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The Metabolism of Dietary Polyphenols and the Relevance to Circulating Levels of Conjugated Metabolites

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Berry extracts rich in anthocyanins have been linked to protective effects including the modulation of age-related neurological dysfunction and the improvement of the resistance of red blood cells against oxidative stress *in vitro*. In this study the bioavailability, metabolism and elimination of polyphenols from blackcurrant juice, rich in anthocyanins, flavonols, and hydroxycinnamates, were investigated. The four major native anthocyanidin glycosides of blackcurrant juice, delphinidin-3-glucoside, delphinidin-3-rutinoside, cyanidin-3-glucoside and cyanidin-3-rutinoside, were detected and identified in low amounts by HPLC and LC-MS in plasma and urine post-ingestion. Elimination of the anthocyanins was fast (maximum excretion after 1 h) and plasma levels (0–128.6 nmol/l) and total urinary excretion (0.07–1.35 mg; 0.007–0.133% of the dose ingested) were low. Most significantly, of the hydroxycinnamates, conjugated and free ferulic, isoferulic, *p*-coumaric, sinapic and vanillic acids were identified in plasma and urine, using GC-MS techniques. Quercetin and kaempferol (as glucuronides) and the proposed colonic metabolite of quercetin, 3-hydroxyphenylacetic acid, were detectable in a minority of subjects. Increased daily urinary hippuric, 4-hydroxyhippuric and 3-hydroxyhippuric acid levels were also observed post-ingestion in all volunteers.

Keywords: Blackcurrant; Anthocyanins; Hydroxycinnamates; Flavonols; Absorption; Metabolism

INTRODUCTION

Berry extracts rich in anthocyanins, such as blueberry and elderberry, have recently been linked to the improvement of age-related neurological dysfunction,[1] as well as the protection of red blood cells[2] and cultured stratial neurones[3] against oxidative stress induced damage. These observations contribute to the already established epidemiological correlation of a high intake of fruits and vegetables and lower risks of cardiovascular disease and cancer.[4–6] The anthocyanins, major phenolic constituents in dark coloured berries and red grapes,[7–9] have been implicated as the likely bioactive compounds for the observed protective and preventive effects of berry extracts and red wine, although no direct evidence has yet been provided. The metabolic events in the gastro-intestinal tract following the administration of berry extracts such as degradation in the colonic environment have not been studied. Berries also contain other phenolic compounds than anthocyanins, as well as L-ascorbic acid, and their extracts display high antioxidant activities *in vitro*.[10–12] However, recent studies on other dietary flavonoids have implicated roles for their *in vivo* metabolites in the modulation of cell signaling pathways.[13] Crucial for the understanding of the modes of action anthocyanins is a detailed knowledge of their achievable levels in the circulation (and tissues) and the metabolic events following the ingestion of dark coloured berries. Blackcurrant juice is a good dietary source of anthocyanins containing up to 3 g/l,[14] including delphinidin-3-glucoside, delphinidin-3-rutinoside, cyanidin-3-glucoside,

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and cyanidin-3-rutinoside.[7–9,14] In addition, blackcurrants and also their juice contain high amounts of L-ascorbic acid (around 1 g/l) as well as flavonol glycosides and hydroxycinnamates.[7,15–18]

Absorption and excretion of very low proportions of the intact anthocyanin glycosides has been reported after ingestion of anthocyanin rich berry or wine extracts,[19–25] but little information is available concerning further metabolism such as colonic degradation. The only reported metabolite associated with dietary intake of anthocyanins is protocatechuic acid in rats.[21] In contrast, studies have described the further metabolism of flavonols, flavan-3-ols, citrus flavonoids and hydroxycinnamates.[26–31]

The purpose of this study was to investigate the bioavailability of anthocyanins and biomarkers of the biotransformation of all the ingested dietary polyphenols from blackcurrant juice in humans. In contrast to other researchers[19–25] the focus was on the metabolism of all the polyphenols of the juice with an emphasis on their colonic metabolism, rather than on the absorption and elimination of the anthocyanins of the juice. The balance between the absorption of dietary polyphenols as native compounds and specific conjugated compounds versus their metabolism through colonic degradation, resulting in specific simple phenolic metabolites, was examined and the structural assignments confirmed by LC-MS/MS and GC-MS techniques.

MATERIAL AND METHODS

Chemicals

Ferulic acid, isoferulic acid, chlorogenic acid, sinapic acid, hippuric acid, cyanidin-3-glucoside, cyanidin-3-rutinoside, quercetin-3-glucoside, kaempferol-3-glucoside, kaempferol-3-rutinoside, myricetin, myricitrin, kaempferol were obtained from Extrasynthese, Genay, France. Methanol (HPLC grade), acetonitrile (HPLC grade), acetic acid, acetone were obtained by Rathburn Chemicals LTD, Walkerburn, Scotland. Orthophosphoric acid (85%) was from BDH Laboratory Supplies, Poole, England. Quercetin, *p*-coumaric acid, 3-hydroxyphenylacetic acid, vanillic acid, homovanillic acid, thymol, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, trichloroacetic acid, simulated gastric juice (without pepsin), *N*-(*t*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide chlorosilane, undecane, β-glucuronidase (EC 3.2.1.31) Type L-II from Limpets and Sulfatase (EC 3.1.6.1) Type V from Limpets were purchased from Sigma Chemicals Co., Steinheim, Germany. Delphinidin-3-glucoside and delphinidin-3-rutinoside were obtained from Polyphenols, Norway.

Blackcurrant Juice

The juice was produced from blackcurrant juice concentrate (GlaxoSmithKline, Coleford, UK), which was reconstituted to 150% of the original strength of the juice the concentrate was originally made from. The high acidity of the juice was reduced adding sodium hydroxide and acesulfam K () was used as a sweetener. The juice was then pasteurised for preservation. No preservatives were added.

Study Design

Ethical permission was obtained from the Guy's Research Ethics Committee.

Ten healthy male and female subjects, aged between 22 and 36 years (mean age 28 ± 4.7 years) with a BMI between 17.9 and 25.9 kg/m^2 (mean BMI 23.0 ± 3.2 kg/m^2), were recruited. Exclusion criteria were pregnancy, any form of known liver disease, diabetes mellitus or a previous myocardial infarction, gall bladder problems or abnormalities of fat metabolism, involvement in a weight-reducing dietary regimens, ingestion of any dietary supplements (including dietary fatty acids), consuming more than 15 units of alcohol per week.

The study design and the collection of samples are depicted schematically in Fig. 1. For the first two days of the study, the volunteers consumed a polyphenol-free diet and their 24-h urine was collected each day. The volunteers were instructed to sustain the polyphenol-free diet and to consume approx. 3 l of water per day throughout the entire study. After administration of 330 ml of blackcurrant juice in the morning, collection of the individual urine samples commenced. During this time the subjects were cannulated, and blood samples (10 ml) were taken throughout the day. Baseline blood samples were taken before the ingestion of the juice, and at 1, 2, 4 and 6 h after the ingestion. Urine collection also continued until the following day at 4 p.m. in the evening. The polyphenol-free diet was sustained until the end of the study.

HPLC Analysis

HPLC analysis was undertaken using the Waters system consisting of controller 600, autosampler 717 plus, photodiode array detector 996, on-line degasser. Samples were analysed on a Fluofix column (NEOS Company Ltd., Kobe, Japan), 4.6 × 250 mm, with 5 μm particle size and a guard column of the same material 4.6 × 15 mm. Mobile phase A consisted of water/85% *o*-phosphoric acid (99.5/0.5, v/v) and mobile phase B of acetonitrile/water/85% *o*-phosphoric acid (50/49.5/0.5, v/v/v). The gradient applied was as follows: from 0 to 5 min 100% A, from 5 to 40 min to 70% A and 30% B, from

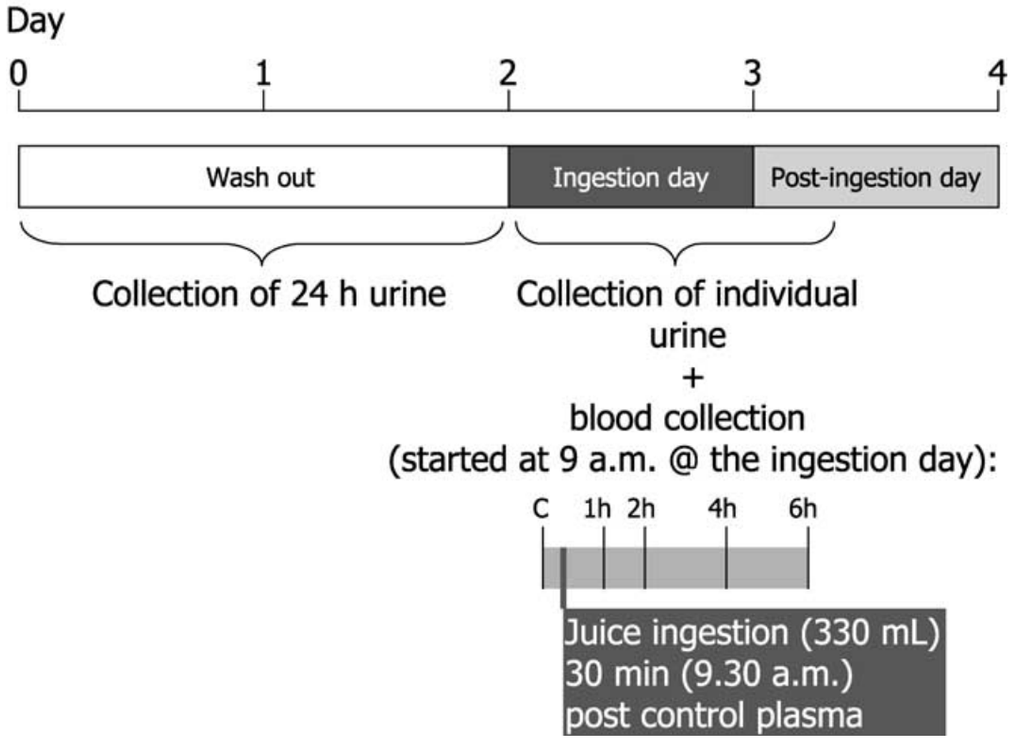

FIGURE 1 Scheme of the study design.

40 to 60 min to 100% B, from 60 to 65 min 100% B and from 65.1 min 100% A. Run time was 70 min followed by a 10 min delay prior to the next injection. Injection volume was 50 μl for treated urine samples and 100 μl for plasma samples and untreated urine. Components in urine and plasma were preliminarily identified according to retention time, UV/visible spectra and spiking with commercially available relevant standards (quercetin, kaempferol, quercetin-3-rutinoside, kaempferol-3-glucoside, kaempferol-3-rutinoside, delphinidin-3-glucoside, delphinidin-3-rutinoside, cyanidin-3-glucoside, cyaniding-3-rutinoside, ferulic acid, isoferulic acid, sinapic acid, *p*-coumaric acid, vanillic acid, 3-hydroxyphenylacetic acid, and hippuric acid). All standard curves were obtained from the authentic standard compounds. 3- and 4-Hydroxyhippuric acids were quantified relative to 3- and 4-hydroxybenzoic acids, respectively. Wavelengths used for quantification were: anthocyanins (520 nm), quercetin, myricetin and kaempferol (370 nm), flavonol glycosides (354 nm), caffeic acid derivatives, chlorogenic acid, ferulic, sinapic and isoferulic acid (324 nm), *p*-coumaric acid (310 nm), 3-hydroxyphenylacetic acid (272 nm), vanillic acid (260 nm), 3-hydroxyhippuric acid (288 nm), 4-hydroxyhippuric acid (254 nm), hippuric acid (225 nm). Limits of quantification in plasma were 2.5 nmol/l for *p*-coumaric acid, 5 nmol/l for anthocyanins, ferulic, sinapic and isoferulic acid, 10 nmol/l for vanillic acid, quercetin and kaempferol, 20 nmol/l for hippuric acid. Coefficient of variance for all standards was $< \pm 3\%$.

LC/MS Analysis

The samples were analysed using a ThermoFinnigan LCQ Deca XP quadrupole ion trap mass spectrometer and a ThermoFinnigan HPLC equipment. Separation was performed using a Phenomenex Aqua C18 column (150×2 mm, 3 μm) with the following gradient (Phase A: 5% methanol in water, 1% trifluoracetic acid, phase B: 50% acetonitrile in water, 1% trifluoracetic acid) 0–5 min 100% A, 5–40 min from 100% A to 50% A, 40–60 min to 0% A, 60–65 min 0% A. Compounds were detected using a full ion scan and identified by a product ion scan.

GC-MS Analysis

Standard solutions were prepared as follows: 1 mg of each standard compound was dissolved in 50 μl dry acetonitrile and 20 μl *N*-(*t*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (TBDMS) containing 1% *N*-(*t*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide chlorosilane (TBDMSCl). After 30 min, 10 μl of the derivatised samples were dried under nitrogen and dissolved in 20 μl undecane. This stock solution was diluted prior to GC/MS analysis. The samples were purified as described before and dried

under nitrogen. The samples were subsequently derivatised with TBDMS as described above.

The samples and standards were analysed on a Fisons GC8000 gas chromatograph, applying a DB-1701 column (column length for plasma 12 m and for urine 15 m, resulting in different retention times) and a Fisons Trio 1000 using EI positive and full scan mode. 1 μl of sample was injected and the following temperature gradient applied: 0–1 min: 150°C, 1–16 min: 20°C/min.

Sample Preparation

Blackcurrant Juice for HPLC Analysis

The blackcurrant juice was filtered through a 0.45 μm membrane prior to HPLC analysis.

Plasma for HPLC Analysis

Plasma (1 ml) was immediately treated with 5 mg L-ascorbic acid and deproteinised with 200 μl 10% trichloracetic acid in methanol (w/w). The sample was then extracted with 1 ml acetone and centrifuged. The resulting supernatant was freeze-dried and the residue dissolved in 250 μl 20% methanol for quantitative HPLC analysis.

For β-glucuronidase treatment 1 ml plasma was acidified with 5 μl acetic acid, prior to the addition of β-glucuronidase, and incubated for 2 h at 37°C. Following this deproteinisation and extraction were undertaken as described above. *trans*-Cinnamic acid was used as internal standard. The precision of the method was $< \pm 5\%$, intra-run variance was $< \pm 15\%$ and the recoveries before and after β-glucuronidase treatment were >80 and $>75\%$, respectively.

Urine for HPLC Analysis

Untreated urine samples were filtered through a 0.45 μm membrane prior to quantitative HPLC analysis. For β-glucuronidase treatment, 2 ml urine was acidified with 5 μl acetic acid prior to the addition of β-glucuronidase and incubated for 2 h at 37°C. The precision of the method was $< \pm 5\%$, intra-run variance was $< \pm 15\%$ and inter run variance $< \pm 30\%$, while the recovery after β-glucuronidase treatment was $>90\%$.

Urine for GC-MS and LC-MS Analysis

Samples of urine (each 10 ml), untreated for the anthocyanins and β-glucuronidase treated, were acidified with 200 μl acetic acid and extract three times with 10 ml ethylacetate. The ethylacetate extract was blown to dryness under a nitrogen gas stream. The residue was dissolved in 1 ml water/methanol/acetic acid (94/5/1, v/v/v) and the solution was applied to a C-18 cartridge (Waters Sep-Pak C18 cartridge), pre-washed with 3 ml methanol and pre-conditioned with 6 ml water/methanol/acetic acid (94/5/1, v/v/v). Following the application of the urine extracts the cartridge was washed with 6 ml water/methanol/acetic acid (94/5/1, v/v/v) and the polyphenols were eluted from the cartridge material with 3 ml methanol/water/acetic acid (60/39/1, v/v/v). The SPE extract was freeze-dried and the residue was used for qualitative GC-MS analysis. For LC-MS analysis the above described SPE procedure was applied to a representative urine sample. The residue of the freeze-dried extract was dissolved in 1% acetic acid and used for qualitative LC-MS analysis.

Gastric Juice Treatment

Two dilutions (1:10) of the blackcurrant juice, one with simulated gastric juice and another one with water (control), were prepared and incubated at 37°C for 3 h. After cooling, the filtered solutions were analysed by HPLC.

RESULTS

Volunteers were supplement with a single dose of blackcurrant juice following a washout period of two days. During the entire study a strictly polyphenol-free diet was followed.

Urinary Excretion

Urine samples were collected on two 24 h cycles prior to the supplementation day, on which the collection of single elimination samples was started and continued for 32 h. Urine samples were analysed by HPLC with PDA detection, after acidification and following treatment with β-glucuronidase/sulfatase to cleave conjugates. The four major anthocyanins of the blackcurrant juice, delphinidin-3-glucoside, delphinidin-3-rutinoside, cyanidin-3-glucoside, cyanidin-3-rutinoside, were detected intact and unmetabolised in the acidified urine of all volunteers post-ingestion of the blackcurrant juice (Fig. 2a). In addition to the anthocyanins, the hydroxycinnamates ferulic, isoferulic, sinapic and *p*-coumaric acid were detected in urine post-β-glucuronidase/sulfatase treatment, and retention time and comparison with standards also suggested vanillic acid (Fig. 2b,c). Increased daily amounts excreted of the common urinary metabolites, 4-hydroxyhippuric, 3-hydroxyhippuric, and hippuric acid were also observed following the ingestion of the blackcurrant juice. Quantification of the identified anthocyanins, conjugates and metabolites was undertaken by HPLC/DAD. Peak identification by HPLC was

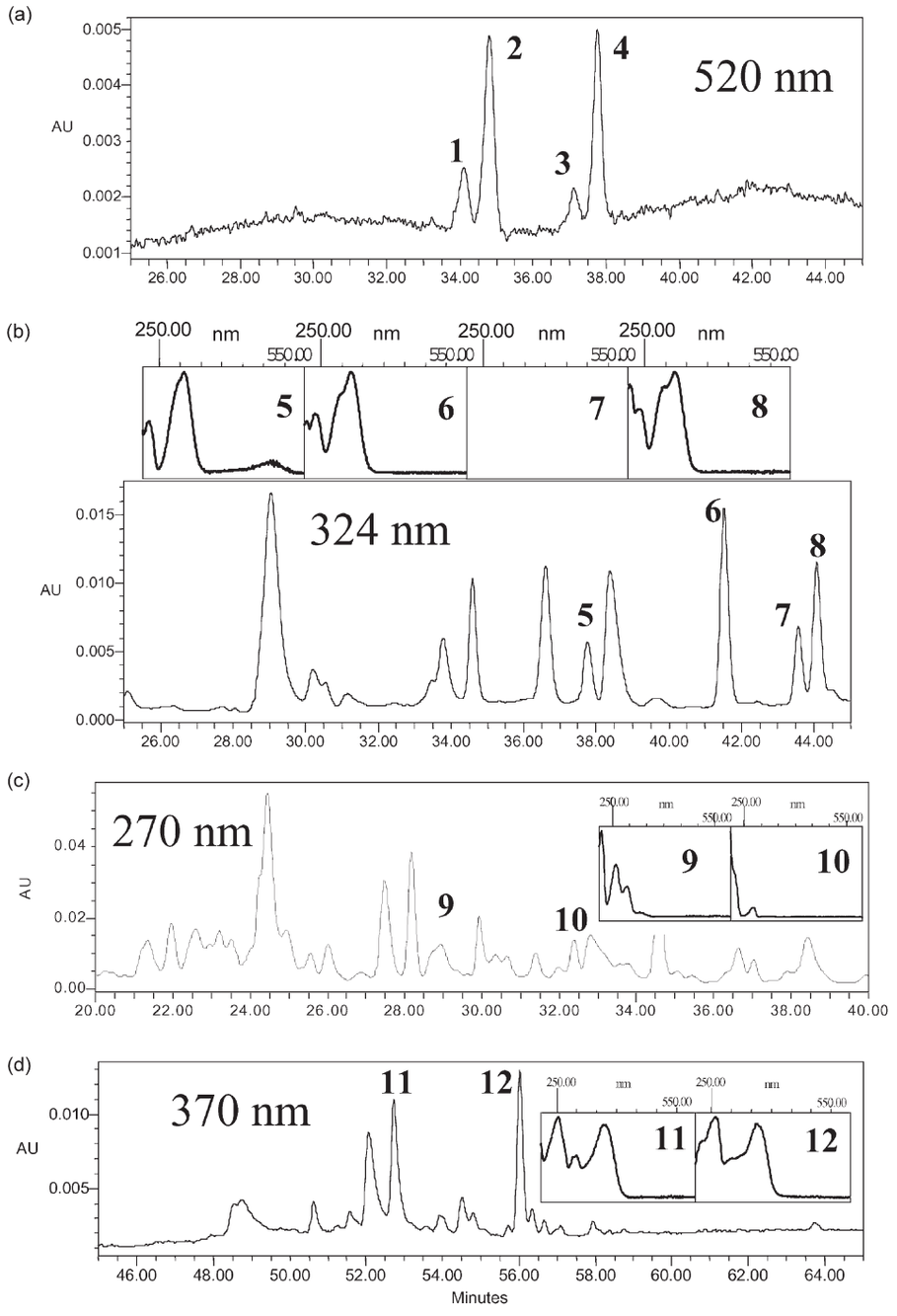

FIGURE 2 Exemplary chromatograms of urine. (a) Acidified urine at 520 nm. Peaks: [1] delphidin-3-glucoside, [2] delphinidin-3-rutinoside, [3] cyanidin-3-glucoside, [4] cyanidin-3-rutinoside. (b) Enzyme treated urine at 324 nm. Peaks including UV-spectra: [5] *p*-coumaric acid, [6] ferulic acid, [7] sinapic acid, [8] isoferulic acid. (c) Enzyme treated urine 270 nm. Peaks including UV-spectra: [9] vanillic acid, [10] 3-hydroxyphenylacetic acid. (d) Enzyme treated urine at 370 nm. Peaks including UV-spectra: [11] quercetin, [12] kaempferol.

based on retention time relative to pure standards, UV-spectra and spiking with authentic commercial standards. The identity of the anthocyanins was then verified using LC/MS analysis of a purified urine sample. Beside the molecular mass ion spectrum of the four anthocyanins (delphinidin-3-glucoside: $m/z = 465$; delphinidin-3-rutinoside: $m/z = 611$; cyanidin-3-glucoside: $m/z = 449$; cyanidin-3-rutinoside: $m/z = 595$) the product ion spectrum of each compound showed a neutral loss of glucose or rutinose resulting in the mass of the anticipated aglycone (Fig. 3). The identity of the identified phenolic acids was directly verified by GC-MS comparing the mass spectra and retention time of a representative extract of β-glucuronidase/sulfatase treated urine with standard data (Fig. 4).

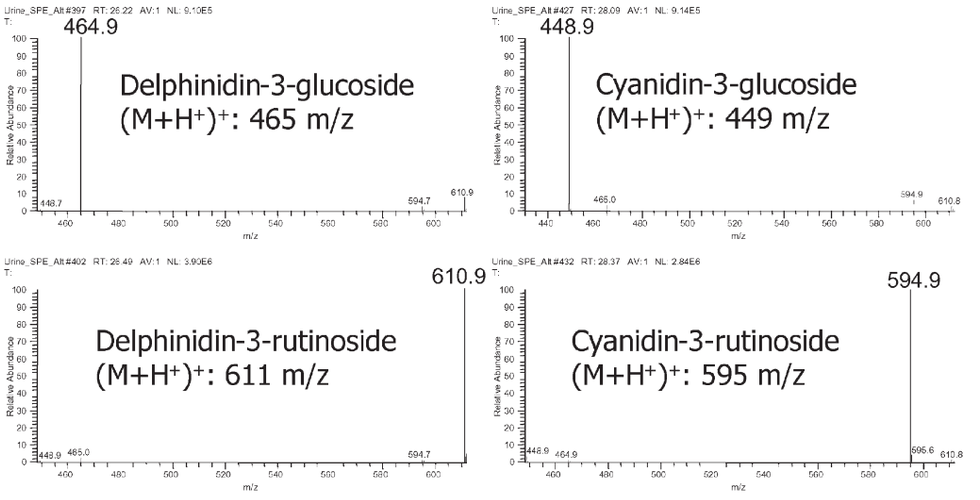

FIGURE 3 Urinary elimination of anthocyanins: Mass spectra of [1] delphidin-3-glucoside, [2] delphinidin-3-rutinoside, [3] cyanidin-3-glucoside, [4] cyanidin-3-rutinoside in urine.

The 3- and 4-hydroxyhippuric acid (base peak *m/z*: 366; RT (retention time): 10.6 and 12.6 min, respectively) were identified using commercially available 2-hydroxyhippuric acid to obtain a comparable mass spectra. The high urinary concentration of hippuric acid (base peak *m/z*: 236; RT: 5.6 min) resulted in strong signal. The hydroxycinnamates ferulic acid (base peak *m/z*: 365; RT: 11.4 min), isoferulic acid (base peak *m/z*: 365; RT: 10.9 min), *p*-coumaric acid (base peak *m/z*: 335; RT: 7.2 min), sinapic acid (base peak *m/z*: 395; RT: 12.7 min) and caffeic acid (base peak *m/z*: 465; RT: 13.5 min) were identified using authentic standards. The phenolic acids vanillic acid (base peak *m/z*: 339; RT: 7.1 min) and 3-hydroxyphenylacetic acid (base peak *m/z*: 323; RT: 5.7 min) were also detected in the analysed urine extract post-β-glucuronidase treatment.

The total daily amounts excreted as well as the time profile of the amounts excreted of the anthocyanins and the identified metabolites varied widely in the cohort of the study. The total daily amounts excreted of the anthocyanins and the identified metabolites during the study are summarised in Table I.

The pattern of the amount-time profile of the identified polyphenols and metabolites appeared to be similar for all volunteers whereas the amounts excreted varied considerably. The total amounts of anthocyanins excreted by all volunteers did not exceed 1.33 mg, which is very low compared to the dose administered of approx. 1 g. The maximum excretion of the anthocyanins occurred 30–90 min after the juice consumption indicating a relatively fast absorption and elimination. An exemplary amount-time profile of the four blackcurrant anthocyanins is shown in Fig. 5a. Based on the total urinary excretion of the anthocyanins, the absorption rate would be as low as 0.007–0.135%. This absorption rate represents an estimate, most likely to low because the extent of tissue distribution and/or bilary excretion could not be measured or estimated due to a general lack of data.

An exemplary amount-time profile of the hydroxycinnamic acids and vanillic acid is shown in Fig. 5b. As for the anthocyanins great individual variation in amount-time profile as well as the total daily amounts excreted were observed. The maximum excretion of the hydroxycinnamic acids and vanillic acid occurred 60–180 min post-ingestion of the juice, which was significantly later than for the anthocyanins.

The two common urinary metabolites of phenolic compounds, hippuric and 4-hydroxyhippuric acid, showed a great increase in the amounts excreted less than 2 h post-ingestion of the blackcurrant juice. In contrast, the maximum excretion of 3-hydroxyhippuric acid appeared 8–16 h post-ingestion suggesting an independent metabolic pathways, most likely colonic degradation of ingested polyphenols, resulting in the different response to the ingestion of the juice. Quercetin and kaempferol were only detectable in urine post-β-glucuronidase/sulfatase treatment of two volunteers of Southeast Asian origin adding to a total amount excreted of 0.11 and 0.44 mg of quercetin and 0.09 and 0.30 mg kaempferol (Fig. 2d). The proposed colonic metabolite of quercetin, 3-hydroxyphenylacetic acid,[32,33] was present in urine of four volunteers (total excretion of 0.71–3.35 mg) (Fig. 2c). An exemplary amount-time profile of the two flavonols and 3-hydroxyphenylacetic acid is shown in Fig. 5c. The amounts excreted of homovanillic and 4-hydroxyphenylacetic acid were also monitored but remained relatively steady throughout the study

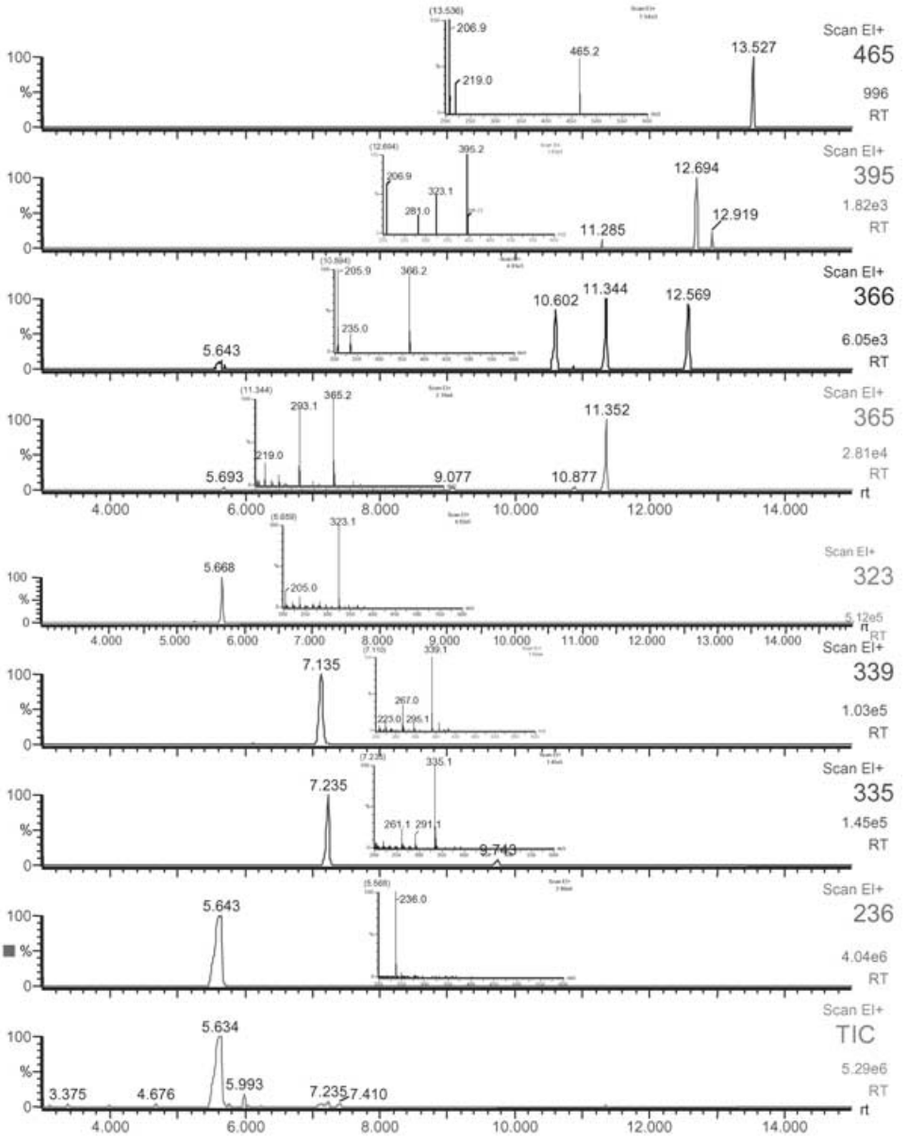

FIGURE 4 Selective ion chromatograms and mass spectra of the TBDMS derivates of the identified phenolic acid isolated from β-glucuronidase treated urine post-supplementation. [5.6 min] hippuric acid (BP: 236 m/z); [5.7 min] 3-hydroxyphenylacetic acid (BP: 323 m/z); [7.1 min] vanillic acid (BP: 339 m/z); [7.2 min] *p*-coumaric acid (BP: 335 m/z); [10.6 min] 3-hydroxyhippuric acid (BP: 366 m/z), [10.9 min] isoferulic acid (BP: 365 m/z); [11.4 min] ferulic acid (BP: 365 m/z); [12.6 min] 4-hydroxyhippuric acid (BP: 366 m/z); [12.7 min] sinapic acid (BP: 395 m/z).

and displayed no correlation to the ingestion of the blackcurrant juice.

Plasma Levels

Plasma samples were drawn prior to the blackcurrant juice administration and 1, 2, 4 and 6 h post-administration and extracted as described in the "Material and Methods" section. The resulting extracts, without and with β-glucuronidase/sulfatase treatment, were analysed by HPLC/DAD. The four intact blackcurrant anthocyanins were detected in the plasma extracts (1-h post-ingestion) of 9 out of 10 volunteers (Fig. 6a). The anthocyanin composition in the plasma was almost identical to the anthocyanins composition of the blackcurrant juice indicating a similar bioavailability for the anthocyanins despite the difference in structure and sugars attached. The hydroxycinnamates ferulic, isoferulic, sinapic and *p*-coumaric acids as well as the benzoate vanillic acid were all detected in plasma post-β-glucuronidase/sulfatase following the ingestion of the blackcurrant juice (Fig. 6b,c). Again, as with the studies, the flavonols quercetin and kaempferol were detectable in plasma post-β-glucuronidase/sulfatase urine of the two volunteers of South-East Asian origin

TABLE I Means and range (in brackets) of the total daily urinary excretion of the identified anthocyanins, conjugates and metabolites during the study ($n = 10$)

	Mean (range) [mg]		
	Washout day 1	Washout day 2	Study day
Delphinidin-3-glucoside	n.d.	n.d.	0.02 (n.d.–0.08)
Delphinidin-3-rutinoside	n.d.	n.d.	0.23 (0.03–0.78)
Cyanidin-3-glucoside	n.d.	n.d.	0.01 (n.d.–0.05)
Cyanidin-3-rutinoside	n.d.	n.d.	0.16 (0.03–0.49)
p-Coumaric acid	0.01 (0–0.12)	n.d.	0.44 (0.30–0.71)
Sinapic acid	0.02 (0–0.18)	n.d.	0.55 (0.17–0.86)
Ferulic acid	0.37 (0–0.87)	0.24 (n.d.–0.70)	1.21 (0.46–1.76)
Isoferulic acid	0.02 (0–0.18)	n.d.	0.46 (0.14–0.73)
Vanillic acid	0.35 (0–1.95)	0.45 (n.d.–2.65)	2.09 (1.16–2.66)
3-Hydroxyhippuric acid	25.97 (0.30–82.14)	3.80 (0.30–17.87)	7.30 (0.68–14.90)
4-Hydroxyhippuric acid	9.02 (0.80–22.29)	6.66 (0.55–33.45)	25.21 (14.29–60.32)
Hippuric acid	399.50 (48.04–1309.83)	117.20 (21.05–294.84)	514.45 (337.63–778.81)

n.d.: Not detectable.

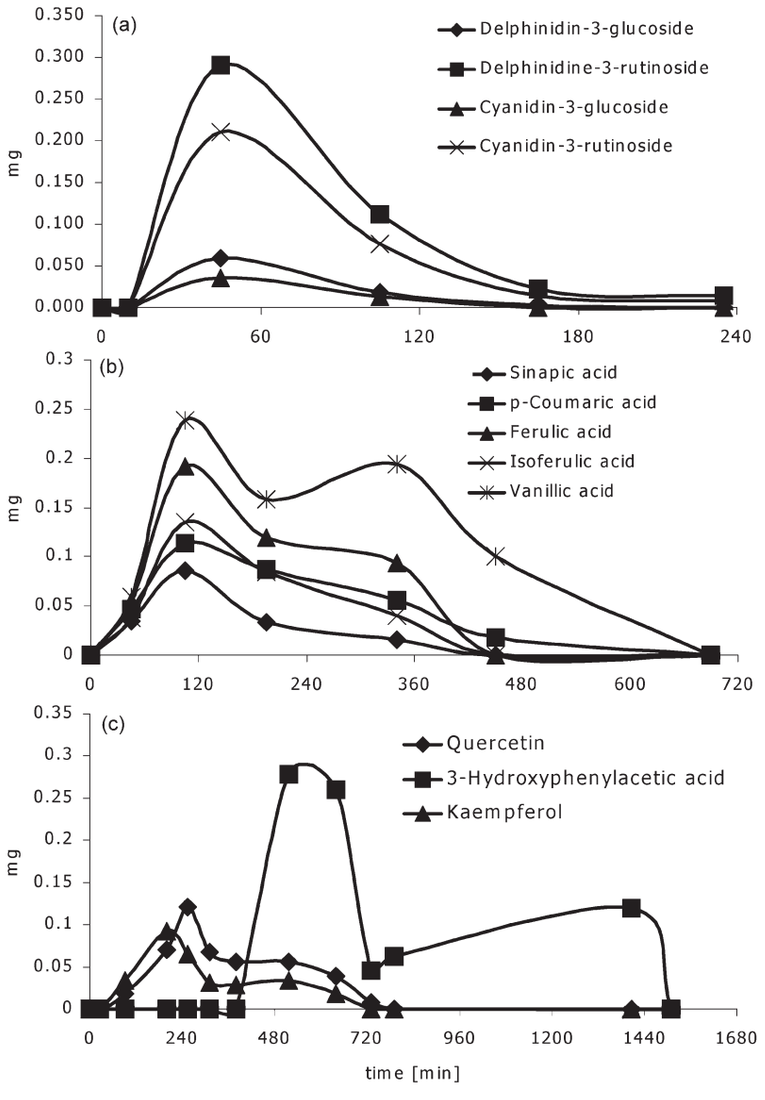

FIGURE 5 Exemplary amount-time-profiles of the identified anthocyanins in acidified urine (a), the hydroxycinnamic acids and vanillic acid in enzyme treated urine (b) and flavonols and 3-hydroxyphenylacetic acid in enzyme treated urine (c).

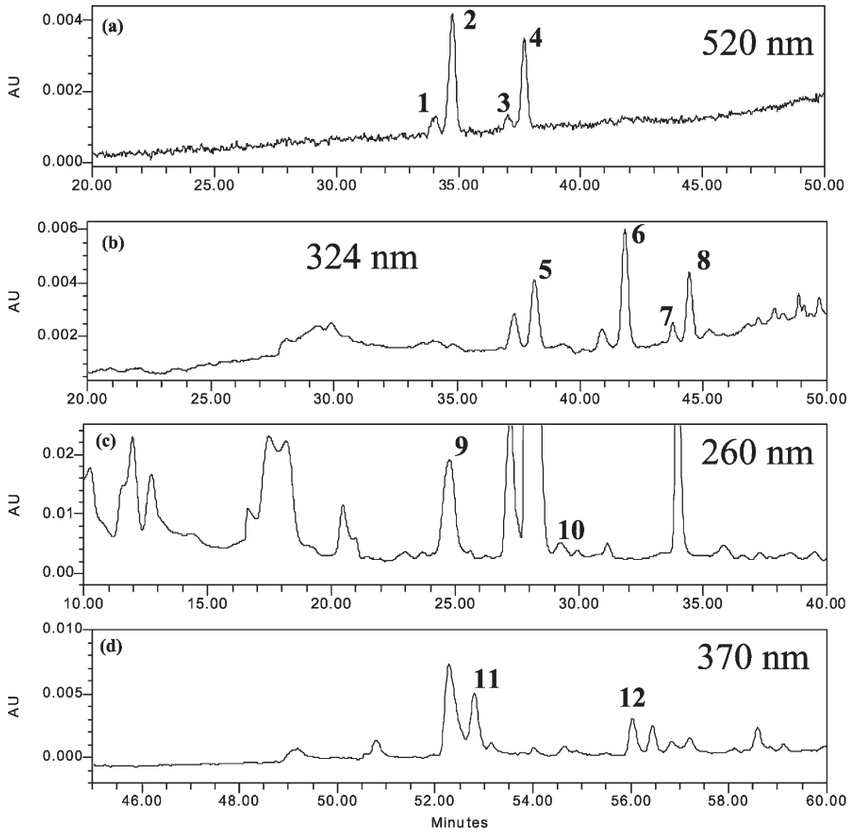

FIGURE 6 Exemplary chromatograms of extracted plasma. (a) Untreated plasma at 520 nm. Peaks: [1] delphidin-3-glucoside, [2] delphinidin-3-rutinoside, [3] cyanidin-3-glucoside, [4] cyanidin-3-rutinoside. (b) Enzyme treated plasma at 324 nm. Peaks: [5] *p*-coumaric acid, [6] ferulic acid, [7] sinapic acid, [8] isoferulic acid. (c) Enzyme treated plasma at 260 nm. Peaks: [9] hippuric acid, [10] vanillic acid. (d) Enzyme treated plasma at 370 nm. Peaks: [11] quercetin, [12] kaempferol.

(Fig. 6d). The quantitative results of the plasma samples are summarised in Table II.

The highest plasma concentrations of the anthocyanins (delphinidin-3-glucoside 6.1 nmol/l, delphinidin-3-rutinoside 51.3 nmol/l, cyanidin-3-glucoside 3.5 nmol/l, cyanidin-3-rutinoside 24.4 nmol/l; $n = 10$) were achieved in the 1-h post-ingestion sample (Fig. 7a). The plasma concentrations of the anthocyanins strongly decreased in the 2 and 4 h post-ingestion samples implying rapid absorption and elimination of the anthocyanins in the circulation. No anthocyanins were present in plasma sample 6 h post-ingestion, apart from one volunteer with residual level of delphinidin-3-rutinoside. The hydroxycinnamic acids and vanillic acid reached their highest plasma concentration 1 or 2 h post-ingestion (*p*-coumaric acid 37.2 nmol/l after 1 h, ferulic acid 121.8 nmol/l after 1 h, isoferulic acid 73.9 nmol/l after 1 h, sinapic acid 9.8 nmol/l after 1 h, vanillic acid 150.0 nmol/l after 2 h; $n = 10$), which indicates a prolonged pathway of absorption and elimination in comparison to the anthocyanins (Fig. 7b). The highest plasma concentration of quercetin and kaempferol concentration in the two Southeast Asian volunteers (quercetin 139.1 nmol/l, kaempferol 145.2 nmol/l after 4 h and quercetin 22.5 nmol/l, kaempferol 56.3 nmol/l after 2 h) appeared 2 and 4 h post-ingestion of the blackcurrant juice implicating a more prolonged pathway of absorption and elimination than those of the identified metabolites and the anthocyanins (Fig. 7c). The plasma concentration of hippuric acid (basal level 0.52 μmol/l; $n = 10$) increased approx. 25 fold (12.64 μmol/l; $n = 10$) in relation to the basal level 1-h post-ingestion and declined thereafter to reach approximately three times the basal levels 6 h post-ingestion (1.44 μmol/l; $n = 10$). Great individual variations in the plasma levels of the anthocyanins and the identified metabolites were observed in the cohort of the study, i.e. delphinidin-3-rutinoside varied between not detectable and 124 nmol/l in 1 h post-ingestion plasma sample. The variations of the plasma levels at the different sampling time points are also displayed in Table II.

TABLE II Mean concentrations and concentration ranges of the identified anthocyanins, conjugates and metabolites in plasma at 0, 1, 2, 4 and 6 h post-ingestion of 330 ml blackcurrant juice ($n = 10$)

	Control (range)	1 h (range)	2 h (range)	4 h (range)	6 h (range)
Delphinidin-3-glucoside [nmol/l]	n.d.	6.1 (n.d.–17.8)	1.1 (n.d.–7.1)	n.d.	n.d.
Delphinidin-3-rutinoside [nmol/l]	n.d.	51.3 (9.8–128.6)	29.3 (n.d.–84.9)	10.1 (n.d.–36.2)	1.4 (n.d.–12.3)
Cyanidin-3-glucoside [nmol/l]	n.d.	3.5 (n.d.–14.3)	0.6 (n.d.–3.8)	n.d.	n.d.
Cyanidin-3-rutinoside [nmol/l]	n.d.	24.4 (6.9–57.1)	12.9 (n.d.–34.2)	3.6 (n.d.–12.2)	n.d.
p-Coumaric acid [nmol/l]	n.d.	37.2 (21.3–55.0)	27.1 (9.7–47.4)	9.5 (4.0–24.6)	3.3 (n.d.–17.9)
Sinapic acid [nmol/l]	n.d.	9.8 (n.d.–24.7)	6.5 (n.d.–16.2)	0.5 (n.d.–5.5)	n.d.
Ferulic acid [nmol/l]	n.d.	122 (48.9–241.5)	115 (42.2–188.9)	52 (19.2–114.2)	28.9 (n.d.–101.0)
Isoferulic acid [nmol/l]	n.d.	73.9 (22.0–166.9)	75.6 (14.0–125.3)	27.5 (n.d.–53.8)	10.6 (n.d.–36.6)
Vanillic acid [nmol/l]	n.d.	126 (54.7–196.9)	150 (63.2–251.9)	62.7 (n.d.–158.4)	28 (n.d.–112.5)
Hippuric acid [μmol/l]	0.52 (0.07–1.05)	12.6 (6.62–19.52)	3.83 (270–7.02)	1.96 (1.12–3.69)	1.44 (0.39–3.11)

n.d.: not detectable.

FIGURE 7 Time-concentration profiles ($n = 10$) of the identified anthocyanins in plasma (a), the hydroxycinnamic acids and vanillic acid in enzyme treated plasma (b) and flavonols in enzyme treated plasma (c).

Juice Analysis

The blackcurrant juice ingested (single dose of 330 ml) was directly analysed by HPLC/DAD, which revealed a complex composition and high amounts of anthocyanins, hydroxycinnamates and flavonol glycosides. The identified and tentatively assigned polyphenols and their amounts expressed in mg/330 or mg/ingested dose in the blackcurrant juice are listed in Table III. The four major anthocyanins and three unidentified minor anthocyanins of the blackcurrant juice added to an ingestion of more than 1 g of total anthocyanins per single dose. Based on the total urinary excretion and the amounts ingested of the individual anthocyanins absorption rates of 0.046% for delphindin-3-rutinoside, 0.045% for cyanidin-3-rutinoside, 0.039% for delphinidin-3-glucoside, and 0.032% for cyanidin-3-glucoside were calculated.

TABLE III Amounts of identified and tentatively assigned polyphenols in 330 ml of the blackcurrant juice ingested ($n = 3$)

Anthocyanins	
Delphinidin-3-glucoside	98.2 mg/330 ml
Delphinidin-3-rutinoside	493.3 mg/330 ml
Cyanidin-3-glucoside	60.2 mg/330 ml
Cyanidin-3-rutinoside	358.2 mg/330 ml
4 unidentified anthocyanins*	29.3 mg/330 ml
Flavonols	
Myricetin-3-rutinoside†	31.46 mg/330 ml
Myricetin-3-glucoside†	34.84 mg/330 ml
Quercetin-3-rutinoside	14.83 mg/330 ml
Quercetin-3-glucoside	8.20 mg/330 ml
Kaempferol-3-rutinoside	2.52 mg/330 ml
Kaempferol-3-glucoside	0.20 mg/330 ml
Myricetin	8.22 mg/330 ml
Quercetin	1.83 mg/330 ml
Kaempferol	0.34 mg/330 ml
Hydroxycinnamates	
Caffeic acid derivatives‡	22.2 mg/330 ml
p-Coumaric acid derivatives‡	32.0 mg/330 ml
Hydroxybenzoic acids	
Protocatechuic acid	1.07 mg/330 ml
4-hydroxybenzoic acid glucoside¶	0.94 mg/330 ml
Total anthocyanins	1029.2 mg/330 ml
Total polyphenols HPLC	1197.9 mg/330 ml

*Quantified relative to cyanidin-3-glucoside. †Tentative assignments; quantified relative to myricetin-3-rhamnoside (myricetin-3-glucoside) and quercetin-3-rutinoside (myricetin-3-rutinoside). ‡Tentative assignments; quantified relative to chlorogenic acid. ¶Tentative assignment; quantified relative to 4-hydroxybenzoic acid.

Gastric Juice Treatment

In order to ascertain the potential effects of the acidic environment of the gastric lumen, the blackcurrant juice was incubated with simulated gastric juice. The incubation of the blackcurrant juice (1:10 dilution) with simulated gastric juice (pH 2.0) had no effect on the polyphenol composition.

DISCUSSION

The absorption, metabolism and elimination of all three major groups of polyphenols in blackcurrant juice, namely anthocyanins, hydroxycinnamates and flavonols has been demonstrated following the ingestion of a single dose of blackcurrant juice. The absorption of intact anthocyanins in very low amounts in humans has previously been shown by other researchers[19–25] using purified compounds or highly concentrated extracts from berries or wine but not dietary sources. Our studies revealed that the blackcurrant anthocyanins, delphinidin-3-glucoside, delphinidin-3-rutinoside, cyanidin-3-glucoside, cyanidin-3-rutinoside, were rapidly absorbed and eliminated intact directly from the juice consistent with these findings but in very low amounts (combined average of 0.42 mg). The plasma levels, pharmacokinetic behaviour and absorption rates of the four anthocyanins achieved in this study are consistent with the results of Matsumoto *et al.*,[23] involving administration of purified anthocyanins from blackcurrants, and the results obtained in other studies.[20,24] The focus of the latter studies was exclusively on the anthocyanins while the metabolic fate of other polyphenolic constituents of berries or the aspects of colonic metabolism were not investigated. However, conjugated hydroxycinnamic acids and flavonols (two volunteers) deriving from other polyphenols in the blackcurrant juice as well as accompanied the anthocyanins, but reached significantly higher plasma and urinary concentrations (maximum plasma levels of up to 250 nmol/l) than the anthocyanins. These hydroxycinnamic acids, namely sinapic, *p*-coumaric acid and possibly also ferulic acid, which are detected in the glucuronidated form and the caffeic acid metabolites isoferulic and also ferulic acid, emerged as quantitatively more important metabolites than the anthocyanins, despite much lower amounts of hydroxycinnamates relative to the anthocyanins in the original blackcurrant juice. The maximum plasma levels of the conjugated hydroxycinnamic acids and the metabolite vanillic acid were significantly higher than the maximum levels reported in studies following the ingestion of hydroxycinnamate-rich meal[35] or prunes[36] while their concentration-time profiles showed great similarity. Significant variations in both plasma urine levels of conjugates and metabolites between the individuals in the cohort of the study were observed throughout the study. Contributing factors might be individual variations in levels and activities of small intestinal enzymes and transporters as well as the characteristic individual compositions of the colonic microflora. Identified secondary metabolites derived from colonic interactions were also identified in plasma and urine. In particular, vanillic acid with maximum plasma levels being in the range of 63.2–251.9 nmol/l and daily urinary levels in the range of 1.16–2.66 mg/day, and 3-hydroxyphenylacetic acid with daily urinary levels in the range of n.d.–3.35 mg/day. 3-Hydroxyphenylacetic acid, a colonic metabolite associated with quercetin and its glycosides,[32,33] was only detected in four volunteers, and the flavonols quercetin and kaempferol only in two volunteers indicating a low and variable bioavailability of flavonol glycoside from the blackcurrant juice in humans. The low bioavailability as well as slow absorption and elimination of flavonol glycosides have been previously described.[34] The major metabolic events associated with the metabolism of hydroxycinnamates and the flavonol glycosides are the release and absorption of the free hydroxycinnamic acids and flavonol aglycones in the gastro-intestinal tract, followed by glucuronidation and, in some instances, *O*-methylation.

The anthocyanins are not susceptible to Phase I or Phase II metabolism in the small intestine since neither the corresponding aglycones nor *O*-methylation products of the anthocyanins were detectable in plasma or urine, both previously identified in rats following the administration of purified anthocyanins.[20,21] Glucuronidation of the anthocyanins was also not evident.

The quantitative results derived in this study indicate that the majority of the ingested polyphenols from the blackcurrant juice are subjected to metabolism in the colon. The ingestion of high amounts of polyphenols have also been linked with increased urinary excretion of hippuric acids, such as hippuric acid, 3-hydroxyhippuric acid, and 4-hydroxyhippuric acid.[35,37–39] Following the ingestion of the blackcurrant juice significant increases in the daily amounts excreted of all three hippuric acids were detected but great differences in the amount-time profiles of the three hippuric acids observed mirroring differences in metabolic origin and pathways of formation. The relatively immediate increase of hippuric acid and 4-hydroxyhippuric acid following the ingestion of the juice cannot be explained by colonic degradation of polyphenols from the juice. This necessary time for colonic degradation is mirrored in the amount-time profile of the 3-hydroxyhippuric acid excretion, which has been strongly associated with the colonic degradation of hydroxycinnamates, such as caffeic and ferulic acid derivatives,[39] peaking after more than 12 h post-ingestion. The colonic metabolite of the flavonols, 3-hydroxyphenylacetic acid, displayed a similar excretion pattern to 3-hydroxyhippuric acid reaching its maximal excretion more than 8 h post-ingestion.

Following the ingestion the complex polyphenol pattern of the blackcurrant juice is metabolically reduced to a small number of conjugates and metabolites present in plasma and urine, apart from the small amounts of anthocyanins absorbed and excreted. The results provide crucial information for future investigations of potential bioactivities of dietary blackcurrant polyphenols and their *in vivo* forms and concentrations following the consumption of the juice.

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