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Human colonic catabolism of dietary flavan-3-ol bioactives

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ABSTRACT

Understanding the fate of ingested polyphenols is crucial in elucidating the molecular mechanisms underlying the beneficial effects of a fruit and vegetable-based diet. This review focuses on the colon microbiota-mediated transformation of the flavan-3-ols and the structurally related procyanidins found in dietary plant foods and beverages, plus the flavan-3-ol-derived theaflavins of black tea, and the post-absorption phase II metabolism of the gut microbiota catabolites. Despite significant advances in the last decade major analytical challenges remain. Strategies to address them are presented.

1. Introduction

Since the pioneering report of Hertog et al. (1993), which has been cited more than 6000 times, there has been increasing evidence of the involvement of dietary polyphenols, in particular flavan-3-ols, in protective effects against non-communicatable conditions including coronary heart disease, inflammation, cancer and declining cognitive function (Schroeter et al., 2006, 2010; Loke et al., 2008; Heiss et al., 2010; Curtis et al., 2012; Del Rio et al., 2013; Brickman et al., 2014; Rodriguez-Mateos et al., 2014; Ottaviani et al., 2018, 2020; Sloan et al., 2021). *In planta* C_6–C_3–C_6 polyphenols (aka flavonoids) occur principally as glycosylated conjugates, and gallate esters in the case of tea, grapes and wine (Crozier et al., 2006). Following ingestion of polyphenols the attached sugar or gallic acid is removed, initially in the upper gastrointestinal (GI) tract and the released aglycones undergo phase II metabolism in epithelial/hepatic cells appearing in the systemic circulation as the sulfate, glucuronide and methylated derivatives (Williamson et al., 2018).

Nonetheless polyphenol glycosides and gallates are not fully absorbed in the upper GI tract and, together with substantial amounts of those conjugated with disaccharides, pass from the small intestine to colon (Stalmach et al., 2010; Borges et al., 2013) where they are subjected to the action of the microbiota and, after cleavage of the conjugating moiety, the released C_6–C_3–C_6 aglycones enter the systemic circulation as phase II metabolites prior to being excreted in urine. Ring fission of the aglycones also takes place yielding a mixture of low molecular weight phenolics, a portion of which is subject to phase II metabolism. However, their identification and quantification in biofluids and an evaluation of their role in polyphenol bioavailability is, for a number of reasons, less straight forward than that of C_6–C_3–C_6 phase II metabolites (Clifford et al., 2020; Clifford and Crozier, 2012).

HPLC-HR-MS analysis of urine and plasma collected in a human bioavailability study identified and quantified 67 phenolic catabolites (Pereira-Caro et al., 2016). Even this figure is likely to be very much an underestimate (Rubio-Aliaga et al., 2011; Kuhnert and Clifford, 2022). However, many of these catabolites are likely to be present in very low concentrations that are unlikely to impact on bioactivity unless there are substantial synergistic interactions. A complication of more importance is that a number of the compounds present in sizable amounts are background products that are not derived exclusively from dietary polyphenols. There are, for instance, mammalian pathways to hippuric acid **1** (*N*-benzoyl-glycine) from benzoic acid **2** (Clifford et al., 2000), phenylalanine **3** and tyrosine **4** (Self et al., 1960; Grumer 1961; Bridges et al., 1970). In addition, hepatic metabolism of surplus aromatic amino

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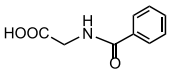
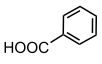
acids produces hippuric acid **1**, benzoic acid **2**, 3-(3'-hydroxyphenyl) propanoic acid **5**, 2-hydroxy-3-(4'-hydroxyphenyl)propanoic acid [aka 3-(4′-hydroxyphenyl)lactic acid] **6,** 3-hydroxy-3-(3'-hydroxyphenyl) propanoic acid [aka 3-(3′-hydroxyphenyl)hydracrylic acid] **7**, 2-hydroxy-(4'-hydroxyphenyl)acetic acid (aka 4′-hydroxymandelic acid) **8,** 3-hydroxybenzoic acid **9**, 4-hydroxybenzoic acid **10**, 3'-hydroxyhippuric acid **11,** and 4'-hydroxyhippuric acid **12** (Curtius et al., 1976). Other sources of phenolics include medicinal drugs, such as aspirin, and food additives including benzoic acid and hydroxybenzoic acid esters.


Hippuric acid **1**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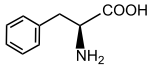
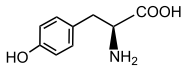
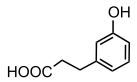
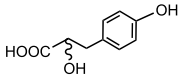
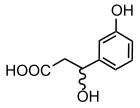
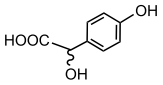

Phenylalanine **3**

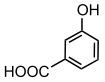

Tyrosine **4**

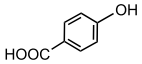

3-(3'-Hydroxyphenyl)propanoic acid **5**

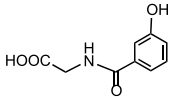

2-Hydroxy-(4'-hydroxyphenyl)propanoic acid **6**

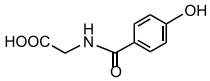

3-Hydroxy-(3'-hydroxyphenyl)propanoic acid **7**


2-Hydroxy-(4'-hydroxyphenyl)acetic acid **8**


3-Hydroxybenzoic acid **9**


4-Hydroxybenzoic acid **10**


3'-Hydroxyhippuric acid **11**


4'-Hydroxyhippuric acid **12**

Clearly, it is of paramount importance that investigations into the microbiota-mediated catabolism of dietary phenolics take measures to distinguish between genuine polyphenol catabolites and background phenolics derived from other sources. The gold standard for feeding studies requires the use of isotopically labelled substrates. Potential alternative protocols to include:

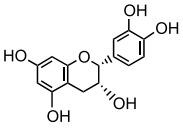
- After a wash-out period on a low (poly)phenol diet, comparison of phenolics in plasma and urine with and without the consumption of the test products.
- After a wash-out period on a low (poly)phenol diet, comparison of phenolics in plasma and urine before and after the consumption of test products by ileostomists and volunteers with a functioning colon.
- Incubations with and without specific (poly)phenol substrates and analysis of catabolites produced by microbiota and/or fecal slurries.
- Incubation of (poly)phenol substrates in vitro with colonocyte/hepatocyte cell systems in presence/absence of appropriate co-factors.

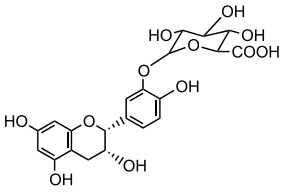
This review will focus on flavan-3-ols and a gold standard feeding study with 2-^{14}C-labelled (−)-epicatechin ([2–^{14}C]EC) which, as well as providing data on the structurally-related (−)-epicatechin metabolites (SREMs) absorbed in the small intestine, produced much clearer view of events occurring in the lower bowel than feeds with unlabelled polyphenols as the radiolabel enabled phenolic catabolites to be identified and quantified without the complications of having to distinguished them from a diversity of unlabelled phenolics originating from other substrates. Information on the colonic fate of the oligomeric flavan-3-ols, the procyanidins (PCs), and the black tea fermentation products, theaflavins, are also discussed.

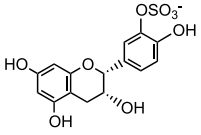
2. Colonic catabolism of dietary flavan-3-ols

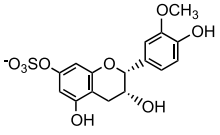
2.1. [2–^{14}C](−)-Epicatechin

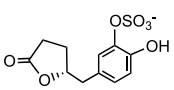
The flavan-3-ol monomer (−)-epicatechin **13** is found widely in fruits and vegetables, and occurs in especially high amounts in cocoa (Crozier et al., 2006). Ottaviani et al. (2016) have carried out a feeding study in which volunteers ingested radiolabelled (−)-epicatechin. Eight male subjects ingested 300 μCi (60 mg, 270 μmol) of [2–^{14}C]EC after which radioactivity in blood, urine and feces was monitored at intervals over a period of up to 72 h. Total recovery of radioactivity in urine and feces was almost 100% of the ingested [2–^{14}C]EC indicating minimal long term tissue deposition of the compounds derived from the ingested monomer (Borges et al., 2018).

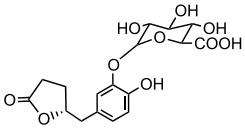

(−)-Epicatechin **13**

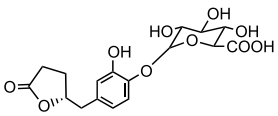

(−)-Epicatechin-3'-*O*-glucuronide **14**


(−)-Epicatechin-3'-*O*-sulfate **15**


3'-Methoxy-(−)-epicatechin-7-*O*-sulfate **16**


5-(4'-Hydroxyphenyl)-γ-valerolactone-3'-sulfate **17**


5-(4'-Hydroxyphenyl)-γ-valerolactone-3'-glucuronide **18**


5-(3'-Hydroxyphenyl)-γ-valerolactone-4'-glucuronide **19**

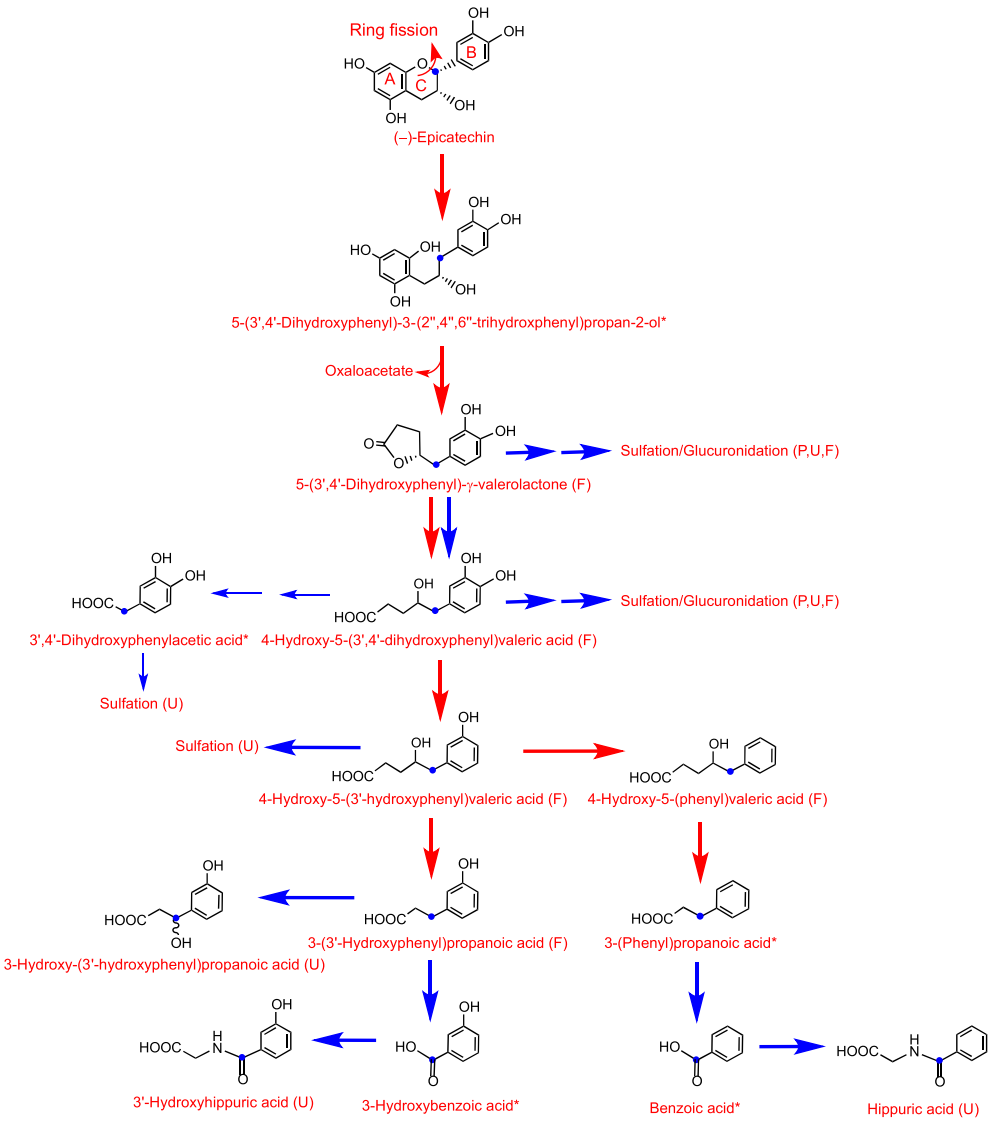


Fig. 1. Proposed pathways for the catabolism by colonic microbiota of [2–^{14}C](−)-epicatechin passing from the small to the large intestine (red arrows), and potential steps catalysed by mammalian enzymes in colonocytes and/or hepatocytes (blue arrows). Thin arrows indicate minor routes. Location of metabolites: P – plasma; U – urine; F – feces. Asterisks indicate potential intermediates that do not accumulate in detectable quantities in either plasma, urine or feces. The blue circle indicates the position of ^{14}C-label. Radiolabelled (−)-epicatechin-3′-sufate, as well as [2–^{14}C](−)-epicatechin, is likely to move from the small to the large intestine (Actis-Goretta et al. 2013). Although it is feasible that the sulfate moiety remains intact, for simplicity it is assumed that it is removed by the colonic bacteria releasing (−)-epicatechin. Based on the data of Ottaviani et al. (2016) and Borges et al. (2018). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Twelve ^{14}C-labelled SREMs, including (−)-epicatechin-3′-glucuronide **14**, (−)-epicatechin-3′-sulfate **15**, and 3′-methoxy-(−)-epicatechin-7-sulfate **16**, were detected in plasma. After attaining an overall peak plasma concentration (C_{max}) of 1223 nmol/L, 1.0 h after [2–^{14}C]EC intake the SREMs declined rapidly with an apparent elimination half-life ($AT_{1/2}$) of 1.9 h and, in almost all instances, had disappeared from the circulatory system within 8 h. The ~1 h C_{max} indicates absorption in the upper GI tract. Urinary excretion of the [^{14}C]EC phase II metabolites was equivalent to 20% of intake.

A series of microbiota-derived 5-carbon ring fission catabolites (5C-RFCs) with a summed C_{max} of 588 nmol/L appeared later in plasma with T_{max} of ~6 h. They were present in the circulatory systems for longer than the SREMs having a $AT_{1/2}$ of 5.7 h. They also had an area under the curve concentration ~3-fold higher than that of the SREMs. The main 5C-RFCs were 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-sulfate **17** and 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-glucuronide **18**. Lower concentrations of other 5C-RFCs were also detected, in the form of 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-glucuronide **19**, two 5-(phenyl)-γ-valerolactone-glucuronide-sulfates and three 4-hydroxy-5-(hydroxyphenyl)valeric acid phase II conjugates.

5C-RFCs were also detected in urine along with ^{14}C-labelled hippuric acids, 3-hydroxy-3-(3′-hydroxyphenyl)propanoic acid **7** and a hydroxyphenylacetic acid-sulfate. Voided feces contained 10% of the radioactivity intake principally in the form of phenylvaleric acids and smaller quantities of phenyl-γ-valerolactones and 3-(3′-hydroxyphenyl)propanoic acid **5**. There was an almost total recovery of radioactivity in urine and feces in the form of SREMs (20%), 5C-RFCs (42%), 2/3C-RFCs (7%) and hippuric acids (21%) along with a number of minor unidentified radiolabelled products. Such estimates without the use of a radiolabeled substrate would be very much an approximation.

As a result of their prolonged presence in the circulatory system and substantial excretion in urine, 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-sulfate **17** and 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-glucuronide **18** are good biomarkers of the intake of flavan-3-ol-containing dietary products (Ottaviani et al., 2020), especially as they are also catabolites of PCs (Anesi et al., 2019; Mena et al., 2019) (see Section 2.2.) which typically occur in quantity along with flavan-3-ol monomers in nuts, fruits and vegetables including almonds, apples, banana, berries, pears, grapes, cocoa, and legumes (Crozier et al., 2006; Del Rio et al., 2013). C_6–C_5 valerolactones are microbiota catabolites only of flavan-3-ols (Williamson et al., 2018) although similar C_6–C_5 valeric acids lacking the side chain hydroxyl are also microbiota catabolites of cereal grain alkyl-resorcinols (Landberg et al., 2009).

The proposed fate of [2–^{14}C]EC on moving from the small to the

large intestine, where ring fission is followed by post-absorption phase II metabolism, is illustrated in Fig. 1. There are multiple routes for the gut microbiota catabolism of flavan-3-ols which may proceed simultaneously with the pathways varying depending upon the precise composition of the microbiome. The balance between these routes determines which intermediates accumulate sufficiently to be detected in biofluids, and it is improbable that all intermediates would be detected in a single study, even if they are produced.

It is proposed that [2–^{14}C]EC microbiota-mediated catabolism would begin with opening of the C-ring and the A-ring which convert the C_6–C_3–C_6 substrate to a transient C_6–C_9 metabolite, that in turn is converted to oxaloacetate (C_4) and the C_6–C_5 5C-RFCs, particularly 5-(3',4'-dihydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(3',4'-dihydroxyphenyl)valeric acid. The greater portion of these 3',4'-dihydroxy catabolites is absorbed and converted to sulfate and glucuronide conjugates by coloncoycte and/or hepatic enzymes as shown in Fig. 1.

Analysis of feces indicated that the smaller unabsorbed portion of the phenylvaleric acids is subject to dehydroxylation of the phenyl ring yielding 3′-hydroxy-, and side-chain dehydroxylated [^{14}C]catabolites, together with the residual 3′,4′-dihydroxyphenylvaleric acid. In addition, microbiota-mediated-side chain shortening and the removal of the 4′-hydroxyl group resulted in the formation of [^{14}C]3-(3′-hydroxyphenyl)propanoic acid (Borges et al., 2018). This 3C-RFC, which was partially absorbed and excreted in urine, is also produced from (−)-epicatechin and PCs *in vitro* by fecal microbiota (Roowi et al., 2010; Stoupi et al., 2010a). These discoveries throw further light on the findings of a combination of earlier *in vitro* bacterial incubations and animal feeding studies (Groenewoud and Hundt, 1984; Krumholz and Bryant, 1988; Stoupi et al., 2010b; Kutschera et al., 2011; Margalef et al., 2015; Serra et al., 2011).

Fecal incubations with (−)-epicatechin by Roowi et al. (2010) and Di Pede et al. (2022) yielded 3C-RFCs but not 2C-RFCs. In contrast, Stoupi et al. (2010a) did detect fecal conversions of (−)-epicatechin to 3′-hydroxyphenylacetic acid and phenylacetic acid. Potentially, an α-oxidation of 3-(3′-hydroxyphenyl)propanoic acid resulted in the production of the radiolabelled phenylacetic acids while most further metabolism of [^{14}C]3-(3′-hydroxyphenyl)propanoic acid also involves mammalian enzymes with a β-oxidation catalysing the removal of two carbons from the side chain yielding 3′-hydroxybenzoic acid, which glycination would convert to 3′-hydroxyhippuric acid. A parallel pathway from 4-hydroxy-5-(phenyl)valeric acid could result in the production of [^{14}C]hippuric acid (Fig. 1). The glycination step involves hepatic enzymes and urinary excretion indicated there was a 21% conversion of the ingested [^{14}C]EC to hippuric acids.

A radiolabeled hydracrylic acid, 3-hydroxy-3-(3′-hydroxyphenyl) propanoic acid **7**, was excreted in urine after ingestion of [2–^{14}C]EC. This 3C-RFC is not formed *in vitro* when (−)-epicatechin **13** is incubated with fecal bacteria (Roowi et al., 2010). It is, therefore, likely originates from a 3-hydroxylation of the 3-(3′-hydroxyphenyl)propanoic acid side chain during β-oxidation (Fig. 1).

The use of the labelled substrate allowed the yield of catabolites and their conjugates to be accurately determined as 3-(3'-hydroxyphenyl) propanoic acid **5**, 3-hydroxy-3-(3'-hydroxyphenyl)propanoic acid **7**, 3'-hydroxyhippuric acid **11**, and especially hippuric acid **1**, are produced from aromatic amino acids, among other sources, and are always present in urine even after a low-polyphenol diet and a wash-out (Roowi et al., 2010; Stalmach et al., 2013). The magnitude of this effect was clearly illustrated in a study where nine habitual black tea-drinking volunteers followed a low polyphenol diet for 3 days and still excreted hippuric acid (313–1162 μmol/24 h) (Clifford et al., 2000). That part of this production is hepatic without the involvement of colon-derived benzoic acid is clearly illustrated by ileostomists who excreted 72 ± 43 μmol/24 h of hippuric acid while on the low polyphenol diet and the water control stage of a green tea beverage study (Roowi et al., 2010).

Fig. 1 gives an overall view of [2–^{14}C]EC catabolism, but it should be recognized that while the pathways that are illustrated are well supported by the evidence, there may be others and a full elucidation of the catabolic routes remains a major analytical challange.

2.2. Procyanidins

Oligomeric and polymeric PCs are known as condensed tannins. PCs consist exclusively of (−)-epicatechin and (+)-catechin units and can occur as polymers of up to 50 flavan-3-ol monomer units. Proanthocyanidins containing other flavan-3-ol monomers have a more restricted occurrence in dietary plant products (Gu et al., 2003). B-type PCs consist of (−)-epicatchin and (+)-catechin units with oxidative coupling occurring between the C-4 of the heterocycle and C-6 or C-8 positions of the adjacent unit to create oligomers and polymers. A-type PCs have an additional ether bond between C-2 and C-7 of adjacent monomeric units (Fig. 2).

Studies with ileostomists indicate that up to 90% of the ingested PCs reach the lower bowel intact and become substrates for catabolic reactions mediated by the colonic microbiota (Kahle et al., 2007; Hagl et al., 2011). *In vitro* C-ring cleavage of A- and B-type PCs has been reported (Stoupi et al., 2010a; Engemann et al., 2012; Ge et al., 2015; Chen et al., 2021; Di Pede et al., 2022). A-type PCs are more resistant to fission of the interflavan bond than B-type PCs presumably because of the additional C2–C7 bond (Engemann et al., 2012; Li et al., 2019; Chen et al., 2021; Di Pede et al., 2022).

Phenyl-γ-valerolactones and phenylvaleric acids are the main colonic catabolites of both flavan-3-ol monomers and PCs. They appear

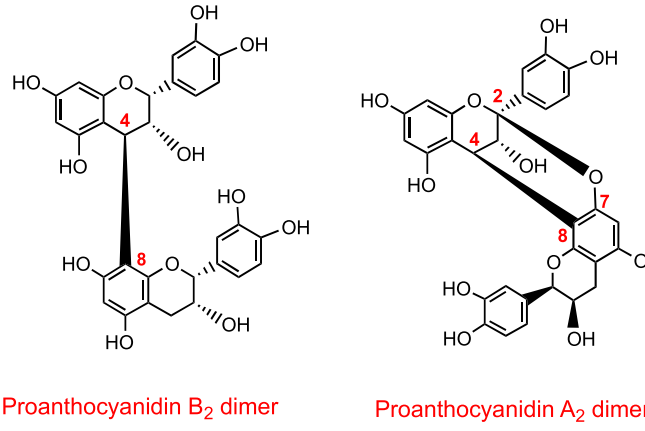


Fig. 2. Structures of A- and B-type proanthocyanidins.

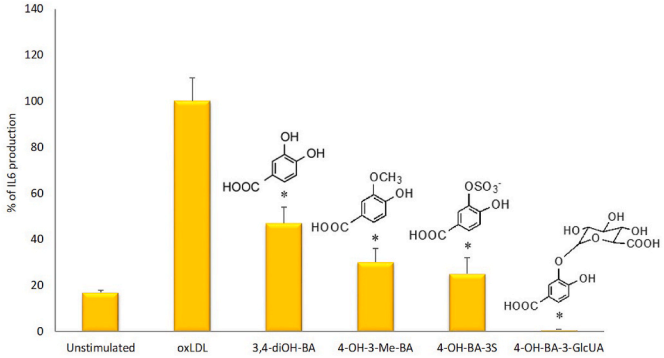


Fig. 3. Oxidised LDL (Ox-LDL) induced IL6 production. Data expressed as % relative to OxLDL treated endothelial cells. * Significant relative to oxLDL-treated cells, $p < 0.05$, ANOVA with Tukey post-hoc t-test, n = 3. 3,4-dihydroxybenzoic acid (3,4-diOHBA), 3-methoxy-4-hydroxybenzoic acid (4-OH-3-Me-BA), 4-hydroxybenzoic acid-3-sulfate (4-OH-BA-3S), 4-hydroxybenzoic acid-3-glucuronide (4-OH-BA-3-GlcUA). (Williamson et al., 2018, based on data of Amin et al., 2015).

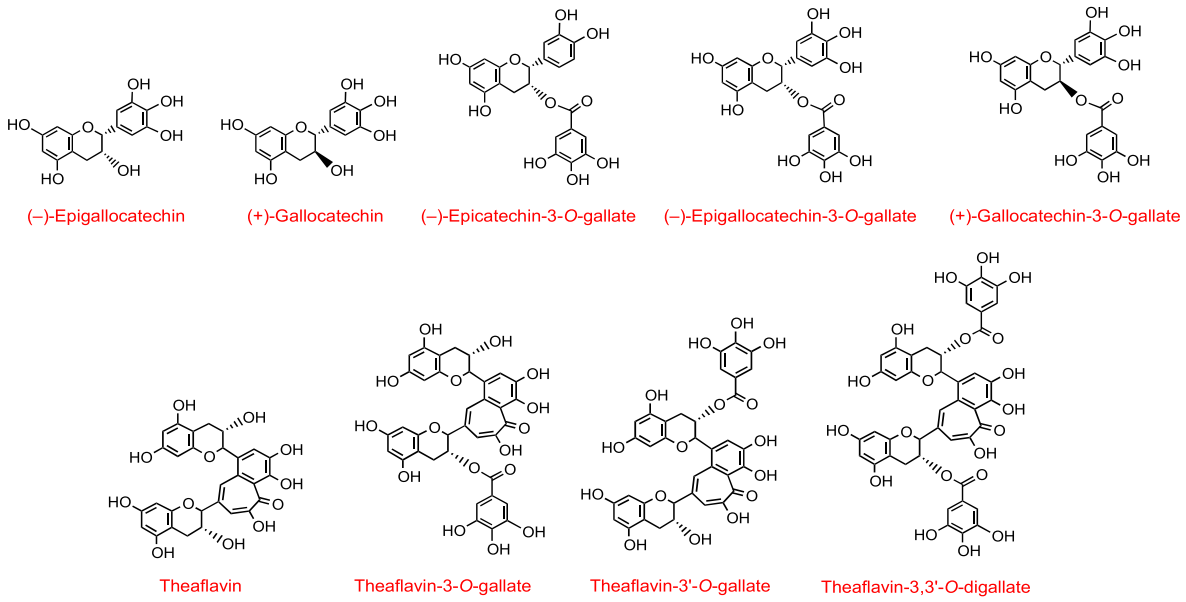
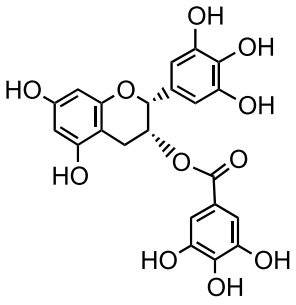


Fig. 4. (-)-Epigallocatechin, (+)-gallocatechin, their 3-O-gallate esters and theaflavin and their gallate esters.

to be produced *in vivo* through (i) ring fission of unabsorbed monomers, (ii) interflavan bond cleavage of PCs and catabolism of the released monomer unit, (iii) direct ring fission of the lower unit of the oligomer PC skeleton, and (iv) in the case of green tea galloylated flavan-3-ols, such as (−)-epigallocatechin-3-*O*-gallate **20**, cleavage of the gallic acid moiety followed by ring fission for the flavan-3-ol monomer unit (Mena et al., 2019; Tao et al., 2019). Pathways ii) and iii) are particularly relevant for the metabolism of PCs, as direct ring fission of the lower unit of the oligomer PC skeleton is likely to be the most representative catabolic pathway leading to the production of 5C-RFCs (Mena et al., 2019).



(–)-Epigallocatechin-3-*O*-gallate **20**

Some, although not all, *in vitro* studies have reported low level release of free monomer units from B-type PCs (Spencer et al., 2000; Appeldoorn et al., 2009; Stoupi et al., 2010a; Di Pede et al., 2022), while *in vivo* studies with human and rodent models have not (Donovan et al., 2002; Holt et al., 2002; Tsang et al., 2005; Ottaviani et al., 2012; Wiese et al., 2015) arguably because monomers are rapidly turned over being converted to 5C-RFCs as illustrated in Fig. 1.

Incubations of oligomeric PCs with human colonic microbiota achieved limited fission of inter-flavan bonds. Dimer A2, at relatively very low levels, and B-type PCs with a DP of up to 5, underwent A- and C-ring fission yielding 5C-RFCs and 3-(3'-hydroxyphenyl)propanoic acid **5** (Di Pede et al., 2022). There was a ca. 14% conversion of B2 dimer to 5C-RFCs after a 24 h fecal incubation, significantly higher than the 5.4% yield observed with the B-type PC trimer, tetramer and pentamer. Dimer A2 yielded only 0.3% whereas the recovery from monomers was 29–40%. These results suggest ring fission primarily of the lower unit of oligomeric PCs with little or no depolymerization which would provide additional monomers as a facile source of 5C-RFCs (Di Pede et al., 2022).

Volunteer feeding studies delivering oligomeric PCs in apple or cocoa (Ottaviani et al., 2012; Wiese et al., 2015; Hollands et al., 2020) or gallated oligomeric PCs in tea (Calani et al., 2012; van Duynhoven et al., 2014; Pereira-Caro et al., 2017), cranberry (Feliciano et al., 2016, 2017; Favari et al., 2020) and almonds (Urpi-Sarda et al., 2009; Bartolomé et al., 2010; Garrido et al., 2010) are all consistent with oligomers being subject to only limited ring fission *in vivo*. Even the low yields of 5C-RFCs recorded in these studies will be over-estimates of the true yield from the oligomers because some free monomers would also have been available for catabolism.

PC dimers are found in human plasma at only transient low nmol/L concentrations (Holt et al., 2002; Sano et al., 2003; Ottaviani et al., 2012). Although reported in rodent urine following ingestion of grape seed (Tsang et al., 2005) and red wine extracts (Pereira-Caro et al., 2020) and in human urine following cranberry consumption (Peron et al., 2017) PCs have more often been below the limit of detection (Baba et al., 2002; Donovan et al., 2002; Wiese et al., 2015). These observations, plus the recovery of only trace amounts of PCs and their catabolites from fecal samples associated with feeding studies (Stoupi et al., 2010a; Choy et al., 2014; 2014; Pereira-Caro et al., 2016; 2020) suggest that (i) *in vivo* the catabolism of oligomeric PCs is greater than implied by the results of the *in vitro* studies and (ii) the efficient absorption of PC breakdown products in the colon, raising the potential for the PCs to contribute to the bioactivity of the flavan-3-ol monomers to a greater extent than initially envisaged. For example, benzoic acid phase II metabolites are reported to have anti-inflammatory effects, reducing IL-6 secretion by vascular endothelial cells treated with oxidized LDL (Fig. 3) (Amin et al., 2015) albeit at 100 nmol/L, well above transient *in vivo* concentrations.

Endothelin-1 is peptide with a vasoconstricting effect and its synthesis is inhibited in cultured bovine endothelial cells by red wine PCs (Corder et al., 2001). Enhanced male longevity in the *département* of Gers region in the south west France has been linked to improved vascular health brought about by the consumption of Madiran red wines with a high PC content, that are produced locally from Tannat grapes (Corder

Table 1
Concentration of flavan-3-ol monomers, theaflavins and thearubigins in green and black tea infusions. Data expressed as mg/L ± standard error (n = 3).[a].

Compound	Green tea	Black tea	Black tea content as a % of green tea content
(−)-epicatechin	738 ± 17	11 ± 0.2	1.5
(−)-epigallocatechin	1565 ± 18	33 ± 0.8	2.1
(−)-epicatechin-3-*O*-gallate	361 ± 12	26 ± 0.1	7.2
(−)-epigallocatechin-3-*O*-gallate	1255 ± 63	19 ± 0.0	1.5
(+)-catechin	270 ± 9.5	12 ± 0.1	4.4
(+)-gallocatechin	383 ± 3.1	n.d.	0
Total flavan-3-ol monomers	4572	100	2.2
theaflavin	n.d.	64 ± 0.2	∞
theaflavin-3-*O*-gallate	n.d.	63 ± 0.6	∞
theaflavin-3′-*O*-gallate	n.d.	36 ± 0.8	∞
theaflavin-3,3′-*O*-digallate	n.d.	62 ± 0.1	∞
Total theaflavins	n.d.	224	∞
Total thearubigins	n.d.	1681	∞

[a] Black tea made from the same batch of leaves as the green tea. Stock solutions of the tea infusions were prepared by adding 18 mL of boiling water to 1 g of leaves. After 3 min, the brew was filtered to remove particulate matter prior to analysis of the filtrate. n.d. − not detected. After Del Rio et al. (2004).

et al., 2006). However, PCs are not absorbed to any extent *in vivo* and the proposed link between male longevity and the consumption of Madiran wines, while interesting, is speculative. A similar point can be made with regard to the anti-adhesive uropathogenic activity of cranberry PCs in the context of alleviating of urinary tract infection. The physiological evidence for the positive effects of 5C-RFCs is much more convincing (Mena et al., 2017).

It should be pointed out that the identification of SREMs and 5C-RFCs derived from cocoa flavan-3-ol monomers and PCs (Ottaviani et al., 2012, 2016; Borges et al., 2018), covered in Sections 2.1 and 2.2 of this review, is of importance in view of the findings of the COSMOS trial (Sesso et al., 2022) discussed elsewhere in this edition of the Molecular Aspects of Medicine, as it opens the door to meaningful studies on the mechanisms underlying the protective effects of flavan-3-ol-rich cocoa on cardiovascular disease.

2.3. Theaflavins

Green tea (*Camellia sinsensis*) is a rich source of flavan-3-ols, principally as (epi)gallocatechins and their gallic acid (3,4,5-trihydroxybenzoic acid) esters (Fig. 4). The levels of these flavan-3-ol monomers decline as a consequence of loss of cellular compartmentation and the

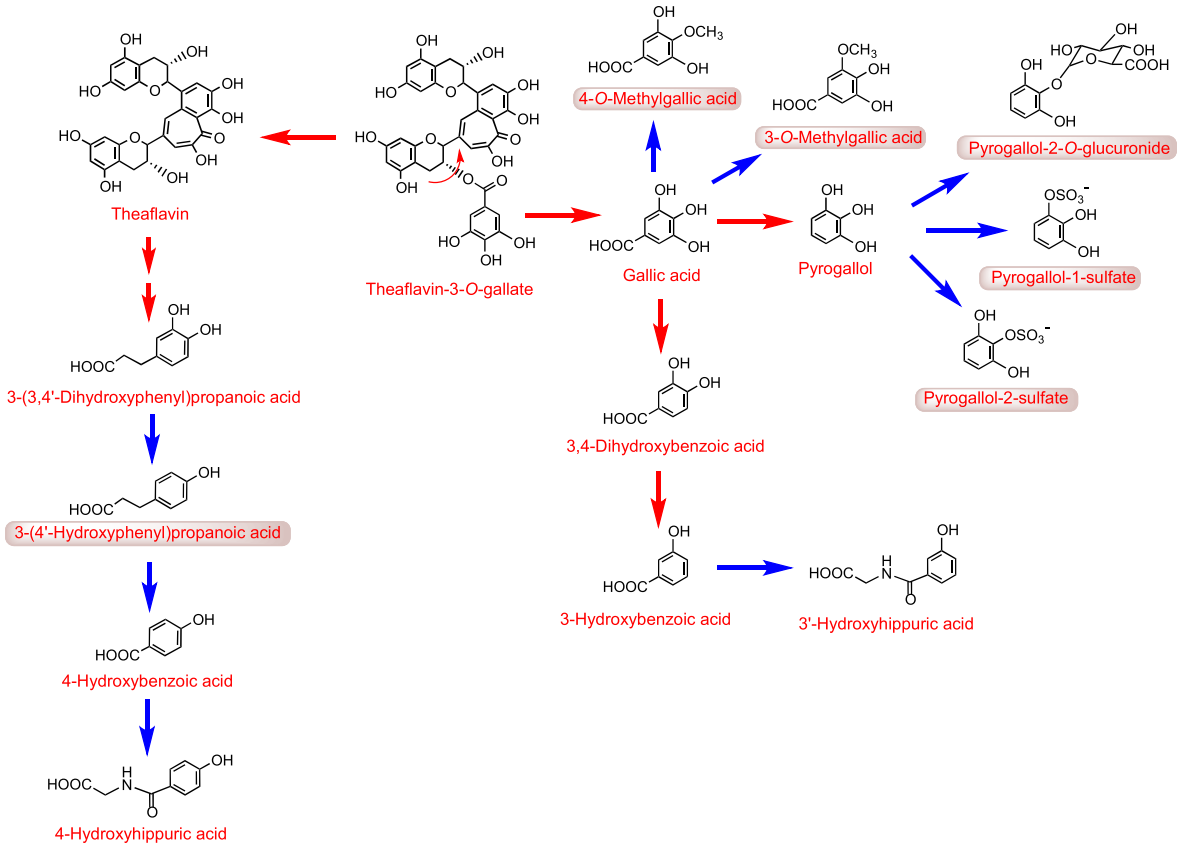


Fig. 5. Proposed principle pathways for the catabolism of theaflavins by colonic microbiota and mammalian phase II metabolism. Red arrows indicate microbiota-mediated steps, and blue arrows represent mammalian enzyme-mediated conversions. The illustrated theaflavin is theaflavin-3-*O*-gallate. It assumed that the 3′ gallate and the 3,3′-digallate will be similarly subject to degalloylation releasing theaflavin. Boxed names indicate the main products to accummulate in urine after theaflavin intake. After Pereira-Caro et al. (2017). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

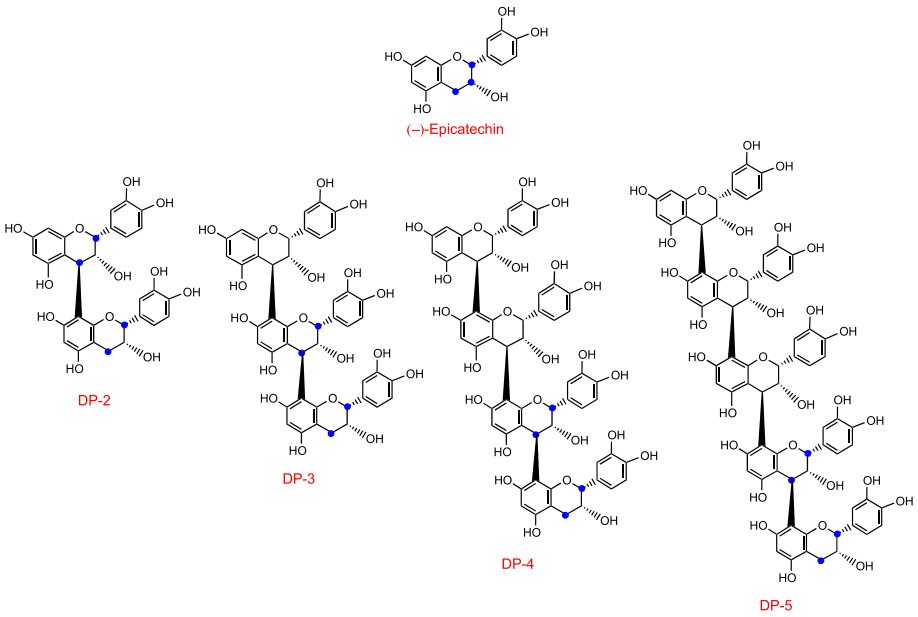


Fig. 6. Structures of ^{13}C-labelled (−)-epicatechin and procyanidins with a degree of polymerisation (DP) of 2–5. Blue circles indicate the positions of ^{13}C label. (After Bussy et al., 2021). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

action of polyphenol oxidase and peroxidases during black tea manufacture and as a result the main components in black tea leaves are the high molecular weight thearubigins and smaller amounts of theaflavins (Table 1) (Del Rio et al., 2004). Theaflavins are derived from two flavan-3-ol monomer subunits and during the production of black tea the A-ring and C-ring of the two monomers remain intact, but the two B-rings are converted to a benztropolone structure (Drynan et al., 2010). Black tea contains theaflavin, theaflavin-3-*O*-gallate, theaflavin-3′-*O*-gallate and theaflavin-3,3′-*O*-digallate (Fig. 4).

In a human feeding study with theaflavins, urinary excretion 0–24 h after intake was equivalent to 0.0006% of intake indicating that black tea theaflavins have poor systematic bioavailabilty (Mulder et al., 2001). In a later investigation two volunteers ingested a 1 g supplement containing 3.4 μmol of flavan-3-ol monomers and 22.7 μmol of dimers, and 988 μmol of mixed theaflavins (see Fig. 4), equivalent to 10 cups of black tea (Pereira-Caro et al., 2017). No theaflavins were detected in urine collected over a 30 h period post-ingestion confirming they are poorly absorbed and that most of the intake will pass to the colon. In keeping with this possibility the urine contained 60 nmol of flavan-3-ol phase-2 conjugates, and 1.5 μmol of 5C-RFCs including phase II conjugates. These were accompanied by 169 μmol of C_6–C_3 metabolites, of which 166 μmol was 3-(4'-hydroxyphenyl)propanoic acid, 19 μmol of C_6–C_2 metabolites, 54 μmol of C_6–C_1 metabolites of which 39 μmol were gallic acid derivatives. Also detected was 723 μmol of C_6–C_0 metabolites, of which 713 μmol retained the *vic*-trihydroxy pattern of substitution characteristic of gallic acid. The 169 μmol yield of C_6–C_3 metabolites exceeds the amount of flavan-3-ols and PCs (26 μmol) in the consumed theaflavin-rich extract strongly suggesting that the C_6–C_3 metabolites, and specifically 3-(4'-hydroxyphenyl)propanoic acid, are at least in part, derived from the theaflavin benztropolone skeleton, (Pereira-Caro et al., 2017).

The theaflavin extract was also subjected to *ex vivo* incubations with fecal material (Pereira-Caro et al., 2017). The theaflavin skeleton was comparatively resistant to degradation by colonic bacteria as there was a 67% recovery after a 24 h fecal incubation which yielded a number of phenolic catabolites. The theaflavin galloyl moiety was removed by the microbiota and the released theaflavin accumulated, intially along with low levels of gallic acid which declined to be replaced by small amounts of benzoic acid, 3-hydroxybenzoic acid and pyrogallol (1,2,3-trihydroxybenzene). The gut microbiota catabolism of the predominant polyhydroxylated thearubigin benztropolone skeleton in black tea has been little studied. Conversion to a naphthoquinone, a known component of black tea, has been reported, but further rupture of this moiety was not detected (Liu et al., 2021).

Proposed pathways for the catabolism of theaflavins by colonic microbiota and mammalian phase II metabolism are presented in Fig. 5, which like the routes shown in Fig. 1, are plausible but unproven and a full elucidation of the catabolic routes represents another major analytical undertaking. However, some clarification could be achieved by carrying out separate fecal incubations with gallic acid and non-galloylated theaflavins.

3. Current challenges

3.1. Bioavailability studies using isotopically-labelled substrates

To make significant advances future feeding studies require the use of isotopically labelled substrates. The use of [2–^{14}C]EC in a human feeding study facilitated an evaluation of colonic catabolism with a greatly improved degree of clarity as it possible to readily differentiate radiolabelled phenolic catabolites from their ^{12}C-counterparts originating from other sources, as outlined in Section 2.1. The specific activity of the EC was 14.5 μCi μmol^{-1} and as such its metabolites were readily detected by HPLC with an on-line radioactivity detector. However, the ^{14}C label was difficult to distinguish from ^{12}C ions by mass spectrometric detection as the intensity of at M^{+2} was only ~1% of that of the M^{+} ion. A further limitation is that because of the intermittent emission of low energy β-particle radiation, an HPLC radioactivity detector requires a ~10 s time constant (Reeve and Crozier 1977) which impacts adversely on chromatographic resolution, necessitating the use of long shallow mobile phase gradients which preclude the use of rapid UHPLC analysis.

Investigations making use of ^{13}C-labelled substrates where there is much more extensive labelling, which is readily distinguished from ^{12}C fragments by mass spectrometry, have a number of advantages as shown in an anthocyanin bioavailability study by Czank et al. (2013). In this investigation 0.5 g (1114 μmol) of [6,8,10,3′,5′-$^{13}C_5$]cyanidin-3-*O*-glucoside was ingested by human subjects after which plasma and urine were collected over a 48 h period. The particular advantage of using the [$^{13}C_5$]anthocyanin was that it had three ^{13}C molecules incorporated into

the A-ring and two into the B-ring. An array of $^{13}C_{2/3}$-labelled phenolics was detected in plasma and urine, and because of the label it was possible to ascertain whether they were derived from the A- or B-ring of cyanidin. The capacity of MS to determine relatively low levels of incorporation of the ^{13}C-label into the ^{12}C-pools of phenolic compounds enabled trace amounts of labelled catabolites to be identified and quantified in much greater detail than is possible in feeding studies with dietary products and unlabelled substrates.

A number of stable isotope labelled flavan-3-ols have been produced for use as internal analytical standards, namely [$^{13}C_3$]EC, and $^{13}C_4$-labelled B-type PCs with DPs of 2–5 (Fig. 6) (Bussy et al., 2021). If [$^{13}C_3$]EC was available in sufficient quantity it might be realistic to prepare labelled theaflavins and thearubigins *in vitro* using, respectively, polyphenol oxidase and peroxidase. Alternatively, a simpler and cheaper approach would be to prepare benzotropolone mimics using 3,4-dihydroxybenzoic acid (aka protocatechuic acid) in place of (−)-epicatechin, and gallic acid in place of (−)-epigallocatechin for *in vitro* and *in vivo* bioavailability studies and if promising results were obtained to progress to the use of ^{13}C precursors.

The most serious limitation with feeding studies involving the use of isotopically-labelled flavan-3-ols, is the expense of synthesizing isotopically labelled substrates. This is further compounded by the difficulties in obtaining ethical permission for human investigations with ^{14}C-labelled substrates coupled with the rigorous conditions under which the feeding study would have to be carried out.

3.2. Analysis of procyanidins

PCs can be analysed by reversed phase HPLC but this is limited to DP 3–4 because of band broadening with DP > 4. This does not present problems when analysing plasma and urine because the higher molecular weight PCs, as noted above, are not absorbed. Separation of DP2-10 cocoa PCs was achieved by several groups using silica and/or diol HPLC columns with fluorescence and MS/MS detection (Prior et al., 2001; Gu et al., 2003). Subsequently, normal phase HPLC with a diol stationary phase was used to quantify DP-2-10 PCs in cocoa and chocolate, employing reference PCs isolated from cocoa (Robbins et al., 2009). Recently, more robust hydrophilic interaction chromatography (HILIC) has been used to analyse oligomeric PCs in apple extracts (Hollands et al., 2017) while Bussy et al. (2021) developed and validated purification protocols and HILIC-based HPLC-MS/MS methodology, which discriminated between A- and B-type PCs with the same degree of polymerisation, using primary standards and ^{13}C-labelled PCs as internal standards.

As yet there is limited information on the amounts of DP2-10 and higher molecular weight PCs recovered from ileal fluid. This would provide information of the extent to which they pass from the small intestine and enter the lower bowel where they are subjected to the action of the colonic microbiota. It is also remains to be determined to what extent existing HPLC-MS methodology, as well as gel permeation chromatography (Kennedy and Taylor, 2003) and phloroglucinolysis (Bindon and Kennedy, 2011), can be used to accurately analyse the levels of unabsorbed PC oligomers and polymers and their partially degraded chains in feces and *in vitro* fecal incubates.

In depth and informed discussion of analytical protocols employed to investigate (poly)phenols in various plant products, most notably tea, coffee and berries, and their metabolites and catabolites, as well the pitfalls, limitations and potential for further development, can be found in recent reviews by Clifford and Kuhnert (2022), Kuhnert and Clifford (2022) and Kay et al. (2022).

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Conflicts of interested

The author declare no conflicts of interest.

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