RESEARCH ARTICLE

Absorption, metabolism, and excretion of green tea flavan-3-ols in humans with an ileostomy

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Green tea containing 634 μmol of flavan-3-ols was ingested by human subjects with an ileostomy. Ileal fluid, plasma, and urine collected 0–24 h after ingestion were analysed by HPLC-MS. The ileal fluid contained 70% of the ingested flavan-3-ols in the form of parent compounds (33%) and 23 metabolites (37%). The main metabolites effluxed back into the lumen of the small intestine were *O*-linked sulphates and methyl-sulphates of (epi)catechin and (epi)gallocatechin. Thus, in subjects with a functioning colon substantial quantities of flavan-3-ols would pass from the small to the large intestine. Plasma contained 16 metabolites, principally methylated, sulphated, and glucuronidated conjugates of (epi)catechin and (epi)gallocatechin, exhibiting 101–256 nM peak plasma concentration and the time to reach peak plasma concentration ranging from 0.8 to 2.2 h. Plasma pharmacokinetic profiles were similar to those obtained with healthy subjects, indicating that flavan-3-ol absorption occurs in the small intestine. Ileostomists had earlier plasma time to reach peak plasma concentration values than subjects with an intact colon, indicating the absence of an ileal brake. Urine contained 18 metabolites of (epi)catechin and (epi)gallocatechin in amounts corresponding to 6.8 ± 0.6% of total flavan-3-ol intake. However, excretion of (epi)catechin metabolites was equivalent to 27% of the ingested (−)-epicatechin and (+)-catechin.

Keywords:
Glucuronide and sulphate metabolites / Green tea flavan-3-ol bioavailability / Ileal and urinary excretion / Ileostomists / Plasma pharmacokinetics

1 Introduction

Green tea infusions contain high levels of flavan-3-ols with (−)-epigallocatechin-3-*O*-gallate, (–)-epigallocatechin, and (−)-epicatechin-3-*O*-gallate typically being present in highest concentrations (Fig. 1) [1, 2]. Green tea catechins have been reported to exert a wide range of beneficial effects *in vitro* having antioxidant, antimutagenic, anticarcinogenic, and antihypertensive properties [3, 4]. However, epidemiology studies have produced conflicting evidence on the protective role of green tea against chronic diseases such as cancer [5–7], cardiovascular diseases ([8, 9], and diabetes [10]. Nonetheless, if flavan-3-ols are to exert protective effects on human health, they need to be absorbed and reach specific target tissues.

The use of *in vitro* studies and animal models has provided useful information on the bioavailability and established the small intestine as the site for the absorption

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Abbreviations: AUC, area-under-the-curve; C_{max}, peak plasma concentration; **PDA,** photodiode array; T_{max}, the time at which peak plasma concentration is reached; $T_{1/2}$, elimination half-life

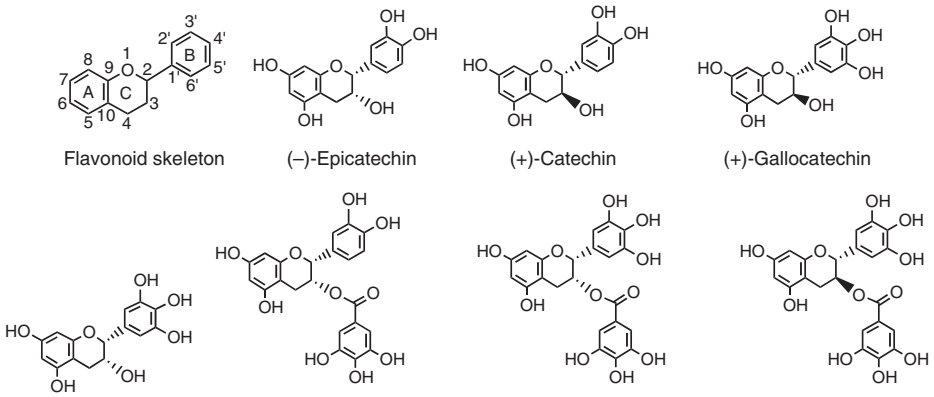

Figure 1. Structures of flavan-3-ols in green tea.

of flavan-3-ol monomers [11–14]. At present, intervention studies with healthy human subjects have investigated the absorption of green tea flavan-3-ols by examining their presence in plasma and urine after acute ingestion [15–17]. However, in these studies HPLC coupled with electrochemical detection was used to analyse samples after treatment of plasma and urine with glucuronidase/sulphatase to convert metabolites to the native aglycone flavan-3-ol structures and, as a consequence, only indirect information on the metabolites was obtained [18]. Recently, HPLC with multistage MS detection (*i.e.* MS^2 and MS^3) has been used for the analysis of flavan-3-ols in body fluids [19, 20] and this has facilitated identification of the metabolites.

This study investigated the fate of flavan-3-ols in human volunteers with an ileostomy by using HPLC with photodiode array (PDA) and MS detection, to analyse ileal fluid, plasma, and urine collected 0–24 h after the acute ingestion of 300 mL of green tea. The use of ileostomists to study the bioavailability of flavan-3-ols offers a number of advantages. By comparing intake with the content of ileal fluid it is possible to assess metabolism and absorption occurring in the small intestine from a different perspective to that gained by analysing plasma and urine from subjects with an intact functioning colon. In addition, the appearance of compounds in the circulatory system of ileostomists establishes that absorption occurs in the small intestine while analysis of ileal fluid provides information on the compounds, which in healthy subjects will pass from the small to the large intestine where they have the potential to impact on colonic health and will also be subject to degradation to phenolic acids by the colonic microflora.

2 Material and methods

2.1 Chemicals

Sodium diethyldithiocarbamate, l-ascorbic acid, (−)-gallocatechin, (−)-epicatechin, (+)-catechin, and (−)-gallocatechin-3-*O*-gallate were obtained from Sigma Aldrich (Poole, Dorset, UK), and (−)-epigallocatechin, (−)-epigallocatechin-3-*O*-gallate, and (−)-epicatechin-3-*O*-gallate were purchased from Apin Chemicals (Abingdon, UK). Ethyl gallate was obtained from Fluka (Sigma Aldrich). (−)-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).

2.2 Green tea beverage

The beverage was prepared by adding 300 mL of boiled distilled water to 3 g of Indonesian green tea leaves (Tetley GB, Greenford, Middlesex, UK), which were left to brew for 3 min with continuous stirring before filtering. The green tea brew was then cooled to room temperature prior to analysis of 5 μL volumes in triplicate by HPLC-PDA-MS^n.

2.3 Human feeding study

The study protocol was approved by the Glasgow Royal infirmary Ethics Committee (REC reference number: 04/S070/48). The inclusion criteria for the participants were to be in good health, non-smoker, not pregnant, aged 18–70 years, and have had an ileostomy. Five volunteers participated in the study (three male and two female). The participants had an average age of 48 years (SD 6.1), average height of 166 cm (SD 10), average weight of 71 kg (SD 11), and a mean body mass index of 25.5 kg/m^2 (SD 2.4). The volunteers have had an ileostomy for 17 years (SD 9.8), due to ulcerative colitis ($n = 4$) and Crohn's disease ($n = 1$). The participants were, otherwise, healthy. For 2 days prior to the study and for 24 h after drinking green tea, volunteers followed a diet low in flavonoids and phenolic compounds by avoiding fruits, vegetables, whole grains, nuts, wine, coffee, tea, chocolate, fruit juices, and dietary antioxidant supplements. On the day

of the study, after an overnight fast, each subject drank 300 mL of green tea and after 3 h, a standard lunch containing low levels of phenolic compounds was provided. The amount consumed was not limited, water was available all day, and the subjects were encouraged to drink. All urine excreted for 24 h, as well as ileal fluid contained in the ileostomy bag over the periods 0–2, 2–5, and 5–24 h, were collected. The volumes of urine and weights of ileal fluid samples were measured and aliquots were stored at −80°C prior to analyses. Blood samples were collected at 0 h, prior to drinking the green tea and after 0.5, 1, 2, 3, 4, 5, 6, and 24 h, in heparinised tubes. Plasma was separated following centrifugation at 16 110 *g* for 10 min at 4°C, and 30 μL of 50% aqueous formic acid, and 100 μL of 10 mM ascorbic acid added to 1 mL aliquots prior to storage at −80°C.

2.4 Ileal fluid extraction

Extraction consisted in placing 2.5 g of ileal fluid in each of two 50 mL Falcon tubes and adding 10 mL of 1% formic acid in 50% v/v aqueous methanol containing 20 mM of sodium diethyldithiocarbamate and 50 μg of ethyl gallate as an internal standard. After homogenising with an Ultra-Turrax® (T25 basic, IKA® Werke KG, Staufen, Germany) at 24 000 rpm for 1 min, the contents of the two tubes were then mixed using a flat shaker at a speed of 400 rpm for 30 min, before being centrifuged at 5600 *g* for 20 min at 4°C. The supernatant was collected and the pellet re-extracted using a solution of 1% formic acid in methanol, containing 20 mM of sodium diethyldithiocarbamate. The two supernatants were pooled and reduced in volume *in vacuo* for 30 min using a rotary evaporator at 35°C. The samples were then dried for a further 30 min under a flow of nitrogen, before being freeze-dried at −60°C. The lyophilised residues were re-suspended in 2 mL of 10% aqueous methanol containing 0.1% formic acid and centrifuged at 16 110 *g* for 30 min at 4°C, in a 0.2 μm Micro-Spin™ Eppendorf filter (Alltech Associates Applied Sciences, Lancashire, UK). The filtered extracts were diluted tenfold and 5 μL volumes analysed in triplicate by HPLC-PDA-MS^n.

2.5 Plasma extraction

The extraction of flavan-3-ols in plasma was based on the method by Day *et al.* [21], and consisted in adding 450 μL of plasma drop-wise to 1.0 mL of ACN in a 2 mL Eppendorf. The plasma was primarily spiked with 1 μg of ethyl gallate, used as an internal standard, and 20 μL of 10% w/v of ascorbic acid containing 0.5 mM EDTA (adapted from the method by Lee *et al.* [15]). Samples were vortexed five times for 30 s every 2 min, and then centrifuged at 1500 *g* for 20 min at 4°C. The supernatant was removed and the pellet re-extracted under the same condition using 1.0 mL of methanol. The two supernatants were combined and dried under a flow of nitrogen in a heated block at 35°C before being re-suspended in 250 μL of mobile phase containing 10% of methanol, and centrifuged at 16 110 *g* for 10 min at 4°C in a 0.2 μm Micro-Spin™ Eppendorf filter. A total of 100 μL of the extracts were injected in duplicate into the LC-MS^n system.

2.6 Extractions efficiencies

The efficiency of the extraction of flavan-ols was evaluated by spiking blank ileal fluid and plasma with equal amounts of ethyl gallate and (−)-epicatechin, (−)-epigallocatechin, (−)-epigallocatechin-3-*O*-gallate, and (−)-epicatechin-3-*O*-gallate. The spiked samples were extracted as described above and analysed in triplicate by HPLC with MS in the SIM mode. The recoveries of the different types of flavan-3-ols were calculated as a ratio relative to the recovery of ethyl gallate. The ratios were subsequently used to quantify the flavan-3-ols and their metabolites based on the recovery of the ethyl gallate internal standard from the individual samples of plasma and ileal fluid as described by Stalmach *et al.* [22].

2.7 Urine analysis

Urine samples were centrifuged at 16 110 *g* for 3 min at 4°C, after which 50 μL aliquots of the supernatant were analysed in triplicate by HPLC-PDA-MS^n.

2.8 HPLC-PDA-MS^n

Flavan-3-ols and their metabolites in green tea, plasma, ileal fluid extracts, and urine were analysed using a Surveyor HPLC with a PDA detector and an LCQ Duo ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Separations were performed at 40°C using a Phenomenex Synergi 4 μm RP-MAX 80 Å 250 × 4.6 mm (id) reverse phase 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 (for the analysis of metabolites of (epi)gallocatechin-3-*O*-gallate and (epi)catechin-3-*O*-gallate, 60-min gradients of 5–40% and 5–50% ACN in 0.1% formic acid were also used). The column eluant passed through the flow cell of the PDA and was then split and 0.3 mL/min directed to the mass spectrometer with an ESI source operating in full scan negative ionisation mode. 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, dissolved in the initial HPLC mobile phase, into the source 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, and source voltage was 3 kV, collision energy set at 35%, and in-source fragmentation set at 5 V.

Following HPLC separation, flavan-3-ols in green tea were detected with the PDA monitor at 280 nm. Identification of flavan-3-ols and their metabolites in the tea, plasma, ileal fluid extracts, and urine was based on HPLC with MS using consecutive reaction monitoring, while HPLC with MS-SIM was utilised for quantification.

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

Maximum plasma concentration of the flavan-3-ol derivatives from 0 to 6 h post-dose was defined as C_{max}, with time to reach peak plasma concentration (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 Fisher Scientific).

3 Results

3.1 Quantities of flavan-3-ols in the green tea infusate

The green tea beverage consumed by the ileostomy volunteers contained 634 ± 14 μmol of flavan-3-ols. The main component was (−)-epigallocatechin-3-*O*-gallate, accounting for 38% of the total flavan-3-ols, followed by (−)-epigallocatechin (30%) and (−)-epicatechin and (−)-epicatechin-3-*O*-gallate (11 and 10%, respectively). The remaining 11% of the flavan-3-ols consisted in (+)-gallocatechin, (+)-catechin, and (+)-gallocatechin-3-*O*-gallate.

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

HPLC-MS^2 was used to identify the native green tea flavan-3-ols, (+)-gallocatechin, (−)-epigallocatechin, (+)-catechin, (−)-epicatechin, (−)-epigallocatechin gallate, (+)-gallocatechin gallate, and (−)-epicatechin gallate, in ileal fluid (data not shown). The same methodology was also used to identify a range of flavan-3-ol sulphate, glucuronide, methyl sulphate, and methyl glucuronide metabolites in ileal fluid, plasma, and urine (MS^3). Twenty-three metabolites were identified in ileal fluid (Fig. 2), 16 in plasma, and 18 in urine.

Analysis of flavan-3-ols and their metabolites is somewhat more subtle than is generally appreciated. For instance, without reference compounds that can be separated by reversed phase HPLC, MS is unable to distinguish between epicatechin and catechin metabolites and also epigallocatechin and gallocatechin metabolites. We therefore refer to metabolites as (epi)catechins or (epi)gallocatechins. To complicate matters further, although chiral chromatography, using a mobile phase that is not compatible with on-line MS, can separate (+) and (−) flavan-3-ol enantiomers, they co-chromatograph when analysed by reversed phase HPLC [23]. Although, some degree of interconversion may occur between optical isomers with, for

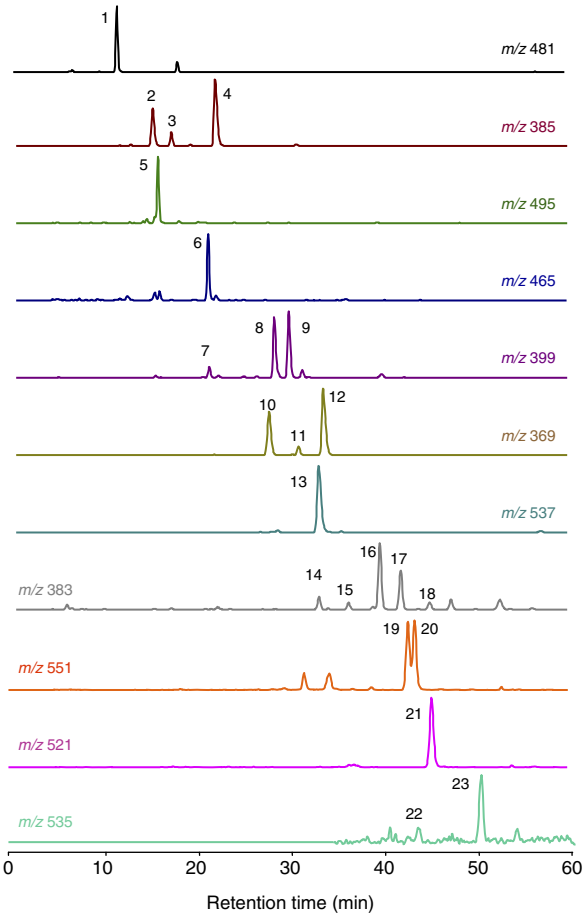

Figure 2. HPLC-SIM analysis of flavan-3-ol metabolites ileal fluid collected 2–5 h after the ingestion of 300 mL of green tea. Chromatograms represent gradient reversed phase HPLC analysis with detection of flavan-3-ols metabolites by MS using SIM at: *m/z* 481 for (epi)gallocatechin-*O*-glucuronides; *m/z* 385 for (epi)gallocatechin-*O*-sulphates; *m/z* 495 for *O*-methyl-(epi)gallocatechin-*O*-glucuronides; *m/z* 465 for (epi)catechin-*O*-glucuronides; *m/z* 399 for *O*-methyl-(epi)gallocatechin-*O*-sulphates; *m/z* 369 for (epi)catechin-*O*-sulphates; *m/z* 537 for (epi)gallocatechin-3-*O*-gallate-*O*-sulphates; *m/z* 383 for *O*-methyl-(epi)catechin-*O*-sulphates; *m/z* 551 for *O*-methyl-(epi)gallocatechin-3-*O*-gallate-*O*-sulphates; *m/z* 521 for (epi)catechin-3-*O*-gallate-*O*-sulphate; and *m/z* 535 for (epi)catechin-3-*O*-gallate-*O*-sulphates. For identification of peaks 1–23 see Table 1.

instance (−)-epicatechin forming (+)-epicatechin [24], for simplicity we have assumed that the unmetabolised green tea flavon-3-ols detected in plasma and ileal fluid have not undergone such a change.

The identification of the complex array of sulphate, glucuronide, methyl sulphate, and methyl glucuronide flavan-3-ol metabolites is summarized in Table 1, with the basis of the identifications being as follows:

Peak 1 (Retention time (R_t) = 11.2 min) was identified by its negatively charged molecular ion ($[M\text{-}H]^-$) at *m/z* 481, which on MS^2 fragmentation produced an ion at *m/z* 305, corresponding to a 176 *amu* loss of a glucuronide moiety. The subsequent MS^3 fragmentation of the daughter ion at *m/z* 305 matched the fragmentation pattern of a (epi)gallocatechin. Peak 1 is, therefore, an (epi)gallocatechin-*O*-glucuronide.

Peaks 2, 3, and 4 (R_t = 14.8, 16.9, 21.7 min) produced $[M\text{-}H]^-$ at *m/z* 385, and a daughter fragment at *m/z* 305 indicative of an (epi)gallocatechin that was confirmed by MS^3. The initial 80 *amu* loss corresponds to the cleavage of a sulphate. Peaks 2, 3, and 4 are, therefore, (epi)gallocatechin-*O*-sulphates.

Peak 5 (R_t = 15.4 min) was identified by its $[M\text{-}H]^-$ at *m/z* 495, with MS^2 loss of 176 *amu* glucuronide unit producing an ion at *m/z* 319. Further fragmentation revealed an MS^3 profile of ions at *m/z* 301, 275, 260, 233, and 137, indicating a methylated (epi)gallocatechin with the *m/z* 137 ion being characteristic of 4′-*O*-methylation [25]. Peak 5 is therefore a 4′-*O*-methyl-(epi)gallocatechin-*O*-glucuronide.

Peak 6 (R_t = 20.8 min) had an $[M\text{-}H]^-$ at *m/z* 465, which MS^2 fragmentation resulted in a 176 *amu* loss of a glucuronyl group. Subsequent MS^3 fragmentation of the ion at *m/z* 289 confirmed peak 6 as an (epi)catechin-*O*-glucuronide. This metabolite that had a different R_t to an (−)-epicatechin-7-*O*-glucuronide standard may be (−)-epicatechin-3′-*O*-glucuronide, which has been identified in urine collected after oral ingestion of (−)-epicatechin by humans [26].

Peaks 7, 8, and 9 (R_t = 21.0, 28.2, 29.8 min) all produced an $[M\text{-}H]^-$ at *m/z* 399, which on MS^2 fragmentation underwent an 80 *amu* loss of a sulphate group producing a daughter ion at *m/z* 319. The MS^3 profile allowed the identification of these peaks as 4′-*O*-methyl-(epi)gallocatechin-*O*-sulphates.

Peaks 10, 11, and 12 (R_t = 27.5, 30.8, 33.4 min) had an $[M\text{-}H]^-$ at *m/z* 369. The MS^2 loss of a sulphate moiety resulted in an ion at *m/z* 289, demonstrating the presence of (epi)catechin-*O*-sulphates.

Peak 13 (R_t = 32.9 min) was identified by its $[M\text{-}H]^-$ at *m/z* 537, which on MS^2 yielded an *m/z* 457 fragment with an 80 *amu* loss. MS^3 of the *m/z* 457 ion produced ions at *m/z* 331, 305, and 169 characteristic of an (epi)gallocatechin-3-*O*-gallate. This metabolite is, therefore, an *O*-sulphate of (epi)gallocatechin-3-*O*-gallate.

Peaks 14, 15, 16, and 17 (R_t = 33.0, 36.2, 39.6, and 41.9) all had an $[M\text{-}H]^-$ at *m/z* 383, which produced a daughter ion at *m/z* 303 corresponding to a loss of an 80 *amu* sulphate group. The resulting MS^3 fragments at *m/z* 259, 285, 244, and 219 corresponded to the spectrum of a standard of

Table 1. Identification of flavan-3-ols metabolites in ileal fluid, plasma and urine, based on HPLC retention times and MS fragmentation patterns[a)]

Flavan-3-ol metabolites (*HPLC peak no.*)	Occurrence	R_t (min)[b)]	$[M\text{-}H]^-$ (*m/z*)	MS^2 (*m/z*)	MS^3 (*m/z*)
(Epi)gallocatechin-*O*-glucuronide (*1*)	IF, P, U	11.2	481	305 (M-GlcUA)	179, 221, 219, 261
(Epi)gallocatechin-*O*-sulphates (*2–4*)	IF, U	14.8, 16.9, 21.7	385	305 (M-S)	179, 221, 219, 261
4′-*O*-methyl-(epi)gallocatechin-*O*-glucuronide (*5*)	IF, P, U	15.4	495	319 (M-GlcUA)	275, 301, 260, 233, 137
(Epi)catechin-*O*-glucuronide (*6*)	IF, P, U	20.8	465	289 (M-GlcUA)	245, 205
4′-*O*-methyl-(epi)gallocatechin-*O*-sulphates (*7–9*)	IF, P, U	21.0, 28.2, 29.8	399	319 (M-S)	275, 301, 260, 233, 137
(Epi)catechin-*O*-sulphates (*10–12*)	IF, P, U	27.5, 30.8, 33.4	369	289 (M-S)	245, 205
(Epi)gallocatechin-3-*O*-gallate-*O*-sulphate (*13*)	IF, P	32.9[c)]	537	457 (M-S)	331, 305, 169
3′-*O*-methyl-(epi)catechin-*O*-sulphates (*14–17*)	IF, P, U	33.0, 36.2, 39.6, 41.9	383	303 (M-S)	259, 285, 244, 219
4′-*O*-methyl-(epi)catechin-*O*-sulphate (*18*)	IF, P, U	45.0	383	303 (M-S)	259, 285, 244, 219, 137
O-methyl-(epi)gallocatechin-3-*O*-gallate-*O*-sulphates (*19*, *20*)	IF	42.9, 43.6[c)]	551	471 (M-S)	319, 169
(Epi)catechin-3-*O*-gallate-*O*-sulphate (*21*)	IF, P	45.4[c)]	521	441 (M-S)	289, 331, 169
O-methyl-(epi)catechin-3-*O*-gallate-*O*-sulphates (*22*, *23*)	IF	43.1, 49.9[d)]	535	455 (M-S)	303, 285, 169

a) R_t, Retention time (min); IF, ileal fluid; P, plasma; U, urine; S, sulphate; GlcUA, glucuronide.
b) Retention time on 60 min of 4–25% ACN, unless stated otherwise.
c) 60 min gradient of 5–40% ACN.
d) 60 min gradient of 5–50% ACN.

3′-*O*-methyl-(−)-epicatechin. These metabolites are therefore 3′-*O*-methyl-(epi)catechin-*O*-sulphates.

Peak 18 ($R_t = 45.0$ min) yielded a similar MS fragmentation as peaks 14–17 but had an additional major MS^3 fragment at *m/z* 137, which corresponded with the spectrum of a standard of 4′-*O*-methyl-(−)-epicatechin, also described by Cren-Olive *et al.* [27]. Peak 18 is thus identified as a 4′-*O*-methyl-(epi)catechin-*O*-sulphate.

Peaks 19 and 20 ($R_t = 42.9$, 43.6 min) had an $[M\text{-}H]^-$ at *m/z* 551, which produced an MS^2 ion at *m/z* 471, which on MS^3 yielded ions at *m/z* 319 and 169. This fragmentation is in keeping with the presence of *O*-methyl-(epi)gallocatechin-3-*O*-gallate-*O*-sulphates.

Peak 21 ($R_t = 45.4$ min) had an $[M\text{-}H]^-$ at *m/z* 521 and a daughter ion at *m/z* 441. The loss of an 80 *amu* corresponds to the cleavage of a sulphate moiety, and the resulting MS^3 profile indicated the presence of an (epi)catechin-3-gallate-*O*-sulphate.

Peaks 22 and 23 ($R_t = 43.1$, 49.9 min) had an $[M\text{-}H]^-$ at *m/z* 535, which loss of an 80 *amu* resulted in an MS^2 ion at *m/z* 455. MS^3 fragmentation produced ions at *m/z* 303, 285, and 169, indicating the presence of *O*-methyl-(epi)catechin-3-*O*-gallate-*O*-sulphates.

After the initial qualitative analysis, ileal fluid, plasma, and urine samples were analysed quantitatively by HPLC with MS in the SIM mode. Figure 2 illustrates typical HPLC-SIM traces obtained with a 2–5 h ileal fluid extract. it should be noted that in the qualitative analyses outlined above only the designated *peaks 1– 23* had yielded the appropriate ions when subjected to further fragmentation. Thus, for instance, the two early eluting, unlabelled peaks in the *m/z* 551 trace are not *O*-methyl-(epi)gallocatechin-3-*O*-gallate-*O*-sulphates as, unlike *peaks 19* and *20*, the *m/z* 551 ion did not produce an MS^2 daughter ion at *m/z* 471.

3.3 Quantitative analysis of ileal fluid

All of the metabolites listed in Table 1, together with (+)-gallocatechin, (−)-epigallocatechin, (+)-catechin, (−)-epicatechin, (−)-epigallocatechin-3-*O*-gallate, (+)-gallocatechin-3-*O*-gallate, and (−)-epicatechin-3-*O*-gallate, were identified and quantified in the ileal fluid samples of the five volunteers (Table 2). A total of 206 ± 11 μmol of unmetabolised green tea flavan-3-ols were recovered in the 0–24 h samples of ileal fluid, which is equivalent to 33 ± 1.8% of intake. The (−)-epicatechin and (+)-catechin content of the ileal fluid was equivalent to 11 and 6.8%, respectively, of the amounts ingested while there were recoveries of 18% for (−)-epigallocatechin and 27% for (+)-gallocatechin. There were higher recoveries of the 3-galloylated flavan-3-ols: 89% for (+)-gallocatechin-3-*O*-gallate, a minor component in the green tea, 49% for (−)-epigallocatechin-3-*O*-gallate, the major green tea flavan-3-ol, and 45% for (−)-epicatechin-3-*O*-gallate.

Substantial quantities of flavan-3-ol metabolites were also detected in the ileal fluid mainly in the form of (epi)catechin-*O*-sulphates, which were equivalent to 82% of the (+)-catechin/(−)-epicatechin ingested, and (epi)gallocatechin-*O*-sulphates, which corresponded to 45% of the (+)-gallocatechin/(−)-epigallocatechin intake. Smaller quantities of (epi)gallocatechin-3-*O*-gallate and (−)-epicatechin-3-*O*-gallate metabolites, accounting for 1.1 and 1.4% of the respective doses, were also detected. The amount of metabolites recovered in the 24 h ileal effluent following the ingestion of 634 μmol of flavan-3-ols accounted for 37% (232 μmol), and together with the recovery of non-absorbed parent compounds, represented 69% (439 μmol) of the amount initially present in the green tea (Table 2). This implies an overall absorption of 31% of the ingested green tea flavan-3-ols.

3.4 Quantitative analysis of plasma

Plasma collected 0–24 h after the ingestion of the 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)gallocatechin together with the native green tea flavan-3-ols (−)-epicatechin-3-*O*-gallate and (−)-epigallocatechin-3-*O*-gallate as well as (−)-epigallocatechin, which has not previously been detected in plasma (Table 1). *O*-sulphates of (epi)gallocatechin-3-*O*-gallate (*peak 13*) and (epi)catechin-3-*O*-gallate (*peak 21*) were also detected in the plasma (Table 1) of some of the volunteers but in low quantities that precluded obtaining reliable quantitative pharmacokinetic data. The pharmacokinetic profiles of the nine main 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 glucuronide (*peak 1*), which reached a C_{max} of 256 ± 41 nM after 1.1 ± 0.5 h while an (epi)catechin-*O*-glucuronide (*peak 6*) was present in lower quantities with a C_{max} of 101 ± 20 nM and a T_{max} of 0.8 ± 0.1 h. The unmetabolised flavan-3-ols, (−)-epigallocatechin-3-*O*-gallate, (−)-epicatechin-3-*O*-gallate, and (−)-epigallocatechin attained C_{max} values of 35 ± 5, 17 ± 6, and 18 ± 1.8 nM after 0.6 ± 0.1, 1.0 ± 0.3, and 0.5 ± 0.0 h, respectively. All the flavan-3-ols and their metabolites were only present in trace amounts after 6 h and were not present in detectable amounts in the 24 h plasma samples.

3.5 Quantitative analysis of urine

A total of 43 ± 3.8 μmol of flavan-3-ol metabolites were excreted in the 24 h urine samples of the five volunteers (Table 4). This corresponds to 6.8 ± 0.6% of the flavan-3-ol

Table 2. Quantities of flavan-3-ols and metabolites recovered in ileal fluid 0–24 h after the ingestion of 300 mL of green tea

Flavan-3-ols and metabolites (*HPLC peak no.*)	Amount ingested (μmol)	Recovered in ileal fluid	
		(μmol)	% of amount ingested
(+)-Catechin	18	1.2±0.2	6.8±1.2
(–)-Epicatechin	69	7.9±1.7	11±2.5
(+)-Gallocatechin	50	13±2.1	27±4.2
(–)-Epigallocatechin	190	35±5.8	18±3.1
(–)-Epigallocatechin-3-*O*-gallate	238	116±5.1	49±2.1
(+)-Gallocatechin-3-*O*-gallate	5.2	4.6±0.3	89±5.2
(–)-Epicatechin-*O*-gallate	64	29±3.3	45±5.2
Total parent flavan-3-ols	**634**	**207±11**	**33±1.8**
(Epi)catechin-*O*-sulphates (*10–12*)		72±4.1	
O-methyl-(epi)catechin-*O*-sulphates (*14–18*)		5.4±0.5	
(Epi)catechin-*O*-glucuronide (6)		0.5±0.2	
	Total (epi)catechin metabolites	*78±4.3*	*90±5.0*
(Epi)gallocatechin-*O*-sulphates (*2–4*)		108±5.2	
O-methyl-(epi)gallocatechin-*O*-sulphates (*7–9*)		40±4.3	
O-methyl-(epi)gallocatechin-*O*-glucuronide (*5*)		1.5±0.5	
(Epi)gallocatechin-*O*-glucuronide (*1*)		1.5±0.4	
	Total (epi)gallocatechin metabolites	*151±8.9*	*63±3.7*
(Epi)gallocatechin-3-*O*-gallate-*O*-sulphate (*13*)		1.8±0.4	
O-methyl-(epi)gallocatechin-3-*O*-gallate-*O*-sulphates (*19, 20*) (*19, 20*)		0.9±0.2	
	Total (–)-epigallocatechin-3-O-gallate metabolites	*2.7±0.5*	*1.1±0.2*
(Epi)catechin-3-*O*-gallate-*O*-sulphate (*21*)		0.4±0.1	
Methyl-(epi)catechin-3-*O*-gallate-*O*-sulphates (*22, 23*)		*0.4±0.1*	
	Total (–)-epicatechin-3-O-gallate metabolites	*0.8±0.2*	*1.4±0.3*
Total metabolites		**232±13**	**37±2.1**
Total parent flavan-3-ols and metabolites		**439±13**	**69±2.0**

Data expressed as mean values±SE ($n = 5$).
For HPLC peak numbers see Fig. 2. and Table 1.

content of the ingested green tea. The main compounds excreted in urine were 4′-*O*-methyl-(epi)gallocatechin-*O*-sulphates, *O*-methyl-(epi)catechin-*O*-sulphates, and (epi)catechin-*O*-sulphates (Table 4). The total amount of (epi)catechin and (epi)gallocatechin metabolites excreted were 24±2.0 and 19±2.0 μmol, respectively. When compared with the dose of the parent compounds ingested, the percentages excreted accounted for 27±2.3% and 8.0±0.8% for the (epi)catechin and (epi)gallocatechin metabolites, respectively (Table 4).

4 Discussion

The array of flavan-3-ols detected in plasma, and their pharmacokinetic profiles, after ingestion of green tea by subjects with an ileostomy were similar to what was obtained with volunteers with an intact colon [22], indicating absorption in the small intestine. The C_{max}, and as a consequence the *AUC* values, observed with the ileostomists were higher but this is probably a reflection of the fact that different green teas were used in the two studies. The ileostomists had earlier T_{max} values than subjects with colon, for all the absorbed flavan-3-ols and their metabolites (Table 5). This is probably due to more rapid transit through the small intestine due to the absence of an ileal brake [28, 29]. Once absorbed into the circulatory system, the various flavan-3-ols appeared to behave in a similar manner as the presence or absence of a colon did not impact on plasma $T_{1/2}$ values (Table 5).

The appearance of (−)-epigallocatechin-3-*O*-gallate, (−)-epicatechin-3-*O*-gallate, and (−)-epigallocatechin in plasma, albeit in low concentrations, is unusual, as flavonoids typically pass from the gastrointestinal tract into the circulatory systems as glucuronide and/or sulphated metabolites. Despite their presence in plasma, none of these unmetabolised flavan-3-ols were excreted in urine. It is possible that the kidneys are unable to remove them from the bloodstream in which case other mechanism(s) must be involved in their rapid decline after reaching C_{max}. Studies

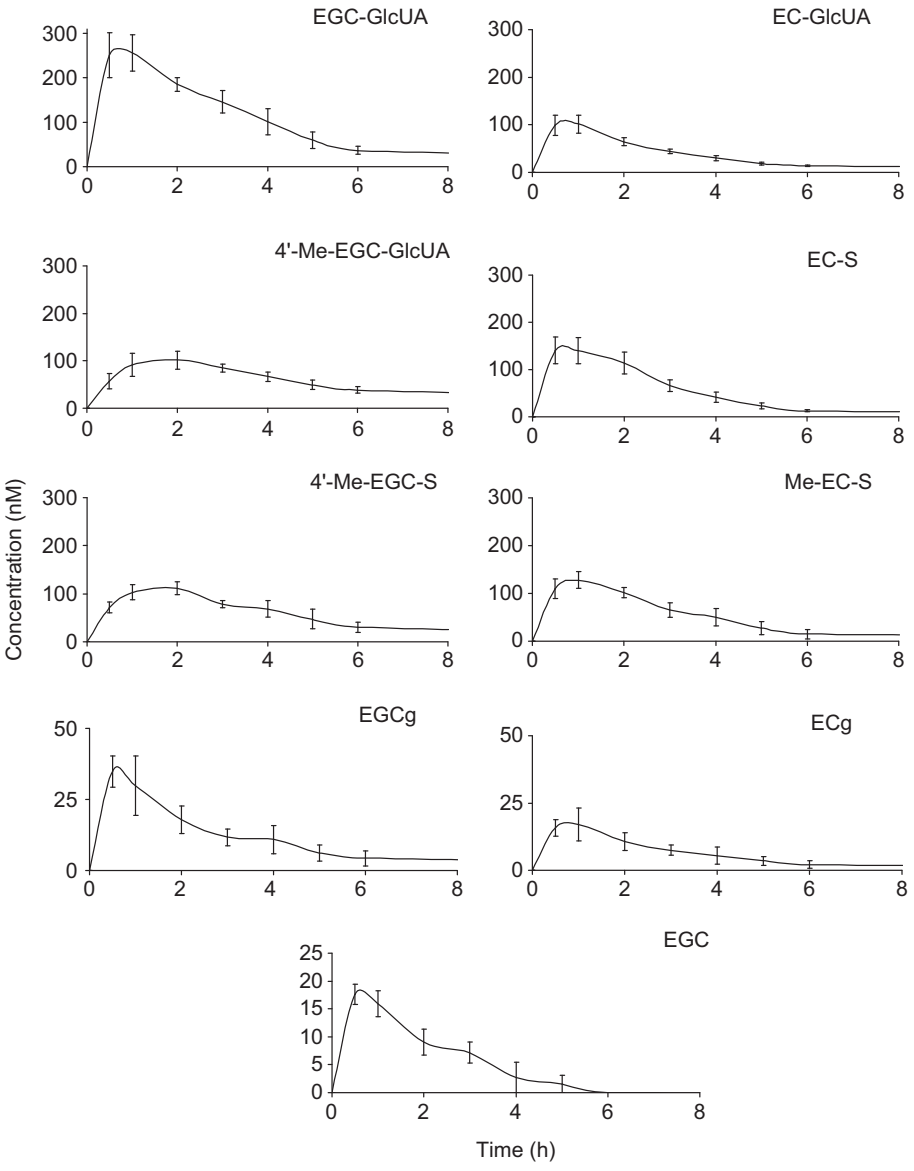

Figure 3. Concentrations of an (epi)gallocatechin-*O*-glucuronide (EGC-GlcUA) (*peak 1*), 4′-*O*-methyl-(epi)gallocatechin-*O*-glucuronide (4′-Me-EGC-GlcUA) (*peak 5*), 4′-*O*-methyl-(epi)gallocatechin-*O*-sulphates (4′-Me-EGC-S) (*peaks 7–9*), (epi)catechin-*O*-glucuronide (EC-GlcUA) (*peak 6*), (epi)catechin-O-sulphates (*peaks 10–12*), 3′-and 4′-*O*-methyl-(epi)catechin-*O*-sulphates (Me-EC-S) (*peaks 14–18*), (−)-epigallocatechin-3-gallate (EGCg), (−)-epicatechin-3-*O*-gallate (ECg), and (−)-epigallocatechin (EGC) in the plasma of human subjects with an ileostomy 0–6 h after the ingestion of 300 mL of green tea. Data expressed as mean values with their SEs ($n = 5$) 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 1.

Table 3. Pharmacokinetic analysis of flavan-3-ols and their metabolites detected in plasma of ileostomy volunteers following the ingestion of 300 mL of green tea

Flavan-3-ols (*HPLC peak no.*)	C_{max} (nM)	T_{max} (h)	*AUC* (μmol.h.L^{-1})	$T_{1/2}$ (h)
(Epi)gallocatechin-*O*-glucuronide (*1*)	256 ± 41	1.1 ± 0.5	0.83 ± 0.15	1.8
4′-*O*-methyl-(epi)gallocatechin-*O*-glucuronide (*5*)	102 ± 19	2.2 ± 0.4	0.42 ± 0.08	2.8
4′-*O*-methyl-(epi)gallocatechin-*O*-sulphates (*8*, *9*)	111 ± 14	1.5 ± 0.3	0.43 ± 0.08	2.2
(Epi)catechin-*O*-glucuronide (*6*)	101 ± 20	0.8 ± 0.1	0.29 ± 0.05	1.7
(Epi)catechin-*O*-sulphates (*10*, *12*)	140 ± 28	1.3 ± 0.3	0.43 ± 0.09	1.5
O-methyl-(epi)catechin-*O*-sulphates (*14*, *16*–*18*)	128 ± 18	1.3 ± 0.3	0.40 ± 0.09	1.6
(–)-Epigallocatechin-3-*O*-gallate	35 ± 5.4	0.6 ± 0.1	0.09 ± 0.03	1.9
(–)-Epicatechin-3-*O*-gallate	17 ± 6.1	1.0 ± 0.3	0.05 ± 0.02	1.7
(–)-Epigallocatechin	18 ± 1.8	0.5 ± 0.0	0.04 ± 0.01	1.3

Data expressed as mean values ± SE ($n = 5$).
For HPLC peak numbers see Fig. 2. and Table 1.

with rats have led to speculation that (−)-epigallocatechin-3-*O*-gallate is removed from the blood stream in the liver and returned to the small intestine in the bile. The evidence, however, is equivocal. In one study (−)-epigallocatechin-3-*O*-gallate was fed to rats by gavage and only *ca*. 3% of intake was detected in bile principally as metabolites [30]. When tritium-labelled (−)-epigallocatechin-3-*O*-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 with 2% in urine over the 48 h period [31]. This involved extensive metabolism of the (−)-epigallocatechin-3-*O*-gallate as it was present in bile as sulpho and/or glucuronide conjugates of 4″-*O*-methyl- and 4′,4″-*O*-dimethyl-(−)-epigallocatechin-3-*O*-gallate. To what extent enterohepatic recirculation of (−)-epigallocatechin-3-*O*-gallate metabolites occurs in humans is difficult to establish without direct experimentation. If it was a significant event, then in the present study (−)-epigallocatechin-3-*O*-gallate metabolites would be expected to be detected in substantial amounts in ileal fluid. The data presented in Table 2 indicate that this is not the case as 0–24 h ileal fluid contained an (epi)gallocatechin-3-*O*-gallate-*O*-sulphate and two *O*-methyl-(epi)gallocatechin-3-*O*-gallate-*O*-sulphates in amounts corresponding to only 1.1% of (−)-epigallocatechin-3-*O*-gallate intake.

In contrast to the 3-*O*-galloylated flavan-3-ols, the (epi)catechins and (epi)gallocatechins were both present in ileal fluid principally as sulphate and methylated metabolites together with trace amounts of glucuronides (Table 2). *In vitro* incubations with gastric juice and ileal fluid have indicated that flavan-3-ols are stable and are not metabolised or degraded over a 4 h period at 37°C [32]. This suggests that the presence of substantial metabolites of (epi)catechin and (epi)gallocatechin in ileal fluid is likely to be the result of conversions occurring in the enterocytes of the small intestine, with a portion of the metabolites being transported into the circulatory system and the remainder being subjected to efflux back into the lumen of the small intestine.

The data presented in Table 6, which in part summarises the more detailed information in Table 2, shows differential recoveries of the four major classes of flavan-3-ols in ileal fluid collected 0–24 h after drinking green tea. There was a 100 ± 5% recovery of the (epi)catechins principally as metabolites while the overall recovery of (epi)gallocatechin and its metabolites was 83 ± 5% of intake. The urinary excretion of (epi)catechin metabolites was 27 ± 2% of intake while that of (epi)gallocatechin metabolites was 8 ± 1%, figures that are in keeping with those obtained in previous studies and which imply that (epi)catechin in particular is highly bioavailable [22, 32].

Table 4. Flavan-3-ol metabolites excreted in urine of ileostomy volunteers 0–24 h after the ingestion of 300 mL of green tea

Flavan-3-ol metabolites (*HPLC peak no.*)	Urinary excretion (μmol)
(Epi)gallocatechin-*O*-glucuronide (*1*)	3.3 ± 0.6
(Epi)gallocatechin-*O*-sulphates (*2–4*)	1.8 ± 0.6
4′-*O*-methyl-(epi)gallocatechin-*O*-sulphates (*7–9*)	13 ± 1.1
4′-*O*-methyl-(epi)gallocatechin-*O*-glucuronide (*5*)	1.0 ± 0.2
Total (epi)gallocatechin metabolites	*19.1 ± 2.0 (8.0 ± 0.8%)*
(Epi)catechin-*O*-glucuronide (*6*)	0.8 ± 0.1
(Epi)catechin-*O*-sulphates (*10–12*)	11 ± 0.9
O-methyl-(epi)catechin-*O*-sulphate (*14–18*)	12 ± 1.1
Total (epi)catechin metabolites	*23.8 ± 2.0 (27.4 ± 2.3%)*
Total flavan-3-ol metabolites	**43 ± 3.8 *(6.8 ± 0.6%)***

Data expressed as mean values ± SE ($n = 5$).
For HPLC peak numbers see Fig. 2. and Table 1. Italicised figures in parentheses represent excretion as a percentage of intake.

Table 5. A comparison of plasma T_{max} and $T_{1/2}$ values of flavan-3-ols and their metabolites obtained after the ingestion of green tea by healthy humans with a colon and subjects with an ileostomy

Flavan-3-ols (*HPLC peak no.*)	T_{max} (h)		$T_{1/2}$ (h)	
	Ileostomists	Healthy	Ileostomists	Healthy
(Epi)gallocatechin-*O*-glucuronide (*1*)	1.1 ± 0.5	2.2 ± 0.2	1.8	1.6
4′-*O*-Methyl-(epi)gallocatechin-*O*-glucuronide (*5*)	2.2 ± 0.4	2.3 ± 0.3	2.8	3.1
4′-*O*-Methyl-(epi)gallocatechin-*O*-sulphates (*7-9*)	1.5 ± 0.3	2.2 ± 0.2	2.2	2.2
(Epi)catechin-*O*-glucuronide (*6*)	0.8 ± 0.1	1.7 ± 0.2	1.7	1.6
(Epi)catechin-*O*-sulphates (*10-12*)	1.3 ± 0.3	1.6 ± 0.2	1.5	1.9
O-Methyl-(epi)catechin-*O*-sulphates (*14-18*)	1.3 ± 0.3	1.7 ± 0.2	1.6	1.5
(–)-Epigallocatechin-3-*O*-gallate	0.6 ± 0.1	1.9 ± 0.1	1.9	1.0
(–)-Epicatechin-3-*O*-gallate	1.0 ± 0.3	1.6 ± 0.2	1.7	1.5
(–)-Epigallocatechin	0.5 ± 0.0	-	1.3	-

Data on healthy subjects from Stalmach *et al.* [22]. Data expressed as mean values ± SE ($n = 10$ for healthy subjects and $n = 5$ for ileostomy volunteers)
For HPLC peak numbers see Fig. 2. and Table 1.

Table 6. Recovery of flavan-3-ols and their metabolites in ileal fluid and urine collected 0–24 h after the ingestion of 300 mL of green tea by human subjects with an ileostomy

Green tea flavan-3-ols	Intake	Ileal fluid			Urine			Total recovered
		Flavan-3-ols	Metabolites	Total	Flavan-3-ols	Metabolites	Total	in ileal fluid and urine
(Epi)catechins	87 ± 3	9.1 ± 1.9	78 ± 4	87 ± 5 (*100 ± 5%*)	n.d.	24 ± 2	24 ± 2 (*27 ± 2%*)	111 ± 4 (*128 ± 5%*)
(Epi)gallocatechins	240 ± 7	48 ± 7	151 ± 9	199 ± 11 (*83 ± 5%*)	n.d.	19 ± 2	19 ± 2 (*8 ± 1%*)	219 ± 10 (*91 ± 4%*)
(Epi)gallocatechin-3-*O*-gallate	243 ± 4	121 ± 5	2.8 ± 0.5	123 ± 5 (*51 ± 2%*)	n.d.	n.d.	n.d. (*0%*)	124 ± 5 (*51 ± 2%*)
(−)-Epicatechin-3-*O*-gallate	64 ± 1	29 ± 3	0.9 ± 0.2	30 ± 3 (*46 ± 5%*)	n.d.	n.d.	n.d. (*0%*)	30 ± 3 (*46 ± 5%*)

Data presented in μmol ± standard error ($n = 5$). Figures in italicised parentheses are flavan-3-ols and their metabolites recovered in ileal fluid and urine, expressed as a percentage of intake. n.d. – not detected

Combining the amount in ileal fluid with that excreted in urine gives a 128 ± 5% recovery of (epi)catechin intake and a 91 ± 4% of (epi)gallocatechin. The most likely explanation for these unusually high recoveries is that at some stage during passage from the mouth to the small intestine 3-*O*-degalloylation is occurring, resulting in the conversion of (−)-epicatechin-3-O-gallate to (−)-epicatechin and (−)-epigallocatechin-3-O-gallate to (−)-epigallocatechin. In keeping with this possibility the recoveries of the two galloylated flavan-3-ols in ileal fluid was *ca.* 50% (Table 6).

It has been reported that holding green tea in the mouth results in the conversion of (−)-epigallocatechin-3-*O*-gallate to (−)-epigallocatechin as a result of the action of a human salivary esterase [33]. In the present study, the tea was not held in the mouth for any period of time; so it would seem improbable that the salivary esterase could be responsible for such conversions on a substantial scale. It seems more plausible that they occur as a result of the multitude of enzyme activities in the wall of the small intestine. In a previous study with volunteers with an ileostomy, analysis of ileal fluid found no evidence of post-ingestion conversion of (−)-epigallocatechin-3-*O*-gallate to (−)-epigallocatechin [32]. However, the (−)-epigallocatechin-3-*O*-gallate was ingested as a powder in a gelatine capsule and this may have impacted on its fate of the flavan-3-ol. This matter needs to be revisited with the (−)-epigallocatechin-3-*O*-gallate being fed dissolved in an aqueous solution in a concentration that is comparable to that found in a green tea infusion.

There are claims in the literature that green tea flavan-3-ols are poorly bioavailable because of instability under digestive conditions with >80% losses being observed with *in vitro* digestion models simulating gastric and small intestine conditions [34, 35]. It is clear that the data obtained in these investigations do not accurately reflect the *in vivo* fate of flavan-3-ols following ingestion as they are at variance with the high urinary excretion observed in green tea feeding studies [22, 36] and the substantial recovery of flavan-3-ols in ileal fluid observed in the present study and also after the consumption of Polyphenon E, a green tea extract [32].

Finally, it is of note that despite (epi)catechins being absorbed in quantity with urinary excretion of their metabolites being equivalent to 27% of intake (Table 4), overall 69% of the green tea flavan-3-ol intake was present in ileal fluid (Table 2). In healthy subjects with a functioning colon this would pass from the small to the large intestine where it would be subjected to the action of the microflora and be degraded to phenolic acids including hydroxyphenyl-γ-valerolactones [37]. These processes, and whether or not they are involved in the protective effects of green tea consumption, merit further investigation.

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The authors have declared no conflict of interest.

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