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Research Article

Absorption, metabolism and excretion of Choladi green tea flavan-3-ols by humans

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Ten healthy human subjects consumed 500 mL of Choladi green tea, containing 648 μmol of flavan-3-ols after which plasma and urine were collected over a 24 h period and analysed by HPLC-MS. Plasma contained a total of ten metabolites, in the form of *O*-methylated, sulphated and glucuronide conjugates of (epi)catechin and (epi)gallocatechin, with 29 – 126 nM peak plasma concentrations (C_{max}) occurring 1.6 – 2.3 h after ingestion, indicative of absorption in the small intestine. Plasma also contained unmetabolised (–)-epigallocatechin-3-gallate and (–)-epicatechin-3-gallate with respective C_{max} values of 55 and 25 nM. Urine excreted 0 – 24 h after consumption of green tea contained 15 metabolites of (epi)catechin and (epi)gallocatechin, but (–)-epigallocatechin-3-gallate and (–)-epicatechin-3-gallate were not detected. Overall flavan-3-ol metabolite excretion was equivalent to 8.1% of intake, however, urinary (epi)gallocatechin metabolites corresponded to 11.4% of (epi)gallocatechin ingestion while (epi)catechin metabolites were detected in amounts equivalent to 28.5% of (epi)catechin intake. These findings imply that (epi)catechins are highly bioavailable, being absorbed and excreted to a much greater extent than most other flavonoids. It is also evident that flavan-3-ol metabolites are rapidly turned over in the circulatory system and as a consequence C_{max} values are not an accurate quantitative indicator of the extent to which absorption occurs.

Keywords: Flavan-3-ols / Glucuronide / Green tea / Methyl / Urinary excretion

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1 Introduction

Tea produced mainly from infusions of dried leaves of *Camellia sinensis* is a popular beverage throughout the world. There is much interest in the potential health benefits of green tea as animal models and *in vitro* studies have demonstrated that it has antioxidant, antimutagenic, anticarcinogenic and antihypertensive properties [1 – 4]. However, epidemiological studies present conflicting reports on the protective role of green tea against cancer [5 – 7], cardiovascular disease [8, 9] and diabetes [10]. The protective effects of green tea have been attributed to its high flavan-3-ol (aka catechins) content (Fig. 1), the most abundant component being (–)-epigallocatechin-3-gallate [11, 12] although in some less common teas (–)-epicatechin-3-gallate predominates [13].

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In order for green tea flavan-3-ols to exert protective effects on human health *in vivo*, they need to be absorbed and reach target specific tissues in a bioactive form. After acute ingestion flavan-3-ols are absorbed and metabolised, and appear in blood and urine [14 – 16]. There are reports of catechins, including (–)-epigallocatechin-3-gallate, being detected in blood as both intact forms and metabolites, and reaching micromolar concentrations in plasma [17 – 19]. Typically, HPLC with absorbance or electrochemical detection has been used to analyse plasma and/or urine after treatment with glucuronidase/sulphatase to convert metabolites to the native aglycone flavan-3-ol structures [16, 19, 20]. As a consequence, only indirect information on the metabolites has been obtained. Recently, HPLC with multistage mass MS detection (*i.e.* MS^2 and MS^3) has been used to analyse flavan-3-ols in body fluids and this has facilitated the identification of metabolites [21, 22].

This paper reports on a quantitative pharmacokinetic study of the absorption and metabolism of flavan-3-ols in healthy human volunteers after the ingestion of 500 mL of Choladi green tea. This was achieved by analysing flavan-3-ols and their metabolites in plasma and urine samples col-

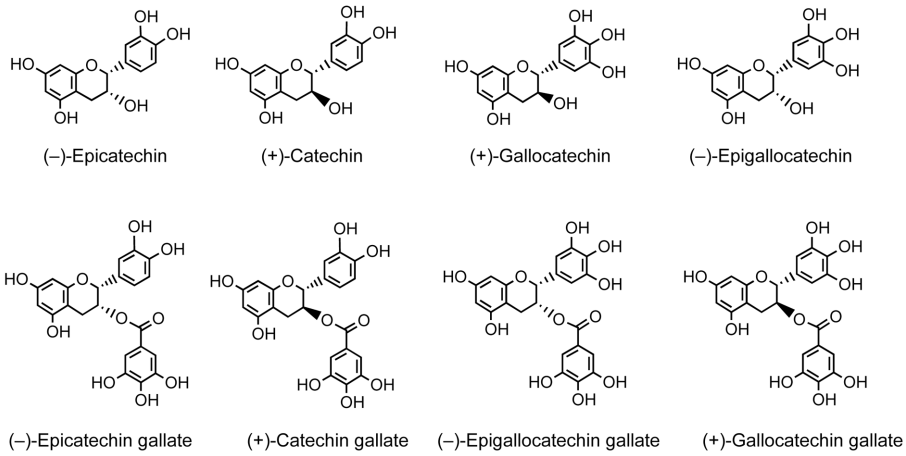

Figure 1. Structures of green tea flavan-3-ols.

lected over a 24 h period after intake. Compounds were identified by HPLC-MS^3 and subsequently quantified using HPLC with MS in the selective ion monitoring (SIM) mode.

2 Materials and methods

2.1 Tea and chemicals

Five hundred millilitre bottles of Choladi green tea beverage were supplied by Beverage Partners Worldwide (Zürich, Switzerland). The green tea (Choladi GTFT; Nestlé, Choladi, India) is a cold, water-soluble extract prepared from hand picked leaves. The tea factory is located in the midst of tea gardens in the Nilgiris (Blue Mountain) district of Tamil Nadu state in South India so as to enable processing to take place within 24 h of the leaves being picked. Choladi is located at an altitude of 850 m above sea level. (–)-Gallocatechin, (–)-epicatechin, (+)-catechin and (–)-gallocatechin-3-gallate were obtained from Sigma (Poole, Dorset, UK), and (–)-epigallocatechin, (–)-epigallocatechin-3-gallate and (–)-epicatechin-3-gallate were purchased from Apin Chemicals (Abingdon, UK). Ethyl gallate was obtained from Fluka (Sigma Aldrich, Poole, Dorset, UK). (–)-Epicatechin-7-*O*-glucuronide was a gift from Professor Junji Terao and Dr. Yoshichika Kawai (University of Tokushima, Japan). Dr. Yukihiko Hara (Mitsui Norin, Tokyo, Japan) kindly supplied standards of 3′- and 4′-*O*-methyl-(–)-epicatechin. HPLC grade solvents were obtained from Rathburn Chemicals (Walkerburn, Pebbles, Scotland, UK). Formic acid was supplied by Fisher Chemicals.

2.2 Human feedings studies

The feeding study was carried out with ten human volunteers. For 2 days prior to, and 24 h after the ingestion of green tea, the subjects followed a diet low in flavonoids and phenolic compounds by avoiding fruit, nuts, vegetables, tea, coffee, fruit juices, wine and dietary antioxidant supplements. On the day of the study, after an overnight fast, each subject drank 500 mL of Choladi green tea. Basal venous blood samples were collected at 0 h and, after drinking the tea, further blood samples were collected at 0.5, 1, 2, 4, 6, 8 and 24 h time points. The blood was collected in heparin tubes and centrifuged immediately at $3000 \times g$ for 15 min at 4°C, after which the plasma was divided into 1 mL aliquots to which was added 30 μL of 50% aqueous formic acid and 100 μL of 10 mM ascorbic acid, before being stored at −80°C prior to extraction and analysis. Urine was collected before the volunteers drank the tea and 0–5, 5–12, 12–24 h after consumption. The volume of urine collected during each period was measured and aliquots stored at −80°C prior to analysis by HPLC-DAD-MS^3 without further processing.

2.3 Extraction of plasma

Plasma samples were extracted using a method developed by Day *et al.* [23], and consisted of adding dropwise 450 μL of plasma to 1125 μL of ACN in a 2 mL Eppendorf tube. Each sample was spiked with 20 μL of 10% aqueous ascorbic acid containing 0.5 mM EDTA and 1 μg of ethyl gallate as an internal standard (method adapted from ref. [19]). The samples were vortexed for 30 s every 2 min, and after 10 min were centrifuged for 20 min at $1500 \times g$ at 4°C. The supernatant was decanted and the pellet re-extracted with 1125 μL of methanol; after centrifugation the two supernatants were combined and reduced to dryness under a stream of nitrogen at 35°C. The residues were resuspended in 25 μL of methanol to which was added 225 μL of 0.1% aqueous formic acid. Once resuspended, the plasma extracts were centrifuged at $16\,100 \times g$ for 10 min at 4°C, in a 0.2 μm Micro-Spin™ Eppendorf filter (Alltech Associ-

ates Applied Sciences, Lancashire, UK) prior to analysis by HPLC-DAD-MS.

2.4 HPLC-DAD-ESI-MS analysis

Flavan-ols and their metabolites in green tea, plasma and urine were analysed using a Surveyor HPLC with a DAD and a Finnigan LCQ Duo IT mass spectrometer fitted with an electrospray interface (ESI) (Thermo Finnigan, San Jose, USA). Separations were performed at 40°C using a Phenomenex Synergi 4 µm RP-MAX 80 Å 250 × 4.6 mm (id) RP column (Phenomenex, Macclesfield, UK). Injections were carried out using an autosampler maintained at 4°C. The mobile phase, pumped at a flow rate of 1 mL/min, was a 60 min gradient of 4 – 25% ACN in 0.1% aqueous formic acid. The column eluant passed through the flow cell of the DAD and was then split and 0.3 mL/min directed to the mass spectrometer with ESI operating in full scan negative ionisation mode (100 – 1000 *m*/*z*). Analyses of samples were initially carried out using full scan, data dependant MS scanning from *m*/*z* 100 to 1000. The tuning of the mass spectrometer was optimised by infusing a standard of (–)-epicatechin into the source along with the 4% ACN in 0.1% aqueous formic acid, the initial HPLC mobile phase, at a flow rate of 0.3 mL/min. Capillary temperature was 275°C, sheath gas and auxiliary gas were 80 and 60 U/min, respectively, source voltage was 3 kV, with collision energy set at 35%, and in-source fragmentation at 5 V.

Following HPLC separation and MS^3 identification, flavan-3-ols and their metabolites were quantified using HPLC with the MS operating in the SIM mode. Unmetabolised flavan-3-ols were quantified by reference to the appropriate standard compound while metabolites of (epi)catechin and (epi)gallocatechin in urine and plasma were quantified using (–)-epicatechin and (–)-epigallocatechin, respectively.

2.5 Plasma extraction efficiency

The efficiency of the extraction of flavan-3-ols from plasma was evaluated by spiking blank plasma with 1 µg of ethyl gallate and 1 µg of (–)-epicatechin, (–)-epigallocatechin, (–)-epigallocatechin-3-gallate and (–)-epicatechin-3-gallate. The spiked samples were extracted as described above and analysed in triplicate by HPLC-SIM. The percentage recoveries of the individual flavan-3-ols and ethyl gallate are presented in Table 1. The recovery of the flavan-3-ols is also expressed as a ratio relative to the recovery of ethyl gallate. This ratio was subsequently used in combination with the recovery of the ethyl gallate internal standard to assess recoveries of (–)-epigallocatechin-3-gallate and (–)-epicatechin-3-gallate as well as metabolites of (–)-epicatechin and (–)-epigallocatechin from individual plasma samples.

Table 1. Recoveries of flavan-3-ols from plasma relative to the recovery of ethyl gallate

	% Recovery[a)]	Ratio[b)]
(–)-Epigallocatechin	74 ± 5	1.0
(–)-Epicatechin	55 ± 2	1.3
(–)-Epigallocatechin-3-gallate	41 ± 4	1.7
(–)-Epicatechin-3-gallate	40 ± 2	1.8
Ethyl gallate	71 ± 1	1.0

a) Data expressed as mean values ± SE (n = 3).
b) Expressed as a ratio relative to the recovery of ethyl gallate.

2.6 Pharmacokinetic analysis of flavan-3-ols and their metabolites in plasma

Maximum plasma concentration of the flavan-3-ol derivatives from 0 to 24 h postdose was defined as C_{max}, with T_{max} being the time at which C_{max} was reached. The elimination half-life ($T_{1/2}$) for the metabolites was computed by using the following formula $T_{1/2} = 0.693/Ke$ where Ke is the slope of the linear regression of the plasma metabolite concentrations. Area-under-the-curve (AUC) calculations were determined using a Kinetica software package (Thermo Electron Corporation).

3 Results

3.1 Analysis of green tea

The flavan-3-ol content of 500 mL of Choladi green tea was 257 ± 4 µmol (standard error n = 3) (–)-epigallocatechin; 230 ± 6 µmol (–)-epigallocatechin-3-gallate; 58 ± 1 µmol (–)-epicatechin; 49 ± 1 µmol (–)-epicatechin-3-gallate; 36 ± 1 µmol (+)-gallocatechin; 9 ± 1 µmol (+)-catechin and 8 ± 1 µmol (+)-gallocatechin-3-gallate; making a total of 648 ± 6 µmol of total flavan-3-ols. The tea has a faint citrus flavour but no flavanones were detected.

3.2 Identification of flavan-3-ols and their metabolites in plasma and urine

Plasma and urine samples collected after the ingestion of 500 mL of green tea were analysed by HPLC-DAD-MS^3 (Table 2, Fig. 2). Two out of the seven green tea flavan-3-ols, (–)-epigallocatechin-3-gallate and (–)-epicatechin-3-gallate, were detected in plasma along with 10 metabolites, while 15 metabolites of (epi)catechin and (epi)gallocatechin were detected in urine. Note that without reference compounds MS^3 is unable to distinguish between (–)-epicatechin and (+)-catechin metabolites and also (–)-epigallocatechin and (+)-gallocatechin derivatives. No metabolites of either (epi)catechin-3-gallate or (epi)gallocatechin-3-gallate were detected in urine. The identification of the complex array of glucuronide, sulphate and methyl metabolites

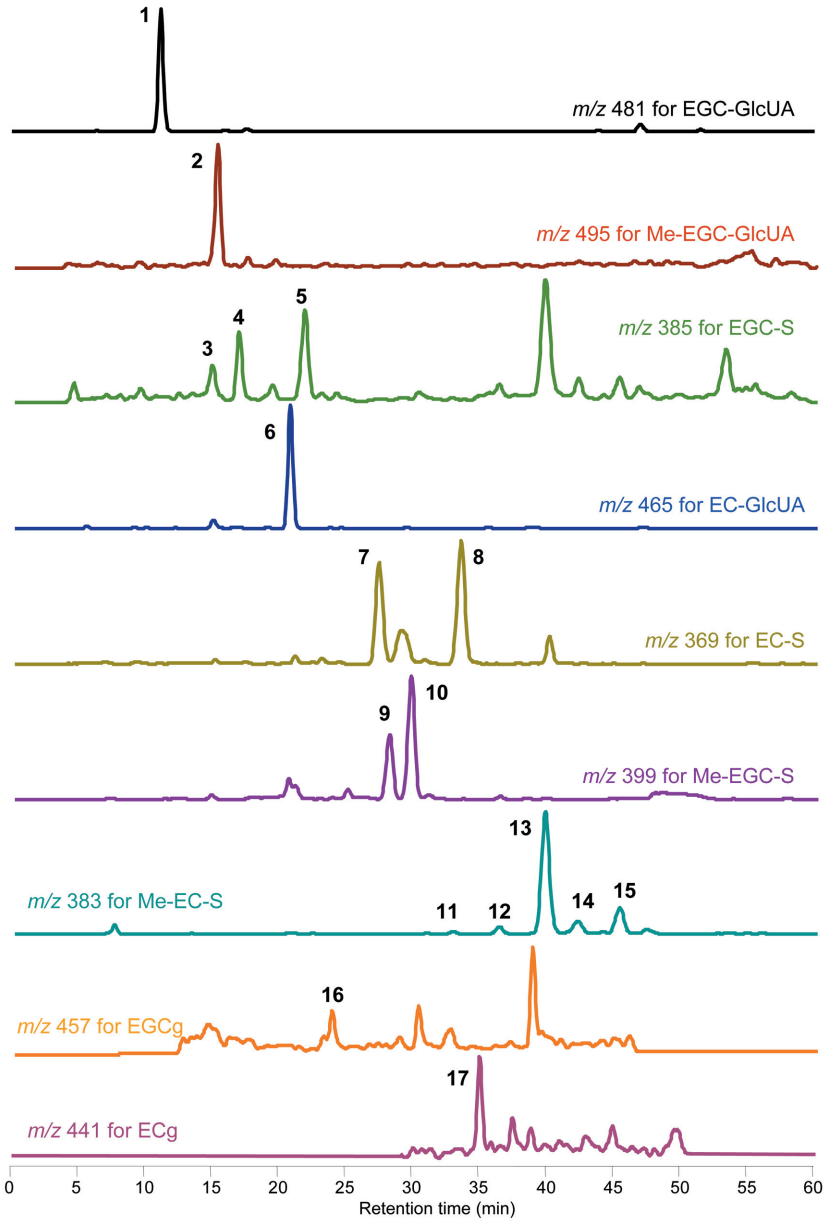

Figure 2. HPLC-SIM quantification of flavan-3-ol metabolites in plasma and urine collected after the ingestion of 500 mL of choladi green tea. Chromatograms represent gradient RP-HPLC analysis with detection of flavan-3-ols and their metabolites by MS using SIM at: *m/z* 481 for (epi)gallocatechin-*O*-glucuronides (EGC-GlcUA); *m/z* 495 for *O*-methyl-(epi)gallocatechin-*O*-glucuronides (Me-EGC-GlcUA); *m/z* 385 for (epi)gallocatechin-*O*-sulphates (EGC-S); *m/z* 465 for (epi)catechin-*O*-glucuronides (EC-GlcUA); *m/z* 369 for (epi)catechin-*O*-sulphates (EC-S); *m/z* 399, *O*-methyl-(epi)gallocatechin-*O*-sulphates (Me-EGC-S); *m/z* 383 for *O*-methyl-(epi)catechin-*O*-sulphates (Me-EC-S); *m/z* 457 for (−)-epigallocatechin-3-gallate (EGCg); and *m/z* 441 for (−)-epicatechin-3-gallate (ECg). Traces at *m/z* 457 and 441 obtained with plasma, all other traces are urine samples. For identification of peaks 1 – 17 see Table 2.

of (epi)catechin and (epi)gallocatechin, based on the following criteria, is summarized in Table 2.

Peak 1 (t_R = 11.1 min) had a negatively charged molecular ion ($[M - H]^-$) at *m/z* 481, which on MS^2 produced a fragment ion corresponding to (epi)gallocatechin at *m/z* 305. The 176 *amu* loss equates with cleavage of a glucuronide moiety. Moreover, the MS^3 fragmentation of *m/z* 305 ions produced daughters ions at *m/z* 261, 221, 219 179, 165 and 125 in keeping with the presence of (epi)gallocatechin. This peak is, therefore, identified as an (epi)gallocatechin-*O*-glucuronide.

Peak 2 (t_R = 15.2 min) had a $[M - H]^-$ at *m/z* 495, with MS^2 yielding an ion at *m/z* 319. This 176 *amu* loss indicates cleavage of a glucuronyl unit. MS^3 of the ion at *m/z* 319 corresponded to a methyl-(epi)gallocatechin, with a fragment at *m/z* 137 indicative of 4′-*O*-methylation [22]. This MS fragment pattern, thus, demonstrates that peak 2 is a 4′-*O*-methyl-(epi)gallocatechin-*O*-glucuronide.

Peaks 3 – 5 (t_R = 14.8, 16.8 and 21.8 min), detected in urine but not plasma, had a $[M - H]^-$ at *m/z* 385, which on MS^2 produced an (epi)gallocatechin daughter ion at *m/z* 305 through loss of an 80 *amu* sulphate moiety. The *m/z* 305 fragment yielded MS^3 ions at *m/z* 261, 221, 219, 179, 165 and 125 confirming the identity of the (epi)gallocatechin unit. Peaks 3 – 5 are therefore, (epi)gallocatechin-*O*-sulphates.

Table 2. HPLC-MS^3 identification of flavan-3-ol metabolites in human plasma and urine collected 0 – 24 h after the ingestion of 500 mL of Choladi green tea[a)]

Flavan-3-ols	HPLC peak number	HPLC R_t (min)	$[M-H]^-$ (*m/z*)	MS^2 ions (*m/z*)	MS^3 ions (*m/z*)	Location
(Epi)gallocatechin-*O*-glucuronide	1	11.1	481	305	261, 221, 219, 179, 165, 125	Plasma, urine
4′-*O*-Methyl-(epi)gallocatechin-*O*-glucuronide	2	15.2	495	319	304, 301, 275, 260, 235, 233, 137	Plasma, urine
(Epi)gallocatechin-*O*-sulphates	3 – 5	14.8, 16.8, 21.8	385	305	261, 221, 219, 179, 165, 125	Urine
(–)-Epicatechin-3′-*O*-glucuronide	6	20.6	465	289	245, 205, 179	Plasma, urine
(Epi)catechin-*O*-sulphates	7, 8	27.2, 33.4	369	289	245, 205, 179	Plasma, urine
4′-*O*-Methyl-(epi)gallocatechin-*O*-sulphates	9, 10	28.1, 29.7	399	319	304, 301, 275, 260, 235, 233, 137	Plasma, urine
3′-*O*-Methyl-(epi)catechin-*O*-sulphates	11 – 14	32.7, 36.2, 39.7, 42.1	383	303	285, 259, 244, 219, 217	Plasma, urine
4′-*O*-Methyl-(epi)catechin-*O*-sulphate	15	45.3	383	303	285, 259, 244, 219, 217, 137	Plasma, urine
(–)-Epigallocatechin-3-gallate	16	23.1	457	331, 305, 169, 193	–	Plasma
(–)-Epicatechin-3-gallate	17	35.1	441	289, 331, 169, 271	–	Plasma

a) R_t retention time. For peak numbers see Fig. 2. [M – H],$^-$ negatively charged molecular ion.

Peak 6 (t_R = 20.6 min) had a $[M - H]^-$ at *m/z* 465, which on loss of 176 *amu* (cleavage of a glucuronyl unit) yielded a MS^2 ion at *m/z* 289. MS^3 of the *m/z* 289 ion yielded fragments characteristic of (epi)catechin indicating that this peak is an (epi)catechin glucuronide. It did not cochromatograph with (–)-epicatechin-7-*O*-glucuronide and is possibly (–)-epicatechin-3′-*O*-glucuronide, which has been identified in urine, collected after oral ingestion of (–)-epicatechin by humans [24].

Peaks 7 and 8 (t_R = 27.2 and 33.4 min, respectively) both had a $[M - H]^-$ at *m/z* 369 which yielded an MS^2 ion at *m/z* 289. This 80 *amu* loss indicates cleavage of a SO_3^- unit. MS^3 of the *m/z* 289 ion provided a mass spectrum corresponding to that of (epi)catechin. This indicates that peaks 7 and 8 are (epi)catechin-*O*-sulphates.

Peaks 9 and 10 (t_R = 28.1 and 29.7 min) both had a $[M - H]^-$ at *m/z* 399 which with an 80 *amu* loss (cleavage of a SO_3^- unit) yielded an MS^2 ion at *m/z* 319. The MS^3 produced fragments consistent with an *O*-methyl-(epi)gallocatechin. The presence of a fragment at *m/z* 137 also indicates that peaks 9 and 10 are, 4′-*O*-methyl-(epi)gallocatechin sulphates.

Peaks 11 – 15 (t_R = 32.7, 36.2, 39.7, 42.1 and 45.3 min, respectively) all had a *m/z* 383 $[M - H]^-$ which on MS^2 produced an *m/z* 303 fragment indicative of the cleavage of a SO_3^- unit. MS^3 of the *m/z* 303 ion yielded a mass spectrum corresponding to that of a methyl-(epi)catechin. Moreover, with peak 15, MS^3 of *m/z* 303 produced an ion at *m/z* 137, specific for 4′-*O*-methylation [22]. Peak 15 is, therefore, a 4′-*O*-methyl-(epi)catechin sulphate. With peaks 11, 12, 13 and 14, MS^3 of *m/z* 303 did not yield a *m/z* 137 fragment but did produce ions at *m/z* 285, 259, 244, 219 and 217 which is indicative of a 3′-*O*-methyl-(epi)catechin. These peaks are, therefore, are identified as 3′-*O*-methyl-(epi)catechin sulphates.

Peak 16 (t_R = 23.1 min), which was detected in plasma but not urine, cochromatographed with (–)-epigallocatechin-3-gallate. It produced a $[M - H]^-$ at *m/z* 457 and MS^2 ions at *m/z* 331, 305, 193 and 169 to further confirm the presence of (–)-epigallocatechin-3-gallate.

Peak 17 (t_R = 35.1 min), which was also found only in plasma, was (–)-epicatechin-3-gallate based on cochromatography with a standard and a $[M - H]^-$ at *m/z* 441 and MS^2 ions at *m/z* 331, 289, 271 and 169.

After the initial qualitative analysis, plasma and urine samples were analysed by HPLC with MS in the SIM mode and typical HPLC-SIM traces obtained and used to quantify (–)-epigallocatechin-3-gallate, (–)-epicatechin-3-gallate and the 15 (epi)gallocatechin and (epi)catechin metabolites, as illustrated in Fig. 2. Note, in the qualitative analyses outlined above only the designated peaks 1 – 17 had yielded the appropriate ions when subjected to further fragmentation. Thus, for instance, the late eluting, unlabelled peaks in the *m/z* 385 trace are not (epi)gallocatechin-*O*-sulphates as, unlike peaks 3 – 5, the *m/z* 385 ion did not produce a daughter ion at *m/z* 305.

3.3 Quantitative analysis of plasma

As described in the previous section, plasma collected 0 – 24 h after the ingestion of the Choladi green tea was found to contain glucuronide, sulphate and methyl-sulphate metabolites of (epi)catechin and glucuronide, methyl-glucuronide and methyl-sulphate metabolites of (epi)galloca-

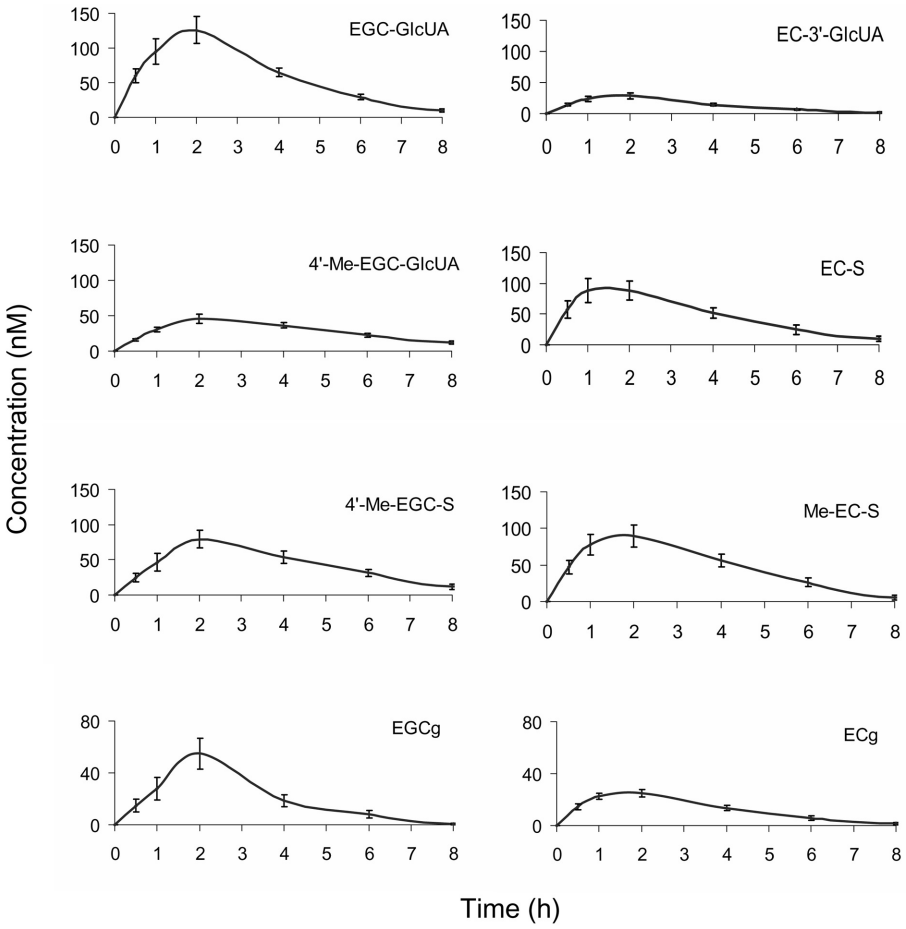

Figure 3. Concentrations of (epi)gallocatechin-*O*-glucuronide (EGC–GlcUA) (peak 1), 4′-*O*-methyl-(epi)-gallocatechin-*O*-glucuronide (4′-Me-EGC-GlcUA) (peak 2), 4′-*O*-methyl-(epi)gallocatechin-*O*-sulphates (4′-Me-EGC-S) (peaks 9 and 10), (–)-epicatechin-3′-*O*-glucuronide (EC-3′-GlcUA) (peak 6), 3′- and 4′-*O*-methyl-(epi)catechin-*O*-sulphates (Me-EC-S) (peaks 11–15), (–)-epigallocatechin-3-gallate (EGCg) (peak 16) and (-)-epicatechin-3-gallate (ECg) (peak 17) in the plasma of human subjects 0–8 h after the ingestion of 500 mL of green tea. Data expressed as mean values with their standard errors ($n = 10$) depicted by vertical bars. Note that no flavan-3-ols or their metabolites were detected in plasma collected 24 h after ingestion of the green tea. For peak numbers see Fig. 2 and Table 2.

Table 3. Pharmacokinetic analysis of flavan-3-ols and their metabolites detected in plasma of healthy volunteers following the ingestion of 500 mL of green tea

Flavan-3-ols (HPLC peak number)	C_{max} (nM)	T_{max} (h)	*AUC* (μM/h)	$T_{1/2}$ (h)
(Epi)gallocatechin-*O*-glucuronide (1)	126 ± 19	2.2 ± 0.2	0.56 ± 0.09	1.6
4′-*O*-Methyl-(epi)gallocatechin-*O*-glucuronide (2)	46 ± 6.3	2.3 ± 0.3	0.32 ± 0.05	3.1
(–)-Epicatechin-3′-*O*-glucuronide (6)	29 ± 4.7	1.7 ± 0.2	0.13 ± 0.02	1.6
(Epi)catechin-*O*-sulphates (7,8)	89 ± 15	1.6 ± 0.2	0.47 ± 0.11	1.9
4′-*O*-Methyl-(epi)gallocatechin-*O*-sulphates (9, 10)	79 ± 12	2.2 ± 0.2	0.44 ± 0.09	2.2
O-Methyl-(epi)catechin-*O*-sulphates (11–15)	90 ± 15	1.7 ± 0.2	0.42 ± 0.10	1.5
(–)-Epigallocatechin-3-gallate (16)	55 ± 12	1.9 ± 0.1	0.17 ± 0.05	1.0
(–)-Epicatechin-3-gallate (17)	25 ± 3.0	1.6 ± 0.2	0.12 ± 0.02	1.5

Data expressed as mean values ± SE ($n = 10$).

techin together with the native green tea flavan-3-ols (–)-epicatechin-3-gallate and (–)-epigallocatechin-3-gallate (Table 2). The pharmacokinetic profiles of the eight groups of flavan-3-ols and their metabolites are illustrated in Fig. 3 and an analysis of the pharmacokinetic parameters is presented in Table 3.

None of the compounds were present in the circulatory system at 0 h but they were present in detectable quantities 30 min after consumption of the green tea (Fig. 3). The main component to accumulate in plasma was an (epi)gallocatechin-*O*-glucuronide (peak 1), which reached a C_{max} of 126 ± 19 nM after 2.2 ± 0.2 h while (–)-epicatechin-3′-*O*-glucuronide (peak 6) was present in lower quantities with a C_{max} of 29 ± 4.7 nM and a T_{max} of 1.7 ± 0.2 h. The unmetabolised flavan-3-ols, (–)-epigallocatechin-3-gallate and (–)-epicatechin-3-gallate attained C_{max} values of 55 ± 12 and 25 ± 3.0 nM after 1.9 ± 0.1 and 1.6 ± 0.2 h, respectively. Mean T_{max} values ranged from 1.6 to 2.3 h with times for $T_{1/2}$ extending from 1.0 to 3.1 h (Table 3). All the flavan-3-ols and their metabolites were present in only trace amounts

Table 4. Quantification of the major groups of flavan-3-ol metabolites excreted in urine 0–5, 5–12 and 12–24 h after the ingestion of 500 mL of Choladi green tea by ten human volunteers

Flavan-3-ol metabolites (HPLC peak number)	0–5 h	5–12 h	12–24 h	Total (0–24 h)
(Epi)gallocatechin-*O*-glucuronide *(1)*	3.8 ± 0.8	2.3 ± 0.7	0.4 ± 0.1	6.5 ± 1.2
4′-*O*-Methyl-(epi)gallocatechin-O-glucuronide *(2)*	1.8 ± 0.6	1.5 ± 0.5	1.1 ± 0.6	4.4 ± 1.5
(Epi)gallocatechin-*O*-sulphates (3–5)	0.9 ± 0.1	0.7 ± 0.1	1.0 ± 0.2	2.6 ± 0.3
4′-*O*-Methyl-(epi)gallocatechin-*O*-sulphates *(9,10)*	6.6 ± 1.5	10.3 ± 2.1	2.9 ± 0.6	19.8 ± 3.0
Total (epi)gallocatechin metabolites				33.3 ± 5.0 *(11.4 ± 1.7%)*
(–)-Epicatechin-3-*O*-glucuronide (6)	0.7 ± 0.1	0.6 ± 0.2	0.2 ± 0.1	1.5 ± 0.3
(Epi)catechin-*O*-sulphates (7, 8)	3.2 ± 0.4	2.4 ± 0.6	1.1 ± 0.3	6.7 ± 0.7
O-Methyl-(epi)catechin-*O*-sulphates (11–15)	5.2 ± 0.7	4.1 ± 0.9	1.6 ± 0.4	10.9 ± 1.2
Total (epi)catechin metabolites				19.1 ± 2.2 (*28.5 ± 3.3%*)
Total flavan-3-ol metabolites	22.2 ± 3.8 *(3.4 ± 0.6%)*	21.9 ± 4.5 *(3.4 ± 0.7%)*	8.3 ± 1.6 *(1.3 ± 0.2%)*	52.4 ± 7.2 *(8.1 ± 1.1%)*

Data expressed as mean values in μmol ± standard error ($n = 10$). Italicised figures in parentheses indicate amount excreted as a percentage of intake.

after 8 h and were not present in detectable amounts in the 24 h plasma samples.

3.4 Quantitative analysis of urine

Urine samples were collected following an overnight fast (0 h) and 0–5, 5–12 and 12–24 h after ingestion of the green tea and, like plasma, the flavan-3-ol content was analysed quantitatively by HPLC-SIM. The urine did not contain any of the original green tea flavan-3-ols but 15 metabolites of (epi)catechin and (epi)gallocatechin were detected, including three (epi)gallocatechin-*O*-sulphates, peaks 3–5, that did not accumulate in plasma (Table 2). Quantitative estimates of the seven metabolite groups are presented in Table 4. The main metabolites to be excreted were 4′-*O*-methyl-(epi)gallocatechin-*O*-sulphates (19.8 μmol), peaks 9 and 10, and *O*-methyl-(epi)catechin-*O*-sulphates (10.9 μmol), predominantly in the form of peak 13 (Fig. 2).

Most excretion of the flavan-3-ol metabolites occurred during the 0–5 and 5–12 h collection periods. The 0 h urine samples contained no detectable flavan-3-ols or flavan-3-ol metabolites. The total amount of metabolites excreted over the 0–24 h period by the individual subjects varied from 25.8–87.6 μmol (data not shown). This corresponds to 4.0–13.5% of the 648 μmol of flavan-3-ols in the ingested 500 mL of green tea. The mean level of excretion was 52.4 ± 7.2 μmol, equivalent to 8.1% of intake (Table 4). However, the 33.3 ± 5.0 μmol excretion of (epi)gallocatechin metabolites relative to the 293 μmol of (–)-epigallocatechin and (+)-gallocatechin in the green tea, is 11.4% of intake while the 19.1 ± 2.2 μmol recovery of (epi)catechin represents 28.5 ± 3.3% of the (+)-epicatechin and (+)-catechin intake.

4 Discussion

The Choladi green tea is a rich source of flavan-3-ol monomers, and contained 648 ± 6 μmol/500 mL. Following ingestion by human volunteers, unmetabolised (–)-epigallocatechin-3-gallate and (–)-epicatechin gallate appear in the circulatory system together with glucuronide, methyl and sulphate metabolites of (epi)catechin and (epi)gallocatechin with C_{max} values ranging from 25 to 126 nM and T_{max} values of 1.6–2.3 h. These T_{max} values and the pharmacokinetic profiles illustrated in Fig. 3 are indicative of absorption in the small intestine. The appearance of unmetabolised flavonoids in plasma is unusual. The passage of the (–)-epigallocatechin-3-gallate and (–)-epicatechin-3-gallate through the wall of the small intestine into the circulatory system without metabolic modification could be a consequence of the presence of the 3-*O*-galloyl moiety, as gallic acid *per se* is readily absorbed with a reported urinary excretion of 37% of intake [25, 26].

Urine excreted over a 24 h period after ingestion of the Choladi green tea contained a similar array of flavan-3-ol metabolites to that detected in plasma except for the presence of three additional (epi)gallocatechin-*O*-sulphates (*peaks 3–5*) and an absence of (-)-epigallocatechin-3-gallate and (–)-epicatechin-3-gallate. In total, 52.4 ± 7.2 μmol of metabolites were excreted, which is equivalent to 8.1% of the ingested green tea flavan-3-ols. When the urinary (epi)gallocatechin and (epi)catchin metabolites are considered separately, a somewhat different picture emerges. The 33.3 ± 5.0 μmol excretion of (epi)gallocatechin metabolites is 11.4% of the ingested (–)-epigallocatechin and (+)-gallocatechin while the 19.1 ± 2.2 μmol recovery of (epi)catechin represents 28.5 ± 3.3% of intake. These are high fig-

Table 5. Plasma C_{max} and 0 – 24 h urinary excretion of flavonoid metabolites, and the excretion/C_{max} ratio after the ingestion of green tea, onions, orange juice and strawberries containing, respectively, flavan-3-ol monomers, quercetin glucosides, hesperetin-7-rutinoside and pelargonidin-3-glucoside

Feed	Flavonoids ingested and dose	Flavonoid metabolites		Excretion/C_{max} ratio (nmol/nM)
		C_{max} (nM)	Excretion (nmol)	
Green tea	Flavan-3-ol monomers (648 μmol)	538	52 400 (8.1%)	97
Onions [33]	Quercetin-*O*-glucosides (275 mmol)	1313	12 900 (4.7%)	9.8
Orange juice [34]	Hesperetin-7-*O*-rutinoside (168 μmol)	646	8 800 (5.3%)	13.6
Strawberries [35]	Pelargonidin-3-*O*-glucoside (222 μmol)	274	1 700 (0.8%)	6.2

All feeds carried out under identical conditions following a 2-day low flavonoid diet and an overnight fast.

ures, in keeping with recoveries obtained in earlier studies with green tea and cocoa products [27], confirming that (–)-epicatechin and (+)-catechin, in particular, are highly bioavailable being absorbed and excreted to a much greater extent than other flavonoids (see Table 5), with the possible exception of isoflavones [27, 28].

In a previous study, feeding (–)-epigallocatechin-3-gallate powder in a capsule to humans did not result in the appearance of detectable quantities of either (–)-epigallocatechin-3-gallate or any flavan-3-ol metabolites in urine [29]. This indicates that postingestion, (–)-epigallocatechin-3-gallate is not subjected to loss of either the 3-*O*-galloyl moiety or the 5′-hydroxyl group and conversion to either (–)-epigallocatechin or (–)-epicatechin during passage through the body. There is, evidence, however, that human saliva contains an esterase and that holding green tea in the mouth can result in the conversion of (–)-epigallocatechin-3-gallate to (–)-epigallocatechin [30]. In the present study the tea was not held in the mouth for any period of time so such a conversion is likely to be small, at best, and not impact to any extent on the quantities of (–)-epigallocatechin metabolites excreted. It follows that the high excretion of (epi)catechin metabolites (Table 4) is also a genuine event and is unlikely to be an indirect consequence of the salivary esterase removing the 3-*O*-galloyl group from substantial amounts of (–)-epicatechin-3-gallate and (+)-catechin-3-gallate.

The absence of detectable amounts of (–)-epigallocatechin-3-gallate in urine, despite its presence in plasma, an event observed by other investigators [14, 16, 18], is difficult to explain. It is possible that the kidneys are unable to remove (–)-epigallocatechin-3-gallate from the bloodstream, but if this is the case there must be other mechanisms that result in its rapid decline after reaching C_{max}. Studies with rats have lead to a speculation that (–)-epigallocatechin-3-gallate is removed from the blood stream in the liver and returned to the small intestine in the bile. The evidence, however, is far from compelling. In one study (–)-epigallocatechin-3-gallate was fed to rats by gavage; only *ca.* 3% of intake was detected in bile, principally as metabolites [31]. When tritium labelled (–)-epigallocatechin-3-gallate was injected intravenously into bile-duct-cannulated rats 57% of the injected radioactivity was excreted in bile within 4 h and 77% within 48 h compared to 2% in urine over the 48 h period [32]. This involved extensive metabolism of the (–)-epigallocatechin-3-gallate as it was present in bile as sulpho and/or glucuronide conjugates of 4″-methyl- and 4′,4″-dimethyl-(–)-epigallocatechin-3-gallate. To what extent enterohepatic recirculation of (–)-epigallocatechin-3-gallate metabolites occurs in humans has not been established. If it was a significant event, then (–)-epigallocatechin-3-gallate metabolites would be expected to be detected in substantial amounts in ileal fluid. This seems not to occur as in studies in which Polyphenon E, which contains sizable amounts of (–)-epigallocatechin-3-gallate, was fed to human volunteers with an ileostomy, ileal fluid collected 0 – 24 h after ingestion contained only trace quantities of (–)-epigallocatechin-3-gallate metabolites, principally as sulphated derivatives [29].

Summing the C_{max} values for the individual flavan-3-ols and metabolites in Table 3 results in an overall maximum plasma concentration of 538 nM being attained after the ingestion of green tea. This is lower than the 1313 nM C_{max} of quercetin metabolites obtained following the ingestion of onions containing 275 μmol of quercetin-4′-*O*-glucoside and quercetin-3,4′-*O*-diglucoside [33] and is also less than the 646 nM C_{max} of the 7- and 3′-*O*-glucuronides of hesperetin that appear in plasma after the ingestion of orange juice containing 168 μmol of hesperetin-7-*O*-rutinoside [34] (see Table 5). Despite the low concentration of the green tea flavan-3-ol metabolites in plasma, the data on urinary excretion, as discussed earlier, demonstrate that they are absorbed in substantial quantities. Their failure to accumulate in comparable concentrations in plasma suggests that they are in a state of flux and are being rapidly turned over in the circulatory system and, rather than accumulating, are excreted *via* the kidneys. The dynamics of these processes appear to vary somewhat with individual flavonoids as indicated in Table 5, which contains values of urinary excretion/plasma C_{max} ratios (nmol/nM). In the present study with Choladi green tea a ratio of 97 was obtained, indicating high excretion and rapid turnover of flavan-3-ol metabolites

in the circulatory system. This compares with a figure of 9.8 obtained when flavonol-rich onions were fed to human volunteers, 13.6 after drinking orange juice containing principally the flavanone, hesperetin-7-*O*-rutinoside [34], and 6.2 obtained following the ingestion of strawberry anthocyanins [35].

While analysis of plasma provides valuable information on the identity, C_{max} and T_{max} values of circulating flavonoid metabolites after acute supplementation, the estimates of *AUC* and $T_{1/2}$, however, do not provide an accurate quantitative assessment of uptake for the gastrointestinal tract, due to their rapid turnover in the circulatory system. Urinary excretion provides a more realistic figure but, as this does not include the possibility of metabolites being sequestered in body tissues, this too is an underestimate of absorption, but to what degree remains to be determined.

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5 References

[1] Barbosa, D. S., Green tea polyphenolic compounds and human health, *J. Verbr. Lebensm.* 2007, *2*, 407 – 413.

[2] Cabrera, C., Artacho, R., Giménez, R., Beneficial effects of green tea – A review, *J. Am. Coll. Nutr.* 2006, *25*, 79 – 99.

[3] Zaveri, N. T., Green tea and its polyphenolic catechins: Medicinal uses in cancer and noncancer applications, *Life Sci.* 2006, *78*, 2073 – 2080.

[4] Yang, C. S., Lambert, J. D., Ju, J., Lu, G., *et al*., Tea and cancer prevention: Molecular mechanisms and human relevance, *Toxicol. Appl. Pharmacol.* 2006, *224*, 265 – 273.

[5] Gao, Y. T., McLaughlin, J. K., Blot, W. J., Ji, B. T., *et al*., Reduced risk of oesophageal cancer associated with green tea consumption, *J. Nat. Cancer Inst*. 1994, *86*, 855 – 858.

[6] Ji, B. T., Chow, W. H., Hsing, A. W., McLaughlin, J. K., *et al*., Green tea consumption and the risk of pancreatic and colorectal cancers, *Int. J. Cancer* 1997, *70*, 255 – 258.

[7] Suzuki, Y., Tsubono, Y., Nakaya, N., Koizumi, Y., *et al*., Green tea and the risk of colorectal cancer: Pooled analysis of two prospective studies in Japan, *J. Epidemiol*. 2005, *15*, 118 – 124.

[8] Kuriyama, S., Shimazu, T., Ohmori, K., Kikuchi, N., *et al*., Green tea consumption and mortality due to cardiovascular disease, cancer, and all causes in Japan: The Ohsaki study, *JAMA* 2006, *296*, 1255 – 1265.

[9] Stangl, V., Lorenz, M., Stangl, K., The role of tea and tea flavonoids in cardiovascular health, *Mol. Nutr. Food Res*. 2006, *50*, 218 – 228.

[10] Iso, H., Date, C., Wakai, K., Fukui, M., *et al*., The relationship between green tea and total caffeine intake and risk for self-reported type-2 diabetes among Japanese adults, *Ann. Int. Med.* 2006, *144*, 554 – 562.

[11] Del Rio, D., Stewart, A. J., Mullen, W., Burns, J., *et al*., HPLC-MS^n analysis of phenolic compounds and purine alkaloids in green and black tea, *J. Agric. Food Chem.* 2004, *52*, 2807 – 2015.

[12] Stewart, A. J., Mullen, W., Crozier, A., On-line high-performance liquid chromatography analysis of the antioxidant activity of phenolic compounds in green and black tea, *Mol. Nutr. Food Res.* 2005, *49*, 52 – 60.

[13] Shao, W., Powell, C., Clifford, M. N., The analysis by HPLC of green, black and Pu'er teas produced in Yemen, *J. Sci. Food Agric.* 1995, *69*, 535 – 540.

[14] Henning, S. M., Niu, Y., Liu, Y., Lee, N. H., *et al*., Bioavailability and antioxidant effect of epigallocatechin gallate administered in purified form versus as green tea extract in healthy individuals, *J. Nutr. Biochem.* 2005, *16*, 610 – 616.

[15] van het Hof, K. H., Wiseman, S. A., Yang, C. S., Tijburg, L. B., Plasma and lipoprotein levels of tea catechins following repeated tea consumption, *Proc. Soc. Exp. Biol. Med.* 1999, *220*, 203 – 209.

[16] Chow, H. H., Cai, Y., Alberts, D. S., Hakim, I., *et al*., Phase I pharmacokinetic study of tea polyphenols following single-dose administration of epigallocatechin gallate and polyphenon E, *Cancer Epidemiol. Biomark. Prev.* 2001, *10*, 53 – 58.

[17] Unno, T., Kondo, K., Itakura, H., Takeo, T., Analysis of (–)-epigallocatechin gallate in human serum obtained after ingesting green tea, *Biosci. Biotechnol. Biochem.* 1996, *60*, 2066 – 2068.

[18] Ullmann, U., Haller, J., Decourt, J. P., Girault, N., *et al*., A single ascending dose study of epigallocatechin gallate in healthy volunteers, *J. Int. Med. Res.* 2003, *31*, 88 – 101.

[19] Lee, M. J., Wang, Z. Y., Li, H., Chen, L., *et al*., Analysis of plasma and urinary tea polyphenols in human subjects, *Cancer Epidemiol. Biomark. Prev.* 1995, *4*, 393 – 399.

[20] Henning, S. M., Niu, Y. T., Lee, N. H., Thames, G. D., *et al.*, Bioavailability and antioxidant activity of tea flavanols after consumption of green tea, black tea, or a green tea extract supplement, *Am. J. Clin. Nutr.* 2004, *80*, 1558 – 1564.

[21] Li, C., Lee, M. J., Sheng, S., Meng, X., *et al*., Structural identification of two metabolites of catechins and their kinetics in human urine and blood after tea ingestion, *Chem. Res. Toxicol.* 2000, *13*, 177 – 184.

[22] Meng, X., Lee, M. J., Li, C., Sheng, S., *et al.*, Formation and identification of 4′-*O*-methyl-(–)-epigallocatechin in humans. *Drug Metab. Dispos.* 2001, *29*, 789 – 793.

[23] Day, A. J., Mellon, F., Barron, D., Sarrazin, G., *et al*., Human metabolism of dietary flavonoids: Identification of plasma metabolites of quercetin, *Free Rad. Res.* 2001, *35*, 941 – 952.

[24] Natsume, M., Osakabe, N., Oyama, M., Sasaki, M., *et al*., Structures of (–)-epicatechin glucuronide identified from plasma and urine after oral ingestion of (–)-epicatechin: differences between human and rat, *Free Rad. Biol. Med.* 2003, *34*, 840 – 849.

[25] Shahrzad, S., Bitsch, I., Determination of gallic acid and its metabolites in human plasma and urine by high-performance liquid chromatography, *J. Chromatogr. B* 1998, *705*, 87 – 95.

[26] Shahrzad, S., Aoyagi, K., Winter, A., Koyama, A., Bitsch, I., Pharmacokinetics of gallic acid and its relative bioavailability from tea in humans, *J. Nutr.* 2001, *131*, 1207 – 1210.

[27] Manach, C., Williamson, G., Morand, C., Scalbert, A., Rémésy, C., Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies, *Am. J. Clin. Nutr.* 2005, *81*, 230S – 242S.

[28] Donovan, J. L., Manach, C., Fauks, R. M., Kroon, P. A., in: Crozier, A., Clifford, M. N., Ashihara, H. (Eds.), *Plant Secondary Metabolites: Occurrence, Structure and Role in the Human Diet*, Blackwell Publishing, Oxford 2006, pp. 303 – 351.

[29] Auger, C., Hara, Y., Crozier, A., Bioavailability of Polyphenon E flavan-3-ols in humans with an ileostomy, *J. Nutr.* 2008, *138*, 1535S – 1542S.

[30] Yang, C. S., Lee, M.-J., Chen, L., Human salivary tea catechin levels and catechin esterase activities: Implication in human cancer prevention studies, *Cancer Epidemiol. Biomark. Prev.* 1999, *8*, 83 – 89.

[31] Kida, T., Suzuki, Y., Matsumoto, N., Nanjo, F., *et al*., Identification of biliary metabolites of (–)-epigallocatechin gallate in rats, *J. Agric. Food Chem.* 2000, *48*, 4151 – 4155.

[32] Kohri, T., Nanjo, F., Suziki, M., Seto, R., *et al*., Synthesis of (–)-[4-^{3}H]epigallocatechin gallate and its metabolic fate in rats after intravenous administration, *J. Agric. Food Chem.* 2001, *49*, 1042 – 1048.

[33] Mullen, W., Edwards, C. A., Crozier, A., Absorption, excretion and metabolic profiling of methyl-, glucuronyl-, glucosyl and sulpho-conjugates of quercetin in human plasma and urine after ingestion of onions, *Brit. J. Nutr.* 2006, *96*, 107 – 116.

[34] Mullen, W., Archeveque, M.-A., Edwards, C. A., Crozier, A., Bioavailability and metabolites of orange juice flavones in humans: impact of a full fat yoghurt, *J. Agric. Food Chem.*, in press.

[35] Mullen, W., Edwards, C. A., Serafini, M., Crozier, A., Bioavailability of pelargonidin-3-glucoside and its metabolites in humans following the ingestion of strawberries with and without cream, *J. Agric. Food Chem.* 2008, *56*, 713 – 719.