-sampsi) (StataCorp). Power calculation estimated the required sample size to be ten (five per arm). Healthy subjects (*n* 10: five females and five males) between 23 and 27 years of age

(25.0 (SEM 2.8) years) and with a BMI between 19.6 and 25.1 kg/m<sup>2</sup> (22.4 (SEM 3.9) kg/m<sup>2</sup>) were recruited. They were deemed eligible for the study in consideration of specific exclusion criteria (3 months without medications, vitamin and mineral supplementation). In a double-blind fashion, the included subjects were randomly assigned to the initial arm of the study for juice or smoothie. After randomisation, a 10-d washout period was followed by the intervention day. After an overnight fast, the participants ingested 0.33 litres of juice or smoothie; blood (0, 10, 20, 30, 60, 90 and 120 min) and urine (fractions of 0–3, 3–6, 6–9, 9–12, 12–15 and 15–24 h) samples were taken. Participants were allowed to consume only water within 3 h after ingestion of the study juice or smoothie. A 14 d run-out finished the first cycle. This procedure was performed twice for juice or smoothie. The present study design is shown in Fig. 1.

**Source of anthocyanin-rich juice, smoothie and extract.** Juice, smoothie and extract were produced at the Geisenheim University (Department of Wine Chemistry and Beverage Technology, Research Center Geisenheim) and made from an eighty/twenty mixture of red grapes (grape varieties *Dakapo* and *Accent*) and blueberries (*Vaccinium myrtillus*). Briefly, grapes were extruded, and the resulting juice was separated, blended with blueberry juice from concentrate, pasteurised and hot-filled into 0.33 litres of brown glass bottles. Smoothie was produced from juice being blended with 20% blueberry puree instead of blueberry juice. Juice was analysed directly after membrane filtration (pore size 0.45 µm) for basic analytical parameters such as total phenolics, concentrations of ACN and Trolox equivalent antioxidant capacity (TEAC)<sup>(29,30)</sup>. For HPLC and TEAC analyses, smoothie was extracted twice with 80% methanol. Corresponding extract for *in vitro* studies was made from juice. Briefly, grape/blueberry juice was loaded onto a pilot glass column (Kronlab/Schott, 200 × 750 mm) filled with adsorber resin (Resindion/Mitsubishi). Water-soluble juice constituents such as sugars, organic acids and minerals were washed out with two bed volumes of distilled water. The first eluate was slightly coloured due to low ACN levels. ACN and colourless polyphenols were eluted with two bed volumes of 96% ethanol. The ethanolic fraction was concentrated using a rotary evaporator and lyophilised.

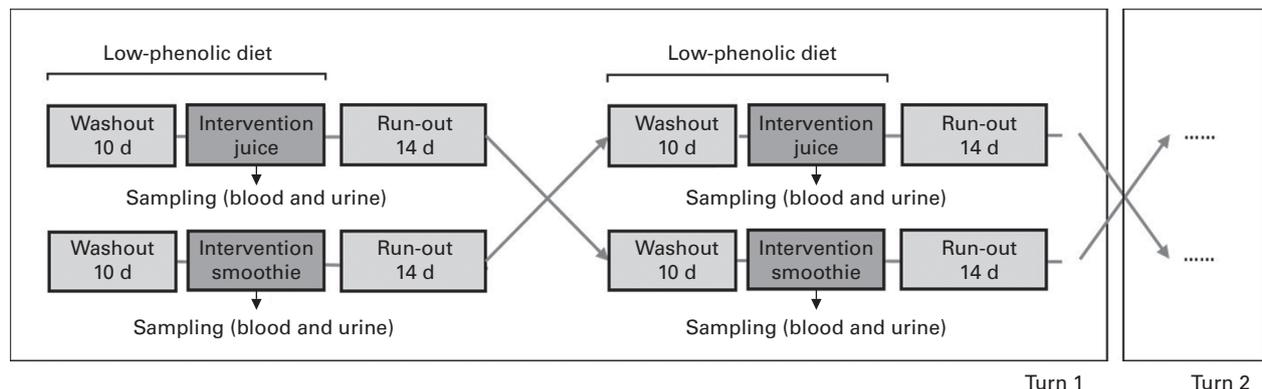
Analysis of ACN as well as macro- and micro-nutrient content in juice and smoothie is described elsewhere<sup>(31)</sup>. 3,4-Dihydroxybenzoic acid (3,4-DHB) was analysed by UPLC-MS (see 'Analysis of *in vitro* and *in vivo* samples' section). The concentrations of ACN and 3,4-DHB are listed in Table 1.

After an overnight fast, volunteers consumed 0.33 litres of juice or smoothie. Volunteers ate a standardised lunch 5 h after drinking the beverage and thereafter remained on a low-polyphenol diet for a further 20 h until the final urine samples were collected. Venous blood samples (30 ml) were collected in EDTA tubes from all volunteers at 0, 10, 20, 30, 40, 60, 90 and 120 min post-ingestion, and plasma was separated by centrifugation at 3000 g for 10 min at 4°C. Plasma was divided into 1 ml aliquots, each of which was acidified using 30 µl of 50% aqueous formic acid and stored at –80°C until further analysis. Urine was collected at baseline and over six time periods, 0–3, 3–6, 6–9, 9–12, 12–15 and 15–24 h, after ingestion of the test drink. The total volume for each period was recorded, acidified with 1% formic acid and aliquots were stored at –80°C until further analysis.

## Analysis of *in vitro* and *in vivo* samples

### Sample extraction

Solid-phase extraction (SPE) was used for basal cell compartment samples as well as for plasma and urine samples due to lower concentrations of ACNs and 3,4 DHB. Before SPE, pelargonidin-3-*O*-rutinoside and 2-chloro-5-nitrobenzoic acid were added to the samples as internal standards. Plasma samples were extracted using a slightly modified method developed by Esselen *et al.*<sup>(32)</sup>. Briefly, SPE Waters Oasis<sup>®</sup> HLB (Waters) cartridges were conditioned with 1 ml methanol and 1 ml 1.5 M-formic acid before 1 ml plasma sample was loaded onto the column. The column was then washed with 1 ml of 1.5 M-formic acid and 1 ml methanol (5%). Retained ACN were eluted with 1 ml of 0.1% formic acid in methanol. The eluate was reduced to dryness under a stream of N at 20°C. Plasma preparations were reconstituted with 100 µl of trifluoroacetic acid (5% v:v) in water. Reconstituted samples were stored at –80°C until further analysis. Urine and basal



**Fig. 1.** Design of the bioavailability study. Study participants were randomly assigned to consume the test beverages. They passed two cycles getting juice and smoothie in a double-blind, cross-over design. One cycle was intermitted by a 10 d washout phase and a 14 d run-out phase. At the intervention day, the volunteers consumed the beverages after an overnight fast. Blood and urine samples were taken as described in the 'Materials and methods' section. All the subjects completed the study (*n* 10).

**Table 1.** Anthocyanins (ACN) and 3,4-dihydroxybenzoic acid (3,4-DHB) in beverages (ACN-rich juice and smoothie) and the corresponding extract\* (Mean values and standard deviations, *n* 2)

Phenolic	Juice (mg/l)		Juice (μmol/l)		Smoothie (mg/l)		Smoothie (μmol/l)		Extract (mg/l)		Extract (μmol/l)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
3,4-DHB	11.5	0.72	71.8	4.8	9.26	0.15	60.1	0.4	0.94	0.09	6.16	0.68
Malvidin-3-glucoside	273	4	554	8	274	10	556	20	419	2	849	3
Malvidin-3-(6''-p-coumaroyl)-glucoside	66.4	0.3	104	1	46.5	1.9	72.7	3.0	103	1	161	1
Malvidin-3-(6''-O-acetyl)-glucoside	51.4	0.7	96.6	1.3	46.4	2.1	86.7	3.9	87.8	0.5	164	1
Malvidin-3-arabinoside	4.22	0.21	9.1	0.4	5.58	0.12	11.9	0.2	5.49	0.27	11.7	0.4
Σ Malvidin	396	5	764	11	373	14	728	27	615	3	1185	5
Peonidin-3-glucoside	165	2	356	4	163	6	352	13	248	1	537	1
Peonidin-3-(6''-O-acetyl)-glucoside	18.3	0.1	36.2	0.2	14.7	1.2	29.1	2.4	31.2	0.0	61.7	0.1
Peonidin-3,5-diglucoside	5.94	0.45	9.4	0.6	8.04	0.18	12.8	0.2	7.48	0.82	11.8	1.3
Peonidin-3-galactoside	ND		ND		4.40	0.06	9.54	0.08	ND		ND	
Σ Peonidin	189	2	402	5	190	7	403	15	287	1	610	2
Delphinidin-3-glucoside	67.4	1.1	144	2	105	3	225	7	96.4	1.3	207	3
Delphinidin-3-arabinoside	10.9	0.1	25.0	0.2	30.9	1.1	71.0	2.5	14.2	1.0	32.6	2.3
Delphinidin-3-galactoside	9.37	0.16	20.0	0.2	43.1	1.9	92.6	4.1	15.5	0.2	33.3	0.4
Delphinidin-3-(6''-O-acetyl)-glucoside	5.15	0.26	10.1	0.4	6.59	0.64	12.8	1.2	9.16	0.32	17.9	0.6
Σ Delphinidin	92.7	1.5	199	3	185	7	402	15	135	3	291	6
Petunidin-3-glucoside	69.8	1.4	145	2	87.0	3.3	181	6	103	1	216	2
Petunidin-3-galactoside	ND		ND		11.9	0.5	24.8	1.0	ND		ND	
Petunidin-3-(6''-p-coumaroyl)-glucoside	15.3	0.1	24.5	0.2	12.7	0.8	20.3	1.3	29.9	0.2	47.8	0.3
Petunidin-3-(6''-O-acetyl)-glucoside	10.9	0.1	20.9	0.6	8.44	1.36	16.1	2.5	17.0	0.1	32.6	0.2
Σ Petunidin	96	1	191	3	120	5	242	11	150	1	296	2
Cyanidin-3-glucoside	41.1	0.5	91.4	1.4	58.4	2.1	129	5	62.9	0.5	140	1
Cyanidin-3-galactoside	13.4	0.1	29.8	0.2	30.2	0.9	67.2	2.0	21.8	0.6	48.5	1.3
Cyanidin-3-arabinoside	12.0	0.2	28.6	0.5	18.6	0.6	44.3	1.4	17.4	0.0	41.5	0.0
Cyanidin-3,5-diglucoside	ND		ND		4.97	0.11	8.07	0.23	ND		ND	
Cyanidin-3-(6''-O-acetyl)-glucoside	ND		ND		2.55	0.59	5.14	1.03	ND		ND	
Σ Cyanidin	66.5	0.8	149	1	114	4	254	9	102	1	230	2
Sum ACN	840	11	1707	23	983	38	2031	79	1290	9	2613	18

ND, not detectable.

\*Beverages were analysed by HPLC-photodiode array/electrospray ionisation-MS or ultra-performance liquid chromatography-MS; for more details, see 'Materials and methods' section.

cell culture samples were performed according to Urpi-Sarda *et al.*<sup>(33)</sup> with slight modifications. Briefly, samples were centrifuged at 1500 *g* for 5 min at 20°C. Samples (1 ml) were loaded onto a preconditioned SPE Waters Oasis<sup>®</sup> HLB Cartridge (Waters GmbH). Metabolites were eluted with 1 ml acidified methanol after washing the column with 1 ml of 1.5 M-formic acid and 1 ml of 5% methanol. The eluate was reduced to dryness under a stream of N at 20°C. Urine and basal cell culture samples were reconstituted with 100 μl formic acid (5% v:v) and methanol (10%) in water. Again, reconstituted samples were stored at -80°C until further analysis.

### Identification and quantification of anthocyanins and 3,4-dihydroxybenzoic acid

Apical cell culture samples were analysed by HPLC-UV. Due to lower concentrations, basal cell culture compartments, urine and plasma samples were analysed by UPLC-MS. Calibration curves in both cases were generated by using a grape extract with a known amount of Mal-3-glc and 3,4-DHB, which was used for the quantification of all ACN and 3,4-DHB.

**HPLC-UV.** A capillary HPLC system (Summit<sup>®</sup> Dionex GmbH) equipped with a pump (P580 A HPG), an autosampler (ASI-100T Automated Sample Injector) and a reversed-phase

column (type: inner diameter 6.35 mm, pore size 0.4 μm, NUCLEOSIL 300 C18; Macherey-Nagel GmbH + Company KG) as well as an UVD 340U (Dionex GmbH) were used for analysis of apical cell culture compartments. Detection was carried out at a wavelength of 520 nm. Mobile phases consisted of 5% v:v formic acid in water (solvent A) and methanol (solvent B). The solvent gradient was 5% B at 0–2 min, 20% B at 10–15 min, 30% B at 30 min, 37% B at 35–40 min, 40% B at 41–43 min, 95% B at 50–62 min and 5% B at 70–80 min at a flow rate of 70 μl/min. Injection volume was 15 μl.

**UPLC-MS.** UPLC separation was performed using an Ultimate 3000 Rapid Separation LC system (Dionex, Thermo Fisher Scientific Germany BV & Co KG) equipped with a pump (HPG 3200 RS), an autosampler (WPS-3000 TRS) and a reversed phase (C18) column (type: 100 mm × 2.1 mm, particle size 2.6 μm, Kinetex; Phenomenex). Mobile phases consisted of 0.7% v:v formic acid in water (solvent A) and methanol (solvent B). The injection volume was 40 μl, and the solvent gradient was 2% B at 0–0.5 min, 7% B at 1 min, 15% B at 5 min, 40% B at 10 min, 70% B at 13–16 min at a flow rate of 400 μl/min (*p* (max) = 550 bar). UPLC system was coupled to an Exactive Orbitrap Mass Spectrometer (Thermo Fisher Scientific) by an electrospray ion source. Capillary temperature was set to 350°C, and spray voltage was 4.4 kV in positive ion mode and 3.5 kV in negative ion

mode. Measurements were performed by continuously switching between positive and negative ion mode. Mass spectra were acquired in the range of  $m/z$  200–850 for positive ion mode and  $m/z$  100–850 for negative ion mode.

**Statistical and pharmacokinetic analyses of metabolites in cell culture, plasma and urine samples**

*In vitro analysis*

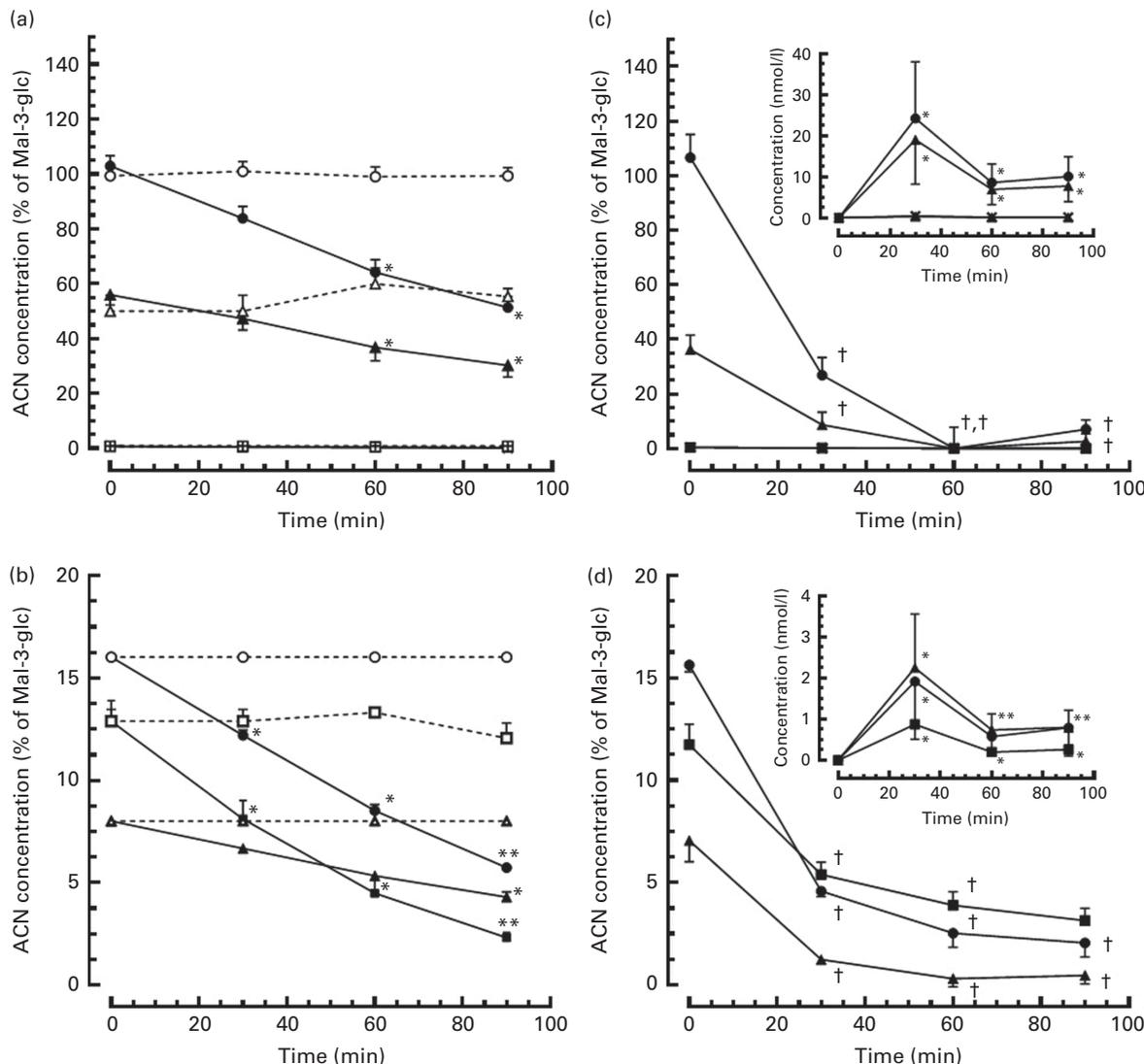
All data are presented as mean values with their standard errors ( $n$  3 in duplicates). Significant differences between the means of ACN degradation and incubation in transport chambers and the respective control chambers were assessed by

one-way ANOVA. The significance of differences for ACN degradation and incubation was assessed using  $t$  test. Differences were considered to be significant at  $P < 0.05$  and  $P < 0.01$ .

*In vivo analysis*

Data on ACN and their metabolites are presented as mean values with their standard errors ( $n$  10 in duplicates). The Kolmogorov–Smirnov test was used to test the data for normal distribution, and significant differences between juice and smoothie were analysed by one-way ANOVA or paired  $t$  test. Differences were considered to be significant at  $P < 0.05$ .

The statistical IBM software SPSS for Windows (version 19.0.0; IBM Deutschland GmbH) and GraphPad Prism



**Fig. 2.** Individual anthocyanin (ACN, in percentage of initial malvidin-3-glucoside (Mal-3-glc) concentration (50  $\mu$ M Mal-3-glc equivalents)) over time in the apical compartments during degradation (a and b) at pH 2.0 (.....) and pH 7.4 (—) and incubation (c and d) experiments (pH 7.4). Basal appearance (nmol/l) of individual ACN is shown in insets. Values are means ( $n$  3 experiments each run in duplicates), with their standard errors represented by vertical bars. Mean value was significantly different to the initial concentrations: \*  $P < 0.05$ , \*\*  $P < 0.01$ . † Mean value was significantly different between incubation (with cells) and degradation (without cells) chambers ( $P < 0.05$ ). (a) ●, Mal-3-glc (7.4); ▲, peonidin-3-glucoside (Peo-3-glc (7.4)); ■, malvidin-3,5-diglucoside (Mal-3,5-dglc (7.4)); ✕, peonidin-3,5-diglucoside (Peo-3,5-dglc (7.4)); ○, Mal-3-glc (2.0); △, Peo-3-glc (2.0); □, Mal-3,5-dglc (2.0); +, Peo-3,5-dglc (2.0). (b) ●, Petunidin-3-glucoside (Pet-3-glc (7.4)); ▲, cyanidin-3-glucoside (Cya-3-glc (7.4)); ■, delphinidin-3-glucoside (Del-3-glc (7.4)); ○, Pet-3-glc (2.0); △, Cya-3-glc (2.0); □, Del-3-glc (2.0). (c) ●, Mal-3-glc; ▲, Peo-3-glc; ■, Mal-3,5-dglc; ✕, Peo-3,5-dglc. (d) ●, Pet-3-glc; ▲, Cya-3-glc; ■, Del-3-glc.

(version 6.0.2; GraphPad Software, Inc.) were used for pharmacokinetic data analysis.

## Results

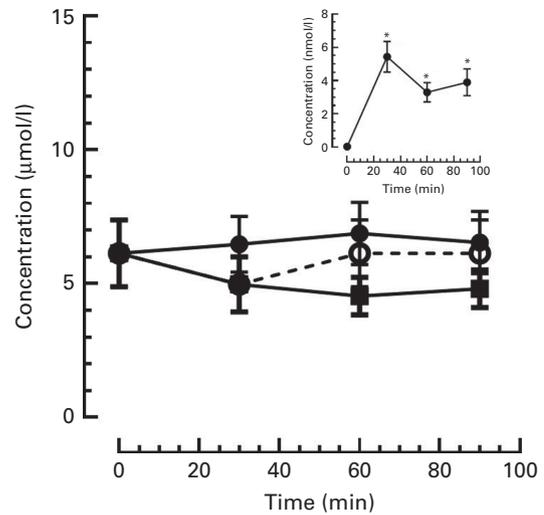
### In vitro study

After application of the grape/blueberry extract to the apical compartments of transwell chambers, ACN (Mal-3-glc, peonidin-3-glucoside (Peo-3-glc), petunidin-3-glucoside (Pet-3-glc), delphinidin-3-glucoside (Del-3-glc) and Cya-3-glc) degradation at pH 2.0 and 7.4 (without cells) was determined by HPLC-UV. Cellular transport of ACN from the apical to the basal compartment at pH 7.4 was determined by UPLC-MS. As shown in Fig. 2(a) and (b), all ACN concentrations decreased significantly over time in the degradation chambers (without cells) at pH 7.4, but not at pH 2.0, thus reflecting the stability at lower pH. Significant differences between incubation media (pH 7.4, with cells; Fig. 2(c) and (d) and the respective degradation media (pH 7.4, without cells) became significant after 30 min; correspondent enrichment in the basal compartment was observed for both native ACN, Mal-3-glc and Peo-3-glc, reaching a maximum after 30 min of incubation time (insets in Fig. 2(c) and (d)). Despite the higher concentration of Mal-3-glc in the extract compared with Peo-3-glc (50 and 31  $\mu\text{mol/l}$ , respectively), basal concentrations after 30 min were in the same range (24.5 (SEM 12.4) and 19.6 (SEM 13.4) nmol/l, respectively) revealing absorption rates of 0.05% for Mal-3-glc and 0.06% for Peo-3-glc. In contrast to the monoglucosylated ACN, the diglucosylated Mal-3,5-dglc and Peo-3,5-dglc were not transported in quantifiable concentrations (Fig. 2(a), inset). Regarding the minor ACN, petunidin (Pet-3-glc) and delphinidin (Del-3-glc) showed higher instability than cyanidin (Cya-3-glc) over time. Significant differences in the disappearance of Pet-3-glc, Del-3-glc and Cya-3-glc between degradation (without cells) and incubation (with cells) chamber were observed at 30 min. Thus, absorption efficiencies appeared to be influenced by the structural skeleton since basal appearance of Cya-3-glc was much higher than that of Pet-3-glc or Del-3-glc (2.5 (SEM 1.3) *v.* 1.9 (SEM 1.1) and 0.8 (SEM 0.4) nmol/l) corresponding to recovery rates of 0.03, 0.01 and 0.005% of initial apical concentration for Cya-3-glc, Pet-3-glc and Del-3-glc, respectively.

For 3,4-DHB, no degradation was observed in the apical compartment of the transwell chambers (Fig. 3); however, cellular transport from the apical to the basal compartment was observed after 30 min of incubation time (5.5 (SEM 2.4) nmol/l; 2.5% of the apical concentration).

### In vivo study

The total amount of ACN in 0.33 litres juice and smoothie was 277.4 (SEM 3.8) and 324.5 (SEM 12.8) mg with the major ACN Mal-3-glc (90.2 (SEM 1.3) and 90.6 (SEM 3.3) mg) and Peo-3-glc (54.5 (SEM 0.7) and 53.8 (SEM 2.0) mg) and the minor ACN Del-3-glc (22.2 (SEM 0.4) and 34.6 (SEM 1.1) mg), Cya-3-glc (13.6 (SEM 0.2) and 19.1 (SEM 0.7) mg) and Pet-3-glc (22.8 (SEM 0.5) and 28.7 (SEM 1.1) mg) (Table 1). These five

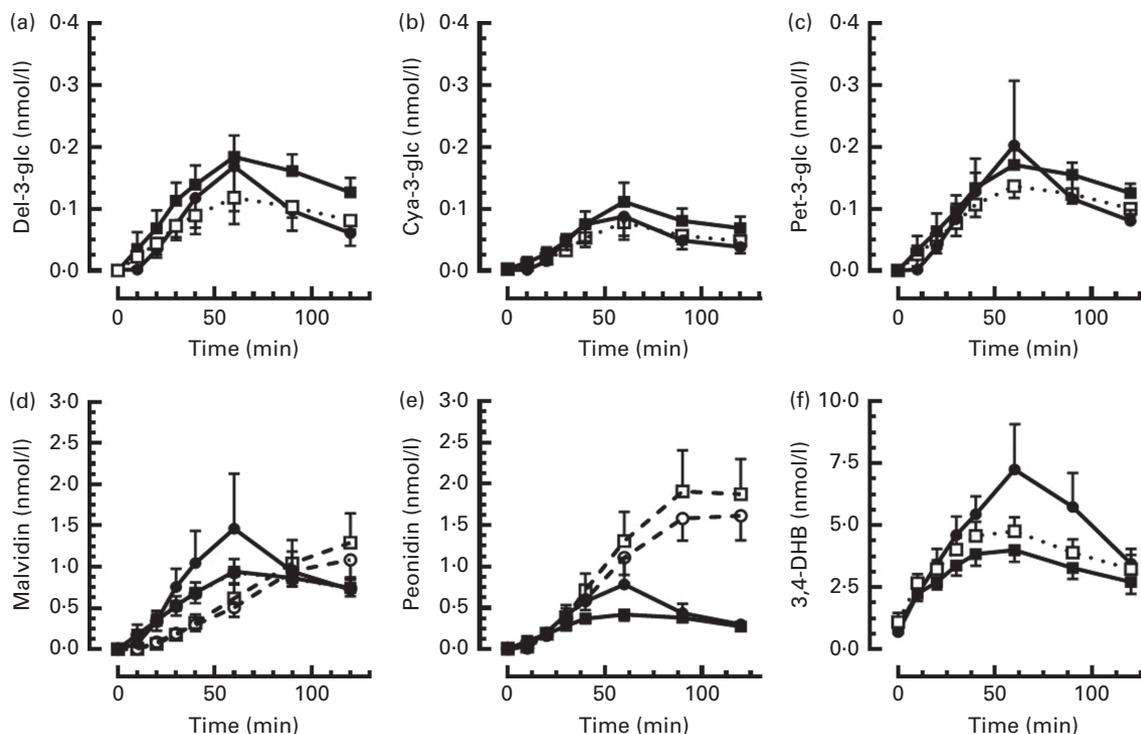


**Fig. 3.** 3,4-Dihydroxybenzoic acid (3,4-DHB) ( $\mu\text{mol/l}$ ) over time in apical compartments during degradation at pH 2.0 (.....) and pH 7.4 (—) and incubation experiments (pH 7.4); basal appearance (nmol/l) of 3,4-DHB is shown in the inset. Values are means ( $n$  3 experiments each run in duplicates), with their standard errors represented by vertical bars. \*Mean value was significantly different to the initial concentrations ( $P < 0.05$ ). ●—, 3,4-DHB (pH 7.4, degradation); ○—, 3,4-DHB (pH 2.0, degradation); ■—, 3,4-DHB (pH 7.4, incubation).

ACN accounted for about 80% of the ACN ingested via juice or smoothie, and were detected in plasma as well as in urine samples after consumption of the beverage by young and healthy volunteers (Fig. 4). ACN in baseline plasma and urine samples were absent before the ingestion of juice and smoothie, demonstrating the compliance of our subjects with dietary rules within the washout period. The occurrence of the five ACN named above in plasma reflected their concentration in juice and smoothie with Del-3-glc, Cya-3-glc and Pet-3-glc (Fig. 4(a)–(c)) as minor and Mal-3-glc and Peo-3-glc (Fig. 4(d) and (e)) as major compounds. UPLC-MS analysis of plasma samples not only revealed the occurrence of ACN glucosides in all subjects ( $n$  10 in duplicate), but also glucuronidated (glucuro) metabolites of malvidin and peonidin (Fig. 4(d) and (e)), although with wide individual variations. In addition to glucuronides, neither further phase II metabolites nor degradation products were found in plasma and urine.

Pharmacokinetic profiles of plasma ACN glucosides and glucuronides are shown in Fig. 4 and summarised in Table 2. After ACN intake, Mal-3-glc and Peo-3-glc were detected in plasma with maximal concentration ( $C_{\text{max}}$ ) of 1.54 (SEM 0.66) and 0.80 (SEM 0.32) nmol/l for juice and 1.00 (SEM 0.14) and 0.46 (SEM 0.07) nmol/l for smoothie, respectively. Both ACN had a similar time to maximal concentration ( $t_{\text{max}}$ ) after juice or smoothie intake with a slightly delayed peak after smoothie ingestion (68 (SEM 8) *v.* 75 (SEM 8) and 55 (SEM 3) *v.* 69 (SEM 6) min).

Although the AUC for Mal-3-glc and Peo-3-glc revealed higher values after juice (103.1 (SEM 34.6) and 52.5 (SEM 16.6) nmol  $\times$  min/l) than after smoothie (81.5 (SEM 11.4) and 37.4 (SEM 5.5) nmol  $\times$  min/l) ingestion, there were no significant differences with regard to the bioavailability of these



**Fig. 4.** Plasma concentrations of anthocyanin and 3,4-dihydroxybenzoic acid (3,4-DHB) from juice (●) or smoothie (non-adjusted (■) and adjusted (□)) and their glucuronides (juice (○) and smoothie (□)) *in vivo*. Values are means (*n* 10 in duplicates), with their standard errors represented by vertical bars for (a) delphinidin-3-glucoside (Del-3-glc), (b) cyanidin-3-glucoside (Cya-3-glc), (c) petunidin-3-glucoside (Pet-3-glc), (d) malvidin-3-glucoside/glucuronide, (e) peonidin-3-glucoside/glucuronide as well as (f) 3,4-DHB.

ACN from juice and smoothie. This was shown for both, non-adjusted and dose-adjusted Mal-3-glc and Peo-3-glc values. The pharmacokinetic profiles of glucuronides were different compared with those of the glucosides. Malvidin-3-glucuronide (Mal-3-glucuro) had a  $C_{max}$  of 1.1 (SEM 0.2) and 1.3 (SEM 0.3) nmol/l for the juice and smoothie, a  $t_{max}$  of 114 (SEM 4) and 117 (SEM 3) min and an AUC of 64 (SEM 12) and 73 (SEM 20), respectively. Peonidin-3-glucuronide (Peo-3-glucuro) had a higher  $C_{max}$  of 1.7 (SEM 0.3) and 2.1 (SEM 0.5) nmol/l for the juice and smoothie, a lower  $t_{max}$  of

105 (SEM 5) and 108 (SEM 5) min and a higher AUC of 114 (SEM 20) and 135 (SEM 34) nmol × min/l, respectively. It was not possible to determine the  $t_{1/2}$  because the levels had not yet declined to baseline after reaching  $C_{max}$ . This is most evident considering the pharmacokinetic profiles of glucuronides. In contrast to the two major ACN, malvidin and peonidin, the minor ACN were only detected as glucosides with concentrations near the detection limit. Again, no significant differences were observed concerning the bioavailability of ACN from juice and smoothie (Table 2).

**Table 2.** Pharmacokinetic parameters of anthocyanins and phenolic acid in plasma (Mean values with their standard errors, *n* 10 in duplicate)

	$C_{max}$ (nmol/l)				$t_{max}$ (min)				AUC (nmol × min/l)				AUC <sub>adj</sub> (nmol × min/l)		Relative BV Smoothie (%)
	Juice		Smoothie		Juice		Smoothie		Juice		Smoothie		Smoothie		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
Del-3-glc	0.18	0.09	0.19	0.03	58	5	67	8	10	4	15	2	10	2	91.7
Cya-3-glc	0.10	0.04	0.12	0.03	55	7	68	8	6.0	2.1	8.2	2.1	5.8	1.5	96.5
Pet-3-glc	0.21	0.10	0.19	0.03	69	5	71	10	12	5	14	2.2	11	2	91.6
Mal-3-glc	1.5	0.6	1.0	0.1	68	8	75	8	103	34	81	11	82	11	80.0
Peo-3-glc	0.80	0.32	0.46	0.07	55	3	69	6	52	16	37	5	37	5	70.9
Mal-3-glucuro	1.1	0.2	1.3	0.3	114	4	117	3	64	12	73	20			
Peo-3-glucuro	1.7	0.3	2.1	0.5	105	5	108	5	114	20	135	34			
3,4-DHB	7.6	1.8	4.5	0.5	57	5	63	8	568	79	285*	38	314*	47	79.2*

$C_{max}$ , maximal concentration;  $t_{max}$ , time to maximal concentration; AUC<sub>adj</sub>, AUC adjusted to ingested dose; BV, bioavailability; Del-3-glc, delphinidin-3-glucoside; Cya-3-glc, cyanidin-3-glucoside; Pet-3-glc, petunidin-3-glucoside; Mal-3-glc, malvidin-3-glucoside; Peo-3-glc, peonidin-3-glucoside; Mal-3-glucuro, malvidin-3-glucuronides; Peo-3-glucuro, peonidin-3-glucuronides; 3,4-DHB, 3,4-dihydroxybenzoic acid.

\* Mean value was significantly different ( $P < 0.05$ ).

In contrast to the ACN and their metabolites, there was a significant difference in the bioavailability of the PA 3,4-DHB from juice and smoothie. The pharmacokinetic profiles (Fig. 4(f)) and parameters (Table 2) showed that 3,4-DHB was better bioavailable from juice than from smoothie.  $C_{max}$ ,  $t_{max}$  and AUC values for juice were 7.6 (SEM 1.8) nmol/l, 57 (SEM 5) min and 568 (SEM 79) nmol × min/l, respectively. In contrast,  $C_{max}$ ,  $t_{max}$  and AUC values for smoothie were 4.5 (SEM 0.5) nmol/l, 63 (SEM 8) min and 285 (SEM 38) nmol × min/l, respectively, i.e. after adjustment to the dose in smoothie, the AUC value was 314 (SEM 47) nmol × min/l.

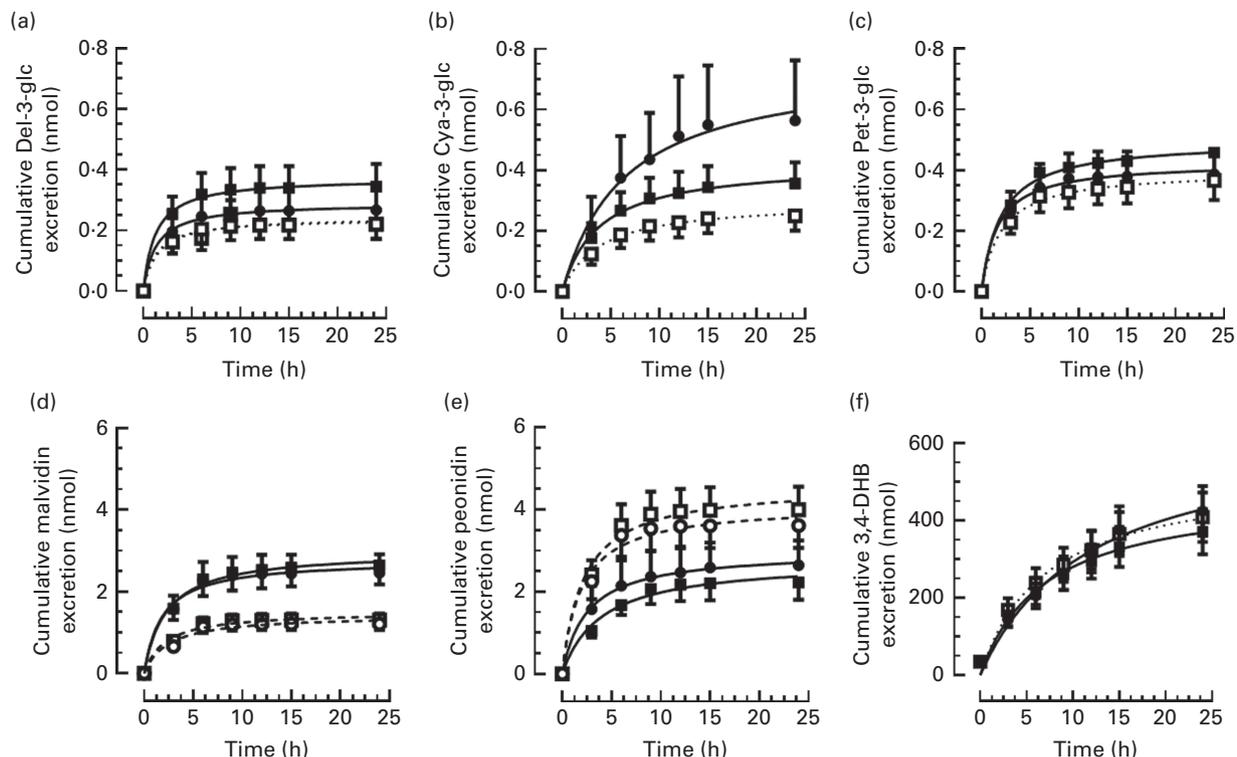
All ACN and their metabolites as well as the PA identified in the plasma were also detected in urine after the ingestion of juice or smoothie. Urinary samples were collected at intervals of 0–3, 3–6, 6–9, 9–12, 12–15 and 15–24 h, and the excretion of both glucosides and glucuronides peaked within the first two time intervals (0–3 and 3–6 h) and were detectable until 12 h after ACN intake. The cumulative excretion curves of the ACN and their glucuronidated metabolites are shown in Fig. 5.

The main urinary ACN in both juice and smoothie were Mal-3-glc with 2.46 (SEM 0.44) and 2.64 (SEM 0.44) nmol/l and Peo-3-glc with 2.65 (SEM 0.57) and 2.22 (SEM 0.40) nmol/l being excreted until 24 h after ACN intake (Table 3). Substantial amounts of the minor ACN Del-3-glc, Cya-3-glc and Pet-3-glc were also detected, although in lower concentrations and without significant differences between juice and smoothie (0.27 (SEM 0.08) *v.* 0.34 (SEM 0.07), 0.56 (SEM 0.19)

*v.* 0.36 (SEM 0.07) and 0.39 (SEM 0.08) *v.* 0.46 (SEM 0.08) nmol/l, respectively). Glucuronidated metabolites of malvidin and peonidin occurred in urine with Peo-3-glucuro revealing the highest concentrations (3.61 (SEM 0.52) and 4.01 (SEM 0.53) nmol/l after juice and smoothie intake, respectively) compared with Mal-3-glucuro with 1.2 (SEM 0.2) and 1.3 (SEM 0.2) nmol/l after juice and smoothie intake, respectively. In contrast to plasma, urinary excretion of 3,4-DHB reached high values after juice and smoothie ingestion, but without significant difference (23 (SEM 20) *v.* 10 (SEM 8) nmol/l) after baseline correction.

## Discussion

Since ACN from juice or smoothie have a great beneficial potential concerning health and possibly served as an additional portion to increase the FV intake, it is of great importance to investigate their bioavailability in humans. Thus, the present study investigated the bioavailability of phenolic compounds from an ACN-rich juice and smoothie *in vivo* as well as the cellular uptake from a corresponding extract *in vitro*. This involved the assessment of (1) the stability, degradation and transport rates of ACN using Caco-2 cells as absorptive intestinal epithelial cells, (2) the stability, the degradation and transport rates of PA 3,4-DHB *in vitro*, (3) the identification and quantification of ACN and their metabolites in plasma and urine as well as (4) the quantification of 3,4-DHB in



**Fig. 5.** Cumulative urinary concentrations of anthocyanin and 3,4-dihydroxybenzoic acid (3,4-DHB) from juice (—●—) or smoothie (non-adjusted (—■—) and adjusted (—□—)) and their glucuronides (juice (—○—) and smoothie (—□—)) *in vivo*. Values are means (*n* 10 in duplicate), with their standard errors represented by vertical bars for (a) delphinidin-3-glucoside (Del-3-glc), (b) cyanidin-3-glucoside (Cya-3-glc), (c) petunidin-3-glucoside (Pet-3-glc), (d) malvidin-3-glucoside/glycoside and (e) peonidin-3-glucoside/glycoside.

**Table 3.** Pharmacokinetic parameters of anthocyanins and phenolic acid in urine

 (Mean values with their standard errors, *n* 10 in duplicate)

Parameters	Cumulative $C_{\max}$ (nmol/l)				Cumulative $C_{\max \text{ adj}}$ (nmol/l)	
	Juice		Smoothie		Smoothie	
	Mean	SEM	Mean	SEM	Mean	SEM
Del-3-glc	0.27	0.08	0.34	0.07	0.22	0.05
Cya-3-glc	0.56	0.19	0.36	0.07	0.25	0.05
Pet-3-glc	0.39	0.08	0.46	0.08	0.37	0.06
Mal-3-glc	2.4	0.4	2.6	0.4	2.6	0.4
Peo-3-glc	2.6	0.6	2.2	0.4	2.2	0.4
Mal-3-glucuro	1.2	0.2	1.3	0.2	1.3	0.2
Peo-3-glucuro	3.6	0.5	4.0	0.5	4.0	0.5
3,4-DHB	23	20	10	8	12	10

$C_{\max}$ , maximal concentration;  $C_{\max \text{ adj}}$ , maximal concentration adjusted to ingested dose; Del-3-glc, delphinidin-3-glucoside; Cya-3-glc, cyanidin-3-glucoside; Pet-3-glc, petunidin-3-glucoside; Mal-3-glc, malvidin-3-glucoside; Peo-3-glc, peonidin-3-glucoside; Mal-3-glucuro, malvidin-3-glucuronides; Peo-3-glucuro, peonidin-3-glucuronides; 3,4-DHB, 3,4-dihydroxybenzoic acid.

plasma and urine *in vivo* after ingestion of an ACN-rich juice or smoothie.

1. *In vitro*, we evaluated the transport of ACN from a grape/blueberry extract in Caco-2 cells using a two-compartment model. We showed that the five main glycones Mal-3-glc, Peo-3-glc, Pet-3-glc, Del-3-glc and Cya-3-glc in the extract were transported across the Caco-2 cell monolayers in their intact glycone form, although with low transport efficiency, which was in accordance with previous *in vitro* studies. While Steinert *et al.*<sup>(34)</sup> demonstrated the disappearance of black currant ACN from the apical compartment, but no occurrence in the basal compartment, Yi *et al.*<sup>(35)</sup> and Liu *et al.*<sup>(22)</sup> showed that ACN from blueberry extract were detected in the basal compartment. Similar to our data, transport efficiencies were dependent on the individual ACN with the highest recovery rates found in the basal compartments of Peo-3-glc > Cya-3-glc > Mal-3-glc > Pet-3-glc > Del-3-glc. We observed no correlation between structure and recovery rates, although facilitated uptake into cells was shown for ACN with chemical structures exerting greater hydrophobicity (e.g. malvidin and peonidin) than those with higher numbers of hydroxyl groups (e.g. delphinidin)<sup>(35)</sup>. In our study, we found different recovery rates with Peo-3-glc and Pet-3-glc despite their similar structure (four hydroxyl and one methyl group). In contrast, Cya-3-glc, for example, has four hydroxyl groups and no methyl group; however, the recovery rates were higher than that of Pet-3-glc. The fast disappearance of ACN in the apical solution and relatively low concentrations in the basal compartment could also be due to chemically induced degradation that may occur not only under *in vitro* conditions but also *in vivo*. Under acidic (pH 2.0) and neutral (pH 7.4) conditions in the absence of Caco-2 cells, ACN were observed to be stable at pH 2.0, but were degraded at pH 7.4 with more than a 40% loss within the

first 30 min for Mal-3-glc. Since the decreasing ACN rates at pH 7.4 were lower in the degradation studies (without cells) than in the incubation studies (with cells), it is assumed that a part of the apical disappearance of ACN was a result of their uptake across the cells. Due to these observations and the occurrence of ACN in the basal compartment, it was speculated that ACN were transported by active transport systems, which has been reported for other flavonoids<sup>(36–39)</sup>. Recently, Manzano & Williamson<sup>(40)</sup> demonstrated that ACN such as pelargonidin-3-glucoside inhibited glucose uptake. Using Na-containing (GLUT sodium-glucose co-transporter 1 (SGLT1) and GLUT2, both active) and Na-free (only GLUT2, active) conditions, they showed that the inhibition of GLUT2 was greater than that of SGLT1. They further observed that PPT (polyphenols, PA and tannins) inhibited the glucose transport from the intestinal lumen into cells and the GLUT2-facilitated exit to the basolateral side. Thus, although the chemically induced degradation seemed to be substantial, ACN could be transported across the epithelial monolayer. It should be noted that no transitional increase in the concentration of their aglycones was observed in the present study because of the possible hydrolysis of the glucose unit, probably due to the rapid turnover into other degradation products.

2. Because of the low bioavailability, it is speculated that ACN are likely to be degraded either spontaneously or enzymatically into phenolic degradation products, which are then further metabolised<sup>(41–43)</sup>. However, it is difficult to discriminate between PA deriving from the product or from intestinal and microbial degradation processes. 3,4-DHB, for example, which was present in high amounts in our test juice, smoothie and extract could have also been a degradation product of Cya-3-glc<sup>(44–46)</sup>. Hidalgo *et al.*<sup>(47)</sup> detected degradation products of ACN in low amounts after incubation with human gut microbiota for 4–6 h. Thus, under our conditions, we would exclude that the occurrence of PA in the basal compartment was a result of the cleavage of ACN, but revealed 3,4-DHB transport from the extract, since PA transport takes place in the upper part of the gastrointestinal tract with a low microbe density in healthy subjects. Recently, Wang *et al.*<sup>(48)</sup> demonstrated for the first time the simultaneous quantification of six PA after their oral administration to rats in plasma. They showed that 3,4-DHB was rapidly absorbed reaching  $C_{\max}$  in less than 0.37 h with  $t_{1/2}$  values of 5.75 h and mean  $AUC_{0 \rightarrow \infty}$  values of 789.7 ng × min/ml. This indicates that 3,4-DHB was rapidly absorbed without further fermentation by the gut microbiota and reached high amounts in plasma. In accordance with these observations, we have shown in our *in vitro* study that ACN and the PA 3,4-DHB can be transported in their intact forms, albeit in low concentrations in comparison to their initial apical concentrations.
3. Existing literature on the bioavailability of ACN *in vivo* considering the effect of food matrix are scarce. If smoothie intake is an alternative to increase FV intake,

we, therefore, compared the bioavailability of ACN from beverages with two different food matrices as ACN-rich juice and smoothie. The ACN profiles of the two beverages were nearly similar. In the case of the two main ACN, Mal-3-glc and Peo-3-glc, there was no difference between their content in juice and smoothie; however, Mal-3-glc concentration was higher than the Peo-3-glc concentration. After drinking 0.33 litres of juice or smoothie the AUC for Mal-3-glc obtained from plasma were 103 and 81 nmol  $\times$  min/l and  $C_{\max}$  values were 1.54 and 1.0 nmol/l, respectively. Furthermore, the  $t_{\max}$  value of Mal-3-glc after juice intake was lower than that after smoothie ingestion, indicating that Mal-3-glc from juice was better available (80% relative bioavailability); however, because of the high individual variation, effects were not significant. Urinary excretion of Mal-3-glc was similar with 2.56 and 2.65 nmol/l for intact Mal-3-glc from juice and smoothie, respectively. Peo-3-glc seemed to be better available from juice than from smoothie indicated by the relative bioavailability of Peo-3-glc from smoothie with 71%. In order to compare the bioavailability of the ACN from juice and smoothie, plasma concentrations were corrected for the actual amounts of ACN ingested via juice and smoothie. We could show that after adjustment of the pharmacokinetic parameters to the ACN dosages from juice and smoothie, the bioavailability of Del-3-glc, Cya-3-glc and Pet-3-glc was found to be about 5–10% lower after smoothie intake than after juice ingestion. Similar to Stalmach *et al.*<sup>(49)</sup> who analysed ACN and their metabolites in plasma and urine after ingestion of Concord grape juice (350 ml, 528  $\mu$ mol total phenolic compounds), we showed detectable quantities of glucuronides of Mal-3-glc and Peo-3-glc in plasma and urine, whereas no glucuronides were found for the minor ACN Del-3-glc, Pet-3-glc and Cya-3-glc. The delayed occurrence of Mal-3-glucuro and Peo-3-glucuro in plasma may be a consequence of post-absorption phase II metabolism converting the parental ACN structures to glucuronides in the gut and liver. Furthermore, although plasma malvidin and peonidin levels were in a similar range, Peo-3-glucuro was detected in higher amounts not only in plasma but also in urine compared with Mal-3-glucuro. In contrast to Stalmach *et al.*<sup>(49)</sup> who speculated that the low petunidin:delphinidin ratio in plasma and an enhanced ratio in urine were a consequence of 5'-methylation of delphinidin in the kidney, our results indicate that the Peo-3-glc turnover in the circulatory system is faster than that of its malvidin counterparts as being reflected in increased plasma  $C_{\max}$  levels. Alternatively, Cya-3-glc may have been methylated to Peo-3-glc and has been found as Peo-3-glucuro in plasma and urine after liver glucuronidation<sup>(50,51)</sup>. Although phase II glucuronidation takes place at any free hydroxyl position in the gut or liver, malvidin and peonidin glucuronides were identified as monoglucuronides.

4. In addition, structural differences of ACN interference factors such as food matrices or processing are important

determinants of ACN bioavailability<sup>(52,53)</sup>. Del Bo' *et al.*<sup>(54)</sup> investigated the bioavailability of two different blueberry purees in healthy volunteers and showed that ACN absorption was higher from blanched berries than from a raw berry preparation. In a further study, investigating apple polyphenols, Hagl *et al.*<sup>(27)</sup> have shown that substantially more polyphenols from apple smoothie reached the lower colon compared with those from apple juice. They speculated that absorption efficiencies were reduced after smoothie consumption because they contain more matrix components than juice. Hence, these components probably bind more polyphenols and thus reduce the bioavailability of the substances in the small intestine. In addition, the considerably higher polyphenol content of the apple smoothie could have reduced their relative bioavailability.

5. Interestingly, in this context, Kahle *et al.*<sup>(55)</sup> investigate PA absorption in ileostomy volunteers and indicate that the amounts of hydroxycinnamic acids absorbed or metabolised are smaller after consumption of apple smoothie than after consumption of apple juice. Although in the present study no hydroxycinnamic acids were detected in the beverages or plasma, we have shown for 3,4-DHB that the bioavailability of this PA was significantly lower after smoothie than after juice consumption. Plasma AUC values were 568 (SEM 79) after juice and 285 (SEM 38) nmol  $\times$  min/l after smoothie consumption, respectively 314 (SEM 47) nmol  $\times$  min/l after adjustment (AUC<sub>adj</sub>), with a relative bioavailability of 79% ( $P < 0.036$ ). As 3,4-DHB could also be a degradation product of Cya-3-glc, it cannot be distinguished between 3,4-DHB deriving from the beverage and 3,4-DHB as a degradation product. In contrast,  $t_{\max}$  values of the 3,4-DHB were in a similar range as Cya-3-glc, indicating that observed plasma levels were not a result of degradation processes in the colon.

In conclusion, the present study demonstrated that, despite their instability at neutral physiological pH and intense colonic metabolism, ACN from a berry extract were found at the serosal side in their native forms *in vitro* and their native and glucuronated forms *in vivo* after ingestion of the corresponding ACN-rich juice or smoothie. The liver seems to be the major site of post-absorptive ACN metabolism resulting in ACN monoglucuronides of the main ACN malvidin and peonidin. However, both *in vitro* and *in vivo* studies indicated that ACN absorption was below 0.1% of the initial doses, indicating their intense degradation and fermentation. In contrast to ACN and their glucuronides, we detected a food-matrix effect for the hydroxybenzoic acid 3,4-DHB, resulting in a significant lower bioavailability after smoothie compared with juice consumption. We previously reported that both beverages improved antioxidative parameters such as antioxidative enzyme activities or plasma antioxidative capacity in a human intervention study. Nevertheless, it remains to be discussed whether smoothies and juice could be recommended as a substitute to improve FV intake, particularly for children. Comparable studies investigating the bioavailability of

berry-derived ACN are scarce as well as the evidence of their health-promoting effects. This is a limitation of the present study, and should be investigated in further trials. Despite the acceptance of these beverages in adolescents and children, this source of health-promoting ACN has relatively high sugar content, which needs to be considered in the context of the growing problem of exceeding energy intake.

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The authors' contributions are as follows: S. K., S. R. and C. K. were the principal investigators of the present study, were responsible for the research questions, and involved in the study design and in the writing of the manuscript; H. A. contributed to the *in vitro* study, and C. B. contributed to the randomisation strategy and the handling of human samples; B. F. produced ACN-rich and ACN-depleted beverages and extracts, and measured ACN content; S. D., F. U., A. R. and B. S. were responsible for quantification and identification of ACN/ACN metabolites and PA in plasma, urine and cell culture samples.

The authors declare that there are no conflicts of interest.

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