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Absorption, distribution, metabolism and excretion of apigenin and its glycosides in healthy male adults

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ABSTRACT

The bioavailability of apigenin and its *O*-glycosides in humans was investigated with apigenin-4′-glucuronide (Ap-4′-GlcUA), apigenin-7-glucuronide and apigenin-7-sulfate being identified as in vivo metabolites. Apigenin *per se* was poorly absorbed with metabolites equivalent to 0.5% of intake excreted in urine 0–24 h post-intake. Consumption of a parsley drink containing apigenin-7-*O*-(2″-*O*-apiosyl)glucoside resulted in the peak plasma concentration (C_{max}) of Ap-4′-GlcUA occurring after 4 h, indicative of absorption in the lower gastrointestinal tract (GIT). Urinary excretion of the three metabolites corresponded to 11.2% of intake. Ingestion of dried powdered parsley leaves with yogurt extended the C_{max} of Ap-4′-GlcUA to 6 h. Consumption of chamomile tea containing apigenin-7′-*O*-glucoside resulted in a 2 h C_{max} of Ap-4′-GlcUA, in keeping with absorption in the upper GIT. Urinary excretion was equivalent to 34% of intake. Intake of the parsley drink provided information on intra- and inter-individual variations in the level of excretion of the apigenin metabolites.
Clinical trail registration number: This trail was registered at clinicaltrials.gov as NCT03526081.

1. Introduction

Apigenin (**1**) is a flavone occurring in plants of dietary interest, such as parsley (*Petroselinum crispum)*, celery (*Apium graveloens)* and artichoke *(Cynara cardunculus)*, mainly as the diglycoside apigenin-7-*O*-(2″-*O*-apiosyl)glucoside (**2,** apiin) and to a lesser extent, as the monosaccharide apigenin-7-*O*-glucoside (**3**) [1–3]. A range of C-linked flavone glycosides have also been identified. Potential beneficial effects of apigenin on health have been described [4–8]. However, relatively little is known about the absorption, distribution, metabolism and excretion (ADME) (aka bioavailability) of the flavone. Current reports on the ADME of apigenin in in humans have significant limitations, including the use of analytical methods that did not provide information on the identity of apigenin metabolites appearing in plasma and urine [9–11]. A number of ADME studies in animal models used complex mixtures of diverse flavones [12] or the aglycone apigenin, although in the context of the habitual dietary intake apigenin occurs mostly in the form of its conjugated derivatives [1,2]. Other limitations are based on the extremely high amounts that were ingested [13,14], the low-resolution analysis of flavone metabolites [15], and the use of a radiolabeled apigenin in which the tritium label would be readily exchanged and converted to ^3H_2O [16]. Furthermore, studies on the flavan-3-ol, (−)-epicatechin, indicate clear differences ADME differences between animal models and humans [17,18].

Abbreviations: ADME, absorption; distribution, metabolism and excretion; Ap-4′-GlcUA, apigenin-4′-glucuronide; Ap-7-GlcUA, apigenin-7-glucuronide; Ap-7-S, apigenin-7-sulfate; CBG, cytosolic β-glucosidase; CV, coefficient of variance; GIT, gastrointestinal tract; LPH, lactase phloridzin hydrolase; SRAMs, structurally-related apigenin metabolites.

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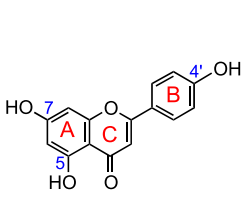
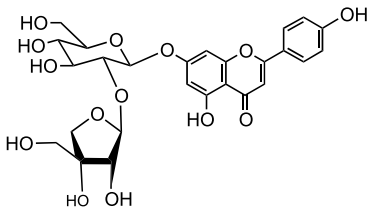
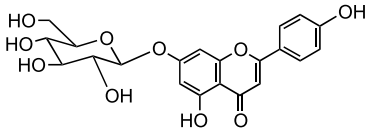


Apigenin (**1**)



Apigenin-7-*O*-(2"-*O*-apiosyl)glucoside (**2**)



Apigenin-7-*O*-glucoside (**3**)

There is little information on the impact of different types of glycosylation on the ADME of apigenin in humans. However, studies with other flavonoids have shown that the sugar moiety can have a substantial effect [19]. Data from Day et al. [20,21] demonstrated that flavonoid mono-glycosides are likely to be subjected to hydrolysis by lactase phloridzin hydrolase (LPH) and cytosolic β-glucosidase (CBG) in the proximal gastrointestinal tract (GIT) with the released aglycone undergoing phase II metabolism and being absorbed in the small intestine. In contrast, the diglycosides hesperetin-7-*O*-rutinoside and quercetin-3-*O*-rutinoside undergo limited hydrolysis in the small intestine, restricting their absorption in the upper GIT. Instead, the rutinose moiety is cleaved by the gut microbiota with the released flavonoid aglycone undergoing metabolism in colonocytes prior to absorption in the systemic circulatory [22].

The current study used HPLC-MS and authentic analytical standards to identify structurally-related apigenin metabolites (SRAMs) in human plasma and urine. The impact of the glycosylation pattern of apigenin on the ADME of the flavone was also investigated in acute absorption studies with healthy male adults (n = 4). The findings from this initial study provided the basis for an investigation with a parsley tea, containing apigenin-7-*O*-(2″-*O*-apiosyl)glucoside (**2**), in which urinary excretion of SRAMs in a total of 17 feeds was used to assess intra- and inter-volunteer variations in the bioavailability of the diglycoside.

2. Experimental

2.1. Chemicals

Apigenin, apigenin-7-*O*-glucoside, apigenin-7-*O*-(2″-*O*-apiosyl) glucoside, and apigenin-7-glucuronide (Ap-7-GlcUA) were obtained from Sigma-Aldrich (St Louis, MO). Apigenin-4′-glucuronide (Ap-4′-GlcUA) and apigenin-7-sulfate (Ap-7-S) were purchased from Toronto Research Chemicals (Toronto, ON, Canada). 3′-Methoxyhippuric acid and other reagents were supplied by Sigma-Aldrich. The nomenclature used for (poly)phenols and their metabolites is that of Kay et al. [23].

2.2. Apigenin- and apigenin glycosides-containing test materials

Apigenin, sold in capsule form as a dietary supplement, was obtained from Swanson Ultra (Fargo, ND, USA). Powdered parsley leaf capsules were purchased from Nature's Way (Lehi, UT, USA) and Twinings "Pure Chamomile Herbal Tea" bags, which along with plain, unsweetened yogurt, were obtained from a local supermarket.

2.3. Study design

The study and protocol were approved by the Institutional Review Board at the University of California, Davis on 06/25/2015. This study was registered at clinicaltrials.gov as NCT03526081. Volunteers gave their written consent to participate in the study. Healthy, male adults, 25–60 years of age, were recruited by public advertisement in the city of Davis, CA, and surrounding areas. Exclusion criteria included a body mass index >30 kg/m^2, blood pressure (BP) > 140/90 mm Hg, allergies to peanut or cocoa, avoidance of caffeinated food products and beverages, a history of cardiovascular disease, stroke, renal, hepatic, or thyroid disease, GIT disorders, previous GIT surgery (except appendectomy), a current intake of plant-derived dietary supplements, persons following vegan/vegetarian diet, and those adhering to an uncommon dietary régime or a weight loss program. To determine eligibility, participants were asked to complete health and lifestyle questionnaires, have their height, weight, and in-office BP determined, and to provide a blood sample for complete blood count, liver, lipid and metabolic panel assessments. Volunteer characteristics are presented in Table S1 in the Supplementary Information.

Enrolled participants commenced the study protocol between 1 and 3 weeks after eligibility was determined. While participating in the study, volunteers were asked to maintain their typical daily activities and diet. All subjects were instructed on adhering to a low-flavone diet, and received a list of recommended foods with a low or negligible flavone content. In addition, volunteers were provided with low (poly) phenol, flavone-free foods for their supper on the day prior to the study day, as well as for all the meals for the duration of the study. Volunteers were also asked to fast for 12 h before each study day.

The initial investigation consisted of an open-label, cross-over, single intake study, in which volunteers consumed the different apigenin-containing products in four different visits:

- Visit 1. The contents of one apigenin capsule dissolved in 450 mL hot water was ingested by three volunteers.
- Visit 2. A 3.2 g sample of the dried parsley leaves was infused for 3 min in 450 mL water at ~65 °C. After the initial ingestion by four subjects, 30 mL of water was added to the container to remove any residual parsley powder and this too was consumed by the volunteers.
- Visit 3. A 3.2 g sample of the dried parsley leaves was mixed with 100 g of plain, unsweetened yogurt prior to intake by the same four subjects. Yogurt was added to evaluate its potential impact as a matrix effect on flavone ADME.
- Visit 4. Three bags of chamomile tea were added in 450 mL of water at ~65 °C for 3 min prior to intake of the infusion by four volunteers.

All the ingested products were freshly prepared on the day of the study, and consumed in less than 5 min. After ingestion, no other food or drink was allowed, except for water, for the following 4 h after which a low flavone lunch was provided. All urine voids excreted during the 12 h before and the 24 h after ingestion were collected. For preservation, 2 mL 0.5% thymol and 20 mL 2 M sodium acetate were added to the urine collection containers. Blood samples were collected in heparin tubes before (0 h) and 2, 4 and 6 h after ingestion. Plasma, obtained by centrifugation of whole blood at 1800*g* for 15 min, was aliquoted in 1 mL volumes and ascorbic acid added for a final concentration of 1 mg/mL before being stored at −80 °C.

The more detailed absorption study consisted of a single intake, open label study, in which 11 healthy male subjects consumed parsley tea, and urine voids were collected 0–24 h post-ingestion. Four of the 11 volunteers repeated the study, resulting in a total of 15 intakes. Two volunteers participating in this study had also completed the initial investigation with the parsley drink, so data obtained with their 0–24 h urine were included, making a total of 17 intakes.

2.4. Processing of plasma and urine

Aliquots of 300 μL of plasma were added to a 96-well Phree (Phenomenex, Torrence, CA) plate containing 1 mL of acetonitrile acidified with 0.1% formic acid and lightly vortexed for 1 min. The Phree plate was centrifuged

at 240*g* for 15 min after which 300 μL of methanol containing 0.1% formic acid was added, and the supernatant revortexed and centrifuged. The filtrate was transferred from the collection plate to 1.5 mL tubes and placed in a Speedvac concentrator and reduced to *ca.* 50 μL after which 50 μL of a solution 100 μM of 3′-methoxyhippuric acid in 0.1% aqueous formic acid was added as an internal standard.

Aliquots of 100 μL of urine were filtered using a 96-well Impact protein precipitation plate and combined with 100 μM of 3′-methoxyhippuric acid as an internal standard. The mixture was then vortexed for 1 min after which the plate was centrifuged for 15 min at 240*g*. A 25 μL volume of the filtrate was analysed by HPLC-MS^2.

2.5. HPLC-MS^2 analysis

Aliquots (20 mg) of the content of apigenin and dried parsley capsules (20 mg) were extract twice with 50% aqueous methanol and Chamomile tea infusions were injected on a Waters Acquity UPLC (Waters, Milford, MA) consisting of an autosampler cooled at 5 °C, a column oven at 40 °C and a binary solvent manager. Separations utilized a 100 × 4.6 mm C18 column (Phenomenex, Torrance, CA) eluted at a flow rate of 1.0 mL/min with a 40 min, 10–50% gradient of increasing amounts of acetonitrile/methanol (90:10, v/v) in 0.5% formic acid in 10 mmol/L aqueous ammonium formate. The column eluate was directed to a Quattro Micro QAA1229 triple quadrupole mass spectrometer fitted with an electrospray interface (Micromass, Milford, MA) operated in negative ionization mode. Apigenin-7-*O*-(2″-*O*-apiosyl) glucoside was used for tuning. Capillary temperature was 350 °C, sheath gas flow was 60 units, while the auxiliary gas was set to 20 units. The spray voltage was 4.5 kV. Flavones were quantified in μmol by reference to apigenin (*m/z* 269 → 225), apigenin-7-*O*-glucoside (*m/z* 431 → 269 and apigenin-7-*O*-(2″-*O*-apiosyl)glucoside (*m/z* 579 → 269).

Plasma and urine extracts were analysed with the same HPLC-MS system using a 100 × 4.6 mm 2.6 μm Kinetex F5 HPLC column (Phenomenex) eluted, respectively, at a flow rate of 0.5 mL/min with a 22 min gradient of 25–32% acetonitrile/methanol (90:10, v/v) in 0.1% aqueous formic acid. Targeted analysis was carried out in MS^2. Ap-7-GlcUA was used for tuning. Capillary temperature was 350 °C, sheath gas flow was 60 units, while the auxiliary gas was set to 20 units. The spray voltage was 4.5 kV. Analysis was performed using multiple reaction monitoring. The specific transitions followed were as follows: Ap-4′-GlcUA, and Ap-7-GlcUA *m/z* 445 → 269, Ap-7-S *m/z* 349 → 269, and apigenin *m/z* 269 → 225. Quantifications of apigenin phase II metabolites were based on standard curves of authentic reference compounds.

3. Results and discussion

3.1. Identification and quantification of apigenin and apigenin-glycosides in test products

Apigenin capsules contained apigenin aglycone whilst the parsley powder was rich in the di-glycosylated conjugate, apigenin-7-*O*-(2″-*O*-apiosyl)glucoside. The 450 mL of chamomile tea contained relatively small amounts of the flavone principally in the form of the mono-glycoside apigenin-7-*O*-glucoside and smaller amounts of apigenin (Table 1). Chamomile is also a rich source of sesquiterpenoids [24].

Table 1
Quantification of apigenin and its glycosides in the ingested products[a].

Product	Apigenin	Apigenin -7-*O*-glucoside	Apigenin-7-*O*-(2″-*O*-apiosyl) glucoside	Total apigenin content
Apigenin capsules (μmol/capsule)	191	–	–	191
Parsley (μmol/3.2 g)	Trace	–	118	118
Chamomile tea ((μmol/infusion of 3 bags)	1.2	13.4	–	14.6

[a] –, not detected.

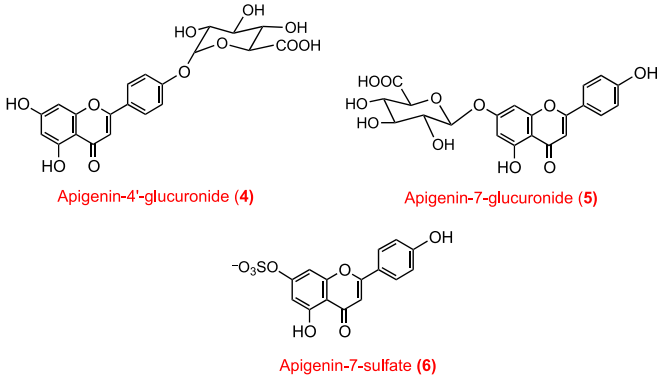
Table 2
HPLC-MS^2 identification of apigenin metabolites in urine and plasma[a].

Metabolites	Rt (min)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)
Apigenin-7-glucuronide (U)	14.4	445	269, 225, 175
Apigenin-4′-glucuronide (U,P)	15.0	445	269, 225, 175
Apigenin-7-sulfate (U, P)	22.4	349	269, 225

[a] U, urine; P, plasma.

3.2. Identification of apigenin metabolites

HPLC-MS^2 analysis of urine collected after the ingestion of the parsley drink revealed the presence of three major SRAMs, comprising two apigenin glucuronides and one apigenin sulfate. The use of authentic standards enabled these metabolites to be identified as Ap-4′-GlcUA (**4**), Ap-7-GlcUA (**5**) and Ap-7-S (**6**) (Table 2). Plasma contained both Ap-4′-GlcUA and Ap-7-S, but Ap-7-GlcUA was not present in detectable quantities.



3.3. Initial absorption studies

3.3.1. Apigenin

Neither apigenin nor its metabolites were detected in plasma collected 0, 2, 4 and 6 h after the ingestion of 191 μmol of apigenin. However, a total of 0.9 μmol of Ap-4′-GlcUA and Ap-7-GlcUA were present in urine excreted 0–24 h after intake (Table 3). This is equivalent to 0.5% of the ingested flavone. As a consequence of low apigenin

Table 3
Urinary excretion of apigenin metabolites after acute intake of 191 μmol of apigenin. Data expressed in μmol ± S.D. (n = 3).[a].

Time	Ap-7-GlcUA	Ap-4′-GlcUA	Ap-7-sulfate	Apigenin	Total
−12-0 h	n.d.	n.d	n.d.	n.d.	n.d.
0–2 h	0.1 ± 0.1	n.d	n.d.	n.d.	0.1 ± 0.1
2–4 h	0.1 ± 0.0	0.1 ± 0.0	n.d.	n.d.	0.2 ± 0.0
4–8 h	0.1 ± 0.0	0.1 ± 0.1	n.d.	n.d.	0.2 ± 0.1
8–12 h	0.3 ± 0.1	0.1 ± 0.1	n.d.	n.d.	0.4 ± 0.1
Total 0–24 h	0.6 ± 0.4	0.3 ± 0.2	n.d.	n.d.	0.9 ± 0.6

[a] n.d., not detected; Ap-7-GlcUA, apigenin-7-glucuronide; Ap-4′-GlcUA, apigenin-4′-glucuronide; Ap-7-S, apigenin-7-sulfate.

aqueous solubility [25] its limited absorption, arguably, resulted in most of the apigenin passing from the small intestine to the colon where it would either undergo phase II metabolism, or microbiota-mediated ring fission yielding low molecular weight catabolites such as 4′-hydroxycinnamic acid, 3-(4′-hydroxyphenyl)propanoic acid and 4′-hydroxyphenylacetic acid. These phenolic compounds were not investigated in the current study as their limited MS ionization adversely impacts HPLC-MS limits of detection.

3.3.2. Parsley drink

Following the consumption of a parsley drink containing apigenin-7-*O*-(2″-*O*-apiosyl)glucoside, Ap-4'-GlcUA and Ap-7-S appeared in plasma. The sulfate had a peak plasma concentration (C_{max}) of 253 ± 202 nmol/L 4 h after intake (T_{max}). The glucuronide had a C_{max} of 153 ± 61 nmol/L and a 4 h T_{max} (Fig. 1). Urine excreted over a 24 h period after parsley intake contained mainly Ap-4'-GlcUA with small quantities of Ap-7-GlcUA neither of which were present in urine excreted 12 h prior to supplementation. A small amount of Ap-7-S, which was a major metabolite in plasma was also detected in urine, but only in the 4–8 h sample (Table 4).

From the plasma profile it is evident that the diglycoside was absorbed and metabolized much more readily than its aglycone, with 0–24 h urine containing 13.2 μmoL of apigenin metabolites (Table 4), equivalent to 11.2% of intake. The timing of the plasma C_{max} at 4 h indicates that the apiosylglucoside is likely to be cleaved in the distal rather than the proximal GIT by the colonic microbiota with the released apigenin being absorbed and metabolized by phase II conjugation reactions.

3.3.3. Parsley with yogurt

Consumption of dried parsley with 100 g of plain, unsweetened yogurt changed the plasma pharmacokinetic profile with the peak concentration of Ap-4′-GlcUA being delayed by at least 2 h to the last time point at 6 h. The appearance of Ap-7-S was similarly delayed (Fig. 2). The delayed C_{max} could be a consequence of consuming the parsley as a powder in a yogurt matrix, which potentially could have slowed gastric emptying and delayed the mouth to cecum transit time of the head of the drink [26,27]. The release of the flavone from the

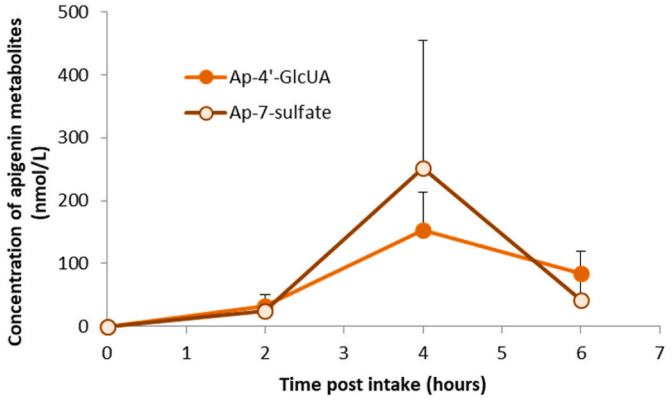


Fig. 1. Pharmacokinetics of apigenin-4′-glucuronide (Ap-4′-GlcUA) and apigenin-7-sulfate (Ap-7-S) in plasma 0–6 h after the ingestion of a parsley drink containing 118 μmol of apigenin-7-*O*-(2″-*O*-apiosyl)glucoside (n = 4).

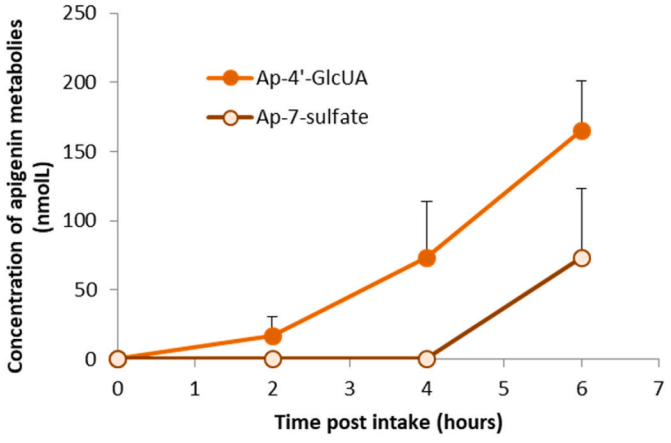


Fig. 2. Pharmacokinetics of apigenin-4′-glucuronide (Ap-4′-GlcUA) and apigenin-7-sulfate (Ap-7-S) in plasma 0–6 h after the ingestion of yogurt with dried, powdered parsley leaves containing 118 μmol of apigenin-7-*O*-(2″-*O*-apiosyl)glucoside (n = 4).

Table 4
Urinary excretion of apigenin metabolites after acute of 450 mL of a parsley drink containing 118 μmol of apigenin-7-*O*-(2″-*O*-apiosyl)glucoside. Data expressed in μmol ± S.D. (n = 4).[a]

Time	Ap-7-GlcUA	Ap-4′-GlcUA	Ap-7-sulfate	Apigenin	Total
−12-0 h	–	–	–	–	n.d.
0–4 h	0.6 ± 0.6	5.6 ± 3.0	–	–	6.2 ± 3.5
4–8 h	0.5 ± 0.4	4.3 ± 4.4	0.1 ± 0.2	–	4.9 ± 4.8
8–12 h	0.2 ± 0.2	1.4 ± 1.7	–	–	1.7 ± 1.9
12–24 h	0.1 ± 0.1	0.3 ± 0.5	–	n.d.	0.4 ± 0.6
Total 0–24 h	1.5 ± 1.2	11.5 ± 7.7	0.1 ± 0.2	n.d.	13.2 ± 8.8

[a] n.d., not detected; Ap-7-GlcUA, apigenin-7-glucuronide; Ap-4′-GlcUA, apigenin-4′-glucuronide; Ap-7-S, apigenin-7-sulfate.

Table 5
Urinary excretion of apigenin metabolites after acute intake of 100 mL of yogurt with dried, powdered parsley leaves containing 118 μmol of apigenin-7-*O*-(2″-*O*-apiosyl)glucoside. Data expressed in μmol ± S.D. (n = 4)[a].

Time	Ap-7-GlcUA	Ap-4′-GlcUA	Ap-7-sulfate	Apigenin	Total
−12-0 h	0.1 ± 0.1	–	–	–	0.1 ± 0.1
0–4 h	0.6 ± 0.8	5.2 ± 8.2	–	–	5.8 ± 9.0
4–8 h	0.8 ± 0.4	10.3 ± 3.1	–	0.2 ± 0.2	11.4 ± 3.3
8–12 h	0.4 ± 0.3	2.7 ± 2.9	–	0.1 ± 0.1	3.1 ± 3.3
12–24 h	0.2 ± 0.3	1.8 ± 3.2	–	0.0 ± 0.1	2.0 ± 3.5
Total 0–24 h	2.0 ± 2.0	20.1 ± 16.3	–	0.3 ± 0.3	22.4 ± 18.2

[a] n.d., not detected; Ap-7-GlcUA, apigenin-7-glucuronide; Ap-4′-GlcUA, apigenin-4′-glucuronide; Ap-7-S, apigenin-7-sulfate.

powered leaf could have been an additional factor slowing absorption. These events could explain the delayed flavone peak plasma concentration, which may be longer than the 6 h time point (Fig. 2), the last collection point. The 0–24 h urinary excretion of 20.1 ± 16.3 μmol metabolites (Table 5) is equivalent to 19% of intake. However, this figure is not statistically different from the 11% excretion of metabolites after ingestion of the parsley drink without yogurt.

3.3.4. Chamomile tea

Feeds were carried out with chamomile tea, which had a much lower flavone content than the parsley drink, containing 13.4 μmol of the monosaccharide apigenin-7-*O*-glucoside and 1.2 μmol of apigenin (Table 1). The metabolites appearing in plasma were the same as after parsley intake, namely Ap-4′-GlcUA and Ap-7-S. However, the pharmacokinetic profile was different as the C_{max} of the metabolites was at 2 h rather than 4 h after intake (Fig. 3). Urinary excretion metabolites over the 24 h period post-ingestion totaled 5.0 μmol, equivalent to 34% of intake, and occurred mainly in the 0–4 h collection period (Table 6). These finding are in keeping with the apigenin-7-*O*-glucoside being hydrolysed by LPH and/or CBG in the proximal GIT, with absorption of the released apigenin occurring principally via enterocytes.

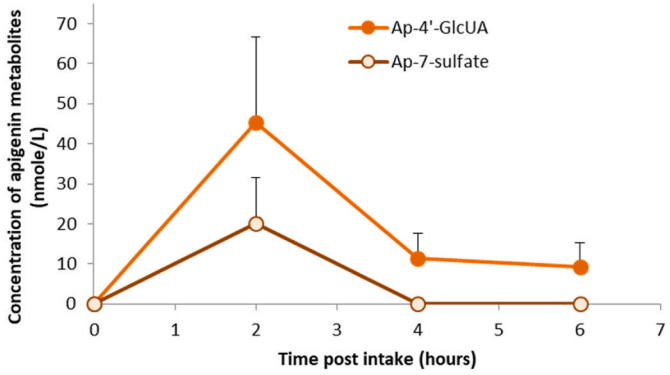


Fig. 3. Pharmacokinetics of apigenin-4′-glucuronide (Ap-4′-GlcUA) and apigenin-7-sulfate (Ap-7-S) in plasma 0–6 h after the ingestion of a chamomile tea containing 1.2 μmol of apigenin and 13.4 μmol of apigenin-7-*O*-glucoside (n = 4).

Table 6
Urinary excretion of apigenin metabolites after acute intake of 450 mL of chamomile tea containing 1.2 μmol of apigenin and 13.4 μmol apigenin-7-*O*-glucoside. Data expressed in μmol ± S.D. (n = 4).[a].

Time	A-7-GlcUA	Ap-4′-GlcUA	Ap-7-sulfate	Apigenin	Total
−12-0 h	–	–	–	–	–
0–4 h	0.3 ± 0.2	2.8 ± 1.9	–	–	3.1 ± 2.1
4–8 h	0.2 ± 0.2	1.5 ± 2.0	–	–	1.7 ± 2.2
8–12 h	0.0 ± 0.0	0.1 ± 0.1	–	–	0.1 ± 0.1
12–24 h	0.1 ± 0.1	0.1 ± 0.2	–	–	0.1 ± 0.2
Total 0–24 h	0.6 ± 0.5	4.4 ± 3.5	–	n.d.	5.0 ± 4.0

[a] –, not detected; Ap-7-GlcUA, apigenin-7-*O*-glucuronide; Ap-4′-GlcUA, apigenin-4′-glucuronide; Ap-7-S, apigenin-7-sulfate.

3.4. Inter- and intra-variations in excretion of apigenin metabolites following ingestion of a parsley drink

Data on 0–24 h urinary excretion of apigenin metabolites in the study with 17 intakes of the parsley drink containing 118 μmol of apigenin-7-*O*-(2″-*O*-apiosyl)glucoside are presented in Table 7. Again the main metabolite was Ap-4′-GlcUA (18.1 ± 10.5 μmol) (SD) followed by Ap-7-GlcUA (2.1 ± 1.4 μmol), which appeared in the urine collected following all 17 intake occasions. Smaller amounts of Ap-7-S (0.12 ± 0.06 μmol) were excreted by 6 volunteers.

The mean total 0–24 h excretion of SRAMs was 20.3 ± 11.6 μmol, which corresponds to 17.2 ± 9.8% of intake (Table 7). The inter-individual variation of excreted metabolites expressed as a coefficient of variation (CV) was 57%. This variation is likely the consequence of volunteer-to-volunteer differences in the gut microbiota responsible for cleavage of sugar moiety of apigenin-7-*O*-(2″-*O*-apiosyl)glucoside releasing the apigenin aglycone prior to absorption and phase II metabolism in the colonocyte. Of note, following ingestion of orange juice, containing flavanones-7-*O*-rutinosides, which also undergo colonic cleavage of the sugar moiety prior to absorption, the inter-volunteer variation of 0–24 h urinary excretion of hesperetin and naringenin phase II metabolites also had a CV of 57% (see Table S2 in Supplementary Information) [28]. The CV of 20% for intra-volunteer variation in excretion of SRAMs was substantially lower than the inter-volunteer %CV at 57%. This is likely to be a consequence of small, fluctuations in volunteer microbiota coupled with other minor physiological and life style changes.

Table 7
Apigenin metabolites in μmol excreted in urine by individual volunteers 0–24 h after ingestion of a parsley drink containing 118 μmol of apigenin-7-*O*-(2″-*O*-apiosyl)glucoside. Data expressed in μmol (n = 4).[a].

Volunteer (intakes)	Ap-7-GlcUA	Ap-4′-GlcUA	Ap-7-S	Total	% of Intake
1 (1)	4.1	31.1	–	35.2	29.8
(2)	2.2	15.1	–	17.3	14.7
(3)	1.3	9.0	0.9	11.2	9.5
2 (1)	1.2	13.7	–	14.9	12.6
(2)	1.5	16.3	0.06	17.8	15.1
(3)	1.5	16.8	0.6	18.9	16.0
3 (1)	2.7	31.7	–	34.4	29.2
(2)	3.1	28.5	–	31.6	26.8
4 (1)	1.7	15.5	0.12	17.3	14.7
(2)	2.3	16.3	0.41	19.0	16.1
5 (1)	0.8	16.8	–	17.6	14.9
6 (1)	2.4	20.0	–	22.4	19.0
7 (1)	2.7	7.9	–	10.6	9.0
8 (1)	0.5	10.0	–	10.5	8.9
9 (1)	0.3	4.7	0.02	5.0	4.2
10 (1)	1.3	9.4	–	10.7	9.1
11 (1)	5.8	45.1	–	50.9	43.1
Mean ± SD	2.1 ± 1.4	18.1 ± 10.5	0.12 ± 0.26	20.3 ± 11.6	17.2 ± 9.8

[a] –, not detected; Ap-7-GlcUA, apigenin-7-glucuronide; Ap-4′-GlcUA, apigenin-4′-glucuronide; Ap-7-S, apigenin-7-sulfate. Intakes 1 and 2, n = 16, intake 3, n = 2.

The most abundant SRAM detected in 0–24 h urine corresponded to Api-4′-GlcUA, accounting for 89% of all SRAMs detected in urine. The other metabolites, Api-7-GlcUA and Api-7-S, represented 10% and 0.6% of all SRAMs in urine, respectively. While apigenin glucuronides were detected in all volunteers, only 6 volunteers excreted detectable amounts of Ap-7-S.

3.5. Analysis, bioavailability and potential bioactivity of apigenin metabolites

For Ap-4′-GlcUA, Ap-7-GlcUA and Ap-7-S, HPLC-MS quantification in plasma and urine used authentic reference compounds. This is critical if accurate estimates of SRAMs are to be obtained as, for instance, quantification of Ap-7-GlcUA by reference to the aglycone apigenin can result in a ~50-fold over-estimate [29].

The data obtained in the current study are in marked contrast to those obtained in an earlier human ADME study on parsley which concluded 66 μmol of ingested apigenin-7-*O*-(2″-*O*-apiosyl)glucoside had limited bioavailability [10]. Rather than identifying and quantifying apigenin metabolites in plasma and urine by HPLC-MS, samples were subjected to glucuronidase/sulfate treatment and the released apigenin aglycone analysed by HPLC with diode array and electrochemical detection The plasma pharmacokinetic profile obtained differed markedly from that illustrated in Fig. 1, reaching a level of 100–120 nmol/L after 2 h which did not begin to decline until 10 h after parsley intake. This could have been a consequence of a matrix effect as parsley was blanched in butter and served either hot or cold with wheat bread toast, salt and pepper. However, urinary excretion 0–24 h after parsley intake was estimated to be 144 ± 110 nmol, equivalent to 0.22% of the ingested dose [10]. This is substantially lower than the 20.3 ± 11.6 μmol, corresponding to 17.2 ± 9.8% of intake, obtained in the current study (Table 7).

Arango et al. [30] in a PNAS paper, previously claimed to have revealed the molecular basis for the action of apigenin by the identification of 160 human target proteins. The apigenin targeted hnRNPA2, a factor associated with numerous cellular malignancies and involved in mRNA metabolism/splicing. It was shown that by inhibiting hnRNPA2 dimerisation, apigenin affected the alternative splicing of key RNAs. However, the study only indicates what happens if apigenin enters cancer cells in relevant concentrations. In practice, apigenin was immobilized on amino polyethyleneglycol-polyacrylamide copolymer beads which would alter its structural ability to interact with specific sites. A further compounding factor is that the vast majority of apigenin molecules in the proximity or inside cells would be in the form of apigenin phase II metabolites (Table 2) casting further doubts as to the relevance of the claims of Arango et al. [30].

4. Conclusions

This is the first identification of Ap-4′-GlcUA, Ap-7-GlcUA and Ap-7-S as post-ingestion metabolites of apigenin-*O*-glycosides. As a consequence, *in vitro* and *ex vivo* studies with human cell-based test systems aiming at elucidating the mechanisms underlying potential protective effects of the dietary flavone should make use of these metabolites and not the aglycone apigenin or its *in planta* glycosides which does not accumulate in the circulatory system. The study demonstrated the impact of yogurt on the ADME of parsley apigenin-7-*O*-(2″-*O*-apiosyl) glucoside which delayed the plasma T_{max} of Ap-4′-GlcUA and Ap-7-S. Such matrix effects raise the need for investigations on the bioavailability of dietary flavonoids as part of normal meals with chronic as well as acute intakes. It is also important to acknowledge that flavonoid ADME can be expected to be amended by environmental modulators such as diet, stress, supplement intake and participation in exercise [31, 32]. Future studies should, therefore, consider the impact of these compounding factors on flavonoid ADME and bioactivity.

Authors' contribution

G.B.: Methodology, Validation, Formal analysis, Investigation, Writing - Review and Editing; R.Y.F.: Methodology, Validation, Formal analysis, Investigation J.I.L., J.K., V.M.: Methodology, Resources, Investigation; J.I.O., A.C.: Conceptualization, Preparing the original draft, Visualization, Supervision, Project administration.

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Declaration of competing interest

J.I.O. is employed by Mars Inc., a company engaged in flavanol research and flavanol-related commercial activities. A.C. is a consultant for Mars Inc.

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Appendix A. Supplementary data

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