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CICLO XXXV

Metabolites from dietary (poly)phenols in humans: interaction with gut microbiota and comprehensive nutrikinetics

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To my family and friends – to my future wife and my mother, this Doctoral Thesis is for you

Contents

Abstract

(Poly)phenols are the most consumed phytochemicals in the western diet, and their intake, mainly associated with that of fruits and vegetables, has been correlated with a multitude of preventive and beneficial actions against several chronic diseases. The potential health benefits of (poly)phenols are strongly attributable to their capability to be efficiently absorbed and metabolized, and in producing bioavailable metabolites able to reach target districts at concentration levels high enough to be bioactive. This Doctoral Thesis comprehensively assessed the production of metabolites from the main dietary (poly)phenols in the framework of their absorption, distribution, metabolism and excretion (ADME) in humans. This work investigated: I) the interaction of flavan-3-ols, hydroxycinnamic acids (HCAs), quercetin and cranberry (poly)phenols with gut microbiota; II) blood nutrikinetics and urinary excretion profiles of flavan-3-ol and HCA metabolites through a systematic review based-approach; III) the metabolic efficiency (*aka* stoichiometry) in the production of the main flavan-3-ol colonic catabolites and main urinary HCA metabolites; IV) the bioavailability of ingested (poly)phenols. The results of this Doctoral Thesis highlighted that: I) *in vivo*, flavan-3-ols and HCAs are metabolized in up to 97 and 41 phase-2 conjugates of colonic catabolites and 22 and 8 colonic catabolites, respectively. If the flavan-3-ol structure (i.e. degree of polymerization, different subunit linkages (A-/B-type), and the presence of galloyl moieties) seems to influence the profile of (phenyl)-γ-valerolactones (PVLs) and phenylvaleric acids (PVAs) produced *in vitro*, the presence of a delivery system, namely phytosome, did not affect the profile of these C_6-C_5 catabolites resulted from flavan-3-ol colonic metabolism of cranberry. On the other hand, phytosome affected the quantitative profile of phenolic catabolites produced from the interaction of quercetin with gut microbiota *in vitro*; II) data analysis of 96 intervention studies (n=1267, total sample size) demonstrated that after flavan-3-ol and HCA intake, up to 180 and 105 metabolites circulate in biofluids, respectively. Their blood concentration levels ranged from 37 to 1493 nmol/L, and metabolites were excreted in urine in amounts ranging from 0.3 to 11 % of intake; III) stoichiometric balances calculated in the production of PVLs and PVAs from cumulative urinary data were in line with those obtained from *in vitro* colonic fermentation of flavan-3-ol monomers and B-type dimers. Monomers are more prone to producing metabolites than oligomer flavan-3-ols. Instead, a specific heterogeneity was found in the stoichiometry for the main urinary metabolites of HCAs (n=16), with phenylpropanoic acids having the highest metabolic yield; IV) although flavan-3-ols and HCAs appear to be moderately bioavailable (31% and 25%, respectively), intra- and inter-source differences in bioavailability values for both (poly)phenol classes emerged. In conclusion, this work represents a starting point for improving and harmonising the available knowledge on the interaction of dietary (poly)phenols with gut microbiota and their nutrikinetic profiles in humans. This Doctoral Thesis could strongly support understanding the fate of ingested (poly)phenols in humans, besides metabolic pathways associated with the production of (poly)phenol metabolites, and their circulating levels in biofluids, which represent pivotal steps for underlying the beneficial effects observed after a higher adherence to plant-based dietary pattern.

Keywords: ADME; bioavailability; colonic catabolites; plant diet; (poly)phenols; phenolic compounds

Chapter 1. Background and aim of the Doctoral Thesis

1.1 Classification and occurrence of phytochemicals in plant foods

Plant phytochemicals include more than 30,000.0 secondary metabolites synthesized *in planta* for protecting plants against infections, as attractants for pollinators and seed-dispersing animals, as allelopathic agents, UV protectants and signal molecules in the formation of nitrogen-fixing root nodules in legumes (Crozier et al., 2010; Del Rio et al., 2013; Rodriguez-Mateos et al., 2014). Some phytochemicals also provide colour to stems, leaves, flowers and fruits. Based on their biosynthetic origins, plant secondary metabolites are classified into (poly)phenols, terpenoids (i.e. carotenoids, phytosterols, and essential oil components), nitrogen-containing alkaloids (i.e. purines, pyrimidines) and sulphur compounds (i.e. glucosinolates and allyl sulfinates) (**Figure 1**) (Crozier et al., 2006).

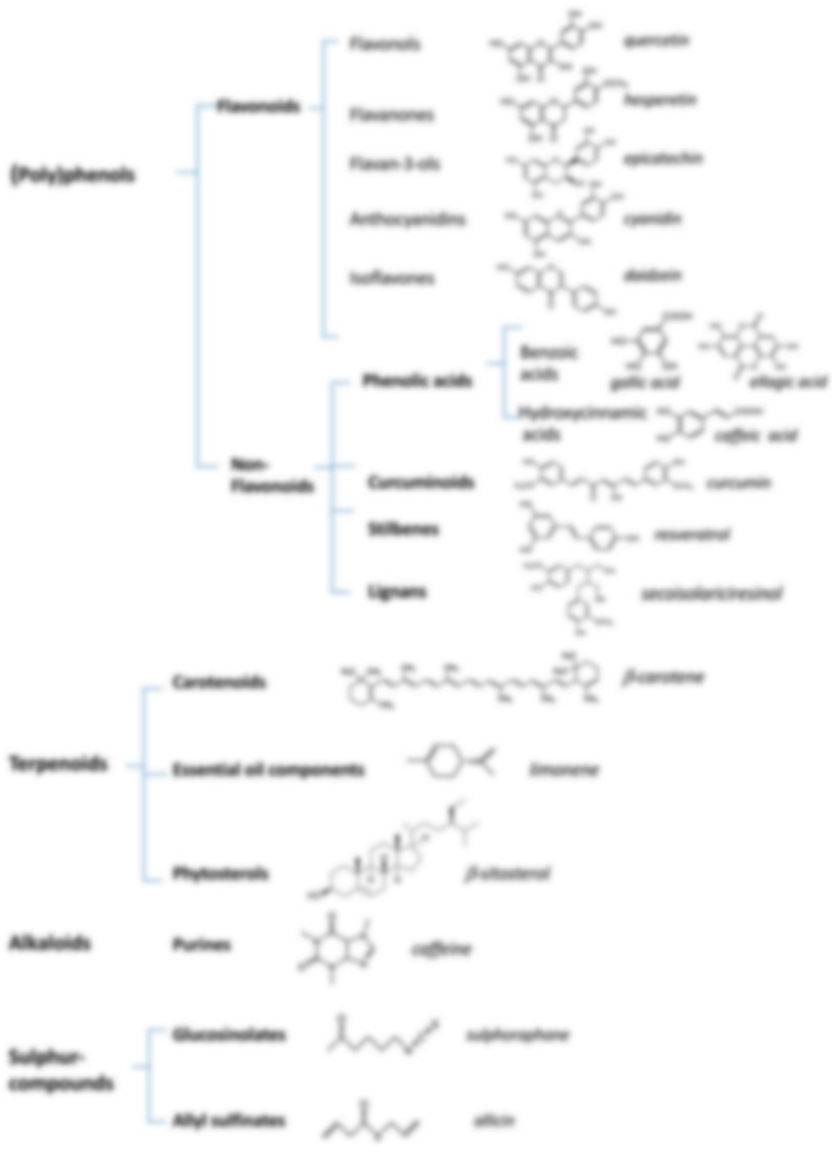

Figure 1. Main categories of dietary phytochemicals. Figure modified from Morand et al. (Morand et al., 2020)

(Poly)phenols can be grouped into flavonoids and non-flavonoids based on the numeral phenol rings and the essential structural elements in the side chain that binds to these phenol rings. Flavonoids are phenolic compounds with a 15-carbon skeleton structure containing two aromatic rings connected by a three-carbon bridge and various hydroxyl groups, which are usually present at the 4′, 5 and 7 positions (Crozier et al., 2007; Del Rio et al., 2013; Rodriguez-Mateos et al., 2014). Further details on classification, dietary intake, and main sources of (poly)phenols will be described in sections 1.2 and 1.3 of the present Doctoral Thesis.

Chemically, terpenoids are usually cyclic unsaturated hydrocarbons, with oxygen groups attached to the basic isoprene skeleton. The main subclasses of terpenoids, which are derived from a common isopentenyl precursor, include monoterpenes (i.e. limonene, carvone or carveol - containing up to ten carbons (two isoprene units)), diterpenes (i.e. retinoids, with twenty carbons (four isoprene units)), sesquiterpenes (containing fifteen carbons (three isoprene units)), triterpenes (containing thirty carbons), and tetraterpenes (i.e. carotenoids such as β-carotene, lutein, lycopene, zeaxanthin and cryptoxanthin – containing forty carbons) (**Figure 1**). Essential oils of herbs and spices (i.e. peppermint, lavender) of flower scents (rose) contain monoterpenes and sesquiterpenes (Patra et al., 2021; Rabi and Gupta, 2008; Wagner and Elmadfa, 2003). Alkaloids are a group of nitrogen-containing compounds that may consist of one or more nitrogen atoms (within a heterocyclic ring). Based on their biosynthetic pathway (from ornithine, lysine, tyrosine, tryptophan or a non-amino acid precursor such as nicotinic acid, or purine analogues), alkaloids may be classified into indole, tropane, piperidine, purine, imidazole, pyrrolizidine, pyrrolidine, quinolizidine and isoquinoline alkaloids (Kaur and Arora, 2015; Koleva et al., 2012), with up to 12,000 alkaloids which have been identified in the plant kingdom (Ashihara et al., 2008; Debnath et al., 2018). Sulphur compounds in the diet are mainly derived from sulphur-containing amino acids such as methionine and cysteine, vitamins (i.e. thiamine and biotin) and other organic sulphur compounds that accumulate in *Alliums* (onions, garlic, leeks) and *Brassicas* (broccoli, cabbage, cauliflower, Brussels sprouts) such as alliin, glucosinolates, glutathione and methylsulfonylmethane (Doleman et al., 2017; Prasad, 2014; Williamson, 1996) (**Figure 1**). The occurrence of phytochemicals of interest in plant foods varies throughout the plant kingdom. Various factors such as variety, soil, climatic conditions, agricultural methods, growing and storage conditions and degree of ripeness, and storage time before consumption may affect the phytochemical content in plant foods (Crozier et al., 2006).

The main sources of phytochemicals and related containing compounds in the diet are as follows (Clifford, 1985, 2000; Clifford et al., 2003; Clifford and Ramirez-Martinez, 1990; Clifford and Scalbert, 2000; Crozier et al., 2006):

- ***fruits*** - apples (*Malus* × domestica) [i.e. 5-*O*-caffeoylquinic acid, 4-*O*-*p*-coumaroylquinic acid, caffeic acid, phloretin-2′-*O*-glucoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-rhamnoside, (−)-epicatechin and procyanidins], pears (*Pyrus communis*) [i.e. 5-*O*-caffeoylquinic acid, 4-*O*-*p*-coumaroylquinic acid, procyanidins and quercetin glycosides], apricots (*Prunus armeniaca*), nectarines (*P. persica* var. *nectarina*) and peaches (*Prunus persica*) [i.e. cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside, (+)-catechin, (−)-epicatechin and proanthocyanidins, β-carotene], cherries (*Prunus avium* and *Prunus cerasus*) [i.e. cyanidin-3-*O*-rutinoside, 3-*O*-caffeoylquinic acid, 3-*O*-*p*-coumaroylquinic acid], plums (*Prunus domestica*) [i.e. anthocyanins, 3-*O*- and 5-*O*-caffeoylquinic acid and procyanidins], citrus fruits [i.e. grapefruit (*Citrus paradisi*), orange (*Citrus sinensis*), lemons (*Citrus limon*), limes (*Citrus aurantifolia*) – flavanones, flavones, monoterpenes (+)- and (−)-limonene], pineapple (*Ananas comosus*) [i.e. *S*-sinapyl-ʟ-cysteine, *N*-ʟ-γ-glutamyl-*S*-sinapyl-ʟ-cysteine and *S*-sinapylgluthathione, glyceryl esters of caffeic and *p*-coumaric acids], dates (*Phoenix dactylifera*) [i.e. carotenoids and anthocyanins], mango (*Mangifera indica*) [i.e. β-carotene, cyanidin-3-*O*-galactoside], papaya (*Carica papaya*) [i.e. β-crytoxanthin], fig (*Ficus carica*), olive (*Olea europa*) [i.e. benzoic and cinnamate derivatives, luteolin and apigenin, oleuropein, hydroxytyrosol], berries (please see section 1.3.1.), melons (*Cucumis melo*) [i.e. carotenes], grapes (*Vitis labrusca*, *Vitis vinifera*) [i.e. anthocyanins, flavan-3-ols and procyanidins], kiwi (*Actinidia deliciosa*) [i.e.

kaempferol-3-*O*-rutinoside and quercetin-3-*O*-rutinoside, hesperetin-7-*O*-rutinoside, (−)-epicatechin], bananas (*Musa cavendishii*) and plantains [i.e. lutein, α-carotene and β-carotene, naringenin-7-*O*-neohesperidoside and quercetin-3-*O*-rutinoside], pomegranate (*Punica granatum* L.) [i.e. gallagic acid, punicalin, punicalagin, 3-*O*-glucosides and 3,5-*O*-diglucosides of cyanidin and delphinidin];

- ***root crops*** – carrots (*Daucus carota*) [i.e. α-carotene and β-carotene, 3-*O*- and 5-*O*-caffeoylquinic acids, 3-*O*-*p*-coumaroylquinic acid, 5-*O*-feruloylquinic acid and 3,5-*O*-dicaffeoylquinic acids], beetroot (*Beta vulgaris*) [i.e. β-carotene, betanin and isobetanin], turnip and swede (*Brassica campestris*, *Brassica napus*) [i.e. glucosinolates], parsnip (*Pastinaca sativa*) and Jerusalem artichoke (*Helianthus tuberosus*);
- ***cabbage and greens*** – varieties of *Brassica oleracea* (broccoli, Brussels sprouts, cabbage, cauliflower) [i.e. isothiocyanates, thiocyanates, nitriles and indole derivatives, glucosinolates [quercetin-3-*O*-sophoroside and kaempferol-3-*O*-sophoroside], hydroxycinnamoyl derivatives], spinach (*Spinaceae oleraceae*) [i.e. cinnamates, carotenoids, lutein, β-carotene, conjugates of quercetin and kaempferol] and leaves of cauliflower [i.e. kaempferol-3,7-*O*-diglucoside, sinapoyl and feruloyl derivatives of kaempferol];
- ***vegetables*** - lettuce (*Lactuca sativa*) [i.e. carotenoids containing both lutein and β-carotene, with some varieties (i.e. red-leaved lettuce Lollo Rosso) containing cyanidin-3-*O*-(6″-malonyl)glucoside, quercetin-3-*O*-(6″-malonyl)glucoside and hydroxycinnamate derivatives], celery (*Apium graveolens*), asparagus (*Asparagus officinalis*) [i.e. β-carotene, quercetin-3-*O*-rutinoside], avocados (*Persea americana*) [i.e. carotenoids lutein, zeaxanthin, α-carotene and β-carotene, phytosterols], artichoke (*Cynara scolymus*) [i.e. 5-*O*-caffeoylquinic acid, luteolin derivatives, apigenin-7-*O*-rutinoside and naringenin-7-*O*-rutinoside], tomato (*Lycopersicon esculentum*) [i.e. steroidal alkaloid tomatine, carotenoids lycopene, β-carotene and lutein, quercetin-3-*O*-rutinoside], peppers (*Capsicum annuum*) [i.e. lutein and β-carotene, quercetin-3-*O*-rhamnoside and luteolin-7-*O*-(2″-*O*-apiosyl-6″-*O*-malonyl)glucoside] and aubergines (*Solanum melongena*) [i.e. delphinidin glycosides, 5-*O*-caffeoylquinic acid], squashes [courgette (zucchini), melons (*Cucumis melo*), Asian pumpkin (*Cucurbita moschata*) - β-carotene, lycopene and lutein], rhubarb (*Rheum rhaponticum*);
- ***onions and garlic*** – onions (*Allium cepa*) [i.e. quercetin-4′-*O*-glucoside, quercetin-3,4′-*O*-diglucoside, isorhamnetin-4′-*O*-glucoside], leeks (*Allium porrum*) and garlic (*Allium sativum*) [i.e. allyl sulfinates such as *S*-Alkyl cysteine sulphoxides, *S*-propyl cysteine sulphoxide, 1-propenyl (vinyl-methyl) derivative, methyl-vinyl or 2-propenyl) derivative; hydroxycinnamates]. Red onions like their yellow counterparts are rich in flavonols and some anthocyanins (i.e. cyanidin derivatives);
- ***beverages*** - tea, matè, coffee, wines, beer, cider, Scotch whisky [i.e. galloyl flavan-3-ols, chlorogenic acids, proanthocyanidins, quercetin-3-*O*-rutinoside, glycosylated derivatives of luteolin, caffeine, trigonelline, cafestol, kahweol and 16-*O*-methyl cafestol, anthocyanins, monoterpene β-myrcene, humulone, cohumulone and adhupulone];
- ***cocoa*** (*Theobroma cacao*) [flavan-3-ol derivatives, theobromine];
- ***cereals*** – wheat (*Triticum spp.*), barley (*Hordeum vulgare*), rice (*Oryza sativa*), maize (*Zea mays*), rye (*Secale cereale*), oats (*Avena sativa*) [i.e. bound cinnamates, avenanthramides, avenulamides, proanthocyanidins, luteolin, lignans, resorcinol derivatives, alkylresorcinols];
- ***legumes*** - soybean (*Glycine max*) [i.e. daidzein-7-*O*-(6″-*O*-malonyl)glucoside and genistein-7-*O*-(6″-O-malonyl)glucoside]; peanuts (*Arachis hypogaea*) [i.e. 5,7-dimethoxyisoflavone]; broad beans (*Vicia faba*) [i.e. flavan-3-ols]; French beans (*Phaseolus vulgaris*) [i.e. quercetin-3-glucuronide]; Pinto beans and red kidney beans (*Phaseolus vulgaris*) [i.e. proanthocyanidins];

- ***algae*** - red alga *(Gelidium* spp., *Pterocladia* spp. *and Gracilaria* spp.*)* [i.e. 2,4,6-Tribromophenol];
- ***herbs and spices*** - [i.e. hydroxybenzoic acids, cinnamic acid derivatives, estragole, eugenol, curcuminoids, capsaicins and piperines, terpenes and terpenoid phenols, and flavonoids];
- ***nuts*** - hazelnuts (*Corylus avellana*), almonds (*Prunus dulcis*), walnuts (*Juglans* spp.), cashew (*Anacardium occidentale*), pecans (*Carya illinoensis*) [i.e. flavan-3-ols and proanthocyanidins, isoflavones, alkylresorcinols].

1.2 Classification, main sources and dietary intake of (poly)phenols

(Poly)phenols are the most consumed phytochemicals in the western diet, with their intake ranging from 150 up to 1000 mg/day, mainly provided by flavonoids (Manach et al., 2004; Scalbert and Williamson, 2000a; Zamora-Ros et al., 2016, 2013b; Ziauddeen et al., 2018). Based on their chemical structure, (poly)phenols are classified as flavonoids, non-flavonoids and tannins (**Figure 2**). Basically, the biosynthesis of flavonoids, stilbenes, hydroxycinnamates and phenolic acids involves shikimate, phenylpropanoid and flavonoid pathways (Crozier et al., 2007).

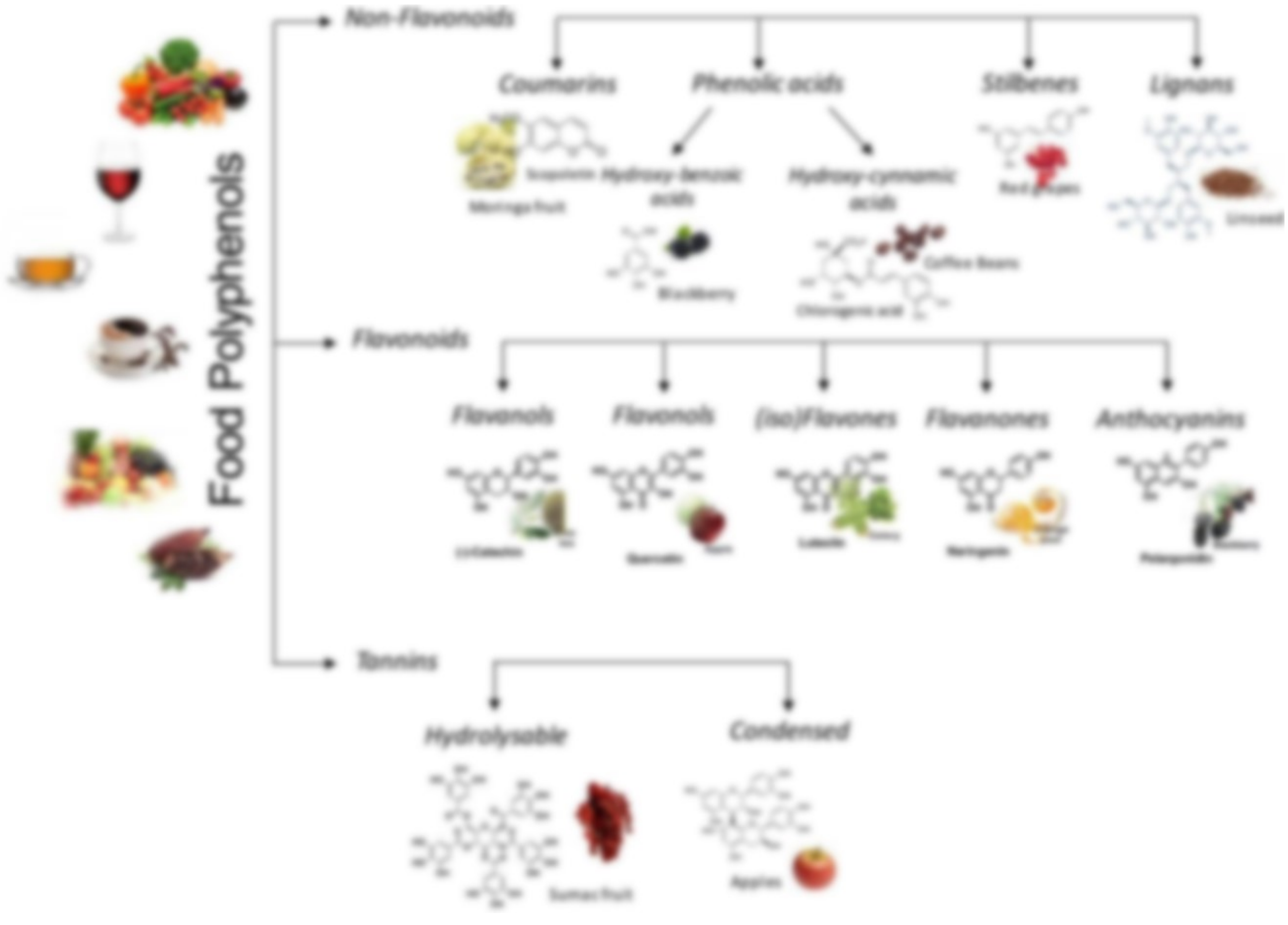

Figure 2. Most important categories of dietary (poly)phenols and their distribution into different classesand subclasses. Figure modified from Reis et al. (Reis et al., 2020)

Flavonoids have a C_6–C_3–C_6 structure, and in accordance with the type of heterocycle involved, they may be divided into flavan-3-ols (or flavanols), flavonols, anthocyanins, flavones, isoflavones, and flavanones (**Figure 2**), occurring naturally as glycosides rather than aglycones *in planta*. Other flavonoid groups that are minor dietary components are chalcones, dihydrochalcones, dihydroflavonols, flavan-3,4-diols, coumarins, and aurones (Del Rio et al., 2013). Flavonoids are involved in UV protection, pigmentation, stimulation of nitrogen-fixing nodules and disease resistance *in planta* (Crozier et al., 2007). Flavan-3-ols are the most consumed flavonoids in the western diet (intake ~ 123-483 mg/day), followed by flavonols and anthocyanins (intake ~ 10-74 and 32-45 mg/day for flavanols and anthocyanins, respectively) (Castro-Barquero et al., 2020; Rosi et al., 2021; Zamora-Ros et al., 2013a; Ziauddeen et al., 2018) (**Figure 3**). Flavan-3-ols are the most complex subgroup of flavonoids, and they may range from simple monomers to polymeric proanthocyanidins, also known as condensed tannins, which may occur as polymers of up to 50 units (Del Rio et al., 2013). B-type procyanidins have C_4−C_8 and/or C_4−C_6 linkages, while A-type procyanidins contain an additional ether linkage between the C_2 in the B-ring of the adjacent unit and the oxygen-bearing C_7 in the A-ring of the terminal (epi)catechin unit (Mena et al., 2019). The most widespread flavan-3-ols in nature include (+)-catechin and (−)-epicatechin, and their galloyl conjugates [i.e. (−)-epigallocatechin, (+)-gallocatechin, (−)-epicatechin-3-*O*-gallate and (−)-epigallocatechin-3-*O*-gallate], while procyanidins, which are proanthocyanidins consisting exclusively of (epi)catechin units, are the most abundant proanthocyanidins in the plant kingdom (Del Rio et al., 2013; Rodriguez-Mateos et al., 2014). Flavan-3-ols (as monomers and proanthocyanidins) accumulate in chocolate, beans, some fruits (i.e. apricot, cherry, grape, peach, apple, kakis (*Diospyros kaki*), berries) and beverages (i.e. tea, red wine, cider, beer) (Bhagwat et al., 2004; Manach et al., 2004). The main dietary sources of flavan-3-ols in the western diet are green tea, red wine, dark chocolate, some fruits and nuts (Mena et al., 2019; Ziauddeen et al., 2018).

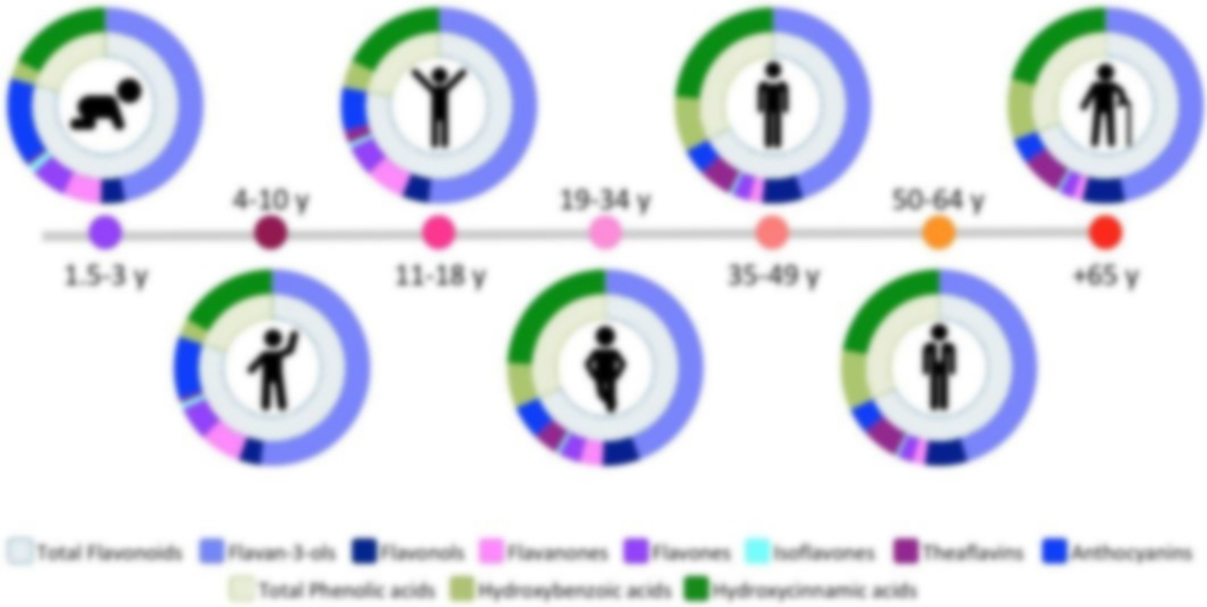

Figure 3. Relative contribution of each class and subclass of phenolic compounds to the total (poly)phenolintake by age group. Figure modified from Ziauddeen et al. (Ziauddeen et al., 2018)

The main dietary flavonols are quercetin, kaempferol, isorhamnetin and myricetin, and they are typically found as found as *O*-glycosides with conjugation occurring at the most frequently at the 3 position of the C-ring but also at the 5, 7, 4′, 3′ and 5′ positions of the carbon ring (Crozier et al., 2007; Del Rio et al., 2013). Up to 200 sugar conjugates of kaempferol have been identified in the plant kingdom. The richest sources of flavonols are yellow onion, curly kale (*Brassica* *oleracea*), leek (*Allium porrum*), cherry tomato, broccoli and blueberry (*Vaccinium caesariense*) (Manach et al., 2004), while their main dietary sources are spinach, beans, apples, tea, and red wine (Godos et al., 2017). Anthocyanidins, principally as their conjugated derivatives, namely anthocyanins, comprise mainly cyanidin, pelargonidin, peonidin, delphinidin, malvidin, which form conjugates (on carbons 3, 5, 7, 3′ and 5′, and most often on C3) with sugars, cinnamates and organic acids such as malic and acetic acids (Del Rio et al., 2013). These phytochemicals accumulate in fruit and flower tissue and vegetables, where they are responsible for red, blue and purple colours. *In planta*, anthocyanidins have an important role to play in attracting pollinating insects (Crozier et al., 2007). Aubergines, berries (i.e. blackberry (*Rubus allegheniensis*), blackcurrant (*Ribes nigrum*), blueberry), black grape, cherry and rhubarb (Manach et al., 2004). The main dietary sources of anthocyanins include cherries, strawberries (*Fragaria × ananassa*), red wine, black olives and melon (Godos et al., 2017; Kay et al., 2017). Flavones, such as apigenin, luteolin, wogonin, and baicalein, are similar structurally to flavonols but lack oxygenation at C-3. Although this flavonoid class mainly occurs as 7-*O*-glycosides, they may be subjected to substitution with hydroxylation, methylation, *O*- and *C*-alkylation, and glycosylation. Flavones are not distributed widely, with significant occurrences reported in celery, parsley and some herbs (Crozier et al., 2007; Del Rio et al., 2013). Flavanones such as naringenin and hesperetin are characterized by the absence of $\Delta^{2,3}$ double bond and the presence of a chiral center at C-2, where the C-ring is attached to the B-ring at C2 in the α-configuration (Crozier et al., 2007; Rodriguez-Mateos et al., 2014). Citrus fruits are main sources of flavanones, with hesperetin-7-*O*-rutinoside (hesperidin) being the most common flavanone glycoside in citrus peel. Isoflavones such as genistein and daidzein are characterized by having the B-ring attached at C3 rather than the C2 position, and leguminous plants (i.e. soya products) are the main sources of these (poly)phenols occurring principally as 7-O-(6"-*O*-malonyl)glucosides with lower amounts of the corresponding 7-O-(6"-*O*-acetyl)glucosides, 7-*O*-glucosides, and the aglycones. The structure of these isoflavonoids is such that they mimic the steroidal hormone oestradiol which blocks ovulation and for this reason isoflavones are classified as phytoestrogens (Crozier et al., 2007; Del Rio et al., 2013; Rodriguez-Mateos et al., 2014).

Non-flavonoid compounds of dietary significance include C_6–C_1 phenolic acids (i.e. gallic acid, which is the precursor of hydrolysable tannins, also called gallotannins and ellagitannins), the C_6–C_3 hydroxycinammates and the polyphenolic C_6–C_2–C_6 stilbenes, besides lignans and coumarins (**Figure 2**) (Crozier et al., 2007; Reis et al., 2020).

Hydroxycinnamic acids [i.e. 3′,4′-dihydroxycinnamic acid (*aka* caffeic acid), 4′-hydroxy-3′-methoxycinnamic acid (*aka* ferulic acid), 3′,5′-dimethoxy-4′-hydroxycinnamic acid (*aka* sinapic acid) and 4′-hydroxycinnamic acid (*aka p*-coumaric acid)] may undergo esterification with tartaric acid or 1_L-(−)-quinic acid *in planta* forming chlorogenic acids (Clifford et al., 2017). Hydroxycinnamic acids are the most consumed phenolic acids in our diet (intake ~ 200 mg/day), with coffee, cereals, potatoes and artichoke being the main dietary sources (El-Seedi et al., 2012; Farah and Lima, 2019; Zamora-Ros et al., 2013b; Ziauddeen et al., 2018) (**Figure 3**). Gallic acid occurs widely as complex sugar esters in gallotannins, such as 2-*O*-digalloyl-tetra-*O*-galloyl-glucose. Gallotannins and ellagitannins are also called hydrolysable tannins due to their ability to be readily broken down, releasing gallic acid and/or ellagic acid, by treatment with dilute acid with respect to condensed tannins (i.e. polymeric proanthocyanidins). The related ellagic acid-based ellagitannins, such as sanguiin H-6 and punicalagin, accumulate in a huge variety of fruits, mainly raspberries and pomegranate (Crozier et al., 2007; Del Rio et al., 2013). Stilbenes have a C_6–C_2–C_6 structure and are phytoalexins produced by plants in response to disease, injury, and stress, with resveratrol (3,5,4′-trihdroxystilbene) being the main representative, which occurs as *cis* and *trans* isomers as well as conjugated derivatives, including *trans*-resveratrol-3-*O*-glucoside (*trans*-piceid). The primary dietary sources of stilbenes include grapes, wine, soya and peanut products (Crozier et al., 2007).

Lignans belong to the group of diphenolic compounds derived from the combination of two phenylpropanoid C_6–C_3 units at the β and β′ carbon, and can be linked to additional ether, lactone, or carbon bonds. Main lignans are secoisolariciresinol, lariciresinol, matairesinol, pinoresinol, medioresinol, and syringaresinol, with flaxseed and sesame seeds representing the richest sources (Durazzo et al., 2018; Rodríguez-García et al., 2019). Coumarins belong to the chemical class of 1-benzopyran derivatives (Sarker and Nahar, 2021), and major compounds include simple coumarins, furanocumarins (linear type and angular type), pyranocoumarins (linear type and angular type), bisoumarins, benzocoumarins and coumestans. Naturally, coumarins accumulate mainly in plant families such as *Apiaceae*, *Asteraceae*, *Rosaceae*, *Rutaceae*, *Rubiaceae*, *Solanaceae*, and *Lamiaceae*, with high coumarin concentration in Cassia cinnamon, besides in some tea, propolis, oils (olive, soy, peanuts corn), coffee, nuts, and wine (Lončar et al., 2020).

1.3 Metabolism of (poly)phenols in humans

After ingestion by humans, (poly)phenols are metabolised following the typical detoxification pathway common to xenobiotics and drugs (Del Rio et al., 2013; Rodriguez-Mateos et al., 2014) (**Figure 4**), which restricts the potentially toxic effects of ingested phytochemicals and facilitates their biliary and urinary elimination by increasing their hydrophilicity (Manach et al., 2004; Scalbert et al., 2002).

1. Polyphenols interact with salivary proteins causing astringency. They are not metabolized in the oral cavity.

2. Most polyphenols can resist to acidic conditions in the human stomach and may be transported bound to coingested plant polysaccharides.

3. Polyphenols can form complexes with proteins and digestive enzymes. Their absorption in the small intestine is limited.

4. Remnant monomeric, oligomeric and polymeric polyphenols reach the caecum without suffering extensive depolymerization.

5. Intact and partially hydrolyzed polyphenols undergo heavy biotransformation by resident colonic microflora and are degraded to phenolic acids, hippuric acids, simple phenols, urolithins and valerolactones, that are effectively absorbed.

6. Polyphenols bound to proteins and unabsorbed catabolites are excreted.

7. Colonic catabolites are further metabolized at hepatic level producing glucuronidated, methylated and sulphated metabolites. Some compounds may undergo enterohepatic circulation.

Figure 4. Metabolic fate of ingested dietary (poly)phenols along the human gastrointestinal tract. Figure modified from Zanotti et al. (Zanotti et al., 2015)

The first step for (poly)phenol absorption and metabolism is the removal of the sugar moiety by glycosidase activities (Scalbert and Williamson, 2000a). Although (poly)phenols glycosides are not extensively metabolized in the oral cavity, their structure can be modified by the hydrolysing activity of saliva and they were found interacting with salivary proteins causing astringency (Lee et al., 2004; Reis et al., 2020; Rodriguez-Mateos et al., 2014; Walle et al., 2005). After reaching the stomach, (poly)phenols are not metabolized in that district being able to resist to acidic conditions, thus reaching the small intestine in their glycosylated form at a different extent (Scalbert and Williamson, 2000a). The small intestine might be considered the first organ where ingested (poly)phenols are extensively metabolized (**Figure 5**).

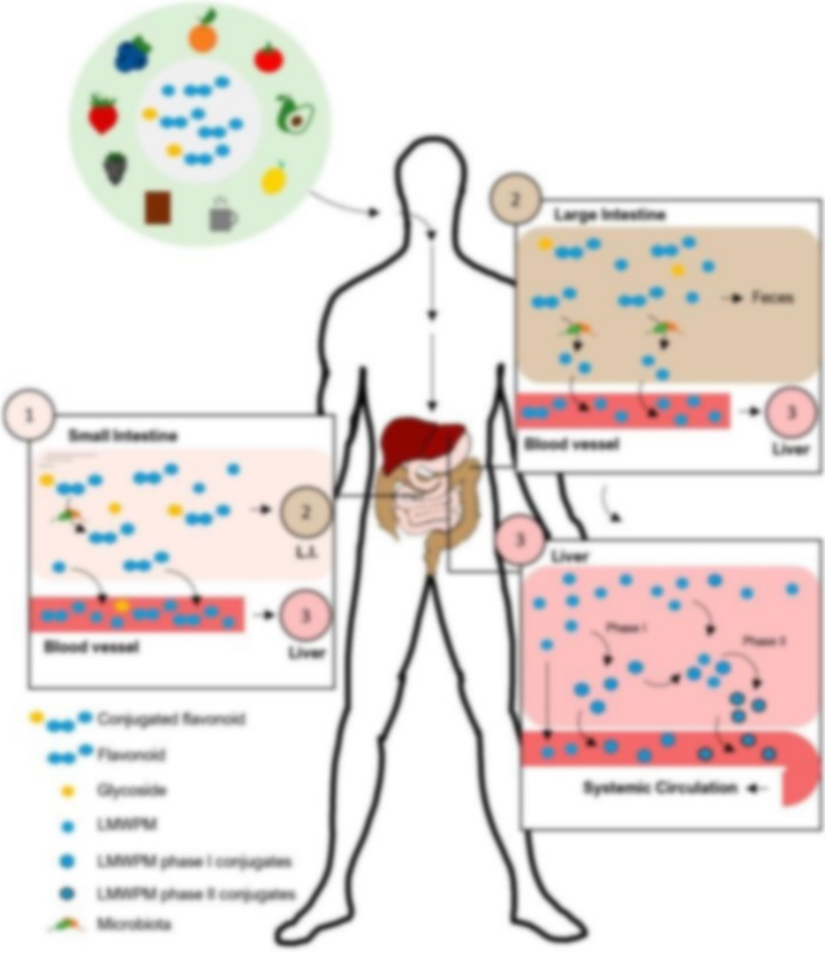

Figure 5. Diets rich in (poly)phenols provide large amounts of flavonoids and flavonoid conjugate reaching the digestive system. Figure modified from Carregosa et al. (Carregosa et al., 2021a)

The absorption step of (poly)phenols in the small intestine begins with the cleavage of the sugar moiety, releasing the aglycones through the action of the lactase phloridzin hydrolase in the brush border of the small intestine epithelial cells. The resulting aglycones will enter the epithelial cells by passive diffusion due to their higher lipophilicity. The cytosolic *β*-glucosidase may catalyze an alternative hydrolytic step within the epithelial cells. Aglycones undergo phase 2 conjugation steps in the wall of the small intestine before reaching the liver through the portal vein (Del Rio et al., 2013; Rodriguez-Mateos et al., 2014). Phase 2 conjugation steps include the addition of sulfate, glucuronide and methoxy groups to the aglycone skeleton through the activity of sulfotransferases, uridine-5′-diphosphate glucuronosyltransferases and catechol-*O*-methyltransferases, respectively. Efflux of some phase 2 metabolites back into the lumen of the small intestine may also occur. Metabolites resulting from absorption in the small intestine rapidly reach the liver to be further subjected to phase 2 conjugation steps before entering the systemic circulation and eventually undergoing renal excretion. Enterohepatic recirculation with the recycling back of hepatic metabolites to the small intestine through bile excretion is possible (Crozier et al., 2010; Del Rio et al., 2013). Studies in ileostomy subjects demonstrated that ~ 30 % of ingested (poly)phenols are absorbed in the upper gastrointestinal tract, while the remaining ~ 70 % (including (poly)phenols that were unmetabolised and/or phase 2 conjugates excreted through the bile by enterohepathic recirculation) reach the colon to be further modified by colon microflora activity (Borges et al., 2013; Catalkaya et al., 2020; Hagl et al., 2011; Kahle et al., 2007, 2005; Olthof et al., 2001; Stalmach et al., 2010b, 2010a). Colon microflora consists of over 1000 bacterial species (Rowland et al., 2018), with *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* being the most dominant bacterial phyla (Williamson and Clifford, 2017). Gut microbiota enzymes catalyze the hydrolysis of glycosides, glucuronides, sulphates, amides, esters and lactones through (poly)phenol skeleton forming simple aglycones. The residual aglycon skeletons undergo ring fission and may be further subjected to catabolic steps of α and/or β-oxidation, reduction, isomerization, decarboxylation, demethylation, dehydroxylation, and fission of ring and/or lactone forming aromatic and phenolic acids with zero to three aromatic hydroxyls forming 5C-ring fission catabolites namely phenyl-γ-valerolatones and their related phenylvaleric acids, C_6-C_3 (i.e. unsaturated compounds such as cinnamates and their derived hydrogenated derivative namely phenylpropanoic acids), C_6-C_2 phenylacetic acid, C_6-C_1 benzoic acid catabolites, besides benzene derivatives (Aura, 2008; Calani et al., 2012; Dall'Asta et al., 2012; Ludwig et al., 2013; Mena et al., 2019; Moreno-Ortega et al., 2022b, 2022a; Mosele et al., 2015; Ordoñez-Díaz et al., 2020; Selma et al., 2009; Stevens and Maier, 2016; Williamson and Clifford, 2017). Catabolic pathways for the main dietary (poly)phenols are illustrated in sections 1.3.1 and 1.3.2 of the present Doctoral Thesis. Catabolites resulting from colon enzyme activity are absorbed in the distal gastrointestinal tract to undergo further phase II metabolism locally and/or in the liver before entering the circulation and being excreted in urine (Manach et al., 2005, 2004; Scalbert and Williamson, 2000b). (Poly)phenols and their related catabolites that will be unabsorbed at colonic level will be voided in the faeces. As a consequence of metabolic reactions occurring both at the small and large intestine level, (poly)phenol metabolites appear in biofluids either as free or conjugated forms (Borges et al., 2018; Monagas et al., 2010; Pereira-Caro et al., 2014; Roowi et al., 2010; Stalmach et al., 2014, 2009). On the other hand, since phase 2 conjugates of (poly)phenol metabolites are recognised by the human body as xenobiotics, they will be rapidly excreted through kidneys to accumulate in the circulatory system to a less extent (Del Rio et al., 2013; Rodriguez-Mateos et al., 2014).

1.3.1 Occurrence, Bioavailability and Metabolism of Berry (Poly)phenols

This is a book chapter of "*Berries and Berry Bioactive Compounds in Promoting Health*" - the Royal Society of Chemistry.

Occurrence, Bioavailability and Metabolism of Berry (Poly)phenols

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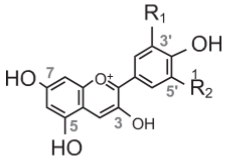
2.1 (Poly)phenolic Compounds in Berries

Berries are a rich source of micronutrients and phytochemicals, and the most notable of which are (poly)phenolic compounds.[1,2] (Poly)phenols found in berries include phenolic acids, flavonoids and polymeric tannins, which comprise ellagitannins (ETs) and proanthocyanidins (PACs), respectively also known as hydrolyzable and condensed tannins. The levels of these constituents are influenced by plant age and environmental and agronomic factors.[3] The vibrant red, purple and dark blue colors of berries are due to anthocyanins; the aglycones, namely cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin, referred to as anthocyanidins, differ by virtue of the presence and absence of hydroxy or methoxy groups at the C-3′ and C-5′ positions (Table 2.1). Because of their instability, anthocyanidins *per se* are not found *in planta* but exist as anthocyanins in which the anthocyanidin moiety is *O*-linked to a range of mono-, di-, and tri- saccharides which can be acetylated to a range of aromatic and aliphatic acids (Figure 2.1). This provides structural stability[4–6] and results in a diverse array of anthocyanin structures being found in berries and other plant sources.[3,7] Anthocyanin contents are typically high in blueberry (*Vaccinium corymbosum*), blackberry (*Rubus fruticosus* L.) and bilberry (*Vaccinium myrtillus*), which cancontain up to 495 mg kg^{-1} fresh weight (FW).[8]

Table 2.1 Structures of major anthocyanins.



Anthocyanidin	R_1	R_2	MW
Pelargonidin	H	H	271
Cyanidin	OH	H	287
Delphinidin	OH	OH	303
Peonidin	OCH_3	H	301
Petunidin	OCH_3	OH	317
Malvidin	OCH_3	OCH_3	331

Levels can be influenced by agronomic conditions and genotype.[3,9,10] Delphinidin-3-*O*-rutinoside is the main anthocyanin in blackcurrants (*Ribes nigrum*). Cyanidin glycosides are found in blackberry, elderberry (*Sambucus nigra*), American cranberry (*Vaccinium macrocarpon*), raspberry (*Rubus idaeus*) and redcurrant (*Ribes rubrum*).[9] The predominant anthocyanin in strawberry (*Fragaria ananassa*) is pelargonidin-3-*O*-glucoside. Blueberries have a complex anthocyanin profile, which includes malvidin-3-*O*-arabinoside and the 3-*O*-galactosides of delphinidin, cyanidin, petunidin and malvidin, with many other minor anthocyanins also being present.[7,9] Cranberries, like blueberries, are a member of the *Ericaceae* but have a very different spectrum of anthocyanins with cyanidin and peonidin-based compounds predominating.[7,9]

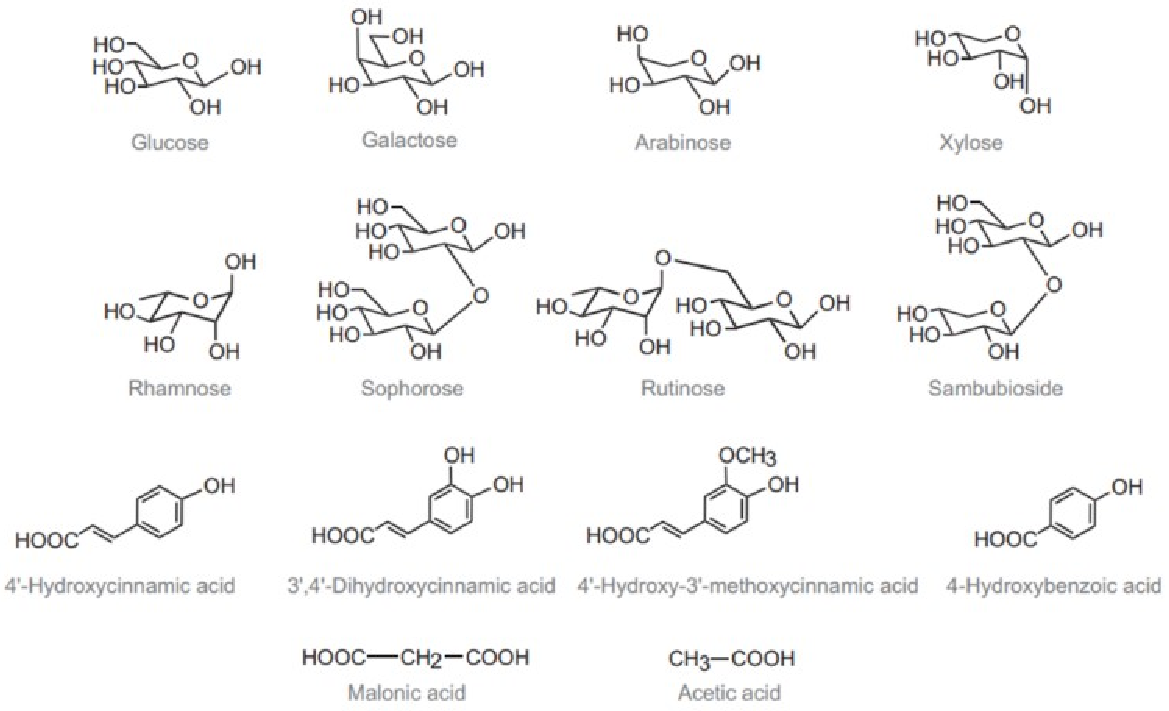
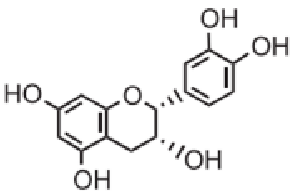
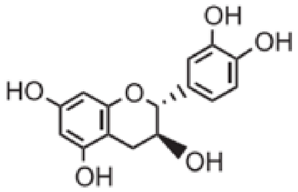


Figure 2.1 Selected sugars and aromatic and aliphatic acids that commonly occur inanthocyanin structures.

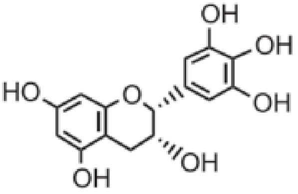
Other flavonoids of dietary relevance occurring in berries include flavan-3-ols.[1] Although less abundant than in fresh cocoa (*Theobroma cacao*) beans and young leaves of green tea (*Camellia sinensis*),[11] American cranberry (73 mg kg^{-1} FW), red raspberry (44 mg kg^{-1} FW) and strawberry (42 mg kg^{-1} FW) contain the monomeric flavan-3-ols (−)-epicatechin **1** and its enantiomer (+)-catechin **2**. (−)-Epigallocatechin **3** and (−)-epicatechin-3-*O*-gallate **4** occur less widely and in lower amounts.[1,9,12] Among ten native varieties of Canadian berries, Alpine blueberry (*Vaccinium uliginosum L.*) contains the highest level of flavan-3-ols (1020 mg kg^{-1} FW).[12] PAC dimers and trimers have been quantified in genotypes of Saskatoon berry (*Amelanchier alnifolia Nutt.*) and American cranberry (259 and 189 mg kg^{-1} FW for dimer and trimer contents, respectively).[9,13] Chokeberry (*Aronia melanocarpa*) and American cranberry are rich sources of PACs (~690 mg kg^{-1} FW).[12,14] American cranberry represents a source of both A- and B-type PACs.[9,14] B-type PACs are formed by oxidative coupling between the C-4 of the upper monomer and the C-6 or C-8 of the adjacent lower unit to create oligomers or polymers. A-type PACs have an additional ether bond between C-2 in the C-ring of one monomer and C-7 in the A-ring of the other monomer (Figure 2.2).



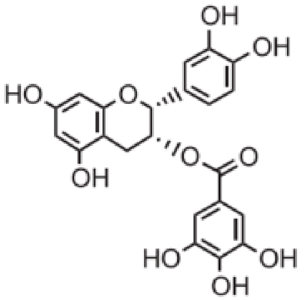
(–)-Epicatechin **1**



(+)-Catechin **2**



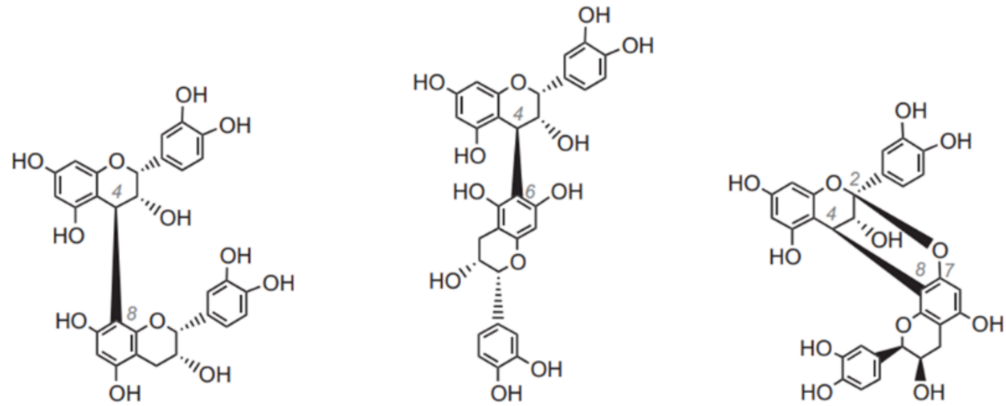
(–)-Epigallocatechin **3**



(–)-Epicatechin-3-*O*-gallate **4**

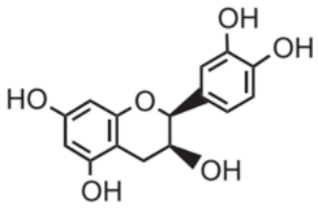
PACs can occur as polymers up to 50 units. Most quantitative estimates of flavan-3-ols in berries provide a simplified assessment of what exists *in planta*. Firstly, the monomers (−)-epicatechin and (+)-catechin also exist as (+)-epicatechin **5** and (−)-catechin **6**. The stereoisomers can be separated by chiral chromatography but not reversed-phase HPLC. A- and B-type PAC dimers and trimers can be separated by reversed-phase HPLC but PACs with a higher degree of polymerization are retained on the column. As a consequence, analyses of many berry PACs are limited to dimers and trimers.

Separation of PACs with a degree of polymerization (DP) of 2–7 subunits can be achieved using hydrophobic interaction chromatography (HILIC).[15] Details of a robust HILIC method with a Torus diol HPLC column have recently been reported.[16] Wang and colleagues have recently identified 304 individual A-type and B-type PACs from cranberry through reversed-phase HPLC coupled with ion-mobility quadrupole time-of-flight mass spectrometry.[17,18] Analysis of higher molecular weight PACs, which can exist insubstantial amounts, is not straightforward but has been accomplished with grape seed PACs using acid-catalyzed depolymerization in the presence of anucleophile and gel permeation chromatography based on the procedures of Kennedy and Taylor and Bindon and Kennedy.[19–21] Estimation of the overall levels of type A cranberry PACs using the colorimetric 4-(dimethylamino)cinnamaldehyde (DMAC) assay has been evaluated by Feliciano *et al.*[22] For the analysis of polymeric PACs, various techniques can be employed making use of their structural properties, *i.e.* binding for proteins and/or polysaccharides,[23,24] or their conversion to cyanidin aglycones upon thermal depolymerization in acid.[25,26]

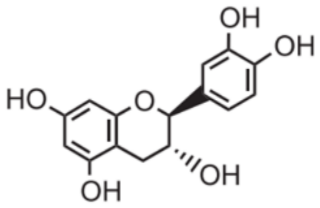


Procyanidin B_2 dimer Procyanidin B_5 dimer Procyanidin A_2 dimer

Figure 2.2 Selected structures of type A and B procyanidins.

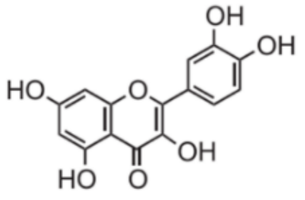


(+)-Epicatechin **5**

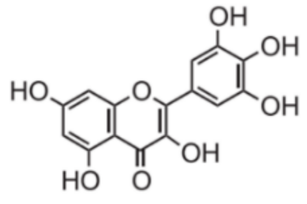


(–)-Catechin **6**

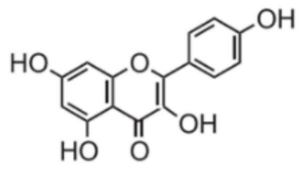
Flavonols are mostly found in berries as glycosides of quercetin **7**, myricetin **8** and, to a lesser extent, kaempferol **9** and isorhamnetin **10**.[1] Eight glycosides of quercetin were quantified in Polish and Canadian berry cultivars, among which quercetin-3-*O*-arabinoglucoside, quercetin-3-*O*-rutino- side and quercetin-3-*O*-galactoside were the most abundant.[13] American cranberries contain approximately 1000 mg total flavonols per kilogram of FW, with quercetin-*O*-glycosides being the main representatives.[2] Myricetin- *O*-glycosides (3-*O*-glucoside, -galactoside and -rutinoside derivatives) are the most abundant flavonols in blackcurrant cultivars (89–203 mg kg^{-1} FW),[9] while kaempferol glycosides accumulate in cherry (*Prunus avium*) (39–50 mg kg^{-1} FW) and blueberry (23–30 mg kg^{-1} FW).[27–29] The ETs are derivatives of ellagic acid **11**, made up of one or more hexahydroxydiphenoyl (HHDP) moieties esterified to a sugar, typically glucose.[30,31] Due to the many possibilities for the HHDP moieties to link withthe sugar and to their marked tendency to form ET oligomers and polymers,a great structural variability and complexity exist.[30] They are found in many berries, including raspberry, strawberry, blackberry, blueberry, American cranberry and cloudberry (*Rubus chamaemorus*).[30] Ellagic acid can accountfor more than 50 % of the total phenolic content of berries, principally in the form of ETs,[3] which are considered to be the main contributors to astringency in strawberries and raspberries.[3] Red raspberries contain approximately 1 mg kg^{-1} of ellagic acid compared to B300 mg kg^{-1} of ETs, mainly sanguiin H-6 **12** and lambertianin C **13**.[9]



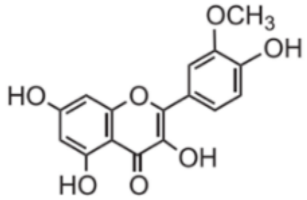
Quercetin **7**



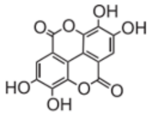
Myricetin **8**



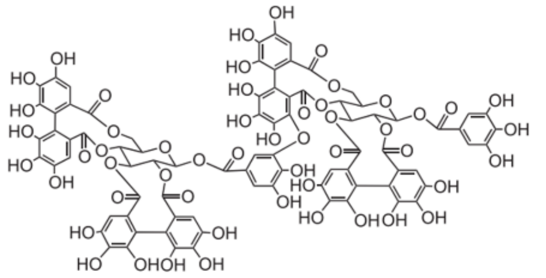
Kaempferol **9**



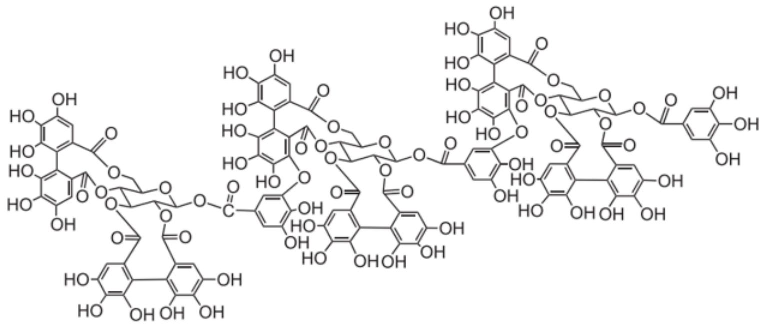
Isorhamnetin **10**



Ellagic acid **11**

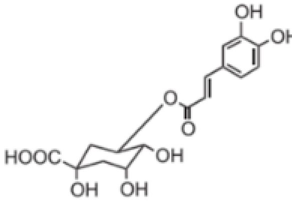


Sanguiin H-6 **12**

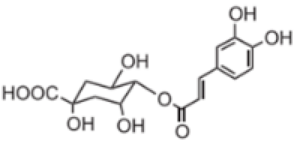


Lambertianin C **13**

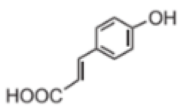
Hydroxycinnamic acid and hydroxybenzoic acid derivatives are the most abundant phenolic acids in berries[1] and have been detected in amounts up to 1390 mg kg^{-1} FW.[12] Chokeberry and chokecherry (*Prunus virginiana*) fruits have a similar phenolic acid content (630 mg kg^{-1} FW), followed by wild blueberry (*aka* low-bush blueberry) (*Vaccinium angustifolium*) (510 mg kg^{-1} of FW), containing mainly 5-*O*-caffeoylquinic acid **14** (chlorogenic acid) and 4-*O*-caffeoylquinic acid **15** (neochlorogenic acid).[12] Among the hydroxycinnamic acid derivatives, 4'-hydroxycinnamic acid **16** (*p*-coumaric acid) has been found in the highest concentrations in Swedish bilberries (510 mg kg^{-1} dry weight [DW]). The 4'-hydroxy-3'-methoxycinnamic acid **17** (ferulic acid) content of cloudberries is similar to that of bilberries (390 mg kg^{-1} DW), while a 3',4'-dihydroxycinnamic acid **18** (caffeic acid) content of 690 and 830 mg kg^{-1} DW has been reported, respectively, in bilberries and lingonberries. 4-Hydroxybenzoic acid **19**, 3,4,5-trihydroxybenzoic acid **20** (gallic acid), 2-hydroxybenzoic acid **21** (salicylic acid) and 4-hydroxy-3-methoxybenzoic acid **22** (vanillic acid) are the main hydroxybenzoic acid derivatives in berry fruits. 3,4,5-Trihydroxybenzoic acid and 4-hydroxy-3-methoxybenzoic acid are found in lingonberries and cloudberries, at respective concentrations of 120 and 2730 mg kg^{-1} DW.[32] The (poly)phenol content of berries can be affected by post-harvest UV light, organic farming and location.[3,33] Stilbenes accumulate in berries to a much more limited extent than other (poly)phenols, with *trans*-resveratrol **23**, *trans*-pterostilbene **24** and *trans*-piceatannol **25** being found mainly as glycosides in deerberry (*Vaccinium stamineum*), blueberry, cowberry and lingonberry (*Vaccinium vitis-idaea*).[34] Although berries are usually consumed as fresh fruit which are perishable and, thus, have limited season availability, at least from local growers, a range of frozen, freeze-dried fruit, juices, capsules, purees, jams and canned products have been developed to facilitate ‘‘all year round’’ consumption. The technology employed for the manufacture of these products has not been investigated in detail, but processing may well affect the natural (poly)phenol content.[14,35,36]



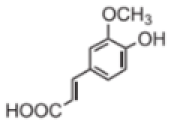
5-*O*-Caffeoylquinic acid **14**



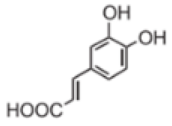
4-*O*-Caffeoylquinic acid **15**



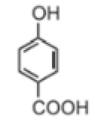
4'-Hydroxycinnamic acid **16**



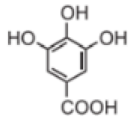
4'-Hydroxy-3'-methoxycinnamic acid **17**



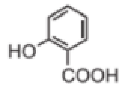
3',4'-Dihydroxycinnamic acid **18**



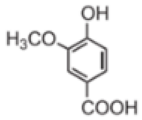
4-Hydroxybenzoic acid **19**



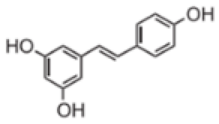
3,4,5-Trihydroxybenzoic acid **20**



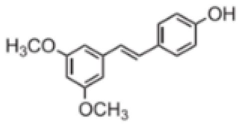
2-Hydroxybenzoic acid **21**



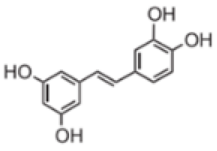
4-Hydroxy-3-methoxybenzoic acid **22**



trans-Resveratrol **23**



trans-Pterostilbene **24**



trans-Piceatannol **25**

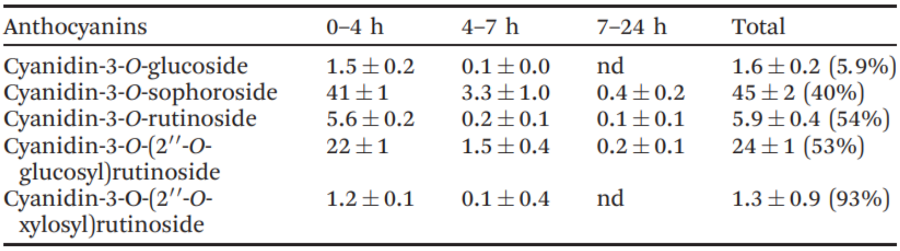
2.2 Absorption, Disposition, Metabolism and Excretion of Berry (Poly)phenols

Typically, absorption of (poly)phenol glycosides is associated with hydrolysis releasing aglycones as a result of the action of lactase phlorizin hydrolase (LPH) in the brush border of the small intestine epithelial cells. LPH exhibits broad substrate specificity for flavonoid-*O*-β-D-glucosides, and the released aglycone can enter the epithelial cells by passive diffusion, as a result of its increased lipophilicity and its proximity to the enterocyte membrane.[37] An alternative hydrolytic step is mediated by cytosolic β-glucosidase (CBG) within the epithelial cells. In order for CBG-catalyzed hydrolysis to occur, the polar glucosides must be transported into the epithelial cells, possibly with the involvement of the active sodium-dependent glucose transporter SGLT1.[38] Thus, there are two possible routes by which the glucoside conjugates are hydrolyzed, namely LPH-diffusion and transport-CBG, with the resultant aglycones appearing in the epithelial cells.[39] Prior to passage into the bloodstream, (poly)phenol aglycones undergo phase II metabolism in the enterocytes, forming sulfate, glucuronide and/or methylated metabolites through the respective action of sulfotransferases, uridine-5′-diphosphate glucuronosyltransferases and catechol-*O*-methyl- transferases. There is an efflux of at least some of the metabolites back into the lumen of the small intestine,[40,41] and this is thought to involve members of the ATP-binding cassette family of transporters, including multidrug resistance protein and P-glycoprotein. Once in the portal bloodstream, metabolites pass to the liver, where they may be subjected to further phase II metabolism.[39] The considerable amounts of (poly)phenols not absorbed from the small intestine pass to the colon,[42–44] where the microbiota can cleave conjugating moieties and the resultant aglycones undergo ring fission, leadingto the production of phenolic catabolites and aromatic compounds. These low-molecular-weight catabolites can be absorbed, and a portion is subjected to phase II metabolism before being excreted in urine in substantial quantities, which in most instances is well in excess of that of metabolites that enter the circulatory system *via* the small intestine.[45,46] When these metabolites and catabolites are included in the overall estimation of absorption, disposition, metabolism and excretion (ADME), which is synonymous with bioavailability in nutrition research (European Food Information Council 2010),[47] it is evident that many (poly)phenols are highly bioavailable. Although there is speculation,[48] as yet there is no compelling evidence that enterohepatic recirculation in humans results in substantial recycling of (poly)phenol metabolites back into the small intestine *via* biliary excretion.[49] In bioavailability studies, it is of note that while plasma profiles can supply useful information, they are not an accurate quantitative guide of absorption because the presence of metabolites and catabolites in the circulatory system is transient as they are rapidly removed from the bloodstream *via* renal excretion. More appropriate estimates of absorption are obtained by determining cumulative urinary excretion, typically over a 24–48 h period, post-intake.[46,50,51] Analysis of (poly)phenol phase II glucuronide, sulfate and methylated metabolites in biofluids has evolved markedly over the last 20 years. Initially, in the absence of reference compounds, samples were subjected to acid or enzyme hydrolysis and the released aglycones were analyzed by HPLC with absorbance, fluorescence or electrochemical detection. This provided, at best, very limited information on the identity of the phase II metabolites. Gradually this methodology was replaced by HPLC–MS, which, even in the absence of reference compounds, facilitated the partial identification of metabolites as methyl, sulfate or glucuronide derivatives,[39] although it now realized that therecould be major inaccuracies with quantitative estimates.[52] In recent years, a number of methods have been devised for the synthesis of (poly)phenol metabolites, a number of which can now be obtained from commercial sources. This has also been accompanied by the increasing use of ultra- high-performance liquid chromatography (uHPLC) and high-resolution mass spectrometry (HRMS). These developments are discussed in Chapter 1.[53]

2.2.1 Anthocyanins

The majority of studies on the post-absorption fate of anthocyanins have focussed on berries. The pioneering studies were carried out at the end of the 1990s.[54] For many years, anthocyanins were considered to be poorly bioavailable in humans,[55] with the trace quantities of unchanged glycosylated structures being observed in plasma within 30–90 min of intake and inurine collected 0–24 h after ingestion.[56–58] However, as noted above, the current perceived wisdom is that (poly)phenols, including anthocyanins, aremore bioavailable than previously envisaged when ADME takes into deglycosylation, formation of phase II glucuronide, sulfate and methoxy metabolites, and to a much greater extent, ring fission and the formation ofphenolic catabolites during passage along the gastrointestinal (GI) tract, as well as post-absorption metabolism following entry into the circulatory system.[6,39,59] The low-level absorption of parent anthocyanin glycosides compared to their phase II metabolites has been reviewed by Cassidy & Minihane and is exemplified in a study by McKay et al.[60,61] After the consumption of cranberry juice, peonidin derivatives, including the 3-*O*-galactoside, arabinoside and glucoside conjugates, were detected in plasma by HPLC with absorbance detection at 520 nm, together with cyanidin-3-*O*-arabinoside, cyanidin-3-*O*-galactoside and cyanidin-3-*O*-glucoside. They reached their maximum concentration (C_{max}) 0.9–4.7 h after juice intake (T_{max}). The C_{max} of these native anthocyanins, expressed as cyanidin-3-*O*-glucoside equivalents, ranged between 77 and 981 pg mL^{-1}, which was at least 1000-fold lower than the C_{max} of anthocyanin glucuronide metabolites. In parallel, urinary excretion of parent anthocyanins was 430-fold lower than that of their phase II glucuronide metabolites.[61] Similar findings emerged from a study in which volunteers ingested strawberries where the predominant anthocyanin was pelargonidin-3-*O*-glucoside. The only anthocyanin to be detected in plasma was a pelargonidin glucuronide with a T_{max} of 1.1 h and a C_{max} of 274 nmol L^{-1}, quantified as pelargonidin-3-*O*-glucoside equivalents. The amount of the glucuronide excreted in urine over a 24 h period after strawberry intake was 1.5 mmol compared to 55 nmol of the ingested pelargonidin-3-*O*-glucoside, a 27-fold difference.[62] Hydroxylation of the anthocyanidin B-ring impacts absorption with the excretion of metabolites of strawberry pelargonidin-3-*O*-glucoside (Figure 2.1) being equivalent to 1.8% of intake,[63] whereas metabolites of blackberry cyanidin-3-*O*-glucoside are reported at 10-fold lower concentrations.[64] Ileal fluid collected from ileostomists following the ingestion of bilberries showed that malvidin and petunidin glycosides, characterized by methoxy-groups on the B-ring, were recovered in greater quantities than cyanidin and delphinidin glycosides which have only B-ring hydroxyls. This would suggest that O-methylation of anthocyanins confers increased stability and/or reduced absorption.[65] Anthocyanin phase II metabolites derived from monosaccharides, most notably, glucosides, appear to be absorbed more readily in the upper GI tract than those originating from di- and trisaccharide conjugates. To some degree, this could be a consequence of the monosaccharides being more readily deglycosylated by LPH and/or CBG. The enhanced susceptibility of monosaccharides to hydrolysis by these enzymes is supported by the data of González-Barrio *et al.*, which showed that after the consumption of raspberries by ileostomists, there was a 5.9% recovery of cyanidin-3-*O*-glucoside and a 40–93% recovery of cyanidin di- and trisaccharides in ileal fluid (Table 2.2).[66] A subsequent study, in which urine and plasma were collected after volunteers with a functioning colon had consumed raspberries, indicated that cyanidin released in the small intestine undergoes only level conversion to aglucuronide metabolite which appeared in plasma 1 h after berry intake with a C_{max} of 0.1 nmol L^{-1}.[67] Most of the anthocyanidin probably underwent a pH-dependent transformation to a retro-chalcone structure that was converted to 3′,4′-dihydroxycinnamic acid **18**.[68] The cinnamic acid did not accumulate in detectable amounts in either plasma or urine, probably as a result of rapid phase II metabolism to 3′-methoxycinnamic acid-4′-sulfate **26** (ferulic acid-4′-sulfate), 3′-methoxycinnamic acid-4′-glucuronide **27** (ferulic acid-4′-glucuronide) and 4′-methoxycinnamic acid-3′-glucuronide **28** (isoferulic acid-3′-glucuronide) all of which appeared in plasma 1.0–1.5 h after raspberry intake with C_{max} values ranging from 14 to 47 nmol L^{-1} before undergoing a series of conversions potentially by the pathways illustrated in Figure 2.3.[67]

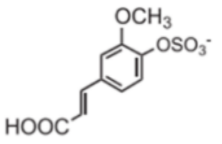
Table 2.2 Recovery of the cyanidin-derived anthocyanins in ileal fluid collected 0–4 h, 4–7 h and 7–24 h after the consumption of 300 g raspberries. After González-Barrio *et al.*[66,a]



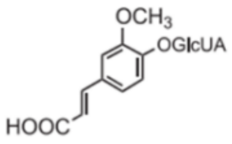
Anthocyanins	0–4 h	4–7 h	7–24 h	Total
Cyanidin-3-*O*-glucoside	1.5 ± 0.2	0.1 ± 0.0	nd	1.6 ± 0.2 (5.9%)
Cyanidin-3-*O*-sophoroside	41 ± 1	3.3 ± 1.0	0.4 ± 0.2	45 ± 2 (40%)
Cyanidin-3-*O*-rutinoside	5.6 ± 0.2	0.2 ± 0.1	0.1 ± 0.1	5.9 ± 0.4 (54%)
Cyanidin-3-*O*-(2′′-*O*-glucosyl)rutinoside	22 ± 1	1.5 ± 0.4	0.2 ± 0.1	24 ± 1 (53%)
Cyanidin-3-O-(2′′-*O*-xylosyl)rutinoside	1.2 ± 0.1	0.1 ± 0.4	nd	1.3 ± 0.9 (93%)

[a] Data presented as means values in µmol ± standard error (*n* =4). Italicized figures in paren- theses represent the amounts recovered as a percentage of the quantity ingested. nd – not detected. Anthocyanins were not detected in ileal fluid samples collected 24 h after raspberry intake.

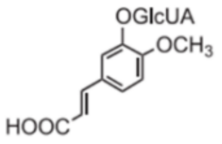
Other cyanidin breakdown products arising, arguably, mainly from the A-ring of the anthocyanin, namely 1,2-dihydroxybenzene **29** (catechol), 1,3-dihydroxybenzene **30** (resorcinol) and 1,2,3- trihydroxy-benzene **31**, were identified by González-Barrio *et al.*[69] following *ex vivo* anaerobic fecal incubations with raspberry extracts. These products are included in Figure 2.3.



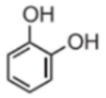
3'-Methoxycinnamic acid-4'-sulfate **26**



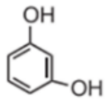
3'-Methoxycinnamic acid-4'-glucuronide **27**



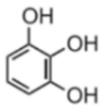
4'-Methoxycinnamic acid-3'-glucuronide **28**



1,2-Dihydroxybenzene **29**



1,3-Dihydroxybenzene **30**

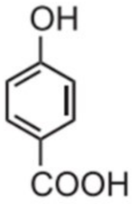


1,2,3-Trihydroxybenzene **31**

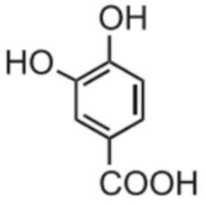
In the preparation of the proposed pathways illustrated in Figure 2.3, the following points were taken into consideration. The conversion of cyanidin to 3′,4′-dihydroxycinnamic acid could involve pH-mediated degradation as well as microbial and mammalian enzymes. Subsequent methylation and glucuronide, sulfate and glycine conjugation are mammalian in origin. Dehydroxylation and demethoxylation are almost certainly mediated by the gutmicroflora, whereas demethylation and hydrogenation steps can be mediated by both microbial and mammalian enzymes. For convenience, the pathways in Figure 2.3 show C_6–C_3 catabolites being converted by two α-oxidations to C_6–C_1 compounds by microflora and/or mammalian enzymes. However, it is plausible that the C_6–C_3 catabolites progress directly to C_6–C_1 structures *via* β-oxidation and that C_6–C_3 catabolites arise independently, possibly by α-oxidation. Alternatively, C_6–C_2 structures could arise from the intact anthocyanidin by an independent gut flora-mediated pathway. In reality, further complexity is introduced as there are multiple pointsat which catabolites might be absorbed. For example, a percentage of some C_6–C_3 catabolites could be absorbed and undergo β-oxidation and/or mammalian phase II conjugation while the balance is subjected to microbial hydrogenation and/or β-oxidation prior to absorption and mammalian conjugation. Also, for some catabolites, mammalian conjugation either does not occur or is incomplete. The production of benzene derivatives almost certainly involves microbial enzymes. An intervention study by de Ferrars *et al.* provided further evidence that, following ingestion, anthocyanins were converted predominantly to phenolic catabolites.[70] Post-menopausal women ingested an elderberry extract containing 500 mg of anthocyanins, mainly in the form of cyanidin-3-*O*-glucoside and cyanidin-3-*O*-sambubioside. In total, 21 metabolites/catabolites were detected. The phenolic catabolites, which included sulfates of 4-hydroxybenzoic acid **32**, 3,4-dihydroxybenzoic acid **33** and 4-hydroxy-3-methoxybenzoic acid **22**, attaineda C_{max} of 1237 nmol L^{-1}, well in excess of the 34 nmol L^{-1} of the parent anthocyanin glycosides, while excretion of the phenolic catabolites in urine was 60-fold higher than that of anthocyanin conjugates.

The most notable anthocyanin bioavailability investigation to date is that of Czank *et al.* and de Ferrars *et al.*, who fed $^{13}C_5$-labeled cyanidin-3-*O*-glucoside (Figure 2.4) to human participants.[71,72] After 48 h, recovery was established as 44 % of the ^{13}C dose, with 6.9 % in breath, 32 % in feces and 5.4 % urine. The particular advantage of using [6,8,10,3',5'-$^{13}C_5$]cyanidin-3-*O*-glucoside was that it had three ^{13}C molecules incorporated into the A-ring and two into the B-ring. Numerous phenolic products were detected in plasma and urine, and because of the ^{13}C label, it was possible to ascertain whether they were derived from the A- or B-ring of cyanidin. The capacity to determine relatively low levels of incorporation of the ^{13}C label into the pools of phenolic compounds enabled cyanidin-derived catabolites to be identified and quantified in much more detail than the raspberry feeding study of Ludwig *et al.*,[67] where catabolites derived from cyanidin were reported only where there was a statistically significant increase in theurinary pool after supplementation. Nonetheless, the structural conversions illustrated in Figure 2.3, based primarily on outputs from raspberry interventions, are broadly in line with the array of ^{13}C-labeled products detected by Czank *et al.* and de Ferrars *et al.*, who in their studies observed conversions to additional benzoic acid and benzene derivatives.[71,72]

There are reports that anthocyanins exist as hundreds of derivatives of phase II conjugates that persist for prolonged periods in tissues and the circulation.[73–75] These inferences are based on an untargeted metabolomics approach using single-ion monitoring and low-resolution triple-quadrupole mass spectrometry and are not supported by more appropriate analyses based on high-resolution time-of-flight or orbitrap mass spectrometry.[76–79] Until more convincing evidence is forthcoming, proposals that following ingestion anthocyanins exist as hundreds of long-lived phase II metabolites, none of which have been structurally identified, lack credibility.



4-Hydroxybenzoic acid **32**



3,4-Dihydroxybenzoic acid **33**

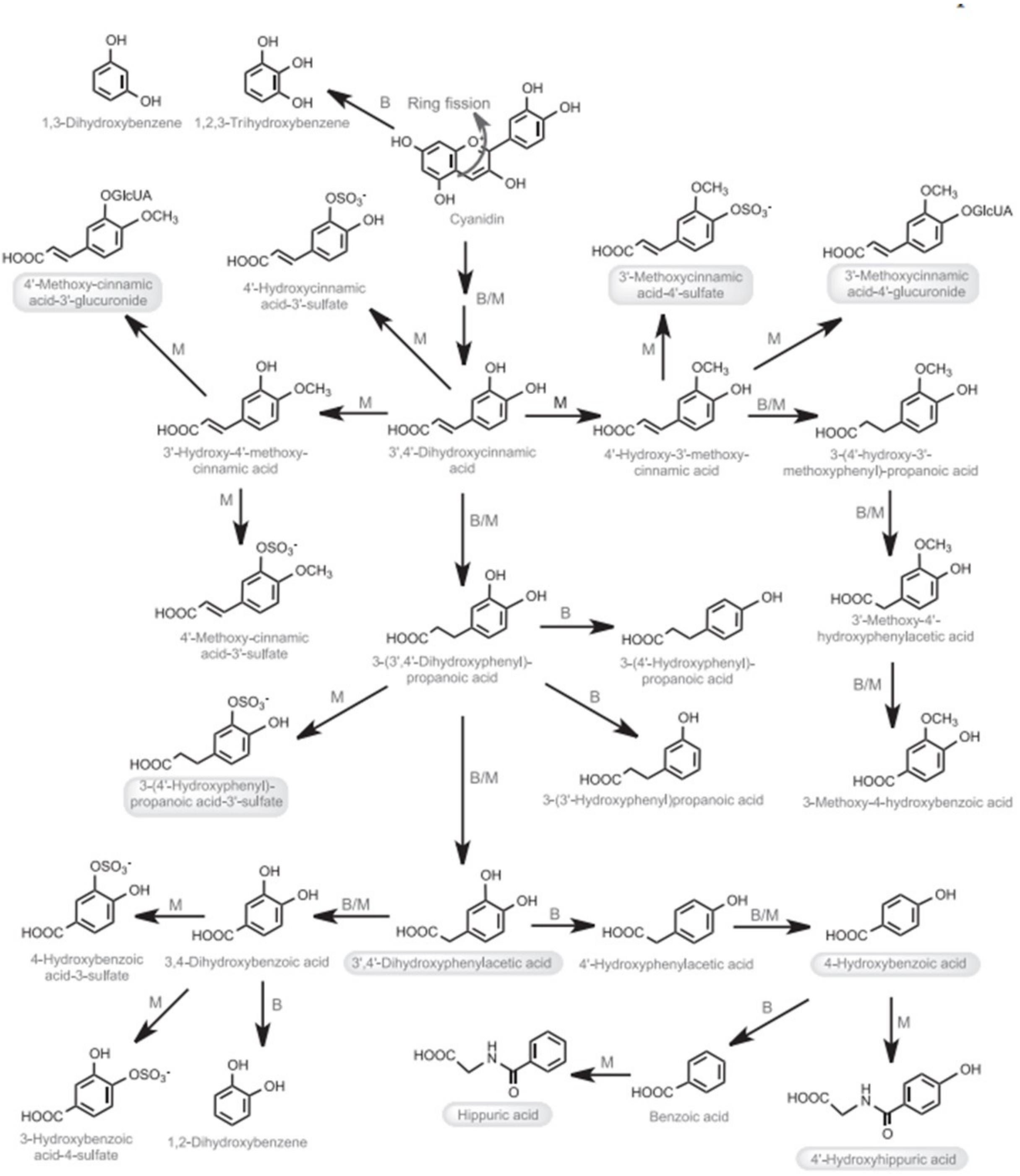


Figure 2.3 Proposed pathways for the conversion of cyanidin-based anthocyanins to phenolic acids and related compounds based on urinary excretion in feeds with red raspberries and *in vitro* fecal incubations.[67,69] M – steps catalyzed by mammalian enzymes. B – steps probably catalyzed by bacterial enzymes. M/B – conversions that could involve mammalian and/or bacterial enzymes. Abbreviation: GlcUA, glucuronic acid. Compounds with boxed names indicate major components in 0–24 h urine.

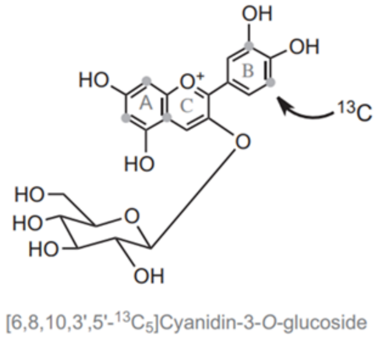


Figure 2.4 Structure of [6,8,10,3′,5′-$^{13}C_5$]cyanidin-3-*O*-glucoside. The five high- lighted the positions of the ^{13}C-label.

2.2.2 Flavan-3-ols

As noted in Section 2.1, flavan-3-ols are found *in planta* in a variety of forms. Most bioavailability research with berries has focused on the monomer (−)-epicatechin, which occurs in comparatively low amounts and this has hindered the identification and quantification of specific metabolites, a task that has been further compounded by difficulties in obtaining reference compounds of glucuronide, sulfate and methyl phase II metabolites.[80,81] Synthesis procedures have been devised, but they are complex and as a result, obtaining metabolites is a specialized and expensive process.[82–84] There is one report on the ADME of [2-^{14}C](−)-epicatechin in humans, which provides useful insights into the likely fate of the monomer in berry-based studies.[85] Measurement of radioactivity in plasma indicated a biphasic profile with maxima at 1 h and 6 h (Figure 2.5). The total radioactivity in plasma never exceeded 2% of intake. The radioactivity witha T_{max} of 1 h consisted of 12 structurally related (−)-epicatechin metabolites (SREMs), principally (−)-epicatechin-3′-glucuronide **34**, (−)-epicatechin-7-glucuronide **35**, (−)-epicatechin-3′-sulfate **36** and 3′-methoxy-(−)-epicatechin-5-sulfate **37**, which, the T_{max} indicates, were absorbed in the upper GI tract.

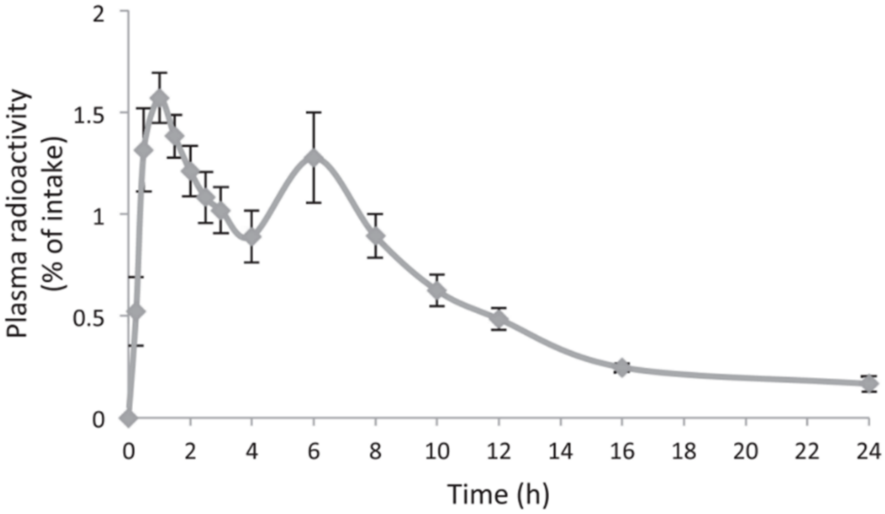
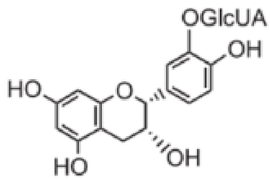
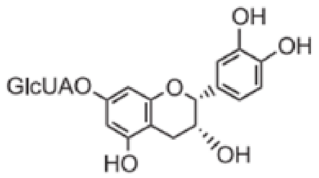


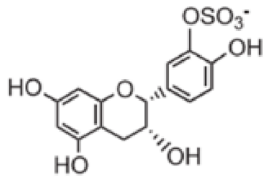
Figure 2.5 Radioactivity detected in plasma collected 0–24 h after the ingestionof 300 µCi (207 µmoles) of [2-^{14}C](−)-epicatechin by volunteers.[85] Data presented as mean values standard error (n= 8) and expressedas a percentage of the ingested radioactivity.



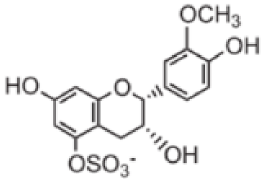
(−)-Epicatechin-3'-glucuronide **34**



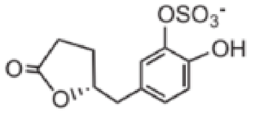
(−)-Epicatechin-7-glucuronide **35**



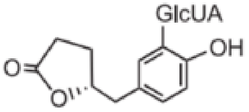
(−)-Epicatechin-3'-sulfate **36**



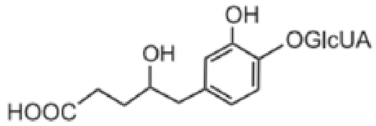
3'-Methoxy-(−)-epicatechin-5-sulfate **37**



5-(4'-Hydroxyphenyl)-γ-valerolactone-3'-sulfate **38**

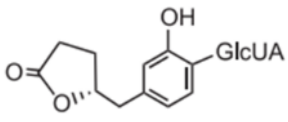


5-(4'-Hydroxyphenyl)-γ-valerolactone-3'-glucuronide **39**

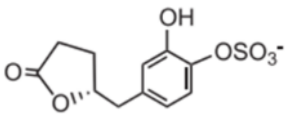


4-Hydroxy-5-(3'-Hydroxyphenyl)valeric acid-4'-glucuronide **40**

(−)-Epicatechin that was not absorbed in the small intestine, and its metabolites effluxed back in the lumen of the GI tract, passed to the colon where they were subjected to the action of the microbiota. Hence, radioactivity appearing in the circulatory system with a T_{max} of 6 h (Figure 2.5) was associated with the resultant 5C-ring fission metabolites (5C-RFMs), principally in the form of 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-sulfate **38** and 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-glucuronide **39**. Other 5C- RFMs detected in plasma included 4-hydroxy-5-(3′-hydroxyphenyl)valeric acid-4′-glucuronide **40**. Urinary recovery 0–48 h after intake indicated excretion of SREMs and 5C-RFMs that was equivalent, respectively, to 20% and 42% of (−)-epicatechin intake, while there was also a 7% excretion of 2C- and/or 3C-ring fission metabolites (2C/3C-RFMs) and 21% excretion of hippuric acids. Thus, the recovery of metabolites absorbed in the colon was 70%. Voided feces contained 10% of the ingested radioactivity as a diversity of ring fission metabolites.[86] Potential routes for the degradation of (−)-epicatechin by microbiota in the colon and subsequent phase II metabolism in colonocytes and/or hepatocytes are summarizedin Figure 2.6. Additional details can be found in papers by Monagas *et al.*,[87] Rodriguez-Mateos *et al.*,[50] Borges *et al.*,[86] Mena *et al.*[88] and Liu *et al.*[89] A further study with (−)-epicatechin by Actis-Goretta *et al.* made use of a Loc-I-gut intestinal technique to introduce (−)-epicatechin into an isolated section of the jejunum.[40] This revealed that there was an efflux of absorbed metabolites back into the intestinal lumen and that the main metabolite effluxing back into the jejunum was (−)-epicatechin-3′-sulfate **36**, while (−)-epicatechin-3′-glucuronide **34** was absorbed in the circulatory system and excreted in urine to a greater extent. The data obtained in this study have also indicated that enterohepatic recirculation of flavan-3-ol metabolites was, at most, a very minor event. Bioavailability studies on flavan-3-ols using berries include that of Pimpão *et al.*,[79] who investigated the 24 h metabolic fate of (−)-epicatechin **1** and (+)-catechin **2**, following acute intake by healthy volunteers of a (poly)phenol-rich fruit puree consisting of blueberries, blackberries, raspberries, strawberry tree (*Arbutus unedo*) and crowberries (*Corema album* L.). Structurally related (−)-epicatechin metabolites in the form of two (epi)catechin sulfate and two glucuronide conjugates were excreted in urine at high levels after 2 h, suggesting their absorption in the upper GI tract. Post-ingestion of a grape and wild blueberry mix, the circulating levels of SREMs was investigated by Philip *et al.*[90] SREMs, including an (epi)catechin-glucuronide, two (epi)catechin-sulfate isomers and a methoxy(epi)catechin-sulfate, had a C_{max} in plasma 3 h post-intake, arguably indicative of flavan-3-ol monomer absorption in the proximal GI tract. Neither of these studies had access to SREMs as reference compounds, so the metabolites could only be partially identified, nor could they be accurately quantified.[91] Following the intake of cranberry juice containing 1741 mg of total flavan-3-ols, 22 free and phase-II derivatives of the colon-derived 5C- RFMs were identified in plasma and urine samples, of which 13 were quantified.[92] 5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-glucuronide **39**, 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-glucuronide **41** and two 5-(hydroxyphenyl)-γ-valerolactone-sulfate isomers were the most abundant plasma 5C-RFMs.[92] 5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-glucuronide was reported to be the main metabolite to be excreted in urine post-cranberry intake followed by 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-glucuronide and a 5-(hydroxyphenyl)-γ-valerolactone-sulfate.[92]



5-(3'-Hydroxyphenyl)-γ-valerolactone-4'-glucuronide **41**



5-(3'-Hydroxyphenyl)-γ-valerolactone-4'-sulfate **42**

After strawberry tree ingestion, containing principally (epi)catechin and dimeric PACs, neither native flavan-3-ols nor SREMs were detected in plasma,[93] whereas an (epi)catechin-sulfate was the most abundant metabolite in urine along with lower levels of several sulfated and glucuronidated derivatives.[93] Similarly, (epi)catechin and PACs were not detected in plasma or urine collected after consumption of cranberry juice, although PACs were the most abundant flavan-3-ols in the drink.[94] Generally, the amount of native cranberry flavan-3-ols detected in the circulatory system and urine is negligible compared to that of colonic metabolites principally in the form of 5C-RFMs. Following the ingestion of cranberry PACs, or wild blueberry PACs with DPs of 3–10, only one 5C-RFM derivative, namely 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate **42**, was quantified in plasma and urine.[95,96] PACs have not been detected in plasma or urine in most human bioavailability investigations. Among the exceptions, the study by McKay et al. detected the accumulation of a variety of metabolites, including trace amounts of PAC A2 dimer (see Figure 2.1), in urine after the ingestion of a cranberry juice.[61] Low levels of PAC dimers in biofluids have also been reported by other investigators.[97–101] It is evident that high-molecular-weight PACs are not absorbed and that there is extremely limited absorption of dimers and trimers into the circulatory systems. The substantial amounts of PACs that occur in many berries, therefore, pass into the lower bowel. The available evidence obtained by Appeldoorn *et al.*[102] and Stoupi *et al.*[103] indicates theyare degraded by the colonic microbiota, with scission of the interflavanbond releasing tail units which, like monomers, undergo ring fission, being converted *via* 5-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol **43** to 5C-RFMs.[89] The 5C-RFMs, as shown in Figure 2.5, can be further degraded to lower-molecular-weight phenolic acids and aromatic compounds, which are also derived from the catabolism of the upper and lower PAC extension units. These catabolites, unlike the parent PACs, are readily absorbed *in situ*.[50,88] Voided feces are likely to containunabsorbed PACs and, although as yet they have not been analyzed, partially degraded PAC chains.[19]

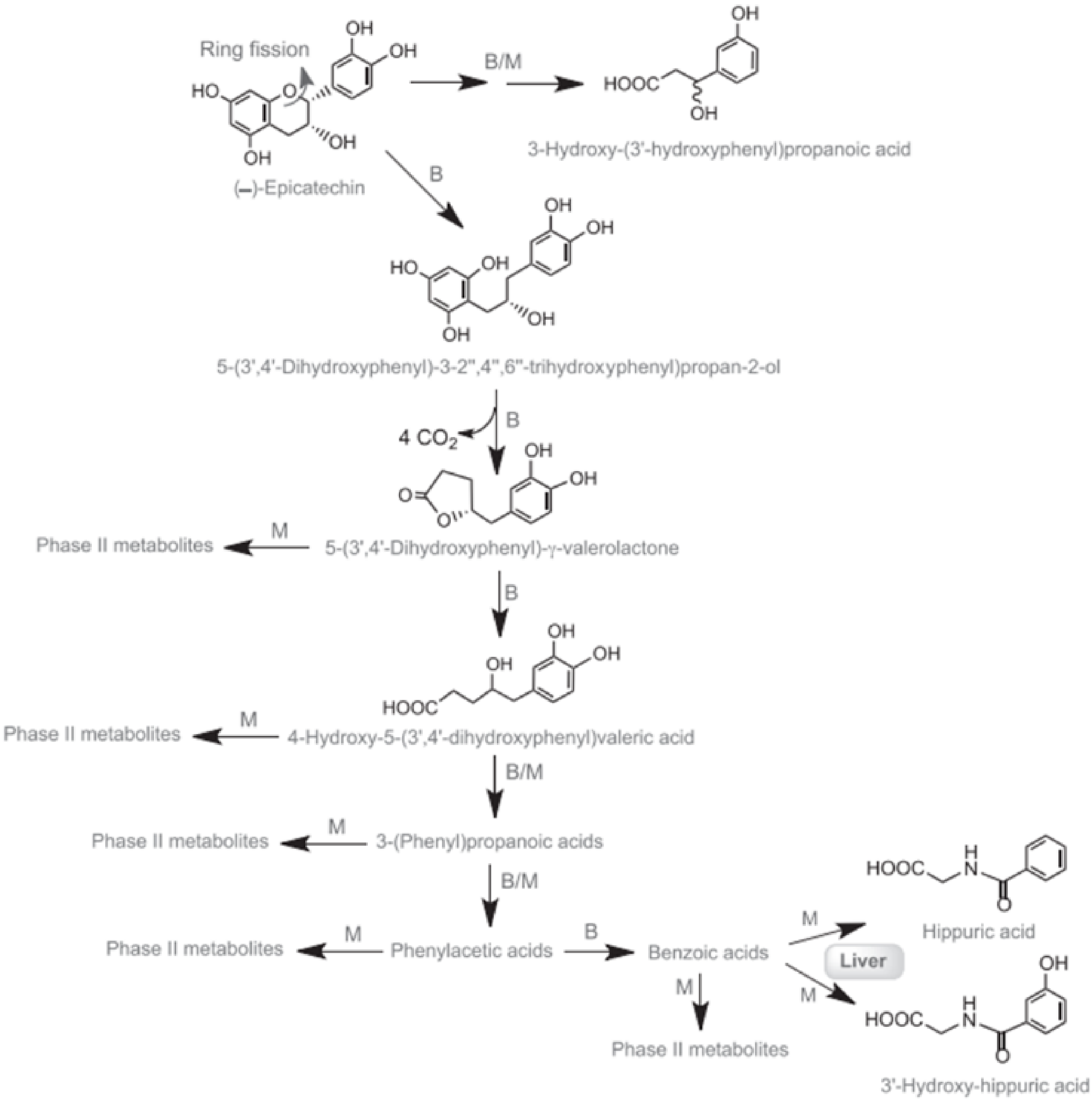
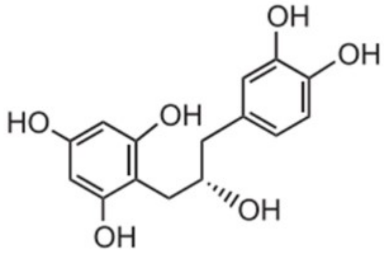


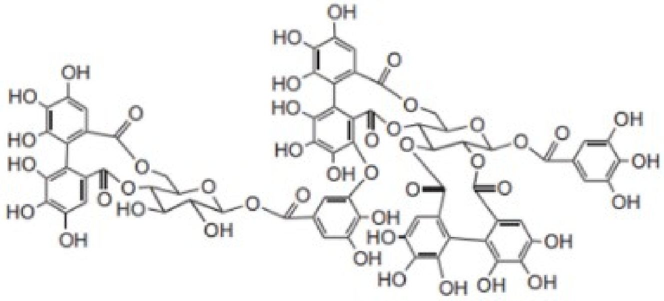
Figure 2.6 Proposed routes for the metabolism of (−)-epicatechin passing from the small to the large intestine. M – steps catalyzed by mammalian enzymes. B – steps probably catalyzed by bacterial enzymes. M/B – conversions that could involve mammalian and/or bacterial enzymes. There is evidence that tail units of proanthocyanidins, like (−)-epicatechin, are converted to 5-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-tri-hydroxyphenyl)propan-2-ol.



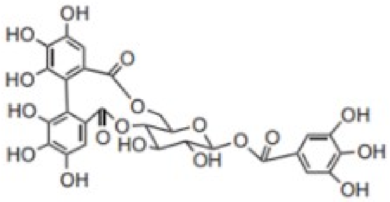
5-(3',4'-Dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)propan-2-ol **43**

2.2.3 Ellagitannins

Studies on the ADME of **11** acid and ETs with berries have focussed principally on strawberries and raspberries.[31,66,67,69,93,104,105] Both types of berries contain sanguiin H-6 **12** and ellagic acid, both as the aglycone and conjugated with a sugar. Raspberries also contain sanguiin H-10 **44** and lambertianin C **10**,[66] and strawberries strictinin **45**,[93] casuarictin **46** and agrimoniin **47**.[106] Much of the non-berry research on the bioavailability of ETs has involved pomegranate juice, which is rich in ETs, including punicalagin **48**.[107,108]

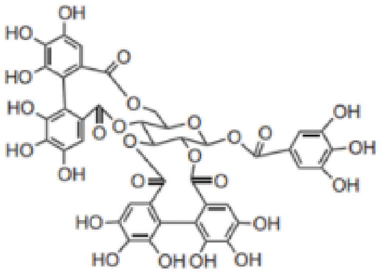


Sanguiin H-10 **44**

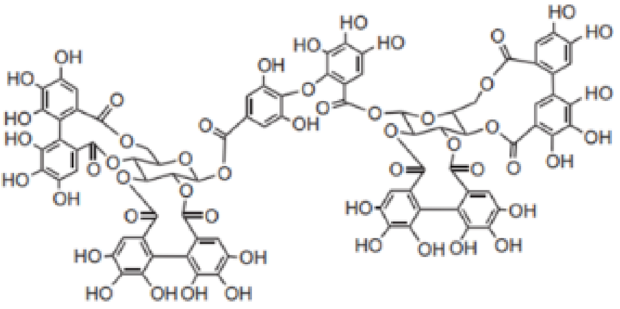


Strictinin **45**

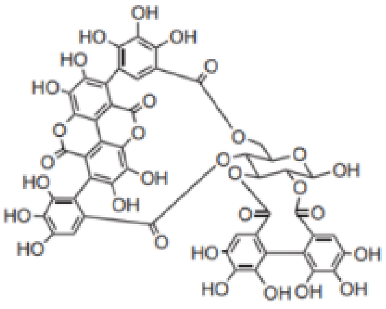
When ingested, ETs remain stable in the stomach and are either hydrolyzed in the small intestine due to pH changes or remain in their intact form. ETs and ellagic acid are poorly absorbed in the small intestine and reach the colon, as observed in a parallel intervention study with healthy participants and ileostomists who ingested 300 g of raspberries.[66] The 0–48 h ileal fluids contained only sanguiin H-6 **12** of the three ETs identified in raspberries, with a recovery corresponding to the 23 % of intake. Ellagic acid recovery was 241 % of intake, indicating ET hydrolysis in the upper GI tract.[66] No ETs and ellagic acid or ellagic acid glycosides were detected in plasma samples of healthy subjects,[66] an observation confirmed by Mosele *et al.*[93] However, in pomegranate juice intervention studies with healthy volunteers with an intact colon, ETs did not accumulate in plasma, although nmol L^{-1} levels of free ellagic acid were detected 1 h post-ingestion.[107,108] Similar findings were recently obtained in a trial that correlated ET metabolites from raspberries with improvements in endothelial function.[109] These results suggest low-level absorption of ellagic acid, either from the dietary source *per se* or released from ETs or ellagic acid glycosides, in the upper bowel. Ellagic acid appears in the circulatory system mainly as the free acid,[108] but it can also undergo phase II metabolism: ellagic acid-glucuronide, dimethylellagic acid (DMEA) and DMEA-glucuronide have been detected in plasma in nmol L^{-1} concentrations and in urine in mmol amount.[66,67,93,107,108] Ellagic acid and ETs reaching the large intestine are substrates for the colonic microbiota-mediated conversion into urolithins (6*H*-di- benzo[*b*,*d*]pyran-6-one).



Casuarictin **46**



Agrimoniin **47**



Punicalagin **48**

Firstly, ETs are hydrolyzed into ellagic acid by tannin-hydrolase.[110] *Ex vivo* fecal incubations suggest that the conversion of ETs to ellagic acid may be the rate-limiting step in the formation of urolithins.[69] Ellagic acid undergoes lactone-ring cleavage and decarboxylation, yielding an intermediate 3,4,8,9,10-pentahydroxy-urolithin (urolithin M5). Subsequent dehydroxylation reactions occur, generating tetrahydroxy- urolithins, namely 3,4,8,10-tetrahydroxy-urolithin (urolithin E), 3,8,9,10-tetrahydroxy-urolithin (urolithin M6) and 3,4,8,9-tetrahydroxy-urolithin (urolithin D), trihydroxy-urolithins, namely 3,8,10-trihydroxy-urolithin (urolithin M7) and 3,8,9-trihydroxy-urolithin (urolithin C) and finally dihydroxy-urolithins, namely 3,8-dihydroxy-urolithin (urolithin A) and its isomer 3,9-dihydroxy-urolithin (isourolithin A) and 3-hydroxy-urolithin (urolithin B) (Figure 2.7).[110] Urolithin production from ETs and ellagic acid appears to be linked to individual gut microbiota composition, although there is limited information on the bacterial species that are involved. Currently, bacteria species from human feces, belonging to the *Coriobacteriaceae*, *Gordonibacter urolithinfaciens* and *Gordonibacter pamelaeae*, have been shown to carry out the first catabolic steps resulting in 3,8,9-trihydroxy-urolithin formation.[110] Other microbial species and environmental conditions leading to di- and monohydroxy-urolithins remain to be unraveled. The progressive dehydroxylation steps from penta- to di- and monohydroxy-urolithins lead to an increase in lipophilicity and consequently an increase in absorption. Typically, 3,8-dihydroxy-urolithin, 3,9- dihydroxy-urolithin and 3-hydroxy-urolithin are the main urolithins found inhuman plasma and urine, as phase II glucuronide metabolites.[110] These metabolites first appear in human plasma ~8 h after ET and ellagic acid ingestion and reach C_{max} after 48–72 h.[48,67] Urinary excretion starts ~16 h post-ingestion of intake and can continue for a further 4 days.[31,104] In a recent review, Tomás-Barberán and colleagues estimated that the main urolithins attain C_{max} in human plasma in the µmol L^{-1} range (0.024–35 µmol L^{-1} and 0.0045–0.745 µmol L^{-1} for 3,8-dihydroxy-urolithin and for 3,9-dihydroxy-urolithin glucuronides, respectively, and 0.012–7.3 µmol L^{-1} for urolithin-3-glucuronide).[110] Lower levels, around 0.005 µmol L^{-1}, of free urolithins have also been detected in plasma usually when analysis has made use of uHPLC–HRMS techniques with low limits of detection.[104,110] Other minor metabolites identified and quantified in plasma and urine include a hydroxy-urolithin-sulfate, a urolithin-sulfoglucuronide and a urolithin-sulfate.[67,109,110] The highest urinary recovery after berry consumption was describedby Truchado and colleagues,[104] who reported a mean 92-h urinary excretionof urolithin conjugates of 58± 48% of the administered 62 mg of ellagic acid in a strawberry supplement. In a previous study, Cerdá and colleagues observed lower-mean recovery of ETs in 0–56 h urine, equal to 2.8±4.4% for strawberry, 3.4± 4.4% for raspberry, 6.5± 5.7% for oak-aged red wine and 16.6±28% for walnuts.[31] In agreement with this study, recoveries of urolithins in 0–48 h urine after raspberry consumption by ten individual volunteers ranged from 0 to 8.6% of ellagic acid and ET intake.[66] The effect of ellagic acid/ET ratio on urolithin production is controversial. González-Sarrías *et al.* showed that higher free ellagic acid promotes higher urolithin production after the consumption of two pomegranate extracts containing different ellagic acid/ETs ratios.[108] This is in keeping with the fecal incubations of González-Barrio *et al.*, which showed that ellagic acid was converted to urolithins more readily than ETs.[69] In contrast, Truchado and colleagues reported that urolithin production was not affected by an increase in ellagic acid/ETs ratio promoted by thermal processing.[104] This, however, could be a matrix effect as urolithin excretion was compared after the ingestion of fresh strawberries and a strawberry purée. A further complicating factor could be the release of ETs from achenes in the processed purée. The analysis of human feces has unveiled relatively low but variable concentrations of ETs, ellagic acid and urolithin aglycones.[48,110]

Tomás-Barberán and colleagues reported fecal concentrations of unconjugated urolithins in the range of 7–7180 mg g^{-1} for 3,8-dihydroxy-urolithin, 13–1894 mg g^{-1} for 3-hydroxy-urolithin and 36–207 mg g^{-1} for 3,9-dihydroxy- urolithin.[110] This is in line with the variable conversions of ellagic acid and pomegranate punicalagin **48** to urolithins by fecal samples from different subjects reported by González-Barrio *et al.*[69] It is clear that there is a large inter-individual variability (IIV) with urolithin production, and this has been associated with varying microbiota compositions. This variability is not only quantitative with low vs. high producers[31] but also qualitative with more subjects excreting 3,8-dihydroxy-urolithin than 3-hydroxy-urolithin derivatives,[66,69] giving birth to the concept of metabolic phenotypes, which is discussed further in Section 2.3.[111]

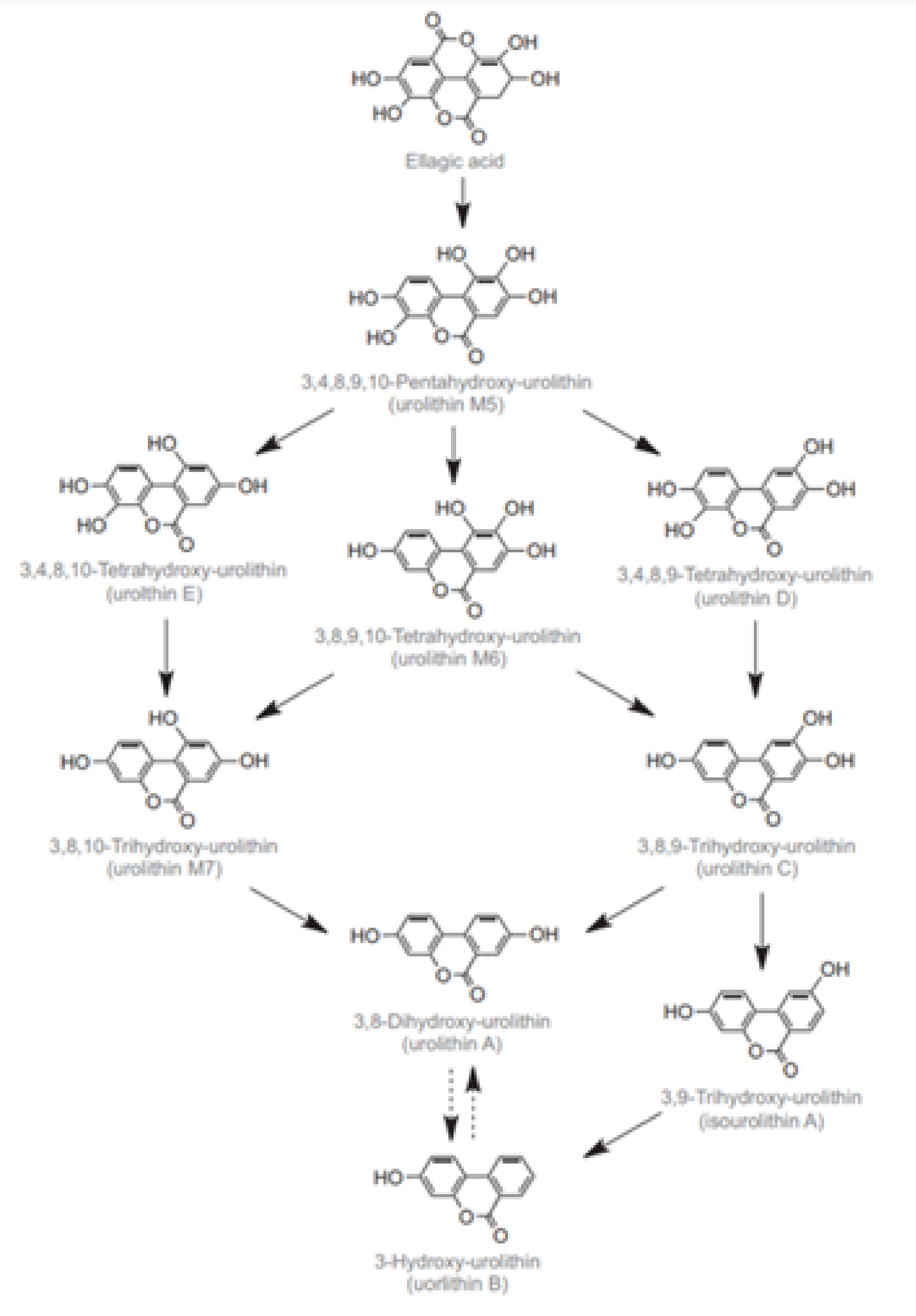
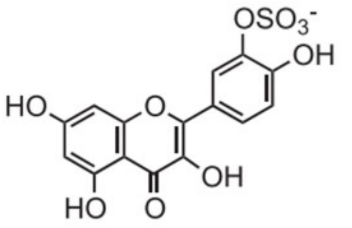


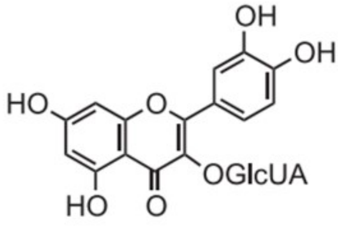
Figure 2.7 In the colon, ellagitannins (ETs) are converted to ellagic acid, whichthe resident microbiota can convert to urolithins by the routes illustrated

2.2.4 Flavonols

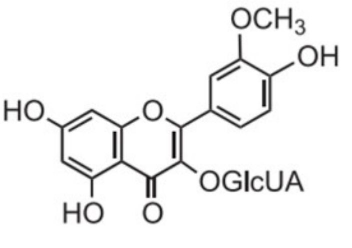
Flavonol glycosides of quercetin **7**, myricetin **8**, kaempferol **9** and isorhamnetin **10** occur widely throughout the plant kingdom.[7,34] Feeding with onions (where about 200 g of fried onions were consumed) has shown that quercetin-*O*-glucosides are bioavailable with the aglycone being absorbed inthe small intestine and appearing in the bloodstream as glucuronide, sulfate and methyl metabolites, the main ones being quercetin-3′-sulfate **49** and quercetin-3-glucuronide **50**.[112,113] Urinary excretion 0–24 h after intake was equivalent to 4.7% of the consumed flavonol glucosides. In contrast, the disaccharide, quercetin-3-*O*-rutinoside (rutin) in tomatoes, passes to the distal GI tract where it is deglycosylated by microbial enzymes and appears in plasma in ~500-fold lower concentrations than after quercetin glucoside intake, as quercetin 3-glucuronide and isorhamnetin-3-glucuronide **51**.[45] Most of the quercetin released in the colon undergoes microbiota-mediated ring fission giving rise to phenylpropanoic, phenylacetic and hippuric acids, which were excreted in urine in amounts equivalent to 23% of flavonol intake.[45] A subsequent study with fecal incubations showed that the quercetin catabolites were further degraded to benzoic acids and hydroxybenzenederivatives.[114]



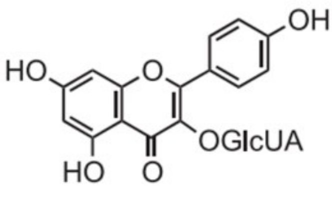
Quercetin-3'-sulfate **49**



Quercetin-3-glucuronide **50**



Isorhamnetin-3-glucuronide **51**



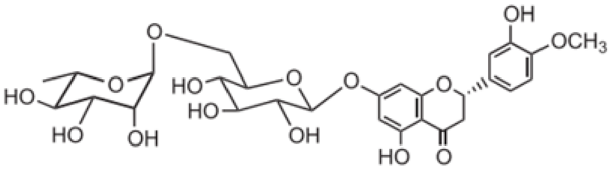
Kaempferol-3-glucuronide **52**

While flavonols do occur in berries, they are not major constituents and are present along with high concentrations of other (poly)phenols.[115,116] For instance, a wild blueberry drink used in an acute bioavailability study contained 302 mg of total (poly)phenols, of which 31 mg were flavonols,[95] consisting of a mixture of myricetin and quercetin glycosides.[116] Also present were 150 mg anthocyanins, 64 mg caffeoylquinic esters, 52 mg of PACs, 4 mg of flavan-3-ol monomers and 1 mg of benzoic and cinnamic acids.[95] At baseline, prior to intake, plasma contained 3±1 nmol L^{-1} of quercetin-3-glucuronide, which after 2 h increased significantly to 5±1 nmol L^{-1}. Substantial amounts of an assortment of (poly)phenolic compounds were also detected in plasma 2 h after blueberry intake, but most were present prior to supplementation and likewise with the profile of phenolics excretedin urine. In the circumstances, elucidating which of the diversity of phenolic catabolites originate from what constituents in the drink is not feasiblein the majority of instances. The one exception was 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate **42**, which can originate from flavan-3-ol monomers and the tail unit of PACs (Section 2.2.2). In a bioavailability study with cranberry juice, volunteers ingested 787 mg of (poly)phenols comprising 710 mg of PACs, 16 mg anthocyanins, 5 mg flavan-3-ol monomers, 31 mg flavonols, quercetin and myricetin aglyconesand glycosides, 5 mg 5-*O*-

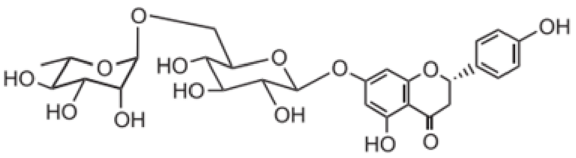
caffeoylquinic acid and 19 mg phenolic acids.[94] Along with low levels of kaempferol **9**, kaempferol-3-glucuronide **52** and quercetin-3-glucuronide, 5-(3'-hydroxyphenyl)-γ-valerolactone-4'-sulfate and numerous phenolics and their phase II metabolites were detected in plasma and urine and a detailed metabolic map was presented summarizing their potential origins.[94] Along with many other (poly)phenols, chokeberries (*A. melanocarpa*) contain flavonol aglycones and quercetin-, kaempferol- and myricetin-*O*-glycosides. After feeding a chokeberry extract to healthy men, analysis of plasma and urine detected low levels of kaempferol-3-glucuronide, quercetin-3-sulfate **49** and quercetin-3-glucuronide **50**.[117]

2.2.5 Other (Poly)phenols

Flavanones, most notably the 7-*O*-rutinosides of hesperetin **53** and naringenin **54** occur predominately in citrus fruits.[7] They are, however, found in very small amounts in chokeberries (*Aronia* spp.).[117,118] The bioavailability of these flavanones, which are absorbed in the distal GI tract as phase II metabolites after microbiota-mediated aglycone release, has been investigated principally with orange juice.[51,119–121] Unsurprisingly, no specific flavanone phase II metabolites were detected in either plasma or urine after ingestion of a 500 mg *A. melanocarpa* extract that contained only 5 nmol of hesperetinand 20 nmol of naringenin.[117]

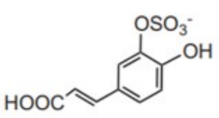


Hesperetin-7-*O*-rutinoside **53**

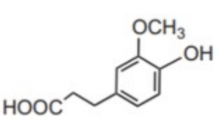


Naringenin-7-*O*-rutinoside **54**

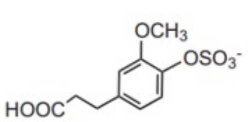
The majority of studies on the bioavailability of 5-*O*-caffeoylquinic acid **14** have used coffee beverages that contain especially high levels of chlorogenic acid together with smaller amounts of other acyl quinic acid esters.[7] The 3C-RFMs that accumulate transiently in plasma after ingestion of 412 µmol of chlorogenic acids include 3'-methoxycinnamic acid-4'-sulfate **26**, 4'-hydroxycinnamic acid-3'-sulfate **55** (caffeic acid-3'-sulfate), 3-(4'-hydroxy-3'-methoxyphenyl)propanoic acid **56** (dihydroferulic acid) and 3-(3'-methoxy-phenyl)propanoic acid-4'-sulfate **57** (dihydroferulic acid-4'-sulfate). 5-*O*-Caffeoylquinic acid and related acyl-quinic acids have been detected in low amounts in a number of berry species.[34] The exceptions, which contain substantial amounts, are wild blueberry and highbush blueberry (*V. corymbosum*). In the study where a wild blueberry drink was consumed, as mentioned in Section 2.2.4, the supplement contained 64 mg (181 mmol) of chlorogenic acids per 100 g FW of berries.[95]



4'-Hydroxycinnamic acid-3'-sulfate **55**



3-(4'-Hydroxy-3'-methoxyphenyl)propanoic acid **56**

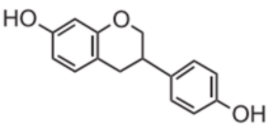


3-(3'-Methoxyphenyl)propanoic acid-4'-sulfate **57**

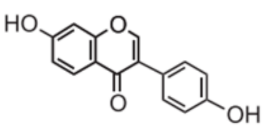
Post-intake, numerous phenolic compounds were detected and quantified in plasma and urine, including 3C-RFMs, that could be catabolites of the ingested chlorogenic acids. However, many of these compounds were also detected in plasma and urine after ingestion of cranberries which contained much lower amounts of chlorogenic acids.[94] Ascertaining their origins is, therefore, not feasible, especially as they could also be degradation products of other (poly)phenols,including anthocyanins, flavan-3-ols and flavonols. There have been a few dietary studies on the bioavailability of the stilbene *trans*-resveratrol **23**, principally because it is a very minor component in the human diet and is found, at best, in trace levels in berries. The findings of ADME studies that have been carried out with stilbenes, none of which involved berries, have been reviewed by Vitaglione *et al.*[123]

2.3 Inter-individual Variability in the Metabolism of Berry (Poly)phenolics

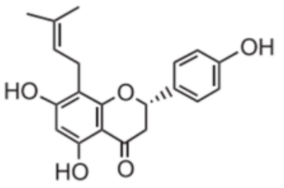
The beneficial effects of (poly)phenol-rich foods on the promotion of cardio-metabolic health have been widely demonstrated on some key risk factors.[124–128]Nevertheless, despite this large body of evidence, the role of (poly)phenols incardiometabolic protection has, as yet, not been demonstrated consistently.[129,130] This could be due to the heterogeneity in individuals' response to theconsumption of dietary (poly)phenols.[130,131] However, this IIV and its impact on physiological outcomes have, as yet, not been investigated in depth.[132] IIV in the production of metabolites has been observed with many bioactives, including ETs, flavanones, (−)-epicatechin and ETs.[83,108,117] Some factors, including sex, age and dietary habits, may influence (poly)phenol ADME. However, due to the colonic metabolism of a large fraction of ingested (poly)phenols, the microbial population in the lower bowel isarguably the most important factor modulating IIV.[131] Particularly well-established examples of IIV are the formation of urolithins from ETs, equol **58** from the isoflavone daidzein **59** and 8-prenylnaringenin **60**, a hop prenylflavonoid, for which metabolic phenotypes (*aka* metabotypes) with different microbial functionalities have been proposed.[111,133,134] Metabotypes have been proposed for the conversion of flavan-3-ols to 5C- RFMs.[135] Three phenotypes have been observed with urolithins independent of volunteers' health status and demographic characteristics, including age, gender and body mass index. A higher percentage of 3-hydroxy-urolithin **61** producers was observed in volunteers with chronic illness associated with gut microbial imbalance (dysbiosis).[111] This is in keeping with the proposed role of microbial composition in inter-individual differences and highlightsthe need for a new generation of intervention studies designed to captureevents underlying this and other IIVs.[136]



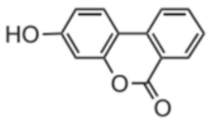
Equol **58**



Daidzein **59**



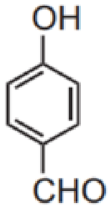
8-Prenylnaringenin **60**



3-Hydroxy-urolithin **61**

IIV in the metabolism of berry (poly)phenols began with studies on bioavailability with raspberry and strawberry, which identified high and low 3-hydroxy-urolithin producers from ET and ellagic acid.[31,66,118,137]

After strawberry consumption, the excretion of 3-hydroxy-urolithin ranged from 0.05 to 6.3% and from 0.21 to 7.6% after raspberry ingestion.[31] Feliciano *et al.* assessed (poly)phenol IIV after blueberry consumption.[95] 1,2-Dihydroxybenzene **29** derivatives and benzoic acids showed the lowest IIV in plasma (36%–58% and 50%–63%, respectively), whereas benzaldehydes, phenylacetic acids and benzoic acids were excreted with more uniformity (47%–69%, 50%–54% and 40%–71%, respectively). Phenylpropanoic acids, 1,2,3-trihydroxybenzene **31** and 5C-RFMs in plasma and urine exhibited IIV. The IIV for the sum of (poly)phenol catabolites was marginally lower in plasma (40%–48%) than urine (47%–54%). In general, bioavailability pharmacokinetic parameters are expressed as mean or median values accompanied by figures for standard deviation or standard error. Recently, the coefficient of variation (CV or CV%) has begun to be used in relation to IIV. The CV of the plasma area under the curve (AUC) of total (poly)phenol metabolites quantified after consumption of cranberry juice was 53% and the CV for C_{max} was 51%. The CV for the AUC varied between 48% for 4-hydroxybenzaldehyde **62** and 163% for a 1-methoxy-hydroxybenzene-sulfate. The CV for C_{max} varied between 43% for 3-(3'-methoxyphenyl)propanoic acid-4'-sulfate **57** and 216% for 4-hydroxy-3- methoxybenzoic acid **22**.[138]



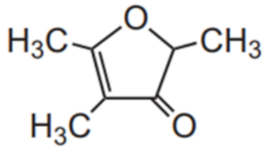
4-Hydroxybenzaldehyde **62**

The targeted analysis of 5C-RFMs after consumption of increasing doses of flavan-3-ols in cranberry juice highlighted a high variability among subjects. CV for plasma C_{max} ranged from 32% to 297%, similar to those reported for the 0–24 h AUC (32% to 277%). CV calculated for 24 h-cumulative urinary excretion for the sum of 5C-RFMs varied from 138% to 289% depending on the treatment.[92]

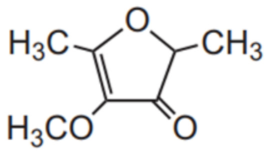
2.4 Biomarkers of Berry Intake and Future Research

In recent years, biomarkers of food intake have attracted interest as apromising tool to objectively assess the consumption of certain food items orfood groups. In order to be validated and defined as such, candidate biomarkers of food intake must meet certain criteria.[139] In particular, a biomarker of intake has to be specific to a particular food and be detectable in plasma or urine in a manner reflective of intake. Berries contain high levels of micronutrients and fibers and are among the richest sources of (poly)phenolic compounds, none of which are exclusive to berries, whether considering individual berry species or berries as a group.[140,141] Urolithins are produced specifically from ETs and ellagic acid (Section 2.2.2), which unfortunately are not exclusive to berries. The same applies to flavan-3-ol-derived 5C-RFMs, which, while biomarkers of flavan-3-ol intake, are not specific biomarkers of berry consumption. In this more restrictive role, urinary SREMs and 5C-RFMs were used as biomarkers of flavan- 3-ol intake in a study involving the analysis of urine from about 25 000 participants in the EPIC Norfolk study.[142] This revealed that high flavan-3-ol intake from tea, pome fruits, berries, cocoa-derived products and nuts was associated with significantly lower systolic blood pressure in men andwomen.

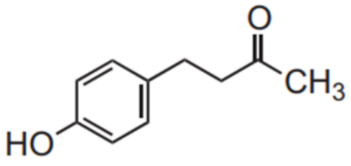
Furthermore, hypertensive participants had a stronger inverse association between the biomarkers and systolic blood pressure compared to normotensive participants. Flavan-3-ol intake could, therefore, have a role inthe maintenance of cardiovascular health on a population scale. In a recent review, aroma compound metabolites have been proposed as potential biomarkers of intake of specific berries, including furaneol **63** and mesifurane **64** phase II metabolites of strawberry and 4-(4'-hydroxyphenyl)-2-butanone **65** metabolites of raspberry.[141] However, furaneol is found in tropical fruits such as kiwi and pineapple and is also a product of Maillard reactions produced during cooking. To further compound matters, 4-(4'-hydroxyphenyl)-2-butanone is being used increasingly as a food additive and sois found in many aromatized foods and, up to now, its bioavailability and metabolism in humans have not been investigated.[141] It has been suggested that these compounds, in combination with other metabolites, such as pelargonidin metabolites and/or urolithins, could be used as part of a multi- metabolite biomarker panel.[143,144] This approach to predict intake would appear to be more appropriate than focussing on a single biomarker.[141] However, much research remains to be carried out to validate potential bio- markers and evaluate their applicability. More untargeted metabolomics studies aimed at discovering specific biomarkers of berry intake are requiredand efforts need to be devoted to identifying possible discriminating metab- olites, since this step remains the major bottleneck of biomarker research.



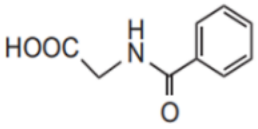
Furaneol **63**



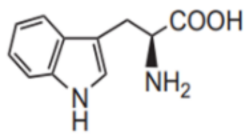
mesifurane **64**



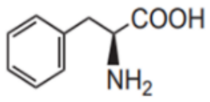
4-(4'-Hydroxyphenyl)-2-butanone **65**



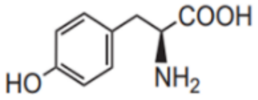
Hippuric acid **66**



Tryptophan **67**



Phenylalanine **68**



Tyrosine **69**

As outlined in Section 2.2, with the exception of the 5C-RFMs andurolithins, most 2/3C-RFMs, benzoic acid and benzene derivatives are colonic degradation products that can be derived from many (poly)phenols. To further complicate matters, some of these catabolites, such as hippuric acid **66**, are produced by the body independent of dietary intake, from benzoic acid **32**, tryptophan **67**, phenylalanine **68** and tyrosine **69**.[145–148] Determining to what degree individual catabolites are derived from the (poly)phenol intake is, therefore, less than straightforward. Clarification could be achieved using ^{13}C-labeled (poly)phenols in ADME studies. Indeed,as noted in Section 2.2.1, this approach has been used by Czank *et al.* and de Ferrars *et al.*, who fed [6,8,10,3',5'-$^{13}C_5$] cyanidin-3-*O*-glucoside (Figure 2.5) to human volunteers.[71,72] The particular advantage of using the $^{13}C_5$-labeled cyanidin-3-*O*-glucoside was that it was possible to determine what proportion of individual metabolites/catabolites were endogenous as opposed to originating from the labeled anthocyanin.

Furthermore, as the cyanidin-3-*O*-glucoside had three ^{13}C molecules incorporated into the A-ring and two intothe B-ring, it was also possible to ascertain whether metabolites/catabolites detected in plasma and urine were derived from the A- or B-ring of cyanidin.

2.5 Concluding Remarks

In conclusion, berries represent important dietary sources of many types of (poly)phenols, which following ingestions initially undergo a variety of patterns metabolism in enterocytes of the small intestine prior to absorption into the circulatory system. (Poly)phenols not absorbed in the upper GI tract enter the colon where microbiota-mediated breakdown yields a variety of potentially bioactive phenolics that can undergo phase II metabolism in colonocytes prior to absorption into the bloodstream, which can be followed by further modification by hepatocytes. For many years, it was believed that dietary (poly)phenols were poorly absorbed; however, it is now realized that when their diverse metabolites and catabolites are taken into account,they are highly bioavailable and yield a diversity of potentially bioactive phenolic compounds. Elucidating the pathways involved in these events represents a major analytical challenge, and progress is being made on a number of fronts with acute and chronic feeding studies and *in vitro* and *ex vivo* model test systems coupled with major refinements in analytical methodology. Key biomarkers of intake, including phase II metabolites of 5-(phenyl)-γ-valerolactones and urolithins, are beginning to be identified. Despite some gaps, due to inaccurate quantification of (poly)phenol metabolites, new insights into the colonic fate of ingested berry (poly)phenols and metabolomic approaches may eventually be useful in accurately determining levels of consumption. However, IIV may represent a challenge that needs to be taken into account when evaluating the beneficial effects of (poly)phenol-rich berries, and other plant-based foods, on the promotion of cardiometabolic health.

Abbreviations

5C-RFMs	5C-ring fission metabolites
3C-RFMs	3C-ring fission metabolites
2C-RFMs	2C-ring fission metabolites
ADME	absorption, disposition, metabolism and excretion
AUC	area under the curve
CBG	cytosolic β-glucosidase
C_{max}	maximum concentration
CV	coefficient of variation
DMAC	4-(dimethylamino)cinnamaldehyde
DMEA	dimethylellagic acid
DP	degree of polymerization
DW	dry weight
ETs	ellagitannins
FW	fresh weight
GI	gastrointestinal
HHDP	hexahydroxydiphenoyl

HILIC	hydrophobic interaction chromatography
HRMS	high-resolution mass spectrometry
IIV	Inter-individual variability
LPH	lactase phlorizin hydrolase
PACs	proanthocyanidins
SREMs	structurally related (−)-epicatechin metabolites
T_{max}	time to reach C_{max}
uHPLC	ultra-high-performance liquid chromatography

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1.3.2 Human colonic catabolism of dietary flavan-3-ol bioactives

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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.

Keywords: Flavan-3-ols; Colonic catabolic and phase II metabolism; [2–^{14}C](−)-Epicatechin; Procyanidins; Theaflavins

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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).

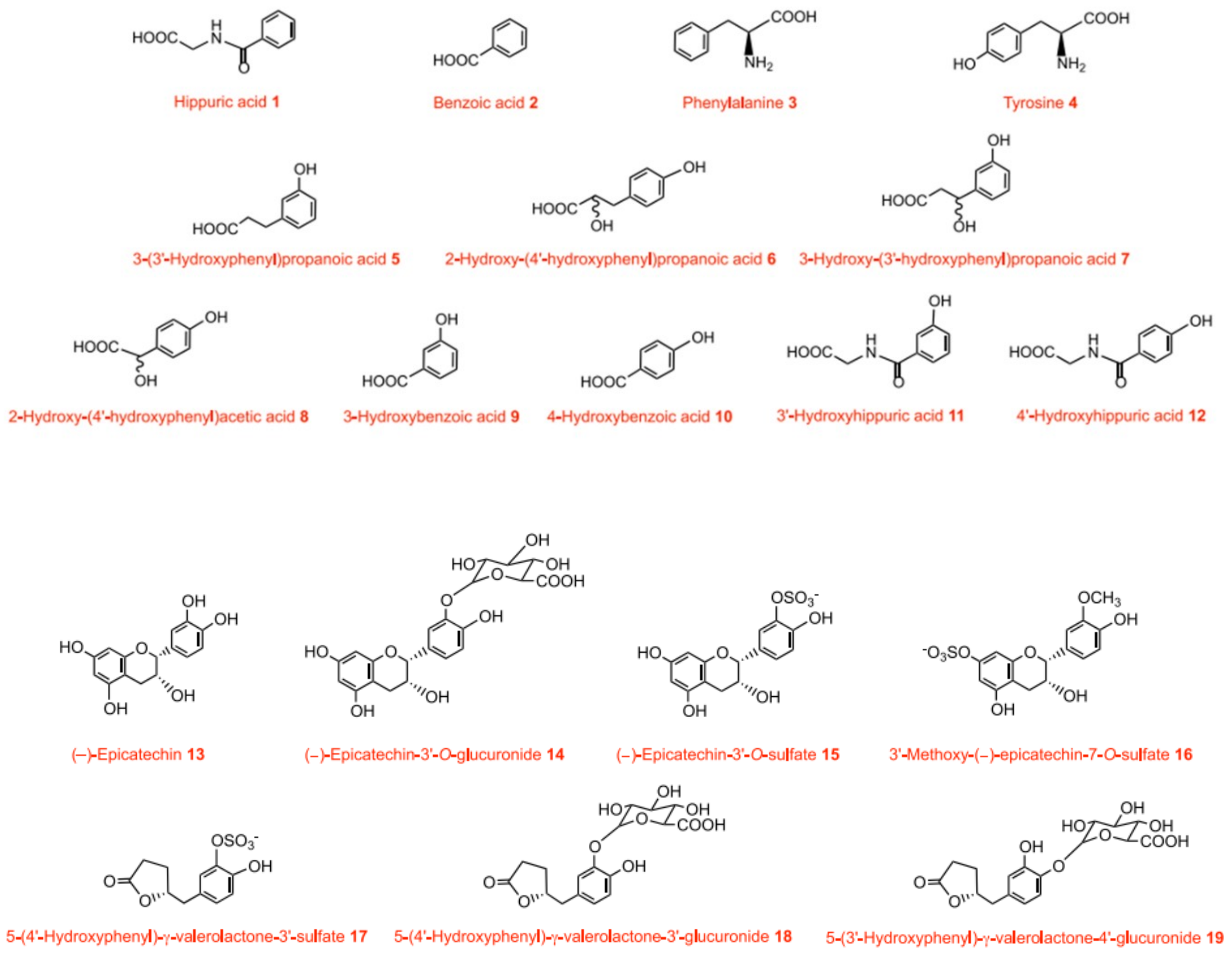
Nonetheless polyphenol glycosides and gallates are not fully absorbed in the upper GI tract and, together with substantial amounts of those conjugated with disaccharides, pass from the small intestine to colon (Stalmach et al., 2010; Borges et al., 2013) where they are subjected to the action of the 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 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. 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). 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 (5CRFCs) 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 (C4) 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 ^{3}C-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.

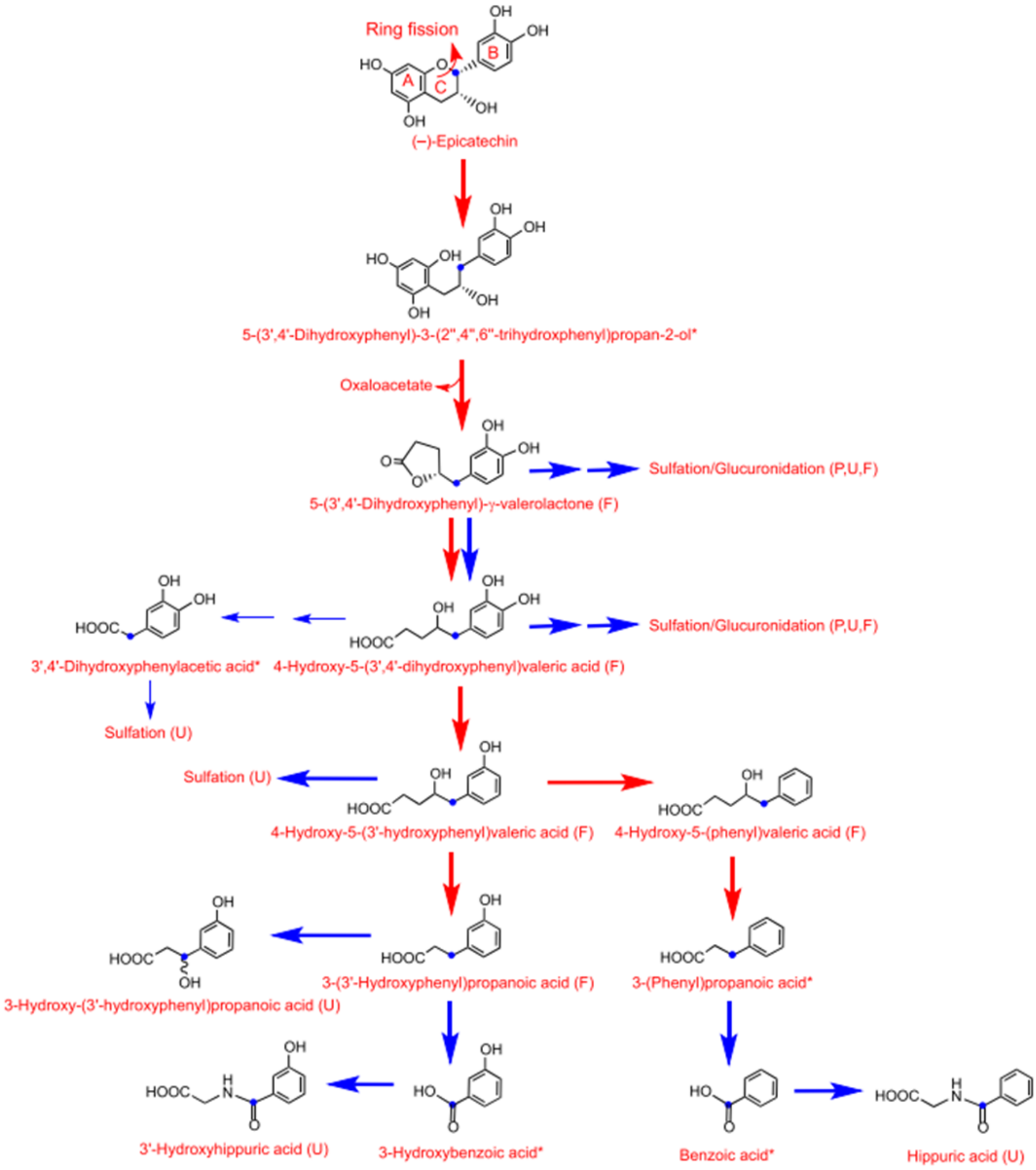


Fig. 1. Proposed pathways for the catabolism by colonic microbiota of [2–^{14}C](−)-epicatechin passing from the small to the large intestine (red arrows), and potential steps catalysed by mammalian enzymes in colonocytes and/or hepatocytes (blue arrows). Thin arrows indicate minor routes. Location of 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.)

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 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).

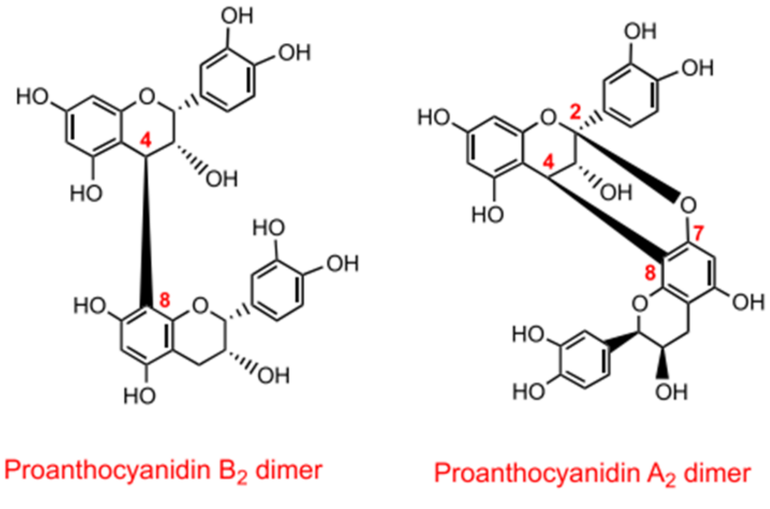


Fig. 2. Structures of A- and B-type proanthocyanidins.

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 A_2, 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 A_2 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; Bartolom´e 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.

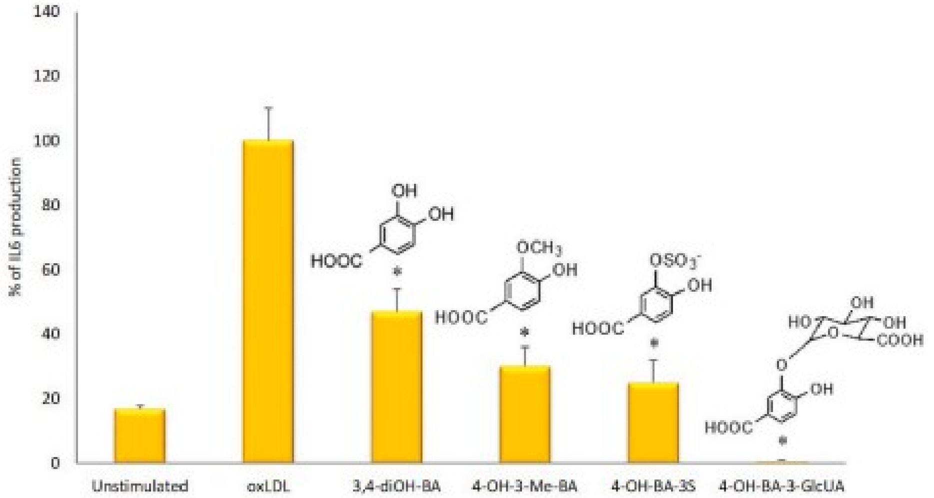
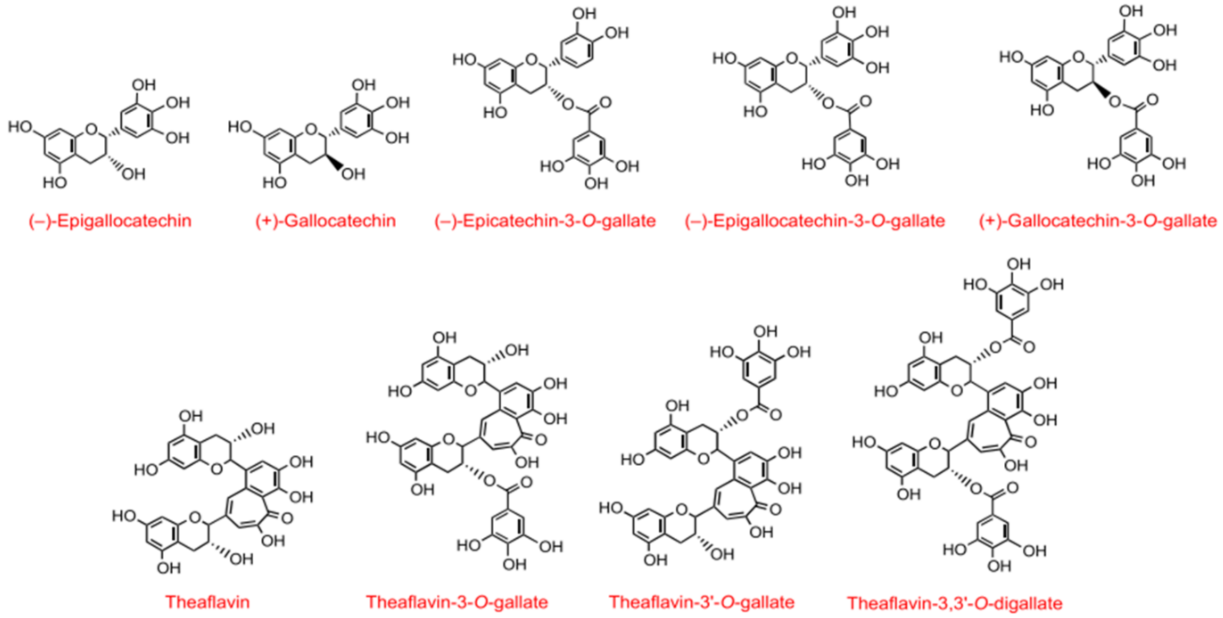


Fig. 3. Oxidised LDL (Ox-LDL) induced IL6 production. Data expressed as % relative to OxLDL treated endothelial cells. * Significant relative to oxLDLtreated cells, $p < 0.05$, ANOVA with Tukey post-hoc *t*-test, n = 3. 3,4-dihydroxybenzoic acid (3,4-diOHBA), 3-methoxy-4-hydroxybenzoic acid (4-OH-3-Me-BA), 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).

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 department 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 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 5CRFCs 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.



(–)-Epigallocatechin

(+)-Gallocatechin

(–)-Epicatechin-3-*O*-gallate

(–)-Epigallocatechin-3-*O*-gallate

(+)-Gallocatechin-3-*O*-gallate

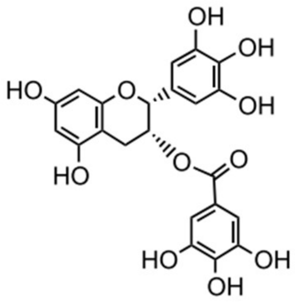
Theaflavin

Theaflavin-3-*O*-gallate

Theaflavin-3'-*O*-gallate

Theaflavin-3,3'-*O*-digallate

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



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

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 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 nongalloylated theaflavins.

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).

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.

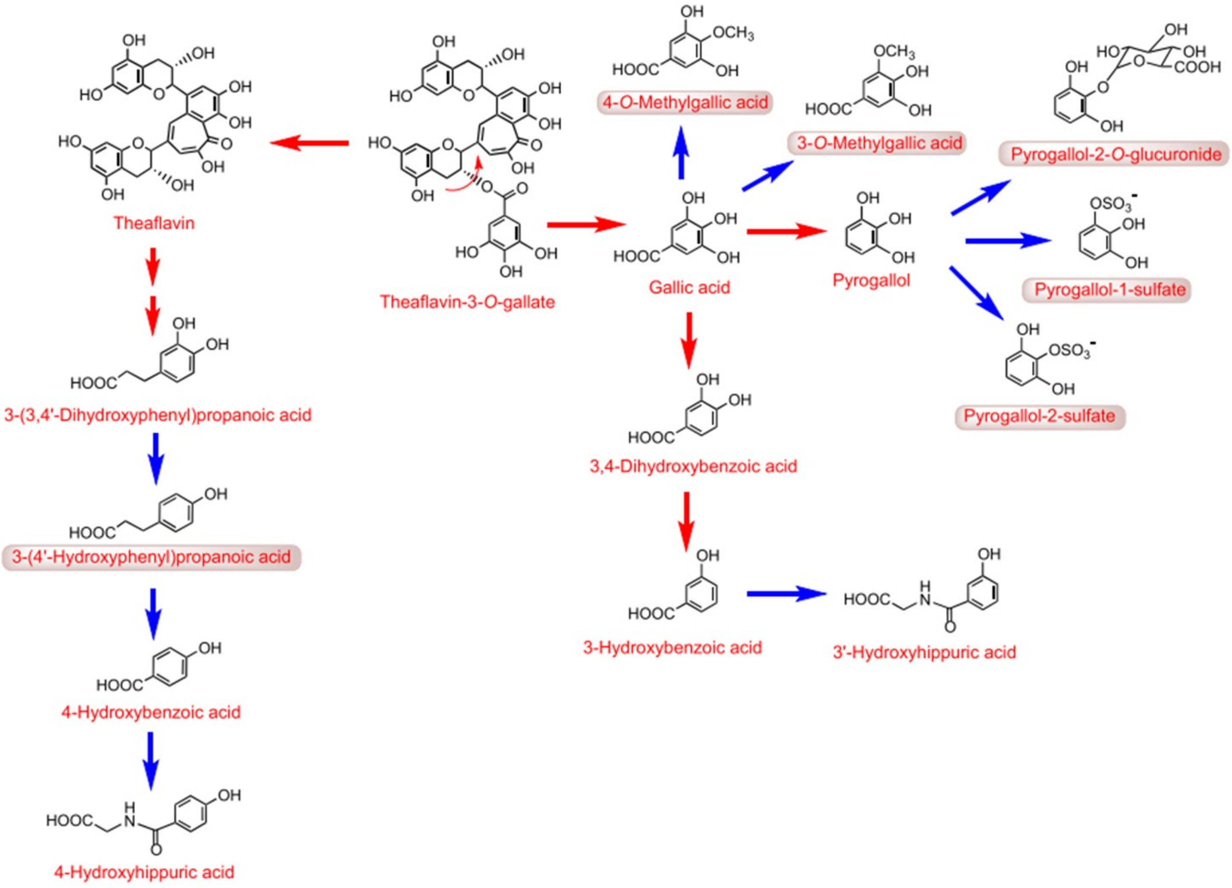


Fig. 5. Proposed principle pathways for the catabolism of theaflavins by colonic microbiota and mammalian phase II metabolism. Red arrows indicate microbiotamediated steps, and blue arrows represent mammalian enzyme-mediated conversions. The illustrated theaflavin is theaflavin-3-*O*-gallate. It assumed 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.)

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.

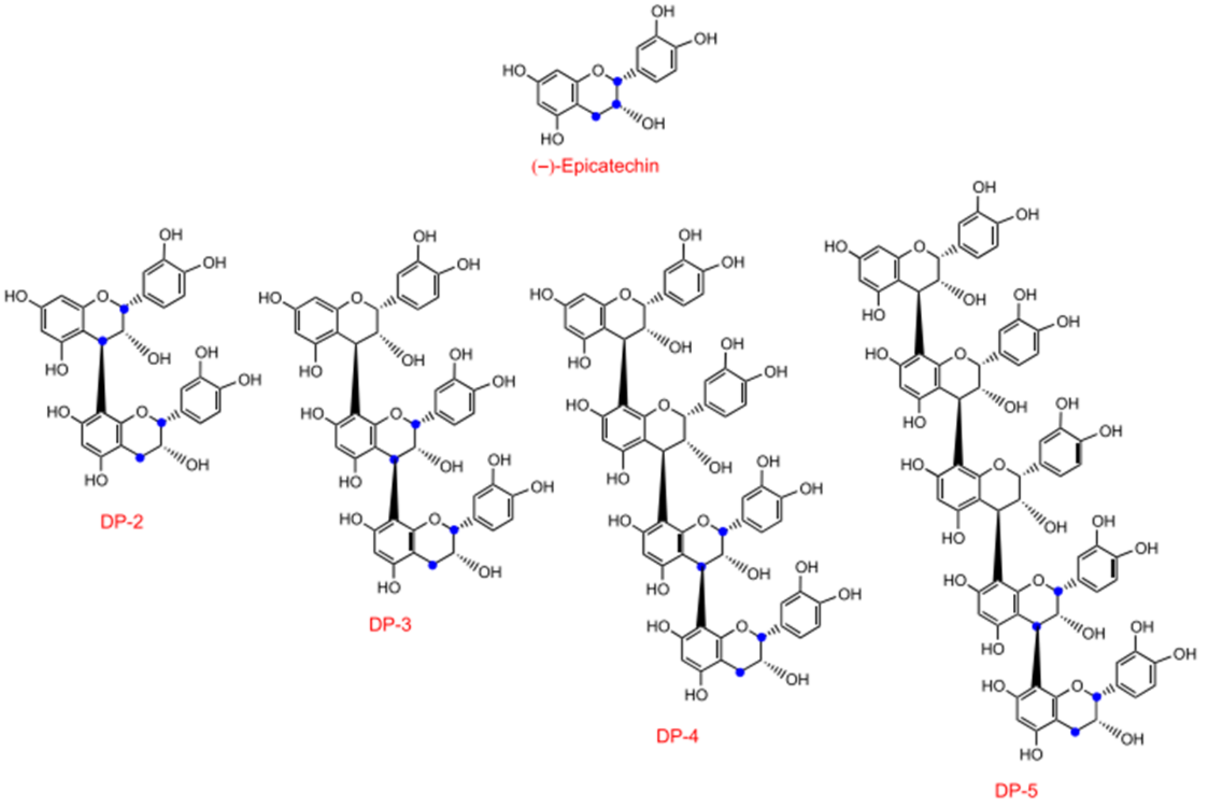


Fig. 6. Structures of ^{13}C-labelled (−)-epicatechin and procyanidins with a degree of polymerisation (DP) of 2–5. Blue circles indicate the positions of ^{13}C label. (After Bussy et al., 2021). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web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

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 DP 2-10 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 DP 2-10 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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2.1 ADME of (poly)phenols and their derived metabolites are key actors for the potential bioactivity recognized to (poly)phenol-rich food intake

2.1.1 Plant-based food intake and health benefits on human health

It has been demonstrated that plant-based diets, which include a wide variety of dietary patterns containing lower amounts of animal source foods (i.e. dairy and meat) and higher amounts of plant-source foods including fruits, vegetables, legumes, nuts, seeds, legumes, and grains (Hemler and Hu, 2019a; Marsh et al., 2012) are:

- able to exert beneficial effects on cardiovascular risk factors (Dinu et al., 2020, 2017; Hemler and Hu, 2019a; Huang et al., 2016; Kottler et al., 2009; Meslier et al., 2020; Sofi et al., 2018b; Tang et al., 2017; Vauzour et al., 2010; Wang et al., 2015; Yokoyama et al., 2014a, 2014b; Zhao et al., 2017);
- associated with a substantially lower risk of developing type-2 diabetes and reducing key diabetes-related macrovascular and microvascular complications (McMacken and Shah, 2017; Satija et al., 2016);
- useful for childhood and/or adult overweight and obesity prevention and treatment (Medawar et al., 2019; Newby, 2009; Turner-Mcgrievy et al., 2017; Turner-McGrievy et al., 2015);
- recommended for cancer prevention besides improving prognosis in cancer survivors (Hardt et al., 2022; Hemler and Hu, 2019b);
- involved in managing risks and complications of chronic kidney disease (Carrero et al., 2020; Joshi et al., 2021) and neuroinflammation (Pistollato and Battino, 2014);
- more sustainable than diets rich in animal products (Lynch et al., 2018; Pimentel and Pimentel, 2003; Sabaté and Soret, 2014).

The potential health benefits of plant-based foods on human health are associated with a lower intake of saturated fat and cholesterol, and with higher levels of dietary fiber, magnesium and potassium, vitamins C and E, folate, bioactive phytochemicals including (poly)phenols, carotenoids, glucosinolates, and alkaloids, and unsaturated fatty acid content (Aydar et al., 2020; Craig and Mangels, 2009; Tuso et al., 2015). Moreover, plant-based diets use fewer natural resources and are less impacting on the environment (Sabaté and Soret, 2014). Studies on individual plant-based foods showed that consumption of some fruits and vegetables is associated with cardiovascular disease protection (Angelino et al., 2019b; Awrence et al., 1997; Gillman et al., 1995; Hu, 2003; John et al., 2002; Joshipura et al., 2001; Knekt et al., 1996, 1994; Liu et al., 2001; Luís et al., 2018; Lupoli et al., 2020), positively affected inflammatory markers (Buscemi et al., 2012; Riso et al., 2014b), improved resistance to oxidative stress (Riso et al., 2014a, 2009, 2005; Sánchez-Moreno et al., 2003) and may reduce risk of type-2 diabetes (Mursu et al., 2014) and cancer (Burton-Freeman and Reimers, 2010; Xu et al., 2014). An inverse association between whole grain intake and risk of type-2 diabetes and colorectal cancer and risk of cardiovascular disease was demonstrated (Jacobs et al., 1998; Liu et al., 2000; Reynolds et al., 2019; Tieri et al., 2020). Some ancient wheat species such as einkorn (*Triticum monococcum*), emmer (*Triticum dicoccum*), khorasan (*Triticum turgidum ssp. turanicum*) and spelt (*Triticum spelta*) showed exhaustive effects on lipid and glycaemic profiles, and inflammatory and oxidative status *in vivo* (Dinu et al., 2018; Sofi et al., 2018a; Whittaker et al., 2017) (**Figure 6**).

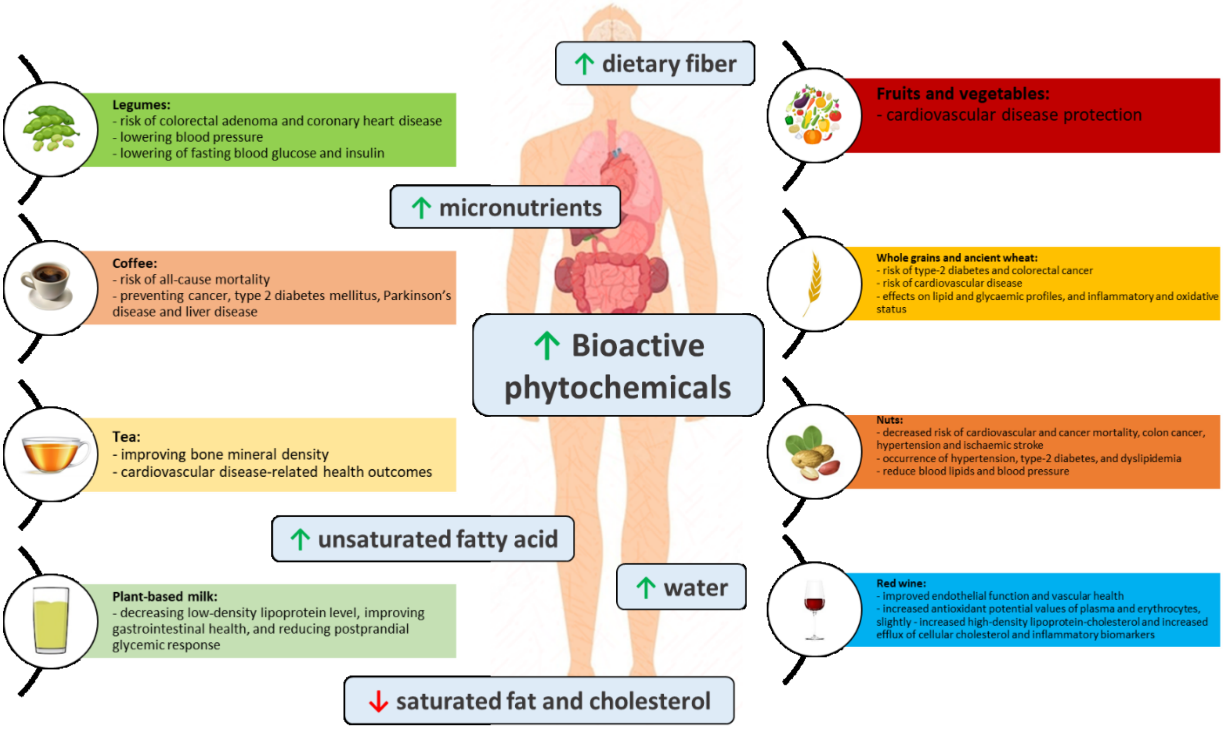


Figure 6. Plant-based food intake and human health.

On the other hand, an umbrella review of observational studies found a decreased risk of cardiovascular and cancer mortality, colon cancer, hypertension, and ischaemic stroke for higher nut consumption (Martini et al., 2021). Nut intake was inversely associated with the occurrence of hypertension, type-2 diabetes, and dyslipidemia in Southern Italian Adults (Micek et al., 2021). On the other hand, a systematic review, meta-analysis, and dose-response of 61 controlled intervention trials showed that tree nut intake lowers total and low-density lipoprotein cholesterol, apolipoprotein B, and triglycerides (Del Gobbo et al., 2015). In another systematic review and meta-analysis of 26 clinical trials with a total of 1059 participants, Guasch-Ferré and colleagues (Guasch-Ferré et al., 2018) demonstrated that incorporating walnuts into the diet improved blood lipid profile without adversely affecting body weight or blood pressure. Pistachios and mixed nuts (including walnuts, almonds, pistachios, cashews, hazelnuts, macadamia nuts, pecans, peanuts, and soy nuts) were found to have a significant reducing effect on diastolic blood pressure (Mohammadifard et al., 2015), while Viguiliouk and colleagues (Viguiliouk et al., 2014) found that tree nuts improved glycemic control in individuals with type-2 diabetes. A possible association with decreased risk of colorectal adenoma and coronary heart disease was found for higher legume consumption (Martini et al., 2021). Intake of beans, peas, chickpeas, and lentils was found to lower blood pressure in a meta-analysis of randomized controlled trials from 554 participants with and without hypertension (Jayalath et al., 2014), while Sievenpiper and colleagues (Sievenpiper et al., 2009) demonstrated that non-oil-seed pulses such as chickpeas, beans, peas, and lentils, alone or in low-glycaemic-index or high-fibre diets, improve markers of longer term glycaemic control in humans. Higher legumes and fruits and vegetables intake were associated with a lower risk of non-cardiovascular and total mortality in a prospective cohort study in 18 countries (PURE) (Miller et al., 2017). Coffee and tea, prepared from beans of *Coffea arabica* and *Coffea canephora* var. Robusta (two of the most species of economic importance), and from the leaves of *Camellia* spp., for coffee and tea, respectively, are the most widely consumed beverages in the world (Crozier et al., 2009; Ludwig et al., 2014). A 22% lower risk of all-cause mortality for every

2 additional cups of total coffee per day was found in a Mediterranean cohort of more than 19,000 participants, with the risk being most substantial among participants older than 54 years (Navarro et al., 2018). Coffee intake was associated with preventing action against cancer (breast, colorectal, colon, endometrial, and prostate), type-2 diabetes mellitus, Parkinson's disease and liver disease (Grosso et al., 2017a; Higdon and Frei, 2007; Ludwig et al., 2014; Martini et al., 2016). Grosso and colleagues (Grosso et al., 2017b) found that increased coffee consumption is associated with a modest decrease in the risk of hypertension in prospective cohort studies (**Figure 6**).

Tea consumption decreased the risk of liver conditions, including hepatocellular carcinoma, liver steatosis, hepatitis and liver cirrhosis (Yin et al., 2015) and fracture by improving bone mineral density (Shen et al., 2009). The literature also supports an inverse association with various cardiovascular disease-related health outcomes (Abe and Inoue, 2020; Basu and Lucas, 2007; Hodgson and Croft, 2010). Green tea intake was found to reduce the risk of dementia, Alzheimer's disease, mild cognitive impairment, or cognitive impairment in systematic reviews of observational studies (Kakutani et al., 2019; Qing-Ping et al., 2016). A double-blind, placebo-controlled trial showed that chronic tea consumption reduces platelet activation and plasma C-reactive protein in healthy men (Steptoe et al., 2007).

Red wine improved endothelial function and vascular health (Agewall et al., 2000; Corder et al., 2006) and protected against certain chronic health conditions such as increased antioxidant potential values of plasma and erythrocytes, slightly increased high-density lipoprotein-cholesterol and increased efflux of cellular cholesterol and inflammatory biomarkers (Guilford and Pezzuto, 2011) (**Figure 6**). Moreover, an *in vitro* study demonstrated that red wine phenolics blocked human platelet aggregation and eicosanoid synthesis (Pace-Asciak et al., 1995).

Plant-based milk substitutes, water-soluble extracts of legumes, oilseeds, cereals, pseudocereals, vegetables, and nuts, were found to decrease low-density lipoprotein levels, improve gastrointestinal health, and reduce postprandial glycemic response (Reyes-Jurado et al., 2021).

2.1.2 Biological effects of dietary (poly)phenols

Over the last few years, a considerable number of human studies demonstrated the benefits of (poly)phenol against several cardiometabolic risk factors. Various (poly)phenol classes such as anthocyanins from berries, flavan-3-ols from cocoa and green tea, hydroxycinnamic acids from nuts (i.e. almonds and pistachios) and isoflavone from soy products were found to improve blood pressure levels (Grosso et al., 2022; Ottaviani et al., 2020), besides being involved in regulating cellular lipid metabolism, thereby mitigating atheroprotective effects (Zanotti et al., 2015). Consistent and significant modulatory effects of flavan-3-ol-containing tea, cocoa and apple products on body composition and blood lipids were demonstrated in a systematic review and meta-analysis by González-Sarrías and colleagues (González-Sarrías et al., 2017). A systematic review and meta-analysis of 156 randomized trials, flavan-3-ol intervention resulted in significant improvements in acute/chronic flow-mediated dilation, systolic and diastolic blood pressure, cholesterol, triglycerides, haemoglobin A1c, and homeostasis model assessment of insulin resistance (Raman et al., 2019) (**Figure 7**). Recently, the COSMOS trial demonstrated that cocoa flavan-3-ol supplementation might reduce cardiovascular disease death by 27% (Sesso et al., 2022) and in parallel, *in vitro* and *in vivo* studies using cocoa (poly)phenols (mainly as (−)-epicatechin and procyanidins) supported their beneficial actions on the human cardiovascular system (Sies et al., 2005). The beneficial action of (poly)phenols on cardiovascular health was also demonstrated through *in vitro* and *in vivo* models for anthocyanins (de Pascual-Teresa et al., 2010; De Pascual-Teresa and Sanchez-Ballesta, 2008; Wallace, 2011), flavanones (Barreca et al., 2017; Cassidy et al., 2016; Testai and Calderone, 2017), ellagitannins (Larrosa et al., 2010; Selma et al., 2018), flavanols and chlorogenic acids (Grosso et al., 2018; Kris-Etherton et al., 2002; Lin Li et al., 2020; Menezes et al., 2017; Zhao et al., 2011).

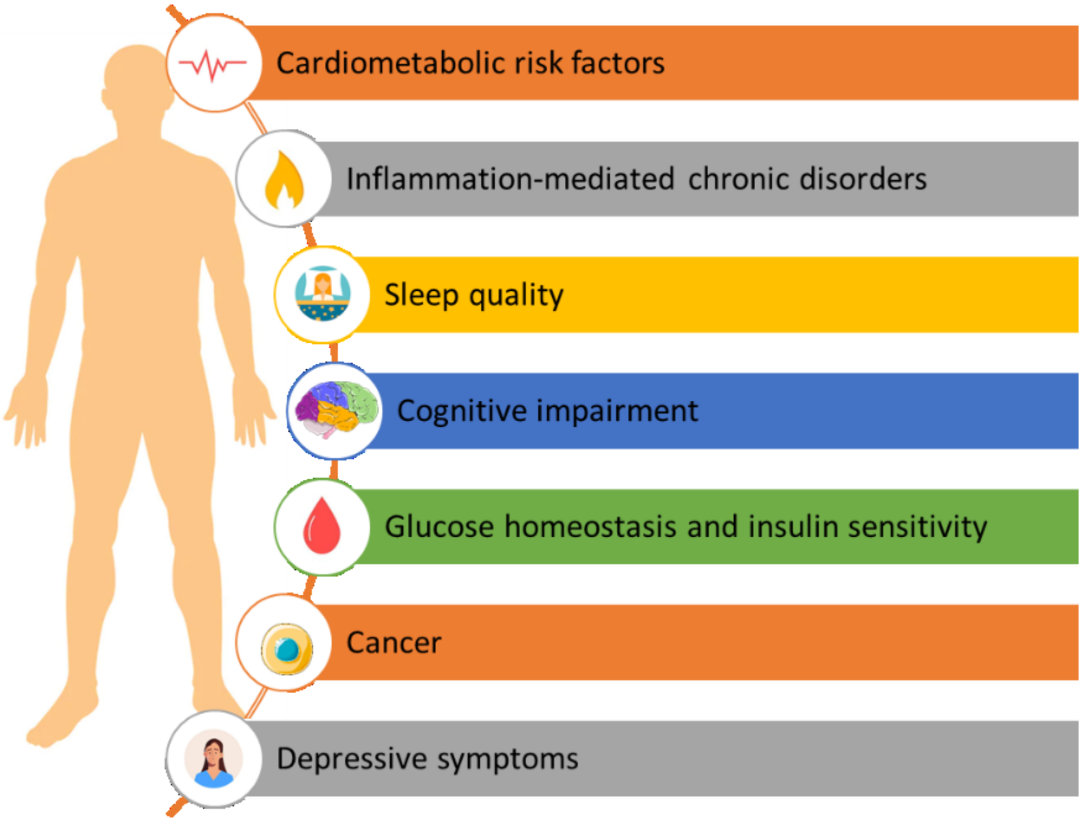


Figure 7. Health benefits of dietary (poly)phenols

(Poly)phenols were also able to mediate oxidative stress mechanisms involved in inflammation-mediated chronic disorders by inhibiting enzymes responsible for eicosanoid production (Hussain et al., 2016; Marhuenda-Muñoz et al., 2019). The role of (−)-epicatechin in the homeostasis of reactive oxygen species and redox balance, and inflammation process has been extensively revised by Shay and colleagues (Shay et al., 2015). A regulation of obesity-derived inflammation and the improvement of antioxidant status were demonstrated for dietary anthocyanins and extra virgin olive oil high in polyphenols in the OLIVAUS study (Del Bo et al., 2015; Y. M. Lee et al., 2017; Sarapis et al., 2022). By virtue of their ability to improve glucose homeostasis and insulin sensitivity, besides reducing postprandial insulin levels, (poly)phenols were also found to be able to regulate carbohydrate metabolism (Bahadoran et al., 2013; Cao et al., 2018; Costabile et al., 2019; Serina and Castilho, 2021; Rienks et al., 2018). In particular, (poly)phenols may inhibit the activity of enzymes involved in carbohydrate digestion, such as α-amylase and/or α-glucosidase affecting the post-prandial glycemic responses after dietary carbohydrate intake (Dall'Asta et al., 2020). The link between (poly)phenol consumption from supplements and foods and insulin resistance was extensively summarized by (Williamson and Sheedy, 2020), showing that the most promising candidates for improving insulin resistance are (−)-epicatechin, (−)-epicatechin-containing foods and anthocyanins (**Figure 7**). Cell and animal models also proved the anticarcinogenic effect of quercetin, some galloylated flavan-3-ols [such as (−)-epigallocatechin-3-*O*-gallate], resveratrol and luteolin through modulation of enzyme activities and apoptosis, gene expression, and P-glycoprotein activation, and various signalling pathways (Briguglio et al., 2020; Duthie et al., 2000; Spagnuolo et al., 2012; Vauzour et al., 2010). Hydroxycinnamic acids, flavan-3-ols and flavonols were found to inhibit the methyltransferase activities of histone deacetylase which usually result downregulated in cancerogenic cells (Briguglio et al., 2020; Dall'Asta et al., 2022). The immunomodulatory activities of (poly)phenols and their modulation of oral and gut microbiota were proposed as preventive mechanisms in the development of colorectal cancer (Cueva et al., 2020). Regarding cognitive health, intake of specific flavonoid classes, namely flavan-3-ols, anthocyanins, and flavonols (i.e. quercetin), besides some phenolic acids classes such as hydroxycinnamic acids was associated with cognitive impairment among adult individuals by Godos and colleagues (Godos et al., 2021, 2020a). Human epidemiological and animal data suggest that tea (poly)phenols may decrease the incidence of dementia, Alzheimer and Parkinson's disease. One of the main tea flavan-3-ol, namely (−)-epigallocatechin-3-*O*-gallate, exerted neuroprotective/neurorescue activities in various models of neurological disorders through radical scavenging and iron chelating activity, besides modulation of cellular survival and signal transduction pathway (Mandel et al., 2008; Weinreb et al., 2004). Finally, looking at other beneficial effects linked to (poly)phenol consumption, Godos and colleagues (Godos et al., 2020b) demonstrated that a higher dietary intake of hydroxycinnamic acids, naringenin and apigenin was associated with better sleep quality in more than 1900 adults living in southern Italy. An inverse association between quercetin and naringenin consumption and depressive symptoms was observed for the highest versus lowest quartile of intake in the Mediterranean Healthy Eating, Lifestyle and Aging (MEAL) Study (Godos et al., 2018) (**Figure 7**).

In this context, European Food Safety Authority (EFSA) approved health claims releated to i) cocoa flavanols and protection of lipids from oxidative damage, and maintenance of normal blood pressure (EFSA, 2012), and maintenance of normal endothelium-dependent vasodilation (Agostoni et al., 2012), ii) (poly)phenols in olive and protection of LDL particles from oxidative damage (EFSA, 2011), and iii) plant sterol-enriched foods for their cholesterol-lowering effects (EFSA, 2014).

2.1.3 ADME of (poly)phenols and their derived metabolites drive the beneficial effects of (poly)phenol-rich foods

Assessing the absorption, distribution, metabolism and excretion (ADME) of dietary (poly)phenols, and subsequently their metabolic fate and bioavailability, are pivotal to fully clarify i) which circulating compounds interact with target cells, organs and tissues, and ii) correlate (poly)phenol intake with the preventive action against several chronic conditions and with the myriads of beneficial effects observed in humans (**Figure 8**).

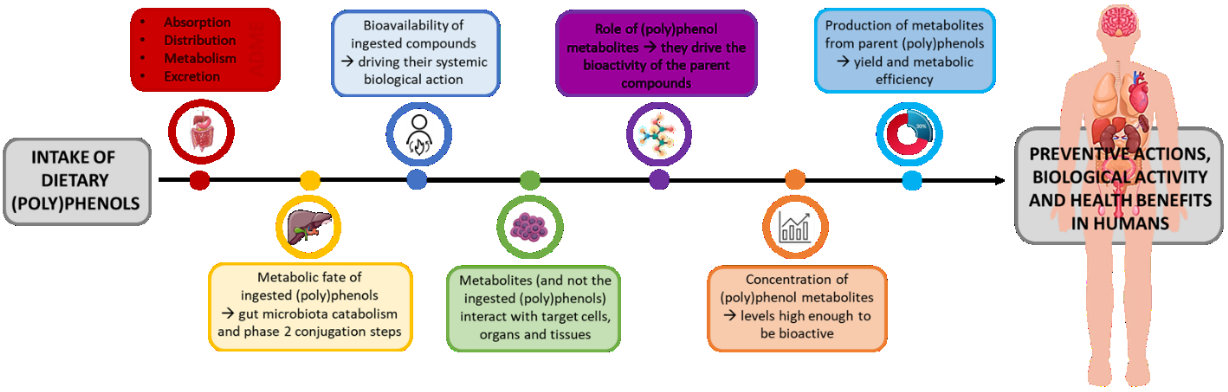


Figure 8. ADME and metabolites of dietary (poly)phenols: key actors for their potential bioactivity in humans

From the clinical nutrition point of view, the concept of bioavailability refers to the ratio between the total amount of compounds excreted in urine following the ingestion of a specific food/supplement and the dose of ingested parent compounds, thus indicating the proportion of the ingested compound that is being absorbed and available for being distributed and metabolized within body organs (Velderrain-Rodríguez et al., 2014). The capability of (poly)phenols to be metabolized in bioavailable molecules in humans is an essential step to exert a specific systemic biological action following their consumption (Grosso, 2018; Velderrain-Rodríguez et al., 2014). On the other hand, since ingested (poly)phenols are extensively metabolized along the human gastrointestinal tract (Del Rio et al., 2013; Mithul Aravind et al., 2021; Rodriguez-Mateos et al., 2014), the potential bioactivity observed after their intake is not related to the parent compounds but to the hundreds of circulating metabolites and/or catabolites which may be more active and better absorbed than their precursors (Mithul Aravind et al., 2021). After their production, (poly)phenol derived metabolites and/or microbial catabolites, which might be considered potential ‘actors’ of the putative health effects derived from intake of (poly)phenol-rich foods, should be able to i) efficiently reach and interact with the target destination, and ii) circulate in biofluids at concentration levels high enough to be bioactive (Carregosa et al., 2022; Velderrain-Rodríguez et al., 2014) (**Figure 8**). On the other hand, (poly)phenol metabolites persist for longer periods of time in circulation, as observed from their high half-elimination time ($t_{1/2}$) values (Carregosa et al., 2021). The actual internal exposure to food phytochemical metabolites is largely modulated by the capacity of individuals to carry out all mechanisms correlated to the ADME of ingested compounds.

In this context, various *in vivo* and *in vitro* models assessed the potential bioactivity of (poly)phenols by studying their i) simple phase 2 conjugates and ii) gut microbiota catabolites and phase 2 conjugate counterparts.

Looking at the putative positive effects of phase 2 conjugates of ingested (poly)phenols on health, plasma flavan-3-ol phase 2 conjugates, namely (epi)catechin-7-glucuronide, 4′-methoxy-(epi)catechin, and 4′-methoxy-(epi)catechin-glucuronide were correlated with nitric oxide increase in a double-blind, crossover and dose-response study with a cocoa drink (Heiss et al., 2005). In a similar setting, (−)-epicatechin and (−)-epicatechin-7-glucuronide were found to be independent predictors of flow-mediated dilation effects in a study on 16 male adults after consuming a cocoa drink (Schroeter et al., 2006). An increase in flow-mediated dilation was also correlated with urinary levels of hesperetin-3′-glucuronide and hesperetin-7-glucuronide after blood orange juice consumption by 15 overweight men and women (Lu Li et al., 2020), while plasma *S*-nitrosothiol with blood quercetin and epicatechin levels in a crossover study on 12 adult males (Loke et al., 2009, 2008). The change in brachial artery diameter was correlated with a phase 2 conjugate of quercetin, namely quercetin-3′-glucuronide, after intake of 400 mg of quercetin by 15 adults (Perez et al., 2014). After consumption of orange juice by 24 overweight male adults, Morand and colleagues (Morand et al., 2011) found a significant correlation between plasma hesperetin and changes in microvascular endothelial reactivity. Also, after orange juice intake, urinary hesperetin and naringenin were correlated with erythrocyte catalase activity in 100 overweight or obese adults (Rangel-Huerta et al., 2015). A study on the biological activity of the main tea monomer flavan-3-ols, namely (−)-epigallocatechin-3-*O*-gallate and (−)-epigallocatechin, showed that some glucuronide counterparts maintained the radical scavenging activity and inhibited the release of arachidonic acid from HT-29 human colon cancer cells (Lu et al., 2003). On the contrary, glucuronide derivatives of (−)-epicatechin had no effect on hydrogen peroxide-induced-cell death in neurones and fibroblasts (Spencer et al., 2001). Glucuronide conjugates of hesperetin and naringenin, derived from the metabolism of bergamot juice (poly)phenols by humans, were found to modulate inflammation in myeloid angiogenic cells (Spigoni et al., 2017). In parallel, blood low molecular weight metabolites, by virtue of their high bioavailability, are strongly able to overcome cellular barriers and reach the brain (Angelino et al., 2019a; Carregosa et al., 2019).

In this context, (poly)phenol metabolites belonging to various classes, namely 3,4-dihydroxybenzoic acid (*aka* protocatechuic acid), 3-methoxybenzoic acid-4-sulfate (*aka* vanillic acid-4-sulfate), 4′-hydroxy-3′-methoxycinnamic acid (*aka* ferulic acid), cinnamic acid, 3-(3′-hydroxyphenyl)propanoic acid and 3′,4′-dihydroxyphenylacetic acid resulted able to mediate age-related disorders such as neuroinflammation, thus involved in the onset of neurodegenerative diseases (Carregosa et al., 2021, 2019; Hano and Tungmunnithum, 2020) (**Figure 9**).

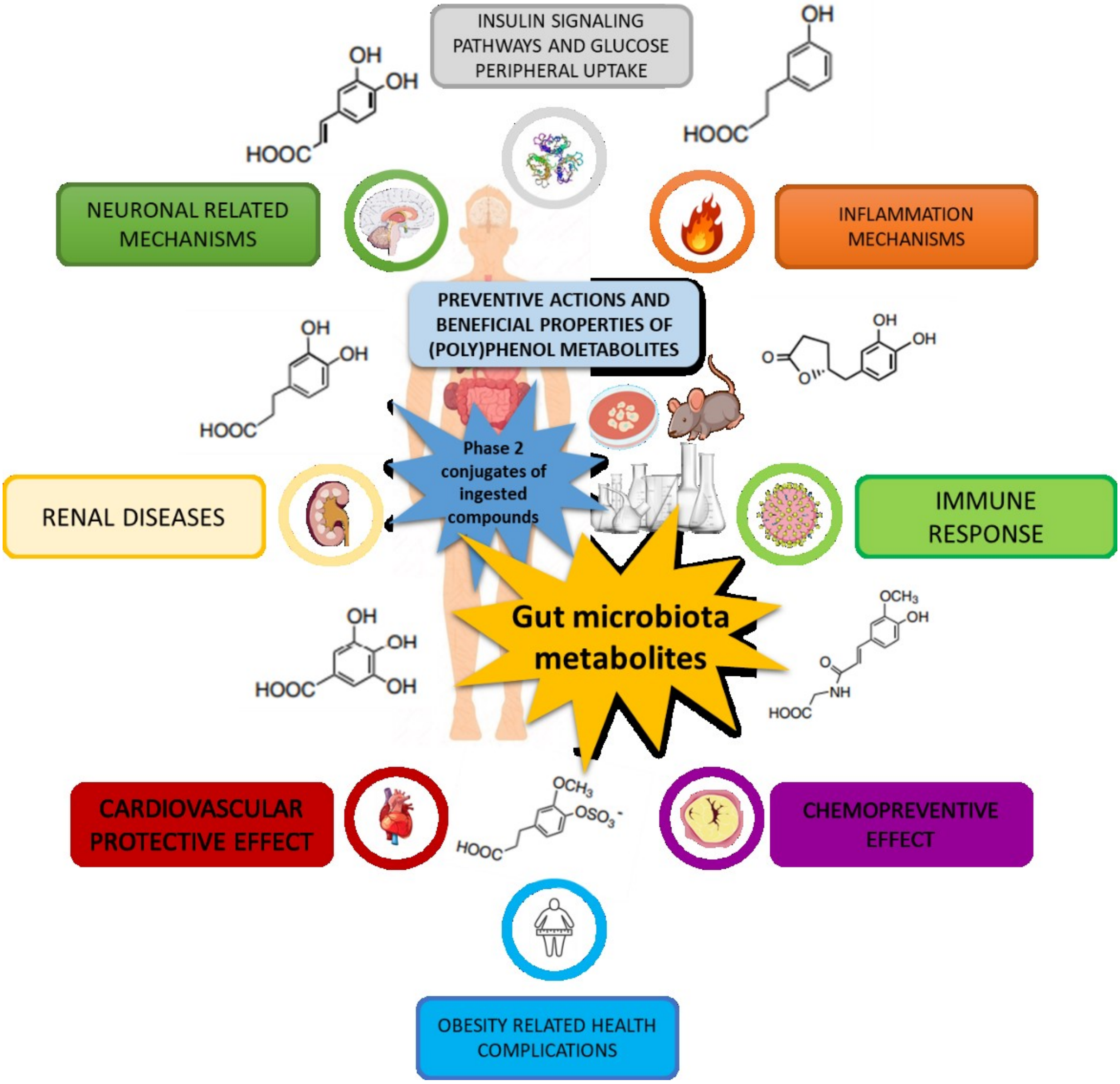


Figure 9. Metabolites of dietary (poly)phenols: preventive actions and biological activity

Chlorogenic acid-derived catabolites [i.e. 3-(3′,4′-dihydroxyphenyl)propanoic acid (*aka* dihydrocaffeic acid), 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid (*aka* dihydroferulic acid) and 3′-methoxy-4′-hydroxycinnamoyl-glycine (*aka* feruloylglycine)] were most effective in protecting neuronal cells to oxidative stress, a feature of the diabetic conditions in an *in vitro* experimental model at physiological concentrations (Verzelloni et al., 2011). In the same experimental model, Verzelloni and colleagues (Verzelloni et al., 2011) demonstrated that two colonic metabolites of ellagitannins, namely urolithins and benzene-1,2,3-triol (*aka* pyrogallol), had anti-glycation properties compared to the parent ellagitannins. The onset of type-2 diabetes and related complications associated with (poly)phenol metabolites were also entensively reviewed by (Marhuenda-Muñoz et al., 2019; Mithul Aravind et al., 2021). Insulin signaling pathways and glucose peripheral uptake were found attenuated by various flavan-3-ol microbial metabolites, namely 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone, 3′,4′-dihydroxyphenylacetic acid, and 2,3-dihydroxybenzoic acid (Álvarez-Cilleros et al., 2018; Márquez Campos et al., 2020; Scazzocchio et al., 2011; Takagaki et al., 2019). Within the same context, the inhibitory activity against the enzyme α-glucosidase was maintained after the *in vitro* fermentation of Chilean wild currants *Ribes magellanicum* and *R. punctatum* (poly)phenols (Burgos-Edwards et al., 2018). Up to 34 physiologically relevant (poly)phenol metabolites including phenylpropanoic acids, phenylacetic acids, cinnamates and benzoic acids were found most likely to be responsible for the kidney protective effects of (poly)phenols in an analysis of the (poly)phenol metabolome conducted using human urine (Guerreiro et al., 2022). Dihydroxylated low molecular weight phenolic compounds, namely 3-(3′,4′-dihydroxyphenyl)propanoic acid and 3′,4′-dihydroxyphenylacetic acid, significantly inhibited the secretion of pro-inflammatory cytokines (i.e. TNF-a, IL-1b and IL-6) in lipopolysaccharide-stimulated peripheral blood mononuclear cells (Monagas et al., 2009). 3,4,5-Trihydroxybenzoic acid (*aka* gallic acid), one of the main benzoic acids in plant-based foods, influenced the gut microbiome activities and modulated the immune response maintaining intestinal health (Yang et al., 2020). Immunomodulatory activity was also observed for (phenyl)-γ-valerolactones and phenylvaleric acids in mouse splenocytes treated *in vivo* (Kim et al., 2016) (**Figure 9**). Also, these peculiar gut microbiota metabolites of flavan-3-ols exerted a chemopreventive effect by inhibiting the proliferation of human cervical cancer cells and those of androgen-dependent human prostate cancer cells (Hara-Terawaki et al., 2017; Stanisławska et al., 2019). Hydroxycinnamic acids were found mediate cellular differentiation, proliferation, and apoptosis, considered important mechanisms involved in the onset of carcinogenesis (Rocha et al., 2012). This class of unsaturated phenolic acids showed potential benefits in experimental diabetes and hyperlipidemia, also serving as molecules for the treatment of obesity-related health complications (Alam et al., 2016). Referring to the cardiovascular protective effect of (poly)phenol metabolites, Lee and colleagues (C. C. Lee et al., 2017) demonstrated that 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone had stronger prevention effect on tumor necrosis factor-α-stimulated adhesion of THP-1 human monocytic cells to human umbilical vein endothelial cells compared to its potential precursors such as dimer and monomer flavan-3-ols and its microbial metabolites including dihydroxylated aglycones of phenylpropanoic and phenylacetic acids. (−)-Epigallocatechin-3-*O*-gallate metabolites, such as mono-, di- and three-idroxylated forms of (phenyl)-γ-valerolactones and phenylvaleric acids showed inhibitory activity against angiotensin I-converting enzyme (Takagaki and Nanjo, 2015). The influence of phenolic acids such as 4′-hydroxyphenylacetic acid, 3-(4′-hydroxyphenyl)propanoic acid and 3′,4′-dihydroxyphenylacetic acid on mitochondria and neutrophils, the main producers of reactive oxygen species in tissues and circulation was assessed by (Beloborodova et al., 2012) (**Figure 9**). Management of an important risk factor of cardiovascular diseases, such as atherosclerosis mechanisms, was found to be mediated by microbial metabolites of proanthocyanidins, which significantly inhibited the proinflammatory cytokines TNF-α, IL-1β and IL-6 secretion (Lambert et al., 2007; Spencer et al., 2004). The urinary excretion of 4-methoxy-3,5-dihydroxybenzoic acid (*aka* 4-methylgallic acid) was inversely associated with the change in flow-mediated dilation responses after chronic (4 weeks) intake of black tea by adults with mild hyperlipidaemia (Hodgson et al., 2006). The increase in flow-mediated dilation was also correlated with higher levels of plasma simple phenolic acids [i.e. 4-hydroxy-3-methoxybenzoic acid (*aka* vanillic acid), and benzoic acids, hippuric and hydroxyhippuric acids, and 4′-hydroxy-3′-

methoxyphenylacetic acid (*aka* homovanillic acid)] after intake of blueberry drinks by healthy male adults (Rodriguez-Mateos et al., 2013). In a double-blind, crossover study on healthy male adults with cranberry drinks, up to 20 metabolites, including 3-(4′-methoxyphenyl)propanoic acid-3′-sulfate (*aka* dihydroisoferulic acid 3-sulfate), 3′-methoxycinnamic acid-4′-sulfate (*aka* ferulic acid 4-sulfate) and 3-methoxyphenylacetic acid-4-sulfate (*aka* homovanillic acid sulfate) were correlated with an increase in flow-mediated dilation (Rodriguez-Mateos et al., 2016). Also, chlorogenic acid metabolites were found to be correlated with flow-mediated dilation after low and high doses of chlorogenic acids from coffee (Mills et al., 2017), together with blueberry anthocyanin metabolites (Rodriguez-Mateos et al., 2019) and aronia plasma metabolites (Istas et al., 2019). Referring to another predictor of cardiovascular risk, namely carotid-femoral pulse-wave velocity change, it was significantly improved and associated with plasma equol, a metabolite of soy isoflavones in an acute study (Hazim et al., 2016). A phenylpropanoic acid catabolite, namely 3-(4′-hydroxyphenyl)propanoic acid, together with other urinary aronia anthocyanins metabolites (i.e. peonidin-3-galactoside and cyanidin-3-galactoside), were associated with reduced fasting plasma total cholesterol and low-density lipoprotein in peripheral blood mononuclear cells (Xie et al., 2016) (**Figure 9**).

3.1 The EU-JPI project ‘FoodPhyt’: background and objectives

According to the World Health Organization (WHO), the worldwide prevalence of obesity nearly tripled between 1975 and 2016. In 2016, out of the 1.9 billion overweight adults, 650 million were obese, and overall, about 13% of the world’s adult population (11% of men and 15% of women) were obese. In 2019, an estimated 38.2 million children under the age of 5 were overweight or obese. The non-communicable diseases Risk Factor Collaboration estimated that by 2025, the prevalence of obesity would reach 18 % in men and 21 % in women (Di Cesare et al., 2016). Overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health (“Obesity and overweight” -https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight). Increased intake of energy-dense foods and physical inactivity are recognised as the leading causes of obesity and overweight, which subsequently represent major risk factors for noncommunicable diseases such as cardiovascular diseases, diabetes, musculoskeletal disorders, especially osteoarthritis, some cancer forms (such as endometrial, breast, ovarian, prostate, liver, gallbladder, kidney and colon) and poor mental health (Chooi et al., 2019; Lafia et al., 2022), exerting enormous pressure on the economic burden: the annual global economic costs of obesity are about US$2 trillion, representing 2.8 % of the world’s gross domestic product (Chu et al., 2018; da Silva, 2019) (**Figure 10**). Cardiovascular diseases, consisting of ischemic heart disease, stroke, heart failure, peripheral arterial disease, and several other cardiac and vascular conditions, constitute the leading cause of worldwide mortality, with over 17 million deaths estimated in 2017 and 35 % of total deaths in women in 2019 (Mensah et al., 2019; Roth et al., 2020; Vogel et al., 2021). The worldwide incident cases of diabetes mellitus have increased by 102.9 % from 1990 to 2017, with an increase in type-2 diabetes mellitus dominating this trend compared to type-1 diabetes mellitus. Besides, the age of type-2 diabetes mellitus onset also becomes lower due to changing in dietary habits and sedentary lifestyle related to economic development (Liu et al., 2020). In 2019, the total number of cancer deaths globally attributable to all estimated risk factors was over 4 million, with behavioural risk factors, such as tobacco and alcohol use, unsafe sex, and dietary risks being the most attributable cancer deaths and disability-adjusted life-years (Tran et al., 2022). Changes in dietary and physical activity patterns might be considered pivotal for the fight against obesity and related conseguences on health (Bray et al., 2016) (**Figure 10**). The increase in fruit and vegetale intake, together with that of legumes, whole grains and nuts, is strongly encouraged from WHO to reduce overweight and obesity at the individual level ("Obesity and overweight"-https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight) (**Figure 10**). Also environmental factors such as sociocultural (eg, cuisines, body size perceptions, and cultural characteristics), economic (eg, national wealth and distribution of wealth), policy (eg, political and regulatory regimes), or physical (eg, built environment) are likely to be moderators of the effects of globalisation on obesity prevalence (Jaacks et al., 2019).

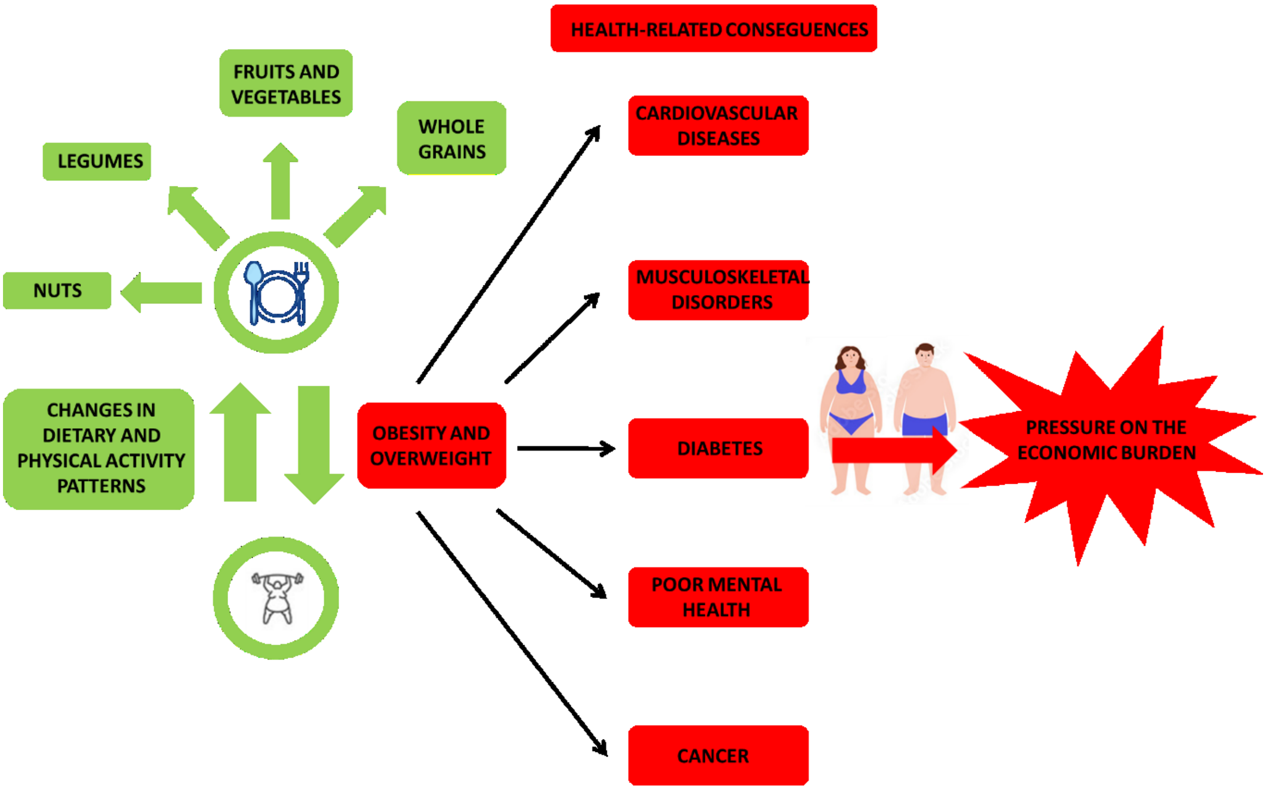


Figure 10. Dietary and physical activity patterns and non-communicable disease development

The main reason for the increase in the prevalence of obesity and overweight is the inability of food systems to deliver healthy diets. The consumption of industrialised and processed foods is growing in most countries (da Silva, 2019). To address the Global Syndemic of obesity, undernutrition, and climate change, a radical rethink of behaviour linked to how people are eating, living, consuming and moving is key, and for fighting this, food systems may play a central role by providing healthy and nutritious food that is accessible and affordable for everyone (da Silva, 2019; Kleinert and Horton, 2019). Food and Agriculture Organization of the United Nations (FAO) and WHO are leading the implementation of the Decade of Action on Nutrition 2016–2025, supporting several countries to address the multiple burdens of malnutrition from different perspectives and actions such as improvement of product labelling, encourage consumption of fresh and local foods, and introduce of nutrition in primary school curriculums (da Silva, 2019). The EAT Lancet commission on Food, Planet, Health recommends a radical transformation of the global food system, with a drastic shift towards more plant-based and fewer animal-source foods. A diet rich in plant-based foods is healthy, sustainable, and suitable for both people and the planet ("EAT-Lancet Commission Brief for Everyone - EAT" - https://eatforum.org/lancet-commission/eatinghealthyandsustainable/; Willett et al., 2019). Global consumption of fruit, vegetables, nuts, whole grains and legumes should double by 2050, while meat and sugar should be severely reduced. Over the last few years, an explosion of research demonstrated the preventive and beneficial effects of plant-based foods and dietary phytochemicals against obesity and correlated diseases (please refer to the previous sections for further details). On the other hand, data on dietary bioactivities and phytochemical metabolites are scattered in the scientific literature and challenging to evaluate. To improve the interpretation and accuracy of scientific results and harmonize data regarding the ADME of dietary bioactives in humans, improved access and understanding of this consolidated knowledge is urgently needed.

The JPI-HDHL FoodPhyt "*Food Phytochemicals matter for cardiometabolic health*" project aims at raising awareness and understanding of the potential of food phytochemicals on human health to support the global fight against obesity and associated cardiometabolic diseases (https://www6.inrae.fr/foodphyt). FoodPhyt gathers a strong multinational consortium of partners and collaborators from Spain (Pr R. Estruch, IDIBAPS, Hospital Clínic Barcelona), Italy (Pr D. Del Rio, University of Parma), Sweden (Pr R. Landberg, Chalmers University of Technology), Germany (Pr S. E. Kulling, Max Rubner-Institut), California (Pr D. Wishart, University of Alberta), and United Kingdom (Dr A. Rodriguez-Mateos, King's College) (**Figure 11**).

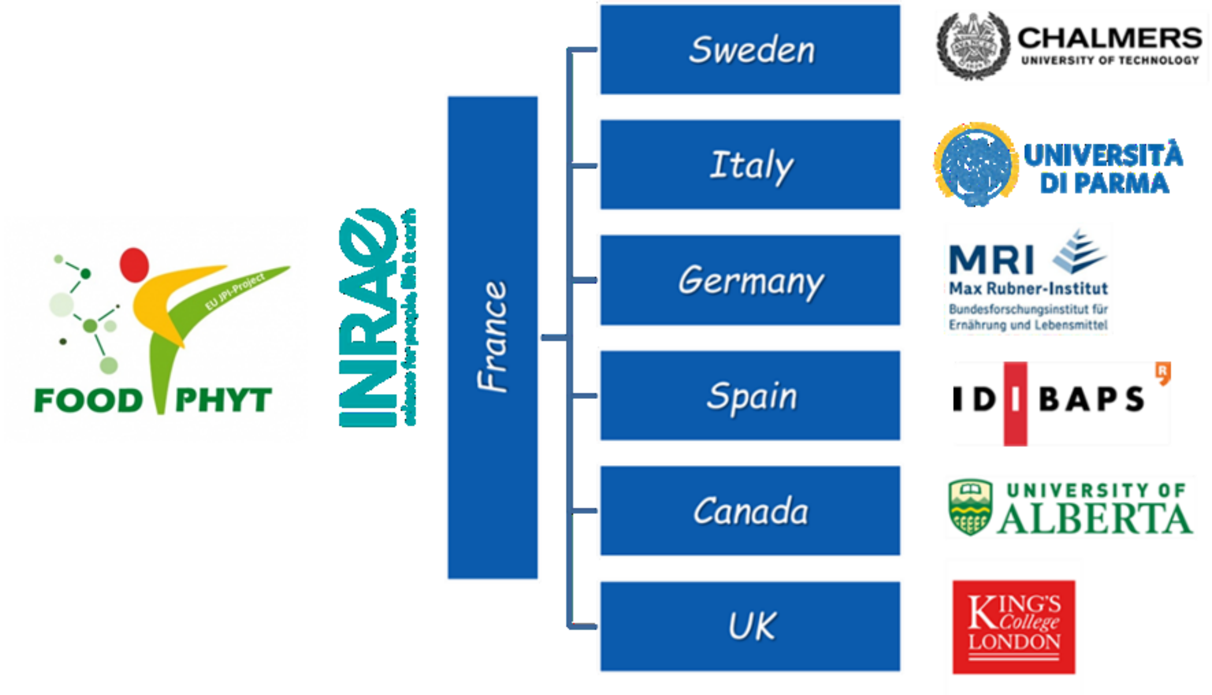


Figure 11. Multinational consortium of partners and collaborators of the FoodPhyt project

FoodPhyt work plan is organised into 3 scientific Work packages (WPs) and 1 WP for coordination, networking and dissemination (**Figure 12**) aiming at i) consolidating knowledge available on metabolism, cardiometabolic health effects and mechanisms of action of food phytochemicals and plant foods in an open-access database (PhytoHub 2.0) to fit the needs of various end-users and ii) validate biomarkers of plant food intake, and to harmonize metabolomic methods for comprehensively phenotyping the actual internal exposure to food phytochemical metabolites. Online databases have proven to be key resources for boosting research and derived applications in a specific field, making data accessible to everyone and searchable in many ways to serve a large variety of purposes.

Moreover, improving the assessment of exposure to dietary phytochemicals might be considered pivotal to better clarify and correlate their intake with cardiometabolic health. FoodPhyt aims at (re)investigating the associations observed between plant food intake biomarkers or specific food phytochemical metabolites present in biofluids with body weight and cardiometabolic outcomes in pre-existing human studies (https://www6.inrae.fr/foodphyt). Through better knowledge and assessment methods for phytochemicals, there is considerable potential for improving the nutritional quality of raw and processed plant foods and end-users such as nutritionists, dieticians and health providers may integrate the knowledge on protective food phytochemicals in their dietary advice and adapt those to personal needs and food preferences. They may recommend, within a balanced diet, some particular plant-based foods for targeting a metabolic dysfunction or gut microbiome dysbiosis, with much greater compliance and potential efficacy.

In detail, the FoodPhyt objectives are:

- To critically review and harmonize the knowledge on the metabolism, health effects, and mechanisms of action of the main dietary phytochemicals by upgrading the open-access database PhytoHub (**Figure 13**) (https://phytohub.eu/);
- To improve the accuracy of dietary assessment for food phytochemicals, by developing new metabolomics methods based on quantification of plant food intake biomarkers, and comprehensive profiling of phytochemical metabolites in human biofluids;
- To demonstrate the usefulness of these new dietary/exposure assessment methods in previous or new human studies to (re)investigate associations observed with body weight, obesity, and cardiometabolic outcomes;
- To foster the capacity building of Early Career Scientists in the fields of food phytochemicals, metabolomics, dietary assessment, databases, and the prevention of obesity and cardiometabolic diseases.

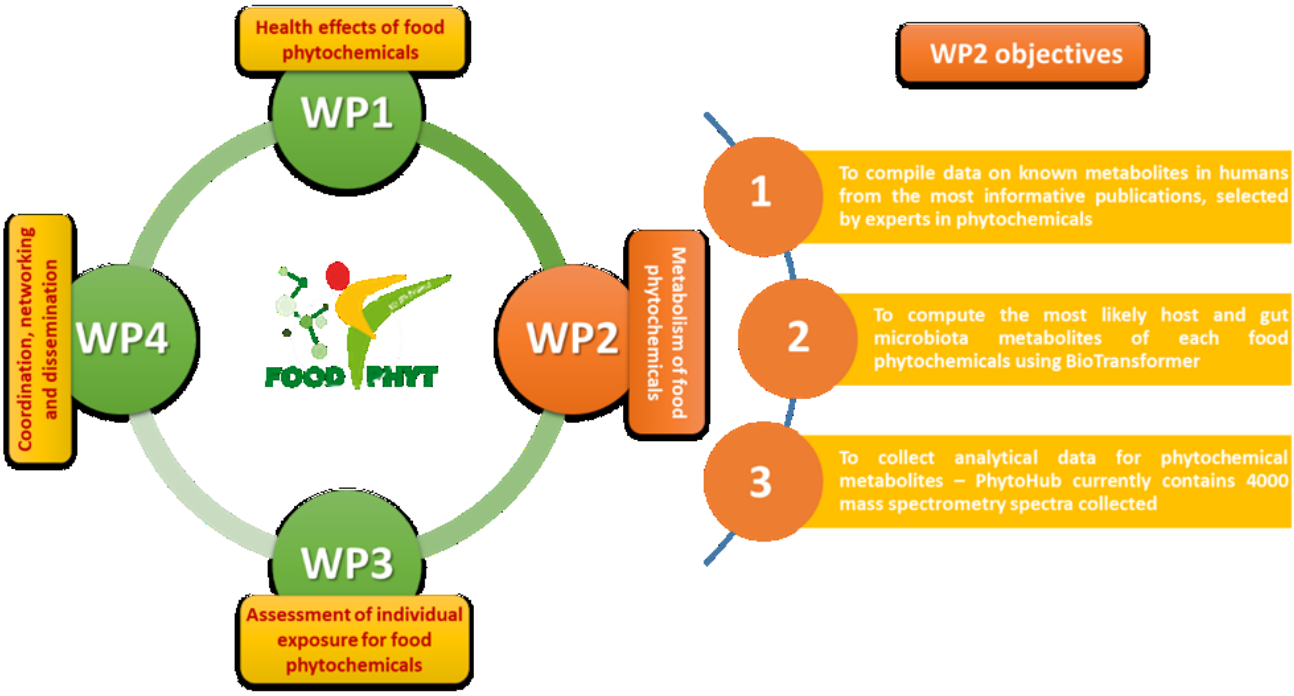
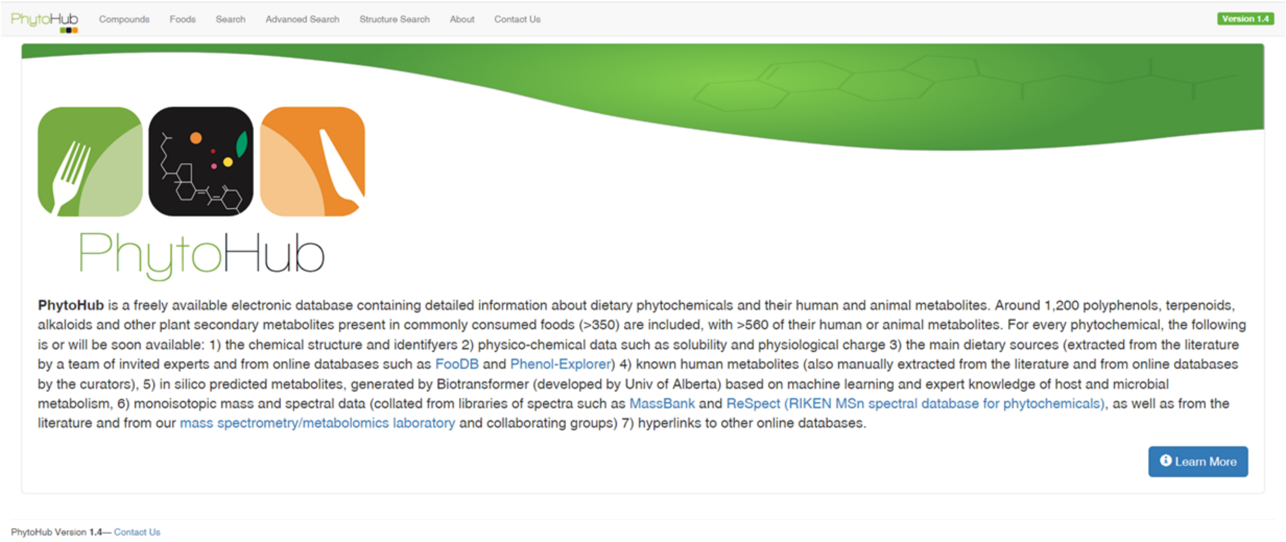


Figure 12. Work package (WP) of the FoodPhyt project and WP2 objectives



PhytoHub is a freely available electronic database containing detailed information about dietary phytochemicals and their human and animal metabolites. Around 1,200 polyphenols, terpenoids, alkaloids and other plant secondary metabolites present in commonly consumed foods (>350) are included, with >560 of their human or animal metabolites. For every phytochemical, the following is or will be soon available: 1) the chemical structure and identifyers 2) physico-chemical data such as solubility and physiological charge 3) the main dietary sources (extracted from the literature by a team of invited experts and from online databases such as FooDB and Phenol-Explorer) 4) known human metabolites (also manually extracted from the literature and from online databases by the curators), 5) in silico predicted metabolites, generated by Biotransformer (developed by Univ of Alberta) based on machine learning and expert knowledge of host and microbial metabolism, 6) monoisotopic mass and spectral data (collated from libraries of spectra such as MassBank and ReSpect (RIKEN MSn spectral database for phytochemicals), as well as from the literature and from our mass spectrometry/metabolomics laboratory and collaborating groups) 7) hyperlinks to other online databases.

Figure 13. PhytoHub interface ((https://phytohub.eu/)

4.1 Aim of the Doctoral Thesis

This Doctoral Thesis comprehensively assessed the production of metabolites from the main dietary (poly)phenols in the framework of their ADME in humans (**Figure 14**).

To this aim, the present Ph.D. project applied four research lines to investigate:

- the *in vivo* and *in vitro* interaction of flavan-3-ols, hydroxycinnamic acids, quercetin and cranberry (poly)phenols with human gut microbiota describing meta- and catabolic pathways associated with the production of their colonic and phase 2 derived metabolites;
- blood nutrikinetics and urinary excretion of metabolites derived from intake of flavan-3-ols and hydroxycinnamic acids through a systematic review based-approach;
- the yield and metabolic efficiency (*aka* stoichiometry) in the *in vitro* and *in vivo* production of the main colonic catabolites of flavan-3-ols and main urinary metabolites of hydroxycinnamic acids through i) analyses of cumulative urinary data, and ii) the application of a robust colonic fermentation model;
- the overall and dietary source-driven differences in the bioavailability of flavan-3-ols and hydroxycinnamic acids.

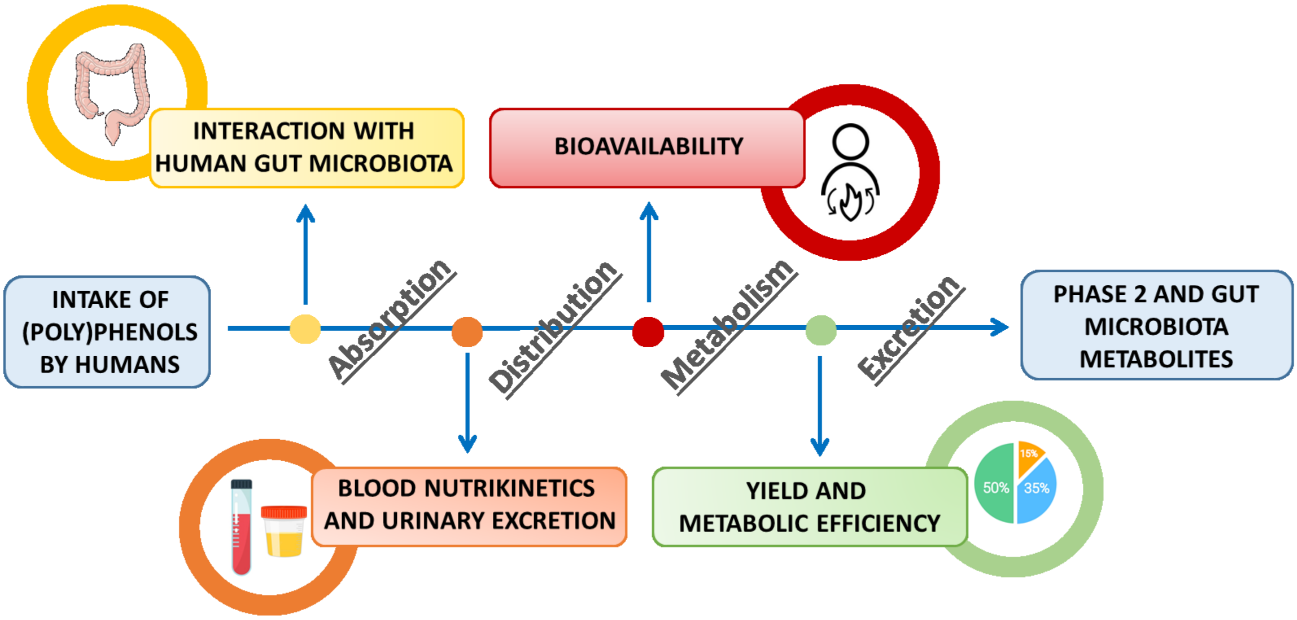


Figure 14. PhD project workflow

Chapter 2. In Vitro Faecal Fermentation of Monomeric and Oligomeric Flavan-3-ols: Catabolic Pathways and Stoichiometry

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In Vitro Faecal Fermentation of Monomeric and Oligomeric Flavan-3-ols: Catabolic Pathways and Stoichiometry

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ABSTRACT

Scope: The study evaluates the influence of flavan-3-ol structure on the production of phenolic catabolites, principally phenyl-γ-valerolactones (PVLs), and phenylvaleric acids (PVAs). Methods and Results: A set of 12 monomeric flavan-3-ols and proanthocyanidins (degree of polymerization (DP) of 2–5), are fermented *in vitro* for 24 h using human faecal microbiota, and catabolism is analyzed by UHPLC-ESI-MS/MS. Up to 32 catabolites strictly related to microbial catabolism of parent compounds are detected. (+)-Catechin and (−)-epicatechin have the highest molar mass recoveries, expressed as a percentage with respect to the incubated concentration (75 µmol L^{-1}) of the parent compound, for total PVLs and PVAs, both at 5 h (about 20%) and 24 h (about 40%) of faecal incubation. Only A-type dimer and B-type procyanidins underwent the ring fission step, and no differences are found in total PVL and PVA production (≃1.5% and 6.0% at 5 and 24 h faecal incubation, respectively) despite the different DPs. Conclusion: The flavan-3-ol structure strongly affects the colonic catabolism of the native compounds, influencing the profile of PVLs and PVAs produced *in vitro*. This study opens new perspectives to further elucidate the colonic fate of oligomeric flavan-3-ols and their availability in producing bioactive catabolites.

Keywords: human gut microbiota, phenolic catabolites, phenyl-γ-valerolactones, procyanidins, stoichiometry

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1. Introduction

Flavan-3-ols are the most consumed flavonoids in the western diet,[1–4] with green tea, red wine, dark chocolate, and some fruits and nuts as the main contributors to their daily intake. Flavan-3-ols range from simple monomers to polymeric proanthocyanidins (PACs), also known as condensed tannins. Procyanidins (PCs) are exclusively composed of (epi)catechin units. B-type PCs have C_4–C_8 and/or C_4–C_6 linkages, while A type PCs contain an additional ether linkage between the C_2 in the B-ring of the adjacent unit and the oxygen-bearing C_7 in the A-ring of the terminal (epi)catechin unit.[5] After ingestion, monomeric flavan-3-ols are rapidly absorbed in the upper gastro-intestinal tract, while up to the 90% of the ingested PACs reach the colon intact where they become substrates for microbial breakdown.[6] The dietary intake of flavan-3-ols has been associated with several beneficial effects on human health,[7,8] and this is associated not with their native structure, but with the bioactivity of their circulating metabolites and catabolites,[5,9–15] among which phenyl-γ-valerolactones (PVLs) and phenylvaleric acids (PVAs) represent the most important C_6–C_5 catabolites.[5] These catabolites are specific to flavan-3-ols[5] and they exhibited various bioactive properties,[16–18] becoming plausible candidates responsible for the recognized biological activity attributed to the intake of their parent compounds. There are reports that the A-type dimer is more bioactive than its B-type counterpart[19] and that the bioactivity at a systemic level tends to be stronger with the increase in the degree of polymerization (DP).[20,21] However, these studies take into account neither the negligible absorption of the native oligomeric structures,[6] nor their colonic catabolism.[5,22,23] The structural properties of parent flavan-3-ol monomers and PACs may affect the native compound-colonic microflora interaction and could represent a key aspect of the beneficial properties associated with the intake of these phytochemicals. The catabolism of PCs has been investigated with *in vivo*[24–26] and *in vitro* studies,[27–29] and the production of PVLs from B-type dimers as well as from PCs contained in complex matrices such as nuts and fruits, has been well-established.[30–36] However, less is known about the interaction of the human gut microbiota with A-type dimers,[27] the ability of the microbiota to catabolize A- and/or B-type oligomers, and the possible production of PVLs and PVAs from these flavan-3-ol structures.

The aim of this study was to elucidate the influence of flavan-3-ol structure, including DP, different subunit linkages (A-/B-type), and the presence of galloyl moieties, on the production of catabolites, in particular PVLs and PVAs. Molar mass recoveries of gut microbiota catabolites were calculated to estimate their contribution to the colonic degradation of parent flavan-3-ols. The study also aimed at defining stoichiometric balances in the production of PVLs and PVAs to estimate the dose of parent compounds to be ingested to achieve a known amount of circulating 5-carbon side chain ring fission catabolites (5C-RFCs). Twelve flavan-3-ols were incubated individually with human faecal microbiota for 5 and 24 h and the resultant catabolites were analyzed by UHPLC-ESI-MS/MS.

2. Experimental Section

2.1. Chemicals and Reagents

Formic acid, bile salts, soluble starch, (+)-arabinogalactan, tryptone, yeast extract, xylan from birchwood, L-cysteine hydrochloride monohydrate, guar gum, inulin, Tween 80, buffered peptone water, Dulbecco's phosphate buffer saline (PBS), casein sodium salt from bovine milk, pectin from citrus fruits, mucin from porcine stomach-type III, $CaCl_2$, KCl, NaCl, $NaHCO_3$, anhydrous K_2HPO_4, KH_2PO_4, $MgSO_4$ monohydrate, $FeSO_4$ heptahydrate, resazurin redox indicator, (+)-catechin, (−)-epicatechin, phenylacetic acid, 4′-hydroxyphenylacetic acid, 3′-

hydroxyphenylacetic acid, 3′,4′-dihydroxyphenylacetic acid, 3-phenylpropanoic acid, 3-(4′-hydroxyphenyl)propanoic acid, 3-(3′-hydroxyphenyl)propanoic acid, 3-(3′,4′-dihydroxyphenyl)propanoic acid, benzoic acid, 4-hydroxybenzoic acid, 3-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 3,4,5-trihydroxybenzoic acid, benzene1,2,3-triol, benzene-1,3,5-triol, 3,4-dihydroxybenzaldehyde, and 4-hydroxybenzaldehyde were purchased from Sigma-Aldrich (St Louis, MO, USA). 5-(4′-Hydroxyphenyl)-valerolactone, 5-(3′-hydroxyphenyl)-valerolactone, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone and 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone were synthesized in house.[37] 5-(4′-Hydroxyphenyl)valeric acid was purchased from Toronto Research Chemicals (Toronto, ON, Canada). (−)-Epigallocatechin-3-*O*-gallate (EGCG), dimer A$_2$, dimer B$_2$ and trimer BB were purchased from Extrasynthese (Genay Cedex, France). Trimer AA, trimer AB, tetramer ABA, tetramer BBB and pentamer BBBB were purchased from PlantaAnalytica (New Milford, CT, USA). Theaflavin-3′-*O*-gallate was purchased from LGC STANDARD (Milan, Italy). Oligomers were checked for possible monomeric flavan-3-ol content by UHPLC-ESI-MS/MS analysis, resulting below 1%, *w/w*. All solvents and reagents were UHPLC-grade and were purchased from VWR International (Milan, Italy), unless otherwise indicated. Ultrapure water from MilliQ system (Millipore, Bedfort, MA, USA) was used throughout the experiment.

2.2. *In Vitro* Faecal Fermentation Substrates

Twelve flavan-3-ols, including the monomers (+)-catechin and (−)-epicatechin, oligomeric PACs with a different DP, including two dimers [dimer A$_2$, and dimer B$_2$], three trimers [trimer AA, trimer AB, and trimer BB], two tetramers [tetramer ABA, and tetramer BBB], and one pentamer, and two galloyl derivatives, namely EGCG and theaflavin-3′-*O*-gallate, were employed individually as substrates for microbial catabolism through *in vitro* faecal fermentations. Structural properties of these compounds are detailed in **Table 1**.

Table 1. Structural properties of monomeric and oligomeric flavan-3-ols used as substrates for microbial breakdown.

Compound	DP	Monomeric units	Linkage type	Galloy lunits
(+)-catechin	1	–	–	–
(−)-epicatechin	1	–	–	–
Dimer A2	2	(−)-epicatechin, (+)-epicatechin	A type	–
Dimer B2	2	(−)-epicatechin, (−)-epicatechin	B type	–
Trimer AA	3	(−)-epicatechin, (−)-epicatechin, (−)-catechin	A type, A type	–
Trimer AB	3	(−)-epicatechin, (−)-epicatechin, (−)-catechin	A type, B type	–
Trimer BB	3	(−)-epicatechin, (−)-epicatechin, (−)-epicatechin	B type, B type	–
Tetramer ABA	4	(−)-epicatechin, (+)-epicatechin, (−)-epicatechin, (−)-catechin	A type, B type, A type	–
Tetramer BBB	4	(−)-epicatechin, (−)-epicatechin, (−)-epicatechin, (−)-epicatechin	B type, B type, B type	–
Pentamer BBBB	5	(−)-epicatechin, (−)-epicatechin, (−)-epicatechin, (−)-epicatechin, (−)-epicatechin	B type, B type, B type, B type	–
(−)-Epigallocatechin-3-*O*-gallate	1	–	-	1
Theaflavin-3′-*O*-gallate	2	–	-	1

DP = degree of polymerization.

2.3. *In Vitro* Colonic Biotransformation Procedure

Growth medium and faecal slurries were prepared, and the *in vitro* incubations performed as described previously.[38–41] Briefly, 1 L of growth medium was prepared, aliquoted, and sterilized at 121 °C for 15 min in glass vessels (12 mL) before sample preparation. Fresh faeces were collected from three healthy volunteers (2 women and 1 man, aged 24.0 ± 5.6, height 1.7 ± 0.1 m, weigh 66.3 ± 15.5 kg, and BMI 21.8 ± 2.9 kg m^{-2} (mean ± SD)) who did not have any intestinal disease and were not treated with antibiotics for the previous 3 months.[38] Donors followed a controlled diet lacking (poly)phenol-containing foods for 2 days prior to faecal collection. After collection, faeces were immediately stored in an anaerobic jar and processed within 2 h. Faeces from donors were pooled in equal amount and homogenized with 1% *w/v* sterilized PBS to obtain a 10% *w/v* faecal slurry used as fermentation starter.[38] The Ethics Committee of Area Vasta Emilia Nord (AVEN) approved the collection and use of the faecal slurries (protocol no. 796/2018/sper/unipr) and all the donors provided informed consent for the collection of faecal slurries. Parent compounds were dissolved in an aqueous bile salt solution[42] and suspensions were left for 2 h at room temperature under constant magnetic stirring.[40] In each fermentation batch, 45% of the growth medium, 45% of faecal slurry, and 10% of substrate suspension were added to reach a total fermentation volume of 4 mL.[43] The faecal slurry and the aqueous product suspension were put into the vessel containing growth medium, were sealed, and flushed with N_2 to create anaerobiosis. Parent compounds were fermented at a final concentration of 75 μmol L^{-1}. This concentration is in line with the concentration of flavan-3-ols found in the ileal fluid of ileostomy patients consuming dietary amounts of flavan-3-ols.[6,44,45] Blank samples containing the growth medium and the faecal slurry, without substrate aqueous suspension, as well as abiotic control samples, containing the growth medium and the substrate suspensions without faecal starter, were also prepared.[43] Vessels were incubated for 24 h at 37 °C at 200 strokes min^{-1} in a Dubnoff bath (JULABO, Seelbach Germany). Samples were collected prior starting the fecal fermentation (0 h) and after 5 h and 24 h incubation. Microbial catabolism was stopped adding 10% *v/v* of acetonitrile,[40,41] and samples were frozen (-20 °C) until extraction and analysis. All experiments were carried out in triplicate.

2.4. Fermented Sample Preparation

Different solvents, including I) acidified methanol (0.1% *v/v* formic acid),[46] II) acidified acetonitrile (0.1% *v/v* formic acid),[47] III) acidified ethyl acetate (0.1% *v/v* formic acid)[30,40]) and a Solid Phase Extraction (SPE) method[48] were employed to determine the extraction efficiency from fermented samples for both parent compounds and their related gut microbiota catabolites, adopting the method of Di Pede et al.[40] with minor modifications. Based on these preliminary analyses, acidified methanol (0.1% *v/v* formic acid) displayed the best extraction recovery for the analytes. Briefly, an aliquot of 300 μL of each fermented sample was finally extracted with acidified methanol (0.1% *v/v* formic acid) (1.2 mL), vortexed for 30 s, sonicated for 10 min in an ultrasonic bath, vortexed for 30 s, and re-sonicated for 5 min. Samples were centrifuged (Centrisart A-14C Refrigerated Micro-Centrifuge and Rotor YCSR-A1C, Sartorius Lab Instruments GmbH and Co. KG, Goettingen, Germany) at 14 460 × g for 10 min and the upper organic layer was transferred into a clean microfuge tube. After the first extraction, the residual pellet of the fermented samples was re-extracted following the same procedure, using 500 μL of the same solvent. Finally, supernatants were pooled, vortexed for 30 s and centrifuged at 14 460 × g for 10 min before UHPLC-ESI-MS/MS analysis.

2.5. UHPLC-ESI-MS/MS Analysis

Samples were analyzed using a UHPLC DIONEX Ultimate 3000 fitted with a TSQ Vantage triple quadrupole mass spectrometer (MS) (Thermo Fisher Scientific Inc., San Jose, CA, USA) equipped with a heated-electrospray ionization source (H-ESIII; Thermo Fisher Scientific Inc.). Chromatographic and ionization parameters were based on the method of Brindani et al.[37] with slight modifications for UHPLC conditions: mobile phase A was 0.01% *v/v* formic acid in water and mobile phase B was acetonitrile containing 0.01% *v/v* formic acid. Substrates and their catabolites were monitored in the SRM mode. The evaluation of the range of calibration curves was based on data fitting to linear regression, and acceptable fitting was estimated by using the coefficient of determination (R^2). The limit of detection (LOD) and limit of quantification (LOQ) for reference standards were determined as the concentration in which the quantifier transition showed a signal-to-noise (S/N) ratio ≥ 3 and ≥ 10, respectively (Table S1, Supporting Information). Chromatograms and mass spectral data were acquired using Xcalibur software 2.1 (Thermo Fisher Scientific Inc.). Parent compounds were quantified by using calibration curves of respective standards in a concentration range of 0.05–10.0 μmol L^{-1}. When standards were not available catabolites were quantified by using calibration curves of structurally similar compounds, in a concentration range of 0.02–75.0 μmol L^{-1}.

2.6. Data and Statistical Analysis

Results were presented as mean values ± SD. In accordance with previous studies,[31,34] molar mass recoveries for catabolites were expressed as percentage (%) with respect to the incubated concentration of parent compound (75 μmol L^{-1}) to facilitate comparisons among the different fermented products. Molar mass recoveries in the production of total 5C-RFCs (PVLs and PVAs) and total catabolites were calculated as the sum of single molar mass recovery of 5C-RFCs and total catabolites, respectively. Stoichiometric balances in the production of 5C-RFCs (PVLs and PVAs) were estimated through molar mass recoveries assuming the production of PVLs and PVAs i) from 1 μmol of incubated parent compound, or ii) from 1 μmol of all the possible monomeric unit released from the oligomeric structure in accordance with the DP (2-5) of parent compound. General Linear Models (GLM) were used to evaluate i) the effect of time, treatment, and treatment of the incubation process (treatment × time) on molar mass recovery of monomeric and dimeric units, diphenylpropan-2-ols, PVLs, PVAs, 3-(phenyl)propanoic acid, benzoic acid, and benzene derivatives produced from parent compounds during the faecal incubations, ii) the effect of time on molar mass recovery of catabolites produced from fission of parent compounds with a DP >1, iii) the effect of time, treatment, and treatment × time on molar mass recoveries of total 5C-RFCs (PVLs and PVAs) and total catabolites. ANOVA with Tukey's post hoc test was applied to detect differences in molar mass recoveries of the catabolites produced from faecal incubations with different substrates over the same incubation period (T5 and T24). *t*-Test was applied to compare data on molar mass recoveries of catabolites considering the same fermented substrate for different incubation periods (T5 and T24), and for benzene derivatives produced from EGCG incubation (T5 *vs* T24) and from fermentation of EGCG versus theaflavin-3′-*O*-gallate at 24 h. A difference was considered significant at $p < 0.05$. PCA with varimax rotation was applied to explore differences in the behavior of parent compounds in the in vitro colonic environment and in the appearance of catabolites over the fecal fermentation. PCA was carried out taking into account the single concentrations of all the catabolites produced over time, with the exception of fission catabolites and theaflavin derived from the degradation of parent compounds having a DP >1 and from catabolism of theaflavin-3′-*O*-gallate, respectively. All statistical analyses were performed with the SPSS statistical software (version 26, SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Degradation of Substrates and Identification of Microbial Catabolites

Twelve substrates comprising monomeric flavan-3-ols and PACs (DP 2–5) were incubated with faecal material for

5 and 24 h at a concentration of 75 µmol L^{-1}. After 5 h faecal fermentation, the decrease of parent compounds ranged from 17% to 100% for trimer AA and (+)-catechin, respectively, and from 33% to >95% for theaflavin-3′-*O*-gallate and for the remaining substrates, respectively, after 24 h. Tetramer ABA was not catabolized by colonic microbiota over the 24 h-*in vitro* incubation. A total of 105 compounds were monitored by UHPLC-ESIMS/MS (Table S2, Supporting Information) and 32 catabolites were identified and quantified in the various samples following faecal fermentation. These comprised mainly diphenylpropan-2-ols, PVLs and their related PVAs, and 3-(phenyl)propanoic acids (**Table 2**).

Table 2. Gut microbiota catabolites identified after 5 and 24 h faecal fermentation of monomeric and oligomeric flavan-3-ols.

Molecular weight (Da)	Gut microbiota catabolites
	Fission catabolites of oligomers
	Dimers
	Derived from catabolic pathway of dimer A2
578	1 fission dimer A2, form 1
	Derived from catabolic pathway of dimer B2
580	1 fission dimer B2, form 1
582	2 fission dimer B2, form 1
	Trimers
	Derived from catabolic pathway of trimer AA
864	1 fission trimer AA, form 1
864	1 fission trimer AA, form 2
	Derived from catabolic pathway of trimer AB
866	1 fission trimer AB, form 1
866	1 fission trimer AB, form 2
	Derived from catabolic pathway of trimer BB
868	1 fission trimer BB, form 1
870	2 fission trimer BB, form 1
	Tetramers
	Derived from catabolic pathway of tetramer BBB
1156	1 fission tetramer BBB, form 1
1156	1 fission tetramer BBB, form 2
1156	1 fission tetramer BBB, form 3
1158	2 fission tetramer BBB, form 1
1160	3 fission tetramer BBB, form 1
1162	4 fission tetramer BBB, form 1
	Diphenylpropan-2-ols
276	1-(Hydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol
292	1-(3′,5′-Dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol
292	1-(3′,4′-Dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol
	Phenyl-γ-valerolactones
192	5-(4′-Hydroxyphenyl)-γ-valerolactone
192	5-(3′-Hydroxyphenyl)-γ-valerolactone
208	5-(3′,5′-Dihydroxyphenyl)-γ-valerolactone
208	5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone
	Phenylvaleric acids

194	5-(3′-Hydroxyphenyl)valeric acid
210	4-Hydroxy-5-(hydroxyphenyl)valeric acid
210	5-(3′,5′-Dihydroxyphenyl)valeric acid
210	5-(3′,4′-Dihydroxyphenyl)valeric acid
	Phenylpropanoic acids
166	3-(3′-Hydroxyphenyl)propanoic acid
182	3-(3′,5′-Dihydroxyphenyl)propanoic acid
	Benzoic acid
170	3,4,5-Trihydroxybenzoic acid
	Benzene derivative
126	Benzene-1,2,3-triol
	Monomer unit
290	(−)-Epicatechin
	Dimer unit
564	Theaflavin

The nomenclature of catabolites was standardized according to Kay et al·[60] Chromatographic and spectrometric information is provided in Table S2, Supporting Information. Structures of fission derivatives of dimer A2 and dimer B2 are reported in Figures S2 and S3, Supporting Information.

Retention time and selective reaction monitoring (SRM) conditions employed for identification and quantification of compounds are reported in Table S2, Supporting Information. In absence of available standards for some catabolites, the criteria for identification were based on previously reported LC-MS analyses.[5,30,34,37,41,49,50] In the control samples, consisting of growth medium and faecal material without flavan-3-ols substrates, or substrates and growth medium without the faecal starter, no more than negligible amounts of catabolites were detected, indicating that the (poly)phenol-free diet produced almost blank faeces, and that the incubation process did not influence the production of the quantified catabolites. A potential pathway of each *in vitro* fermented parent compounds is presented in **Figure 1**, indicating possible routes involved in the gut microbiota-mediated catabolism.

3.2. Molar Mass Recoveries for Catabolites

The main effects of treatment, time and treatment × time ($p < 0.001$) were observed for the production of diphenylpropan-2-ols [1-(hydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol, 1-(3′,5′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan2-ol, 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol], PVLs [5-(3′-hydroxyphenyl)-γ-valerolactone, 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone], PVAs [5-(3′-hydroxyphenyl)valeric acid, 4-hydroxy5-(hydroxyphenyl)valeric acid), 5-(3′,5′-dihydroxyphenyl)valeric acid, 5-(3′,4′-dihydroxyphenyl)valeric acid], 3-(3′-hydroxyphenyl) propanoic acid and 3-(3′,5′-dihydroxyphenyl)propanoic acid, benzoic acid and benzene derivatives [3,4,5-trihydroxybenzoic acid and benzene-1,2,3-triol], (−)-epicatechin and theaflavin (**Table 3**). A substantial significant effect of time ($p < 0.001$) was also observed for the production of oligomers with interflavanic linkage fission and/or C-ring opening (**Table 4**).

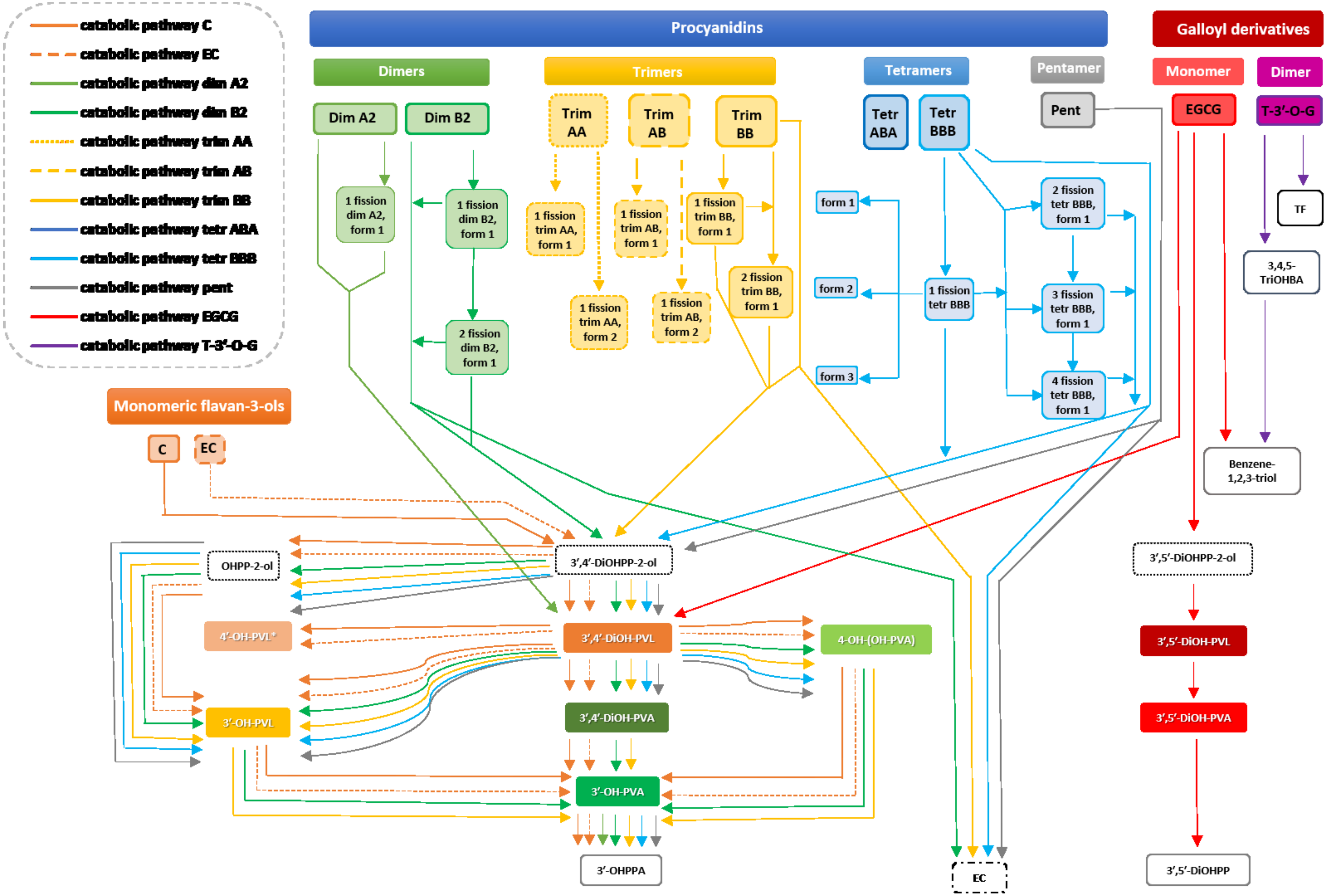
catabolic pathway C
catabolic pathway EC
catabolic pathway dim A2
catabolic pathway dim B2
catabolic pathway trim AA
catabolic pathway trim AB
catabolic pathway trim BB
catabolic pathway tetr ABA
catabolic pathway tetr BBB
catabolic pathway pent
catabolic pathway EGCG
catabolic pathway T-3′-O-G
Procyanidins
Galloyl derivatives
Dimers
Trimers
Tetramers
Pentamer
Monomer
Dimer
Dim A2
Dim B2
Trim AA
Trim AB
Trim BB
Tetr ABA
Tetr BBB
Pent
EGCG
T-3′-O-G
1 fission dim A2, form 1
1 fission dim B2, form 1
2 fission dim B2, form 1
1 fission trim AA, form 1
1 fission trim AA, form 2
1 fission trim AB, form 1
1 fission trim AB, form 2
1 fission trim BB, form 1
2 fission trim BB, form 1
form 1
form 2
form 3
1 fission tetr BBB
2 fission tetr BBB, form 1
3 fission tetr BBB, form 1
4 fission tetr BBB, form 1
TF
3,4,5-TriOHBA
Benzene-1,2,3-triol
Monomeric flavan-3-ols
C
EC
OHPP-2-ol
3′,4′-DiOHPP-2-ol
4′-OH-PVL*
3′,4′-DiOH-PVL
4-OH-(OH-PVA)
3′-OH-PVL
3′,4′-DiOH-PVA
3′-OH-PVA
3′-OHPPA
EC
3′,5′-DiOHPP-2-ol
3′,5′-DiOH-PVL
3′,5′-DiOH-PVA
3′,5′-DiOHPP

Figure 1. Proposed catabolic pathways and catabolites produced after *in vitro* faecal fermentation of monomeric and oligomeric flavan-3-ols. C: (+)-catechin; EC: (−)-epicatechin; Dim: dimer; Trim: trimer; Tetr: tetramer; Pent: pentamer; EGCG: (−)-epigallocatechin-3-*O*-gallate; T-3′-*O*-G: theaflavin-3′-*O*-gallate; OHPP-2-ol: 1-(hydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol; 3′,5′-DiOHPP-2-ol: 1-(3′,5′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol; 3′,4′-DiOHPP-2-ol: 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol; 3′-OH-PVL: 5-(3′-hydroxyphenyl)-γ-valerolactone; 4′-OH-PVL: 5-(4′-hydroxyphenyl)-γ-valerolactone; 3′,5′-DiOH-PVL: 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone; 3′,4′-DiOH-PVL:5-(3′,4′-dihydroxyphenyl)-γ-valerolactone; 3′-OH-PVA: 5-(3′-hydroxyphenyl)valeric acid; 4-OH-(OH-PVA): 4-hydroxy-5-(hydroxyphenyl)valeric acid; 3′,5′-DiOH-PVA: 5-(3′,5′-dihydroxyphenyl)valeric acid; 3′,4′-DiOH-PVA: 5-(3′-4′-dihydroxyphenyl)valeric acid; 3′-OHPPA: 3-(3′-hydroxyphenyl)propanoic acid; 3′,5′-DiOHPPA: 3-(3′,5′-dihydroxyphenyl)propanoic acid; 3,4,5-TriOHBA: 3,4,5-trihydroxybenzoic acid; TF: theaflavin. * means not quantified in fermented samples.

3.2.1. Diphenylpropan-2-ols and Fission Oligomers

Based on the incubated concentration of parent compound (75 μmol L^{-1}), at 5 h, the molar mass recovery of 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol derived from (−)-epicatechin incubation was significantly higher than after (+)-catechin incubation ($p < 0.001$). A significant reduction of 1-(3′,4′ dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol molar mass recovery was observed in parallel with the increase of the DP of the precursors (dimer B2, trimer BB, tetramer BBB, pentamer) at 5 h incubation, whereas an opposite trend was observed at 24 h (Table 3), where the recovery of 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol increased with the DP of oligomers. After 5 h incubation, (+)-catechin was more prone to produce 1-(hydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol, a putative dehydroxylated derivative of 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol, than (−)-epicatechin ($p < 0.001$). No significant differences ($p > 0.05$) emerged for the molar mass recoveries of 1-(hydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol after B-type oligomer incubation (dimer B2, trimer BB, tetramer BBB, pentamer) at 5 h, as well as between dimer B2 and trimer BB after 24 h incubation. Considering the different hydroxyl group pattern, a transient production of 1-(3′,5′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol was observed only after incubation of EGCG (Table 3). Taking into account catabolites derived from fission of oligomeric parent compounds (Table 3), B-type substrates (dimer B2, trimer BB, tetramer BBB) generally underwent various fission reactions with respect to their A and A-B type counterparts (dimer A2, trimer AA, trimer AB), except for tetramer ABA where catabolism did not occur. Fission catabolites might be produced from interflavan cleavage and/or C-ring opening (Figures S2 and S3, Supporting Information). The molar mass recovery for 1-fission tetramer BBB (form 1) was significantly higher after 5 h compared to its concentration at 24 h ($p < 0.001$). Other cleavage catabolites such as 1-fission dimer A2 (form 1), 1-fission trimer AA (form 2), and 1-fission trimer AB (forms 1 and 2) accumulated in significantly higher amounts after 24 h than after 5 h faecal incubation (Table 4).

3.2.2. PVLs and PVAs

The molar mass recovery of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone produced from the faecal biotransformation of (−)-epicatechin was 2.4- and 3.8-fold higher than the quantity recovered after (+)-catechin fermentation at 5 and 24 h incubation ($p < 0.001$ and $p < 0.01$), respectively (Table 3). Considering oligomers, the faecal incubation of dimer A2 led to the formation of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, although in a significant lower amount than after dimer B2 incubation at 24 h ($p < 0.001$). 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone from dimer A2 was the sole 5C-RFM identified after fermentation of A-type oligomers. B-type PACs (trimer BB, tetramer BBB, pentamer) were catabolized to 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (Table 3) regardless the DP and the incubation time. In contrast, 5-(3′-hydroxyphenyl)-γ-valerolactone, the 3′-monohydroxylated product of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, displayed no significant differences between the monomers (+)-catechin and (−)-epicatechin at 24 h, and it also accumulated after the 24 h incubation of all B-type precursors (dimer B2, trimer BB, tetramer and BBB, pentamer). Taking into account PVAs, although (−)-epicatechin led to the highest molar mass recovery for 4-hydroxy-5-(hydroxyphenyl)valeric acid and 5-(3′,4′-dihydroxyphenyl)valeric acid at 24 h, these catabolites were also produced after incubation of B-type PCs, reaching similar recoveries after a 24 h incubation regardless the DP. 5-(3′-Hydroxyphenyl)valeric acid, a monohydroxylated catabolite derived putatively from both 4-hydroxy-5-(hydroxyphenyl)valeric acid and 5-(3′,4′-dihydroxyphenyl)valeric acid, had the highest molar mass recovery when (−)-epicatechin was fermented for 24 h, followed by (+)-catechin, dimer B2 and trimer BB, for which a significant reduction was observed as the DP increased. Among galloyl derivatives, only EGCG was catabolized into two dihydroxylated PVLs, namely 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, and one dihydroxylated PVA (5-(3′,5′-dihydroxyphenyl)valeric acid), the latter increasing 12-fold at 24 h compared to the amount recovered after 5 h faecal incubation ($p < 0.01$) (Table 3). Stoichiometric balances for the production of 5C-RFMs (PVLs and PVAs) calculated after the in vitro fermentation of each parent compound are reported in Table S3.

3.2.3. Phenylpropanoic Acids

After the fermentation of 75 μmol L^{-1} of native compound, no significant differences were found in molar mass recovery for 3-(3′-hydroxyphenyl)propanoic acid derived from either (+)-catechin or (−)-epicatechin after a 5 h incubation or after 24 h when it was present in higher amounts (Table 3). 3-(3′-Hydroxyphenyl)propanoic acid was also one of the two low molecular weight catabolites recovered after dimer A2 faecal fermentation, together with 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, although its 24 h molar mass recovery was significantly lower compared to the amount recovered after dimer B2 catabolism ($p < 0.001$) (Table 3). Indeed, all B-type substrates (dimer B2, trimer BB, tetramer BBB, pentamer) resulted in the production of 3-(3′-hydroxyphenyl)propanoic acid, after 5 h fermentation and accumulated in high amounts after 24 h. Trimer BB also had the highest molar mass recovery of 3-(3′-hydroxyphenyl)propanoic acid after 24 h. A second 3-(hydroxyphenyl)propanoic acid, namely 3-(3′,5′-dihydroxyphenyl)propanoic acid, was produced exclusively by EGCG catabolism, and increased significantly over time with a 13.4-fold increase at 24 h compared to 5 h ($p < 0.001$).

3.2.4. Galloyl Derivatives of the Colonic Catabolism

The gallated substrates (EGCG and theaflavin-3′-*O*-gallate) both yielded benzene-1,2,3-triol but only theaflavin-3′-*O*-gallate yielded detectable 3,4,5-trihydroxybenzoic acid, although it is presumed to be the precursor of the benzene-1,2,3-triol (Table 3). The significant ($p < 0.001$) greater yield of the triol from EGCG at 24 h is consistent. Neither was detected after incubation of the other substrates. Theaflavin was detected following degallation but (−)-epigallocatechin was not.

Table 3. Molar mass recoveries (%) for diphenylpropan-2-ols, phenyl-γ-valerolactones (PVLs), phenylvaleric acids (PVAs), phenylpropanoic acids, benzoic acid and benzene derivatives, (−)- epicatechin (EC) and theaflavin (TF) produced after 5 and 24 h faecal fermentation of monomeric and oligomeric flavan-3-ols.

Substrates	Time (h)	EC	OHPP-2-ol	3′,5′-DiOHPP-2-ol	3′,4′-DiOHPP-2-ol	3′-OH-PVL	3′,5′-DiOH-PVL	3′,4′-DiOH-PVL	3′-OH-PVA	4-OH-(OH-PVA)	3′,5′-DiOH-PVA	3′,4′-DiOH-PVA	3′-OHPPA	3′,5′-DiOHPPA	3,4,5-TriOHBA	Benzene-1,2,3-triol	TF
Flavan-3-ol monomers																	
(+)-Catechin	5	n.d.	44.0 ± 2.0^{a}	-	0.2 ± 0.1^{d}	1.5 ± 0.3^{b}	-	6.1 ± 0.6$^{b;***}$	n.d.	0.2 ± 0.1	-	n.d.	4.5 ± 0.2^{a}	-	-	-	-
	24	n.d.	n.d.	-	n.d.	17.5 ± 5.3$^{a;***}$	-	1.3 ± 0.5^{b,c}	1.1 ± 0.1^{b}	8.7 ± 0.7$^{b;**}$	-	0.3 ± 0.1^{b,c}	15.5 ± 1.6$^{d,e;***}$	-	-	-	-
(−)-Epicatechin	5	n.d.	22.4 ± 2.2^{b}	-	12.6 ± 0.8^{a}	4.7 ± 0.8^{a}	-	14.9 ± 1.4$^{a;**}$	n.d.	0.7 ± 0.1	-	n.d.	4.4 ± 0.3^{a}	-	-	-	-
	24	n.d.	n.d.	-	n.d.	17.9 ± 15.3^{a}	-	5.0 ± 2.9^{a}	2.7 ± 1.0^{a}	12.9 ± 2.1$^{a;**}$	-	1.7 ± 0.4^{a}	12.0 ± 4.3$^{d,e;*}$	-	-	-	-
Flavan-3-ol dimers																	
Dimer A2	5	n.d.	n.d.	-	n.d.	n.d.	-	0.2 ± 0.0^{d,e}	n.d.	n.d.	-	n.d.	0.9 ± 0.3^{c}	-	-	-	-
	24	n.d.	n.d.	-	n.d.	n.d.	-	0.3 ± 0.1$^{c;*}$	n.d.	n.d.	-	n.d.	10.5 ± 3.6$^{e;**}$	-	-	-	-
Dimer B2	5	2.5 ± 1.4^{a}	1.5 ± 0.0$^{c;***}$	-	4.4 ± 2.6^{b}	n.d.	-	1.3 ± 1.3^{c,d,e}	n.d.	n.d.	-	n.d.	1.8 ± 0.5^{b}	-	-	-	-
	24	n.d.	0.1 ± 0.0^{c}	-	0.1 ± 0.0^{b}	5.4 ± 0.4^{b}	-	6.3 ± 0.5$^{a;**}$	0.1 ± 0.0^{c}	1.7 ± 0.2^{c,d}	-	0.2 ± 0.0^{c}	43.5 ± 1.1$^{b;***}$	-	-	-	-
Trimers																	
Trimer AA	5	n.d.	n.d.	-	n.d.	n.d.	-	n.d.	n.d.	n.d.	-	n.d.	n.d.	-	-	-	-
	24	n.d.	n.d.	-	n.d.	n.d.	-	n.d.	n.d.	n.d.	-	n.d.	n.d.	-	-	-	-
Trimer AB	5	n.d.	n.d.	-	n.d.	n.d.	-	n.d.	n.d.	n.d.	-	n.d.	n.d.	-	-	-	-
	24	n.d.	n.d.	-	n.d.	n.d.	-	n.d.	n.d.	n.d.	-	n.d.	n.d.	-	-	-	-
Trimer BB	5	1.1 ± 0.2$^{a,b;**}$	2.0 ± 0.6$^{c;**}$	-	2.5 ± 0.3$^{c;***}$	n.d.	-	1.9 ± 0.1$^{c;***}$	n.d.	n.d.	-	n.d.	4.2 ± 0.1^{a}	-	-	-	-
	24	0.3 ± 0.2^{c}	0.3 ± 0.1^{c}	-	0.1 ± 0.0^{b}	2.1 ± 0.5^{b}	-	1.0 ± 0.2^{b,c}	0.4 ± 0.1^{c}	2.1 ± 0.3^{c}	-	0.6 ± 0.0^{b}	68.6 ± 9.3$^{a;***}$	-	-	-	-
Tetramers																	
Tetramer ABA	5	n.d.	n.d.	--	n.d.	n.d.	-	n.d.	n.d.	n.d.	-	n.d.	n.d.	-	-	-	-
	24	n.d.	n.d.	-	n.d.	n.d.	-	n.d.	n.d.	n.d.	-	n.d.	n.d.	-	-	-	-
Tetramer BBB	5	0.2 ± 0.1^{b}	0.2 ± 0.0^{c}	-	0.8 ± 0.1^{c,d}	n.d.	-	1.9 ± 0.4^{c}	n.d.	n.d.	-	n.d.	4.7 ± 0.6^{a}	-	-	-	-
	24	1.5 ± 0.4$^{b;**}$	5.7 ± 1.7$^{a;**}$	-	3.1 ± 1.4^{a}	1.1 ± 0.6^{b}	-	3.6 ± 0.7$^{a,b;**}$	n.d.	0.2 ± 0.1^{c,d}	-	0.2 ± 0.1^{c}	19.6 ± 4.3$^{d;***}$	-	-	-	-
Pentamer																	
Pentamer BBBB	5	0.1 ± 0.0^{b}	0.1 ± 0.0^{c}	-	0.5 ± 0.0^{d}	n.d.	-	1.6 ± 0.1^{c,d}	n.d.	n.d.	-	n.d.	4.1 ± 0.8^{a}	-	-	-	-
	24	2.0 ± 0.5$^{a;***}$	3.4 ± 0.3$^{b;**}$	-	3.6 ± 1.7$^{a;*}$	0.9 ± 0.6^{b}	-	3.4 ± 0.2^{a}	n.d.	0.3 ± 0.2^{c,d}	-	0.6 ± 0.3^{b}	34.1 ± 1.6$^{c;***}$	-	-	-	-
Galloyl derivatives																	
Monomer																	
EGCG	5	n.d.	n.d.	6.6 ± 0.1	n.d.	n.d.	1.4 ± 0.2	0.7 ± 0.2^{c,d,e}	n.d.	n.d.	0.4 ± 0.0	n.d.	n.d.	3.9 ± 0.3	n.d.	20.2 ± 1.0***	-
	24	n.d.	n.d.	n.d.	n.d.	n.d.	1.7 ± 0.2	n.d.	n.d.	n.d.	4.8 ± 1.4**	n.d.	n.d.	48.5 ± 7.5***	n.d.	12.2 ± 2.0^{a}	-
Dimer																	
T-3′-*O*-G	5	-	-	-	-	-	-	-	-	-	-	-	-	-	3.6 ±	n.d.	3.9 ±

														0.7		0.8
24	-	-	-	-	-	-	-	-	-	-	-	-	-	17.5 ± 3.4**	5.8 ± 0.8^{b}	8.1 ± 2.0**

Molar mass recoveries for catabolites were expressed as percentage with respect to the incubated concentration of parent compound (75 μmol L^{1-}). Data are expressed as mean ± SD (n = 3). n.d.: not detected. EGCG: (−)-epigallocatechin-3-*O*-gallate; T-3′-*O*-G: theaflavin-3′-*O*-gallate; OHPP-2-ol:1-(hydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol; 3′,5′-DiOHPP-2-ol: 1-(3′,5′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol; 3′,4′-DiOHPP-2-ol: 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol; 3′-OH-PVL: 5-(3′-hydroxyphenyl)-γ-valerolactone; 3′,5′-DiOH-PVL: 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone; 3′,4′-DiOH-PVL: 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone; 3′-OH-PVA: 5-(3′-hydroxyphenyl)valeric acid; 4-OH-(OH-PVA): 4-hydroxy-5-(hydroxyphenyl)valeric acid; 3′,5′-DiOH-PVA: 5-(3′,5′-dihydroxyphenyl)valeric acid; 3′,4′-DiOH-PVA: 5-(3′-4′-dihydroxyphenyl)valeric acid; 3′-OHPPA: 3-(3′-hydroxyphenyl)propanoic acid; 3′,5′-DiOHPPA: 3-(3′,5′-dihydroxyphenyl)propanoic acid; 3,4,5-TriOHBA: 3,4,5-trihydroxybenzoic acid; TF: theaflavin. - indicates catabolites which cannot be produced through the colonic degradation pathway of the incubated parent compound. Cell colors range from light to dark blue, indicating the range from low to high molar mass recovery for each catabolite over time: dark color is reported only when light color was indicated. Different lower case letters indicate significant differences among different fermented parent compounds considering the same incubation period ($p < 0.05$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, indicate significant differences comparing the same fermented substrate after different incubation period.

Table 4. Molar mass recoveries (%) for catabolites produced by interflavanic linkage fission and/or C-ring opening of parent compounds having a DP >1 after 5 and 24 h faecal fermentation.

Compounds	5 h	24 h
Dimers		
Derived from catabolic pathway of dimer A2		
1 fission dimer A2, form 1	27.1 ± 4.4	37.0 ± 1.1**
Derived from catabolic pathway of dimer B2		
1 fission dimer B2, form 1	7.3 ± 1.7	n.d.
2 fission dimer B2, form 1	n.d.	4.1 ± 0.7
Trimers		
Derived from catabolic pathway of trimer AA		
1 fission trimer AA, form 1	n.d.	1.8 ± 0.0
1 fission trimer AA, form 2	54.4 ± 8.9	112.8 ± 2.6***
Derived from catabolic pathway of trimer AB		
1 fission trimer AB, form 1	15.8 ± 2.4	25.6 ± 2.3***
1 fission trimer AB, form 2	23.4 ± 2.4	51.2 ± 3.6***
Derived from catabolic pathway of trimer BB		
1 fission trimer BB, form 1	11.7 ± 1.6	n.d.
2 fission trimer BB, form 1	2.7 ± 0.1	n.d.
Tetramers		
Derived from catabolic pathway of tetramer BBB		
1 fission tetramer BBB, form 1	3.3 ± 0.5***	1.1 ± 0.3
1 fission tetramer BBB, form 2	n.d.	2.0 ± 0.5
1 fission tetramer BBB, form 3	n.d.	1.2 ± 0.2
2 fission tetramer BBB, form 1	n.d.	14.9 ± 1.9
3 fission tetramer BBB, form 1	3.2 ± 0.5	n.d.
4 fission tetramer BBB, form 1	n.d.	3.1 ± 0.3

Molar mass recoveries for catabolites were expressed as percentage with respect to the incubated concentration of parent compound (75 μmol L^{1-}). Data are expressed as mean ± SD (n = 3). n.d.: not detected; Cell colors range from light to dark blue, indicating the range from low to high molar mass recovery for each catabolite over time: dark color is reported only when light color was indicated. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, indicate significant differences comparing the same fermented substrate after different incubation period.

3.3. Comprehensive Assessment of the Differences in the Catabolism of Flavan-3-ols Incubated

A principal component analysis (PCA) including the concentrations of catabolites accumulating with most of the fermented substrates, namely diphenylpropan-2-ols (1-(hydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol, 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol, 1-(3′,5′-dihydroxyphenyl)-3-(2″,4″,6″ trihydroxyphenyl)-propan-2-ol), 5C-RFMs (5-(3′-hydroxyphenyl)-γ-valerolactone, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone and 5-(3′-hydroxyphenyl)valeric acid, 4-hydroxy-5-(hydroxyphenyl)valeric acid, 5-(3′,4′-dihydroxyphenyl)valeric acid, 5-(3′,5′-dihydroxyphenyl)valeric acid), 3-(phenyl)propanoic acids (3-(3′-hydroxyphenyl)propanoic acid and 3-(3′,5′-dihydroxyphenyl)propanoic acid), benzoic acid and benzene derivatives (3,4,5-trihydroxybenzoic acid and benzene-1,2,3-triol) and (−)-epicatechin, revealed the existence of some differences regarding both the amounts of catabolites and the time of their appearance, that depend on the substrate incubated (**Figures 2** and S1, Supporting Information).

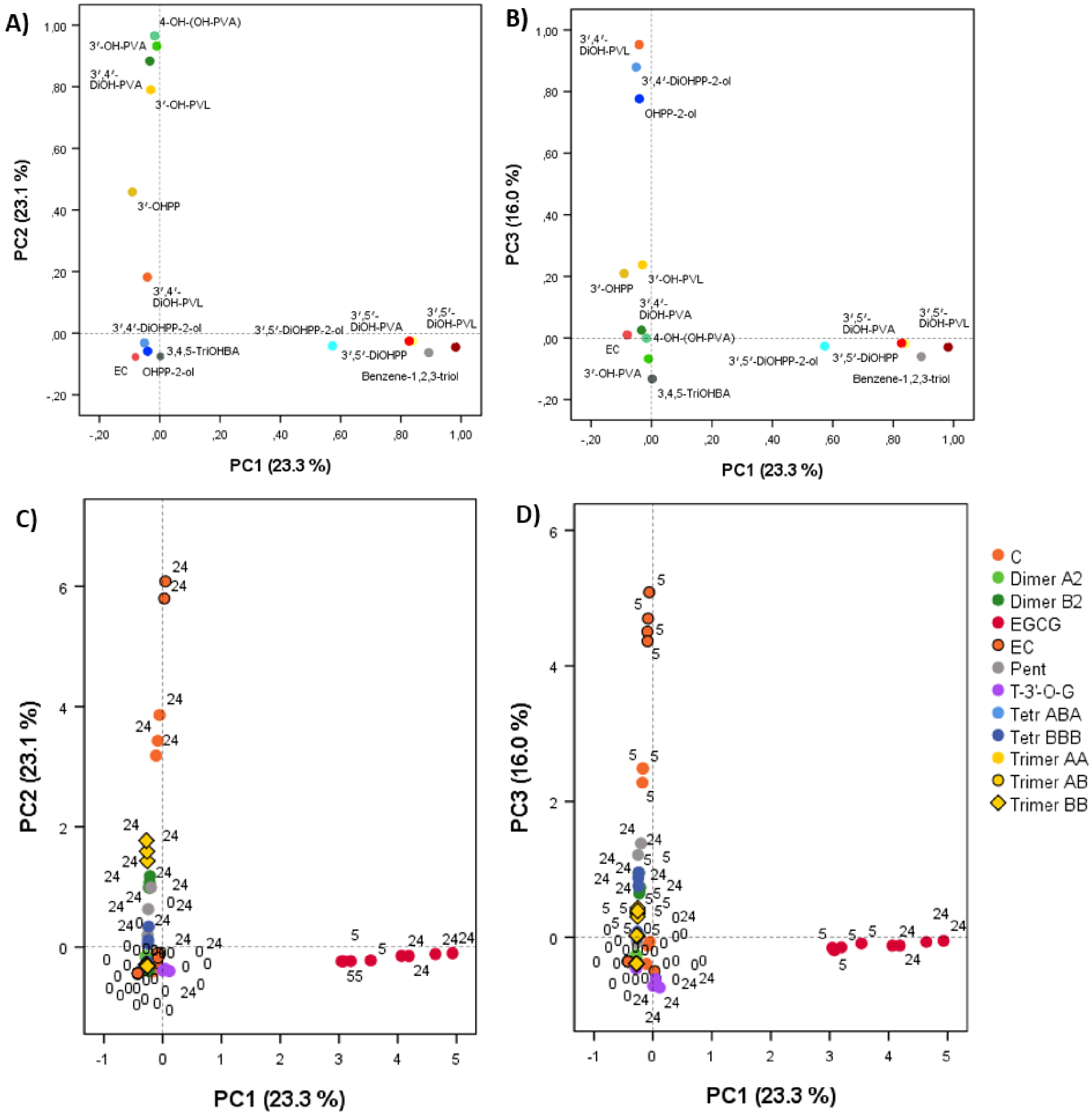

Figure 2. PCA to explore differences in the behavior of parent compounds in the *in vitro* colonic environment and in the appearance of catabolites over the faecal fermentation of monomeric and oligomeric flavan-3-ols. Loading plots of PC1 versus PC2 (A), PC1 versus PC3 (B). Score plots of the concentrations of identified catabolites obtained from PC1 and PC2 (C), PC1 and PC3 (D). The number accounts for the collection time (0, 5, and 24 h). C: (+)-catechin; EC: (−)-epicatechin; Dim: dimer; Trim: trimer; Tetr: tetramer; Pent: pentamer; EGCG: (−)-epigallocatechin-3-*O*-gallate; T-3′-*O*-G: theaflavin-3′-*O*-gallate. OHPP-2-ol: 1-(hydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol; 3′,5′-DiOHPP-2-ol: 1-(3′,5′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol; 3′,4′-DiOHPP-2-ol: 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol; 3′-OH-PVL: 5-(3′-hydroxyphenyl)-γ-valerolactone; 3′,5′-DiOH-PVL: 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone; 3′,4′-DiOH-PVL: 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone; 3′-OH-PVA: 5-(3′-hydroxyphenyl)valeric acid; 4-OH-(OH-PVA): 4-hydroxy-5-(hydroxyphenyl)valeric acid; 3′,5′-DiOH-PVA: 5-(3′,5′-dihydroxyphenyl)valeric acid; 3′,4′-DiOH-PVA: 5-(3′,4′-dihydroxyphenyl)valeric acid; 3′-OHPPA: 3-(3′-hydroxyphenyl)propanoic acid; 3′,5′-DiOHPPA: 3-(3′,5′-dihydroxyphenyl)propanoic acid; 3,4,5-TriOHBA: 3,4,5-

trihydroxybenzoic acid. Too specific compounds as fission catabolites from oligomers were not included.

Three principal components (PCs) explained up to 62.6% of the total variance. The first PC (PC1) accounted for the 23.3% of the total variability (Figure 2A and B) and it was positively loaded mainly by 3′,5′-dihydroxyphenyl catabolites (1-(3′,5′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol, 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone, 5-(3′,5′-dihydroxyphenyl)valeric acid, and 3-(3′,5′-dihydroxyphenyl)propanoic acid) and a benzene derivative (benzene-1,2,3-triol). PC2, representing the 23.1% of the variance (Figure 2A and Figure S1, Supporting Information), was positively linked to 3′,4′-dihydroxy and 3′hydroxy "late-products" of the colonic catabolism, such as some PVAs (5-(3′-hydroxyphenyl)valeric acid, 4-hydroxy-5-(hydroxyphenyl)valeric acid, and 5-(3′,4′-dihydroxyphenyl)valeric acid), a 3-(3′-hydroxyphenyl)propanoic acid and a monohydroxylated PVL (5-(3′-hydroxyphenyl)-γ-valerolactone). In contrast, the third PC (PC3) accounted for the 16.0% of the total variability (Figure 2B and Supporting information Figure S1) and it was positively loaded mainly by "early products" of the catabolic pathway, such as diphenylpropan-2-ols (1-(hydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol and 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan2-ol) and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone. Score plots accounting for the catabolic pathway of each precursor are presented in Figure 2C and D. As expected, EGCG was characterized by the production of 3′,5′-dihydroxyphenyl catabolites, as showed by PC1 scores, being distinct from the rest of the parent compounds (Figure 2C). Monomers elicited a well defined and different behavior in the production of phenolic catabolites in comparison to oligomers. Monomers were clearly distinguished considering the early and late products of their catabolic pathways: at 5 h, (−)-epicatechin had the highest positive values for PC3 (5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and diphenylpropan-2-ols), followed by (+)-catechin (Figure 2D); at 24 h, (−)-epicatechin was more prone to the production of late catabolites (PVAs, 3-(3′-hydroxyphenyl)propanoic acid, and 5-(3′-hydroxyphenyl)-γ-valerolactone) than (+)-catechin (Figure 2C). This indicated that the catabolism of (−)-epicatechin occurred at a faster rate than (+)-catechin. On the other hand, if early products of the catabolic pathway of (−)-epicatechin and (+)-catechin (diphenylpropan-2-ols and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone) appeared at 5 h, these catabolites appeared in lower amounts, and later after B-type oligomer incubation, as shown by PC2 and PC3 scores (Supporting Information Figure S1). Finally, the catabolism of PACs containing A-type linkages was minimal as they showed values close to zero for all the PCs (Figure 2C and D).

3.4. Quantitative Profiles in the Production of Total 5-Carbon Side Chain Ring Fission Catabolites and Total Microbial Catabolites

Significant main effects of treatment, time and treatment × time ($p < 0.001$) were found for the molar mass recovery calculated for total 5C-RFCs, including PVLs and PVAs (**Figure 3**) and for the sum of all quantified catabolites (**Figure 4**), produced after 5 and 24 h faecal fermentation of parent compounds. Considering the incubated concentration, (+)-catechin and (−)-epicatechin led to the highest molar mass recoveries for total 5C-RFCs at 5 h (7% and 20% for (+)-catechin and (−)-epicatechin, respectively) and 24 h (29% and 40% for (+)-catechin and (−)-epicatechin, respectively) of faecal incubation. Total PVLs and PVAs produced from EGCG incubation, beside the significant increase over the incubation process ($p < 0.01$), was in line with 5C-RFCs produced after oligomer incubation (Figure 3A). Despite the absence of a significant difference between dimer A2 and dimer B2 at 5 h, the molar mass recovery for total 5C-RFCs after dimer B2 fermentation was significantly higher than that derived from dimer A2 at the end of the incubation period (24 h, $p < 0.01$) (Figure 3B). The incubation of 75 μmol L^{-1} of B-type oligomers did not lead to significant differences in total 5C-RFC recovery after 5 and 24 h, which resulted ≃1.5% and ≃5.4%, respectively, for trimer BB, tetramer BBB, and pentamer BBBB (Figure 3C and D). Considering the whole set of catabolites, most of the precursors led to a different set of catabolites, with some statistically significant differences in their overall recovery (Figure 4). Of note, trimer AA and trimer AB underwent fission without producing any low molecular weight phenolic catabolites (Figure 4). The molar mass recovery of total catabolites significantly increased over time for almost all the incubated parent flavan-3-ols, ranging from 6% to 59%, for pentamer and (+)-catechin, respectively, at 5 h (Figure 4A), and from 31% to 114%, for theaflavin-3′-*O*-gallate and trimer AA, respectively, at 24 h (Figure 4B). The relative contribution of all the catabolite classes quantified after 5 and 24 h of faecal fermentation of different flavan-3-ols is showed in Figure 5. Fission and diphenylpropan-2-ol derivatives were generally the most abundant catabolites produced at 5 h, ranging from 38% to 100%, and from 7% to 78%, respectively. With the exception of A-type trimers, 3- (3′-hydroxyphenyl)propanoic acid was the most representative catabolite after a 24 h incubation, followed by 5C-RFCs (in particular PVLs) (Figure 5).

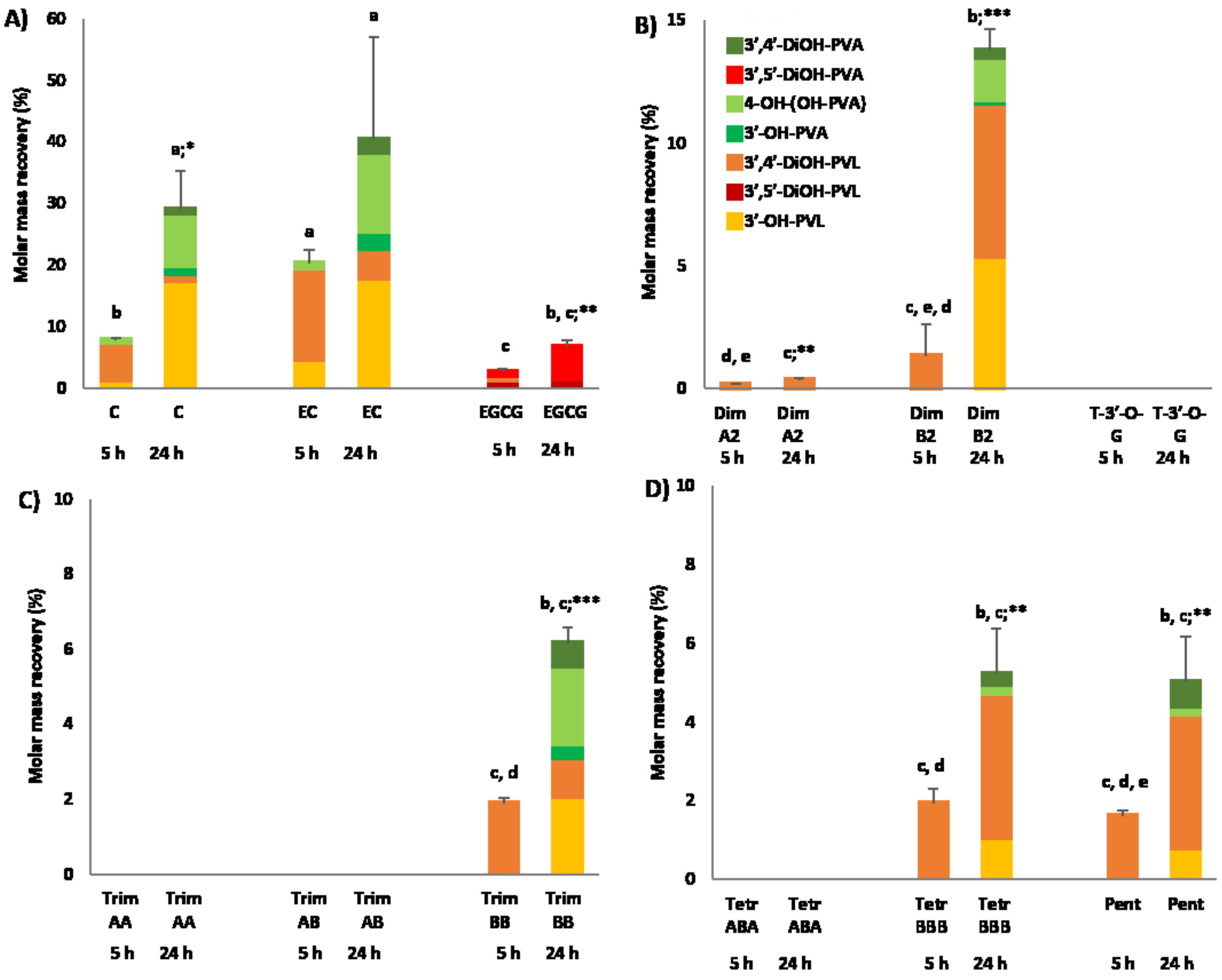

Figure 3. Molar mass recovery (%), reported as mean ± SD (n = 3), for the production of 5C-RFCs (PVLs and PVAs) produced after 5 and 24 h faecal fermentation of C, EC and EGCG (A), dimers and T-3′-*O*-G (B), trimers (C), tetramers and pentamer (D). Different lower case letters indicate significant differences among different fermented parent compounds considering the same incubation period ($p < 0.05$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ indicate significant differences comparing the same fermented substrate after different incubation period. Molar mass recoveries for catabolites were expressed as percentage with respect to the incubated concentration of parent compound (75 µmol L^{-1}). C: (+)-catechin; EC: (−)-epicatechin; EGCG: (−)-epigallocatechin-3-*O*-gallate; Dim: dimer; T-3′-*O*-G: theaflavin-3′-*O*-gallate; Trim: trimer; Tetr: tetramer; Pent: pentamer; 3′,4′-DiOH-PVA:5-(3′,4′-dihydroxyphenyl)valeric acid; 3′,5′-DiOH-

PVA: 5-(3′,5′-dihydroxyphenyl)valeric acid; 4-OH-(OH-PVA): 4-hydroxy-5-(hydroxyphenyl)valeric acid; 3′-OH-PVA: 5-(3′-hydroxyphenyl)valeric acid; 3′,4′-DiOH-PVL: 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone; 3′,5′-DiOH-PVL: 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone; 3′-OH-PVL: 5-(3′-hydroxyphenyl)-γ-valerolactone.

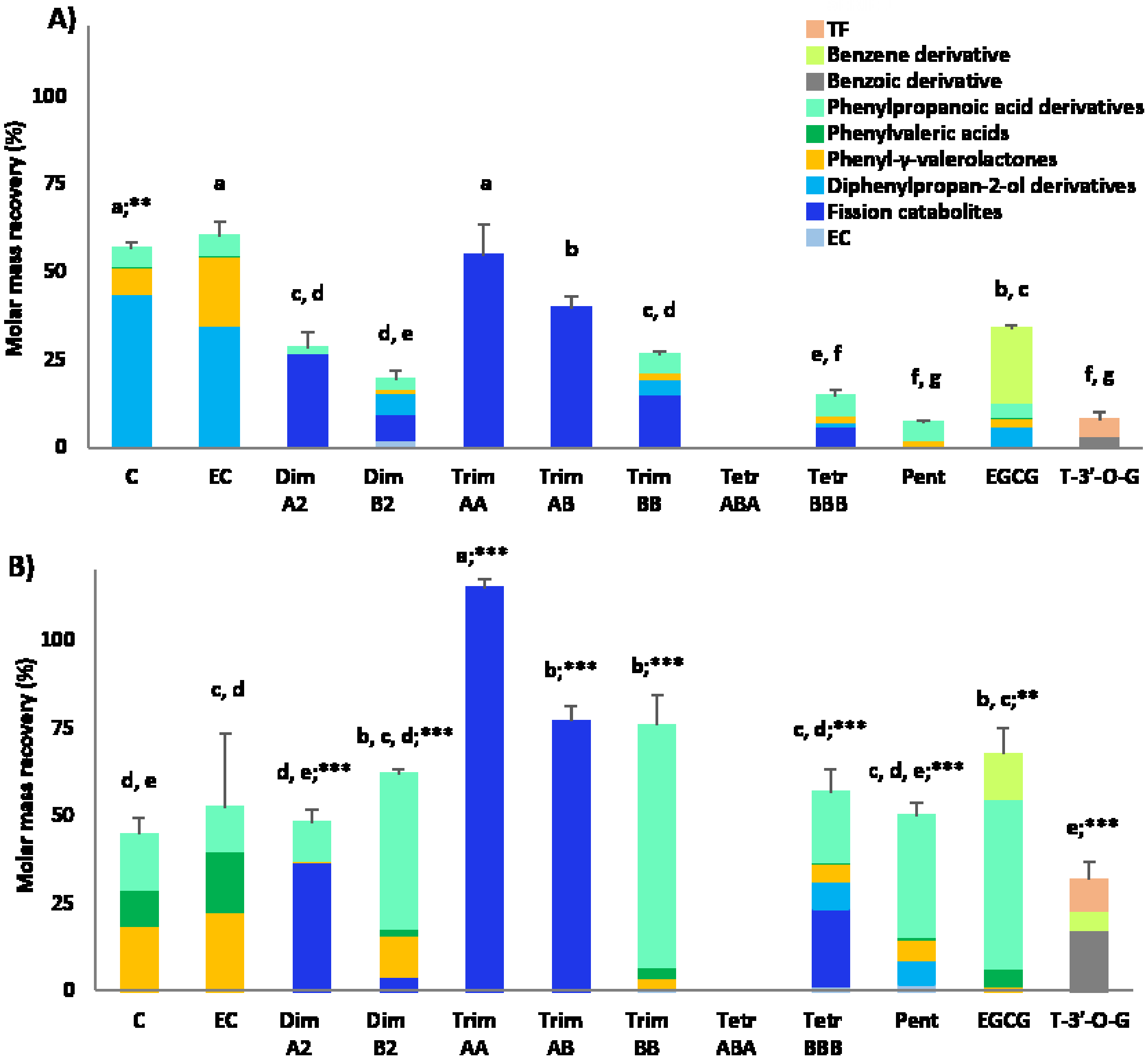

Figure 4. Molar mass recovery (%), reported as mean ± SD (n = 3), for the production of total catabolites produced after 5 (A) and 24 h (B) of faecal fermentation of monomeric and oligomeric flavan-3-ols. Different lower case letters indicate significant differences among different fermented parent compounds considering the same incubation period ($p < 0.05$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ indicate significant differences comparing the same fermented substrate after different incubation period. Molar mass recoveries for catabolites were expressed as percentage with respect to the incubated concentration of parent compound (75 µmol L^{-1}). C: (+)-catechin; EC: (−)-epicatechin; Dim: dimer; Trim: trimer; Tetr: tetramer; Pent: pentamer; EGCG: (−)-epigallocatechin-3-*O*-gallate; T-3′-*O*-G: theaflavin-3′-*O*-gallate; TF: theaflavin. Diphenylpropan-2-ol derivatives include OHPP-2-ol, 3′,5′-diOHPP-2-ol and 3′,4′-diOHPP-2-ol. Fission catabolites include compounds produced by fission reaction of parent compounds having a DP above 1 (it might be either interflavan cleavage or C-ring opening).

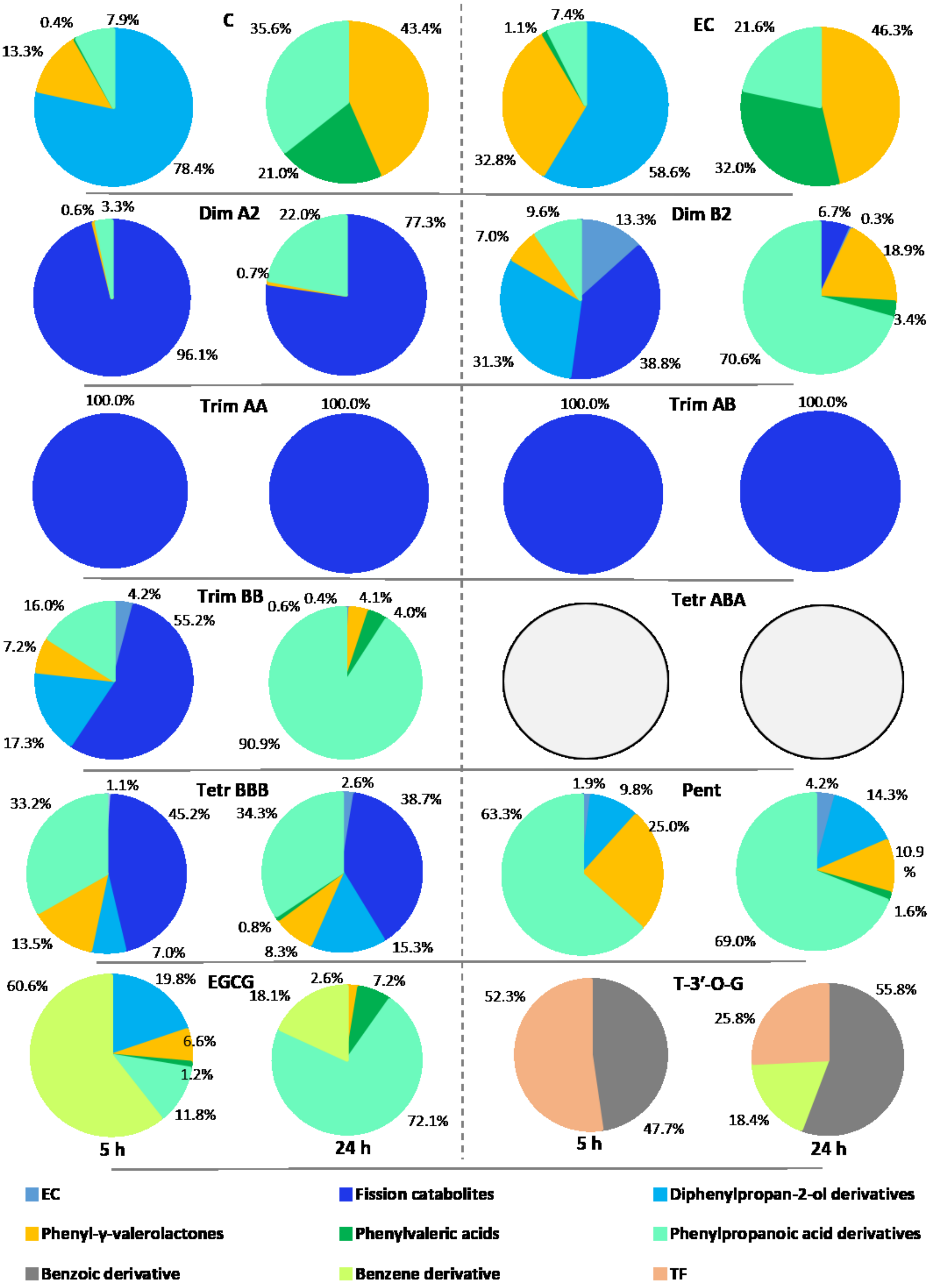

Figure 5. Relative contribution of catabolite classes after 5 and 24 h faecal fermentation of monomeric and oligomeric flavan-3-ols. C: (+)-catechin; EC: (−)-epicatechin; Dim: dimer; Trim: trimer; Tetr: tetramer; Pent: pentamer; EGCG: (−)-epigallocatechin-3-*O*-gallate; T-3′-*O*-G: theaflavin-3′-*O*-gallate; TF: theaflavin. Diphenylpropan-2-ol derivatives include OHPP-2-ol, 3′,5′-diOHPP-2-ol and 3′,4′-diOHPP-2-ol. Fission catabolites include compounds produced by fission reaction of parent compounds having a DP above 1 (it might be either interflavan cleavage or C-ring opening).

4. Discussion

In the present study, a heterogeneous set of flavan-3-ol monomers, dimers and oligomers were fermented *in vitro* to elucidate the impact of the DP and nature of the inter-flavan linkage on the production of colonic catabolites. Up to 32 flavan-3-ol catabolites were identified (Table 2), confirming that, except for tetramer ABA, all the incubated substrates underwent extensive catabolism. Limited fission of interflavan bonds was observed for substrates with only B-type linkages consistent with previous reports.[30,34,51] The major catabolic pathway is via reductive C-ring cleavage, yielding diphenylpropan-2-ol derivatives.[5,52] The subsequent A ring cleavage led to PVLs and further PVAs by γ-valerolactone ring opening,[5] prior to dehydroxylation, primarily at the 4′ position of the B-ring (Figure 1), followed by further oxidation steps. Diphenylpropan-2-ol derivatives were quantified both after monomer ((+)-catechin, (−)-epicatechin, and EGCG) and B type oligomer catabolism. The formation from tetramer BBB of three catabolites with masses 2 amu higher than the substrate suggests that at least three of the monomer units are susceptible to reductive C-ring scission. In contrast, dimer B2 and trimer BB yielded only one such catabolite, but it is possible that some isomers coeluted. The formation from dimer B2 and trimer BB of catabolites having masses 4 amu higher than the substrate indicate that reductive C-ring opening can occur twice in the same substrate. The catabolite 6 amu larger than trimer BB was sought but not found. The detection of catabolites with masses 4, 6, and 8 amu higher than the substrate in the tetramer BBB incubation demonstrates that for this substrate all four monomer units are susceptible, but, surprisingly, the pentamer did not yield any catabolites. In contrast for substrates with A-type linkages (dimer A2, trimer AA, trimer AB) the equivalent catabolites had only increased by 2 amu indicating that only one unit had been reduced in each substrate, although the detection of two trimeric isomers indicates that at least two monomer units are accessible. Accordingly, catabolites 4 amu, and perhaps 6 amu higher, might have been detected if the incubations had continued beyond 24 h.

The C-ring opened catabolite of an (–)-epicatechin-A-type dimer has been reported previously,[27,53] but so far as we are aware, this is the first report of such catabolites from an A-type trimer and of catabolites produced from oligomeric PACs by reduction of more than two C-rings (Table 4). If monomers and end-units of B-type PCs mainly undergo the C-ring fission through the activity of different colonic microbial strains,[30,50,54,55] A-type PCs could potentially be subjected to Cring opening steps, as well as fission of interflavan bonds.[27,28,53] However, it still remains unclear whether, prior to further C-ring catabolism, the catabolic route of A-type PCs involves only the interflavan bond fission or is also responsible for the possible formation a quinone methide derivatives through a rearrangement of the B-ring after interflavan bond fission.[28,53] In contrast, no fission reactions beyond the loss of the galloyl moiety occurred in theaflavin-3′-*O*-gallate faecal incubates. Focusing on PVL and PVA production, although several studies reported the formation of these catabolites after monomer and B-type dimer *in vitro* fermentation,[5] it is evident that the structural configuration of the monomers (Figure 2), as well as the PC structure and the DP, affected the quantitative PVL and PVA profile. The highest molar mass recoveries for total 5C-RFCs found with (+)-catechin and (−)-epicatechin (Figure 3) indicate that they were more efficiently converted into PVLs and PVAs than oligomers, in accordance with previous *in vitro* studies.[31,34,50] The current study demonstrated that B-type oligomers with high DP produce 5C-RFCs. Stoupi and colleagues[34] showed that the cleavage of the interflavan bond represents a minor pathway of the colonic catabolism of dimer B2, as no more than 10% was converted into (−)-epicatechin in an *in vitro* faecal model. Since no significant differences were found in total PVL and PVA molar mass recovery in parallel with the increase of the DP of B type oligomers (less than 10%) (Figure 3), it seems likely that only a small fraction of monomeric units are released from the native structure, and become available for the C-ring opening needed for the production of 5C-RFMs. Previously, Stoupi and colleagues[34] demonstrated that the lower unit of dimer B2 might be more easily accessible to colonic microbiota activity.

Consequently, the main catabolic route of B-type oligomers may involve direct ring fission of the terminal unit of oligomers, without any interflavan bond cleavage and monomer unit release. The low yield of C_6-C_5 catabolites from oligomers compared with monomers and dimers could be due to the steric hindrance limiting access to the C-ring. In keeping with this possibility it has been reported that no microbial catabolites are produced from 3-methoxy-(+)-catechin intake.[56] Regarding 5C-RFM and A-type PACs, this is the first study, to the best of our knowledge, to have demonstrated the production of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone following human faecal incubation of dimer A2. Recently, Chen et al.[28] identified 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone after *in vitro* fermentation of dimer A2 using rat faecal microbiota. However, there is an absence of information with human microbiota. The low molar mass recovery of total PVLs and PVAs calculated for dimer A2 compared to the dimer B2 (Figure 3) highlights that the additional ether C_2-C_7 linkage strongly affects the C-ring cleavage step *in vitro*. The DP also negatively influenced the colonic biotransformation of A-type PCs. While dimer A2 catabolism yielded a few low molecular weight microbial catabolites, trimer AA and trimer AB underwent only a single fission-catabolic reaction, whereas tetramer ABA was not catabolized by gut microbiota over the 24 h incubation period. An absence ring fission was also observed with theaflavin-3′-*O*-gallate, that did not lead to PVLs or PVAs in accordance with previous reports.[57,58]

From a stoichiometric point of view, the results of the present investigation indicate that the ingestion of ≈3 μmol of (+)-catechin and (−)-epicatechin or *ca.* 15 μmol of EGCG would potentially produce 1 μmol of circulating 5C-RFMs (Table S3, Supporting Information). These quantities are in line with the estimation obtained for B-type substrates: ≈7 and 18 μmol of dimer B2 and of the remaining B-type substrates (trimer BB, tetramer BBB, pentamer), respectively, would be needed to achieve 1 μmol of PVL and PVA catabolites. In contrast, based on the reduced ability of gut microbiota to catabolize A-type PCs, more than 290 μmol of dimer A2 are needed to produce 1 μmol of 5CRFMs. However, all (epi)catechin subunits of PAC oligomers are potentially available for microbial catabolism after microbial depolymerization. Consequently, assuming the full release of each (epi)catechin unit, in accordance with the DP (2-5) of the native structure, the production of 1 μmol of 5C-RFMs after the complete breakdown of oligomers drastically decreased (Table S3, Supporting Information), suggesting that oligomers may be a poor source of these C_6-C_5 catabolites (Table S3, Supporting Information). In a human feeding trial with apple extracts containing flavan-3-ol monomers and oligomers (DP 2–10), Hollands et al.[36] found that the 22% of ingested monomers were excreted as PVLs, whereas with oligomers there appeared to be negligible ring fission *in vivo*. Similarly, 202 μmol (representing the 26% of 24 h urinary phenolic catabolites) of PVLs and PVAs were excreted in urine following ingestion of Elstar apples containing 775 μmol of monomers.[59] Following intake of cocoa flavan-3-ols, Ottaviani and colleagues[26] pointed out that monomers represent a more significant source of PVLs than PCs, while Wiese and colleagues[25] highlighted that the 24 h urinary excretion of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone represented, respectively, 6%, 4% and 0.7% of ingested (−)-epicatechin, dimer B1 and oligomers with a mean DP of 5.9.

Among 3-(phenyl)propanoic acids, derived from β-oxidation of the side chain of PVAs,[5] only 3-(3′-hydroxyphenyl)propanoic acid was quantified after monomer and PC fermentation. Appeldorn and colleagues[30] found high amount of 3-(3′-hydroxyphenyl)propanoic acid following *in vitro* faecal incubation of B-type dimers, suggesting a rapid conversion of PVAs into the mono-hydroxylated 3-(phenyl)propanoic acids. The limited number of C_6-C_2 C_6-C_1 and C_6 gut microbiota catabolites detected in the present work beyond C_6-C_3 3-(phenyl)propanoic acids could be due to the incubation periods: the fermentation stopped at 24 h, whereas some low molecular weight catabolites usually increase in later faecal incubation phases.[27,34,35] Secondly, some low molecular weight catabolites have relatively poor MS ionization, resulting in a higher limit of detection and quantification (see Table S1, Supporting Information). The lack of some lower molecular weight catabolites could have partially influenced also the stoichiometric balance, since the molar mass recoveries calculated for total gut microbiota catabolites rarely reached the 100% (Figure 4). In addition, the quantification of some catabolites should be considered as semi-quantification due to the absence of reference standards, possibly resulting in a mis-estimation of their levels.

Finally, although all the expected catabolites related to the known catabolic pathways of flavan-3-ols[5] were monitored, some intermediates and/or unknown re-arranged catabolites whose production was previously hypothesized,[27,28,51,55] might have increased the molar mass recovery for some compounds. Nevertheless, the present study opens new perspectives for an untargeted catabolomic approach addressing a complete, data-driven investigation of the catabolites produced from PACs in the human colonic environment, as well as for NMR investigations to fully understand the structures of possible newly formed catabolites. In conclusion, this study investigated for the first time the behavior of a variety of individual flavan-3-ol substrates in an *in vitro* human colonic environment. New catabolic routes, resulting in PC fission catabolites, have been established. The study sheds light on how structure can affect the interaction between the native flavan-3-ols and colonic microbial catabolic activity. The structural heterogeneity of native substrates strongly affected the profile of PVLs and PVAs produced during the *in vitro* fermentations. The calculated stoichiometric balances in the production of 5C-RFMs could potentially support the experimental design of *in vivo* and *in vitro* models aiming at evaluating the catabolism of flavan-3-ols in different experimental settings (i.e., bioavailability and dose-response studies, cell models, etc.). If catabolites are plausible candidates responsible for the recognized biological activity generally attributed to their native compounds,[9,11,13] further studies are needed to fully clarify the I) catabolic fate of heterogeneous PAC sources and their ability to produce key bioactive catabolites, as well as other possible catabolites not yet identified, and II) which microbial populations might be involved in the catabolic pathway of oligomer flavan-3-ols in the colonic environment and thus, adding evidence on the type and interaction modes of these phytocompounds with the human gut microbiota.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

G.D.P., L.B., D.D.R., and P.M. designed the study. G.D.P. conducted the study and performed the analysis. G.D.P., L.B., and P.M. analyzed and interpreted data. G.D.P. drafted the manuscript. L.B., P.M. F.B., M.N.C., A.C., and D.D.R. edited the manuscript. All authors critically read and approved the final version of the manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting information

Table S1. Limit of detection (LOD) and limit of quantification (LOQ) (µmol L^{-1}) for reference standards used for the identification and quantification of gut microbiota catabolites. The nomenclature of gut microbiota catabolites was standardized according to Kay et al.[1]

Compound	LOD (µmol L^{-1})	LOQ (µmol L^{-1})
(+)-Catechin	0.02	0.05
(−)-Epicatechin	0.02	0.05
Dimer A2	0.02	0.05
Dimer B2	0.02	0.05
Trimer AA	0.02	0.05
Trimer AB	0.05	0.25
Trimer BB	0.05	0.25
Tetramer ABA	0.02	0.05
Tetramer BBB	0.05	0.25
Pentamer BBBB	0.05	0.25
(−)-Epigallocatechin-3-*O*-gallate	0.05	0.25
Theaflavin-3′-*O*-gallate	0.25	1.00
5-(4′-Hydroxyphenyl)-γ-valerolactone	0.06	1.12
5-(3′-Hydroxyphenyl)-γ-valerolactone	0.01	0.03
5-(3′,5′-Dihydroxyphenyl)-γ-valerolactone	0.02	0.06
5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone	0.02	0.06
5-(3′,4′,5′-Trihydroxyphenyl)-γ-valerolactone	0.30	5.00
5-(4′-Hydroxyphenyl)valeric acid	0.25	5.00
3-Phenylpropanoic acid	0.25	2.00
3-(4′-Hydroxyphenyl)propanoic acid	2.00	5.00
3-(3′-Hydroxyphenyl)propanoic acid	2.00	5.00
3-(3′,4′-Dihydroxyphenyl)propanoic acid	0.25	2.00
Phenylacetic acid	0.05	50.00
4′-Hydroxyphenylacetic acid	2.00	5.00
3′-Hydroxyphenylacetic acid	10.00	25.00
3′,4′-Dihydroxyphenylacetic acid	0.05	2.00
Benzoic acid	2.00	5.00
4-Hydroxybenzoic acid	0.25	2.00
3-Hydroxybenzoic acid	0.25	2.00
3,4-Dihydroxybenzoic acid	0.05	0.25
3,4,5-Trihydroxybenzoic acid	0.05	0.25
4-Hydroxybenzaldehyde	0.05	0.25
Benzene-1,3,5-triol	0.25	2.00
Benzene-1,2,3-triol	0.25	2.00
3,4-Dihydroxybenzaldehyde	0.05	0.25

Table S2. Retention times and selective reaction monitoring (SRM) conditions for identification and quantification of monomeric and oligomeric flavan-3-ols and their gut microbiota catabolites by UHPLC-ESI-MS/MS. The nomenclature of gut microbiota catabolites was standardized according to Kay et al.[1]

Compound (Abbreviation)	**RT (min)**	**Parent ion (M - H)$^-$ (*m/z*)**	**S-lens**	**Quantifier**		**Qualifier**		**Standard used for quantification**
				Product ion (*m/z*)	**CE (V)**	**Product ion (*m/z*)**	**CE (V)**	
Parent compounds								
Monomeric flavan-3-ols								
(+)-Catechin	3.65	289	112	245	18	203	23	(+)-Catechin
(−)-Epicatechin	4.32	289	112	245	18	203	23	(−)-Epicatechin
Oligomeric flavan-3-ols								
Dimers								
Dimer A2	4.91	575	131	289	25	449	25	Dimer A2
Dimer B2	4.22	577	131	289	27	407	25	Dimer B2
Trimers								
Trimer AA	4.95	861	140	861	25	-	-	Trimer AA
Trimer AB	4.82	863	140	863	25	289	25	Trimer AB
Trimer BB	4.50	865	140	865	25	289	25	Trimer BB
Tetramers								
Tetramer ABA	5.05	574*	145	289	25	574	25	Tetramer ABA
Tetramer BBB	4.59	576*	145	289	22	125	42	Tetramer BBB
Pentamer								
Pentamer BBBB	4.68	720.6*	204	289	34	125	45	Pentamer BBBB
Galloyl derivatives								
Monomer								
(−)-Epigallocatechin-3-*O*-gallate (EGCG)	4.42	457	86	169	21	125	44	EGCG
Dimer								
Theaflavin-3′-*O*-gallate	5.60	715	98	563	30	715	30	Theaflavin-3′-*O*-gallate
Fission catabolites								
Dimers								
Derived from catabolic pathway of dimer A2								
1 Fission Dimer A2, form 1	5.18	577	131	577	25	291	25	Dimer A2
2 Fission Dimer A2, form 1	-	579	-	-	-	-	-	n.d.
Derived from catabolic pathway of dimer B2								
1 Fission Dimer B2, form 1	4.89	579	131	291	25	289	25	Dimer B2

2 Fission Dimer B2, form 1	5.53	581	131	581	25	291	25	Dimer B2
Trimers								
Derived from catabolic pathway of trimer AA								
1 Fission Trimer AA, form 1	5.06	863	140	863	25	-	-	Trimer AA
1 Fission Trimer AA, form 2	5.32	863	140	863	25	-	-	Trimer AA
2 Fission Trimer AA, form 1	-	865	-	-	-	-	-	n.d.
3 Fission Trimer AA, form 1	-	867	-	-	-	-	-	n.d.
Derived from catabolic pathway of trimer AB								
1 Fission Trimer AB, form 1	5.07	865	140	865	25	291	25	Trimer AB
1 Fission Trimer AB, form 2	5.17	865	140	865	25	291	25	Trimer AB
2 Fission Trimer AB, form 1	-	867	-	-	-	-	-	n.d.
3 Fission Trimer AB, form 1	-	869	-	-	-	-	-	n.d.
Derived from catabolic pathway of trimer BB								
1 Fission Trimer BB, form 1	4.71	867	140	867	25	291	25	Trimer BB
2 Fission Trimer BB, form 1	4.86	869	140	869	25	-	-	Trimer BB
3 Fission Trimer BB, form 1	-	871	-	-	-	-	-	n.d.
Tetramers								
Derived from catabolic pathway of tetramer ABA								
1 Fission Tetramer ABA, form 1	-	576*	-	-	-	-	-	n.d.
2 Fission Tetramer ABA, form 1	-	578*	-	-	-	-	-	n.d.
3 Fission Tetramer ABA, form 1	-	580*	-	-	-	-	-	n.d.
4 Fission Tetramer ABA, form 1	-	582*	-	-	-	-	-	n.d.
Derived from catabolic pathway of tetramer BBB								
1 Fission Tetramer BBB, form 1	4.80	578*	145	291	25	578	25	Tetramer BBB
1 Fission Tetramer BBB, form 2	5.60	578*	145	578	25	289	25	Tetramer BBB
1 Fission Tetramer BBB, form 3	6.21	578*	145	578	25	-	-	Tetramer BBB
2 Fission Tetramer BBB, form 1	5.54	580*	145	580	25	291	25	Tetramer BBB
3 Fission Tetramer BBB, form 1	4.63	582*	145	291	25	289	25	Tetramer BBB
4 Fission Tetramer BBB, form 1	5.23	584*	145	584	25	-	-	Tetramer BBB
Pentamers								
Derived from catabolic pathway of pentamer BBBB								
1 Fission Pentamer, form 1	-	722.6*	-	-	-	-	-	n.d.
2 Fission Pentamer, form 1	-	724.6*	-	-	-	-	-	n.d.

3 Fission Pentamer, form 1	-	726.6*	-	-	-	-	-	n.d.
4 Fission Pentamer, form 1	-	728.8*	-	-	-	-	-	n.d.
5 Fission Pentamer, form 1	-	730.6*	-	-	-	-	-	n.d.
Theaflavin-3′-*O*-gallate derivatives								
Derived from catabolic pathway of theaflavin								
1 Fission Theaflavin, form 1	-	565	-	-	-	-	-	n.d.
2 Fission Theaflavin, form 1	-	567	-	-	-	-	-	n.d.
Derived from catabolic pathway of theaflavin-3′-O-gallate								
1 Fission Theaflavin-3′-*O*-gallate	-	717	-	-	-	-	-	n.d.
2 Fission Theaflavin-3′-*O*-gallate	-	719	-	-	-	-	-	n.d.
Diphenylpropan-2-ol derivatives								
1-(Hydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol	4.84	275	98	231	16	191	30	3′,4′-diOH-PVL
1-(3′,5′-Dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol	4.16	291	98	123	30	167	30	3′,5′-diOH-PVL
1-(3′,4′-Dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol	4.36	291	98	123	30	247	16	3′,4′-diOH-PVL
1-(3′,4′,5′-Triihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol	-	307	-	-	-	-	-	n.d.
1-(3′,4′,5′-Trihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl) propan-2-yl gallate[§]	-	459	-	-	-	-	-	n.d.
Phenyl-γ-valerolactones								
5-Phenyl-γ-valerolactone	-	175	-	-	-	-	-	n.d.
5-(4′-Hydroxyphenyl)-γ-valerolactone	4.92	191	67	147	16	106	31	n.q.
5-(3′-Hydroxyphenyl)-γ-valerolactone (3′-OH-PVL)	5.07	191	67	147	16	106	31	3′-OH-PVL
5-(3′,5′-Dihydroxyphenyl)-γ-valerolactone (3′,5′-diOH-PVL)	3.98	207	81	163	18	123	20	3′,5′-diOH-PVL
5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone (3′,4′-diOH-PVL)	4.33	207	81	163	18	122	21	3′,4′-diOH-PVL
5-(3′,4′,5′-Trihydroxyphenyl)-γ-valerolactone	2.55	223	75	179	21	138	26	n.d.
Phenylvaleric acids								
5-Phenylvaleric acid	-	177	-	-	-	-	-	n.d.
5-(4′-Hydroxyphenyl)valeric acid	5.46	193	71	147	30	175	30	n.d.
5-(3′-Hydroxyphenyl)valeric acid	5.56	193	71	147	30	175	30	3′-OH-PVL
4-Hydroxy-5-(phenyl)valeric acid	-	193	-	-	-	-	-	n.d.
4-Hydroxy-5-(hydroxyphenyl)valeric acid	4.38	209	63	147	20	101	20	3′,4′-diOH-PVL
5-(3′,5′-Dihydroxyphenyl)valeric acid	4.75	209	63	191	15	147	20	3′,5′-diOH-PVL
5-(3′,4′-Dihydroxyphenyl)valeric acid	5.02	209	63	191	15	165	12	3′,4′-diOH-PVL
4-Hydroxy-5-(3′,5′-Dihydroxyphenyl)valeric acid	-	225	-	-	-	-	-	n.d.
4-Hydroxy-5-(3′,4′-Dihydroxyphenyl)valeric	-	225	-	-	-	-	-	n.d.
5-(3′,4′,5′-Trihydroxyphenyl)valeric acid	-	225	-	-	-	-	-	n.d.

4-Hydroxy-5-(3′,4′,5′-Trihydroxyphenyl)valeric acid	-	241	-	-	-	-	-	n.d.
Phenylbutyric acids								
4-Phenylbutyric acid	-	163	-	-	-	-	-	n.d.
4-(4′-Hydroxyphenyl)butyric acid	-	179	-	-	-	-	-	n.d.
4-(3′-Hydroxyphenyl)butyric acid	-	179	-	-	-	-	-	n.d.
4-(3′,4′-Dihydroxyphenyl)butyric acid	-	195	-	-	-	-	-	n.d.
4-(3′,5′-Dihydroxyphenyl)butyric acid	-	195	-	-	-	-	-	n.d.
4-(3′,4′,5′-Trihydroxyphenyl)butyric acid	-	211	-	-	-	-	-	n.d.
Phenylpropanoic acid derivatives								
3-Phenylpropanoic acid	5.64	149	60	149	10	105	13	n.d.
3-(4′-Hydroxyphenyl)propanoic acid	4.37	165	64	121	13	93	14	n.d.
3-(3′-Hydroxyphenyl)propanoic acid (3′-OHPPA)	4.65	165	64	121	13	119	18	3′-OHPPA
2-Hydroxy-3-(phenyl)propanoic acid	-	165	-	-	-	-	-	n.d.
3-(3′,5′-Dihydroxyphenyl)propanoic acid	2.58	181	64	137	14	-	-	3′,4′-diOHPPA
3-(3′,4′-Dihydroxyphenyl)propanoic acid (3′,4′-diOHPPA)	3.27	181	64	137	14	109	18	n.d.
2-Hydroxy-3-(hydroxyphenyl)propanoic acid	-	181	-	-	-	-	-	n.d.
3-(4′-Hydroxy-3′-methoxyphenyl)propanoic acid	-	195	-	-	-	-	-	n.d.
3-(3′,4′,5′-Trihydroxyphenyl)propanoic acid	-	197	-	-	-	-	-	n.d.
Phenylacetic acid derivatives								
Phenylacetic acid	5.04	135	40	91	10	135	10	n.d.
4′-Hydroxyphenylacetic acid	3.14	151	75	107	13	-	-	n.d.
3′-Hydroxyphenylacetic acid	3.79	151	75	107	13	-	-	n.d.
3′,4′-Dihydroxyphenylacetic acid	1.85	167	35	123	13	122	28	n.d.
3′,4′,5′-Trihydroxyphenylacetic acid	-	183	-	-	-	-	-	n.d.
Benzoic acid derivatives								
Benzoic acid	4.98	121	68	77	13	121	10	n.d.
4-Hydroxybenzoic acid	2.73	137	72	93	16	-	-	n.d.
3-Hydroxybenzoic acid	3.62	137	72	93	16	-	-	n.d.
3,4-Dihydroxybenzoic acid	1.62	153	74	109	16	108	26	n.d.
3,5-Dihydroxybenzoic acid	-	153	-	-	-	-	-	n.d.
3,4,5-Trihydroxybenzoic acid (3,4,5-triOHBA)	0.96	169	78	125	17	79	25	3,4,5-triOHBA
Benzene derivatives								
Benzene-1,2-diol	-	109	-	-	-	-	-	n.d.
4-Hydroxybenzaldehyde	3.70	121	68	92	26	120	20	n.d.

Hydroxybenzyl alcohol	-	123	-	-	-	-	-	n.d.
Benzene-1,3,5-triol	0.88	125	62	125	10	57	17	n.d.
Benzene-1,2,3-triol	1.06	125	62	125	10	79	22	benzene-1,2,3-triol
3,4-Dihydroxybenzaldehyde	2.34	137	72	136	22	108	26	n.d.
2,4,6-Trihydroxybenzaldeyde	-	153	-	-	-	-	-	n.d.
Monomer units								
(−)-Epigallocatechin	-	305	-	-	-	-	-	n.d.
(−)-Epicatechin-3-*O*-gallate	-	441	-	-	-	-	-	n.d.
Dimer units								
Theanaphthoquinone[#]	-	533	-	-	-	-	-	n.d.
Theaflavin	5.45	563	98	241	30	269	30	Theaflavin-3′-*O*-gallate

RT = retention time; *m/z* = mass to charge ratio; CE = collision energy; n.d. = not detected; n.q. = not quantified because below the LOQ. §catabolite previously quantified after faecal fermentation of EGCG[2]; ~catabolite previously quantified after faecal fermentation of EGCG[3]; #catabolite previously quantified after faecal fermentation of theaflavin-3,3′-digallate[2]. * Compounds were monitored as $[M-2H]^{2-}$.

Table S3. Stoichiometric balances in the production of total 5C-RFMs (PVLs and PVAs) from each parent compound fermented *in vitro*.

Compound	**24 h MMR for total 5C-RFMs * (%)**	**Quantity of parent compound to achieve 1 µmol of total 5C-RFMs (µmol)**	**24 h MMR for total 5C-RFMs § (%)**	**Quantity of parent compound to achieve 1 µmol of total 5C-RFMs after complete breakdown of each monomeric subunit (µmol)**
Monomeric flavan-3-ols				
(+)-Catechin	29.0	3.5	29.0	3.5
(−)-Epicatechin	40.2	2.5	40.2	2.5
Oligomeric flavan-3-ols				
Dimers				
Dimer A2	0.3	295.3	0.2	590.5
Dimer B2	13.7	7.3	6.9	14.5
Trimers				
Trimer AA	0.0	0.0	0.0	0.0
Trimer AB	0.0	0.0	0.0	0.0
Trimer BB	6.2	16.2	2.1	48.7
Tetramers				
Tetramer ABA	0.0	0.0	0.0	0.0
Tetramer BBB	5.2	19.2	1.3	76.8
Pentamer				
Pentamer BBBB	5.0	20.0	1.0	99.8
Galloyl derivatives				
Monomer				
(−)-Epigallocatechin-3-*O*-gallate	6.6	15.2	6.6	15.2
Dimer				
Theaflavin-3′-*O*-gallate	0.0	0.0	0.0	0.0

MMR: molar mass recovery; *MMR calculated assuming the production of 5C-RFMs from 1 µmol of incubated parent compound; §MMR calculated assuming the production of 5C-RFMs from 1 µmol of all the possible monomeric unit released from the oligomeric structure in accordance with the DP (2-5) of parent compound.

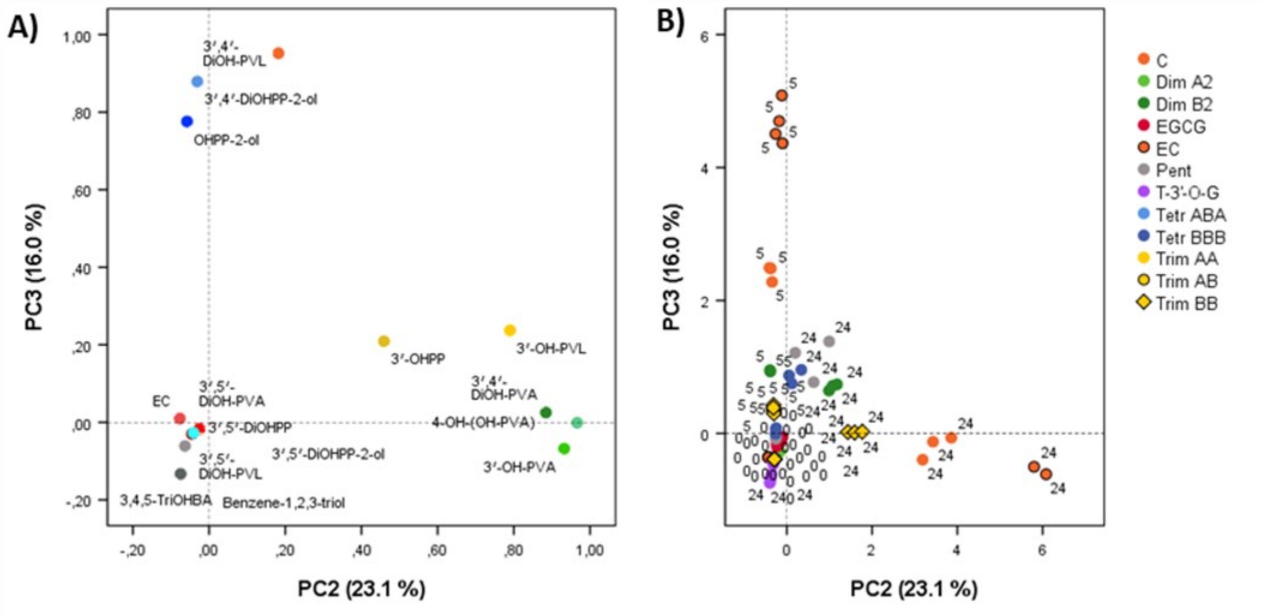
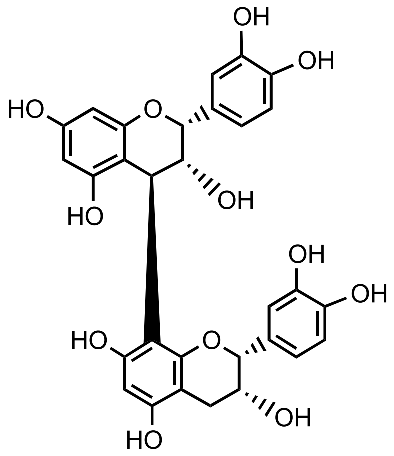
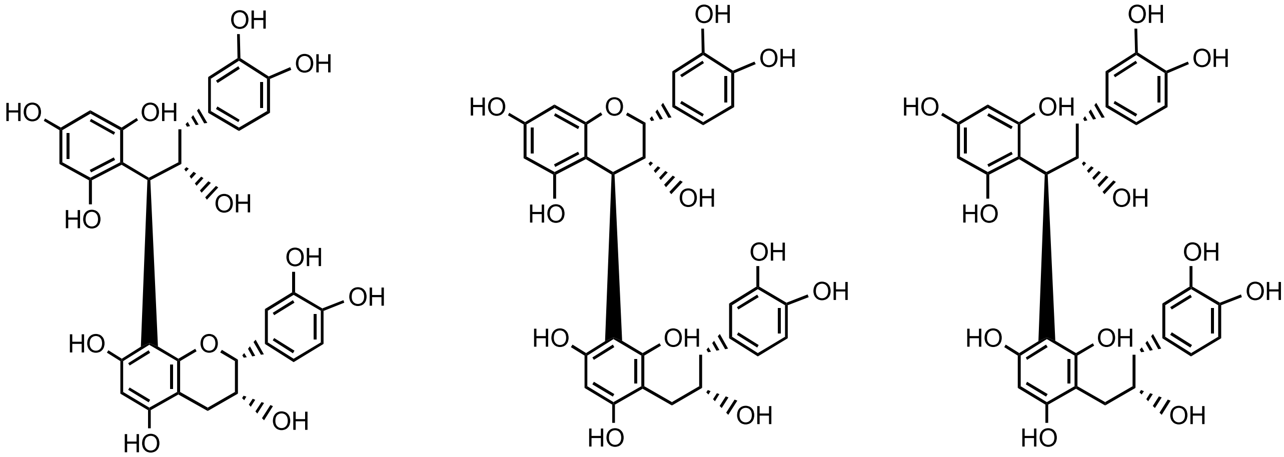


Figure S1. Principal component analysis (PCA) to explore differences in the behavior of parent compounds in the *in vitro* colonic environment and in the appearance of gut microbiota catabolites over the faecal fermentation of monomeric and oligomeric flavan-3-ols. Loading plots of PC2 versus PC3 (A). Score plots of the concentrations of identified gut microbiota catabolites obtained from PC2 and PC3 (B). The number accounts for the collection time (0 h, 5 h and 24 h).

C: (+)-catechin; EC: (−)-epicatechin; Dim: dimer; Trim: trimer; Tetr: tetramer; Pent: pentamer; EGCG: (−)-epigallocatechin-3-*O*-gallate; T-3′-*O*-G: theaflavin-3′-*O*-gallate. OHPP-2-ol: 1-(hydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol; 3′,5′-DiOHPP-2-ol: 1-(3′,5′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol; 3′,4′-DiOHPP-2-ol: 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol; 3′-OH-PVL: 5-(3′-hydroxyphenyl)-γ-valerolactone; 3′,5′-DiOH-PVL: 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone; 3′,4′-DiOH-PVL: 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone; 3′-OHPVA: 5-(3′-hydroxyphenyl)valeric acid; 4-OH-(OH-PVA): 4-hydroxy-5-(hydroxyphenyl)valeric acid; 3′,5′-DiOHPVA: 5-(3′,5′-dihydroxyphenyl)valeric acid; 3′,4′-DiOH-PVA: 5-(3′,4′-dihydroxyphenyl)valeric acid; 3′-OHPP: 3-(3′-hydroxyphenyl)propanoic acid; 3′,5′-DiOHPP: 3-(3′,5′-dihydroxyphenyl)propanoic acid; 3,4,5-TriOHBA: 3,4,5-trihydroxybenzoic acid. Too specific compounds as fission catabolites from oligomers were not included.



Procyanidin B_2 dimer

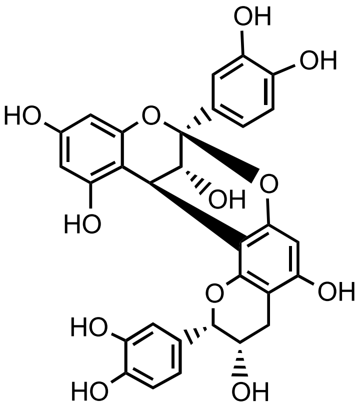


Fission upper unit

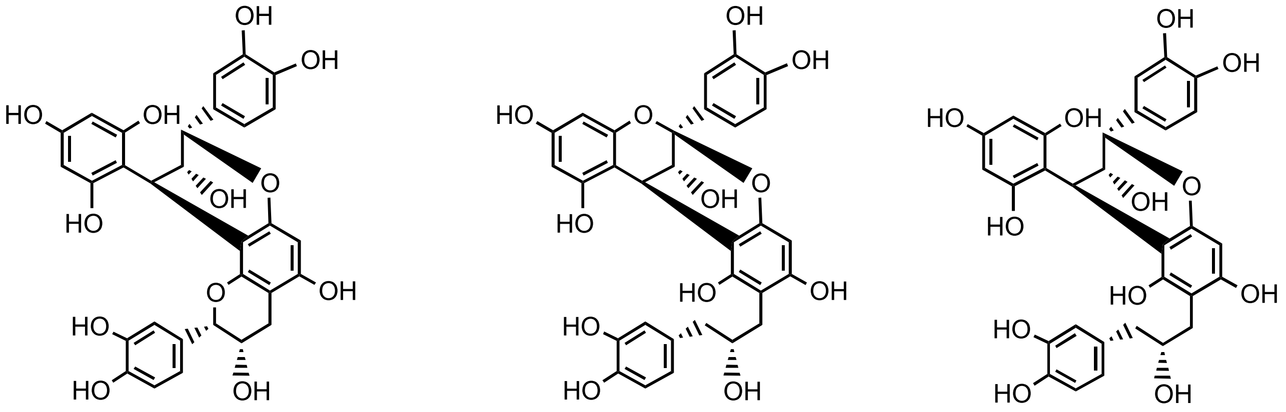
Fission lower unit

Fission both units

Figure S2. Fission derivatives of dimer B2



Procyanidin A_2 dimer



Fission upper unit

Fission lower unit

Fission both units

Figure S3. Fission derivatives of dimer A2

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Chapter 3. The human Microbial Metabolism of Quercetin in Different Formulations: An In Vitro Evaluation

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The human Microbial Metabolism of Quercetin in Different Formulations: An In Vitro Evaluation

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Abstract: Quercetin is one of the main dietary flavonols, but its beneficial properties in disease prevention may be limited due to its scarce bioavailability. For this purpose, delivery systems have been designed to enhance both stability and bioavailability of bioactive compounds. This study aimed at investigating the human microbial metabolism of quercetin derived from unformulated and phytosome-formulated quercetin through an *in vitro* model. Both ingredients were firstly characterized for their profile in native (poly)phenols, and then fermented with human fecal microbiota for 24 h. Quantification of microbial metabolites was performed by ultra-high performance liquid chromatography coupled to mass spectrometry (uHPLC-MS^n) analyses. Native quercetin, the main compound in both products, appeared less prone to microbial degradation in the phytosome-formulated version compared to the unformulated one during fecal incubation.

Quercetin of both products was bioaccessible to colonic microbiota, resulting in the production of phenylpropanoic acid, phenylacetic acid and benzoic acid derivatives. The extent of the microbial metabolism of quercetin was higher in the unformulated ingredient, in a time-dependent manner. This study opened new perspectives to investigate the role of delivery systems on influencing the microbial metabolism of flavonols in the colonic environment, a pivotal step in the presumed bioactivity associated to their intake.

Keywords: flavonol; quercetin; human microbial metabolism; phenolic metabolite; phytosome

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1. Introduction

Dietary flavonoids represent a range of C_6-C_3-C_6 compounds that are widely diffused in fruits, vegetables, grains, herbs, and beverages [1]. The main subclasses of dietary flavonoids are flavonols, flavan-3-ols, anthocyanidins, flavones, isoflavones, and flavanones [2]. Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one) is one of the main dietary flavonols, which are the most ubiquitous flavonoids in foods [3]. Tea, red wine, berries, apples, tomatoes, and onions are theprimary sources of dietary quercetin in the typical Western diet, the daily intake of which is estimated to be up to 30 mg [4]. The role of dietary (poly)phenols is well known in preventing cardiovascular and neurodegenerative diseases, in reducing risk factors of some cancers, and in the management of diabetes [5]. In recent years, there has been a growing trend in using quercetin as a nutraceutical compound, principally due to its health promoting benefits, demonstrated through *in vitro* models and in both animal and human studies [6]. Nevertheless, the protective properties of dietary quercetin may be limited due to its scarce aqueous solubility and stability in the upper gastrointestinal tract (uGIT), rapid metabolism, and short biological half-life [7], which are the main reasons explaining its scarce bioavailability after consumption. For this purpose, delivery systems such as polymeric nanoparticles, liposomes, phytosomes, micelles, and emulsions have been widely applied [8]. Delivery systems aim to encapsulate bioactive compounds from plant extracts into water-based matrixes, improving their chemical stability and water solubility, resulting in a better control of their rate and site of action within the gastrointestinal tract (GIT) by increasing their bioavailability [9]. It has been previously reported that the consumption of quercetin formulated in a food-grade lecithin delivery system significantly improved its oral absorption and bioavailability in healthy subjects [10], resulted in efficient maintenance of physical resistance in triathlon athletes [11], and showed better control of allergy symptoms [12]. Additionally, the quercetin application in colon target delivery systems aimed at the oral therapy of colon disorders has represented an increasing research topic in recent years [13,14]. It is well known that dietary (poly)phenols are mostly unabsorbed in the uGIT, reaching the colon intact, where they are metabolized by fecal microbiota in a wide range of phenolic metabolites [15], suggesting the role of colonic microbiota in influencing the bioactivity of dietary (poly)phenols. Indeed, the microbial bioaccessibility of unabsorbed bioactive compounds in the colonic environment represents a pivotal step in the presumed bioactivity associated with (poly)phenol intake. For this purpose, *in vitro* fecal fermentation models are applied as useful tools to investigate the colonic metabolism of undigested (poly)phenols and their microbial breakdown upon interaction with fecal microbiota. To date, the human microbial metabolism of quercetin has been thoroughly investigated by identifying a series of phenolic acids that may also be involved in the systemic effect of dietary flavonols [16,17]. However, to our knowledge, the influence of delivery systems on quercetin colonic bioaccessibility, as assessed by human fecal fermentation models, is quite unknown, unlike other functionalized plant extracts [18,19].

Thus, the present study aimed to investigate the human microbial metabolism of quercetin derived from the plant *Sophora japonica* L., prepared through two different technologies (unformulated and phytosome-formulated). During *in vitro* fecal incubation, native quercetin appeared to be less prone to microbial degradation in the phytosome-formulated product with respect to the unformulated one. In addition, the colonic bioaccessibility of quercetin changed according to the formulation. These results highlighted the role of the delivery system in affecting the human microbial metabolism of quercetin in a time-dependent manner.

2. Materials and Methods

2.1. Chemicals and Reagents

Formic acid, bile salts, soluble starch, (+)-arabinogalactan, tryptone, yeast extract, xylan from birchwood, L-cysteine hydrochloride monohydrate, guar gum, inulin, Tween 80, buffered peptone water, Dulbecco's phosphate buffer saline (PBS), casein sodium salt from bovine milk, pectin from citrus fruits, mucin from porcine stomach-type III, $CaCl_2$, KCl, NaCl, $NaHCO_3$, anhydrous K_2HPO_4, KH_2PO_4, $MgSO_4$ monohydrate, $FeSO_4$ heptahydrate, resazurin redox indicator, quercetin, kaempferol, quercetin-3-*O*-rutinoside (*aka* rutin), phenylacetic acid, 4′-hydroxyphenylacetic acid, 3′-hydroxyphenylacetic acid, 3′,4′-dihydroxyphenylacetic acid, 3-phenylpropanoic acid, 3-(4′-hydroxyphenyl)propanoic acid, 3-(3′-hydroxyphenyl)propanoic acid, 3-(3′,4′-dihydroxyphenyl)propanoic acid (*aka* dihydrocaffeic acid), 4-hydroxybenzoic acid, 3-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid (*aka* protocatechuic acid), and benzene-1,3,5-triol (*aka* phloroglucinol) were obtained from Sigma-Aldrich (St Louis, MO, USA). Isorhamnetin was from PhytoLab GmbH (Vestenbergsgreuth, Germany). All solvents and water were UHPLC-grade and were purchased from VWR International (Milan, Italy).

2.2. Products

Both unformulated (Quercetin 95%) and phytosome-formulated quercetin (QUERCEFIT™, contains ≥34.0% ≤42.0% of quercetin by hPLC) were provided by Indena S.p.A. (Milan, Italy). Prior ultra-high performance liquid chromatography coupled to mass spectrometry (uHPLC-MS^n) analysis, both ingredients were extracted as previously reported [20]. Briefly, the powder (10 mg) was added to pure methanol and a mixture of acetone/isopropanol/methanol 50:33.33:16.66 (*v/v/v*) (1 mL) for unformulated and phytosome-formulated quercetin. Samples were sonicated for 20 min, centrifuged at 14,460× g for 10 min at 4 °C (Centrisart® A-14C Refrigerated Micro-Centrifuge and Rotor YCSR-A1C, Sartorius Lab Instruments GmbH and Co. KG, Goettingen, Germany), and the supernatants were collected. Both products were subjected to two additional extractions using 0.5 mL of the same solvent, following the same extraction procedure, then the three supernatants were pooled. Finally, unformulated and phytosome-formulated quercetin were adequately diluted (1:1000 and 1:200, respectively) with 50% (*v/v*) aqueous methanol acidified with 0.1% (*v/v*) formic acid before uHPLC-MS^n analyses.

2.3. Growth Medium and Fecal Slurry Preparation

The growth medium (1 L) was prepared as previously reported [21] and sterilized at a temperature of 121 °C for 15 min in 12 mL glass vessels before samples were prepared. Fresh feces were collected from three volunteers who were healthy and without intestinal disease and did not take any antibiotics for the previous 3 months [22]. Donors followed a rigorous diet without (poly)phenol-containing food items for two days before fecal collection. After collection, feces were stored in anaerobic jars and processed within 2 h. Faces from donors were pooled in equal amount and homogenized with 1% (*w/v*) sterilized Dulbecco's PBS to obtain a 10% (*w/w*) fecal slurry that was used as the fermentation starter [22].

2.4. *In Vitro* Fecal Fermentation

The fermentation procedure was performed as previously reported [18,22], with slight modifications. In each fermentation batch, 45% of the growth medium, 45% of the fecal slurry, and 10% of unformulated or phytosome-formulated aqueous suspension were added to reach a total fermentation volume of 4 mL. Unformulated and phytosome-formulated products were fermented at a final concentration of 200 µmol/L. Both unformulated and phytosome-formulated products were dissolved in an aqueous bile salt solution [23] and suspensions were left for 2 h at room temperature under constant magnetic stirring [10]. Blank samples containing the growth medium and the fecal slurry (without botanical ingredient aqueous suspension), as well as abiotic control samples containing the growth medium and the aqueous suspension products (without fecal slurry), were also prepared [24]. The fecal slurry and the aqueous suspension product were put in the vessel containing growth medium, sealed, and flushed with N_2 to create anaerobiosis. Vessels were then incubated for 24 h at 37 °C at 200 strokes per min in a Dubnoff bath (JULABO, Seelbach Germany). Samples were collected at 0 h and after 5 and 24 h incubation. Microbial metabolism was stopped by adding 10% (*v/v*) of acetonitrile [18], and samples were frozen (-18 °C) until extraction and analysis. All experiments were carried out in triplicate.

2.5. Fecal Metabolite Extraction

Fecal metabolites produced during the *in vitro* fecal incubation of unformulated and phytosome-formulated quercetin were extracted adopting the method reported by Bresciani et al. [18], with slight modifications. Briefly, 300 µL of each fermented sample was extracted with 0.1% (*v/v*) formic acid in ethyl acetate, vortexed for 30 s, sonicated for 10 min in an ultrasonic bath, vortexed for 30 s, and re-sonicated for 5 min. Finally, samples were centrifuged at 14,460× g for 10 min and the upper organic layer was transferred to a clean microfuge tube. After the first extraction, the residual pellet of the fermented samples was re-extracted following the same procedure, using 500 µL of the same solvent. Finally, supernatants were pooled and brought to dryness for about 2 h at room temperature through a centrifugal concentrator (SpeedVac Savant SPD121P, Thermo Fisher Scientific Inc., San Jose, CA, USA). Both dried residues were reconstituted in 50% (*v/v*) aqueous methanol acidified with 0.1% (*v/v*) formic acid (dilution factors of 1:10 and 1:2 for the analyses of native quercetin and its fecal metabolites, respectively), vortexed, and centrifuged at 14,460× g for 10 min before uHPLC-MS^n analyses.

2.6. uHPLC/MS^n Analysis

Extracted samples were analyzed by ultra-high performance liquid chromatography (uHPLC) coupled with mass spectrometry (MS), using an Accela uHPLC 1250 apparatus equipped with a linear ion trap MS (LIT-MS) (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA), fitted with a heated-electrospray ionization (H-ESI-II) probe (Thermo Fisher Scientific Inc., San Jose, CA, USA). Separation was carried out by means of a Kinetex Evo C18 column (100 × 2.1 mm; 2.6 µm particle size; Pheneomenex, CA, USA) installed with a precolumn cartridge (Phenomenex). The mobile phase consisted of a mixture of 0.1% (*v/v.*) formic acid in acetonitrile (solvent A) and 0.1% (*v/v*) formic acid in water (solvent B). The flow rate was set at 0.5 mL/min, with the following gradient. Starting from 0 to 0.5 min of 5% solvent A in B, the proportion of A was increased linearly to 40% over a period of 7 min. Solvent A was increased to 80% in 1 min, kept for 2 min, and then the start condition were re-established in 0.5 min and kept for 3 min to re-equilibrate the column (total run: 14 min). Quercetin and fecal metabolites were analyzed by adopting the h-ESI-II parameters reported by Bresciani et al. [18]. Firstly, unformulated and phytosome-formulated products were characterized using full-scan, data-dependent MS^3 experiments from *m/z* 100 to 1000, while the analysis of the fecal metabolites was carried out using full-scan, data-dependent MS^2 experiments from *m/z* 100 to 500. Based on the untargeted analysis and data of the microbial catabolic pathway of quercetin, specific parent ions of quercetin and their main fecal metabolites were monitored through full MS/MS experiments with a collision-induced dissociation (CID) equal to 35, except for benzene-1,3,5-triol, which was further monitored in selected ion monitoring (SIM) mode. The limit of detection (LOD) and quantification (LOQ) for both the parent compound and fecal metabolites were evaluated in both standard solutions and fermented samples. LODs and LOQs were calculated based on the minimal accepted values of the signal-to-noise (S/N) ratio of 3 and 10, respectively. Identified compounds were quantified in fermented samples using calibration curves of the available reference compounds (ranging from 0.01 to 75 µmol/L and from 1.00 to 150 µmol/L for quercetin and its fecal metabolites, respectively). All instrumental data were acquired using Xcalibur software 2.1 (Thermo Fisher Scientific Inc., San Jose, CA, USA).

2.7. Statistical Analysis

Experiments were carried out in triplicate. Results are shown as mean ± SD. One way ANOVA (Tukey's post-hoc test) was applied to detect differences in quercetin concentrations between different fermented samples within the same incubation period (T0, T5, T24) or for the same fermented sample but in a different incubation period ($p < 0.05$). A *t*-test was used to detect significant differences between unformulated and phytosome-formulated fermented samples for each fecal metabolite and their total concentrations, considering the same incubation period ($p < 0.05$). All statistical analyses were carried out using the SPSS statistical software (v25, SPSS, Inc., Chicago, IL, USA).

3. Results and Discussion

3.1. Characterization of Products

Unformulated and phytosome-formulated quercetin were firstly characterized for their profiles in native (poly)phenols. The unambiguous identification of the (poly)phenol fraction was performed based on the retention time and MS fragmentation pattern of the available reference compounds. Chromatographic and mass spectrometric characteristics and the concentrations of native (poly)phenols identified in unformulated and phytosome-formulated products are reported in Table 1.

Table 1. Quantification of native (poly)phenols identified in unformulated and phytosome-formulated products and their mass spectrometric and chromatographic characteristics. Data are expressed as mg/g (mean values ± SD, n = 3).

Compound	**RT (min)**	**$[M - h]^-$ (*m/z*)**	**MS^2 Ions (*m/z*)**	**MS^3 Ions (*m/z*)**	**Unformulated Quercetin**	**Phytosome-Formulated Quercetin**
Quercetin	6.17	301	**179***, 151, 273, 257		959.2 ± 95.0	382.1 ± 25.1
Kaempferol	7.02	285	285, 151		n.q.	n.q.
Isorhamnetin	7.2	315	300		n.q.	n.q.
Rutin	4.27	609	301, 343, 179	301:179, 151, 273, 257	n.q.	n.q.

RT: retention time; *m/z*: mass to charge; * quantifier ions monitored in MS^2 experiment are reported in bold; n.q.: compounds detected but not quantified.

Four flavonoids belonging to the flavonol subclass were identified. As reported in Table 1, free quercetin was the most abundant compound in both preparations, accounting for about 95% and 38% of the total weight in unformulated and phytosome-formulated versions, respectively. Both quercetin products were prepared using flower buds of the plant *Sophora japonica* L., a shrub species of the pea family Fabaceae [25] that is widely employed in the dietary supplements field due to its richness in bioactive compounds [26]. In the present study, where the phytosome-formulated product contained about 1/3 of the quercetin compared to the unformulated one, both products were strictly standardized and characterized for their quercetin content. Phytosome is a food-grade delivery system designed and developed case-by-case for natural products. Quercetin phytosome consists of quercetin and sunflower lecithin in a 1:1 weight ratio, with about 20% of food-grade excipients added to improve the physical state of the product and to standardize it to the hPLC-measured total quercetin content of about 30% [10]. Minor flavonol aglycones, namely kaempferol and isorhamnetin, as well as glycosylated quercetin, namely rutin, were recovered in unformulated and phytosome-formulated products at trace amounts, thus leading their marginal quantification (Table 1).

3.2. Human Colonic Metabolism of Quercetin

After a preliminary uHPLC/MS^n analysis and based on the catabolic pathway reported for quercetin [17,27], a total of twelve colonic metabolites were monitored in unformulated and phytosome-formulated fermented batches. Chromatographic and mass spectral characteristics of phenolic metabolites in fermented samples are reported in Table 2. Quercetin and its breakdown metabolites were quantified in fermented samples using calibration curves of pure commercial standards. The concentration of native quercetin in unformulated and phytosome-formulated ingredients and abiotic controls (containing the growth medium and the product aqueous suspension, without fecal slurry) at baseline (0 h) and after 5 and 24 h incubation are reported in Figure 1.

Table 2. UHPLC-MS^n identification of native quercetin and its phenolic metabolites monitored in fermented samples.

Compound	RT (min)	$[M - h]^-$ (*m/z*)	MS^2 Ions (*m/z*)	LOD (µmol/L)	LOQ (µmol/L)	Quantification
Native compound						
Quercetin	6.17	301	**179** *, 151, 273, 257	0.005	0.01	R.S.
Breakdown metabolites						
3-(3′,4′-Dihydroxyphenyl)propanoic acid	2.35	181	**137**, 119, 109	0.05	1.00	<LOQ
3-(4′-Hydroxyphenyl)propanoic acid	3.18	165	**121**, 93	2.00	25.00	R.S.
3-(3′-Hydroxyphenyl)propanoic acid	3.59	165	**121**, 119	0.05	1.00	R.S.
3-Phenylpropanoic acid	5.54	149	**105**	5.00	50.00	<LOD
3′,4′-Dihydroxyphenylacetic acid	1.41	167	**123**	0.25	1.00	R.S.
4′-Hydroxyphenylacetic acid	2.26	151	**107**	1.00	10.00	R.S.
3′-Hydroxyphenylacetic acid	2.60	151	**107**	0.25	1.00	R.S.
Phenylacetic acid	4.18	135	**91**	2.00	10.00	R.S.
3,4-Dihydroxybenzoic acid	1.20	153	**109**	0.05	1.00	R.S.
4-Hydroxybenzoic acid	2.07	137	**93**	0.25	1.00	<LOQ
3-Hydroxybenzoic acid	2.58	137	**93**	0.25	1.00	<LOD
Benzene-1,3,5-triol	0.65	**125**		5.00	5.00	<LOD

RT: retention time; *m/z*: mass to charge; * quantifier ions monitored in MS^2 experiment are reported in bold; LOD: Limit of detection; LOQ: Limit of quantification; R.S.: quantification with proper reference standard.

The product formulation had an effect on native quercetin microbial degradation, since a significant difference ($p < 0.05$) in quercetin concentration for the unformulated and phytosome-formulated versions emerged after 24 h of fecal incubation (Figure 1A). Instead, both at baseline (0 h) and at 5 h incubation, as expected the levels of the parent flavonol concentration in the unformulated and phytosome-formulated products fermented with fecal slurry were not significantly different ($p > 0.05$), highlighting the action of the human microbiota in promoting a greater quercetin biotransformation after at least 5 h fermentation. Concerning quercetin degradation for the same fermented samples (Figure 1B), the flavonol concentration in unformulated and phytosome-formulated products fermented with fecal slurry significantly changed ($p < 0.05$) over time, while the native quercetin in both abiotic controls was not significantly different ($p > 0.05$) with respect to 0 h. At 5 h of fecal incubation, a significant increase ($p < 0.05$) in parent quercetin concentration with respect to its concentration at 0 h was observed for the sole unformulated product, pointing to the effect of both the employed incubation time and the different product matrix in promoting the flavonol release over time (Figure 1B). On the other hand, the same effect on parent quercetin concentration emerged for the respective abiotic control. At 24 h, the native quercetin concentrations in both ingredients were significantly ($p < 0.05$) reduced upon interaction with fecal microbiota (−93% and −50% compared to quercetin concentration at 0 h for unformulated and phytosome-formulated products, respectively) (Figure

1B). Therefore, the parent flavonol degradation was faster in the unformulated product than in the phytosome-formulated one, pointing to a lower *in vitro* stability of native quercetin derived from the unformulated version with respect to the phytosome-formulated one. Indeed, according to the literature [7,28], phytosome technology has been developed to increase the stability of bioactive compounds, preventing their degradation by digestive enzymes, gut bacterial species, and chemical compounds that are naturally present in the colonic environment.

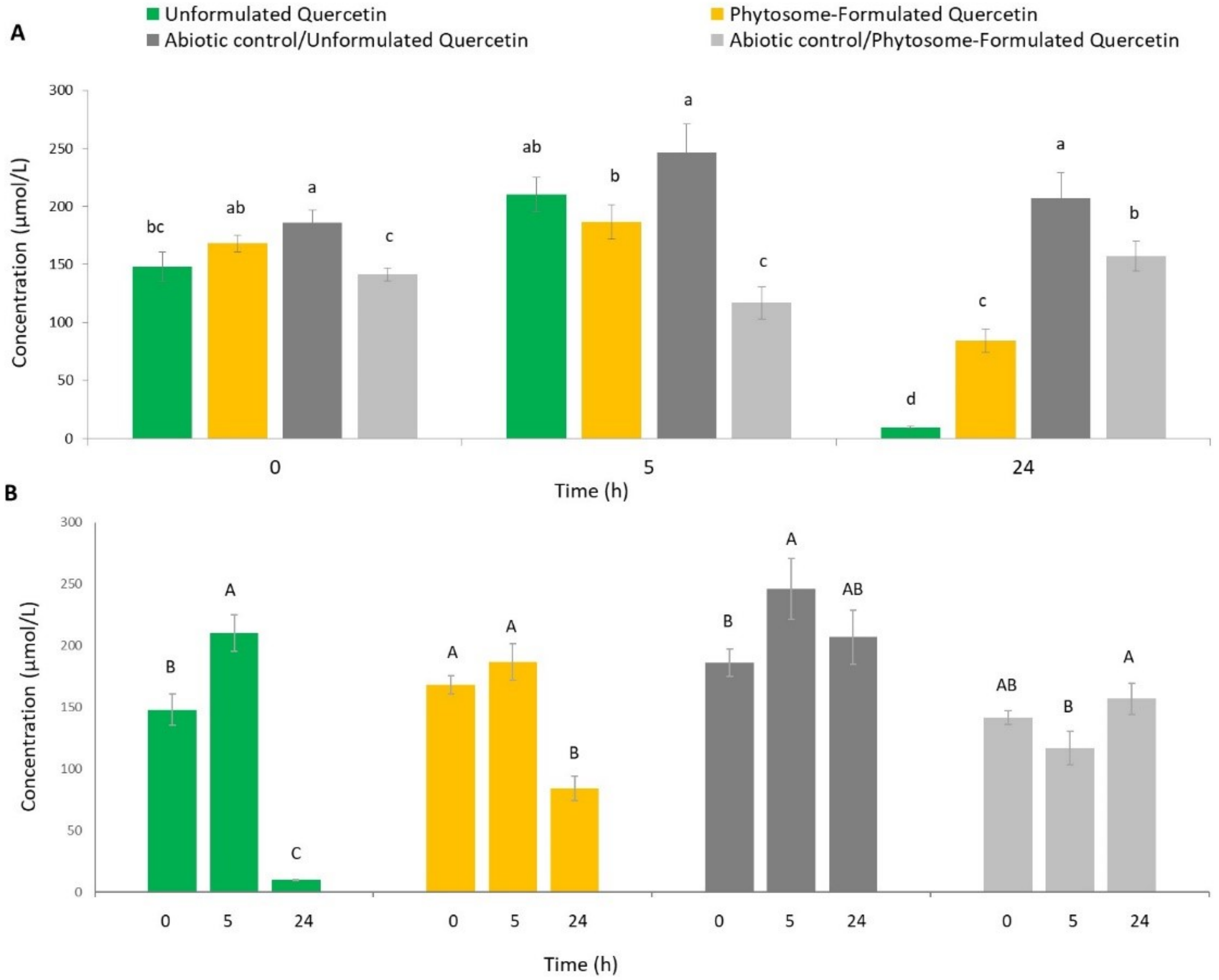

Figure 1. Concentration (μmol/L) of native quercetin in unformulated and phytosome-formulated products and abiotic controls (containing the growth medium and the product aqueous suspension, without fecal slurry) at different time point (0 h, 5 h, and 24 h). Data are expressed as means ± SD (n = 3). (**A**) Different lower case letters indicate significant differences among fermented samples considering the same incubation period ($p < 0.05$). (**B**). Different upper case letters indicate significant differences considering the same incubation period ($p < 0.05$).

A total of nine fecal metabolites were unambiguously identified according to their chromatographic and MS^n characteristics among the targeted quercetin-derived microbial metabolites. In detail, seven phenolic compounds were recovered at quantifiable levels during fecal fermentations of unformulated and phytosome-formulated products, while 3-(3′,4′-dihydroxyphenyl)propanoic acid and 4-hydroxybenzoic acid were identified but not quantified (Table 2). In both abiotic controls, no microbial metabolites were detected, indicating that the *in vitro* incubation process had no effect on the degradation of parent quercetin. Instead, in blank samples containing growth medium and fecal slurry without product suspension, phenylacetic acid and 3-(4′-hydroxyphenyl)propanoic acid were recovered at quantifiable levels during fermentation. Hence, these two phenolic acids were not included among the specific quercetin-derived metabolites. It should be noted that the 48 h (poly)phenol free diet did not fully guarantee blank feces. It was previously reported that phenylacetic acid and 3-(4′-hydroxyphenyl)propanoic acid were recovered at quantifiable concentrations in feces collected from healthy volunteers following a 24 h low-(poly)phenol diet [29]. Additionally, these phenolic acids may arise from the microbial fermentation of aromatic amino acids as tryptophan, phenylalanine, and tyrosine upon consumption of dietary proteins [30,31]. It is well known that unabsorbed flavonoids reach the colon, where they are subjected to the action of gut microbiota enzymes, resulting in a wide range of low-molecular-weight phenolic acids, such as phenylpropanoic, phenylacetic, and benzoic acid derivatives [15]. In the present study, three phenylacetic acid derivatives, one monohydroxyphenylpropanoic acid, and one dihydroxybenzoic acid were identified among the strictly related quercetin-derived metabolites (Figure 2 A,B).

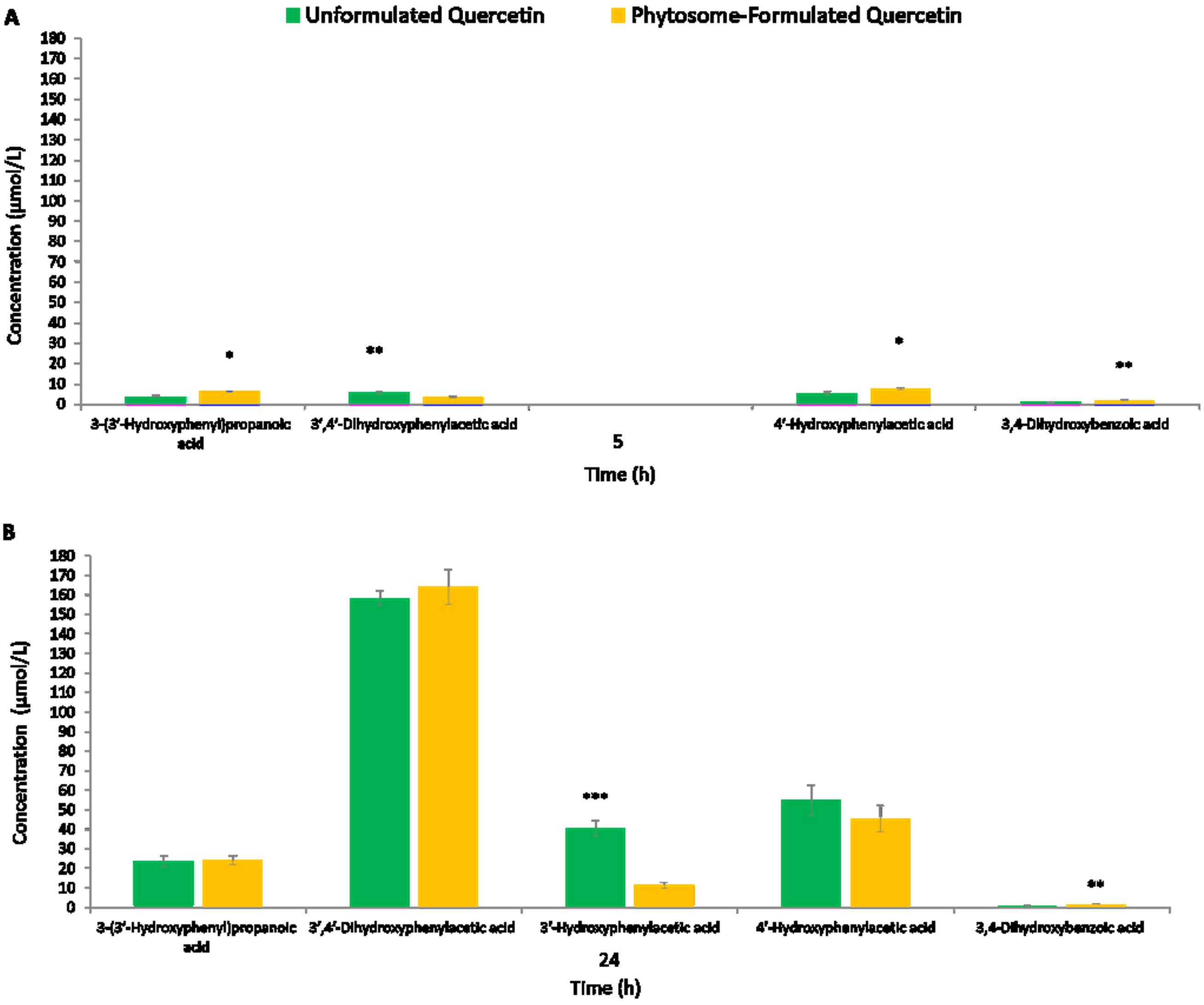

Figure 2. Concentrations (µmol/L) of fecal metabolites after 5 h (**A**) and 24 h (**B**) of *in vitro* fermentation of unformulated and phytosome-formulated products. Data are expressed as mean ± SD (n = 3). Note: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate significant differences between products for a fecal metabolite at the same incubation period. At 5 h, 3′-hydroxyphenylacetic acid was not detected in fermented batches.

At 0 h, no quercetin-derived metabolites were detected in unformulated and phytosome-formulated fermented batches. From a qualitative point of view, both products resulted in the same colonic metabolite profile after 5 and 24 h incubation. However, slight differences in metabolite concentrations emerged between products upon fermentation (Figure 2A,B). Briefly, after 5 h fecal incubation, four fecal metabolites were quantified in unformulated and phytosome-formulated fermented batches, which reached their maximum concentrations at 24 h, suggesting the role of microbiota in quercetin biotransformation during fecal fermentation. These findings were similar to those previously obtained by other authors [17,32,33] upon *in vitro* catabolism of quercetin in the colonic environment. The microbial metabolism of quercetin seemed to be mainly affected by product formulation after 5 h incubation. Indeed, among the four fecal metabolites quantified at 5 h, 3-(3′-hydroxyphenyl)propanoic acid, 4′-hydroxyphenylacetic acid, and 3,4-dihydroxybenzoic acid were quantified at significantly higher concentrations in the phytosome-formulated product with respect to the unformulated one (Figure 2A). During this first colonic catabolism phase, microbiota catalyzed the breakdown of the quercetin skeleton, where C-ring fission firstly occurred [34]. The 3-(3′,4′-dihydroxyphenyl)propanoic acid is one of the first intermediate microbial catabolite of quercetin [17], and after further dehydroxylation or α-oxidation, monohydroxyphenylpropanoic acid and phenylacetic acid derivatives are formed [35,36]. In our experiments, 3-(3′,4′-dihydroxyphenyl)propanoic acid was not quantified (Table 2), which is attributable to its fast degradation into 3-(3′-hydroxyphenyl)propanoic acid and 3′,4′-dihydroxyphenylacetic acid under the action of fecal microbiota, in accordance with the literature [17,37].

At 24 h, an additional metabolite, namely 3′-hydroxyphenylacetic acid, was quantified in both unformulated and phytosome-formulated fermented batches (Figure 2B). Among the five fecal metabolites quantified at the end of the incubation process, significant differences between fermented botanical products were only recovered for 3,4-dihydroxybenzoic acid and 3′-hydroxyphenylacetic acid concentrations ($p < 0.01$ and $p < 0.001$, respectively). These results suggested that the difference in formulation had a minor role on influencing the microbial metabolism of quercetin during the late fecal incubation hours, which equally ensured its *in vitro* breakdown in the colonic environment in both products. The 3′,4′-dihydroxyphenylacetic acid, the main colonic metabolite of quercetin after ring fission and α-oxidation of the side chain [17,27], was recovered at the highest concentration in both products regardless of the formulation (Figure 2B). Its concentrations corresponded to 56% and 66% of the total fecal metabolite concentration recovered in unformulated and phytosome-formulated products at 24 h, respectively (Figure 3). The 3′-hydroxyphenylacetic acid and 4′-hydroxyphenylacetic acid, derived from the loss of a hydroxyl group on the benzene ring of 3′,4′-dihydroxyphenylacetic acid [36], were also major metabolites (Figure 2B) and their concentrations were equal to 34% and 20% of the total fecal metabolite concentrations recovered in unformulated and phytosome-formulated products at the end of incubation period, respectively (Figure 3). According to previous studies, phenylacetic acid derivatives are reported as the main colonic metabolites of quercetin [16,17,38]. These results may suggest that the loss of the hydroxyl group in the 3′-position of to yield 4′-hydroxyphenylacetic acid firstly occurred in the colonic environment *in vitro* in comparison to 4′-dehydroxylation. However, this should be further explored, since Aura and colleagues [32] reported an increase in 3′-hydroxyphenylacetic acid production after dehydroxylation in the 4′-position of 3′,4′-dihydroxyphenylacetic acid upon 8 h fecal incubation of rutin (quercetin-3-*O*-rutinoside).

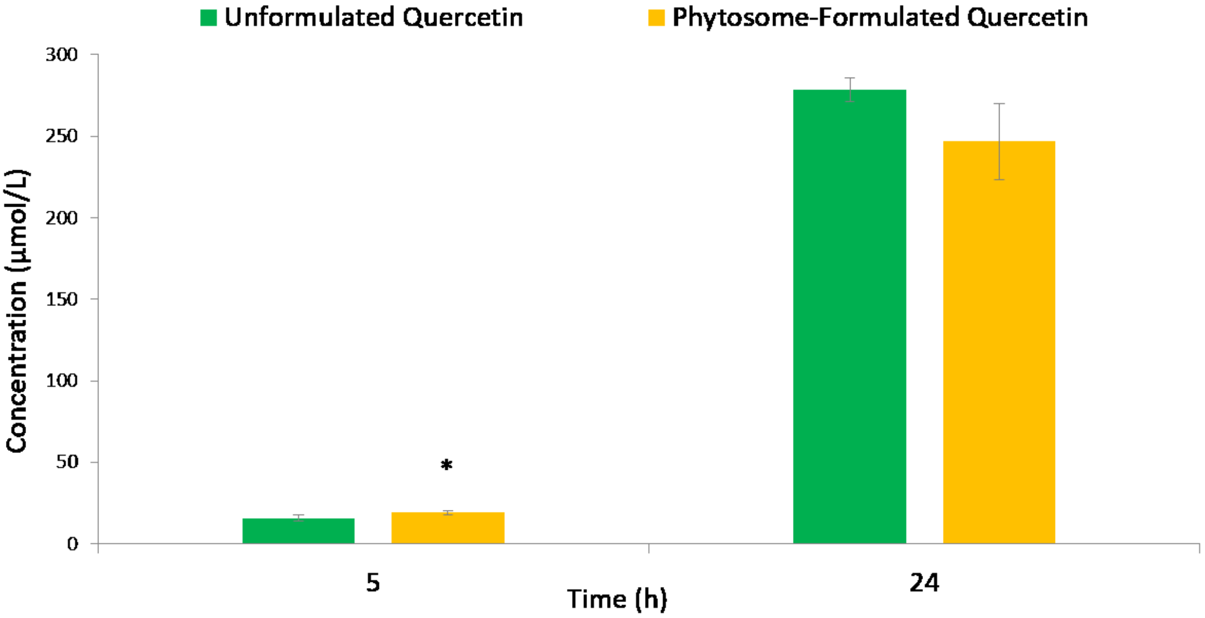

Figure 3. Concentrations (µmol/L) of total fecal phenolic metabolites after 5 h and 24 h of *in vitro* fermentation of unformulated and phytosome-formulated products. Data are expressed as means ± SD (n = 3). Note: * $p < 0.05$, indicate significant differences between products for the same incubation period.

The 3,4-dihydroxybenzoic acid, which could be generated through the rapid α-oxidation of 3-(3′,4′-dihydroxyphenyl)propanoic acid passing via 3′,4′-dihydroxyphenylacetic acid as an intermediate [35], was present at low concentrations, although its production was conditioned by the formulation (Figure 2). Its dehydroxylated derivatives, such as 3- and 4-hydroxybenzoic acids, were not quantified in fermented samples. According to Serra and colleagues [17], 4-hydroxybenzoic acid was the main catabolite derived from the dehydroxylation of the 3,4-dihydroxybenzoic acid benzene ring upon *in vitro* fecal fermentation of quercetin. On the contrary, Jaganath and colleagues [38] reported quantifiable levels of 4-hydroxybenzoic acid only after 30 h of rutin fecal incubation, which may explain the lack of monohydroxybenzoic acids in our fecal fermentations. Other minor compounds related to quercetin catabolism such as 3-phenylpropanoic acid and 3-hydroxybenzoic acid were not detected in fermented batches (Table 2), in line with data on the *in vitro* fecal fermentation of several (poly)phenol sources [17,32,37]. Similarly, the identification of benzene-1,3,5-triol, possibly derived from the cleavage of quercetin A ring [39], was not carried out. As Aura and colleagues reported upon fecal incubation of quercetin [32], benzene-1,3,5-triol is generally rapidly degraded to acetate and butyrate *in vitro*, which may have precluded its identification at high concentrations. On the other hand, the incubation periods applied in this fermentation model may have limited the identification of these phenolic metabolites, while incubations beyond 24 h might lead to quantifiable levels of these compounds, as reported by Sánchez-Patán and colleagues for grape seed flavan-3-ols [40].

When considering the total amount of metabolites upon quercetin fermentation (Figure 3), the cumulative concentration ranged between 15 and 19 µmol/L at 5 h, being significantly ($p < 0.05$) higher (+27%) in the phytosome-formulated product compared to the unformulated one (Figure 3). These findings were in line with the lower concentration of quercetin recovered at 5 h for the phytosome-formulated fermented batches (Figure 1). This result suggested a slight increase of quercetin colonic bioaccessibility in the presence of phytosome in the earlier fecal incubation phase. At 24 h, although the total phenolic metabolite concentration was higher in unformulated fermented batches with respect to phytosome-formulated fermented batches, no significant difference ($p > 0.05$) emerged (Figure 3). Totals of 278 and 246 µmol/L of colonic metabolites were recovered after 24 h fecal incubation of unformulated and phytosome-formulated products, respectively. This observation is stoichiometrically feasible, despite the initial quercetin concentration used to perform the *in vitro* fermentations being 200 µmol/L, as quercetin degradation by gut microbiota results in several phenolic acids derived from ring fission and a series of enzymatic activities involving both A- and B-rings [41]. Additional knowledge on the composition and evolution of the microbial communities belonging to the gut microbiota of the fecal donors would help to better understand which microbial species are behind the reported transformations. Further knowledge on this is needed, as it may help to elucidate the large inter-individual variability existing in metabolite production [42]. The inter-individual variability, whose causes may be related to dietary history, genetic polymorphisms, and variations in gut microbiota metabolism, may play a significant role in influencing quercetin bioavailability [1,42]. On the other hand, strategies improving quercetin absorption or metabolism may be of interest, since the application of this compound in the pharmaceutical field remains limited due to its poor bioavailability [43]. More attention should be paid to increasing the production of bioactive metabolites, as certain specific colonic metabolites (i.e., 3′,4′-dihydroxyphenylacetic acid or 4′-hydroxyphenylacetic acid) have been reported to exert a higher inhibition of platelet aggregation than its precursor quercetin [41]. Additionally, both animal and cell experiments have demonstrated that conjugated phase II metabolites of quercetin are involved in its *in vivo* antihypertensive effect [44]. These findings may encourage further research in developing bioactive compound-rich formulations, aiming for the prevention of specific chronic diseases by paying attention to the metabolites rather than to the parent compounds.

4. Conclusions

Although the microbial metabolism of quercetin has been extensively investigated, this is the first study providing data on the interaction between quercetin derived from different formulations and the human fecal microbiota. Native quercetin was more stable in the phytosome formulation upon 24 h *in vitro* fecal incubation. Quercetin was bioaccessible to human fecal microbiota in both products, showing the same profile for the production of phenolic catabolites. From a quantitative point of view, slight differences in the phenolic metabolite concentration emerged between formulations, mainly in the earlier fecal incubation period (5 h). This small influence of the different formulations on the microbial metabolism of quercetin was observed in a time-dependent manner-3 out of 4 microbial metabolites were quantified at higher concentrations in the phytosome-formulated batch fermentation with respect to the unformulated one after 5 h. In conclusion, several colonic-derived phenolic metabolites probably involved in the systemic effect of dietary (poly)phenols [41] were produced by both products *in vitro*. This study opened new perspectives to investigate the potential of delivery systems regarding influencing the colonic bioaccessibility of flavonols, a pivotal step in the putative bioactivity associated with (poly)phenol intake. Further studies in humans are needed to fully confirm the effective benefits of these promising formulated quercetin-rich ingredients.

Author Contributions: Conceptualization, P.M., D.D.R., A.R., G.P., and P.A.; methodology, G.D.P., L.C., and L.B.; investigation, G.D.P., L.C., L.B., and P.M.; resources, P.M., D.D.R., A.R., G.P., and P.A.; ‘writing—original draft preparation, G.D.P.; writing—review and editing, L.C., L.B., D.D.R., and P.M.; supervision, P.M. and D.D.R. All authors have read and agreed to the published version of the manuscript.

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Chapter 4. In vitro (poly)phenol catabolism of unformulated- and phytosome-formulated cranberry (Vaccinium macrocarpon) extracts

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In vitro (poly)phenol catabolism of unformulated- and phytosome-formulated cranberry (Vaccinium macrocarpon) extracts

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Abstract: Cranberries (*Vaccinium macrocarpon*) represent an important source of anthocyanins, flavan-3-ols and flavonols. This study aimed at investigating *in vitro* the human microbial metabolism of (poly)phenols, principally flavan-3-ols, of unformulated- and phytosome-formulated cranberry extracts. After powder characterization, a 24-h fermentation with human faecal slurries was performed, standardizing the concentration of incubated proanthocyanidins. Cranberry (poly)phenol metabolites were quantified by uHPLC-MS^2 analyses. The native compounds of both unformulated- and phytosome-formulated cranberry extracts were metabolized under faecal microbiota activity, resulting in twenty-four microbial metabolites. Although some differences appeared when considering different classes of colonic metabolites, no significant differences in the total amount of metabolites were established after 24 h of incubation period. These results suggested that a different formulation had no effect on flavan-3-ol colonic metabolism of cranberry and both unformulated- and phytosome-formulated extract. Both formulations displayed the capability to be a potential source of compounds which could lead to a wide array of gut microbiota metabolites *in vitro*.

Keywords: Cranberry; Flavan-3-ols; *In vitro* fermentation; Microbial metabolism; Phytosome formulation; Proanthocyanidins

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1. Introduction

Dietary guidelines worldwide recommend regular daily consumption of fruits and vegetables (FAO/WHO, 2004), choosing different types and different colours. Among fruits, berries are of particular nutritional relevance due to their content in micronutrients and non-nutrient bioactive compounds, namely (poly)phenols (Lavefve et al., 2020). Cranberries (*Vaccinium macrocarpon*) are important sources of anthocyanins, flavan-3-ols, both monomers and A- and B-type proanthocyanidins, and flavonols (Pappas & Schaich, 2009). In recent times, there has been a growing trend in using berry extracts or berry phytochemicals in formulating functional foods and dietary supplements (Nile & Park, 2014), principally due to their potential health benefits (Lavefve et al., 2020). Cranberry consumption has been associated with improvement of intestinal disorders, with a lower risk of cardiovascular disease development, and with glucose control in type-2 diabetic subjects (Rocha et al., 2019; Rodriguez-Mateos et al., 2014, 2016), but cranberries have been mainly investigated for their potential health effect against urinary tract infections (UTI) (Baron et al., 2020; Fu et al., 2017). This effect was initially linked to the native (poly)phenolic compounds, mainly A-type procyanidins (Gonzaíez de Llano et al., 2019). Recent evidence suggested a key role of microbial catabolites of flavan-3-ols, namely phenyl-γ-valerolactones, behind the preventive and prophylactic effect of cranberries against the bacterial colonization and progression of UTI (Gonzaíez de Llano et al., 2019; Mena et al., 2017). However, the efficacy of cranberry supplementation may be affected by the low bioavailability of native phenolic compounds (Del Rio, Borges, et al., 2010; Favari et al., 2020; Feliciano et al., 2017; Mena et al., 2019). For this reason, a food delivery system, namely phytosome, has been developed as technological strategies to create a solid dispersion of insoluble natural products, to enhance the bioavailability of native compounds, improving their intestinal absorption (Petrangolini et al., 2019). *In vitro* fermentation models represent useful tools to investigate the human colonic metabolism of undigested (poly)phenols, providing more comprehensive insights into the true bioavailability of (poly)phenols. Although a few studies investigated the *in vitro* microbial transformation of cranberry (poly)phenols (Ou et al., 2014; Sánchez-Patán et al., 2015), the effect of a different formulation of cranberry extract on the microbial metabolism of its native phenolic compounds is still unknown, contrary to other botanical extracts (Bresciani et al., 2020; Cuomo et al., 2011; Di Pede et al., 2020; Marczylo et al., 2007; Papillo et al., 2019).

Thus, this work aimed at investigating the interaction and the human colonic metabolism of (poly)phenols, principally flavan-3-ols, in unformulated- and phytosome-formulated standardized cranberry extracts, using an *in vitro* faecal fermentation model.

2. Materials and methods

2.1. Chemicals and reagents

Formic acid, bile salts, soluble starch, (+)-arabinogalactan, tryptone, yeast extract, xylan from birchwood, L-cysteine hydrochloride monohydrate, guar gum, inulin, Tween 80, buffered peptone water, Dulbecco′s phosphate buffer saline (PBS), casein sodium salt from bovine milk, pectin from citrus fruits, mucin from porcine stomach-type III, $CaCl_2$, KCl, NaCl, $NaHCO_3$, anhydrous K_2HPO_4, KH_2PO_4, $MgSO_4$ monohydrate, $FeSO_4$ heptahydrate, resazurin redox indicator, 3-(3′,4′-dihydroxyphenyl)propanoic acid, 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid, 3-(3′-hydroxyphenyl)propanoic acid, 3′,4′-dihydroxyphenylacetic acid, 4′-hydroxyphenylacetic acid, 3′-hydroxyphenylacetic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, 3-hydroxybenzoic acid, 4′-hydroxycinnamic acid, 3′,4′-dihydroxycinnamic acid, 4′-hydroxy-3′-methoxycinnamic acid, 5-caffeoylquinic acid, (+)-catechin and (–)-epicatechin were purchased from Sigma-

Aldrich (St Louis, MO, USA). Procyanidin B2 and procyanidin C1 were purchased from PhytoLab (GmbH & Co. KG, Vestenbergsgreuth, Germany). Cyanidin-3-glucoside, kaempferol, quercetin, quercetin-3-glucuronide and rutin were obtained from Extrasynthese (Genay Cedex, France). 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone, 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone and 5-(3′-hydroxyphenyl)-γ-valerolactone were synthesized in house (Brindani et al., 2017). All solvents and reagents were purchased from VWR International (Milan, Italy), unless otherwise indicated. Ultrapure water from MilliQ system (Millipore, Bedford, MA, USA) was used throughout the experiment.

2.2. Cranberry-Based ingredients

Both unformulated cranberry concentrated dry extract (Anthocran®) and phytosome-formulated cranberry extract (Anthocran® Phytosome®) were provided by Indena S.p.A. (Milan, Italy). The unformulated cranberry extract was standardized to contain 27–33% of cranberry proanthocyanidins, quantified by DMAC method. DMAC is a simple, robust and relatively specific spectrophotometric colorimetric assay widely employed to quantify total proanthocyanidins in cranberry (Prior et al., 2010). Phytosome-formulated cranberry extract, containing 1/3 of standardized unformulated cranberry extract (Baron et al., 2020), contained 6–9% of proanthocyanidins, quantified by DMAC method, and the ratio between extract and sunflower lecithin was 1:2 (33.3%:66.7%, *w/w*). Prior uHPLC-MS^n analysis, unformulated cranberry ingredient was extracted as previously reported (Mena et al., 2016). Briefly, 50 mg of powder were added with 1 mL of 50% aqueous methanol acidified with formic acid (0.1%). The samples were sonicated for 25 min, centrifuged at 14,460 × g for 10 min at 4 °C and the supernatants were collected. The extract completely dissolved after the first extraction step and it was diluted with acidified water (0.1 formic acid) (1:20) before uHPLC-MS^n analyses. All experiments were carried out in triplicate.

2.3. *In vitro* faecal fermentation

One litre of growth medium was prepared as previously reported (Bresciani et al., 2018, 2020). The growth medium was sterilized at 121 °C for 15 min in glass vessels (12 mL) before sample preparation. Fresh faeces were collected from three healthy volunteers [2 men and 1 woman, aged 30.6 ± 3.7, with height 1.7 ± 0.4 m, weigh 75.1 ± 6.7 kg and BMI 23.6 ± 1.0 kg m^{-2} (mean ± SD)] who did not have any intestinal disease and were not treated with antibiotics for the previous 3 months (Dall'Asta et al., 2012). Donors followed a strict diet lacking (poly)phenol-containing foods for 2 days prior to faecal collection. After collection, faeces were immediately stored in an anaerobic jar and processed within 2 h. Faeces from donors were pooled in equal amount and homogenized with 1% *w/v* sterilized Dulbecco′s phosphate buffer saline to obtain a 10% (*w/w*) faecal slurry used as fermentation starter (Dall'Asta et al., 2012). The Ethics Committee of Area Vasta Emilia Nord (AVEN) approved the collection and use of the faecal slurries (protocol no. 796/2018/sper/unipr) and all the donors provided informed consent for the collection of faecal slurries. The fermentation procedure was performed as previously reported (Bresciani et al., 2020; Dall'Asta et al.,2012). In each fermentation batch, 45% of the growth medium, 45% of faecal slurry and 10% of unformulated- or phytosome-formulated cranberry aqueous suspension were added to reach a total fermentation volume of 4 mL (Tan et al., 2015). The final concentration of proanthocyanidins in each fermented batch was standardized to 200 µmol/L, based on total proanthocyanidin content (DMAC assay value). Blank samples, containing the culture medium and the faecal slurry, as well as abiotic control samples, containing only the culture medium and the cranberry suspension, were also prepared. The fermentation starter and the cranberry suspension were introduced into the vessel containing sterilized growth medium, sealed with a rubber seal, and flushed through a double-needle with nitrogen to create anaerobic conditions. Vessels were then incubated for 0 h, 5 h and 24 h at 37 ◦C at 200 S per min in a Dubnoff bath (JULABO, Seelbach, Germany). After incubation, microbial metabolism was stopped with 10% of acetonitrile and samples were frozen (-18 °C) until extraction and analysis. All experiments were carried out in triplicate.

2.4. Faecal metabolite extraction

Phenolic compound metabolites generated during the *in vitro* fermentation of unformulated- and phytosome-formulated cranberry were extracted adopting the method reported by Bresciani et al. (Bresciani et al., 2020), with slight modifications. Briefly, 300 µL of fermented samples were extracted with 1.2 mL of acidified ethyl acetate (0.1% formic acid), vortexed for 2 min, sonicated for 10 min in an ultrasonic bath, vortexed for 2 min and re-sonicated for 5 min. Finally, samples were centrifuged at 14,460 × g for 10 min. The upper organic layer was transferred into a clean microfuge tube and dried for about 2 h at room temperature through a centrifugal vacuum concentrator (SpeedVac Savant SPD121P, Thermo Fisher Scientific Inc., San Jos´e, CA, USA). The residual pellet of the fermented sample was re-extracted following the same procedure, using 1 mL of acidified ethyl acetate (0.1% formic acid) as solvent and dried using centrifugal vacuum. Finally, both dried residues were reconstituted in 100 µL of 50% aqueous methanol acidified with formic acid (0.1%), vortexed, sonicated for 10 min, centrifuged at 14,460 × g for 5 min and analysed by uHPLC-MS^n.

2.5. uHPLC/MS^n analysis

Ultra-high performance liquid chromatography coupled to a linear ion trap mass spectrometer (uHPLC-LIT-MS) was used for the analysis of native (poly)phenolic compounds in unformulated cranberry extract, and for the analysis of microbial metabolites generated during the *in vitro* fermentation.

2.5.1. Liquid chromatography analysis

An Accela uHPLC 1250 (Thermo Fisher Scientific Inc.) was used for the chromatographic separation of all the analysis. Separation was carried out by means of Kinetex Evo C18 column (100 × 2.1 mm; 2.6 µm particle size; Phenomenex, CA, USA) installed with a precolumn cartridge (Phenomenex). The oven temperature was set at 40 °C. For the analysis of native (poly)phenolic compounds in cranberry powders, mobile phase, pumped at a flow-rate of 0.4 mL/min, consisted of a mixture of 0.1% (*v/v*) formic acid in acetonitrile (solvent A) and 0.1% (*v/v*) formic acid in water (solvent B). Following 0.5 min of 5% solvent A in B, the proportion of A turned up linearly to 51% over a period of 8.5 min. Solvent A was increased to 80% in 0.5 min, maintained for 2 min and then the start conditions were re-established in 0.5 min and maintained for 5 min to re-equilibrate the column. For the analysis of microbial metabolites in fermented samples, the same elution solvents were used, while the gradient was slightly modified. The mobile phases, pumped at a flow rate of 0.5 mL/min, were kept at 5% A for 0.5 min, then A turned up linearity to 40% keeping this value for 7 min. Solvent A was increased to 80% in 1 min, maintained for 2 min and then the start condition were re-established in 0.5 min and maintained for 3 min to reequilibrate the column.

2.5.2. Mass spectrometry method

A LIT-MS (LTQ XL, Thermo Fisher Scientific Inc.) fitted with a heated ESI probe (H-ESI-II, Thermo Fisher Scientific Inc.) was used to detect and identify compounds. Unformulated cranberry extract, as well as the fermented samples, were firstly analysed using a full-scan, data-dependent MS^3 mode, scanning the mass to charge (*m/z*) range of 100–1000. After this first step, further specific targeted MS^2 analyses were carried out to unambiguously identify the compounds revealed in the first step, by monitoring specific *m/z* transitions. Native phenolic compounds in unformulated powder were analysed in negative ionization mode, with the exception of anthocyanins, which were detected in positive ionization mode. For negative mode, the H-ESI-II interface was set to a capillary temperature of 275 °C and the source heater temperature was 200 °C. The sheath gas (N_2) flow rate was set at 40 (arbitrary units) and the auxiliary gas (N_2) flow rate at 5 (arbitrary units). The source voltage was 4 kV, the capillary voltage was − 42 V and tube lens voltage was − 118 V. For anthocyanin analysis, the H-ESI-II interface was set to a capillary temperature of 275 °C and the source heater temperature was 300 °C. The sheath gas (N_2) flow rate was set at 40 (arbitrary units) and the auxiliary gas (N_2) flow rate at 5 (arbitrary units). The source voltage was 4.5 kV, and the capillary voltage and tube lens voltage were + 20 and + 95 V, respectively. Finally, during the analysis in negative ionization mode of microbial metabolites, the H-ESI-II interface was set to a capillary temperature of 275 °C and the source heater temperature at 250 °C. The sheath gas (N_2) flow rate was set at 40 (arbitrary units), the auxiliary gas (N_2) flow rate at 5 (arbitrary units) and the sweep gas flow was set at 15 (arbitrary units). The source voltage was 3 kV, the capillary voltage and tube lens voltage was − 9 V and − 53 V, respectively. For all negative and positive applied MS methods, ultra-pure helium gas (99.9999%) was used and a collision induced dissociation (CID) energy equal to 35 (arbitrary units) was used to obtain MS^2 fragmentation. Chromatograms and mass spectral data were acquired using Xcalibur software 2.1 (Thermo Fisher Scientific Inc.). Quantification was performed with calibration curves of standards, when available, or using the most structurally similar compound (Table 1 and Supplementary Table S1).

2.6. Statistical analysis

Experiments were carried out in triplicate. Results were shown as mean ± standard deviation (SD). *T*-test was applied to detect significant differences between products within the same time point (T0, T5, T24). All statistical analyses were performed using the SPSS statistical package (version 25, SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. (Poly)phenolic compound characterization in Unformulated Cranberry extract

Unformulated cranberry extract was characterized for its (poly)phenolic profile. Compounds were identified based on the interpretation of their mass spectral behaviour obtained through targeted MS^2 experiments and by comparison with literature. A total of forty-two compounds, belonging to different phenolic classes, namely hydroxybenzoic acids, hydroxycinnamic acids, flavan-3-ols, flavonols, flavones and anthocyanins, were quantified. Chromatographic and mass spectrometric characteristics and the concentration of detected compounds in unformulated cranberry extract are detailed in Table 1. Among fourteen quantified flavonols, quercetin and its glycosylated forms were the most abundant, while, as expected, A-type procyanidins were the main flavan-3-ols in cranberry extract. Only monomers and dimeric to trimeric oligomers of flavan-3-ols were quantified, which corresponded to about 6% of total flavanol content (Table 1), excluding the fraction of high molecular weight proanthocyanidins of cranberry, which are responsible for the 27–33% of total proanthocyanidin content (DMAC method) of standardized cranberry compositions.

Table 1. Quantification of (poly)phenolic compounds in unformulated-cranberry extract and mass spectrometric and chromatographic characteristics. Data are expressed as mg/g (mean values ± SD, n = 3).

Compound	RT (min)	[M− H]$^-$ (*m/z*)	MS^2 ions (*m/z*)	Unformulated Cranberry Extract (mg/g)	Standard used for quantification
Hydroxybenzoic acids					
Benzoic acid	3.75	121	77	1.44 ± 0.15	3-Hydroxybenzoic acid
Hydroxybenzoic acid	3.02	137	93	0.37 ± 0.05	3-Hydroxybenzoic acid
3,4-Dihydroxybenzoic acid	1.36	153	123, 109	12.10 ± 0.25	3,4-Dihydroxybenzoic acid
4-Hydroxy-3-methoxybenzoic acid	4.83	167	123, 152, 108	6.27 ± 0.68	4′-Hydroxy-3′-methoxycinnamic acid
Total Hydroxybenzoic acids				**20.18 ± 0.87**	
Hydroxycinnamic acids					
Cinnamic acid	4.23	147	103	2.49 ± 0.28	4′-Hydroxycinnamic acid
4′-Hydroxycinnamic acid	3.93	163	119	2.63 ± 0.24	4′-Hydroxycinnamic acid
cis-3′,4′-Dihydroxycinnamic acid	2.76	179	135	9.46 ± 0.76	*trans*-3′,4′-Dihydroxycinnamic acid
trans-3′,4′-Dihydroxycinnamic acid	3.10	179	135	3.92 ± 0.18	*trans*-3′,4′-Dihydroxycinnamic acid
Coumaroyl-hexoside	2.76	325	163	2.48 ± 0.38	5-Caffeoylquinic acid
5-Caffeoylquinic acid	2.98	353	191, 179	8.64 ± 0.30	5-Caffeoylquinic acid
3′-Methoxycinnamic acid 4′-hexoside	3.26	355	193, 217, 175	0.49 ± 0.04	5-Caffeoylquinic acid
Feruloylquinic acid	3.93	367	191, 193	0.20 ± 0.03	5-Caffeoylquinic acid
Sinapoyl-hexoside	3.35	385	223, 247, 205	0.81 ± 0.07	5-Caffeoylquinic acid
Total Hydroxycinnamic acids				**31.12 ± 1.29**	
Flavan-3-ols					
(+)-Catechin	2.81	289	245, 205, 179, 271, 165	0.96 ± 0.01	(+)-Catechin
(−)-Epicatechin	3.46	289	245, 205, 179, 271, 165	8.09 ± 0.85	(−)-Epicatechin
Procyanidin dimer (A-type)	4.63	575	449, 423, 289, 407, 539, 453	15.30 ± 1.42	Procyanidin dimer B2
Procyanidin dimer (B-type)	2.63	577	449, 423, 289, 407, 539, 453	0.43 ± 0.05	Procyanidin dimer B2
Procyanidin dimer B2	3.30	577	425, 451, 407, 559, 289, 287	5.67 ± 0.03	Procyanidin dimer B2
Procyanidin dimer (B-type)	4.49	577	425, 451, 407, 559, 289, 287	0.59 ± 0.06	Procyanidin dimer B2
Prodelphinidin [Epicatechin + Epigallocatechin]	2.16	593	425, 467, 407, 441, 575, 289, 303,315	0.84 ± 0.03	Procyanidin dimer B2
Prodelphinidin [Epicatechin + Epigallocatechin]	3.85	593	425, 467, 407, 441, 575, 289, 303, 315	0.14 ± 0.02	Procyanidin dimer B2
Procyanidin trimer (A-type)	3.76	863	711, 451, 411, 573, 531, 289, 693	13.99 ± 1.40	Procyanidin C1
Procyanidin trimer (A-type)	4.13	863	575, 711, 693, 737, 559, 287	7.17 ± 0.58	Procyanidin C1
Procyanidin trimer (A-type)	4.78	863	575, 711, 693, 737, 845, 287	8.15 ± 0.87	Procyanidin C1
Procyanidin trimer (B-type)	3.35	865	739, 695, 713, 847, 577, 575, 449, 407, 425, 287	1.59 ± 0.23	Procyanidin C1
Procyanidin tetramer	4.63	[M− H]^{2}-576			N.Q.
Procyanidin hexamer	2.45	[M− H]^{2}-862			N.Q.
Procyanidin hexamer	3.82	[M− H]^{2}-864			N.Q.
Procyanidin hexamer	4.06	[M− H]^{2}-864			N.Q.
Procyanidin hexamer	4.51	[M− H]^{2}-864			N.Q.
Procyanidin hexamer	4.78	[M− H]^{2}-864			N.Q.
Total Flavan-3-ols				***62.92 ± 3.97***	
Flavonols					
Kaempferol	7.12	285	257	0.27 ± 0.02	Kaempferol
Quercetin	6.23	301	179, 151	40.41 ± 4.31	Quercetin
Isorhamnetin	7.28	315	300	5.15 ± 0.56	Kaempferol
Myricetin	5.35	317	179, 151, 192	4.09 ± 0.15	Kaempferol
Syringetin	7.19	345	330	0.54 ± 0.04	Quercetin-3-glucuronide
Quercetin-pentoside	4.93	433	301	11.59 ± 0.56	Quercetin-3-glucuronide
Quercetin-rhamnoside	5.05	447	301	6.82 ± 0.34	Quercetin-3-glucuronide
Myricetin-pentoside	4.44	449	317	2.17 ± 0.15	Quercetin-3-glucuronide
Quercetin hexoside	4.55	463	301	15.94 ± 2.34	Quercetin-3-glucuronide
Isorhamnetin hexoside	5.07	477	315, 314	0.71 ± 0.03	Quercetin-3-glucuronide
Myricetin hexoside	4.11	479	317, 316	5.29 ± 0.07	Quercetin-3-glucuronide
Syringetin hexoside	5.12	507	345	1.55 ± 0.15	Quercetin-3-glucuronide
Kaempferol rutinoside	6.30	593	285, 447	0.03 ± 0.00	Quercetin-3-rutinoside
Quercetin-3-rutinoside	5.98	609	301	2.43 ± 0.03	Quercetin-3-rutinoside
Total Flavonols				***96.99 ± 7.09***	
Flavones					
Apigenin rutinoside	6.75	577	269	0.02 ± 0.00	Quercetin-3-rutinoside
Total Flavones				***0.02 ± 0.00***	
Anthocyanins		**[M]$^+$(*m/z*)**			
Cyanidin-3-galactoside	2.63	449	287	0.96 ± 0.01	Cyanidin-3-glucoside
Peonidin-3-galactoside	3.10	463	301	2.76 ± 0.04	Cyanidin-3-glucoside
Total Anthocyanins				***3.72 ± 0.05***	

RT: retention time; *m/z*: mass to charge; N.Q. means compound detected but not quantified

3.2. Colonic *in vitro* biotransformation of cranberry (Poly)phenols

Based on the available catabolic pathways reported for cranberry (poly)phenols, especially for flavan-3-ols (Mena et al., 2019), forty-four microbial metabolites were targeted and up to twenty-four were quantified after the *in vitro* fermentation of unformulated- and phytosomeformulated cranberry extracts (Supplementary Table S1).

3.2.1. Identification of cranberry microbial metabolites

In control samples, incubated with growth medium and faecal slurry but without cranberry extracts, no or negligible amount of microbial metabolites were detected, indicating that the (poly)phenol free diet guaranteed blank faeces (data not shown). The same results were obtained when cranberry extracts were incubated with growth medium, without faecal slurry, indicating that the incubation process did not influence the degradation of cranberry (poly)phenols (data not shown). The appearance of microbial-derived metabolites obtained with the incubation of cranberry extracts with growth medium and faecal starter was strictly related to the interaction of parent compounds with colonic microbiota, as shown in Fig. 1. Among the specific colonic metabolites of flavan-3-ols, namely phenyl-γ-valerolactones and phenylvaleric acids, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone and 5-(3′-hydroxyphenyl)-γ-valerolactone were confirmed by comparison with their proper reference standards. On the contrary, 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone, 4-hydroxy-5-(dihydroxyphenyl)valeric acid, 4-hydroxy-5(hydroxyphenyl)valeric acid, 5-(dihydroxyphenyl)valeric acid and the intermediate metabolite 1-(dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol were tentatively identified based on MS^2 experiments, obtained by fragmentation of molecular ions at *m/z* 223, 225, 209, 291, respectively (Supplementary Table S1). Among six identified phenylpropanoic acids (Table 2), 3-(3′,4′-dihydroxyphenyl)propanoic acid was detected in fermented batches (Supplemental Table 1) but it was not included in the set of colonic metabolites because it was quantified also in control samples. The two quantified 3-(dihydroxyphenyl)propanoic acids, which shared the same *m/z* (181) had different chromatographic and MS characteristics (Supplementary Table S1), probably linked to the different position of their hydroxyl groups. Probably for of the same reasons, three and five different acetic and benzoic derivatives, respectively, were identified after cranberry *in vitro* biotransformation (Table 2).

3.2.2. Quantification of cranberry microbial metabolites

Although the *in vitro* fermentation led to the production of the same colonic metabolite profile, the different extract formulations caused slight differences related to the concentration of some colonic metabolites (Table 2). 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone was the main phenyl-γ-valerolactone quantified after unformulated- and phytosomeformulated cranberry fermentation, reaching similar concentrations for both extracts. Moreover, the concentration of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone after 5 h faecal incubation was similar to that obtained after 24 h incubation period, suggesting that the highest amount of this colonic metabolite was produced within 5 h (Table 2). Phenylvaleric acid derivatives were quantified after 24 h of faecal incubation using both cranberry extracts, and 4-hydroxy-5-(dihydroxyphenyl)valeric acid, 4-hydroxy-5-(hydroxyphenyl)valeric acid and 5-(dihydroxyphenyl)valeric acid were significantly higher in batches containing phytosome-formulated cranberry extract ($p < 0.05$ and $p < 0.01$). However, no significant differences emerged in total phenylγ-valerolactone and phenylvaleric acid production neither after 5 h incubation nor after 24 h incubation with faecal starter (Fig. 1). Looking to the two quantified 3-(dihydroxyphenyl)propanoic acids, only one compound clearly showed a trend related to the incubation period. This colonic metabolite was 1.6-fold significantly higher in unformulated extract comparing to phytosome-formulated extract after 24-h faecal incubation (Table 2). Following the same trend, 3-(3′-hydroxyphenyl)propanoic acid significantly increased within 24 h of *in vitro* fermentation, resulting higher in batches containing unformulated cranberry extract (+57% and + 35% at 5 h and 24 h, respectively, compared to 3-(3′ hydroxyphenyl)propanoic acid concentration detected using phytosome-formulated cranberry). The production trend of both 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid and 3-(3′-hydroxy-4′-methoxyphenyl)propanoic acid also reflected the degradation of cranberry native compounds, since their concentration increased within 24-h incubation. Additionally, 3-(3′-hydroxy-4′-methoxyphenyl) propanoic acid concentration was significantly higher in batches incubated with unformulated cranberry extract after 5 h and 24 h incubation period ($p < 0.05$ and $p < 0.001$, respectively). After 5 h incubation, 3′,4′-dihydroxyphenylacetic acid and 4′-hydroxyphenylacetic reached the highest concentrations, with no significant differences between the two cranberry formulations. On the contrary, 3′-hydroxyphenylacetic acid was significantly higher in phytosome-formulated extract after 5 h incubation compared to batches containing unformulated extract ($p < 0.05$) (Table 2). However, the highest concentration for 3′-hydroxyphenylacetic acid was reached after 24 h fermentation, time at which no differences were highlighted based on the different cranberry formulations. It should be noted that 3′-hydroxyphenylacetic acid presented the highest concentration upon cranberry extract fermentation among all the metabolites, reaching up to 156 µmol/L (Table 2). 3,4-Dihydroxybenzoic acid was quantified in the cranberry extract (Table 1) and represented the main hydroxybenzoic acid influencing the concentration of total metabolites. For this reason, although it was obviously detected in fermented batches (Supplementary Table S1), it was not considered among the quantified colonic metabolites derived from cranberry fermentation. As reported for 3-(dihydroxyphenyl) propanoic acid derivatives, two additional dihydroxybenzoic acids were quantified in fermented batches (Supplementary Table S1). The concentration of these two metabolites increased within the incubation period, and the concentration significantly differed ($p < 0.01$) when phytosome-formulated cranberry extract was fermented (Table 2). The total concentration of colonic metabolites was comparable at T0, ideally confirming the incubation of the same concentration of total proanthocyanins. After 5 h, the concentration of total phenolic metabolites was 1.1-fold higher ($p < 0.05$) in the unformulated extract than in the phytosome-formulated extract (Table 2), but no significant differences persisted when the total amount of metabolites was considered at the end of the incubation period (24 h) (Table 2).

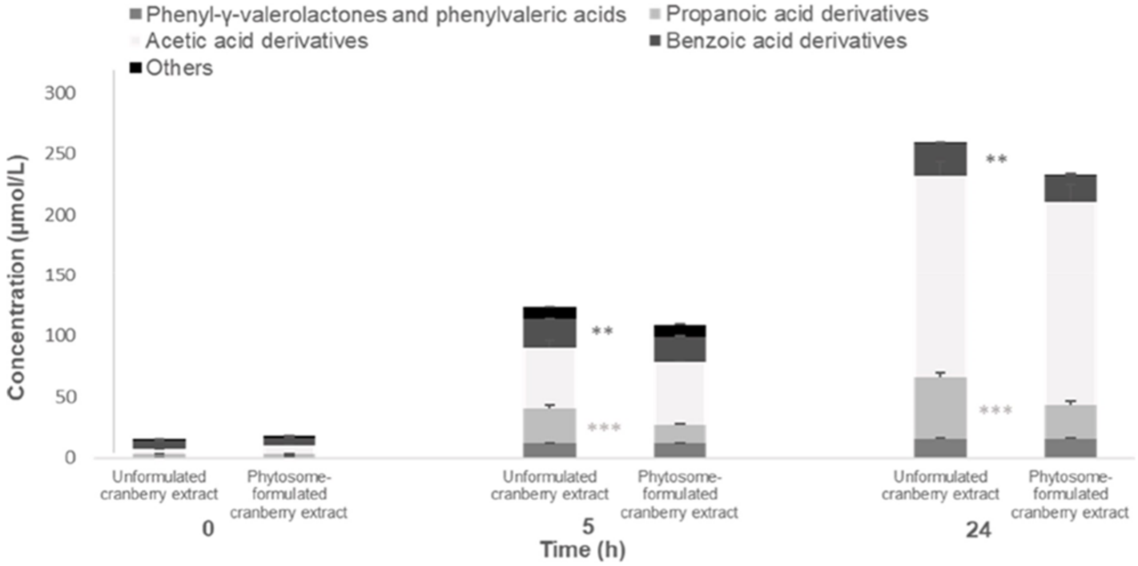

Fig. 1. Total concentration of phenyl-γ-valerolactones and phenylvaleric acids, and phenolic acid derivatives at 0 h, 5 h and 24 h after unformulated- and phytosome-formulated cranberry extract fermentation. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, significant differences between phenolic classes comparing the same incubation period (0 h, 5 h and 24 h).

Table 2
Concentration of faecal metabolites after the fermentation of unformulated- and phytosome-formulated cranberry extracts at different time points. Values are expressed as mean values ± SD (n = 3).

Compound	**Unformulated Cranberry Extract (μmol/L)**			**Phytosome-Formulated Cranberry Extract (μmol/L)**		
	0 h	5 h	24 h	0 h	5 h	24 h
Phenyl-γ-valerolactones and phenylvaleric acids						
1-(Dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol (tentative identification)	n.d.	n.d.	0.19 ± 0.04	n.d.	n.d.	0.11 ± 0.04
4-Hydroxy-5-(dihydroxyphenyl)valeric acid	n.d.	n.d.	0.34 ± 0.01	n.d.	0.05 ± 0.01 *	0.40 ± 0.04 *
5-(Tryhydroxyphenyl)-γ-valerolactone	n.d.	n.d.	0.21 ± 0.03	n.d.	n.d.	0.20 ± 0.03
4-Hydroxy-5-(hydroxyphenyl)valeric acid	n.d.	0.02 ± 0.00	0.05 ± 0.01	n.d.	0.02 ± 0.00	0.09 ± 0.00 **
5-(Dihydroxyphenyl)valeric acid	n.d.	0.03 ± 0.01	0.32 ± 0.04	n.d.	0.03 ± 0.00	0.63 ± 0.14 *
5-(3′,5′-Dihydroxyphenyl)-γ-valerolactone	n.d.	n.d.	0.69 ± 0.01	n.d.	n.d.	0.70 ± 0.11
5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone	0.56 ± 0.02	11.15 ± 0.23	13.36 ± 0.70	0.83 ± 0.41	10.80 ± 0.32	12.63 ± 0.46
5-(4′-Hydroxyphenyl)-γ-valerolactone and 5-(3′-hydroxyphenyl)-γ-valerolactone	n.d.	0.76 ± 0.21	0.97 ± 0.19	n.d.	0.85 ± 0.06	1.13 ± 0.14
Propanoic acid derivatives						
3-(Dihydroxyphenyl)propanoic acid	n.d.	n.d.	2.15 ± 0.29	n.d.	n.d.	1.80 ± 0.16
3-(Dihydroxyphenyl)propanoic acid	1.43 ± 0.20	3.22 ± 0.50	20.35 ± 1.09	1.61 ± 0.10	3.74 ± 0.40	11.99 ± 0.74***
3-(4′-Hydroxy-3′-methoxyphenyl)propanoic acid	n.d.	2.00 ± 0.38	n.d.	n.d.	1.57 ± 0.02	n.d.
3-(3′-Hydroxy-4′-methoxyphenyl)propanoic acid	0.48 ± 0.05	1.41 ± 0.27	11.76 ± 1.33	n.d.	0.95 ± 0.07 *	2.75 ± 0.19***
3-(3′-Hydroxyphenyl)propanoic acid	0.90 ± 0.14	21.56 ± 2.04	16.10 ± 0.66	1.03 ± 0.02	9.32 ± 0.38 **	10.45 ± 3.14 *
Phenylpropanoic acid	n.d.	0.06 ± 0.01	0.16 ± 0.03	n.d.	0.05 ± 0.01	0.15 ± 0.01
Acetic acid derivatives						
3′,4′-Dihydroxyphenylacetic acid	0.51 ± 0.08	29.14 ± 4.22	7.89 ± 0.36	0.62 ± 0.11	25.29 ± 0.87	11.07 ± 2.36
4′-Hydroxyphenylacetic acid	n.d.	4.11 ± 0.20	1.28 ± 0.37	1.81 ± 0.14 **	4.13 ± 0.31	1.46 ± 0.26
3′-Hydroxyphenylacetic acid	3.77 ± 0.07	16.96 ± 2.79	156.00 ± 11.92	4.70 ± 0.34 *	21.93 ± 0.10 *	155.59 ± 11.41
Benzoic acid derivatives						
Dihydroxybenzoic acid	n.d.	12.23 ± 0.23	17.67 ± 0.84	n.d.	9.48 ± 0.43 **	11.45 ± 1.14 **
Dihydroxybenzoic acid	1.72 ± 0.21	3.79 ± 0.21	3.60 ± 0.29	2.13 ± 0.10 *	5.08 ± 0.38 **	5.42 ± 0.35 **
4-Hydroxybenzoic acid	1.98 ± 0.20	2.81 ± 0.13	1.57 ± 0.11	1.74 ± 0.05	2.30 ± 0.12 **	0.68 ± 0.28 **
3-Hydroxybenzoic acid	1.37 ± 0.03	2.79 ± 0.19	2.57 ± 0.17	1.58 ± 0.42	2.25 ± 0.23 **	2.09 ± 0.43
Benzoic acid	1.07 ± 0.12	1.73 ± 0.09	1.82 ± 0.03	0.60 ± 0.13 *	1.01 ± 0.04 ***	1.04 ± 0.19 **
Other low molecular weight metabolites						
Cinnamic acid	n.d.	0.50 ± 0.04	n.d.	n.d.	0.43 ± 0.02	n.d.
Phenyllactic acid	2.09 ± 0.22	9.58 ± 0.45	0.21 ± 0.03	1.71 ± 0.05 *	9.66 ± 0.31	0.39 ± 0.29
Sum of microbial metabolites	***15.87 ± 0.78***	***123.84 ± 8.62***	***259.26 ± 11.75***	***18.35 ± 0.25 *****	***108.94 ± 1.85 ****	***232.22 ± 12.63***

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, significant differences between products comparing the same incubation period (0 h, 5 h and 24 h). n.d.: compounds not detected.

4. Discussion

In the present study, the *in vitro* fermentation of an unformulated and a phytosome-formulated cranberry extract was performed, in order to investigate the possible effect of the formulation on the colonic degradation of cranberry (poly)phenols, mainly flavan-3-ols. Both cranberry extracts were firstly characterized for their native (poly)phenol content, confirming compounds previously identified by Baron and colleagues (Baron et al., 2020). To date, a few studies investigated cranberry colonic *in vitro* biotransformation. Sánchez-Patán and colleagues (Sánchez-Patán et al., 2015) investigated the colonic metabolism of cranberry (poly)phenols, while Ou and colleagues (Ou et al., 2014) focused their attention only on microbial catabolism of cranberry procyanidins. This is the first study that explored the colonic metabolism of cranberry (poly)phenols and, at the same time, the application of a phospholipid delivery system (phytosome) on microbial bioaccessibility. A relevant number of colonic metabolites closely related to microbial degradation of cranberry (poly)phenols were recovered, suggesting that (poly)phenols of both unformulated- and phytosome-formulated cranberry extracts were metabolized by the gut microbiota *in vitro*. Contrary to previous studies (Ou et al., 2014; Sánchez-Patán et al., 2015), 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, a key metabolite in the microbial catabolism of flavan-3-ols (Mena et al., 2019), was identified and quantified. It was the most important compound for this class of colonic metabolites, as previously observed after human consumption of unformulated- and phytosome formulated cranberry extract (Baron et al., 2020). Via C-ring opening and A-ring fission, native cranberry (epi)catechins and procyanidins were transformed into 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and its monohydroxyl-derivatives (Mena et al., 2019; Rowland et al., 2018), whereas prodelphinidins, containing at least one (epi)gallocatechin moiety, were the precursors of 5-(3′,4′,5′-tryhydroxyphenyl)-γ-valerolactone and 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone (Mena et al., 2019) (Table 1). The γ-valerolactone ring could be opened by microbial enzymes producing valeric acid derivatives (Mena et al., 2019). Apart from phenyl-γ-valerolactones and phenylvaleric acids, which are specific microbial metabolites, the colonic degradation of flavan-3-ols, as well as those of other flavonoid classes, led to low molecular weight metabolites. Propanoic, acetic, benzoic and cinnamic acids derivatives are common intermediate and/or final colonic metabolites of almost all (poly)phenol classes (Del Rio et al., 2013; Monagas et al., 2010; Mosele et al., 2015; Rodriguez-Mateos et al., 2014). Together with 3-(3′,4′-dihydroxyphenyl)propanoic acid, which could derive from cyanidins (Kay et al., 2017), caffeoylquinic acid and caffeic acid (Del Rio, Stalmach, et al., 2010), two additional 3-(dihydroxyphenyl)propanoic acids have been detected. It has been reported that (epi)gallocatechins and myricetin could be converted in the colon into 3-(3′,5′-dihydroxyphenyl)propanoic acid and 3-(2′,4′-dihydroxyphenyl)propanoic acid (Mosele et al., 2015; Rowland et al., 2018). Generally, the α-oxidation of phenylpropanoic acids led to the formation of phenylacetic acids (Rodriguez-Mateos et al., 2014). 3′,4′-Dihydroxyphenylacetic acid and its monohydroxylated forms were recovered after the *in vitro* fermentation of both unformulated- and phytosome-formulated cranberry extracts, possibly linked to cranberry flavan-3-ol microbial degradation (Appeldoorn et al., 2009; Ou et al., 2014). However, phenylacetic acid derivatives are also among the main colonic metabolites of flavonols, mainly quercetin (Serra et al., 2012), and anthocyanins, mainly cyanidin (Mosele et al., 2015). Phenylpropanoic acid and phenylacetic acid biotransformation could further lead to the formation of benzoic acid derivatives. Feliciano and colleagues reported 2,3-dihydroxybenzoic acid, 2,4-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid among plasma and urinary metabolites after cranberry consumption (Feliciano et al., 2016). Recently, Baron and colleagues confirmed the presence of these metabolites in human urine after supplementation with unformulated and phytosome-formulated cranberry (Baron et al., 2020). Finally, being cranberry an important source of several (poly)phenol classes, the quantification of 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid and 3-(3′-hydroxy-4′-methoxyphenyl)propanoic acid could be attributed to the colonic biotransformation of hydroxycinnamic acids, as well as the microbial degradation of peonidin (Rodriguez-Mateos et al., 2014). Since the phytosome formulated ingredient contained only one third of the cranberry extract in weight than the unformulated cranberry, the content of incubated proanthocyanidins was standardized based on their DMAC value. After 5 h incubation, the unformulated extract led to a slightly significant higher production of metabolites, principally linked to a higher production of phenylpropanoic and benzoic acid derivatives (Fig. 1), suggesting that phytosome-formulated extract could be metabolized slowly at colonic level compared to the unformulated ingredient. Although some differences persisted considering single metabolites, the *in vitro* fermentation of proanthocyanidins from cranberry extracts led to comparable concentration of metabolites after 24 h, indicating the possibility to obtain the same set of colonic metabolites from flavan-3-ols using both unformulated- and phytosome formulated cranberry extract, with biological activity (Baron et al., 2020).

5. Conclusions

Both unformulated- and phytosome-formulated cranberry extracts can be considered important sources of (poly)phenols in human diet. The native compounds of both ingredients demonstrated a similar profile in faecal microbial catabolites after *in vitro* fermentation, suggesting that the extract formulation technology did not interfere with flavan-3-ol degradation during microbial metabolism. Both cranberry extracts displayed the capability to be a potential source of compounds which could produce a wide array of gut microbiota metabolites *in vitro*. Thus, results of this *in vitro* study can be pivotal for further explorations of phytosome-formulated cranberry on colonic microbiota in humans.

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CRediT authorship contribution statement

Letizia Bresciani: Methodology, Investigation, Writing – original draft, Writing - review & editing. Giuseppe Di Pede: Writing – original draft, Writing - review & editing. Claudia Favari: Methodology, Investigation. Luca Calani: Methodology, Investigation, Writing - review & editing. Veronica Francinelli: Methodology. Antonella Riva: Conceptualization, Resources. Giovanna Petrangolini: Conceptualization, Resources. Pietro Allegrini: Conceptualization, Resources. Pedro Mena: Conceptualization, Investigation, Resources, Writing - review & editing, Supervision. Daniele Del Rio: Conceptualization, Resources, Writing - review & editing, Supervision.

Declaration of interest

Letizia Bresciani, Giuseppe Di Pede, Claudia Favari, Luca Calani and Veronica Francinelli declare no conflict of interest. Pedro Mena and Daniele Del Rio have received research grants from Indena. Antonella Riva, Giovanna Petrangolini and Pietro Allegrini are Indena's employees.

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Appendix A. Supplementary data

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Supplementary data

Table S1: Mass spectrometric and chromatographic characteristics of faecal metabolites identified in unformulated- and phytosome formulated cranberry extracts.

Compound	**RT (min)**	**[M-H]$^-$ (*m/z*)**	**MS^2 ions(*m/z*)**	**Standard used for quantification**
1-(Dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol (tentative identification)	4.72	291		(−)-Epicatechin
1-(Hydroxyphenyl)-3-(2″,″,″-trihydroxyphenyl)-propan-2-ol	n.d.	275		
4-Hydroxy-5-(dihydroxyphenyl)valeric acid	2.12	225	207, 163, 101,123	5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone
5-(3′,4′,5′-Trihydroxyphenyl)-γ-valerolactone	2.46	223	123, 179, 205	5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone
4-Hydroxy-5-(hydroxyphenyl)valeric acid	3.15	209	191, 147, 101, 165	5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone
5-(Dihydroxyphenyl)valeric acid	4.38	209	191, 135, 165	5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone
5-(3′,5′-Dihydroxyphenyl)-γ-valerolactone	2.70	207	163, 123, 189	5-(3′,5′-Dihydroxyphenyl)-γ-valerolactone
5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone	3.05	207	163, 123	5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone
5-(4′-Hydroxyphenyl)-γ-valerolactone and/or 5-(3′- hydroxyphenyl)-γ-valerolactone*	4.27	191	147, 173, 107	5-(3′-Hydroxyphenyl)-γ-valerolactone
4-Hydroxy-5-phenylvaleric acid	n.d.	193		
5-(Hydroxyphenyl)valeric acid	n.d.	193		
5-Phenylvaleric acid	n.d.	177		
5-Phenyl-γ-valerolactone	n.d.	175		
4-Hydroxy-(5- hydroxyphenyl)butanoic acid	n.d.	195		
4-(Dihydroxyphenyl)butanoic acid	n.d.	195		
3-(Dihydroxyphenyl)propanoic acid	1.47	181	137, 151, 163	3-(3′,4′-Dihydroxyphenyl)propanoicacid
3-(Dihydroxyphenyl)propanoic acid	2.00	181	137, 113, 163	3-(3′,4′-Dihydroxyphenyl)propanoic acid
3-(3′,4′-Dihydroxyphenyl)propanoic acid	2.35	181	137	3-(3′,4′-Dihydroxyphenyl)propanoic acid
3-(4′-Hydroxy-3′-methoxyphenyl)propanoic acid	3.60	195	151, 136, 177	3-(4′-Hydroxy-3′-methoxyphenyl)propanoic acid
3-(3′-Hydroxy-4′-methoxyphenyl)propanoic acid	3.94	195	151, 136	3-(4′-Hydroxy-3′-methoxyphenyl)propanoic acid
3-(3′-Hydroxyphenyl)propanoic acid	3.58	165	121	3-(3′-Hydroxyphenyl)propanoic acid
Phenylpropanoic acid	5.51	149	105	3-(3′-Hydroxyphenyl)propanoic acid
3′,4′-Dihydroxyphenylacetic acid	1.48	167	123	3′,4′-Dihydroxyphenylacetic acid
4′-Hydroxyphenylacetic acid	2.33	151	107	4′-Hydroxyphenylacetic acid
3′-Hydroxyphenylacetic acid	2.64	151	107	3′-Hydroxyphenylacetic acid
Phenylacetic acid	n.d.	135		
3,4-Dihydroxybenzoic acid	1.29	153	109, 123	3,4-Dihydroxybenzoic acid
Dihydroxybenzoic acid	1.94	153	138, 109	3,4-Dihydroxybenzoic acid
Dihydroxybenzoic acid	2.22	153	109	3,4-Dihydroxybenzoic acid

4-Hydroxybenzoic acid	2.07	137	93	4-Hydroxybenzoic acid
3-Hydroxybenzoic acid	2.58	137	93	3-Hydroxybenzoic acid
Benzoic acid	4.15	121	77	4-Hydroxybenzoic acid
Cinnamic acid	3.41	147	103	4′-Hydroxycinnamic acid
Phenyllactic acid	3.37	165	147	3-(3′-Hydroxyphenyl)propanoic acid
3,5-Dimethoxy-4-hydroxybenzoicacid	n.d.	197		
3,4,5-Trihydroxybenzoic acid	n.d.	169		
4′-Hydroxy-3′-methoxy-phenylacetic acid	n.d.	181		
4-Hydroxy-3-methoxybenzoic acid	n.d.	167		
3,4-Dihydroxybenzaldehyde	n.d.	137		
Benzene-1,3,5-triol	n.d.	125		
Benzene-1,2,3-triol	n.d.	125		
1-Hydroxy-2-methoxybenzene	n.d.	123		
Hydroxybenzaldehyde	n.d.	121		
Benzene-1,2-diol	n.d.	109		

* This compound coeluated. RT: retention time; *m/z*: mass to charge; n.d.: not detected.

Chapter 5. Revisiting the bioavailability of flavan-3-ols in humans: a systematic review and comprehensive data analysis

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Revisiting the bioavailability of flavan-3-ols in humans: a systematic review and comprehensive data analysis

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Highlights

- Understanding flavan-3-ol bioavailability is essential to unravel their benefits
- Pharmacokinetic profiles of 180 flavan-3-ol metabolites were assessed
- Concentration levels for main metabolites of flavan-3-ols ranged 67-860 nmol/L
- Mean bioavailability for dietary flavan-3-ols is 31%
- Intra- and inter-source differences in flavan-3-ol bioavailability emerged

Abstract

This systematic review summarizes findings from human studies investigating the different routes of absorption, metabolism, distribution and excretion (ADME) of dietary flavan-3-ols and their circulating metabolites in healthy subjects. Literature searches were performed in PubMed, Scopus and the Web of Science. Human intervention studies using single and/or multiple intake of flavan-3-ols from food, extracts, and pure compounds were included. Forty-nine human intervention studies met inclusion criteria. Up to 180 metabolites were quantified from blood and urine samples following intake of flavan-3-ols, mainly as phase 2 conjugates of microbial catabolites (n=97), with phenyl-γ-valerolactones being the most representative ones (n=34). Phase 2 conjugates of monomers and phenyl-γ-valerolactones, the main compounds in both plasma and urine, reached two peak plasma concentrations (C_{max}) of 260 and 88 nmol/L at 1.8 and 5.3 h (T_{max}) after flavan-3-ol intake. They contributed to the bioavailability of flavan-3-ols for over 20 %. Mean bioavailability for flavan-3-ols was moderate (31±23%, n bioavailability values=20), and it seems to be scarcely affected by the amount of ingested compounds. While intra- and inter-source differences in flavan-3-ol bioavailability emerged, mean flavan-3-ol bioavailability was 82 % (n=1) and 63 % (n=2) after (−)-epicatechin and nut (hazelnuts, almonds) intake, respectively, followed by 25 % after consumption of tea (n=7), cocoa (n=5), apples (n=3) and grape (n=2). This highlights the need to better clarify the metabolic yield with which monomer flavan-3-ols and proanthocyanidins are metabolized in humans. This work clarified in a comprehensive way for the first time the ADME of a (poly)phenol family, highlighting the pool of circulating compounds that might be determinants of the putative beneficial effects linked to flavan-3-ol intake. Lastly, methodological inputs for implementing well-designed human and experimental model studies were provided.

Keywords: (Poly)phenols; Phenolics; Phenyl-γ-valerolactones; Pharmacokinetics; Metabolites; Excretion

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1. Introduction

A high adherence to plant-based dietary patterns has been strongly associated with a reduced risk of developing cardiometabolic diseases, type 2 diabetes, and certain cancers, the leading causes of mortality worldwide (FAO/WHO, 2004; Hemler and Hu, 2019; Qiao et al., 2022). The health benefits associated with a high plant-based food intake are attributted to the nutritional profile and the content of a multitude of bioactive compounds synthesized *in planta* in response to various environmental inputs (Crozier et al., 2006). Dietary bioactive compounds include thousands of phytochemicals belonging to different families, among which phenolic compounds (*aka* (poly)phenols) are the most important ones (Del Rio et al., 2013; Rodriguez-Mateos et al., 2014). (Poly)phenols, classified as flavonoids and non-flavonoids, have been identified as potential key mediators of the health benefits of plant-based diets (Manach et al., 2004; Williamson, 2017; Ulaszewska et al., 2020). Flavan-3-ols represent the most complex subclass of flavonoids, ranging from simple monomers to oligomeric and polymeric proanthocyanidins, also known as condensed tannins (Del Rio et al., 2013; Rodriguez-Mateos et al., 2014). The daily intake of flavan-3-ols varies from 200 to 1000 mg, representing together with hydroxycinnamic acids the main contributor of (poly)phenols in Western diet across all age groups, with main sources being tea, cocoa products, red wine, fruits (i.e. apples, pears, berries), and nuts (Vogiatzoglou et al., 2014a,2015; Ziauddeen et al., 2018). Various investigations have pinpointed the capacity of flavan-3-ols to positively affect platelet and endothelial function (Ostertag et al., 2013; Heiss et al., 2015; Rodriguez-Mateos et al., 2015b; Sansone et al., 2015; Saarenhovi et al., 2017; Gröne et al., 2019). The European Food Safety Authority (EFSA) approved a health claim related to cocoa flavan-3-ols and maintenance of normal endothelium-dependent vasodilation (Agostoni et al., 2012). Flavan-3-ol consumption has been associated with significantly lower systolic blood pressure in the EPIC Norfolk cohort (Ottaviani et al., 2020) and with improvements in some biomarkers related to metabolic syndrome development (Yang et al., 2012). Recently, cocoa flavan-3-ol supplementation has been found reducing cardiovascular disease death by 27% in the COSMOS trial (Sesso et al., 2022). After their consumption, only a small fraction of flavan-3-ols is absorbed along the upper gastrointestinal (GI) tract (Monagas et al., 2010). Over 70% of ingested flavan-3-ols reach intact the large bowel (Kahle et al., 2005, 2007; Hagl et al., 2011; Borges et al., 2013) where they are catabolized by gut microbiota into smaller phenolic compounds, which can be further metabolized by phase-2 conjugation reactions occurring in colonocytes or in the liver (Monagas et al., 2010; Mena et al., 2019a). Whereas it is well known that the ingested native compound seldom is the active compound reaching and interacting with organs and tissues but its metabolites (Velderrain-Rodríguez et al., 2014), it becomes clear that metabolites and catabolites of flavan-3-ols may directly drive the bioactivity recognised to these phytochemicals (Campos et al., 2019; Roager and Dragsted, 2019; Márquez Campos et al., 2020). However, to date, comprehensive data on the absorption, metabolism, distribution and excretion (ADME) of these dietary phytochemicals is scarce, and it appears to be largely influenced by the experimental design of the study, target population characteristics, and flavan-3-ol sources (Neilson and Ferruzzi, 2011; Velderrain-Rodríguez et al., 2014). To the best of our knowledge, no systematic and updated data is available on the nutrikinetic features for a comprehensive set of metabolites produced after flavan-3-ol intake.

Thus, this systematic review aims to (i) summarize findings from existing human studies evaluating the ADME of flavan-3-ols in healthy volunteers, (ii) perform a comprehensive data analysis of their circulating metabolites and associated nutrikinetic parameters including urinary recovery, (iii) establish an estimation of flavan-3-ol bioavailability based on published data.

2. Methods

2.1. Search strategy and study selection

This systematic review was reported in line with the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement guidelines (Moher et al., 2009; Page et al., 2021). The systematic literature search was conducted using PubMed, Scopus, and the Web of Science databases in May 2021 and updated in January 2022, using the syntaxes reported in Supplemental Table 1. Any temporal or spatial filters were applied to the search. Studies were included in the present systematic review provided (i) they were human studies investigating the ADME of flavan-3-ols in healthy subjects, (ii) volunteers consumed single or repeated (multiple) dose(s) of flavan-3-ols through a dietary source, an extract or a pure compound, (iii) they provided a quantitative characterization of the total content of ingested flavan-3-ols, (iv) native flavan-3-ols and their derived metabolites were quantified in plasma, serum, and/or urine samples without applying a hydrolysis step to remove phase-2 conjugating moieties, and (v) at least one nutrikinetic parameter is reported, namely peak plasma concentration (C_{max}), time to reach C_{max} (T_{max}), area under the curve (AUC), elimination half-life ($t_{½}$), total cumulative urinary excretion, or urinary excretion (expressed as % of intake), for native flavan-3-ols and their circulating metabolites. Exclusion criteria included (i) the consumption of flavan-3-ols through a mixture of different flavan-3-ol sources, and (ii) studies reported in a non-European languages. No restrictions for the characteristics of study participants for age, sex and ethnicity were applied.

2.2. Data extraction

Two author-pairs independently assessed the studies for their inclusion. Disagreement between authors was resolved through consultation with a third author. Data were extracted from each identified study using a standardized form and the following information was collected: name of the first author; year of publication; type of study (intervention or observational); chemical name, molecular weight, and PhytoHub ID (https://phytohub.eu/) of the circulating compound; type of biofluid(s) (i.e. plasma, serum, urine) in which circulating compounds were quantified; origin of flavan-3-ol metabolite [*unchanged* (when the native flavan-3-ol did not undergo any metabolic step following its ingestion), *host metabolism* (when the compound derived from a biotranformation by small intestine, hepatic or renal phase 1 or phase 2 enzyme), *gut microbiota metabolism* (when the compound derived from flavan-3-ol metabolism through gut microbiota activity), *host and gut microbiota metabolism* (when the compound derived from flavan-3-ol metabolism through gut microbiota activity and further conjugation by a phase 2 enzyme); chemical name of the precursor compound(s) of the metabolite [as i) single compound belonging to the flavan-3-ol class when it was clearly a precursor of that metabolite, or ii) class (i.e. flavan-3-ols) when various compounds belonging to the flavan-3-ol class were putative precursors of the same metabolite]; classification (i.e. food, pure compound, extract) and description of the ingested flavan-3-ol source; type of ingested dose(s) (i.e. single or repeated (multiple)); intervention duration (for studies in which multiple doses were ingested); ingested amount (µmol) of total flavan-3-ols (for multiple dose studies, the total daily dose was provided); description of the study population (i.e. number of subjects, sex, age, body mass index (BMI), and ethnicity, if available); and published values (i.e. mean, concentration unit, dispersion parameter type, dispersion parameter value, and time covered for AUC) for nutrikinetic parameters (i.e. T_{max}, C_{max}, AUC, and $t_{1/2}$) and urinary excretion data (expressed as cumulative excreted amount and/or % of intake) of the circulating compounds. Data on circulating compounds presented as mean and/or sum of metabolites belonging to different chemical species but grouped based on their chemical structure were excluded. On the other hand, data on simple phenolic acids that were not strictly related to flavan-3-ol intake, due to their putative production through the metabolism of other dietary compounds (Del Rio et al., 2013; Rodriguez-Mateos et al., 2014; Selma et al., 2009), were not collected. Finally, only data related to phase-2 conjugates of flavan-3-ols and their specific colonic metabolites, namely phenyl-γ-valerolactones and phenylvaleric acids (Mena et al., 2019a), were collected.

2.3. Data analysis

Chemical names of circulating metabolites were standardized according to Kay et al. (2020). When isomer identification for flavan-3-ol metabolites was not clearly stated in the manuscript, their identification was carried out considering the retention time of unknown isomers in comparison to other metabolites reported in the manuscript and, when possible, the main isomers found in blood fractions following flavan-3-ol intake by previous literature (Männistö and Kaakkola, 199 Stalmach et al., 2009; Actis Goretta et al., 2012; Ottaviani et al., 2012, 2016; van der Hooft et al., 2012; Liang et al., 2014; van Duynhoven et al., 2014; Rodriguez-Mateos et al., 2015a; Brindani et al., 2017; Pereira-Caro et al. 2017b, 2018; Borges et al., 2018; Gómez-Juaristi et al., 2019; Mayorga-Gross and Esquivel, 2019; Favari et al., 2020). If the total amount (µmol) of ingested flavan-3-ols was not reported in the manuscript, the total ingested flavan-3-ol content (including both monomers and oligomer/polymeric forms) was calculated by summing the ingested µmol of individual flavan-3-ols (calculated considering the amount and the molecular weight for each ingested compound), ignoring native compounds which accounted for less than 5 % of the total consumed flavan-3-ols. Nutrikinetic parameters and urinary excretion data for each metabolite were processed to obtain the following parameters (using harmonized units): (i) C_{max} (nmol/L); (ii) T_{max} (h) (values of $T_{max} \leq 2$ h for phenyl-γ-valerolactones and phenylvaleric acids were excluded due to their lack of physiological meaning (Borges et al., 2018; Mena et al., 2019a)); (iii) AUC (nmol/L*h); (iv) $t_{1/2}$ (h); (v) urinary excretion expressed as cumulative excreted amount (µmol), calculated by summing the excreted amounts over different time intervals when it was not reported; (vi) % of intake, calculated as the ratio between the cumulative urinary excretion (µmol) of the metabolite and the total intake (µmol) of ingested flavan-3-ols when no directly reported (urinary excretion data (expressed as % of intake) > 100%, possibly due to underestimations of the ingested dose of flavan-3-ols or to overestimations of the excreted amount occurring when metabolites were quantified without the proper reference standards (Ottaviani et al., 2018b), were excluded); and (vii) average concentration (C_{avg}) (nmol/L) as the ratio between AUC $(\text{nmol/L*h})_{(0\text{-}t)}$ and the total number of hours considered for AUC calculation (Mena et al., 2021) (when the time interval employed for AUC calculation was equal to 0-inf, it was considered as 24 h). When a circulating compound in a publication had a C_{avg} value exceeding its C_{max} value, C_{avg} value was excluded due to its low physiological relevance. C_{max}, AUC and C_{avg} values for each circulating compound were also normalized by dividing their value by the dose (µmol) of ingested flavan-3-ols (Mullen et al., 2009); in the case of multiple-dose studies, values of C_{max}, AUC and C_{avg} were normalized by using the total daily amount (µmol) of consumed flavan-3-ols. Normalized C_{max} values [C_{max} (nmol/L)/ingested µmol of flavan-3-ols] were used for comparisons among studies to determine the main circulating metabolites of flavan-3-ols. This approach avoided any confounding factors related to the putative dose-response relationship existing in the production of phenolic metabolites (Rodriguez-Mateos et al., 2016a, 2016b; Feliciano et al., 2017; Favari et al., 2020). Mean normalized C_{max} values ≥ 1 nmol/L (for unchanged monomer and dimer flavan-3-ols and host metabolites) and ≥ 0.3 nmol/L (for gut microbiota and host-gut microbiota metabolites) were selected as threshold values to define the main circulating forms of flavan-3-ol metabolites; they were established by ranking the metabolites according to their normalized C_{max} values and taking into account C_{max} values reached in the context of habitual flavan-3-ol dietary intake (Ottaviani et al., 2012a, 2012b, 2016; Urpi-Sarda et al., 2009; Borges et al., 2018; Vogiatzoglou et al., 2015, 2014b; Zanotti et al., 2015; Ziauddeen et al., 2018). Finally, to ensure data robustness, the main circulating metabolites of flavan-3-ols were selected when their mean normalized C_{max} values were calculated using at least three biological replicates deriving from at least two manuscripts. When data on flavan-3-ol bioavailability (%) was not reported in the manuscript, it was calculated by computing the ratio between the total flavan-3-ol metabolite urinary excretion (µmol) and the total intake (µmol) of parent flavan-3-ols (both monomers and oligomers/polymers) for each ingested source. Values for flavan-3-ol bioavailability (published and/or estimated) deriving from each study were averaged to provide a mean bioavailability value as accurate as possible, while excluding bioavailability data if they were i) < 1 and/or > 100%, or ii) calculated by not taking into account a complete and appropriate metabolic pathway in terms of metabolites produced following flavan-3-ol intake (i.e. excluding phase 2 conjugates and/or not considering an exhaustive array of colonic metabolites produced after flavan-3-ol intake). Data on plasma and urinary circulating compounds and on the bioavailability of flavan-3-ols were expressed as mean ± standard deviation (SD) and median (25th-75th percentile).

3. Results

3.1. Study Selection

The study selection process is shown in Supplemental Fig. 1. A total of 5517 records were identified through the database search. After removing 1822 duplicates, up to 3695 studies were screened, of which 3458 were excluded based on the title or abstract. A total of 237 eligible records went under the full-text screening process, after which 188 records were excluded. Forty-nine publications met eligibility criteria and were included in the data analysis.

3.2. Characteristics of the included studies

The main characteristics of the studies that met all inclusion criteria are reported in Supplemental Table 2. Out of the 49 included intervention studies (total sample size n = 653), 39 investigated the ADME of flavan-3-ols following a single dose intake. Five publications investigated the ADME of flavan-3-ols following a multiple-dose (1-30 days) intake, while the remaining five publications showed an experimental setting having both single and multiple doses. No observational study met inclusion criteria. Tea (both green and black tea) was the most commonly consumed source of flavan-3-ols in the studies that investigated flavan-3-ol ADME, followed by cocoa and its derived products, then by pure compounds (Supplemental Table 2). The administered doses (µmol) of total flavan-3-ols ranged from 4 to almost 10000 µmol [677.1 (215.6-1335.9) µmol (median (25th-75th percentile))] (Supplemental Table 2 and Supplemental Fig. 2).

3.3. Circulating compounds after flavan-3-ol intake by healthy subjects

Up to 180 quantified metabolites in blood and urine fractions were reported following intake of flavan-3-ols by healthy subjects (Table 1). This included 97 host-gut microbiota metabolites [among which phenyl-γ-valerolactones were the main representatives (n = 34), followed by phenylvaleric acids (19), benzoic acids (12), phenylacetic acids (8), phenylpropanoic acids (7), catechols (7), cinnamates (6), and hippuric acids (4)], 49 host metabolites [phase 2 conjugates of (−)-epicatechin (n = 22), (epi)gallocatechin (7), (−)- epigallocatechin (6), (+)-epicatechin (6), (epi)catechin-3-*O*-gallate (4), (epi)gallocatechin-3-*O*-gallate (2), (+)-catechin (1), and procyanidin dimer B2 (1)], 22 gut microbiota metabolites comprising 6 5C-ring fission catabolites (5 phenyl-γ-valerolactones and 1 phenylvaleric acid), 5 phenylacetic acids, 4 phenylpropanoic acids, 4 benzoic acids and 3 catechols) and 12 unchanged among monomer and dimer flavan-3-ols. The correct name and identifier in the database PhytoHub (www.phytohub.eu), where more chemical data can be found, are provided for each metabolite in Table 1.

Table 1. Metabolites of flavan-3-ols quantified at circulating level following flavan-3-ol intake by healthy humans. Metabolites are classified according to their metabolic origin: (I) *unchanged compounds*: indicates when the native flavan-3-ol did not undergo any metabolic step following its ingestion, (II) *host metabolism*: when the compound derived from a biotranformation by small intestine, hepatic or renal phase 1 or phase 2 enzymes, (III) *gut microbiota metabolism*: when the compound derived from flavan-3-ol metabolism through gut microbiota activity, (IV) *host and gut microbiota metabolism*: when the compound derived from flavan-3-ol metabolism through gut microbiota activity and further conjugation by a phase 2 enzyme. Metabolites are named according to Kay et al., 2020.

Systematic name of metabolite (Abbreviation)	MW (Da) of metabolite	PhytoHub ID of metabolite	Biofluid(s) where metabolite was quantified	Dietary source and precursor(s) of metabolite	Ref.
			Unchanged compounds		
Monomer flavan-3-ols					
(+)-Catechin ((+)-C)	290	PHUB000261	P, U	GT (+)-C; RW (+)-C	(Donovan et al., 2002; Hodgson et al., 2014; Mena et al., 2019b)
(−)-Epicatechin ((−)-EC)	290	PHUB000262	P, U	pure (−)-EC; GT (−)-EC; CHOC (−)-EC; CC (−)-EC	(Barnett et al., 2015; Chow et al., 2001; Hodgson et al., 2014; Mukai et al., 2021; Ottaviani et al., 2012; Yang et al., 2000)
(Epi)catechin[~] ((Epi)C)	290	PHUB001241	S, U	CC f-3-ols[§]	(Vitaglione et al., 2013)
(−)-Catechin ((−)-C)	290	PHUB002190	U	GT (−)-C	(Yang et al., 2000)
(+)-Epicatechin ((+)-EC)	290	PHUB002207	P	pure (+)-EC	(Moreno-Ulloa et al., 2018)
(−)-Epigallocatechin ((−)-EGC)	306	PHUB000264	P, U	GT (−)-EGC	(Chow et al., 2001; Hodgson et al., 2014; Mukai et al., 2021; Yang et al., 2000)
(+)-Gallocatechin ((+)-GC)	306	PHUB000268	P	GT (+)-GC	(Hodgson et al., 2014)
(−)-Gallocatechin ((−)-GC)	306	PHUB002189	U	GT (−)-GC	(Yang et al., 2000)
Galloyl flavan-3-ols					
(−)-Epicatechin-3-*O*-gallate ((−)-ECG)	442	PHUB000263	P	GT (−)-ECG	(Mukai et al., 2021; Stalmach et al., 2009)
(Epi)catechin-3-*O*-gallate[~] ((Epi)CG)	442	PHUB002265	P	BT f-3-ols[§]	(Van Duynhoven et al., 2014)
(−)-Epigallocatechin-3-*O*-gallate ((−)-EGCG)	458	PHUB000265	P	pure (−)-EGCG; GT (−)-EGCG;	(Abe et al., 2018; Chow et al., 2003, 2001; Del Rio et al., 2010b; Fernández et al., 2020; Gawande et al., 2008; Hodgson et al., 2014; Mukai et al., 2021; Nakagawa et al., 2009; Naumovski et al., 2015; Pietta et al., 1998; Stalmach et al., 2009; Ullmann et al., 2004, 2003)
(+)-Gallocatechin-3-*O*-gallate ((+)-GCG)	458	PHUB000269	P	GT (+)-GCG	(Hodgson et al., 2014)
(Epi)gallocatechin-3-*O*-gallate[#] ((Epi)GCG)	458	PHUB002256	P	BT f-3-ols[§]	(van Duynhoven et al., 2014)
Dimer flavan-3-ols					
Procyanidin B2 (Proc B2)	572	PHUB000277	U	CC proc B2	(Vitaglione et al., 2013)
			Host metabolism		
Phase 2 conjugates of (+)-catechin					
with methyl group(s)					
3′-Methoxy-(+)-catechin[*] (3′-Me-(+)-C)	304	PHUB002215	U	RW (+)-C	(Donovan et al., 2002)
Phase 2 conjugates of (−)-epicatechin					
with sulfate group(s)					
(−)-Epicatechin-3′-sulfate ((−)-EC-3′-S)	370	PHUB001030	P, U	CC f-3-ols[§]; pure (−)-EC; CHOC (−)-EC; CC (−)-EC	(Actis-Goretta et al., 2012; Barnett et al., 2015; Gómez-Juaristi et al., 2019; Ottaviani et al., 2012; Ottaviani et al., 2016, 2011; Rodriguez-Mateos et al., 2015a)
(−)-Epicatechin-4′-sulfate ((−)-EC-4′-S)	370	PHUB001032	P, U	CHOC (−)-EC	(Actis-Goretta et al., 2012)
(−)-Epicatechin-5-sulfate ((−)-EC-5-S)	370	PHUB001033	P, U	pure (−)-EC; CHOC (−)-EC; CC	(Actis-Goretta et al., 2012; Javier I. Ottaviani et al., 2012; Ottaviani

				(−)-EC	et al., 2016, 2011)
(−)-Epicatechin-7-sulfate ((−)-EC-7-S)	370	PHUB002147	P	pure (−)-EC; CC (−)-EC	(Ottaviani et al., 2012; Ottaviani et al., 2016)
(−)-Epicatechin-sulfate* ((−)-EC-S)	370	PHUB002150	P, U	CC f-3-ols§; GT f-3-ols§	(Del Rio et al., 2010b; Roura et al., 2008)
(−)-Epicatechin-sulfate*‡ ((−)-EC-S)	370	-	U	BT f-3-ols§	(Del Rio et al., 2010c; Pereira-Caro et al., 2017a)
with methyl and sulfate group(s)					
3′-Methoxy-(−)-epicatechin-4′-sulfate (3′-Me-(−)-EC-4′-S)	384	PHUB001049	P, U	pure (−)-EC; CHOC (−)-EC	(Actis-Goretta et al., 2012; Ottaviani et al., 2016)
3′-Methoxy-(−)-epicatechin-5-sulfate (3′-Me-(−)-EC-5-S)	384	PHUB001050	P, U	CC f-3-ols§; pure (−)-EC; CC (−)-EC; CHOC (−)-EC	(Actis-Goretta et al., 2012; Mullen et al., 2009; Ottaviani et al., 2016; Rodriguez-Mateos et al., 2015a)
3′-Methoxy-(−)-epicatechin-7-sulfate (3′-Me-(−)-EC-7-S)	384	PHUB001051	P, U	CC f-3-ols§; pure (−)-EC; CHOC (−)-EC	(Actis-Goretta et al., 2012; Ottaviani et al., 2016; Rodriguez-Mateos et al., 2015a)
4′-Methoxy-(−)-epicatechin-5-sulfate (4′-Me-(−)-EC-5-S)	384	PHUB001054	P, U	pure (−)-EC; CHOC (−)-EC	(Actis-Goretta et al., 2012; Ottaviani et al., 2016)
4′-Methoxy-(−)-epicatechin-7-sulfate (4′-Me-(−)-EC-7-S)	384	PHUB001055	P, U	pure (−)-EC; CHOC (−)-EC	(Actis-Goretta et al., 2012; Ottaviani et al., 2016)
Methoxy-(−)-epicatechin-sulfate* (Me-(−)-EC-S)	384	PHUB002236	P, U	GT f-3-ols§; pure (−)-EC; CC (−)-EC;	(Barnett et al., 2015; Del Rio et al., 2010b; Gómez-Juaristi et al., 2019)
Methoxy-(−)-epicatechin-sulfate*‡ (Me-(−)-EC-S)	384	-	U	BT f-3-ols§	(Del Rio et al., 2010c; Pereira-Caro et al., 2017a)
with glucuronic acid					
(−)-Epicatechin-3′-glucuronide ((−)-EC-3′-GlcUA)	466	PHUB001029	P, U	CC f-3-ols§; pure (−)-EC; CC (−)-EC; CHOC (−)-EC; GT f-3-ols§	(Actis-Goretta et al., 2012; Barnett et al., 2015; Gómez-Juaristi et al., 2019; Mullen et al., 2009; Ottaviani et al., 2012; Ottaviani et al., 2016, 2011; Rodriguez-Mateos et al., 2015a; Roura et al., 2008; Stalmach et al., 2009)
(−)-Epicatechin-4′-glucuronide ((−)-EC-4′-GlcUA)	466	PHUB001031	P, U	CHOC (−)-EC	(Actis-Goretta et al., 2012)
(−)-Epicatechin-7-glucuronide ((−)-EC-7-GlcUA)	466	PHUB001034	P	pure (−)-EC; CHOC (−)-EC	(Actis-Goretta et al., 2012; Ottaviani et al., 2016)
(−)-Epicatechin-glucuronide* ((−)-EC-GlcUA)	466	PHUB001035	P, U	GT f-3-ols§; BT f-3-ols§	(Del Rio et al., 2010b, 2010c; Pereira-Caro et al., 2017a)
with methyl(s) and glucuronic acid					
4′-Methoxy-(−)-epicatechin-glucuronide* (4′-Me-(−)-EC-GlcUA)	480	PHUB001056	U	CHOC (−)-EC	(Actis-Goretta et al., 2012)
3′-Methoxy-(−)-epicatechin-7-glucuronide (3′-Me-(−)-EC-7-GlcUA)	480	PHUB002148	P	pure (−)-EC	(Ottaviani et al., 2016)
Methoxy-(−)-epicatechin-glucuronide* (Me-(−)-EC-GlcUA)	480	PHUB002153	P, U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
3'-Methoxy-(−)-epicatechin-glucuronide* (3′-Me-(−)-EC-GlcUA)	480	PHUB002232	U	CHOC (−)-EC	(Actis-Goretta et al., 2012)
3′-Methoxy-(−)-epicatechin-5-glucuronide (3′-Me-(−)-EC-5-GlcUA)	480	PHUB002266	P, U	pure (−)-EC; CC (−)-EC	(Gómez-Juaristi et al., 2019; Ottaviani et al., 2016)
4′-methoxy-(−)-epicatechin-7- glucuronide (4′-Me-(−)-EC-7-GlcUA)	480	PHUB002310	P	CC (−)-EC	(JOttaviani et al., 2012)
with sulfate and glucuronic acid					
(−)-Epicatechin-sulfate-glucuronide* ((−)-EC-S-GlcUA)	546	PHUB001037	U	GT f-3-ols§	(Del Rio et al., 2010b)
Phase 2 conjugates of (+)-epicatechin					
with sulfate group(s)					
(+)-Epicatechin-sulfate* ((+)-EC-S)	370	PHUB002210	P	pure (+)-EC	(Moreno-Ulloa et al., 2018)
(+)-Epicatechin-5-sulfate ((+)-EC-5-S)	370	PHUB002312	P	CC (+)-EC	(Ottaviani et al., 2011)

(+)-Epicatechin-3′-sulfate ((+)-EC-3′-S)	370	PHUB002313	P	CC (+)-EC	(Ottaviani et al., 2011)
with methyl and sulfate group(s)					
Methoxy-(+)-epicatechin-sulfate* (Me-(+)-EC-S)	384	PHUB002209	P	pure (+)-EC	(Moreno-Ulloa et al., 2018)
with glucuronic acid					
(+)-Epicatechin-glucuronide* ((+)-EC-GlcUA)	466	PHUB002208	P	pure (+)-EC	(Moreno-Ulloa et al., 2018)
(+)-Epicatechin-3′-glucuronide ((+)-EC-3′-GlcUA)	466	PHUB002311	P	CC (+)-EC	(Ottaviani et al., 2011)
Phase 2 conjugates of (epi)catechin~					
with sulfate group(s)					
(Epi)catechin-sulfate*~ ((Epi)C-S)	370	PHUB001040	P, U	CC f-3-ols§; GT f-3-ols§; RGP f-3-ols§; A f-3-ols§; HZT f-3-ols§; ALM f-3-ols§; BT f-3-ols§; RW f-3-ols§	(Bartolomé et al., 2010; Castello et al., 2018; Clarke et al., 2014; Garrido et al., 2010; Hollands et al., 2020; Mena et al., 2019b; Mocciaro et al., 2019; Motilva et al., 2016; Mullen et al., 2009; Van Duynhoven et al., 2014)
(Epi)catechin-sulfate*‡~ ((Epi)C-S)	370	-	P, U	GT f-3-ols§; G f-3-ols§	(Calani et al., 2012b; Stalmach et al., 2012, 2009)
(Epi)catechin-disulfate*~ ((Epi)C-S-S)	450	PHUB002239	U	A f-3-ols§; HZT f-3-ols§	(Hollands et al., 2020; Mocciaro et al., 2019)
with methyl and sulfate group(s)					
Methoxy-(epi)catechin-sulfate*~ (Me-(epi)C-S)	384	PHUB002164	P, U	GT f-3-ols§; RGP f-3-ols§; A f-3-ols§; HZT f-3-ols§; ALM f-3-ols§; BT f-3-ols§; CC f-3-ols§; RW f-3-ols§	(Castello et al., 2018; Clarke et al., 2014; Garrido et al., 2010; Hollands et al., 2020; Mena et al., 2021, 2019b; Mocciaro et al., 2019; Motilva et al., 2016; van Duynhoven et al., 201
Methoxy-(epi)catechin-sulfate*‡~ (Me-(epi)C-S)	384	-	P, U	GT f-3-ols§; G f-3-ols§	(Calani et al., 2012b; Stalmach et al., 2012, 2009)
with glucuronic acid					
(Epi)catechin-glucuronide*~ ((Epi)C-GlcUA)	466	PHUB002205	P, U	GT f-3-ols§; RGP f-3-ols§; A f-3-ols§; ALM f-3-ols§; G f-3-ols§; CC f-3-ols§; RW f-3-ols§	(Calani et al., 2012b; Castello et al., 2018; Clarke et al., 2014; Garrido et al., 2010; Hollands et al., 2020; Mena et al., 2021, 2019b; Motilva et al., 2016; Stalmach et al., 2012)
with methyl(s) and glucuronic acid					
Methoxy-(epi)catechin-glucuronide*~ (Me-(epi)C-GlcUA)	480	PHUB002206	U	GT f-3-ols§; HZT f-3-ols§; ALM f-3-ols§; G f-3-ols§; RW f-3-ols§; RGP f-3-ols§	(Garrido et al., 2010; Mena et al., 2019b; Mocciaro et al., 2019; Motilva et al., 2016; Stalmach et al., 2012)
with sulfate and glucuronic acid					
(Epi)catechin-sulfate-glucuronide*~ ((Epi)C-S-GlcUA)	546	PHUB002188	P, U	RGP f-3-ols§; A f-3-ols§; GT f-3-ols§; HZT f-3-ols§; CC f-3-ols§	(Castello et al., 2018; Hollands et al., 2020; Mena et al., 2021, 2019b; Mocciaro et al., 2019)
(Epi)catechin-sulfate-glucuronide*‡~ ((Epi)C-S-GlcUA)	546	-	U	GT f-3-ols§	(Calani et al., 2012b)
Phase 2 conjugates of (−)-epigallocatechin					
with methyl group(s)					
Methoxy-(−)-epigallocatechin*‡ (Me-(−)-EGC)	320	-	U	BT f-3-ols§	(Del Rio et al., 2010c)
with sulfate group(s)					
(−)-Epigallocatechin-sulfate* ((−)-EGC-S)	386	PHUB002166	U	GT f-3-ols§	(Clarke et al., 2014; Del Rio et al., 2010b)
(−)-Epigallocatechin-sulfate*‡ ((−)-EGC-S)	386	-	U	BT f-3-ols§	(Del Rio et al., 2010c)
with methyl and sulfate group(s)					
Methoxy-(−)-epigallocatechin-sulfate* (Me-(−)-EGC-S)	400	PHUB002168	P, U	GT f-3-ols§; BT f-3-ols§	(Clarke et al., 2014; Del Rio et al., 2010b, 2010c)
with glucuronic acid					
(−)-Epigallocatechin-glucuronide* ((−)-EGC-GlcUA)	482	PHUB002165	P, U	GT f-3-ols§; BT f-3-ols§	(Clarke et al., 2014; Del Rio et al., 2010b, 2010c)

with methyl(s) and glucuronic acid					
Methoxy-(−)-epigallocatechin-glucuronide[*] (Me-(−)-EGC-GlcUA)	496	PHUB002167	P, U	GT f-3-ols[§]	(Clarke et al., 2014; Del Rio et al., 2010b)
Methoxy-(−)-epigallocatechin-glucuronide[*‡] (Me-(−)-EGC-GlcUA)	496	-	U	BT f-3-ols[§]	(Del Rio et al., 2010c)
with methyl, sulfate and glucuronic acid					
Methoxy-(−)-epigallocatechin-sulfate-glucuronide[*] (Me-(−)-EGC-S-GlcUA)	576	PHUB002181	U	GT f-3-ols[§]	(Del Rio et al., 2010b)
Phase 2 conjugates of (epi)gallocatechin[#]					
with sulfate group(s)					
(Epi)gallocatechin-sulfate[*#] ((Epi)GC-S)	386	PHUB002246	U	HZT f-3-ols[§]	(Mocciaro et al., 2019)
(Epi)gallocatechin-sulfate[*‡#] ((Epi)GC-S)	386	-	U	GT f-3-ols[§]	(Calani et al., 2012b; Stalmach et al., 2009)
with methyl and sulfate group(s)					
Methoxy-(epi)gallocatechin-sulfate[*#] (Me-(epi)GC-S)	400	PHUB002203	P, U	BT f-3-ols[§]; G f-3-ols[§]	(Stalmach et al., 2012; van Duynhoven et al., 2014)
4′-Methoxy-(epi)gallocatechin-sulfate[*‡#] (4′-Me-(epi)GC-S)	400	-	P, U	GT f-3-ols[§]	(Stalmach et al., 2009)
Methoxy-(epi)gallocatechin-sulfate[*‡#] (Me-(epi)GC-S)	400	-	U	GT f-3-ols[§]	(Calani et al., 2012b)
Dimethoxy-(epi)gallocatechin-sulfate[*#] (Di-me-(epi)GC-S)	414	PHUB002204	P	BT f-3-ols[§]	(van Duynhoven et al., 2014)
with glucuronic acid					
(Epi)gallocatechin-glucuronide[*#] ((Epi)GC-GlcUA)	482	PHUB001041	P, U	GT f-3-ols[§]; HZT f-3-ols[§]	(Calani et al., 2012b; Mena et al., 2019b; Mocciaro et al., 2019; Stalmach et al., 2009)
with methyl(s) and glucuronic acid					
4′-Methoxy-(epi)gallocatechin-glucuronide[*#] (4′-Me-(epi)GC-GlcUA)	496	PHUB001058	P, U	GT f-3-ols[§]	(Stalmach et al., 2009)
Methoxy-(epi)gallocatechin-glucuronide[*#] (Me-(epi)GC-GlcUA)	496	PHUB002158	U	GT f-3-ols[§]	(Calani et al., 2012b; Mena et al., 2019b)
with methyl, sulfate and glucuronic acid					
Methoxy-(epi)gallocatechin-sulfate-glucuronide[*#] (Me-(epi)GC-S-GlcUA)	576	PHUB002241	U	GT f-3-ols[§]	(Mena et al., 2019b)
Methoxy-(epi)gallocatechin-sulfate-glucuronide[*‡#] (Me-(epi)GC-S-GlcUA)	576	-	U	GT f-3-ols[§]	(Calani et al., 2012b)
Phase 2 conjugates of (epi)catechin-3-*O*-gallate[~]					
with sulfate group(s)					
(Epi)catechin-3-*O*-gallate-sulfate[*~] ((Epi)CG-S)	522	PHUB002254	P	BT f-3-ols[§]	(van Duynhoven et al., 2014)
with methyl and sulfate group(s)					
Methoxy-(epi)catechin-3-*O*-gallate-sulfate[*~] (Me-(epi)CG-S)	536	PHUB002252	P	BT f-3-ols[§]	(van Duynhoven et al., 2014)
with sulfate and glucuronic acid					
(Epi)catechin-3-*O*-gallate-sulfate-glucuronide[*~] ((Epi)CG-S-GlcUA)	698	PHUB002255	P	BT f-3-ols[§]	(van Duynhoven et al., 2014)
with methyl, sulfate and glucuronic acid					
Methoxy-(epi)catechin-3-*O*-gallate-sulfate-glucuronide[*~] (Me-(epi)CG-S-GlcUA)	712	PHUB002253	P	BT f-3-ols[§]	(van Duynhoven et al., 2014)
Phase 2 conjugates of (epi)gallocatechin-3-*O*-gallate[#]					

with sulfate group(s)					
(Epi)gallocatechin-3-*O*-gallate-sulfate*# ((Epi)GCG-S)	538	PHUB001043	P	BT f-3-ols§	(van Duynhoven et al., 2014)
with methyl and sulfate group(s)					
Methoxy-(epi)gallocatechin-3-*O*-gallate-sulfate*# (Me-(epi)GCG-S)	552	PHUB002257	P	BT f-3-ols§	(van Duynhoven et al., 2014)
Phase 2 conjugates of Procyanidin B-type					
with sulfate group(s)					
Procyanidin B-type sulfate* (Proc B2-S)	658	PHUB002245	U	HZT f-3-ols§	(Mocciaro et al., 2019)
Gut microbiota metabolism					
Phenyl-γ-valerolactones					
5-(3′-Hydroxyphenyl)-γ-valerolactone (3′-OH-VL)	192	PHUB001249	U	CC f-3-ols§	(Gómez-Juaristi et al., 2019)
5-(4′-Hydroxyphenyl)-γ-valerolactone (4′-OH-VL)	192	PHUB001834	U	GT f-3-ols§	(Mena et al., 2019b)
5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone (3′,4′-diOH-VL)	208	PHUB001993	P, S, U	RW f-3-ols§; CC f-3-ols§; CR f-3-ols§; RGP f-3-ols§; A f-3-ols§; GT f-3-ols§	(Castello et al., 2018; Favari et al., 2020; Gómez-Juaristi et al., 2019; Hodgson et al., 2014; Hollands et al., 2020; Mena et al., 2019b; Motilva et al., 2016; Vitaglione et al., 2013)
5-(3′,5′-Dihydroxyphenyl)-γ-valerolactone (3′,5′-diOH-VL)	208	PHUB002235	U	RGP f-3-ols§	(Castello et al., 2018)
5-(3′,4′,5′-Trihydroxyphenyl)-γ-valerolactone (3′,4′,5′-triOH-VL)	224	PHUB001833	U	GT f-3-ols§	(Roowi et al., 2010)
Phenylvaleric acids					
4-Hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid (4-OH-(3′,4′-diOH-VA))	226	PHUB001987	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
Phenylpropanoic acids					
3-(4′-Hydroxyphenyl)propanoic acid (4′-OH-PPA)	166	PHUB000605	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
3-(3′-Hydroxyphenyl)propanoic acid (3′-OH-PPA)	166	PHUB001047	U	CC f-3-ols§	(Gómez-Juaristi et al., 2019)
3-(3′,4′-Dihydroxyphenyl)propanoic acid (3′,4′-diOH-PPA)	182	PHUB000604	U	CC f-3-ols§; BT f-3-ols§	(Gómez-Juaristi et al., 2019; Pereira-Caro et al., 2017a)
3-Hydroxy-3-(3′-hydroxyphenyl)propanoic acid (3-OH-(3′-OH-PPA))	182	PHUB001331	U	GT f-3-ols§; pure (−)-EC	(Ottaviani et al., 2016; Roowi et al., 2010)
Phenylacetic acids					
Phenylacetic acid (PAA)	136	PHUB001068	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
4′-Hydroxyphenylacetic acid (4′-OH-PAA)	152	PHUB000543	U	GT f-3-ols§; BT f-3-ols§	(Pereira-Caro et al., 2017a; Roowi et al., 2010)
3′-Hydroxyphenylacetic acid (3′-OH-PAA)	152	PHUB001048	U	CC f-3-ols§; BT f-3-ols§	(Gómez-Juaristi et al., 2019; Pereira-Caro et al., 2017a)
3′,4′-Dihydroxyphenylacetic acid (3′,4′-diOH-PAA)	168	PHUB000527	U	CC f-3-ols§; BT f-3-ols§	(Gómez-Juaristi et al., 2019; Pereira-Caro et al., 2017a)
2-Hydroxy-2-(4′-hydroxyphenyl)acetic acid (2-OH-(4′-OH-PAA))	168	PHUB001584	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
Benzoic acids					
3-Hydroxybenzoic acid (3-OH-BA)	138	PHUB000294	U	CC f-3-ols§; BT f-3-ols§; GT f-3-ols§	(Clarke et al., 2014; Gómez-Juaristi et al., 2019; Pereira-Caro et al., 2017a)
4-Hydroxybenzoic acid (4-OH-BA)	138	PHUB000295	U	BT f-3-ols§; GT f-3-ols§	(Pereira-Caro et al., 2017a; Roowi et al., 2010)

3,4-Dihydroxybenzoic acid (3,4-diOH-BA)	154	PHUB000310	U	CC f-3-ols§; BT f-3-ols§	(Gómez-Juaristi et al., 2019; Pereira-Caro et al., 2017a)
3,4,5-Trihydroxybenzoic acid (3,4,5-triOH-BA)	170	PHUB000303	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
Catechols					
Benzene-1,2-diol (BE-1,2-diol)	110	PHUB000583	U	GT f-3-ols§; BT f-3-ols§	(Pereira-Caro et al., 2017a; Roowi et al., 2010)
Benzene-1,2,3-triol (BE-1,2,3-triol)	126	PHUB000632	U	GT f-3-ols§; BT f-3-ols§	(Pereira-Caro et al., 2017a; Roowi et al., 2010)
Benzene-1,3,5-triol (BE-1,3,5-triol)	126	PHUB001126	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
Host and gut microbiota metabolism					
Phenyl-γ-valerolactones					
Phase 2 conjugates of 5-(hydroxyphenyl)-γ-valerolactone					
with sulfate group(s)					
5-(Phenyl)-γ-valerolactone-3′-sulfate (VL-3′-S)	272	PHUB001751	P, U	CR f-3-ols§; RGP f-3-ols§; CC f-3-ols§; A f-3-ols§; BT f-3-ols§; GT f-3-ols§; pure (−)-EC; HZT f-3-ols§	(Anesi et al., 2019; Castello et al., 2018; Favari et al., 2020; Gómez-Juaristi et al., 2019; Mena et al., 2021, 2019b; Mocciaro et al., 2019; Ottaviani et al., 2016; Pereira-Caro et al., 2017a; Van Duynhoven et al., 2014)
5-(Phenyl)-γ-valerolactone-4′-sulfate (VL-4′-S)	272	PHUB002182	P	CR f-3-ols§	(Favari et al., 2020)
5-(Phenyl)-γ-valerolactone-sulfate* (VL-S)	272	PHUB002267	U	GT f-3-ols§	(Calani et al., 2012b)
with glucuronic acid					
5-(Phenyl)-γ-valerolactone-glucuronide* (VL-GlcUA)	368	PHUB002159	P, U	GT f-3-ols§; A f-3-ols§	(Calani et al., 2012b; Yuste et al., 2018)
5-(Phenyl)-γ-valerolactone-3′-glucuronide (VL-3′-GlcUA)	368	PHUB002183	P, U	CR f-3-ols§; RGP f-3-ols§; CC f-3-ols§; GT f-3-ols§; BT f-3-ols§	(Anesi et al., 2019; Castello et al., 2018; Favari et al., 2020; Gómez-Juaristi et al., 2019; Mena et al., 2021, 2019b; van Duynhoven et al., 2014)
5-(Phenyl)-γ-valerolactone-4′-glucuronide (VL-4′-GlcUA)	368	PHUB002247	P, U	CR f-3-ols§; BT f-3-ols§; HZT f-3-ols§	(Favari et al., 2020; Mocciaro et al., 2019; van Duynhoven et al., 2014)
Phase 2 conjugates of 5-(dihydroxyphenyl)-γ-valerolactone					
with methyl group(s)					
5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-methoxy (4′-OH-VL-3′-Me)	222	PHUB002145	P	GT f-3-ols§	(Hodgson et al., 2014)
with sulfate group(s)					
5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-sulfate (4′-OH-VL-3′-S)	288	PHUB001755	P, U	pure (−)-EC; A f-3-ols§; ALM f-3-ols§; BT f-3-ols§	(Garrido et al., 2010; Hollands et al., 2020; Ottaviani et al., 2016; Pereira-Caro et al., 2017a; van Duynhoven et al., 2014)
5-(3'-Hydroxyphenyl)-γ-valerolactone-4'-sulfate (3′-OH-VL-4′-S)	288	PHUB001995	P, U	CR f-3-ols§; CC f-3-ols§; A f-3-ols§; RS (−)-EC; BT f-3-ols§	(Anesi et al., 2019; Feliciano et al., 2017, 2016; Gómez-Juaristi et al., 2019; Istas et al., 2018; Pereira-Caro et al., 2017a)
5-(Hydroxyphenyl)-γ-valerolactone-sulfate* (OH-VL-S)	288	PHUB002179	P, U	GT f-3-ols§; CC f-3-ols§	(Clarke et al., 2014; Del Rio et al., 2010b; Mena et al., 2021, 2019b)
5-(5′-Hydroxyphenyl)-γ-valerolactone-3′-sulfate (5′-OH-VL-3′-S)	288	PHUB002185	P, U	CR f-3-ols§; RGP f-3-ols§; BT f-3-ols§; HZT f-3-ols§	(Castello et al., 2018; Favari et al., 2020; Mocciaro et al., 2019; van Duynhoven et al., 2014)
5-(Hydroxyphenyl)-γ-valerolactone-sulfate*‡ (OH-VL-S)	288	-	P, U	CR f-3-ols§; RGP f-3-ols§; HZT f-3-ols§	(Castello et al., 2018; Favari et al., 2020; Mocciaro et al., 2019)
5-(Phenyl)-γ-valerolactone-3′,5′-disulfate (VL-3′,5′-di-S)	368	PHUB002161	U	GT f-3-ols§	(Calani et al., 2012b)
5-(Phenyl)-γ-valerolactone-4′,5′-disulfate (VL-4′,5′-di-S)	368	PHUB002162	U	GT f-3-ols§	(Calani et al., 2012b)
with methyl and sulfate group(s)					

5-(Phenyl)-γ-valerolactone-methoxy-sulfate[*] (VL-Me-S)	302	PHUB002180	P, U	GT f-3-ols[§]; RGP f-3-ols[§]; CC f-3-ols[§]; BT f-3-ols[§]; HZT f-3-ols[§]	(Castello et al., 2018; Clarke et al., 2014; Gómez-Juaristi et al., 2019; Mocciaro et al., 2019; van Duynhoven et al., 2014)
5-(Phenyl)-γ-valerolactone-4′-methoxy-3′-sulfate (VL-4′-Me-3′-S)	302	PHUB002187	P, U	CR f-3-ols[§]; A f-3-ols[§]; ALM f-3-ols[§]	(Anesi et al., 2019; Favari et al., 2020; Garrido et al., 2010)
5-(Phenyl)-γ-valerolactone-3′-methoxy-4′-sulfate (VL-3′-Me-4′-S)	302	PHUB002216	P, U	CR f-3-ols[§]; A f-3-ols[§]; ALM f-3-ols[§]	(Anesi et al., 2019; Bartolomé et al., 2010; Favari et al., 2020)
with glucuronic acid					
5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-glucuronide (4′-OH-VL-3′-GlcUA)	384	PHUB002146	P, U	pure (−)-EC; CR f-3-ols[§]; BT f-3-ols[§]; HZT f-3-ols[§]; RGP f-3-ols[§]; CC f-3-ols[§]; A f-3-ols[§]; ALM f-3-ols[§]	(Castello et al., 2018; Favari et al., 2020; Garrido et al., 2010; Gómez-Juaristi et al., 2019; Hollands et al., 2020; Mena et al., 2021; Mocciaro et al., 2019; Ottaviani et al., 2016; van Duynhoven et al., 2014)
5-(Hydroxyphenyl)-γ-valerolactone-glucuronide[*] (OH-VL-GlcUA)	384	PHUB002160	U	GT f-3-ols[§]; BT f-3-ols[§]	(Clarke et al., 2014; Del Rio et al., 2010b; Pereira-Caro et al., 2017a)
5-(5′-Hydroxyphenyl)-γ-valerolactone-3′-glucuronide (5′-OH-VL-3′-GlcUA)	384	PHUB002186	P, U	GT f-3-ols[§]; BT f-3-ols[§]; CR f-3-ols[§]	(Calani et al., 2012b; Favari et al., 2020; van Duynhoven et al., 2014)
5-(3′-Hydroxyphenyl)-γ-valerolactone-4′-glucuronide (3′-OH-VL-4′-GlcUA)	384	PHUB002234	P, U	CR f-3-ols[§]; RGP f-3-ols[§]; CC f-3-ols[§]; A f-3-ols[§]; ALM f-3-ols[§]; BT f-3-ols[§]; HZT f-3-ols[§]	(Castello et al., 2018; Favari et al., 2020; Garrido et al., 2010; Gómez-Juaristi et al., 2019; Hollands et al., 2020; Mena et al., 2021; Mocciaro et al., 2019; van Duynhoven et al., 2014)
5-(Hydroxyphenyl)-γ-valerolactone-glucuronide[*‡] (OH-VL-GlcUA)	384	-	U	GT f-3-ols[§]; A f-3-ols[§]	(Anesi et al., 2019; Calani et al., 2012b)
with methyl(s) and glucuronic acid					
5-(Phenyl)-γ-valerolactone-methoxy-glucuronide[*] (VL-Me-GlcUA)	398	PHUB002163	P, U	GT f-3-ols[§]; CR f-3-ols[§]; CC f-3-ols[§]; A f-3-ols[§]	(Anesi et al., 2019; Calani et al., 2012b; Favari et al., 2020; Gómez-Juaristi et al., 2019)
5-(Phenyl)-γ-valerolactone-5′-methoxy-3′-glucuronide (VL-5′-Me-3′-GlcUA)	398	PHUB002191	P	BT f-3-ols[§]	(van Duynhoven et al., 2014)
5-(Phenyl)-γ-valerolactone-3′-methoxy-4′-glucuronide (VL-3′-Me-4′-GlcUA)	398	PHUB002212	U	ALM f-3-ols[§]	(Garrido et al., 2010)
5-(Phenyl)-γ-valerolactone-4′-methoxy-3′-glucuronide (VL-4′-Me-3′-GlcUA)	398	PHUB002213	U	ALM f-3-ols[§]	(Garrido et al., 2010)
with sulfate and glucuronic acid					
5-(Phenyl)-γ-valerolactone-sulfate-glucuronide[*] (VL-S-GlcUA)	464	PHUB002156	P, U	pure (−)-EC; GT f-3-ols[§]; CR f-3-ols[§]; RGP f-3-ols[§]; A f-3-ols[§]; BT f-3-ols[§]; HZT f-3-ols[§]; CC f-3-ols[§]	(Anesi et al., 2019; Calani et al., 2012b; Castello et al., 2018; Del Rio et al., 2010b; Favari et al., 2020; Mena et al., 2021, 2019b; Mocciaro et al., 2019; Ottaviani et al., 2016; van Duynhoven et al., 2014)
Phase 2 conjugates of 5-(trihydroxyphenyl)-γ-valerolactone					
with sulphate group(s)					
5-(Dihydroxyphenyl)-γ-valerolactone-sulfate[*] (DiOH-VL-S)	304	PHUB002177	U	GT f-3-ols[§]	(Clarke et al., 2014; Del Rio et al., 2010b)
5-(4′,5′-Dihydroxyphenyl)-γ-valerolactone-3′-sulfate (4′,5′-DiOH-VL-3′-S)	304	PHUB002199	P, U	BT f-3-ols[§]	(Pereira-Caro et al., 2017a; van Duynhoven et al., 2014)
5-(3′,5′-Dihydroxyphenyl)-γ-valerolactone-4′-sulfate (3′,5′-DiOH-VL-4′-S)	304	PHUB002218	U	BT f-3-ols[§]	(Pereira-Caro et al., 2017a)
5-(Dihydroxyphenyl)-γ-valerolactone-sulfate[*‡] (DiOH-VL-S)	304	-	U	GT f-3-ols[§]	(Calani et al., 2012b)
with methyl and sulfate group(s)					
5-(Hydroxyphenyl)-γ-valerolactone-methoxy-	318	PHUB002178	P, U	GT f-3-ols[§]; BT f-3-ols [§]	(Clarke et al., 2014; Del Rio et al., 2010b; Mena et al., 2019b; van

sulfate* (OH-VL-Me-S)					Duynhoven et al., 2014)
5-(Hydroxyphenyl)-γ-valerolactone-methoxy-sulfate*‡ (OH-VL-Me-S)	318	-	U	GT f-3-ols§	(Calani et al., 2012b)
with glucuronic acid					
5-(Dihydroxyphenyl)-γ-valerolactone-glucuronide* (DiOH-VL-GlcUA)	400	PHUB002176	U	GT f-3-ols§	(Clarke et al., 2014; Del Rio et al., 2010b; Mena et al., 2019b)
5-(4′,5′-Dihydroxyphenyl)-γ-valerolactone-3′-glucuronide (4′,5′-DiOH-VL-3′-GlcUA)	400	PHUB002197	P, U	BT f-3-ols§	(Pereira-Caro et al., 2017a; van Duynhoven et al., 2014)
5-(3′,5′-Dihydroxyphenyl)-γ-valerolactone-4′-glucuronide (3′,5′-DiOH-VL-4′-GlcUA)	400	PHUB002198	P	BT f-3-ols§	(van Duynhoven et al., 2014)
5-(Dihydroxyphenyl)-γ-valerolactone-glucuronide*‡ (DiOH-VL-GlcUA)	400	-	U	GT f-3-ols§	(Calani et al., 2012b)
with methyl(s) and glucuronic acid					
5-(Hydroxyphenyl)-γ-valerolactone-methoxy-glucuronide* (OH-VL-Me-GlcUA)	414	PHUB002200	P, U	GT f-3-ols§; BT f-3-ols§	(Clarke et al., 2014; van Duynhoven et al., 2014)
5-(Hydroxyphenyl)-γ-valerolactone-methoxy-glucuronide*‡ (OH-VL-Me-GlcUA)	414	-	U	GT f-3-ols§	(Calani et al., 2012b)
with sulfate and glucuronic acid					
5-(Hydroxyphenyl)-γ-valerolactone-sulfate-glucuronide* (OH-VL-S-GlcUA)	480	PHUB002201	P	BT f-3-ols§	(van Duynhoven et al., 2014)
Phenylvaleric acids					
Phase 2 conjugates of 5-(hydroxyphenyl)valeric acid					
with sulfate group(s)					
5-(Phenyl)valeric acid-sulfate* (PVA-S)	274	PHUB002225	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
5-(Phenyl)valeric acid-3′-sulfate (PVA-3′-S)	274	PHUB002264	U	HZT f-3-ols§	(Mocciaro et al., 2019)
with glucuronic acid					
5-(Phenyl)valeric acid-glucuronide* (PVA-GlcUA)	370	PHUB002226	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
5-(Phenyl)valeric acid-3′-glucuronide (PVA-3′-GlcUA)	370	PHUB002262	U	HZT f-3-ols§	(Mocciaro et al., 2019)
Phase 2 conjugates of 5-(dihydroxyphenyl)valeric acid					
with sulfate group(s)					
5-(4′-Hydroxyphenyl)-valeric acid-3′-sulfate (4′-OH-PVA-3′-S)	290	PHUB002263	U	HZT f-3-ols§	(Mocciaro et al., 2019)
with methyl and sulfate group(s)					
5-(Phenyl)valeric acid-methoxy-sulfate* (PVA-Me-S)	304	PHUB002251	P, U	BT f-3-ols§; HZT f-3-ols§	(Mocciaro et al., 2019; van Duynhoven et al., 2014)
with glucuronic acid					
5-(4′-Hydroxyphenyl)valeric acid-3′-glucuronide (4′-OH-PVA-3′-GlcUA)	386	PHUB002222	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
5-(3′-Hydroxyphenyl)valeric acid-4′-glucuronide (3′-OH-PVA-4′-GlcUA)	386	PHUB002223	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
with sulfate and glucuronic acid					
5-(Phenyl)-valeric acid-sulfate-glucuronide* (PVA-S-GlcUA)	466	PHUB002242	P, U	RGP f-3-ols§	(Castello et al., 2018)
Phase 2 conjugates of 4-hydroxy-5-(hydroxyphenyl)valeric acid					
with sulfate group(s)					

4-Hydroxy-5-(phenyl)valeric acid-3′-sulfate (4-OH-(PVA)-3′-S)	290	PHUB002220	P, U	pure (−)-EC; BT f-3-ols§	(Ottaviani et al., 2016; Pereira-Caro et al., 2017a)
4-Hydroxy-5-(phenyl)valeric acid-4′-sulfate (4-OH-(PVA)-4′-S)	290	PHUB002221	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
Phase 2 conjugates of 4-hydroxy-5-(dihydroxyphenyl)valeric acid					
with sulfate group(s)					
4-Hydroxy-5-(4′-hydroxyphenyl)valeric acid-3′-sulfate (4-OH-(4′-OH-PVA)-3′-S)	306	PHUB001989	P, U	CC f-3-ols§; A f-3-ols§; pure (−)-EC	(Anesi et al., 2019; Gómez-Juaristi et al., 2019; Ottaviani et al., 2016)
4-Hydroxy-5-(hydroxyphenyl)valeric acid-sulfate* (4-OH-(OH-PVA)-S)	306	PHUB002224	P, U	BT f-3-ols§	(Pereira-Caro et al., 2017a; van Duynhoven et al., 2014)
with methyl and sulfate group(s)					
4-Hydroxy-5-(phenyl)valeric acid-methoxy-sulfate* (4-OH-(PVA)-Me-S)	320	PHUB002258	P, U	BT f-3-ols§; A f-3-ols§	(Anesi et al., 2019; van Duynhoven et al., 2014)
with glucuronic acid					
4-Hydroxy-5-(3′-hydroxyphenyl)valeric acid-4′-glucuronide (4-OH-(3′-OH-PVA)-4′-GlcUA)	402	PHUB001745	P, U	pure (−)-EC	(Ottaviani et al., 2016)
4-Hydroxy-5-(4′-hydroxyphenyl)valeric acid-3′-glucuronide (4-OH-(4′-OH-PVA)-3′-GlcUA)	402	PHUB002238	U	CC f-3-ols§; A f-3-ols§	(Anesi et al., 2019; Gómez-Juaristi et al., 2019)
4-Hydroxy-5-(hydroxyphenyl)valeric acid-glucuronide* (4-OH-(OH-PVA)-GlcUA)	402	PHUB002259	P	BT f-3-ols§	(van Duynhoven et al., 2014)
4-Hydroxy-5-(hydroxyphenyl)valeric acid-glucuronide*‡ (4-OH-(OH-PVA)-GlcUA)	402	-	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
with methyl(s) and glucuronic acid					
4-Hydroxy-5-(phenyl)valeric acid-methoxy-glucuronide* (4-OH-(PVA)-Me-GlcUA)	416	PHUB002260	P	BT f-3-ols§	(van Duynhoven et al., 2014)
Phase 2 conjugates of 4-hydroxy-5-(trihydroxyphenyl)valeric acid					
with methyl(s) and glucuronic acid					
4-Hydroxy-5-(hydroxyphenyl)valeric acid-methoxy-glucuronide* (4-OH-(OH-PVA)-Me-GlcUA)	432	PHUB002261	P	BT f-3-ols§	(van Duynhoven et al., 2014)
Phase 2 conjugates of cinnamates					
with methyl group(s)					
4′-Hydroxy-3′-methoxycinnamic acid (4′-OH-3′-Me-CA)	194	PHUB000608	U	CC f-3-ols§; BT f-3-ols§	(Gómez-Juaristi et al., 2019; Pereira-Caro et al., 2017a)
3′-Hydroxy-4′-methoxycinnamic acid (3′-OH-4′-Me-CA)	194	PHUB000622	U	CC f-3-ols§; BT f-3-ols§	(Gómez-Juaristi et al., 2019; Pereira-Caro et al., 2017a)
with sulfate group(s)					
4′-Hydroxycinnamic acid-3′-sulfate (4′-OH-CA-3′-S)	260	PHUB001594	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
with methyl and sulfate group(s)					
3′-Methoxycinnamic acid-4′-sulfate (3′-Me-CA-4′-S)	274	PHUB001171	U	BT f-3-ols§; HZT f-3-ols§	(Mocciaro et al., 2019; Pereira-Caro et al., 2017a)
with methyl(s) and glucuronic acid					
3′-Methoxycinnamic acid-4′-glucuronide (3′-Me-CA-4′-GlcUA)	370	PHUB001170	U	BT f-3-ols§; HZT f-3-ols§	(Mocciaro et al., 2019; Pereira-Caro et al., 2017a)
4′-Methoxycinnamic acid-3′-glucuronide (4′-Me-CA-3′-GlcUA)	370	PHUB001432	U	HZT f-3-ols§	(Mocciaro et al., 2019)
Phase 2 conjugates of phenylpropanoic acids					
with methyl group(s)					
3-(3′-Methoxy-4′-hydroxyphenyl)propanoic acid (3′-	196	PHUB001168	U	CC f-3-ols§	(Gómez-Juaristi et al., 2019)

Me-4′-OH-PPA)					
with sulfate group(s)					
3-(Phenyl)propanoic acid-4′-sulfate (PPA-4′-S)	246	PHUB002227	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
3-(3′-Hydroxyphenyl)propanoic acid-4′-sulfate (3′-OH-PPA-4′-S)	262	PHUB001206	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
3-(4′-Hydroxyphenyl)propanoic acid-3′-sulfate (4′-OH-PPA-3′-S)	262	PHUB001588	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
with methyl and sulfate group(s)					
3-(3′-Methoxyphenyl)propanoic acid-4′-sulfate (3′-Me-PPA-4′-S)	276	PHUB001436	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
with glucuronic acid					
3-(4′-Hydroxyphenyl)propanoic acid-3′-glucuronide (4′-OH-PPA-3′-GlcUA)	358	PHUB001204	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
with methyl(s) and glucuronic acid					
3-(3′-Methoxyphenyl)propanoic acid-4′-glucuronide (3′-Me-PPA-4′-GlcUA)	372	PHUB001435	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
Phase 2 conjugates of phenylacetic acids					
with methyl group(s)					
4′-Hydroxy-3′-methoxyphenylacetic acid (4′-OH-3′-Me-PAA)	182	PHUB000617	U	GT f-3-ols§; CC f-3-ols§	(Gómez-Juaristi et al., 2019; Roowi et al., 2010)
3′-Methoxy-4′-hydroxyphenylacetic acid (3′-Me-4′-OH-PAA)	182	PHUB001920	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
2-Hydroxy-2-(4′-hydroxy-3′-methoxyphenyl)acetic acid (2-OH-(4′-OH-3′-Me-PAA))	198	PHUB001585	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
with sulfate group(s)					
Dihydroxyphenylacetic acid-sulfate* (Di-OH-PAA-S)	248	PHUB002157	U	pure (−)-EC	(Ottaviani et al., 2016)
with methyl and sulfate group(s)					
Methoxy-phenylacetic acid-sulfate* (Me-PAA-S)	262	PHUB001375	P	BT f-3-ols§	(van Duynhoven et al., 2014)
3′-Methoxyphenylacetic acid-4′-sulfate (3′-Me-PAA-4′-S)	262	PHUB002228	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
4′-Methoxyphenylacetic acid-3′-sulfate (4′-Me-PAA-3′-S)	262	PHUB002229	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
with methyl(s) and glucuronic acid					
Methoxyphenylacetic acid-glucuronide* (Me-PAA-GlcUA)	358	PHUB002230	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
Phase 2 conjugates of benzoic acids					
with methyl group(s)					
4-Methoxy-trihydroxybenzoic acid (4-Me-triOH-BA)	184	PHUB001861	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
3-Methoxy-trihydroxybenzoic acid (3-Me-triOH-BA)	184	PHUB002269	U	GT f-3-ols§; BT f-3-ols§	(Clarke et al., 2014; Pereira-Caro et al., 2017a)
Methoxy-trihydroxybenzoic acid*‡ (Me-triOH-BA)	184	-	P	GT f-3-ols§	(Hodgson et al., 2014)
with sulfate group(s)					
Benzoic acid-sulfate* (BA-S)	202	PHUB002173	U	GT f-3-ols§	(Clarke et al., 2014)
Benzoic acid-4-sulfate (BA-4-S)	218	PHUB001583	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
Benzoic acid-3-sulfate (BA-3-S)	218	PHUB002231	U	BT f-3-ols§; GT f-3-ols§	(Clarke et al., 2014; Pereira-Caro et al., 2017a)
4-Hydroxybenzoic acid-3-sulfate (4-OH-BA-3-S)	234	PHUB001289	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)

3-Hydroxybenzoic acid-4-sulfate (3-OH-BA-4-S)	234	PHUB001921	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
with methyl and sulfate group(s)					
Methoxy-dihydroxybenzoic acid-sulfate* (Me-diOH-BA-S)	264	PHUB002270	U	GT f-3-ols§	(Clarke et al., 2014)
3,5-Dimethoxy-hydroxybenzoic acid-4-sulfate (3,5-DiMe-OH-BA-4-S)	278	PHUB002174	U	GT f-3-ols§	(Clarke et al., 2014)
with glucuronic acid					
Trihydroxybenzoic acid-glucuronide* (TriOH-BA-GlcUA)	346	PHUB002271	U	GT f-3-ols§	(Clarke et al., 2014)
with methyl(s) and glucuronic acid					
Methoxy-dihydroxybenzoic acid-glucuronide* (Me-diOH-BA-GlcUA)	360	PHUB002272	U	GT f-3-ols§	(Clarke et al., 2014)
3,5-Dimethoxy-hydroxybenzoic acid-4-glucuronide (3,5-DiMe-OH-BA-4-GlcUA)	374	PHUB001443	U	GT f-3-ols§	(Clarke et al., 2014)
Hippuric acids					
Hippuric acid (HA)	179	PHUB001174	P, U	pure (−)-EC; CHOC f-3-ols§; GT f-3-ols§; BT f-3-ols§	(Clarke et al., 2014; Ottaviani et al., 2016; Rios et al., 2003; Roowi et al., 2010; van Duynhoven et al., 2014)
3′-Hydroxyhippuric acid (3′-OH-HA)	195	PHUB001161	P, U	pure (−)-EC; CC f-3-ols§; BT f-3-ols§	(Gómez-Juaristi et al., 2019; Ottaviani et al., 2016; Pereira-Caro et al., 2017a; van Duynhoven et al., 2014)
4′-Hydroxyhippuric acid (4′-OH-HA)	195	PHUB001334	U	CHOC f-3-ols§; CC f-3-ols§; BT f-3-ols§	(Gómez-Juaristi et al., 2019; Pereira-Caro et al., 2017a; Rios et al., 2003)
with sulfate group(s)					
Hippuric acid-sulfate* (HA-S)	259	PHUB002175	U	GT f-3-ols§	(Clarke et al., 2014)
Phase 2 conjugates of catechols					
with sulfate group(s)					
Hydroxy-benzene-sulfate* (OH-BE-S)	190	PHUB002196	P	BT f-3-ols§	(van Duynhoven et al., 2014)
Benzene-2,3-diol-1-sulfate (BE-2,3-diol-1-S)	206	PHUB001967	U	BT f-3-ols§	(Pereira-Caro et al., 2017a)
Benzene-1,3-diol-2-sulfate (BE-1,3-diol-2-S)	206	PHUB002268	P, U	BT f-3-ols§	(Pereira-Caro et al., 2017a; van Duynhoven et al., 2014)
with methyl and sulfate group(s)					
1-methoxy-benzene-2-sulfate (1-Me-BE-2-S)	204	PHUB002193	P	BT f-3-ols§	(van Duynhoven et al., 2014)
2-methoxy-benzene-1-sulfate (2-Me-BE-1-S)	204	PHUB002194	P	BT f-3-ols§	(van Duynhoven et al., 2014)
with glucuronic acid					
Hydroxy-benzene-glucuronide* (OH-BE-GlcUA)	286	PHUB002195	P	BT f-3-ols§	(van Duynhoven et al., 2014)
Benzene-1,3-diol-2-glucuronide (BE-1,3-diol-2-GlcUA)	302	PHUB002192	P, U	BT f-3-ols§	(Pereira-Caro et al., 2017a; van Duynhoven et al., 2014)

*symbol: when the position of the conjugation is unknown, ‡symbol: this compound is reported as the sum of isomers (in this case, no PhytoHub ID was created), ~symbol: when the compound corresponds to (−/+)-epicatechin or (−/+)-catechin, #symbol: when the compound corresponds to (−/+)-epigallocatechin or (−/+)-gallocatechin, f-3-ols§: when various native flavan-3-ols are possible precursors of the same metabolite. P: plasma, U: urine; S: serum; BT: black tea; GT: green tea; CR: cranberry; RW: red wine; CHOC: chocolate; CC: cocoa; A: apple; RGP: red grape pomace; HZT: hazelnut; ALM: almond; G: grape; RS: raspberry.

Grouping all the flavan-3-ol metabolites based on their metabolic pathway and chemical structure, up to 11 different classes of circulating compounds related strictly to flavan-3-ol intake, namely unchanged monomer and dimer flavan-3-ols, phase 2 conjugates of monomeric and dimeric flavan-3-ols (P2MD), phase 2 conjugates of galloylated flavan-3-ols, phenyl-γ-valerolactones, phenylvaleric acids, cinnamates, phenylpropanoic acids, phenylacetic acids, benzoic acids, hippuric acids, and catechols, were identified in blood and urine fractions following flavan-3-ol intake. Unchanged monomer and dimer flavan-3-ol class was mostly represented by (+/−)-epigallocatechin-*O*-gallate and (+/−)-gallocatechin-*O*-gallate, whereas the main representatives for P2MD class were monomeric flavan-3-ols conjugated with methoxy and sulfate moieties (Supplemental Fig. 3). Phase 2 conjugates of 5-(dihydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(dihydroxyphenyl)valeric acid appeared in biofluids most frequently compared to their aglycones and other hydroxylated forms (Supplemental Fig. 4). Out of the 180 quantified metabolites following intake of flavan-3-ols, 88 of them were recovery only in urine samples, followed by those detected in both plasma/serum and urine (n=56), and only in plasma/serum (n=36) (Supplemental Fig. 5). The intake of tea flavan-3-ols provided the most heterogeneous set of circulating metabolites (n = 134), mainly in the form of phenyl-γ-valerolactones (31), P2MD (18), and phenylvaleric acids (13), followed by cocoa and its derived products (48), pure compounds (30), nuts or oily fruits (hazelnuts, almonds) (28), grape and its derived products (19), apple (19), and berries (14) (Fig. 1).

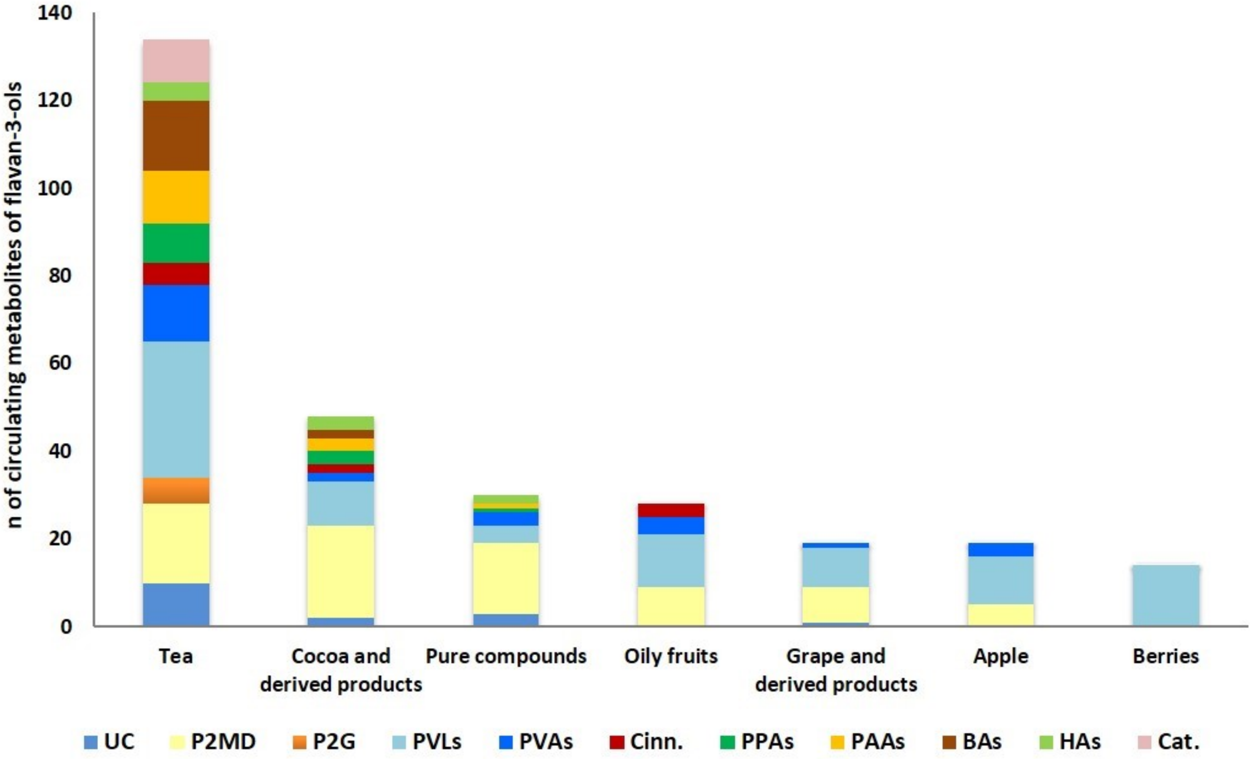

Fig. 1. Number of metabolites of flavan-3-ols, grouped by classes based on their metabolic pathway and chemical structure, quantified at circulating level following intake of the different flavan-3-ol sources by healthy humans. Unchanged monomer and dimer flavan-3-ols (UC), phase 2 conjugates of monomer and dimer flavan-3-ols (P2MD), phase 2 conjugates of galloyl flavan-3-ols (P2G), phenyl-γ-valerolactones (PVLs), phenylvaleric acids (PVAs), cinnamates (Cinn.), phenylpropanoic acids (PPAs), phenylacetic acids (PAAs), benzoic acids (BAs), hippuric acids (HAs), catechols (Cat.). For food sources containing other classes of (poly)phenols yielding also small phenolic metabolites, only P2MD, P2G, PVLs, and PVA were taken into account (i.e. apple, grape and derived products, and berries).

3.4. Nutrikinetics and urinary excretion of flavan-3-ol metabolites in biological samples

3.4.1. Nutrikinetic parameters of the different classes of flavan-3-ol metabolites

The nutrikinetic data for flavan-3-ol metabolites, grouped by classes, are presented in Fig. 2 and Table 2. Except for nutrikinetic parameters that were independent of dose-response with native compounds consumed, such as T_{max} and $t_{1/2}$, an important intra-class variability emerged, as shown from the SD values obtained for C_{max}, AUC and C_{avg} (Table 2). Unchanged monomer and dimer flavan-3-ols reached a C_{max} of 565 ± 1112 (mean ± SD) and 271 (36-597) nmol/L (median (25th-75th percentile) at T_{max} 1.9 ± 1.0 and 1.6 (1.4-2.0) h. P2MD and phenyl-γ-valerolactones, which are the most specific metabolite classes of flavan-3-ols quantified in plasma, reached a C_{max} of 260 ± 483 and 77 (31-256) nmol/L, and 89 ± 211 and 7 (1-59) nmol/L for P2MD and phenyl-γ-valerolactones, respectively. Phase 2 conjugates of galloylated flavan-3-ols reached a T_{max} greater than 2.5 h. Unchanged monomer and dimer flavan-3-ols showed a C_{avg} of 143 ± 181 and 81 (11-221) nmol/L, followed by P2MD (111 ± 235 and 20 (4-99) nmol/L), and phenyl-γ valerolactones (20 ± 49 and 1 (0-9) nmol/L) (Fig. 2 and Table 2). Phenyl-γ-valerolactones and phenylvaleric acids had higher values of $t_{1/2}$ compared to unchanged monomer and dimer flavan-3-ols and P2MD (10.4 ± 11.4 and 6.4 (4.8-11.0) h for phenyl-γ-valerolactones; 16.7 ± 16.7 and 7.6 (7.1-21.8) h for phenylvaleric acids). Although analyses of plasma samples did not reveal in a clear, robust and comprehensive manner the nutrikinetic profile of low molecular weight phenolic compounds upon flavan-3-ol intake (Fig. 2 and Table 2), we found that both hippuric acids and catechols reached C_{max} values exceeding 1000 nmol/L over 6 h (T_{max}), followed by benzoic acids (about 111 nmol/L). Normalized values for C_{max}, AUC and C_{avg}, calculated for the different classes of flavan-3-ol metabolites, are reported in Supplemental Fig. 6 and Table 2. This normalization approach highlighted the higher contribution of P2MD to the plasma concentration of flavan-3-ol metabolites with respect to unchanged monomer and dimer flavan-3-ols. Despite normalization, a large within-class variability was found.

3.4.2. Nutrikinetics of the main circulating flavan-3-ol metabolites

Based on the 68 normalized C_{max} mean values calculated for all the metabolites quantified in blood fractions (serum/plasma) (Supplemental Fig. 7A), nine compounds were established as the most abundant circulating metabolites of flavan-3-ols: one unchanged monomer flavan-3-ol, namely (−)-epigallocatechin-3-*O*-gallate [(−)-EGCG], five P2MD [(−)-epicatechin-3′-sulfate, methoxy-(−)-epicatechin-sulfate, (−)-epicatechin-3′-glucuronide, (epi)catechin-sulfate, (epi)catechin-glucuronide], and three phenyl-γ-valerolactones (5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate, 5-(hydroxyphenyl)-γ-valerolactone-sulfate and 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-glucuronide). The nutrikinetic data for these main flavan-3-ol metabolites are presented in Fig. 3 and Supplemental Table 3, while normalized data is presented in Supplemental Fig. 8 and Supplemental Table 3. (−)-EGCG, representing the main unchanged monomer flavan-3-ol recovered at circulating level following flavan-3-ol consumption, reached a C_{max} of 868 ± 1327 and 476 (272-771) nmol/L (mean ± SD; median (25th-75th percentile) at 2.2 ± 0.9 and 2.0(1.5-2.7) h (T_{max}). Among host metabolites, phase 2 conjugates of (−)-epicatechin showed the highest C_{max} values, ranging from 393 ± 464 and 256 (51-448) nmol/L to 656 ± 878 and 328 (191-649) nmol/L for methoxy-(−)-epicatechin-sulfate and (−)-epicatechin-3′-sulfate, respectively. Overall, the T_{max} of the five phase-2 conjugates of flavan-3-ol monomers was 1.6 ± 0.2 and 1.7 (1.5-1.7) h. Considering the main phenyl-γ-valerolactones found at circulating level after flavan-3-ol intake, 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate presented the highest C_{max} values (368 ± 156 and 332 (305-383) nmol/L, mean ± SD; median (25th-75th percentile), reached at 5.2 ± 1.2 and 5.3 (5.2-6.1) h (T_{max}) (Fig. 3 and Supplemental Table 3). (−)-Epicatechin-

3′-sulfate showed the highest C_{avg} values among phase 2 conjugates of flavan-3-ol monomers (318 ± 481 and 114 (42-439) nmol/L). The C_{avg} of the three main circulating phenyl-γ-valerolactones was 75 ± 20 and 63 (61-83) nmol/L. While (−)-EGCG and the five main phase-2 conjugates of flavan-3-ol monomers showed a mean $t_{½}$ ≈ 2h, the $t_{½}$ of the main phenyl-γ-valerolactones appeared to be higher (≈ 6.3 h) (Fig. 3 and Supplemental Table 3).

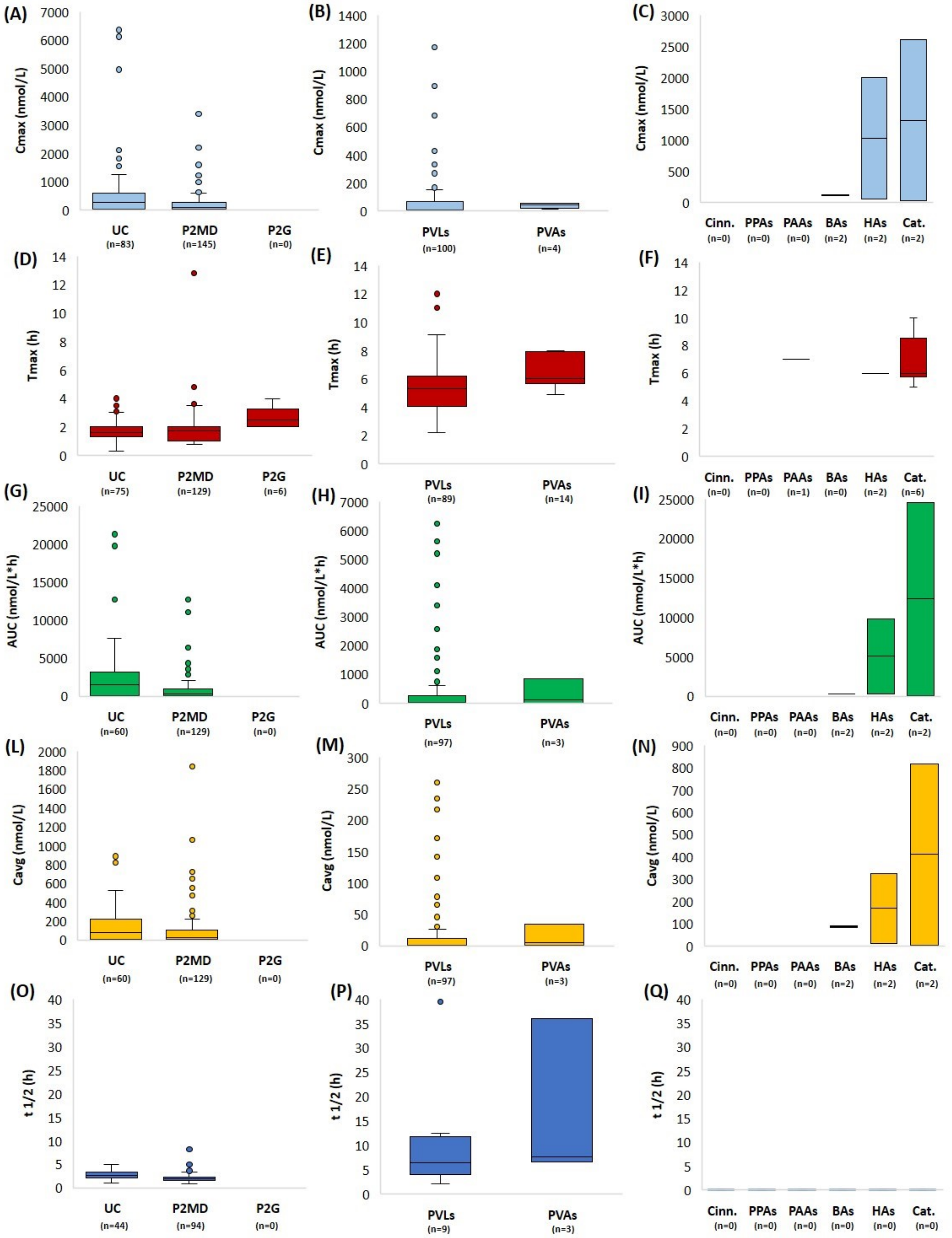

Fig. 2. Box plot for C_{max} (nmol/L) (**A, B, C**), T_{max} (h) (**D, E, F**), AUC (nmol/L*h) (**G, H, I**), C_{avg} (nmol/L) (**L, M, N**), $t_{1/2}$ (h) (**O, P, Q**) of unchanged monomer and dimer flavan-3-ols (UC), phase 2 conjugates of monomer and dimer flavan-3-ols (P2MD), phase 2 conjugates of galloyl flavan-3-ols (P2G), phenyl-γ-valerolactones (PVLs), phenylvaleric acids (PVAs), cinnamates (Cinn.), phenylpropanoic acids (PPAs), phenylacetic acids (PAAs), benzoic acids (BAs), hippuric acids (HAs), catechols (Cat.). Apart for UC, classes of flavan-3-ol metabolites include data derived from both aglycones and their phase 2 conjugates. Maximum plasma concentration (C_{max}), time to reach C_{max} (T_{max}), area under the curve (AUC), average concentration (C_{avg}), apparent half elimination time ($t_{½}$), n indicates the number of biological replicates collected for the same class of flavan-3-ol metabolites and for the same nutrikinetic parameter. No data was available to build panel Q, related to $t_{1/2}$.

3.4.3. Urinary excretion of flavan-3-ol metabolites

Total excretion (µmol) of the different classes of flavan-3-ol metabolites in relation to the ingested amount (µmol) of flavan-3-ols for each study are reported in Supplemental Fig. 9. They were highly variable considering both the same or different sources of flavan-3-ols consumed. Overall, and mainly after tea intake, P2MD and phenyl-γ-valerolactones were excreted in higher amounts than the other classes of metabolites (Supplemental Fig. 9 B/C). As previously observed for other nutrikinetic parameters, a large within-class variability in the urinary excretion of flavan-3-ol metabolites was found (Table 2). Catechols showed the highest mean value of urinary excretion (≈ 10 % of the ingested dose), followed by P2MD and phenyl-γ-valerolactones (≈ 4 %). A similar trend was also observed when median values were considered [9.0 (2.0-11.5) %, 0.7 (0.2-3.8) %, and 0.3 (0.0-2.1) %, median (25th-75th percentile)] for catechols, P2MD and phenyl-γ-valerolactones, respectively (Fig 4A). Even if the urinary excretion, expressed as % of intake, varied widely within the same class of flavan-3-ol metabolites (Fig. 4B), robust data distribution for P2MD and phenyl-γ-valerolactones suggested that both classes were extensively excreted in urine after native flavan-3-ol intake.

To unravel the contribution of each metabolite class to the overall bioavailability of flavan-3-ols, for each study and for each ingested source of flavan-3-ols, the bioavailability (%) of the 10 classes of flavan-3-ol metabolites was calculated by computing the ratio between the total excreted µmol of each metabolite class and the ingested µmol of flavan-3-ols and thus bioavailability values for each metabolite class were averaged (Supplemental Fig. 10). P2MD and phenyl-γ-valerolactones contributed to the bioavailability of flavan-3-ols for about 26 and 24 % for P2MD and phenyl-γ-valerolactones, respectively, followed by phenylvaleric acids and unchanged monomer and dimer flavan-3-ols (2%). Instead, comparing the classes of flavan-3-ol metabolites related to a delayed biotransformation pathway, catechols contributed to flavan-3-ol bioavailability for about 41%, followed by benzoic acids (19%), phenylpropanoic acids (7%), phenylacetic acids and hippuric acids (4%), and cinnamates (2%). Regarding individual main metabolites, while (−)-EGCG was not recovered in urine (Fig. 5A/C), (epi)catechin-sulfate was excreted at higher amounts (12.6 ± 21.3 and 3.8 (1.0-11.8), mean ±SD; median(25th-75th percentile)) than the other main host metabolites (Fig. 5A/C and Supplemental Table 3). Among the main phenyl-γ-valerolactones, 22.0 ± 26.5 and 15.8 (0.5-38.9) % (mean ±SD; median(25th-75th percentile) of the ingested dose of flavan-3-ols was excreted as 5-(hydroxyphenyl)-γ-valerolactone-sulfate (3′,4′ isomers) (Fig. 5B/D and Supplemental Table 3). Values of urinary excretion, expressed as a percentage of intake, for circulating metabolites were pooled according to the same source of ingested flavan-3-ols from which they were derived and are reported in Fig. 6, while the single values of urinary excretion of metabolites produced from each flavan-3-ol source are shown in Fig. 6B.

Table 2. Pharmacokinetic parameters and urinary excretion data for metabolites of flavan-3-ols, grouped by classes based on their metabolic pathway and chemical structure, quantified at circulating level following flavan-3-ol intake by healthy humans. Data are reported as mean ± SD (n indicates the number of biological values collected from literature for each parameter for the flavan-3-ol class).

Classes of flavan-3-ol metabolites	**C_{max} (nmol/L)**	**C_{max} normalized ((nmol/L)/total µmol of ingested flavan-3-ols)**	**T_{max} (h)**	**AUC (nmol/L*h)**	**AUC normalized ((nmol/L*h)/ total µmol of ingested flavan-3-ols)**	**C_{avg} ((nmol/L*h)/n hours)**	**C_{avg} normalized ((nmol/L*h)/ total µmol of ingested flavan-3-ols/n hours)**	**$t_{1/2}$ (h)**	**Urinary excretion (% of intake)**
Unchanged monomer and dimer flavan-3-ols	565.2 ± 1111.5 (*n* = 83)	0.7 ± 0.9 (*n* = 83)	1.9 ± 1.0 (*n* = 75)	2606.5 ± 4118.8 (*n* = 60)	2.8 ± 3.6 (*n* = 60)	143.3 ± 181.3 (*n* = 60)	0.2 ± 0.3 (*n* = 60)	2.8 ± 0.9 (*n* = 44)	1.1 ± 2.0 (*n* = 11)
Phase 2 conjugates of monomer and dimer flavan-3-ols*	259.8 ± 482.5 (*n* = 145)	1.1 ± 1.8 (*n* = 145)	1.8 ± 1.2 (*n* = 129)	892.0 ± 1763.4 (*n* = 129)	4.4 ± 9.0 (*n* = 129)	110.5 ± 234.8 (*n* = 129)	0.4 ± 0.7 (*n* = 129)	2.2 ± 1.1 (*n* = 94)	4.3 ± 10.4 (*n* = 189)
Phase 2 conjugates of galloylated flavan-3-ols*	-	-	2.7 ± 0.8 (*n* = 6)	-	-	-	-	-	-
Phenyl-γ-valerolactones*	88.5 ± 210.5 (*n* = 100)	0.6 ± 3.7 (*n* = 100)	5.3 ± 1.8 (*n* = 89)	479.2 ± 1174.4 (*n* = 97)	0.7 ± 3.2 (*n* = 97)	20.0 ± 48.9 (*n* = 97)	0.0 ± 0.1 (*n* = 97)	10.4 ± 11.4 (*n* = 9)	3.9 ± 10.4 (*n* = 196)
Phenylvaleric acids*	39.5 ± 20.8 (*n* = 4)	0.2 ± 0.1 (*n* = 4)	6.4 ± 1.1 (*n* = 14)	314.7 ± 453.6 (*n* = 3)	1.4 ± 2.3 (*n* = 3)	13.2 ± 18.8 (*n* = 3)	0.1 ± 0.1 (*n* = 3)	16.7 ± 16.7 (*n* = 3)	1.4 ± 2.3 (n = 22)
Cinnamates*	-	-	-	-	-	-	-	-	0.8 ± 1.5 (*n* = 12)
Phenylpropanoic acids*	-	-	-	-	-	-	-	-	2.1 ± 4.4 (*n* = 16)
Phenylacetic acids*	-	-	7 (*n* = 1)	-	-	-	-	-	1.1 ± 1.8 (*n* = 18)
Benzoic acids*	111.4 ± 11.5 (*n* = 2)	0.0 ± 0.0 (*n* = 2)	-	261.9 ± 17.2 (*n* = 2)	0.1 ± 0.0 (*n* = 2)	87.3 ± 5.7 (*n* = 2)	0.0 ± 0.0 (*n* = 2)	-	3.2 ± 5.6 (*n* = 42)
Hippuric acids*	1023.0 ± 1381.7 (*n* = 2)	4.4 ± 6.0 (*n* = 2)	6.0 ± 0.0 (*n* = 2)	5031.0 ± 6679.3 (*n* = 2)	21.9 ± 29.0 (*n* = 2)	167.7 ± 222.6 (*n* = 2)	0.7 ± 1.0 (*n* = 2)	-	2.5 ± 4.0 (*n* = 13)
Catechols*	1309.5 ± 1825.0 (*n* = 2)	5.7 ± 7.9 (*n* = 2)	6.8 ± 1.8 (*n* = 6)	12309.0 ± 17280.3 (*n* = 2)	53.5 ± 75.1 (*n* = 2)	410.3 ± 576.0 (*n* = 2)	1.8 ± 2.5 (*n* = 2)	-	10.3 ± 16.2 (*n* = 8)

C_{max}: maximum plasma concentration; T_{max}: time to reach C_{max}; AUC: area under the curve; C_{avg}: average concentration; $t_{1/2}$: half elimination time; *symbol: when the class includes data derived from both aglycones and their phase 2 conjugates; - means any data was collected for that pharmacokinetic parameter.

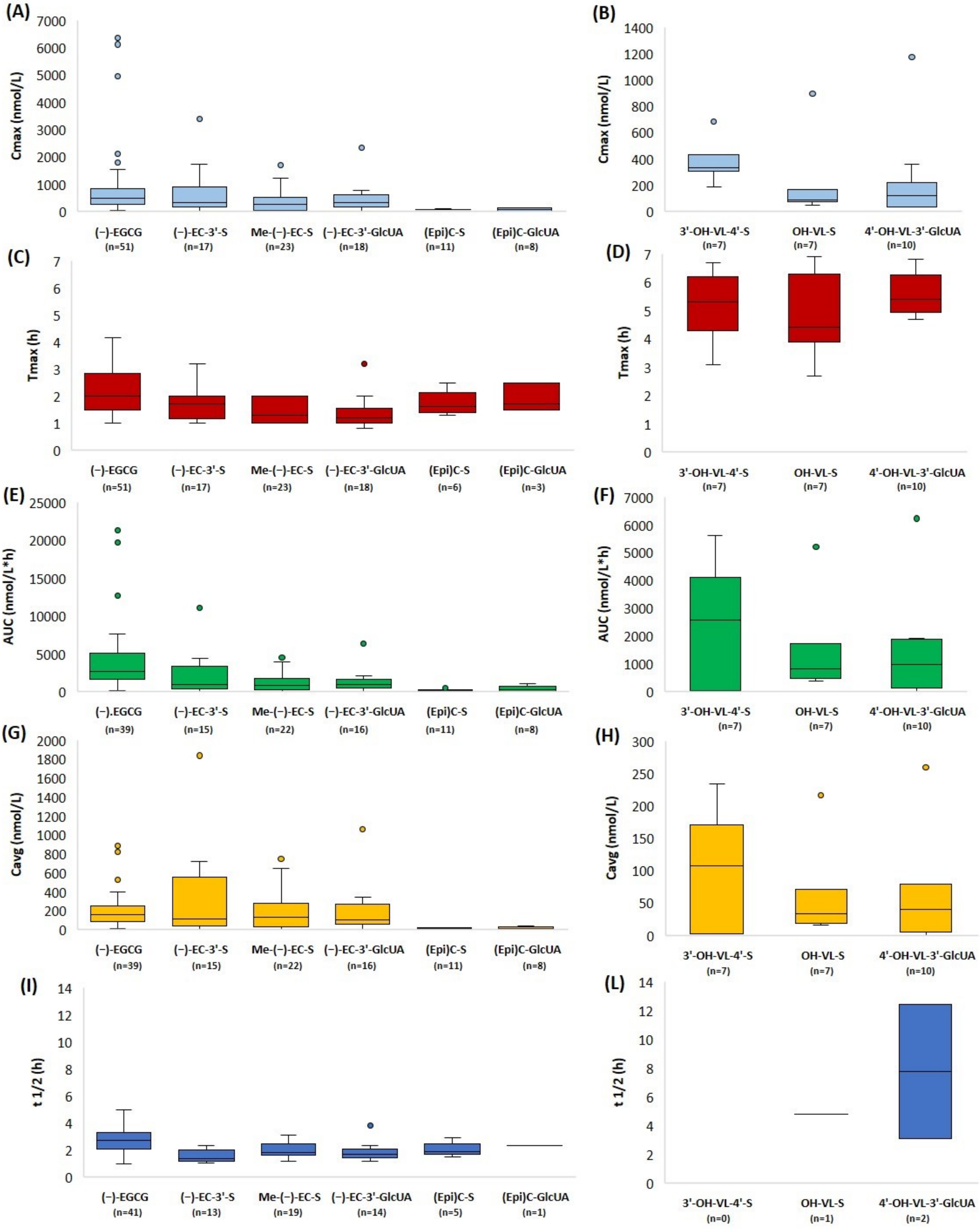

Figure 3. Box plot for C$_{max}$ (nmol/L) (**A, B**), T$_{max}$ (h) (**C, D**), AUC (nmol/L*h) (**E, F**), C$_{avg}$ (nmol/L) (**G, H**), t$_{1/2}$ (h) (**I, L**) of (−)-epigallocatechin-3-*O*-gallate ((−)-EGCG), (−)-epicatechin-3′-sulfate ((−)-EC-3′-S), methoxy-(−)-epicatechin-sulfate (Me-(−)-EC-S), (−)-epicatechin-3′-glucuronide ((−)-EC-3′-GlcUA), (epi)catechin-sulfate ((Epi)C-S), (epi)catechin-glucuronide ((Epi)C-GlcUA), 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate (3′-OH-VL-4′-S), 5-

(hydroxyphenyl)-γ-valerolactone-sulfate (OH-VL-S), 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-glucuronide (4′-OH-VL-3′-GlcUA). (Epi)C-S and (epi)C-GlcUA values include all the (−/+)-epicatechin and (−/+)-catechin isomers quantified at circulating level following flavan-3-ol intake. Me-(−)-EC-S and OH-VL-S values include data derived from both individual and sum of unknown isomers quantified at circulating level following flavan-3-ol intake. Maximum plasma concentration (C_{max}), time to reach C_{max} (T_{max}), area under the curve (AUC), average concentration (C_{avg}), half elimination time ($t_{½}$). Metabolites are named according to (Kay et al., 2020). n indicates the number of biological replicates collected for the same flavan-3-ol metabolite and for the same nutrikinetic parameter.

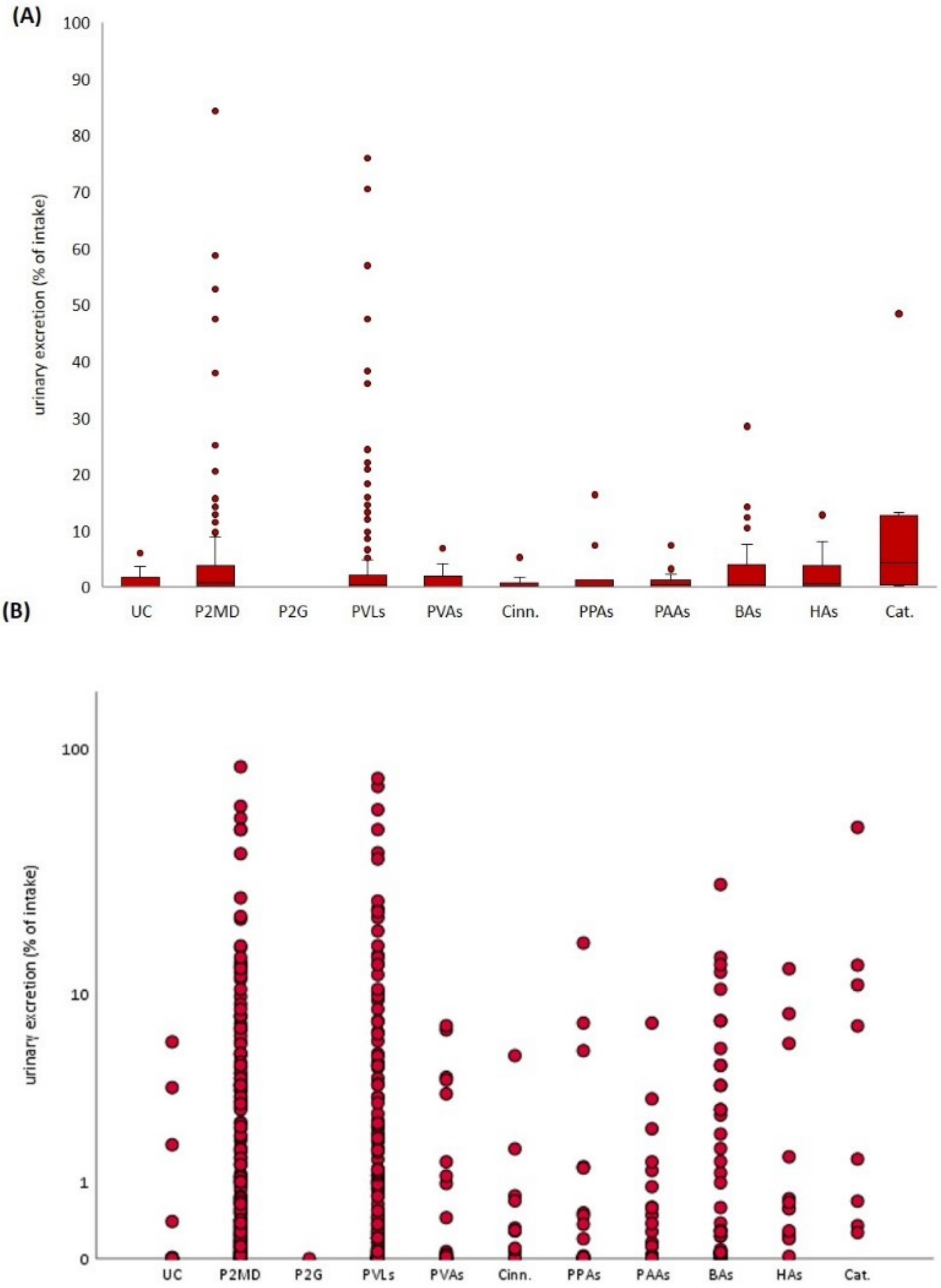

Fig. 4. (**A**) Box plot and single values (**B**) of urinary excretion (% of intake) for metabolites of flavan-3-ols, grouped by classes based on their metabolic pathway and chemical structure, quantified at circulating level following flavan-3-ol intake by healthy humans. Classes of flavan-3-ol metabolites include data derived from both aglycones and their phase 2

conjugates. Unchanged monomer and dimer flavan-3-ols (UC), phase 2 conjugates of flavan-3-ol monomers and dimers (P2MD), phase 2 conjugates of galloylated flavan-3-ols (P2G), phenyl-γ-valerolactones (PVLs), phenylvaleric acids (PVAs), cinnamates (Cinn.), phenylpropanoic acids (PPAs), phenylacetic acids (PAAs), benzoic acids (BAs), hippuric acids (HAs), and catechols (Cat.).

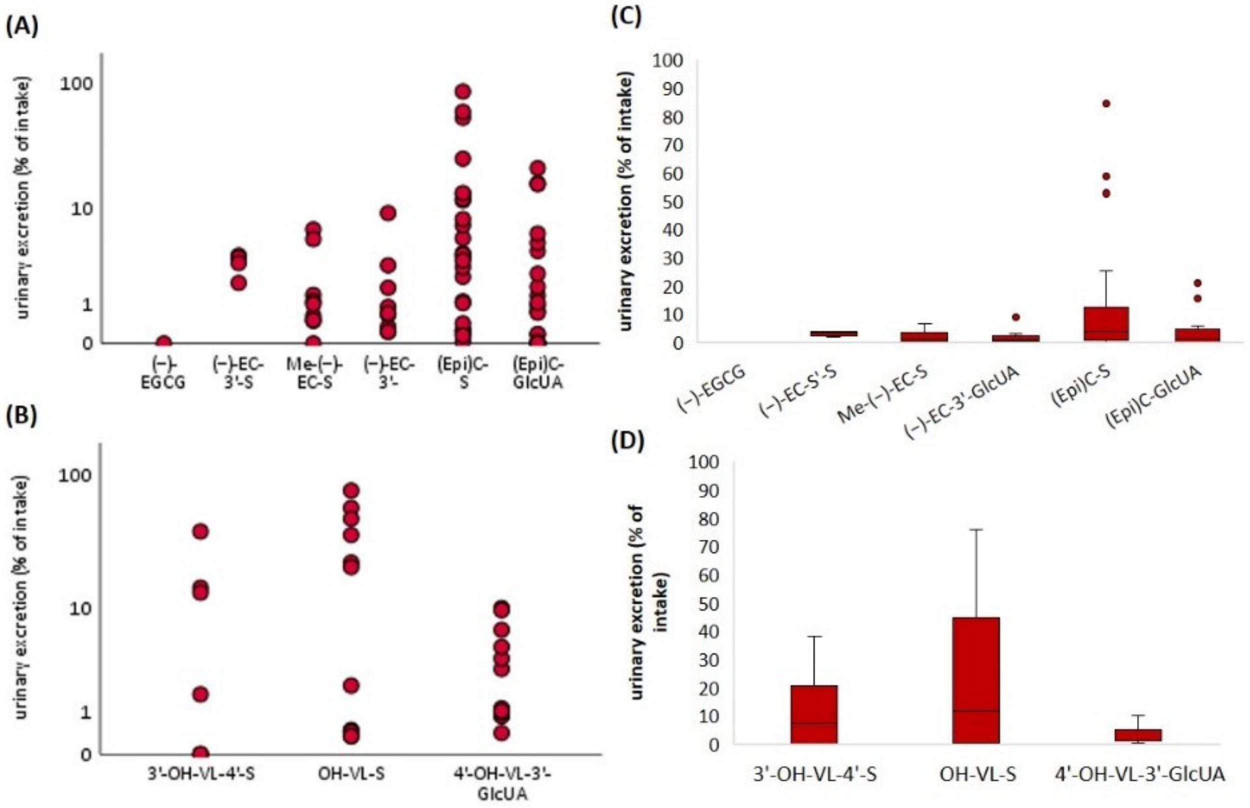

Figure 5. Single values of urinary excretion (% of intake) (**A, B**) and box plot (**C, D**) of urinary excretion (% of intake) for the main flavan-3-ol metabolites quantified following flavan-3-ol intake by healthy humans. (−)-Epigallocatechin-3-*O*-gallate ((−)-EGCG), (−)-epicatechin-3′-sulfate ((−)-EC-3′-S), methoxy-(−)-epicatechin-sulfate (Me-(−)-EC-S), (−)-epicatechin-3′-glucuronide ((−)-EC-3′-GlcUA), (epi)catechin-sulfate ((Epi)C-S), (epi)catechin-glucuronide ((Epi)C-GlcUA), 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate (3′-OH-VL-4′-S), 5-(hydroxyphenyl)-γ-valerolactone-sulfate (OH-VL-S), 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-glucuronide (4′-OH-VL-3′-GlcUA). (Epi)C-S and (epi)C-GlcUA values include all the (−/+)-epicatechin and (−/+)-catechin isomers quantified at circulating level following flavan-3-ol intake. Me-(−)-EC-S and OH-VL-S values include data derived from both individual and sum of unknown isomers quantified at circulating level following flavan-3-ol intake. Metabolites are named according to (Kay et al., 2020).

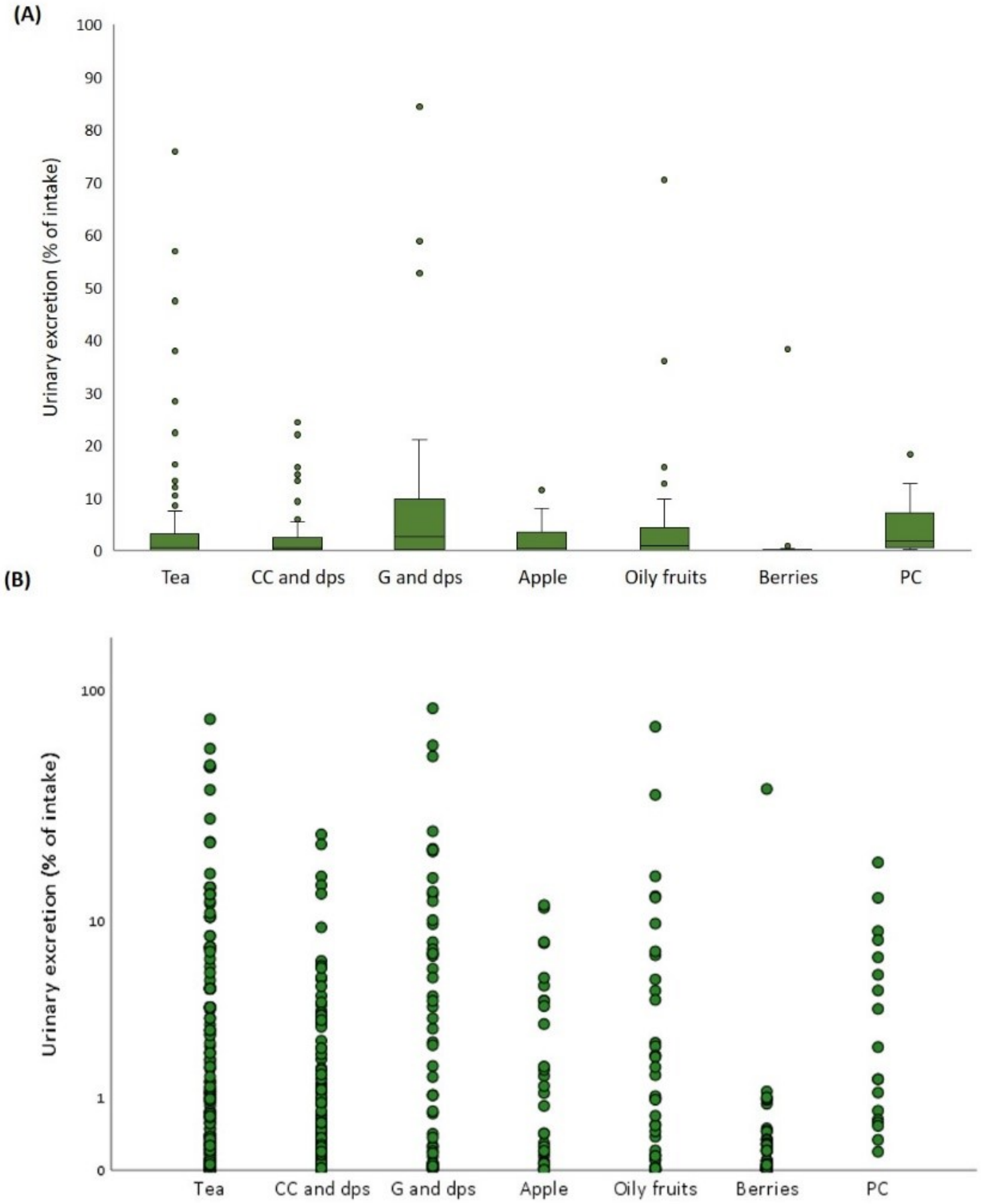

Fig. 6. (**A**) Box plot and single values (**B**) of urinary excretion (% of intake) for all the metabolites quantified following intake of the different sources of flavan-3-ols. Cocoa and derived products (CC and dps), G and dps (grape and derived products), and pure compounds (PC). Flavan-3-ol source (n of biological replicates): tea (210), CC and dps (104), G and dps (50), apple (37), oily fruits (41), berries (67), PC (18).

Flavan-3-ols consumed from grape and derived products were excreted in amounts equal to 8.8 ± 16.2 and 2.7 (0.2-9.3) % of their intake, followed by oily fruits (5.2 ± 12.4 and 1.0 (0.1-4.2) %), pure compounds (4.3 ± 5.0 and 1.8 (0.7-6.5) %) and tea (4.0 ± 10.1 and 0.6 (0.1-3.0) %). Flavan-3-ols ingested from cocoa, apples and berries were excreted in urine to a lesser extent.

3.5. Bioavailability of flavan-3-ols

The mean bioavailability of flavan-3-ols, calculated from the 20 studies which met inclusion criteria for calculating flavan-3-ol bioavailability (see 2.3. section, Supplemental Table 4), was 31 ± 23% [median 25th-75th percentile: 35 (13-40)] (Fig. 7). Differences in these values were associated with the type of study considered, which somehow is related to the period when each article was published (Fig. 7).

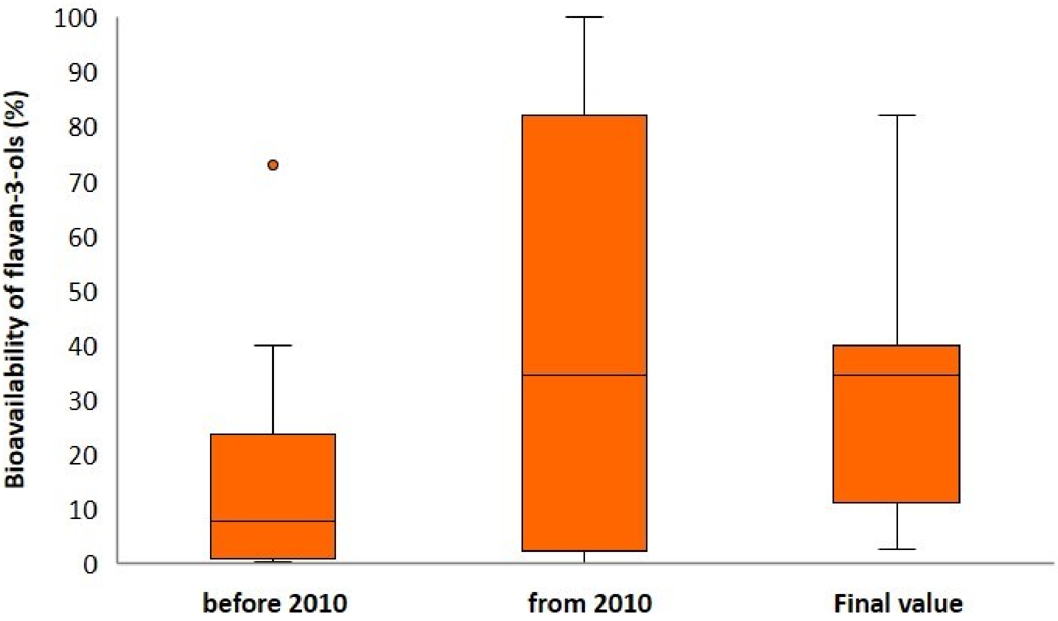

Fig. 7. Box plot for bioavailability (%) of flavan-3-ols calculated taking into account all the values for flavan-3-ol bioavailability collected from literature and/or estimated from urinary excretion data derived from studies published before 2010 (n = 14), from studies published from 2010 onwards (n = 31), from studies which met inclusion criteria [bioavailability values that were <1 and/or >100%, or were calculated by not taking into account a complete and appropriate metabolic pathway in terms of metabolites produced following flavan-3-ol intake were excluded] for calculating the final value of flavan-3-ol bioavailability (%) (n = 20). Details on flavan-3-ol bioavailability (%) values employed to calculate the final value for bioavailability of flavan-3-ols are reported in Supplemental Table 5.

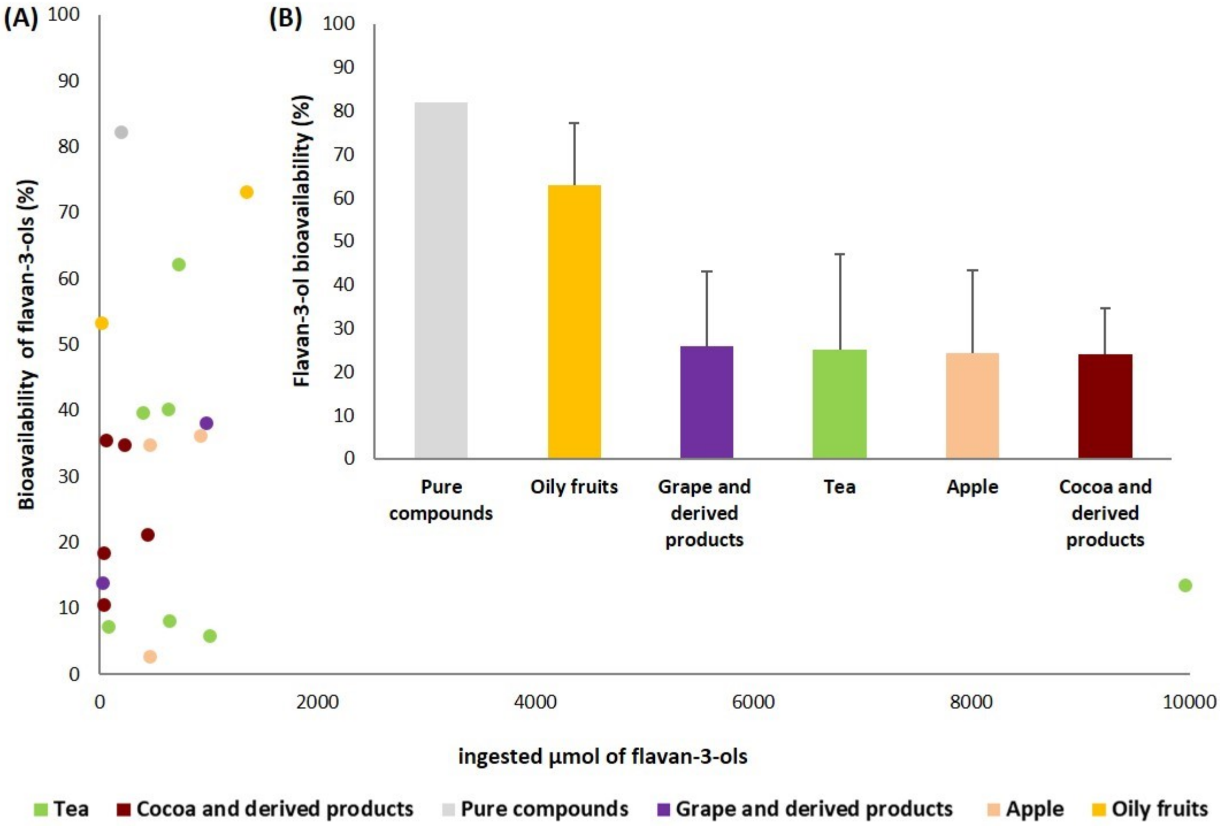

Fig. 8. (**A**) Values of bioavailability (%) for flavan-3-ols, collected from literature and/or estimated from urinary excretion data, and ingested µmol of the different flavan-3-ol sources. Each bullet indicates the bioavailability (%) value for flavan-3-ols, obtained for every single study, and related to each dose of consumed flavan-3-ols in the study. (**B**) Bioavailability of flavan-3-ols calculated for the different food sources employed in the human studies that underwent data analyses. Data are expressed as mean and SD. Flavan-3-ol source (n of values of flavan-3-ol bioavailability (%)): pure compounds (1), oily fruits (2), grape and derived products (2), tea (7), apple (3), cocoa and derived products (5).

The 20 bioavailability values were compared source by source with the ingested amount (µmol) of total flavan-3-ols deriving from each study (Fig. 8A). They were pooled to estimate the mean bioavailability of flavan-3-ols for each source employed in the analysed studies. The bioavailability of flavan-3-ols was 82 % (number -n- of flavan-3-ol bioavailability values collected/estimated for each source=1) and 63 % (n=2) after pure compound (i.e. (−)-epicatechin) and oily fruit (i.e. almond, hazelnut) intake, respectively, followed by 25 % after consumption of tea (n=7), cocoa and derived products (n=5), apple (n=3), and grape and derived products (n=2) (Fig. 8B).

4. Discussion

This is the first work that systematically investigated the nutrikinetics of up to 180 flavan-3-ol derived compounds following their intake by healthy subjects, and estimated the bioavailability of flavan-3-ols. All collected data on the identified metabolites and nutrikinetics data have been made available in the database PhytoHub (www.phytohub.eu) to extend the impact of the work. Tea intake leads the largest number of metabolites (Fig. 1). Although (−)-EGCG is the main unmetabolised compound found at circulating level following tea consumption (Clifford et al., 2013), galloylated flavan-3-ols are easily available to a myriad of metabolic pathways by colonic microflora and mammalian phase 2 enzymes (Calani et al., 2012a, 2012b; Roowi et al., 2010; Stalmach et al., 2009). Flavan-3-ol intake from cocoa elicited a higher increase in circulating compounds (n = 48), prevalently as P2MD (21), and in the appearance of simple phenolic metabolites (Table 1 and Fig. 1), compared to other ingested sources of procyanidins, such as nuts, grape, wine, apple, and berries. These differences might be attributable to the high amount of flavan-3-ol monomers in cocoa (Sorrenti et al., 2020), more prone to gut microbiota metabolism (Crozier et al., 2010; Monagas et al., 2010; Borges et al., 2018; Pede et al., 2022). Indeed, the low number of metabolites recovered after berry intake was probably attributable to their richness in highly polymerized procyanidins (Nile and Park, 2014), which are less susceptible to catabolism by gut microbiota (Mena et al., 2015; Pede et al., 2022). On the other hand, it should be noted that when flavan-3-ols were consumed together with other flavonoids (i.e. anthocyanins, flavonols), considered precursors of various late-products of microbial metabolism, data on simple C_6-C_3, C_6-C_2, C_6-C_1 phenolic acids were not collected. Indeed, berries, apples, oily fruits, grapes and wine contain a huge variety of putative precursors of simple phenolic acids other than flavan-3-ols (Selma et al., 2009; Del Rio et al., 2010a; Teixeira et al., 2014; Trošt et al., 2018; Alasalvar et al., 2020), which may hinder a clear assessment of the bioavailability of flavan-3-ols coming from these dietary sources. But, beyond this aspect related to the co-presence of other precursors of low molecular weight metabolites, the variability in the number of studies collected for each dietary source (Supplementary Table 2) and in the number of metabolites quantified within each study, as well as metabolites coming from the dietary background of volunteers, should be taken into account in the interpretation of these findinds. To have a clear and more realistic scenario on the ability of the different dietary sources of flavan-3-ols in producing metabolites, a large intervention study should be conducted to compare the dietary sources provided at the same dose, with quantification of all produced metabolites using a large-coverage analytical method.

Analyses of data arising from plasma samples proved that unchanged monomer and dimer flavan-3-ols were mainly represented by (−)-EGCG in feeding studies with green/black tea (Fig. 3 and Supplemental Table 3). Tea trials highlighted that unchanged (−)-EGCG circulates at the highest concentration when all the other parent flavan-3-ols and their phase 2 metabolites are taken into account (Stalmach et al., 2009; Del Rio et al., 2010b). The metabolic fate of (−)-EGCG remains to be further investigated, as it is quickly removed from the circulatory system (low $t_{1/2}$ value) but, surprisingly, it is not recovered in urine (Fig. 3 and Supplemental Table 3). It is possible that the galloyl moiety may impact the absorption and subsequent phase 2 conjugation mechanisms of galloylated flavan-3-ols (Henning et al., 2008). With this work, we have supported the large body of evidence (Crozier et al., 2009; Borges et al., 2018;) indicating that flavan-3-ol monomers and dimers are prevalently absorbed in the small intestine, even if some differences in the metabolic yield after ingestion of monomeric, dimeric and oligomeric flavan-3-ols were highlighted (Wiese et al., 2015). The phase 2 conjugates of parent compounds (P2MD), mainly monomers, reached plasma concentration peaks ($C_{max} \approx 260$ nmol/L) at 1.8 h (T_{max}) after flavan-3-ol intake, to rapidly disappear from the circulatory system, as demonstrated from their $t_{1/2}$ values (≈ 2 h postconsumption) (Fig. 2 and Table 2). It has been shown that (−)-epicatechin-3′-sulfate, methoxy-(−)-epicatechin-sulfate, (−)-epicatechin-3′-glucuronide, (epi)catechin-sulfate and (epi)catechin-glucuronide are the most abundant phase 2 conjugates of flavan-3-ol monomers in plasma, even if some quantitative differences in their nutrikinetic profiles were highlighted (Fig. 3 and Supplemental Table 3). Data clearly indicates that the stereochemistry may impact phase 2 sulfation, glucuronidation and methylation of flavan-3-ol monomers, since the three conjugates of (−)-epicatechin attained the highest C_{max} (≈ 500 nmol/L) compared to the other host metabolites (Fig. 3 and Supplemental Table 3). This is not surprising since it has been widely demonstrated that (−)-epicatechin is more bioavailable than (+)-epicatechin and (−/+)-catechin (Ottaviani et al., 2011; Clifford et al., 2013; Borges et al., 2018). This robust data is further supported by the huge efforts in synthesizing pure analytical standards of phase 2 conjugates enabling a more accurate assessment of the circulating levels of (−)-epicatechin metabolites (Sharma et al., 2010; Mull et al., 2012; Ottaviani et al., 2012; Zhang et al., 2013a, 2013b). Although 3′-methoxy-(−)-epicatechin-5-sulfate, methoxy-(epi)catechin-sulfate, methoxy-(epi)catechin glucuronide, and methoxy-(−)-epigallocatechin-sulfate were not selected as main plasma metabolites of flavan-3-ols, they were excreted in urine in amounts equal to 4, 10, 2 and 7 % of the ingested dose, respectively (Supplemental Excel file), indicating the importance of assessing both blood and urine samples when evaluating the bioavailability of flavan-3-ols. On the other hand, (−)-epicatechin was present in the systemic circulation predominantly as sulfate and glucuronide conjugates at the 3′ position (Borges et al., 2018; Ottaviani et al., 2016), proving that phase 2 conjugation mechanisms of flavan-3-ol monomers are stereochemically-dependent. Indeed, Ottaviani et al. (2012) demonstrated that sulfation of (+)-epicatechin took place at 5 position. Nevertheless, the identity of methoxy-(−)-epicatechin-sulfate, as well as that of the two (epi)catechin conjugates [(epi)catechin-sulfate and (epi)catechin-glucuronide] remain unknown for most of the publications. This should encourage further identification initiatives for a complete assessment of the host metabolites occurring in circulation. A point to keep in mind is that the C_{max} values for these (epi)catechin conjugates might suffer from misquantification, commonly occurring in the absence of reference standards (Ottaviani et al., 2018b), or the presence of low bioavailable flavan-3-ol stereoisomers (Donovan et al., 2006; Ottaviani et al., 2011), since nutrikinetic values for these metabolites included all the (−/+)-epicatechin and (−/+)-catechin isomers quantified at circulating level following flavan-3-ol intake (Fig. 3 and Supplemental Table 3).

Flavan-3-ols circulate principally as phase 2 conjugates of colonic catabolites, as up to 97 host-gut microbiota metabolites were found (Table 1), mainly represented by specific ring fission catabolites of flavan-3-ols as phenyl-γ-valerolactones and phenylvaleric acids (Mena et al., 2019a). Unmetabolized flavan-3-ols that reach the colon undergo microbiota-induced transformations, yielding phenyl-γ-valerolactones which may be further converted to phenylvaleric acids (Mena et al., 2019a; Stevens and Maier, 2016), as demonstrated from the T_{max} values obtained for phenyl-γ-valerolactones and phenylvaleric acids (5.3 and 6.4 h, respectively) (Fig. 2 and Table 2). Of note, phenyl-γ-valerolactones were quantified after intake of all the dietary sources of flavan-3-ols. Up to 31 phenyl-γ-valerolactones were recovered after tea intake, followed by berries (n = 14), oily fruits (12), apple (11), cocoa and its derived products (10), grape and its derived products (9), and pure compounds (4) (Fig. 1). These findings support the potential role of phenyl-γ-valerolactones in being considered valuable biomarkers of dietary consumption of flavan-3-ols monomers and procyanidins, although they still require proper validation for that use (Urpi-Sarda et al., 2009; Duynhoven et al., 2011; Ottaviani et al., 2016, 2018a). Phenyl-γ-valerolactones were present in the systemic circulation at a higher concentration than phenylvaleric acids. Moreover, regardless of the conjugation position, phase 2 conjugates of 5-(dihydroxyphenyl)-γ-valerolactones had a higher C_{max} compared to those of 5-(hydroxyphenyl)-γ-valerolactones (≈ 107 and 56 nmol/L, respectively) (Supplemental Excel file), both had a T_{max} of 5 h (T_{max}). Residual aglycones of phenyl-γ-valerolactones had very low C_{max} levels (≈ 19 nmol/L) with a 6 h T_{max}, as a result of the extensive phase II metabolism of these catabolites (Mena et al., 2019a). High $t_{1/2}$ values for phenyl-γ-valerolactones and phenylvaleric acids indicated that these gut microbiota metabolites remain in the systemic circulation for a relatively long time. The exact identity of 5-(hydroxyphenyl)-γ-valerolactone sulfate, one of the main plasma phenyl-γ-valerolactones identified following flavan-3-ol intake, remains unclear in many reports (Fig. 3 and Supplemental Table 3). This can be due to the difficulties in fully identifying these catabolites, although big advances have been made in the synthesis of their aglycones and phase II conjugates (Sánchez-Patán et al., 2011; Curti et al., 2015; Brindani et al., 2017). Nevertheless, it seems likely that most of the work with dihydroxylated flavan-3-ols refer to 3′,4′ isomers, 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-sulfate being the most representative isomer (Ottaviani et al., 2016). In this respect, 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-sulfate was excreted in urine in amounts higher than 15 % of the ingested dose. Additional specific 5C-ring fission catabolites were also excreted in urine in amounts representing over 2 % of flavan-3-ol intake, including 5-(phenyl)-γ-valerolactone-methoxy-sulfate [2.8 ± 5.8 and 0.9 (0.1-1.4) %] [mean ± SD; median (25th-75th percentile)], 5-(hydroxyphenyl)-γ-valerolactone-glucuronide (final values include data derived from both individual and sum of unknown isomers) [3.3 ± 2.8 and 2.2 (1.7-4.9) %], 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-glucuronide [2.5 ± 6.3 and 0.4 (0.3-1.7) %], 5-(phenyl)-γ-valerolactone-sulfate-glucuronide [2.1 ± 6.0 and 0.3 (0.2-0.8) %] and one phenylvaleric acid, 4-hydroxy-5-(4′-hydroxyphenyl)valeric acid-3′-sulfate [2.5 ± 2.1 and 3.4 (1.8-3.7) %] (Supplemental Excel file). Phase II conjugates of phenyl-γ-valerolactones, derived from flavan-3-ol monomers, were excreted in urine prevalently as sulfate conjugates. Different profiles between plasma and urine in the appearance and accumulation of some metabolites might suggest that the kidneys may represent a key organs for the metabolism of some compounds, beyond the phase 2 conjugation mechanisms occurring at small intestine and/or hepatic levels. Comparing the mean value of ingested flavan-3-ols (≈ 1308 µmol) with the mean cumulative urinary excretion of phenyl-γ-valerolactones (≈ 217 µmol) indicates that the ingestion of about 5 µmol of flavan-3-ols would potentially result in excretion of 1 µmol of phenyl-γ-valerolactones. This observation is in line with stoichiometry in the production of these C_5-C_6 colonic metabolites from flavan-3-ol monomers and B type dimers (Pede et al., 2022). Likewise, when comparing the mean cumulative urinary excretion of phenylvaleric acids (≈ 4 µmol) with the mean ingested amount of flavan-3-ols obtained from studies that quantified these catabolites (≈ 1190 µmol), we calculated that 303 µmol of flavan-3-ols would be needed to reach 1 µmol of urinary phenylvaleric acids.

Although plasma sample analyses did not fully reveal robust kinetic profiles of low molecular weight phenolic acids in the systemic circulation (Fig. 2 and Table 2), urine analyses showed that these "late products" of flavan-3-ol metabolism were excreted in average amounts ranging from ≈ 1 % up to 10 % of the ingested flavan-3-ol dose for cinnamates and catechols, respectively, values somewhat comparable to that obtained for P2MD and phenyl-γ-valerolactones (≈ 4 %). Additionally, the contribution of simple phenolic acids to flavan-3-ol bioavailability is underlined by values calculated for their bioavailability (Supplemental Fig. 10). A radiolabel study investigating the ADME of [2-^{14}C](−)-epicatechin in healthy humans (Ottaviani et al., 2016) found that about 57 µmol of C_6-C_3, C_6-C_2, and C_6-C_1 metabolites were excreted in urine, representing 28% of parent compound intake. Our data suggests that low molecular weight phenolic metabolites should be considered as relevant contributor of the ADME of flavan-3-ols. Indeed, although data on C_6-C_3, C_6-C_2, and C_6-C_1 derivatives were collected if they were related to the ingestion of dietary sources providing only flavan-3-ols (Table 1), it is well known that these metabolites have unspecific dietary origin and precursor compounds (Del Rio et al., 2013; Rodriguez-Mateos et al., 2014; Mena et al., 2019a; Carregosa et al., 2022) and their blood and urine profiles might be partially influenced by volunteers' diet.

The mean bioavailability of flavan-3-ols was 31%, which accounts for the moderate bioavailability of dietary flavan-3-ols. Nevertheless, when the mean bioavailability was calculated from studies published before 2010, it was 16 % (Fig. 7). This time cutoff was selected since it coincides with the early development of methods for the synthesis of flavan-3-ol metabolites, allowing proper identification, quantification, and a better knowledge of their metabolic pathways (Borges et al., 2018). As expected, the bioavailability of flavan-3-ols resulted in a higher 38% when studies published from 2010 onwards were considered, providing a clear indication of the impact that the use of reference standards has on the accuracy in the identification and quantification of flavan-3-ols and their whole set of metabolites (Ottaviani et al. 2018b). In accordance with various human trials (Feliciano et al., 2017; Trošt et al., 2018; Gómez-Juaristi et al., 2019; Favari et al., 2020), this systematic review suggested that bioavailability of flavan-3-ols seems to be scarcely affected by the amount of ingested compounds, since 19 out of 20 values considered for assessing the final bioavailability values ranged from 3 up to 82 %, without exceeding 1500 µmol of flavan-3-ol intake (Fig. 8A). In this context, when the source of flavan-3-

ols is taken into account, it should be noted that:

- considering individual bioavailability values, intra- and inter-source differences in flavan-3-ol bioavailability exist and could be explained by the study designs (i.e. degree of processing of the source, period for blood and urine collection), population characteristics, analytical methods used, and the set of metabolites targeted, among other factors;
- pooling bioavailability values for each source employed along the studies analysed, it is possible to observe that principal, flavan-3-ol monomer, (−)-epicatechin, was more bioavailable compared to ingested sources of proanthocyanidins (Crozier et al., 2010; Del Rio et al., 2013), suggesting a role of the structure of ingested flavan-3-ol in affecting the metabolic efficiency in their biotransformation.

Further human studies employing other pure monomer flavan-3-ols are required, also paying attention to the bioavailability of proanthocyanidins from different plant-based foods. Indeed, a trend pointing out a higher bioavailability of flavan-3-ols from almond/hazelnut compared to grapes, apple, tea and cocoa, whose values appear to be entirely comparable, was observed (Fig. 8B). This should be considered a trend, since the mean bioavailability value calculated for each ingested source derived from a highly variable number of replicates, affecting the power of results.

In addition, discriminating blood and urinary profiles of circulating metabolites coming from monomers or proanthocyanidins would be an excellent future turning point to better unravel the ADME of this important class of (poly)phenols and understand differences among food sources. The identification of metabolites strictly related to flavan-3-ol intake (Table 1), their occurrence in the different biofluids where they are detected (Supplemental Fig. 5), and the stoichiometric balances calculated in the production of phenyl-γ-valerolactones and phenylvaleric acids may be valuable tools to potentially support the implementation of i) well-designed human intervention studies and ii) target MS/MS methods able to cover a comprehensive panel of flavan-3-ol metabolites, derived from specific food sources. While plasma analyses provide data on the occurrence, presence, and clearance of the circulating metabolites, urinary excretion gives a more realistic assessment of the uptake and metabolic yield of flavan-3-ols along their biotransformation pathway. Another point worth mentioning is that the C_{max} and C_{avg} values calculated herein may support the design of cell studies firmly in line with more realistic physiological conditions. Finally, this work further support the vital need to use authentic reference compounds to i) fully identify the circulating flavan-3-ol metabolites, ii) improve the accuracy in assessing concentration levels/excreted amounts and so the bioavailability of these phytochemicals, and iii) conduct proper cell or *in vivo* assays, as different isomers may lead to different biological effects (Mele et al., 2017; Mena et al., 2017; Montagnana et al., 2018; Luca et al., 2019; Ruotolo et al., 2020; Cecarini et al., 2021). This systematic review approach, commonly used for health-related observations, has never been previously used to assess the pharmoacokinetics of (poly)phenols, as far as we know. The workflow reported herein may support the scientific community in further gathering evidence on bioactive phenolic metabolites and (poly)phenol bioavailability by using robust methodological approaches related to study design, data processing and result presentation. The presence of data on publicly accessible repositories may also facilitate data processing and harmonization. Applying this systematic approach to other classes of secondary dietary compounds to gain further evidence on their metabolism and bioavailability may pave the way for a better understanding of their bioactivity and the development of sound dietary recommendations. Last but not least, although this work evaluated the ADME of flavan-3-ols starting from a very huge number of studies, our knowledge on dose-response effects, bioavailability of the different flavan-3-ol sources, and how inter-individual variation may affect kinetic profiles and concentration levels of metabolites produced after flavan-3-ol intake remains still incomplete. Understanding how these aspects may condition the health effects attributed to flavan-3-ols is also fully needed. Further research efforts on these aspects are guaranteed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mam.2022.101146.

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018-1862-3, 2018.

Supplementary data

Table 1. Syntaxes used to perform the literature search.

DATABASE	***SYNTAX***
PubMed	(cataboli*[TIAB] OR absor*[TIAB] OR absorption[MeSH] OR excret*[TIAB] OR “Drug Elimination Routes”[MeSH] OR biotransform*[TIAB] OR biotransformation[MeSH] OR bioavailab*[TIAB] OR “Biological Availability”[MeSH] OR *kinetic*[TIAB] OR Pharmacokinetics[MeSH] OR ADME[TIAB] OR conjugat*[TIAB] OR glucuroni*[TIAB] OR sulfate[TIAB] OR sulphate[TIAB] OR (microbi*[TIAB] AND metabolit*[TIAB])) AND (humans[MeSH] OR subjects[TIAB] OR men[TIAB] OR male[MeSH] OR women[TIAB] OR female[MeSH] OR male[TIAB] OR female[TIAB] OR patient*[TIAB] OR volunteer*[TIAB] OR participant*[TIAB] OR population[TIAB] OR cohort [TIAB]) AND ((flavan-3-ol[TIAB] OR "flavan-3-ol"[MeSH] OR "flavan-3-ols"[TIAB] OR flavanol*[TIAB] OR catechin[TIAB] OR catechin[MeSH] OR epicatechin[TIAB] OR procyanidin*[TIAB] OR proanthocyanidin*[TIAB] OR prodelphinidin[TIAB] OR propelargonidin[TIAB]) OR (*phenyl-γ-valer*[TIAB] OR *phenylvaler*[TIAB] OR *phenyl-valer*[TIAB] OR *phenyl-gamma-valer*[TIAB] OR *phenylpentanoic[TIAB]))
Web of Science	(cataboli* OR absor* OR excret* OR biotransform* OR bioavailab* OR *kinetic* OR adme OR conjugat* OR glucuroni* OR sulfate OR sulphate OR (microbi* AND metabolit*)) AND (men OR women OR patient* OR volunteer* OR participant* OR subject* OR male OR female OR population OR cohort) AND (("flavan-3-ol" OR "flavan-3-ols" OR flavanol* OR catechin OR epicatechin OR procyanidin* OR proanthocyanidin* OR prodelphinidin OR propelargonidin) OR (*phenyl-γ-valer* OR *phenylvaler* OR *phenyl-valer* OR *phenyl-gamma-valer* OR *phenylpentanoic*))
Scopus	(cataboli* OR absor* OR excret* OR biotransform* OR bioavailab* OR *kinetic* OR adme OR conjugat* OR glucuroni* OR sulfate OR sulphate OR (microbi* AND metabolit*)) AND (men OR women OR patient* OR volunteer* OR participant* OR subject* OR male OR female OR population OR cohort) AND (("flavan-3-ol" OR "flavan-3-ols" OR flavanol* OR catechin OR epicatechin OR procyanidin* OR proanthocyanidin* OR prodelphinidin OR propelargonidin) OR (*phenyl-γ-valer* OR *phenylvaler* OR *phenyl-valer* OR *phenyl-gamma-valer* OR *phenylpentanoic*))

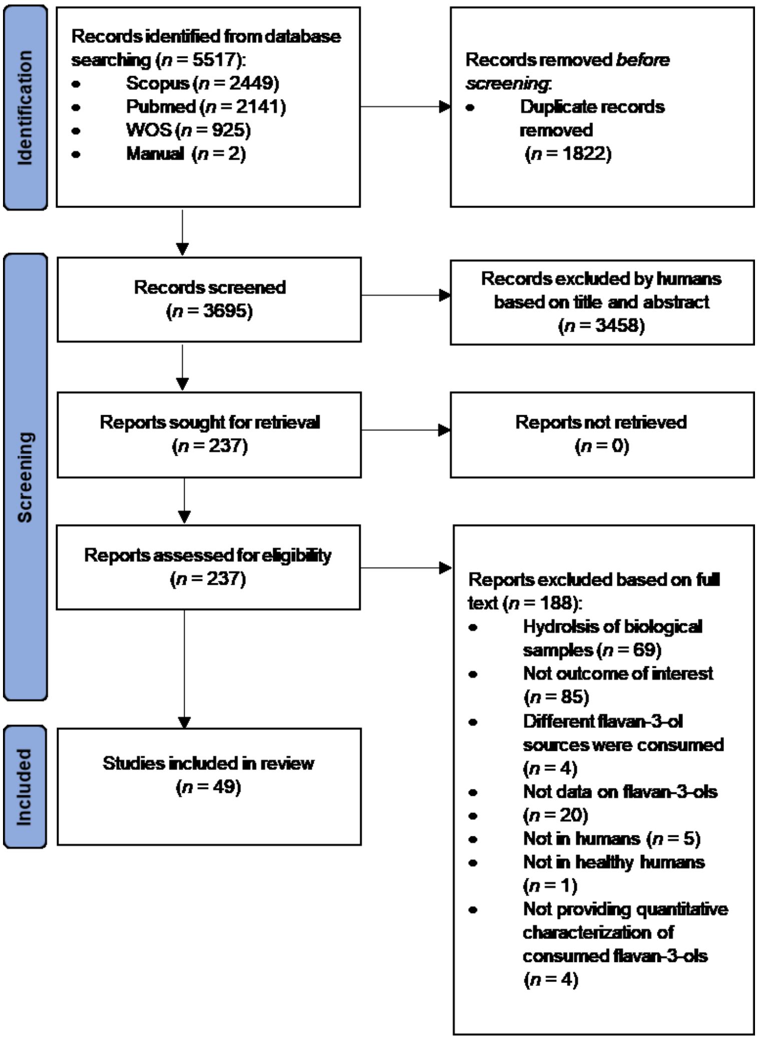

Figure 1. Flowchart of the study selection process. Legend: WOS: Web of Science.

Table 2. Characteristics of the human intervention studies that met inclusion criteria and underwent data analyses.

Dietary source classification	Dietary source description	Total ingested flavan-3-ols (µmol)	Dose type	Study duration	Sample size	Description of population	Ref.
food	100 mL of dealcoholised RW	20.7	S	-	12	6 F; 6 M; Age=19-50 y; BMI for M=26.4±3.5 kg/m^2; BMI for F=26.1±3.8 kg/m^2	(Motilva et al., 2016)
	100 mL of dealcoholised RW + GP extract (1:3)	38.3					
extract	100 mL of dealcoholised RW + encapsulated GP extract (1:3)	38.3					
pure compound	2 capsules of EGCG (2 × 250 mg) + 100 mL of water	1091.7	S	-	4	3 M; 1 F; Age=31.25 ± 9.54 y; BMI=23.1 kg/m^2	(Naumovski et al., 2015)
	2 capsules of EGCG (2 × 250 mg) + 50 g breakfast cereals + 200 mL of milk	1091.7					
	2 capsules of EGCG (2 × 250 mg) + 200 g of strawberry sorbet	1091.7					
food	500 mL of CC powder drink	796.9	S	-	40	20 M; Caucasians; Age=70±4 y; BMI=27±3 kg/m^2; 20 M; Caucasians; Age=26±6 y; BMI=24±2 kg/m^2	(Rodriguez-Mateos et al., 2015)
		1590.3					
food	40 g CC powder + 250 mL whole milk	170.3	S	-	21	9 F; 12 M; Age=25±7 y; BMI=21.59±2.1 kg/m^2	(Roura et al., 2008)
	40 g of CC powder + 250 mL water	170.3					
food	500 mL of Choladi GT	648.0	S	-	10	na	(Stalmach et al., 2009)
food	99 g of nut cream (20 % of CC)	190.0	Mu.	1 day (1 dose at morning + 1 dose at lunch + 1 dose at dinner)	12	4 M; 8 F; Age=24±3 y; BMI=23.1±1.5 kg/m^2	(Vitaglione et al., 2013)
	99 g of CC-nut cream + CC in free form	385.1					
	99 g of CC-nut cream + CC in encapsulated form	385.3					
pure compound	50 mL of solution + 60 mg of $^{14C-}$EC	207.0	S	-	8	8 M; Age=31±3 y; BMI=24.5±3.3 kg/m^2	(Ottaviani et al., 2016)
food	80 g flavanol-rich CHOC + bread and water	749.0	S	-	11	7 M; 4 F; Age=24±3 y	(Rios et al., 2003)
food	250 mL of hot milk + 10 g CC powder	45.0	S	-	9	Age=20-43 y; BMI=24.7±2.7 kg/m^2	(Mullen et al., 2009)
	250 mL of water + 10 g CC powder	45.0					
food	300 mL infusion of GT leaves (3 g)	634.0	S	-	5	4 M; 1 F; Age=41-54 y	(Roowi et al., 2010)
pure compound	capsules of EGCG + 400 mL water	109.2 218.3 436.7 873.4 1746.7 3493.4	S	-	8	na	(Ullmann et al., 2003)
food	500 mL of GT	727.6	S	-	20	Age=25±3 y; BMI=22±3 kg/m^2	(Calani et al., 2012)
food	5 cups GT + 50 mg vitamin C	1054.0	S	-	50	Caucasians; Age=18-65 y	(Clarke et al., 2014)
		1054.0		6 weeks			
		1054.0	Mu.	12 weeks			
food	400 mL of GT	403.9	S	-	20	Age=26±5 y; BMI=23±3 kg/m^2	(Del Rio et al., 2010a)
					5	na	
food	CR drink	641.0 1233.5 1,940.4 2404.3 2993.6	S	-	10	10 M; Age=18-35 y	(Favari et al., 2020)
food	450 mL of CR drink	1233.5	S	-	10	10 M; Age=18-35 y	(Feliciano et al., 2016)
food	450 mL of CR drink	641.0 1233.5 1940.4 2404.3 2993.6	S	-	10	Age=18-35 y	(Feliciano et al., 2017)
extract	300 mg of Teavigo® EGCG GT extract (2 capsules) + 100 mL	305.7	S	-	10	5 M; Age=30.6±5.2 y; BMI=23.7±2.8 kg/m^2	(Fernández et al., 2020)

	of water						
	300 mg of Teavigo® EGCG GT extract (2 capsules) + 100 mL of water + standardized breakfast	305.7					
	266 mg of FontUp® (Teavigo® GT extract with fats, carbohydrates, proteins, vitamins, and minerals) + 200 mL of semi-skimmed milk	545.9				5 F; Age=28.8±3.6 y; BMI=19.9±2.1 kg/m^2	
food	100 g of 70 % dark CHOC	446.8	S	-	5	Age=23.4±3.3 y; BMI=22±2 kg/m^2	(Actis-Goretta et al., 2012)
extract	250 mL of RGP aqueous extract	983.5	S	-	10	10 M; Age=26±2 y; BMI=27±3 kg/m^2	(Castello et al., 2018)
food	15 g of CC + 200 mL of milk	68.2	S	-	13	3 M; 10 F; Age M=26.67±3.21 y; BMI M=22.47±2.97 kg/m^2. Age F=32.60±9.85 y. BMI F=23.36±3.73 kg/m^2	(Gómez-Juaristi et al., 2019)
	25 g of flavanol-rich CC + 200 mL of milk	235.1					
food	330 mL of GT drink	2909.4	Mu.	7 days (1 dose morning + 1 dose evening)	25	13 M; Age=22±5 y; BMI=24.3±3.0 kg/m^2	(Hodgson et al., 2014)
		2909.4		1 day (1 dose morning + 1 dose evening)		12 M; Age=21±2 y; BMI=24.2±1.9 kg/m^2	
extract	Capsules of low dose flavan-3-ol monomers from A	467.0	Mu.	28 days (1 dose daily)	42	15 M; 27 F; Age=50 y and older	(Hollands et al., 2020)
	Capsules of high dose flavan-3-ol monomers from A	934.0					
	Capsules of oligomeric procyanidins from A	472.0					
food	400 g of A + 200 mL of Keto-Drink	5756.8	S	-	11	6 M; 5 F	(Anesi et al., 2019)
extract	Tablets of GT	9972.0	Mu.	4 weeks (6 doses daily)	11	9 F; 2 M; Age=28±6 y; BMI=21.8±1.6 kg/m^2	(Mena et al., 2019)
food	238 mL of HZT skin infusion	66.2	S	-	39	10 M; 29 F; Age=40.2±14.2 y; BMI=22.7±2 kg/m^2	(Mocciaro et al., 2019)
food	500 mL of BT beverage	87.4	S	-	20	17 M; 3 F; Age=31±15 y; BMI=23±3 kg/m^2	(Del Rio et al., 2010b)
food	acqueous extract of 1 g BT leaves	1014.0	S	-	2	2 M; Age=31.0±2.8 y; BMI=26.0±3.0 kg/m^2	(Pereira-Caro et al., 2017)
food	NADOLOL (30 mg) + aqueous solution GT (54 mg in 300 mL)	109.1	S	-	13	6 M; 7 F; Age=21-63 y; BMI=19.3-31.8 kg/m^2	(Abe et al., 2018)
	NADOLOL (30 mg) + aqueous solution GT (162 mg in 300 mL)	327.2					
extract	10 capsules of ALM skin extract	1356.5	S	-	12	Age=29.8±5.2 y	(Bartolomé et al., 2010)
pure compound	capsules of EGCG + water	436.7 873.4 1310.0 1746.7	S	-	5	Age=≥30 y	(Chow et al., 2001)
extract	capsules of Polyphenon E (decaffeinated GT catechin mixture)	664.5 1329.0 1993.5 2658.0					
food	120 mL of Cabernet Sauvignon	120.0	S	-	9	5 M; 4 F; Age=29±3 y	(Donovan et al., 2002)
	120 mL of de-alcoholized Cabernet Sauvignon	120.0					
extract	10 capsules of ALM skin extract	1356.5	S	-	12	7 M; 5 F	(Garrido et al., 2010)
food	RS drink (400 g frozen RS + 100 mL water)	4.1	S	-	10	10 M; Age=27±3 y; BMI=23±2 kg/m^2	(Istas et al., 2018)
pure compound	Encapsulated (+)-EC	34.5 103.4 344.8	S	-	4	2 M; 2 F; Age=60±2 y; BMI=32.1±2.2 kg/m^2	(Moreno-Ulloa et al., 2018)
extract	GT decaffeinated extract	873.4	S	-	6	Age=22+2 y	(Pietta et al., 1998)
	GT decaffeinated extract as phospholipid complex	873.4					
food	350 mL of 100% Concord G juice	32.6	S	-	8	3 M; 5 F; Age=20-40 y; BMI=20.7–26.1 kg/m^2	(Stalmach et al., 2012)
extract	1 capsule of BT (2650 mg) + 250 mL water	230.0	S	-	6	Age=19-65 y;	(Van Duynhoven et al., 2014)

						BMI=18.6-24.2 kg/m^2	
food	80 g of red-fleshed A snack	32.7	S	-	10	5 M; 5 F; Age=37.3±8.4 y	(Yuste et al., 2018)
food	cocoa-based products containing coffee (4x)	15.6	Mu.	1 month (2 doses daily)	21	11 F; 10 M; Age=22.9 ± 0.5 y; BMI: 22.3 ± 1.7 kg/m^2	(Mena et al., 2021)
food	340 mL canned GT	442.3	S	-	1	F; Age=32 y	(Yang et al., 2000)
extract	375 mg of Sunphenon BG-5 (GT extract) + 200 mL of water	714.0	S	-	5	5 F; Age=22 y; BMI=16.2–20.2 kg/m^2	(Mukai et al., 2021)
	375 mg of Sunphenon BG-5 + 200 mL of water + 20 g chocolate	729.0					
food	20 g of milk CHOC	14.0					
food	190 mL of canned tea beverage	207.4	S	-	4	Age=21-28 y	(Nakagawa et al., 2009)
	190 mL of canned tea beverage + 40 mg caffeine	207.4					
	190 mL of canned tea beverage + 180 mg caffeine	207.4					
extract	1 g capsules of GT	917.0	S	-	5	3 M; 2 F; Age=30.6±11.97 y	(Gawande et al., 2008)
	1 g capsules of GT + nutrient mixture	917.0					
	1 g capsules of GT + 250 mL fresh black grapes	917.0					
extract	capsules of GT + 400 mL water	436.7 873.4 1746.7	S	-	9	Age=28.67±6.30 y	(Ullmann et al., 2004)
		436.7 873.4 1746.7	Mu.	10 days (1 dose once daily)			
food	cocoa dairy-based drink	2109.1	S		10	Age=32±1 y; BMI=24±2 kg/m^2	(Ottaviani et al., 2012)
food	low-flavanol cocoa-based dairy drink containing (−)-EC	502.2	S		7	Age = 28 ± 3 y; BMI= 24 ± 1 kg/m^2	(Ottaviani et al., 2011)
	low-flavanol cocoa-based dairy drink containing (+)-EC	502.2					
pure compound	capsules of EGCG	1746.7	S		8	2 M; 6 F; Age=39.5±10.4 y	(Chow et al., 2003)
		1746.7	Mu.	1 dose per day for 4 weeks	8	2 M; 6 F; Age=39.5±10.4	
		1746.7		1 day (1 dose morning + 1 dose evening)		4 M; 4 F; Age=29.4±10.7	
		1746.7		2 dose per day for 4 weeks		4 M; 4 F; Age=29.4±10.7	
extract	capsule of Polyphenon E (decaffeinated GT polyphenol mixture)	1746.7	S		8	3 M; 5 F; Age=32.4±10.1 Y	
		1746.7	Mu.	1 dose per day for 4 weeks	8	3 M; 5 F; Age=32.4±10.1 y	
		1746.7		1 day (1 dose morning + 1 dose evening)		2 M; 6 F; Age=34.1±11.9 y	
		1746.7		2 dose per day for 4 weeks		2 M; 6 F; Age=34.1±11.9 y	
pure compound	(−)-EC powder + 40 mL of water	172.4 344.8 689.7	S	-	3	Age=33 y; BMI=27.2 kg/m^2	(Barnett et al., 2015)
		172.4	S	-	4	4 M; Age=33 y; BMI=27.2±3.7 kg/m^2	
		172.4	Mu.	5 days (1 dose daily)	4	4 M; Age=33 y; BMI=27.2±3.7 kg/m^2	
		344.8		1 day (2 doses daily)		4 M; Age=25.5 y; BMI=23.2± 1kg/m^2	

	344.8	5 days (2 doses daily)	4 M; Age=25.5 y; BMI=23.2± 1kg/m^2

Y: years; BMI: body mass index; S: single; Mu: multiple; na: not available; M: male; F: female; RW: red wine; RGP: red grape pomace; CC: cocoa; GT: green tea; CHOC: chocolate; CR: cranberry; A: apple; HZT: hazelnut; BT: black tea; ALM: almond; RS: raspberry; G: grape; EC: epicatechin.

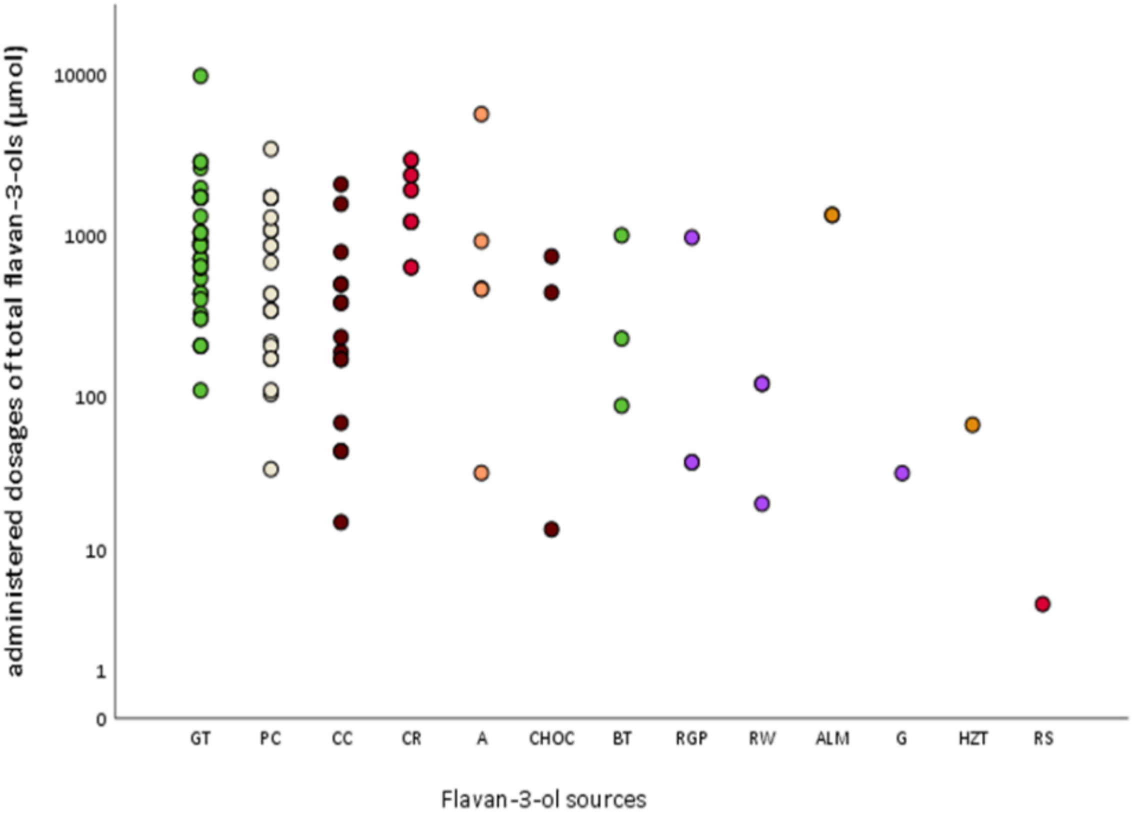

Figure 2. Administered doses (µmol) of total flavan-3-ols through the intervention studies that evaluated flavan-3-ol ADME. Green tea (GT); pure compound (PC); cocoa (CC); cranberry (CR); apple (A); chocolate (CHOC); black tea (BT); red grape pomace (RGP); red wine (RW); almond (ALM); grape (G); hazelnut (HZT); raspberry (RS). Flavan-3-ol sources (n of doses; minimum and maximum values for ingested µmol of total flavan-3-ols): GT (40; 109.1-9972.0 µmol); PC (28; 34.5-3493.4 µmol); CC (15; 15.6-2109.1 µmol); CR (11; 641.0-2993.6 µmol); A (5; 32.7-5756.8 µmol); CHOC (3; 14.0-749.0 µmol); BT (3; 87.4-1014.0 µmol); RGP (3; 38.3-983.5 µmol); RW (3; 20.7-120.0 µmol); ALM (2; 1356.5 µmol); G (1; 32.6 µmol); HZT (1; 66.2 µmol); RS (1; 4.1 µmol).

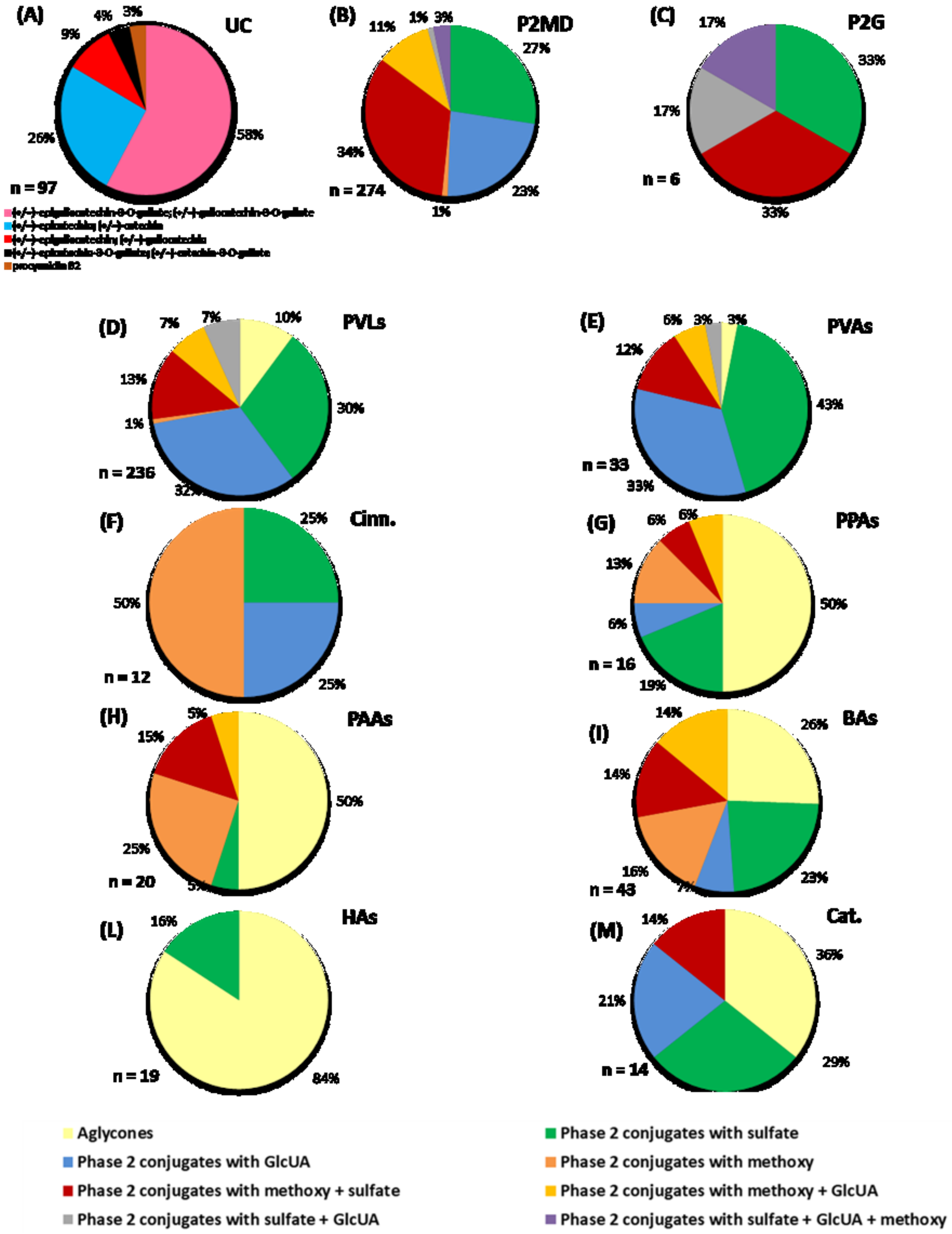

Figure 3. Relative contribution (n of metabolites expressed as % of the total compounds belonging to the same dataset for each metabolite class and divided based on the conjugation moiety) of conjugation group(s) for metabolites of flavan-3-ols grouped by classes based on their metabolic pathway and chemical structure. The relative contribution is calculated taking into account the total metabolites belonging to the whole dataset for the different classes of flavan-3-ol metabolites. Unchanged monomer and dimer flavan-3-ols (UC) (**A**), phase 2 conjugates of monomer and dimer flavan-3-ols (P2MD) (**B**), phase 2 conjugates of galloylated flavan-3-ols (P2G) (**C**), phenyl-γ-valerolactones (PVLs) (**D**), phenylvaleric acids (PVA) (**E**), cinnamates (Cinn.) (**F**), phenylpropanoic acids (PPAs) (**G**), phenylacetic acids (PAAs) (**H**), benzoic acids (BAs) (**I**), hippuric acids (HAs) (**L**), catechols (Cat.) (**M**), glucuronic acid (GlcUA). n indicates the number of metabolites belonging to the dataset of each class of flavan-3-ol metabolites.

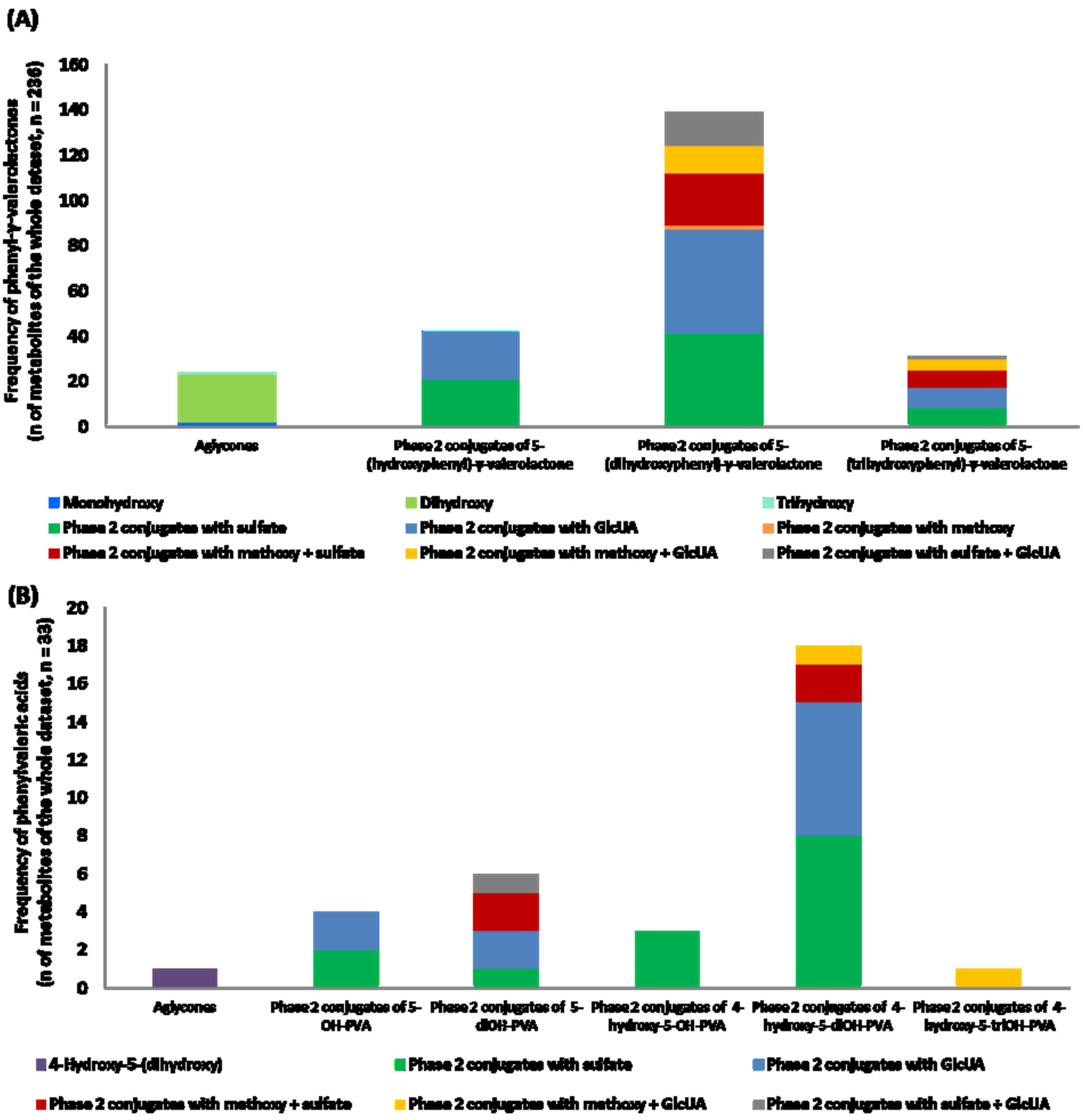

Figure 4. Frequency (number of metabolites quantified in biofluids within each class) of phenyl-γ-valerolactones (**A**) and phenylvaleric acids (**B**), grouped by aglycone skeleton, belonging to the whole dataset for phenyl-γ-valerolactones (n = 236) and phenylvaleric acids (n = 33), respectively. Glucuronic acid (GlcUA), 5-(hydroxyphenyl)valeric acid (5-OH-PVA), 5-(dihydroxyphenyl)valeric acid (5-diOH-PVA), 4-hydroxy-5-(hydroxyphenyl)valeric acid (4-hydroxy-5-OH-PVA), 4-hydroxy-5-(dihydroxyphenyl)valeric acid (4-hydroxy-5-diOH-PVA), 4-hydroxy-5-(trihydroxyphenyl)valeric acid (4-hydroxy-5-triOH-PVA). Metabolites are named according to Kay et al., 2020.

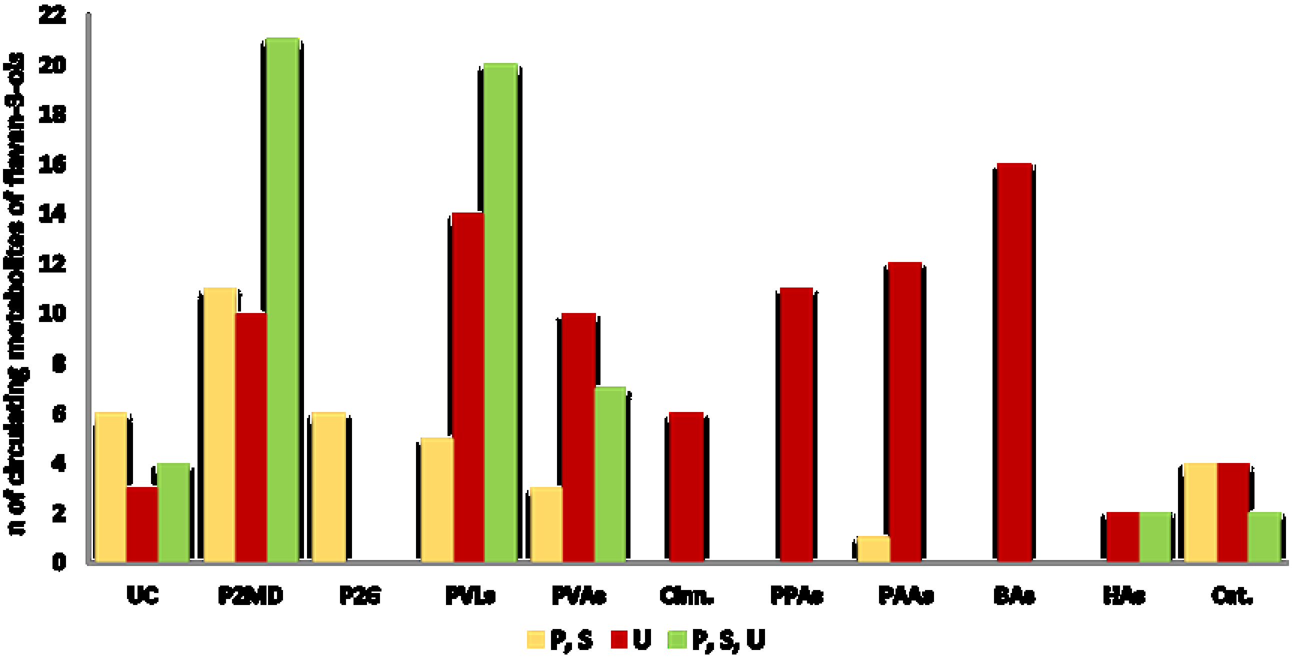

Figure 5. Number of metabolites of flavan-3-ols quantified following flavan-3-ol intake by healthy humans in plasma/serum (P, S) (n=36), urine (U) (n=88), both plasma/serum and urine (P, S, U) (n=56). Unchanged compounds (UC), phase 2 conjugates of monomer and dimer flavan-3-ols (P2MD), phase 2 conjugates of galloyl flavan-3-ols (P2G), phenyl-γ-valerolactones (PVLs), phenylvaleric acids (PVA), cinnamates (Cinn.), phenylpropanoic acids (PPAs), phenylacetic acids (PAAs), benzoic acids (BAs), hippuric acids (HAs), catechols (Cat.).

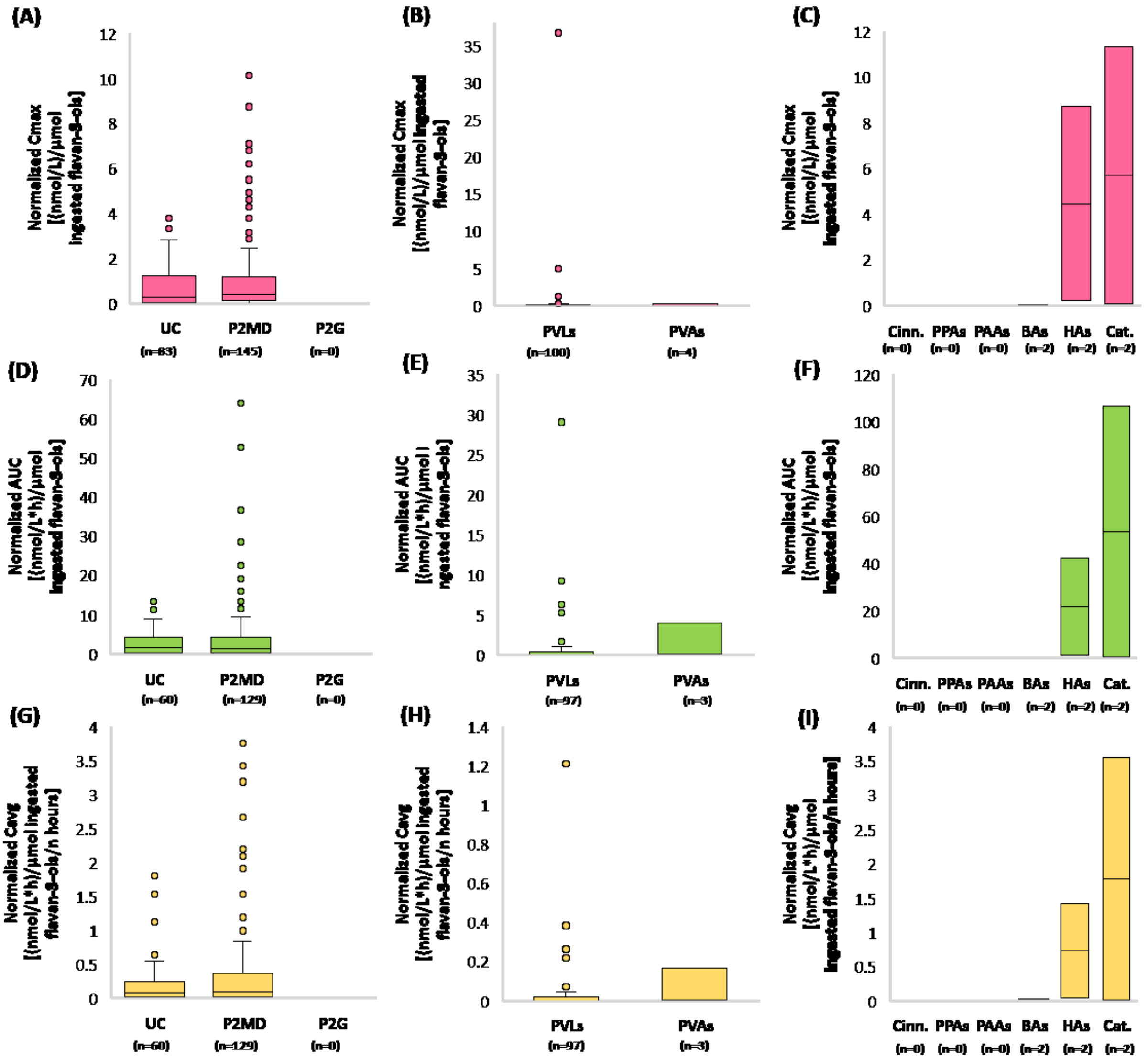

Figure 6. Box plot for normalized C_{max} [(nmol/L)/µmol ingested flavan-3-ols] (**A, B, C**), normalized AUC [(nmol/L*h)/µmol ingested flavan-3-ols] (**D, E, F**) and normalized C_{avg} [(nmol/L*h)/µmol ingested flavan-3-ols/n hours] (**G, H, I**) of unchanged monomer and dimer flavan-3-ols (UC), phase 2 conjugates of monomer and dimer flavan-3-ols (P2MD), phase 2 conjugates of galloyl flavan-3-ols (P2G), phenyl-γ-valerolactones (PVLs), phenylvaleric acids (PVA), cinnamates (Cinn.), phenylpropanoic acids (PPAs), phenylacetic acids (PAAs), benzoic acids (BAs), hippuric acids (HAs), catechols (Cat.). Apart for UC, classes of flavan-3-ol metabolites include data derived from both aglycones and their phase 2 conjugates. Maximum plasma concentration (C_{max}), area under the curve (AUC), average concentration (C_{avg}), n indicates the number of biological replicates collected for the same class of flavan-3-ol metabolites and for the same pharmacokinetic parameter.

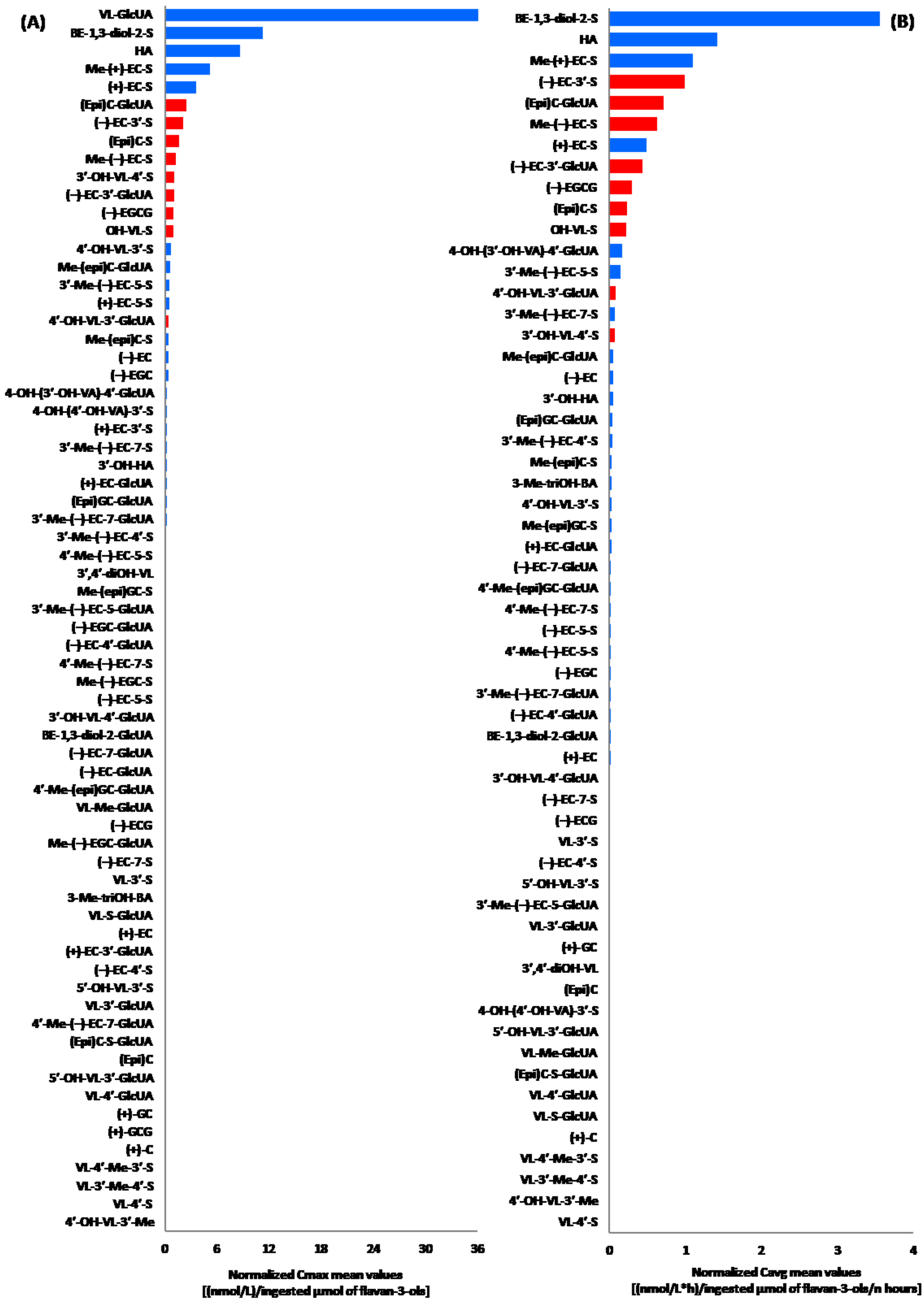

Figure 7. (**A**) Normalized C_{max} [(nmol/L)/µmol ingested flavan-3-ols] and (**B**) normalized C_{avg} [(nmol/L*h)/µmol ingested flavan-3-ols/n hours] mean values for all the metabolites of flavan-3-ols quantified at circulating level following flavan-3-ol intake by healthy humans. Red bars refer to the main circulating metabolites of flavan-3-ols selected based on a normalized C_{max} value ≥ 1 nmol/L for unchanged compounds and host metabolites and ≥ 0.3 nmol/L for gut microbiota and host-gut microbiota metabolites, respectively. The main circulating metabolites of flavan-3-ols were selected if their mean normalized C_{max} values were calculated using at least 3 biological replicates deriving from at least 2 manuscripts. Metabolites are named according to Kay et al., 2020. See Table 1 for full metabolite names.

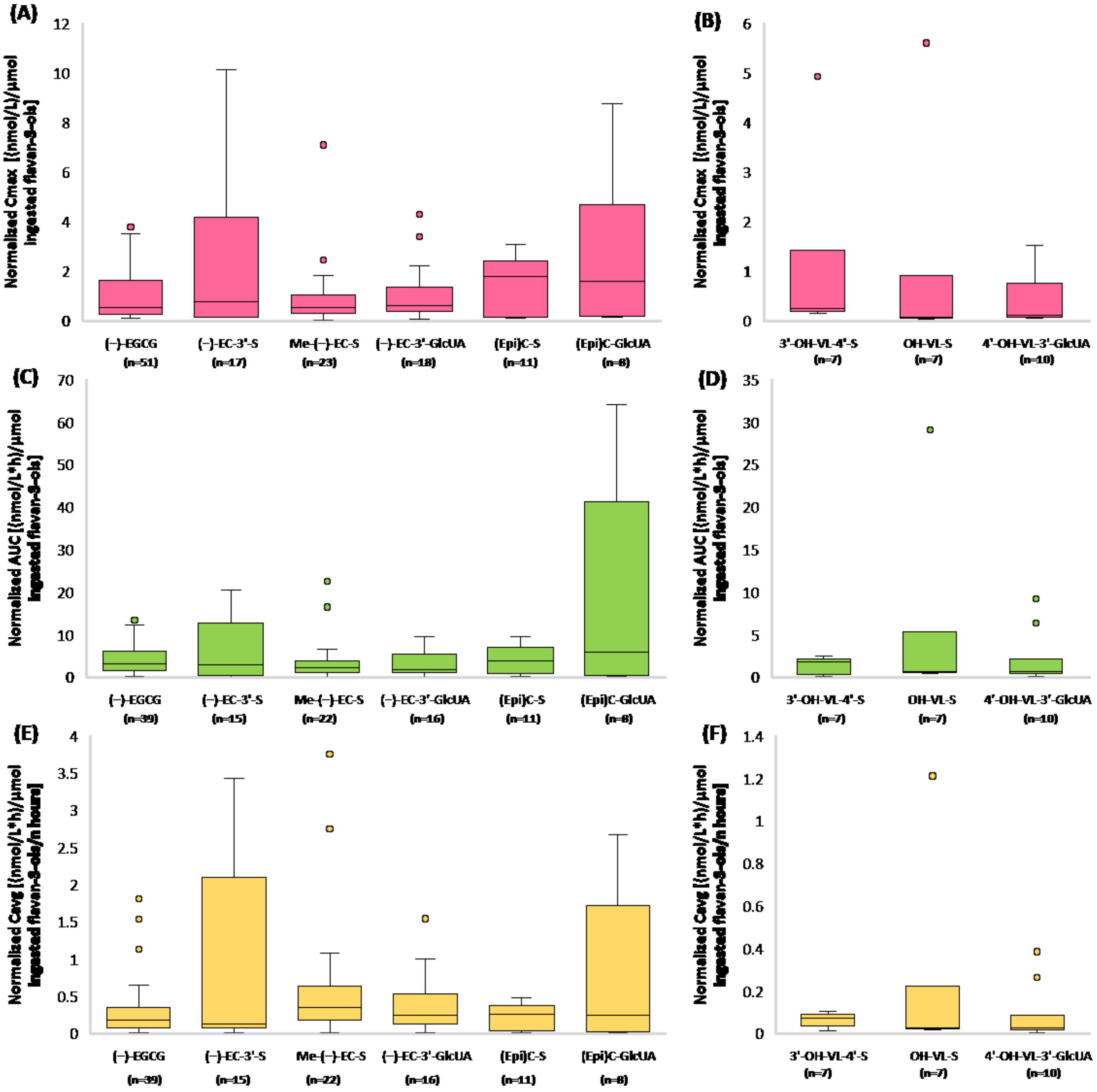

Figure 8. Box plot for normalized C_{max} [(nmol/L)/µmol ingested flavan-3-ols] (**A, B**), normalized AUC [(nmol/L*h)/µmol ingested flavan-3-ols] (**C, D**) and normalized C_{avg} [(nmol/L*h)/µmol ingested flavan-3-ols/n hours] (**E, F**) of (−)-epigallocatechin-3-*O*-gallate ((−)-EGCG), (−)-epicatechin-3′-sulfate ((−)-EC-3′-S), methoxy-(−)-epicatechin-sulfate (Me-(−)-EC-S), (−)-epicatechin-3′- glucuronide ((−)-EC-3′-GlcUA), (epi)catechin-sulfate ((Epi)C-S), (epi)catechin-glucuronide ((Epi)C-GlcUA), 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate (3′-OH-VL-4′-S), 5-(hydroxyphenyl)-γ-valerolactone-sulfate (OH-VL-S), 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-glucuronide (4′-OH-VL-3′-GlcUA). (Epi)C-S and (epi)C-GlcUA values include all the (−/+)-epicatechin and (−/+)-catechin isomers quantified at circulating level following flavan-3-ol intake. Me-(−)-EC-S and OH-VL-S values include data derived from both individual and sum of unknown isomers quantified at circulating level following flavan-3-ol intake. Maximum plasma concentration (C_{max}), area under the curve (AUC), average concentration (C_{avg}), Metabolites are named according to Kay et al., 2020. n indicates the number of biological replicates collected for the same flavan-3-ol metabolite and for the same pharmacokinetic parameter.

Table 3. Pharmacokinetic parameters and urinary excretion data for the main metabolites identified at circulating level following intake of flavan-3-ols by healthy subjects. Data are reported as mean ± SD (n indicates the number of biological values collected from literature for each parameter for the same compound). The main circulating metabolites of flavan-3-ols were selected based on a normalized C_{max} value ≥ 1 nmol/L for unchanged compounds and host metabolites and ≥ 0.3 nmol/L for gut microbiota and host-gut microbiota metabolites, respectively. Metabolites are named according to Kay et al., 2020.

Compounds	**C_{max} (nmol/L)**	**C_{max} normalized ((nmol/L)/total µmol of ingested flavan-3-ols)**	**T_{max} (h)**	**AUC (nmol/L*h)**	**AUC normalized ((nmol/L*h)/ total µmol of ingested flavan-3-ols)**	**C_{avg} ((nmol/L*h)/n hours)**	**C_{avg} normalized ((nmol/L*h)/ total µmol of ingested flavan-3-ols/n hours)**	**$t_{1/2}$ (h)**	**Urinary excretion (% of intake)**
Unchanged compounds									
(−)-Epigallocatechin-3-*O*-gallate	867.7 ± 1327.3 (*n* = 51)	1.0 ± 1.0 (*n* = 51)	2.2 ± 0.9 (*n* = 51)	3974.4 ± 4566.1 (*n* = 39)	4.2 ± 3.7 (*n* = 39)	213.7 ± 190.7 (*n* = 39)	0.3 ± 0.4 (*n* = 39)	2.8 ± 1.0 (*n* = 41)	-
Phase 2 conjugates of monomer flavan-3-ols									
(−)-Epicatechin-3′-sulfate	655.5 ± 878.1 (*n* = 17)	2.1 ± 2.9 (*n* = 17)	1.7 ± 0.6 (*n* = 17)	1988.0 ± 2842.1 (*n* = 15)	6.2 ± 7.5 (*n* = 15)	318.1 ± 480.8 (*n* = 15)	1.0 ± 1.3 (*n* = 15)	1.5 ± 0.4 (*n* = 13)	3.1 ± 0.8 (*n* = 4)
Methoxy-(−)-epicatechin-sulfate*#	392.8 ± 463.7 (*n* = 23)	1.3 ± 1.9 (*n* = 23)	1.5 ± 0.5 (*n* = 23)	1209.6 ± 1321.8 (*n* = 22)	3.8 ± 5.4 (*n* = 22)	201.6 ± 220.3 (*n* = 22)	0.6 ± 0.9 (*n* = 22)	2.0 ± 0.6 (*n* = 19)	1.9 ± 2.3 (n = 9)
(−)-Epicatechin-3′-glucuronide	453.8 ± 525.6 (*n* = 18)	1.0 ± 1.2 (*n* = 18)	1.4 ± 0.6 (*n* = 18)	1299.1 ± 1513.8 (*n* = 16)	3.1 ± 3.2 (*n* = 16)	192.8 ± 258.0 (*n* = 16)	0.4 ± 0.5 (*n* = 16)	1.8 ± 0.7 (*n* = 14)	1.9 ± 2.8 (*n* = 9)
(Epi)catechin-sulfate*~‡	77.6 ± 12.3 (*n* = 11)	1.6 ± 1.1 (*n* = 11)	1.7 ± 0.4 (*n* = 6)	217.8 ± 98.1 (*n* = 11)	4.2 ± 3.1 (*n* = 11)	11.4 ± 5.6 (*n* = 11)	0.2 ± 0.2 (*n* = 11)	2.0 ± 0.5 (*n* = 5)	12.6 ± 21.3 (*n* = 25)
(Epi)catechin-glucuronide*~‡	67.5 ± 50.0 (*n* = 8)	2.5 ± 3.1 (*n* = 8)	1.9 ± 0.5 (*n* = 3)	359.0 ± 371.9 (*n* = 8)	17.0 ± 25.9 (*n* = 8)	15.0 ± 15.5 (*n* = 8)	0.7 ± 1.1 (*n* = 8)	2.3 (*n* = 1)	3.8 ± 6.2 (*n* = 20)
Phenyl-γ-valerolactones									
5-(3′-Hydroxyphenyl)-γ-valerolactone-4′-sulfate	368.4 ± 155.7 (*n* = 7)	1.1 ± 1.8 (*n* = 7)	5.2 ± 1.2 (*n* = 7)	2473.0 ± 2089.7 (*n* = 7)	1.5 ± 0.9 (*n* = 7)	103.5 ± 86.4 (*n* = 7)	0.1 ± 0.0 (*n* = 7)	-	11.3 ± 14.8 (*n* = 6)
5-(Hydroxyphenyl)-γ-valerolactone-sulfate*#	211.8 ± 303.2 (*n* = 7)	1.0 ± 2.1 (*n* = 7)	4.9 ± 1.5 (*n* = 7)	1507.9 ± 1691.7 (*n* = 7)	5.3 ± 10.6 (*n* = 7)	62.8 ± 70.5 (*n* = 7)	0.2 ± 0.4 (*n* = 7)	4.8 (*n* = 1)	22.0 ± 26.5 (*n* = 12)
5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-glucuronide	228.6 ± 345.0 (*n* = 10)	0.4 ± 0.5 (*n* = 10)	5.5 ± 0.7 (*n* = 10)	1397.6 ± 1850.0 (*n* = 10)	1.9 ± 3.2 (*n* = 10)	58.3 ± 77.0 (*n* = 10)	0.1 ± 0.1 (*n* = 10)	7.8 ± 6.6 (*n* = 2)	3.1 ± 3.3 (*n* = 15)

C_{max}: maximum plasma concentration; T_{max}: time to reach C_{max}; AUC: area under the curve; C_{avg}: average concentration; $t_{1/2}$: half elimination time; *symbol: when the position of the conjugation is unknown; ~symbol: when the compound corresponds to (−/+)-epicatechin or (−/+)-catechin; ‡symbol: when the value for this compound includes all the (−/+)-epicatechin and (−/+)-catechin isomers quantified at circulating level following flavan-3-ol intake. # symbol: when the value includes data derived from both individual and sum of unknown isomers quantified at circulating level following flavan-3-ol intake; - means any data was collected for that pharmacokinetic parameter.

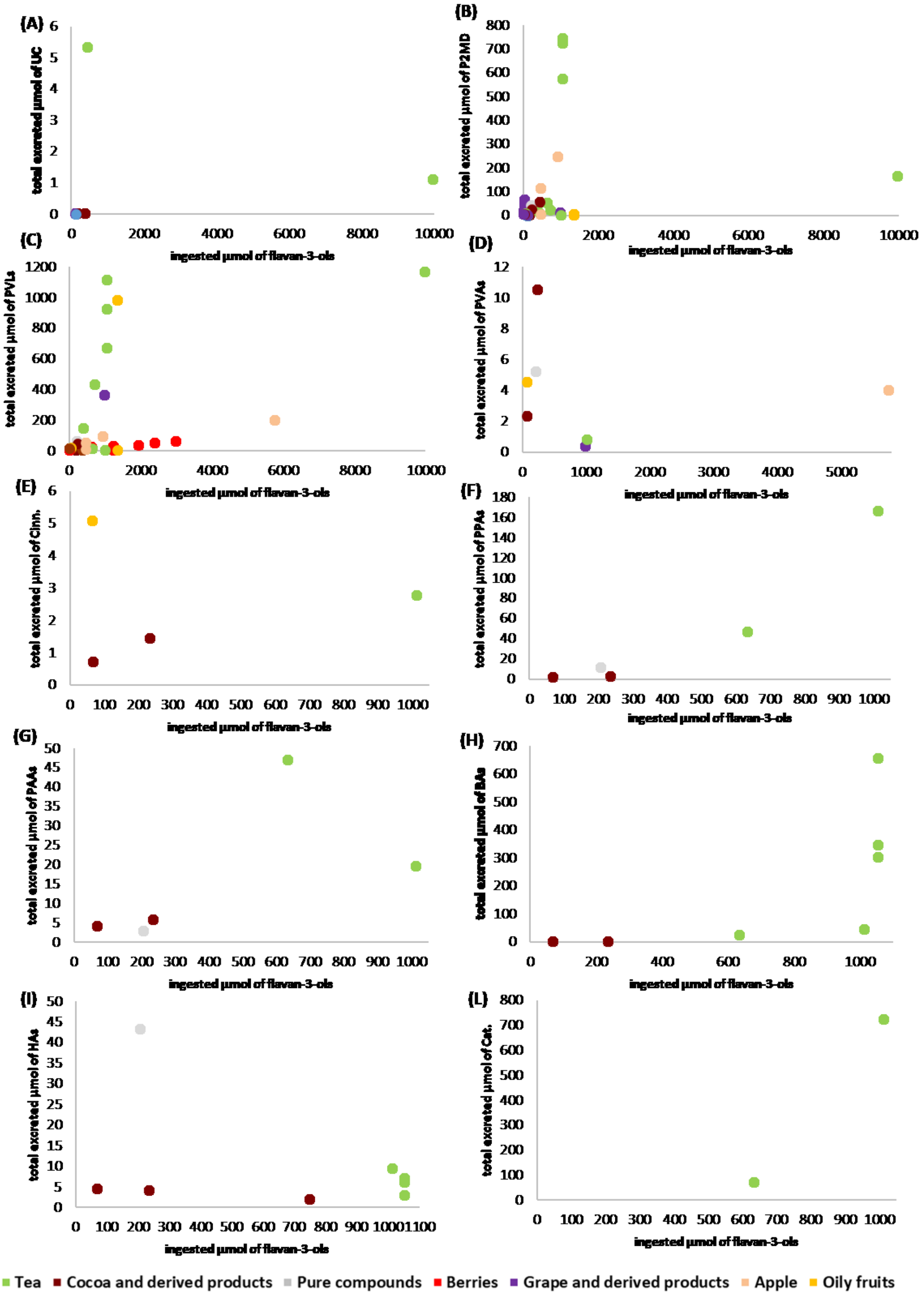

Figure 9. Total excretion (µmol) of the different classes of flavan-3-ol metabolites [unchanged monomer and dimer flavan-3-ols (UC) (**A**), phase 2 conjugates of monomer and dimer flavan-3-ols (P2MD) (**B**), phenyl-γ-valerolactones (PVLs) (**C**), phenylvaleric acids (PVA) (**D**), cinnamates (Cinn.) (**E**), phenylpropanoic acids (PPAs) (**F**), phenylacetic acids (PAAs) (**G**), benzoic acids (BAs) (**H**), hippuric acids (HAs) (**I**), catechols (Cat.) (**L**)] in urine and the ingested amount (µmol) of flavan-3-ol sources. Classes of flavan-3-ol metabolites include data derived from both aglycones and their phase 2 conjugates. Each bullet indicates the total value of excreted µmol for the different classes of flavan-3-ol metabolites in urine, calculated for each single study, and related to each dose of consumed flavan-3-ols in the study. Data on phase 2 conjugates of galloylated flavan-3-ols are not reported due to the absence of urinary data.

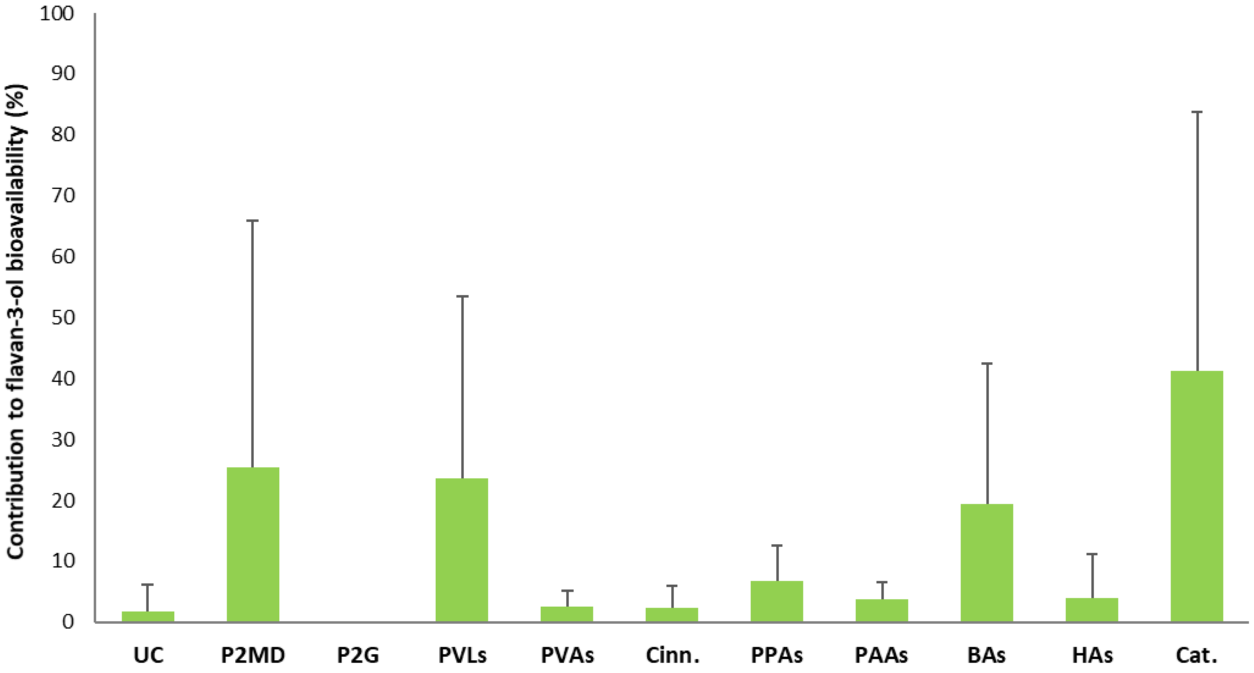

Figure 10. Contribution (%) of each metabolite class to the overall bioavailability of flavan-3-ols [unchanged monomer and dimer flavan-3-ols (UC) (n, number of bioavailability values calculated for each class=7), phase 2 conjugates of monomer and dimer flavan-3-ols (P2MD) (n=31), phenyl-γ-valerolactones (PVLs) (n=30), phenylvaleric acids (PVA) (n=7), cinnamates (Cinn.) (n=4), phenylpropanoic acids (PPAs) (n=5), phenylacetic acids (PAAs) (n=5), benzoic acids (BAs) (n=7), hippuric acids (HAs) (n=8), catechols (Cat.) (n=2)]. Classes of flavan-3-ol metabolites include data derived from both aglycones and their phase 2 conjugates. Data on phase 2 conjugates of galloyl flavan-3-ols (P2G) are not reported due to the absence of urinary data. Data are reported as mean and SD.

Table 4. Flavan-3-ol bioavailability (%) values collected from literature and/or estimated from urinary excretion data employed to calculate the final value for flavan-3-ol bioavailability (%).

Bioavailability of flavan-3-ols (%)	Flavan-3-ol source	Ref
8.1‡	GT	(Stalmach et al., 2009)
82.0‡	pure (−)-EC	(Ottaviani et al., 2016)
10.5‡	CC	(Mullen et al., 2009)
18.3‡	CC	(Mullen et al., 2009)
40.0‡	GT	(Roowi et al., 2010)
62.0‡	GT	(Calani et al., 2012)
39.5‡	GT	(Del Rio et al., 2010a)
21.0‡	CHOC	(Actis-Goretta et al., 2012)
38.1~	RGP	(Castello et al., 2018)
35.3‡	CC	(Gómez-Juaristi et al., 2019)
34.6‡	CC	(Gómez-Juaristi et al., 2019)
34.7~	A	(Hollands et al., 2020)
36.0~	A	(Hollands et al., 2020)
2.6~	A	(Hollands et al., 2020)
13.4~	GT	(Mena et al., 2019)
53.1~	HZT	(Mocciaro et al., 2019)
7.2‡	BT	(Del Rio et al., 2010b)
5.7~*	BT	(Pereira-Caro et al., 2017)
73.0~	ALM	(Garrido et al., 2010)
13.7‡	G	(Stalmach et al., 2012)

‡ symbol: indicates value for flavan-3-ol bioavailability (%) collected from literature; ~ symbol: indicates values for flavan-3-ol bioavailability (%) estimated from urinary excretion data; * symbol: this value was calculated taken into account the urinary excretion of structurally related epicatechin metabolites and 5C-ring fission metabolites originated from the monomers and dimers presented in the theaflavin extract; GT: green tea; EC: epicatechin; CC: cocoa; CHOC: chocolate; RGP: red grape pomace; A: apple; HZT: hazelnut; BT: black tea; ALM: almond; G: grape.

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Chapter 6. A systematic review and comprehensive nutrikinetics, urinary excretion and bioavailability of hydroxycinnamic acids

The article is inserted in the present Doctoral Thesis as manuscript ready for submission at *Antioxidants & Redox Signaling* (IF 7.468)

Forum review article

A systematic review and comprehensive nutrikinetics, urinary excretion and bioavailability of hydroxycinnamic acids

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Running Title: ADME of hydroxycinnamic acids in humans

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Abstract

Significance: Hydroxycinnamic acids (HCAs) are the most consumed phenolic acids in the western diet. Harmonizing the information on the absorption, distribution, metabolism, and excretion (ADME) of HCAs might be fundamental to unravelling which compounds could be responsible for their potential beneficial health effects. This work systematically assessed nutrikinetics and urinary recovery of HCAs, and their metabolites based on literature data.

Recent Advances: HCAs were metabolized to up to 105 new compounds, mainly acyl-quinic and C_6-C_3 unsaturated cinnamic acids. C_6-C_3 unsaturated cinnamic acids reached the highest blood concentration level (C_{max} = 423 nmol/L), with T_{max} values ranging from 2.7 to 4.2 h. C_6-C_3 unsaturated cinnamic acids were excreted in urine in higher amounts than their phenylpropanoic acid derivatives (4 and 1% of intake, respectively), but both in a lower percentage than catechols (11 %). Nutrikinetic and urinary recovery data revealed 18 and 16 main blood and urinary metabolites of HCAs, respectively. HCAs were moderately bioavailable in humans (25 %).

Critical Issues: A relevant intra-class and metabolite variability emerged. It was impossible to assess the bioavailability of HCAs for each ingested source unequivocally. Data on the ADME of HCAs for some plant based-foods were absent or inconsistent.

Future Directions: A comprehensive study investigating the ADME of HCAs deriving from their most important dietary sources is strongly required. Eight ‘golden metabolites’ of HCAs were identified and reached interesting blood concentration and urinary recovery profiles, opening new perspectives to evaluate their potential in bioavailability studies and bioactivity models at physiological concentration levels.

Keywords: (poly)phenols; pharmacokinetics; chlorogenic acids; phenolics; stoichiometry; metabolites

1. Introduction

Plant secondary metabolites are phytochemicals synthesized *in planta* to attract pollinators and seed-dispersing animals, and as a defensive mechanism against herbivores and microbial infections (Del Rio et al., 2013; Rodriguez-Mateos et al., 2014). Dietary phytochemicals include thousands of structures mainly represented by (poly)phenols, followed by terpenoids, alkaloids and sulphur-containing compounds (Crozier et al., 2009; Scalbert et al., 2005). Based on their chemical structure, (poly)phenols are classified into flavonoids (i.e. flavan-3-ols, flavonols, flavones, isoflavones, flavanones, and anthocyanins) and nonflavonoids, including low molecular weight phenolic acids and more complex structures (i.e. stilbenes, lignans and hydrolyzable tannins) (Del Rio et al., 2013). Hydroxycinnamic acids (HCAs) are the most consumed phenolic acids in the western diet, providing, together with flavan-3-ols, the majority of (poly)phenol intake (Zamora-Ros et al., 2013; Ziauddeen et al., 2018). The main dietary HCAs are 3′,4′-dihydroxycinnamic acid (*aka* caffeic acid), 4′-hydroxy-3′-methoxycinnamic acid (*aka* ferulic acid), 3′,5′-dimethoxy-4′-hydroxycinnamic acid (*aka* sinapic acid) and 4′-hydroxycinnamic acid (*aka p*-coumaric acid). *In planta*, these molecules may undergo esterification with 1_{L}-(−)-quinic acid resulting in caffeoylquinic, feruloylquinic and coumaroylquinic acids, along with dicaffeoylquinic acids, also known as ‘chlorogenic acids’ (CGAs) (Clifford et al., 2017). The dietary intake of CGAs in the western diet is estimated to be about 200 mg/day, with coffee, cereals, potatoes and artichokes the most abundant dietary sources (El-Seedi et al., 2012; Farah and Lima, 2019; Zamora-Ros et al., 2013; Ziauddeen et al., 2018). After their consumption, HCAs are partially absorbed in the upper gastrointestinal tract, since up to two-thirds of the ingested dose reaches indigested the colon to be catabolized by gut microbiota action (Calani et al., 2012; Clifford et al., 2020; Kahle et al., 2005; Olthof et al., 2001; Sova and Saso, 2020; Stalmach et al., 2010). The potential health benefits of HCAs include mediation of postprandial glucose and hormonal responses (Ros et al., 2011), and management of some cardiometabolic and cancer risk factors (Coman and Vodnar, 2020; Kajikawa et al., 2019; Kempf et al., 2015; Martini et al., 2019; Mills et al., 2017; Ochiai et al., 2014; Rocha et al., 2012; Rondanelli et al., 2013), lipid metabolism and obesity (Alam et al., 2016). 4′-Hydroxy-3′-methoxycinnamic acid, 3′-methoxycinnamic acid-4′-sulfate (*aka* ferulic acid-4′-sulfate), 3-(3′,4′-dihydroxyphenyl)propanoic acid (*aka* dihydrocaffeic acid), 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid (*aka* dihydroferulic acid), and 3′-methoxy-4′-hydroxycinnamoyl-glycine (*aka* feruloylglycine) were found being bioactives in *in vitro* models at physiological concentrations (Krga et al., 2016; Monagas et al., 2009; Van Rymenant et al., 2017a, 2017b; Verzelloni et al., 2011). An increasing number of human studies assessed the absorption, metabolism, distribution and excretion (ADME) of dietary HCAs showing a substantial inter-study variability in their nutrikinetic and excretion profiles (Clifford et al., 2020, 2017; Sova and Saso, 2020), with maximum plasma concentrations ranging from < 10 to 800 nmol/L, although some compounds were also found circulating at µmol/L levels (Farah et al., 2008; Gómez-Juaristi et al., 2018; Lang et al., 2013; Monteiro et al., 2007; Nardini et al., 2002; Stalmach et al., 2014, 2009). The dietary sources (and its associated matrix effect) of HCAs, the dosages of ingested parent compounds and inter-population differences might be considered major factors explaining the variability observed in blood and urine HCA levels. To our knowledge, any systematic data is available for the nutrikinetic profiles and urinary recovery of compounds quantified in biofluids, and their average blood concentrations, after HCA intake. A harmonized unique value of HCA bioavailability deriving from consumption of the different food sources is also lacking.

Thus, this systematic review aimed at (i) summarize results from existing human studies evaluating the ADME of HCAs in healthy volunteers, (ii) analyze nutrikinetic parameters and urinary recovery of their circulating metabolites, (iii) carry out an estimation of HCA bioavailability based on literature data. After defining the main urinary metabolites of HCAs, this work also aimed at defining stoichiometric balances in their production to estimate the dose of parent compounds to be ingested to achieve a known excreted amount.

2. Results

2.1 Study Selection

The study selection process is shown in Supplementary Figure S1. A total of 8383 records were identified through the database search. After removing 2260 duplicates, up to 6123 studies were screened, of which 5908 were excluded based on the title or abstract. A total of 198 eligible records went under the full-text screening process, after which 151 records were excluded. Forty-seven publications met eligibility criteria and were included in the data analysis.

2.2 Characteristics of the included studies

The main characteristics of the studies that met all inclusion criteria are reported in Supplementary Table S2. Out of the 47 included intervention studies (total sample size n = 614), 43 investigated the ADME of HCAs following a single dose intake. Two publications assessed the ADME of HCAs following a multiple-dose (1-30 days) intake, while the remaining two publications showed an experimental setting with both single and multiple doses. No observational study met the inclusion criteria. The ADME of HCAs was assessed after intake of coffee (both green and roasted) (n = 12 studies), berries (i.e. raspberry, blueberry, cranberry) (6), herbs (i.e. Guizhi Fuling, Melissa Officinalis, Gumiganghwal-tang, guapo, Socheongryong-tang, Shuanghua Baihe) (6), cereals (i.e. wheat, oat) (4), tomato (3), orange (3), pure compounds (i.e. ^{13}C-labelled-cyanidin-3-glucoside, 1,5-dicaffeoylquinic acid) (3), grape products (i.e. red grape pomace, red wine) (3), apple (1), olive oil (1), rosemary tea (1), artichoke (1), yerba mate (1), nuts (i.e. hazelnut) (1) and propolis (1) (Supplementary Table S2). Mean intake of parent compounds ranged from 17 to 5715 µmol for those consumed through olive oil and artichoke, respectively [479.2 (80.5-1096.1) µmol (median (25th-75th percentile)) for all the administered doses of parent compounds] (Supplementary Figure S2 and Supplementary Table S2).

2.3 Circulating compounds after hydroxycinnamic acid intake by healthy subjects

Up to 105 quantified metabolites in blood and urine fractions were reported following intake of HCAs and other phytochemicals [i.e. (poly)phenols (when various flavonoids were precursors of the same metabolite), flavan-3-ols, flavanones, anthocyanins and coumarins] by healthy subjects (Table 1).

Table 1. Acyl-quinic acids, C_6-C_3 unsaturated cinnamic acids and their metabolites quantified in blood/urine samples following HCA intake by healthy humans. C_6-C_3 unsaturated cinnamic acids include compounds quantified in biofluids after consumption of other phytochemicals. *Unchanged compounds*: indicates when the native HCA did not undergo any metabolic step following its ingestion, *host metabolism*: when the compound derived from a biotransformation by small intestine, hepatic or renal phase 1 or phase 2 enzymes, *gut microbiota metabolism*: when the compound derived from HCA metabolism through gut microbiota activity, *host-gut microbiota metabolism*: when the compound derived from HCA metabolism through gut microbiota activity and further conjugation by a phase 2 enzyme.

n°	Chemical name of metabolite	Systematic name of metabolite	MW (Da) of metabolite	PhytoHub ID of metabolite	Metabolic origin of metabolite	Biofluid(s) where metabolite was quantified	Dietary source and precursor(s) of metabolite	Ref.
Acyl-quinic acids								
Caffeoylquinic acid derivatives								
1	4-Caffeoylquinic-1,5-lactone	4-Caffeoylquinic-1,5-lactone	336	PHUB002471	Host metabolism	P	[coffee CGAs]	(Mills et al., 2017)
2	3-Caffeoylquinic-1,5-lactone	3-Caffeoylquinic-1,5-lactone	336	PHUB002479	Host metabolism	P	[coffee CGAs]	(Mills et al., 2017)
3	3-Caffeoylquinic acid	3-Caffeoylquinic acid	354	PHUB000530	Unchanged	P, U, S	[yerba mate, coffee, blueberry 3-caffeoylquinic acid]	(Gómez-juaristi et al., 2018; Mena et al., 2021; Mills et al., 2017; Morton et al., 2018; Zhong et al., 2017)
4	4-Caffeoylquinic acid	4-Caffeoylquinic acid	354	PHUB000537	Unchanged	P, U, S	[coffee, artichoke 4-caffeoylquinic acid]	(Domínguez-Fernández et al., 2022; Gómez-Juaristi et al., 2018; Mills et al., 2017; Morton et al., 2018)
5	5-Caffeoylquinic acid	5-Caffeoylquinic acid	354	PHUB000585	Unchanged	P, U, S	[coffee, yerba mate, tomato, cranberry, artichoke 5-caffeoylquinic acid]	(Domínguez-Fernández et al., 2022; Feliciano et al., 2017, 2016; Gómez-juaristi et al., 2018; Heiss et al., 2022; Lang et al., 2013; Martínez-Huélamo et al., 2016; Martínez-Huélamo et al., 2015; Mena et al., 2021; Mills et al., 2017; Morton et al., 2018; Pan et al., 2016; Scherbl et al., 2017; Stalmach et al., 2014, 2009)
6	3-Dihydrocaffeoylquinic acid	3-Dihydrocaffeoylquinic acid	356	PHUB002455	Gut microbiota metabolite	U	[coffee, yerba mate CGAs]	(Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018)
7	4-Dihydrocaffeoylquinic acid	4-Dihydrocaffeoylquinic acid	356	PHUB002456	Gut microbiota metabolite	U	[coffee CGAs]	(Gómez-Juaristi et al., 2018)
8	5-Dihydrocaffeoylquinic acid	5-Dihydrocaffeoylquinic acid	356	PHUB002457	Gut microbiota metabolite	U	[coffee CGAs]	(Gómez-Juaristi et al., 2018)
9	3-Caffeoylquinic lactone-S*	3-Caffeoylquinic lactone-S*	416	PHUB002441	Host metabolism	P, U	[coffee CGAs]	(Stalmach et al., 2014, 2009)
10	4-Caffeoylquinic lactone-S*	4-Caffeoylquinic lactone-S*	416	PHUB002442	Host metabolism	P, U	[coffee CGAs]	(Stalmach et al., 2014, 2009)
11	Caffeoylquinic lactone-S*	Caffeoylquinic lactone-S*	416	PHUB002458	Host metabolism	U	[coffee, yerba mate CGAs]	(Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018)
12	3-Caffeoylquinic acid-S*	3-Caffeoylquinic acid-S*	434	PHUB002443	Host metabolism	U	[coffee CGAs]	(Stalmach et al., 2014)
13	4-Caffeoylquinic acid-S*	4-Caffeoylquinic acid-S*	434	PHUB002444	Host metabolism	U	[coffee CGAs]	(Stalmach et al., 2014)
14	5-Caffeoylquinic acid-3′-S	5-Caffeoylquinic acid-3′-S	434	PHUB002472	Host metabolism Host-gut microbiota co-metabolite	P	[coffee CGAs]	(Mills et al., 2017)
15	5-Caffeoylquinic acid-4′-S	5-Caffeoylquinic acid-4′-S	434	PHUB002473	Host metabolism Host-gut microbiota co-metabolite	P	[coffee CGAs]	(Mills et al., 2017)
16	1,5-Dicaffeoylquinic acid	1,5-Dicaffeoylquinic acid	516	PHUB000513	Unchanged	P	[1,5-dicaffeoylquinic acid]	(Gu et al., 2007; Liu et al., 2010)
17	Dihydrocaffeoylquinic acid-GlcUA*	Dihydrocaffeoylquinic acid-GlcUA*	532	PHUB002459	Host-gut microbiota co-metabolite	U	[coffee CGAs]	(Gómez-Juaristi et al., 2018)
Feruloylquinic acid derivatives								
18	3-Feruloylquinic-1,5-lactone	3-Feruloylquinic-1,5-lactone	350	PHUB002474	Host metabolism	P	[coffee CGAs]	(Mills et al., 2017)
19	4-Feruloylquinic-1,5-lactone	4-Feruloylquinic-1,5-lactone	350	PHUB002475	Host metabolism	P	[coffee CGAs]	(Mills et al., 2017)
20	3-Feruloylquinic acid	3-Feruloylquinic acid	368	PHUB000531	Unchanged Host metabolism	P, U	[coffee, yerba mate 3-feruloylquinic acid]; [artichoke (poly)phenols]	(Domínguez-Fernández et al., 2022; Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018; Mena et al., 2021; Mills et al., 2017; Scherbl et al., 2017; Stalmach et al., 2014, 2009)
21	4-Feruloylquinic acid	4-Feruloylquinic acid	368	PHUB000541	Unchanged Host metabolism	P, U	[coffee, yerba mate 4-feruloylquinic acid]; [artichoke	(Domínguez-Fernández et al., 2022; Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018; Mena et al.,

							(poly)phenols]	2021; Mills et al., 2017; Scherbl et al., 2017; Stalmach et al., 2014, 2009)
22	5-Feruloylquinic acid	5-Feruloylquinic acid	368	PHUB000550	Unchanged	P, U	[coffee, yerba mate 5-feruloylquinic acid]	(Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018; Mena et al., 2021; Mills et al., 2017; Scherbl et al., 2017; Stalmach et al., 2014, 2009)
23	3-Dihydroferuloylquinic acid	3-Dihydroferuloylquinic acid	370	PHUB002452	Host-gut microbiota co-metabolite	P, U	[coffee, yerba mate CGAs]	(Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018)
24	5-Dihydroferuloylquinic acid	5-Dihydroferuloylquinic acid	370	PHUB002453	Host-gut microbiota co-metabolite	P, U	[coffee, yerba mate CGAs]	(Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018)
25	4-Dihydroferuloylquinic acid	4-Dihydroferuloylquinic acid	370	PHUB002456	Host-gut microbiota co-metabolite	U	[coffee CGAs]	(Gómez-Juaristi et al., 2018)
26	5-Feruloylquinic acid-4′-S	5-Feruloylquinic acid-4′-S	448	PHUB002477	Host-gut microbiota co-metabolite Host metabolism	P	[coffee CGAs]	(Mills et al., 2017)
27	Feruloylquinic lactone-GlcUA*	Feruloylquinic lactone-GlcUA*	526	PHUB002461	Host metabolism	P	[coffee CGAs]	(Gómez-Juaristi et al., 2018)
28	1,5-Diferuloylquinic acid	1,5-Diferuloylquinic acid	544	PHUB002470	Host-gut microbiota co-metabolite	P	[1,5-dicaffeoylquinic acid]	(Gu et al., 2007; Liu et al., 2010)
29	5-Feruloylquinic acid-4′-GlcUA	5-Feruloylquinic acid-4′-GlcUA	544	PHUB002476	Host-gut microbiota co-metabolite Host metabolism	P	[coffee CGAs]	(Mills et al., 2017)
Coumaroylquinic acid derivatives								
30	Coumaroylquinic acid	Coumaroylquinic acid	338	PHUB002460	Unchanged	P, U	[yerba mate, coffee coumaroylquinic acid]	(Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018)
31	Dihydrocoumaroylquinic acid	Dihydrocoumaroylquinic acid	340	PHUB002454	Gut microbiota metabolite	P, U	[coffee, yerba mate CGAs]	(Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018)
32	Coumaroylquinic lactone-GlcUA*	Coumaroylquinic lactone-GlcUA*	496	PHUB002466	Host-gut microbiota co-metabolite	P, U	[coffee CGAs]	(Mena et al., 2021)
C_6-C_3 unsaturated cinnamic acids								
33	t-Cinnamic acid	Cinnamic acid	148	PHUB000586	Gut microbiota metabolite Unchanged	P	[cranberry, blueberry (poly)phenols]; [herb cinnamic acid]	(Feliciano et al., 2017; Jeong et al., 2018; Zhong et al., 2017)
34	m-Coumaric acid	3′-Hydroxycinnamic acid	164	PHUB000588	Gut microbiota metabolite	P, U	[cranberry (poly)phenols]	(Feliciano et al., 2017, 2016; Heiss et al., 2022)
35	o-Coumaric acid	2′-Hydroxycinnamic acid	164	PHUB000589	Host metabolism Gut microbiota metabolite	P, U	[cranberry (poly)phenols]; [herb coumarin]	(Feliciano et al., 2017, 2016; Gasparetto et al., 2015; Heiss et al., 2022)
36	p-Coumaric acid	4′-Hydroxycinnamic acid	164	PHUB000590	Gut microbiota metabolite Host metabolism Unchanged	P, U	[tomato, blueberry, cranberry, propolis, grape (poly)phenols]; [oat 4′-hydroxycinnamic acid]; [coffee CGAs]	(Feliciano et al., 2017, 2016; Heiss et al., 2022; Martínez-Huélamo et al., 2016; Mills et al., 2017; Schär et al., 2018; Stalmach et al., 2012; Yamaga et al., 2021; Zhong et al., 2017)
37	Caffeic acid	3′,4′-Dihydroxycinnamic acid	180	PHUB000574	Unchanged Gut microbiota metabolite Host metabolism	P, U	[apples, herb, red wine 3′,4′-dihydroxycinnamic acid]; [tomato, rosemary tea, blueberry, cranberry, grape, artichoke, olive oil (poly)phenols]; [coffee CGAs]	(Achour et al., 2021; Bitsch et al., 2001; Domínguez-Fernández et al., 2022; Feliciano et al., 2017, 2016; Gómez-Juaristi et al., 2018; Heiss et al., 2022; Martínez-Huélamo et al., 2016; Martínez-Huélamo et al., 2015; Mills et al., 2017; Simonetti et al., 2001; Stalmach et al., 2012; Suárez et al., 2011; Tulipani et al., 2012; Zhong et al., 2017, 2016)
38	Ferulic acid	4′-Hydroxy-3′-methoxycinnamic acid	194	PHUB000608	Unchanged Host-gut microbiota co-metabolite Host metabolism	P, U, S	[wheat, herb, oat 4′-hydroxy-3′-methoxycinnamic acid]; [blueberry, tomato, cranberry, wheat, grape, olive oil, rosemary tea, artichoke (poly)phenols]; [cyanidin-3-glucoside]; [OF]; [coffee CGAs]; [RA]	(Achour et al., 2021; Domínguez-Fernández et al., 2022; Feliciano et al., 2017; Ferrars et al., 2014; Gamel et al., 2019; Gómez-Juaristi et al., 2018; Heiss et al., 2022; Jeong et al., 2021; Lang et al., 2013; Ludwig et al., 2015; Martínez-Huélamo et al., 2016; Martínez-Huélamo et al., 2015; Mills et al., 2017; Pereira-Caro et al., 2020, 2017; Schär et al., 2018; Stalmach et al., 2012; Suárez et al., 2011; Tulipani et al., 2012; Vitaglione et al., 2012; Zhong et al., 2017)

39	isoFerulic acid	3′-Hydroxy-4′-methoxycinnamic acid	194	PHUB000622	Host-gut microbiota co-metabolite Host metabolism	P, U	[coffee, yerba mate CGAs]; [tomato, rosemary tea, blueberry, cranberry, artichoke (poly)phenols]	(Achour et al., 2021; Domínguez-Fernández et al., 2022; Feliciano et al., 2017, 2016; Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018; Heiss et al., 2022; Lang et al., 2013; Martínez-Huélamo et al., 2016; Mills et al., 2017; Scherbl et al., 2017; Zhong et al., 2017)
38 or 39	Hydroxymethoxycinnamic acid	Hydroxymethoxycinnamic acid	194	PHUB002462	Host-gut microbiota co-metabolite	S, U	[cyanidin-3-glucoside]	(Ferrars et al., 2014)
40	Dimethylcaffeic acid	3′,4′-Dimethoxycinnamic acid	208	PHUB002439	Host metabolism	P	[coffee CGAs]	(Farrell et al., 2012; Gómez-Juaristi et al., 2018; Mills et al., 2017; Scherbl et al., 2017)
41	Sinapic acid	3′,5′-Dimethoxy-4′-hydroxycinnamic acid	224	PHUB000638	Host-gut microbiota co-metabolite	P, U	[cranberry (poly)phenols]	(Feliciano et al., 2017, 2016; Heiss et al., 2022)
42	Coumaric acid-4′-S	Cinnamic acid-4′-S	244	PHUB001199	Host-gut microbiota co-metabolite Host metabolism	P, U	[coffee CGAs]; [OF]; [tomato, grape, olive oil, artichoke (poly)phenols]	(Domínguez-Fernández et al., 2022; Gómez-Juaristi et al., 2018; Martínez-Huélamo et al., 2016; Mena et al., 2021; Pereira-Caro et al., 2017; Stalmach et al., 2012; Suárez et al., 2011)
43	Feruloylglycine	3′-Methoxy-4′-hydroxycinnamoyl-glycine	251	PHUB001173	Host metabolism Host-gut microbiota co-metabolite	P, U	[coffee, yerba mate CGAs]; [rosemary tea, red grape pomace, grape, orange, oat (poly)phenols]; [wheat 4′-hydroxy-3′-methoxycinnamic acid]	(Achour et al., 2021; Bresciani et al., 2016; Castello et al., 2020, 2018; Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018; Kerimi et al., 2020; Lang et al., 2013; Mena et al., 2021, 2019; Schär et al., 2018; Stalmach et al., 2014, 2012, 2009)
44	isoFeruloylglycine	4′-Methoxy-3′-hydroxycinnamoyl-glycine	251	PHUB002440	Host-gut microbiota co-metabolite	P, U	[coffee, yerba mate CGAs]; [rosemary tea (poly)phenols]	(Achour et al., 2021; Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018)
45	Caffeic acid-3′-S	4′-Hydroxycinnamic acid-3′-S	260	PHUB001594	Host metabolism Host-gut microbiota co-metabolite	P, U	[coffee, yerba mate CGAs]; [RA]; [grape, artichoke (poly)phenols]; [OF]; [coffee HCAs]	(Domínguez-Fernández et al., 2022; Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018; Ludwig et al., 2015; Mena et al., 2021; Mills et al., 2017; Pereira-Caro et al., 2017; Scherbl et al., 2017; Stalmach et al., 2009, 2014, 2012; Wong et al., 2010)
46	Caffeic acid-4′-S	3′-Hydroxycinnamic acid-4′-S	260	PHUB001918	Host-gut microbiota co-metabolite Host metabolism	P, U	[coffee CGAs]; [coffee HCAs]; [grape, artichoke (poly)phenols]	(Domínguez-Fernández et al., 2022; Mills et al., 2017; Scherbl et al., 2017; Stalmach et al., 2012, 2009; Wong et al., 2010)
45 or 46	Caffeic acid-S*	Hydroxycinnamic acid S*	260	PHUB002438	Host-gut microbiota co-metabolite	P, U	[tomato, olive oil, oat (poly)phenols]; [coffee CGAs]	(Martínez-Huélamo et al., 2016; Mena et al., 2019; Schär et al., 2018; Suárez et al., 2011)
45 and 46	Caffeic acid-S‡	-	260	-	Host-gut microbiota co-metabolite	U	[coffee CGAs]	(Stalmach et al., 2014)
47	Ferulic acid-4′-S	3′-Methoxycinnamic acid-4′-S	274	PHUB001171	Host-gut microbiota co-metabolite Host metabolism	P, U	[wheat 4′-hydroxy-3′-methoxycinnamic acid]; [cranberry, grape, artichoke, orange, olive oil, rosemary tea, red grape pomace, tomato (poly)phenols]; [hazelnut flavan-3-ols]; [OF]; [coffee, yerba mate CGAs]; [RA]; [coffee HCAs]	(Achour et al., 2021; Bresciani et al., 2016; Castello et al., 2020, 2018; Domínguez-Fernández et al., 2022; Feliciano et al., 2017, 2016; Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018; Heiss et al., 2022; Kerimi et al., 2020; Lang et al., 2013; Ludwig et al., 2015; Martínez-Huélamo et al., 2016; Mena et al., 2021, 2019; Mills et al., 2017; Mocciaro et al., 2019; Pereira-Caro et al., 2020, 2017; Rodriguez-Mateos et al., 2016a; Scherbl et al., 2017; Stalmach et al., 2014, 2012, 2009; Suárez et al., 2011; Wong et al., 2010)
48	isoFerulic acid-3′-S	4′-Methoxycinnamic acid-3′-S	274	PHUB001212	Host metabolism Host-gut microbiota co-metabolite	P, U	[coffee CGAs]; [RA]; [coffee HCAs]; [rosemary tea, cranberry, grape, artichoke, oat (poly)phenols]	(Achour et al., 2021; Domínguez-Fernández et al., 2022; Feliciano et al., 2017, 2016; Gómez-Juaristi et al., 2018; Heiss et al., 2022; Lang et al., 2013; Ludwig et al., 2015; Mena et al., 2021; Mills et al., 2017; Schär et al., 2018; Stalmach et al., 2014, 2012, 2009; Wong et al., 2010)
47 or 48	Methoxycinnamic acid-S *	(Iso)ferulic acid-S*	274	PHUB001964	Host-gut microbiota co-metabolite	U	[oat (poly)phenols]	(Schär et al., 2018)

49	Sinapic acid-S	3′,5′-Dimethoxycinnamic acid-4′-S	304	PHUB001431	Host-gut microbiota co-metabolite Host metabolism	U	[oat, red grape pomace (poly)phenols]; [wheat 4′-hydroxy-3′-methoxycinnamic acid]	(Bresciani et al., 2016; Castello et al., 2018; Schär et al., 2018)
50	Trimethoxycinnamic acid-S*	Trimethoxycinnamic acid- S*	318	PHUB002465	Host-gut microbiota co-metabolite	P, U	[coffee CGAs]	(Mena et al., 2021)
51	m-Coumaric acid-3′-GlcUA	Cinnamic acid-3′-GlcUA	340	PHUB001194	Host metabolism	P	[coffee CGAs]	(Mills et al., 2017)
52	p-Coumaric acid-4′-GlcUA	Cinnamic acid-4′-GlcUA	340	PHUB001198	Host metabolism Host-gut microbiota co-metabolite	P, U	[coffee CGAs]; [tomato, cranberry (poly)phenols]	(Gómez-Juaristi et al., 2018; Heiss et al., 2022; Martínez-Huélamo et al., 2016; Mills et al., 2017)
53	Caffeic acid-3′-GlcUA	4′-Hydroxycinnamic acid-3′-GlcUA	356	PHUB001916	Host-gut microbiota co-metabolite Host metabolism	P, U	[coffee CGAs]; [cranberry, artichoke (poly)phenols]	(Domínguez-Fernández et al., 2022; Feliciano et al., 2017, 2016; Heiss et al., 2022; Mena et al., 2021; Mills et al., 2017)
54	Caffeic acid-4′-GlcUA	3′-Hydroxycinnamic acid-4′-GlcUA	356	PHUB001917	Host-gut microbiota co-metabolite Host metabolism	P, U	[coffee CGAs]; [cranberry, artichoke (poly)phenols]	(Domínguez-Fernández et al., 2022; Feliciano et al., 2017, 2016; Heiss et al., 2022; Mena et al., 2021, 2019; Mills et al., 2017; Rodriguez-Mateos et al., 2016a)
53 or 54	Caffeic acid-GlcUA*	Hydroxycinnamic acid GlcUA*	356	PHUB002437	Host-gut microbiota co-metabolite Host metabolism	P, U	[tomato (poly)phenols]	(Martínez-Huélamo et al., 2016; Martínez-Huélamo et al., 2015; Tulipani et al., 2012)
55	Ferulic acid-4′-GlcUA	3′-Methoxycinnamic acid-4′-GlcUA	370	PHUB001170	Host-gut microbiota co-metabolite Host metabolism	P, U	[blueberry, tomato, cranberry, artichoke, orange, olive oil, rosemary tea, red grape pomace, oat (poly)phenols]; [hazelnut flavan-3-ols]; [OF]; [coffee, yerba mate CGAs]; [RA]	(Achour et al., 2021; Castello et al., 2020, 2018; Domínguez-Fernández et al., 2022; Feliciano et al., 2017, 2016; Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018; Heiss et al., 2022; Lang et al., 2013; Ludwig et al., 2015; Martínez-Huélamo et al., 2016; Martínez-Huélamo et al., 2015; Mena et al., 2021; Mills et al., 2017; Mocciaro et al., 2019; Pereira-Caro et al., 2020, 2017; Schär et al., 2018; Scherbl et al., 2017; Suárez et al., 2011; Tulipani et al., 2012; Zhong et al., 2017)
56	isoFerulic acid-3′-GlcUA	4′-Methoxycinnamic acid-3′-GlcUA	370	PHUB001432	Host metabolism Host-gut microbiota co-metabolite	P, U	[coffee, yerba mate CGAs]; [RA]; [OF]; [coffee HCAs]; [hazelnut flavan-3-ols]; [rosemary tea, cranberry, grape, artichoke, orange, oat (poly)phenols]	(Achour et al., 2021; Castello et al., 2020; Domínguez-Fernández et al., 2022; Feliciano et al., 2017, 2016; Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018; Heiss et al., 2022; Ludwig et al., 2015; Mena et al., 2019, 2021; Mills et al., 2017; Mocciaro et al., 2019; Pereira-Caro et al., 2020, 2017; Schär et al., 2018; Scherbl et al., 2017; Stalmach et al., 2014, 2012, 2009; Wong et al., 2010)
Phenylpropanoic acids								
57	Dihydro-m-coumaric acid	3-(3′-Hydroxyphenyl)propanoic acid	166	PHUB001047	Gut microbiota metabolite	P	[coffee CGAs]	(Scherbl et al., 2017)
58	Dihydrocoumaric acid	3-(4′-Hydroxyphenyl)propanoic acid	166	PHUB001177	Gut microbiota metabolite	U	[coffee, yerba mate CGAs]	(Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018)
59	Dihydrocaffeic acid	3-(3′,4′-Dihydroxyphenyl)propanoic acid	182	PHUB000604	Gut microbiota metabolite	P, U	[coffee, yerba mate CGAs];	(Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018; Scherbl et al., 2017; Stalmach et al., 2014, 2009)
60	Dihydroferulic acid	3-(4′-Hydroxy-3′-methoxyphenyl)propanoic acid	196	PHUB001168	Host-gut microbiota co-metabolite	P, U	[coffee, yerba mate CGAs]; [coffee HCAs	(Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018; Kerimi et al., 2020; Lang et al., 2013; Scherbl et al., 2017; Stalmach et al., 2014, 2009; Wong et al., 2010)
61	Dihydroisoferulic acid	3-(3′-Hydroxy-4′-methoxyphenyl)propanoic acid	196	PHUB001433	Host-gut microbiota co-metabolite	P	[coffee, yerba mate CGAs]	(Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018; Scherbl et al., 2017)
62	Dihydrodimethoxycinnamic acid	3′,4′-Dimethoxyphenylpropanoic	210	PHUB002451	Host-gut microbiota co-metabolite	P	[coffee CGAs]	(Gómez-Juaristi et al., 2018)

		acid						
63	Dihydrocoumaric acid-S	3-(Phenyl)propanoic acid-4′-S	246	PHUB002227	Host-gut microbiota co-metabolite	U	[coffee, yerba mate CGAs]	(Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018; Mena et al., 2021)
64	Dihydro-m-coumaric acid-3′-S	3-(Phenyl)propanoic acid-3′- S	246	PHUB002286	Host-gut microbiota co-metabolite	P, U	[coffee CGAs]	(Mena et al., 2021; Scherbl et al., 2017)
65	Dihydrocaffeic acid-4′-S	3-(3′-Hydroxyphenyl)propanoic acid-4′-S	262	PHUB001206	Host-gut microbiota co-metabolite	P, U	[coffee, yerba mate CGAs];	(Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018; Mena et al., 2021)
66	Dihydrocaffeic acid-3′-S	3-(4′-Hydroxyphenyl)propanoic acid-3′-S	262	PHUB001588	Host-gut microbiota co-metabolite	P, U	[coffee, yerba mate CGAs]; [coffee HCAs]; [wheat 4′-hydroxy-3′-methoxycinnamic acid]	(Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018; Kerimi et al., 2020; Mena et al., 2021; Scherbl et al., 2017; Stalmach et al., 2014, 2009; Wong et al., 2010)
65 and 66	Dihydrocaffeoyl- S‡	-	262	-	Host-gut microbiota co-metabolite	P	[coffee CGAs]	(Lang et al., 2013)
67	Dihydroferulic acid-4′-S	3-(3′-Methoxyphenyl)propanoic acid-4′-S	276	PHUB001436	Host-gut microbiota co-metabolite	P, U	[coffee, yerba mate CGAs]; [coffee HCAs]; [wheat 4′-hydroxy-3′-methoxycinnamic acid]	(Bresciani et al., 2016; Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018; Kerimi et al., 2020; Lang et al., 2013; Mena et al., 2021; Scherbl et al., 2017; Stalmach et al., 2014, 2009; Wong et al., 2010)
68	Dihydroisoferulic acid-3′- S	3-(4′-Methoxyphenyl)propanoic acid-3′-S	276	PHUB001592	Host-gut microbiota co-metabolite	P, U	[coffee, yerba mate CGAs]	(Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018; Mena et al., 2021)
69	Dihydrocoumaric acid-GlcUA	3-(Phenyl)propanoic acid-4′-GlcUA	342	PHUB001586	Host-gut microbiota co-metabolite	P, U	[coffee, yerba mate CGAs]	(Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018; Mena et al., 2021)
70	3-(3′-Hydroxyphenyl)propionic acid-GlcUA	3-(Phenyl)propanoic acid-3′-GlcUA	342	PHUB002463	Host-gut microbiota co-metabolite	U	[coffee CGAs]	(Mena et al., 2021)
71	Dihydrocaffeic acid-3′-GlcUA	3-(4′-Hydroxyphenyl)propanoic acid-3′-GlcUA	358	PHUB001204	Host-gut microbiota co-metabolite	U	[coffee, yerba mate CGAs]; [coffee HCAs]	(Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018; Mena et al., 2021; Stalmach et al., 2009; Wong et al., 2010)
72	Dihydroisoferulic acid-3′-GlcUA	3-(4′-Methoxyphenyl)propanoic acid-3′-GlcUA	372	PHUB001434	Host-gut microbiota co-metabolite	P, U	[coffee, yerba mate CGAs];	(Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018; Mena et al., 2021; Scherbl et al., 2017; Stalmach et al., 2009)
73	Dihydroferulic acid-4′-GlcUA	3-(3′-Methoxyphenyl)propanoic acid-4′-GlcUA	372	PHUB001435	Host-gut microbiota co-metabolite	P, U	[coffee, yerba mate CGAs]; [coffee HCAs]; [wheat 4′-hydroxy-3′-methoxycinnamic acid]	(Bresciani et al., 2016; Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018; Lang et al., 2013; Mena et al., 2021; Scherbl et al., 2017; Stalmach et al., 2009; Wong et al., 2010)
Benzoic and benzaldehyde derivatives								
74	4-Hydroxybenzaldheyde	4-Hydroxybenzaldheyde	122	PHUB000542	Host-gut microbiota co-metabolite	P	[coffee CGAs]	(Mena et al., 2021)
75	Benzoic acid-4- S	Benzoic acid-4-S	218	PHUB001583	Host-gut microbiota co-metabolite	U	[coffee CGAs]	(Mena et al., 2021)
76	Vanilloylglycine	3-Methoxy-4-hydroxybenzoyl-glycine	225	PHUB001180	Host-gut microbiota co-metabolite	U	[coffee CGAs]	(Kerimi et al., 2020)
77	Protocatechuic acid- S‡	-	234	-	Host-gut microbiota co-metabolite	P, U	[coffee CGAs]	(Mena et al., 2021)
78	Vanillic acid-S	3-Methoxybenzoic acid-4- S	248	PHUB001294	Host-gut microbiota co-metabolite	U	[coffee CGAs]	(Mena et al., 2021)
79	Syringic acid-S	3,5-Dimethoxy-benzoic acid-4-S	278	PHUB002464	Host-gut microbiota co-metabolite	U	[coffee CGAs]	(Mena et al., 2021)
80	Benzoic acid-4-GlcUA	Benzoic acid-4-GlcUA	314	PHUB001582	Host-gut microbiota co-metabolite	U	[coffee CGAs]	(Mena et al., 2021)
81	Protocatechuic acid-3-GlcUA	4-Hydroxybenzoic acid-3-GlcUA	330	PHUB001288	Host-gut microbiota co-metabolite	P	[coffee CGAs]	(Mena et al., 2021)
82	Isovanillic acid-GlcUA	4-Methoxybenzoic acid-3-GlcUA	344	PHUB001277	Host-gut microbiota co-metabolite	U	[coffee CGAs]	(Mena et al., 2021)

83	Vanillic acid-GlcUA	3-Methoxybenzoic acid-4-GlcUA	344	PHUB001293	Host-gut microbiota co-metabolite	P, U	[coffee CGAs]	(Mena et al., 2021)
Catechol derivatives								
84	Catechol-S*	Hydroxybenzene-S*	190	PHUB002467	Host-gut microbiota co-metabolite	P, U	[coffee CGAs]	(Lang et al., 2013; Mena et al., 2021)
85	Methylcatechol-S*	Methoxybenzene-S*	204	PHUB002468	Host-gut microbiota co-metabolite	P, U	[coffee CGAs]	(Mena et al., 2021)
86	Guaiacol-S	2-Methoxybenzene-1-S	204	PHUB002488	Host-gut microbiota co-metabolite	P	[coffee CGAs]	(Lang et al., 2013)
87	Methoxypyrogallol-S*	Hydroxy-methoxybenzene-S*	220	PHUB001969	Host-gut microbiota co-metabolite	P, U	[coffee CGAs]	(Mena et al., 2021)
88	Catechol-GlcUA*	Hydroxybenzene-GlcUA*	286	PHUB002195	Host-gut microbiota co-metabolite	P	[coffee CGAs]	(Lang et al., 2013)
89	Guaiacol-GlcUA	2-Methoxybenzene-1-GlcUA	300	PHUB002489	Host-gut microbiota co-metabolite	P	[coffee CGAs]	(Lang et al., 2013)
Hippuric acids								
90	Hippuric Acid	Hippuric Acid	179	PHUB001174	Host-gut microbiota co-metabolite	U	[coffee CGAs]	(Mena et al., 2021)
Miscellaneous								
91	Drupanin	Drupanin	232	PHUB002481	Unchanged	P	[propolis drupanin]	(Yamaga et al., 2021)
92	3,4-Dihydroxy-5-prenyl cinnamic acid	3,4-Dihydroxy-5-prenyl cinnamic acid	248	PHUB002487	Unchanged	P	[propolis 3,4-dihydroxy-5-prenyl cinnamic acid]	(Yamaga et al., 2021)
93	Culifolin	Culifolin	298	PHUB002485	Unchanged	P	[propolis culifolin]	(Yamaga et al., 2021)
94	2,2-Dimethylchromene-6-propenoic acid	2,2-Dimethylchromene-6-propenoic acid	298	PHUB002486	Unchanged	P	[propolis 2,2-Dimethylchromene-6-propenoic acid]	(Yamaga et al., 2021)
95	Artepillin C	Artepillin C	300	PHUB002480	Unchanged	P	[propolis artepillin C]	(Yamaga et al., 2021)
96	Capillartemisin A	Capillartemisin A	316	PHUB002482	Unchanged	P	[propolis capillartemisin A]	(Yamaga et al., 2021)
97	Rosmarinic acid	Rosmarinic acid	360	PHUB000634	Unchanged	S, U	[rosemary tea, herb rosmarinic acid]	(Achour et al., 2021; Noguchi-shinohara et al., 2015)
98	Dimethyl-rosmarinic acid*	Dimethyl-rosmarinic acid*	388	PHUB002448	Host metabolism	P	[rosemary tea (poly)phenols]	(Achour et al., 2021)
99	Drupanin-4-GlcUA	Drupanin-4-GlcUA	408	PHUB002484	Host metabolism	P	Drupanin from propolis	(Yamaga et al., 2021)
100	Methyl-rosmarinic acid-S*	Methoxyrosmarinic acid-S*	454	PHUB002450	Host metabolism	U	[rosemary tea (poly)phenols]	(Achour et al., 2021)
101	Dimethyl-rosmarinic acid-S*	Dimethoxyrosmarinic acid-S*	468	PHUB002447	Host metabolism	U	[rosemary tea (poly)phenols]	(Achour et al., 2021)
102	Artepillin C-4-GlcUA	Artepillin C-4-GlcUA	476	PHUB002483	Host metabolism	P	Artepillin C from propolis	(Yamaga et al., 2021)
103	Rosmarinic acid-GlcUA*	Rosmarinic acid-GlcUA*	536	PHUB002449	Host metabolism	P	[rosemary tea (poly)phenols]	(Achour et al., 2021)
104	Methyl-rosmarinic acid-GlcUA*	Methoxyrosmarinic acid-GlcUA*	550	PHUB002446	Host metabolism	P, U	[rosemary tea (poly)phenols]	(Achour et al., 2021)
105	Dimethyl-rosmarinic acid-GlcUA*	Dimethoxyrosmarinic acid-GlcUA*	564	PHUB002445	Host metabolism	P, U	[rosemary tea (poly)phenols]	(Achour et al., 2021)

*symbol: when the position of the conjugation is unknown, ‡symbol: this compound is reported as the sum of isomers (in this case, no PhytoHub ID was created), GlcUA: glucuronide; S: sulfate; CGAs: chlorogenic acids, HCAs: hydroxycinnamic acids; RA: raspberry anthocyanins; OF: orange flavanones

This include 32 acyl-quinic acids [among which caffeoylquinic acids (n=17), feruloylquinic acids (n=12), and coumaroylquinic acids (n=3)], 24 C_6-C_3 unsaturated cinnamic acids [derivatives of (i) 3′,4′-dihydroxycinnamic acid (n=6), (ii) hydroxycinnamic acid (*aka* coumaric acid) (6), (iii) 4′-hydroxy-3′-methoxycinnamic acid (4), (iv) 3′-hydroxy-4′-methoxycinnamic acid (*aka* isoFerulic acid) (4), (v) 3′,5′-dimethoxy-4′-hydroxycinnamic acid (3), (vi) cinnamic acid (1)], 17 phenylpropanoic acids [derivatives of (i) 3-(hydroxyphenyl)propanoic acid (*aka* dihydrocoumaric acid) (n=6), (ii) 3-(3′,4′-dihydroxyphenyl)propanoic acid (5), (iii) 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid (3), 3-(3′-hydroxy-4′-methoxyphenyl)propanoic acid (*aka* dihydroisoferulic acid) (3)], 15 miscellaneous [among which derivatives of rosmarinic acid (n=7)], 9 benzoic acids, 6 catechols, 1 benzaldehyde and 1 hippuric acid. Ranking blood and urinary compounds accordingly to their metabolic origin, a total of 41 host-gut microbiota metabolites [among which 15 phenylpropanoic acids, 9 benzoic acids, 6 acyl-quinic acids, 6 catechols, 3 C_6-C_3 unsaturated cinnamic acids, 1 benzaldehyde, 1 hippuric acid], 20 host metabolites [10 acyl-quinic acids, 8 miscellaneous, 2 C_6-C_3 unsaturated cinnamic acids], 19 unchanged compounds [8 acyl-quinic acids, 7 miscellaneous, 4 C_6-C_3 unsaturated cinnamic acids] and 8 gut microbiota metabolites [4 acyl-quinic acids, 3 phenylpropanoic acids, 1 C_6-C_3 unsaturated cinnamic acid] were found after intake of HCAs and other phytochemicals (Table 1 and Figure 1).

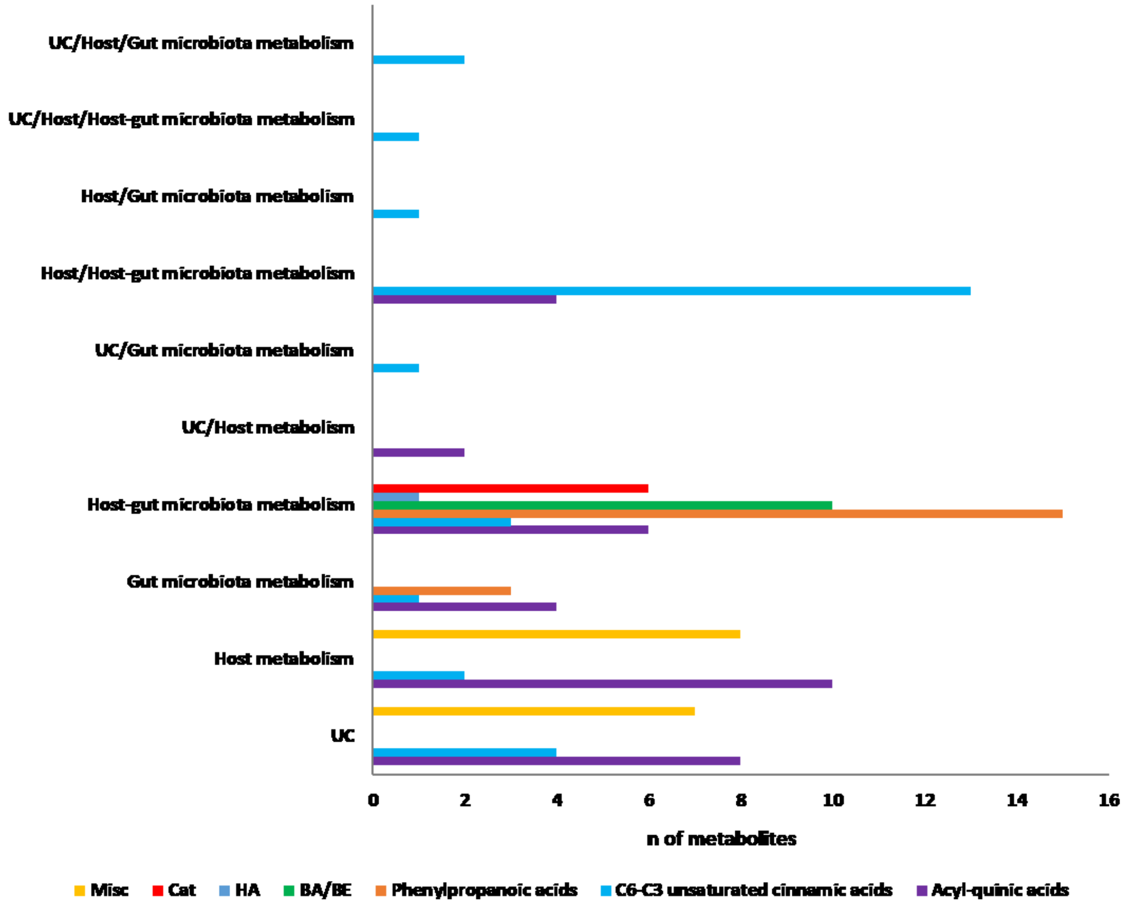


Figure 1. Number of acyl-quinic acids, C_6-C_3 unsaturated cinnamic acids and their metabolites quantified in blood/urine samples following HCA intake by healthy humans and ranked based on their metabolic origin. C_6-C_3 unsaturated cinnamic acids include compounds quantified in biofluids after consumption of other phytochemicals. Unchanged compounds (UC): indicates when the native HCA did not undergo any metabolic step following its ingestion, host metabolism: when the compound derived from a biotransformation by small intestine, hepatic or renal phase 1 or phase 2 enzymes, gut microbiota metabolism: when the compound derived from HCA metabolism through gut microbiota activity, host-gut microbiota metabolism: when the compound derived from HCA metabolism through gut microbiota activity and further conjugation by a phase 2 enzyme. BA/BE: benzoic acids and benzaldehyde derivatives; HA: hippuric acids; Cat:

catechols; Misc: miscellaneous. Acyl-quinic acids include caffeoylquinic acids, feruloylquinic acids, and coumaroylquinic acids. C_6-C_3 unsaturated cinnamic acids include derivatives of 3′,4′-dihydroxycinnamic acid (*aka* caffeic acid), 4′-hydroxy-3′-methoxycinnamic acid (*aka* ferulic acid), 3′-hydroxy-4′-methoxycinnamic acid (*aka* isoFerulic acid), 3′,5′-dimethoxy-4′-hydroxycinnamic acid (*aka* sinapic acid), and hydroxycinnamic acid (*aka* coumaric acid) and cinnamic acid. Phenylpropanoic acids include derivatives of 3-(3′,4′-dihydroxyphenyl)propanoic acid (*aka* dihydrocaffeic acid), 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid (*aka* dihydroferulic acid), 3-(3′-hydroxy-4′-methoxyphenyl)propanoic acid (*aka* dihydroisoferulic acid), and 3-(hydroxyphenyl)propanoic acid (*aka* dihydrocoumaric acid). Misc class include data for unknown forms of methoxycinnamic acid sulfate and hydroxymethoxycinnamic acid, derivatives of rosmarinic acid, artepillin C, drupanin, capillartemisin A, 2,2-dimethylchromene-6-propenoic acid, 3,4-dihydroxy-5-prenyl cinnamic acid and culifolin.

Interestingly, 17 metabolites [13 C_6-C_3 unsaturated cinnamic acids, 4 acyl-quinic acids] attained biphasic responses showing both host and host-gut microbiota metabolism (Figure 1). Out of the 105 quantified metabolites, 51 of them were detected in both plasma/serum and urine samples, followed by those recovered only in plasma/serum (n=32) or urine (n=22) fractions (Table 1). Taking into account circulating compounds strictly related to HCA intake, coffee HCA consumption resulted in up to 82 metabolites [mainly in the form of acyl-quinic acids (n of metabolites=23) and C6-C3 unsaturated cinnamic acids (18)], followed by yerba mate (30), artichoke (16), cereals (i.e. wheat, oat) (12), propolis (8), rosemary tea (7), pure HCAs (3), herbs (2), while unchanged C_6-C_3 unsaturated cinnamic acids were recovered after intake of berries (2), apple (1), tomato (1), and grape products (1) (Figure 2).

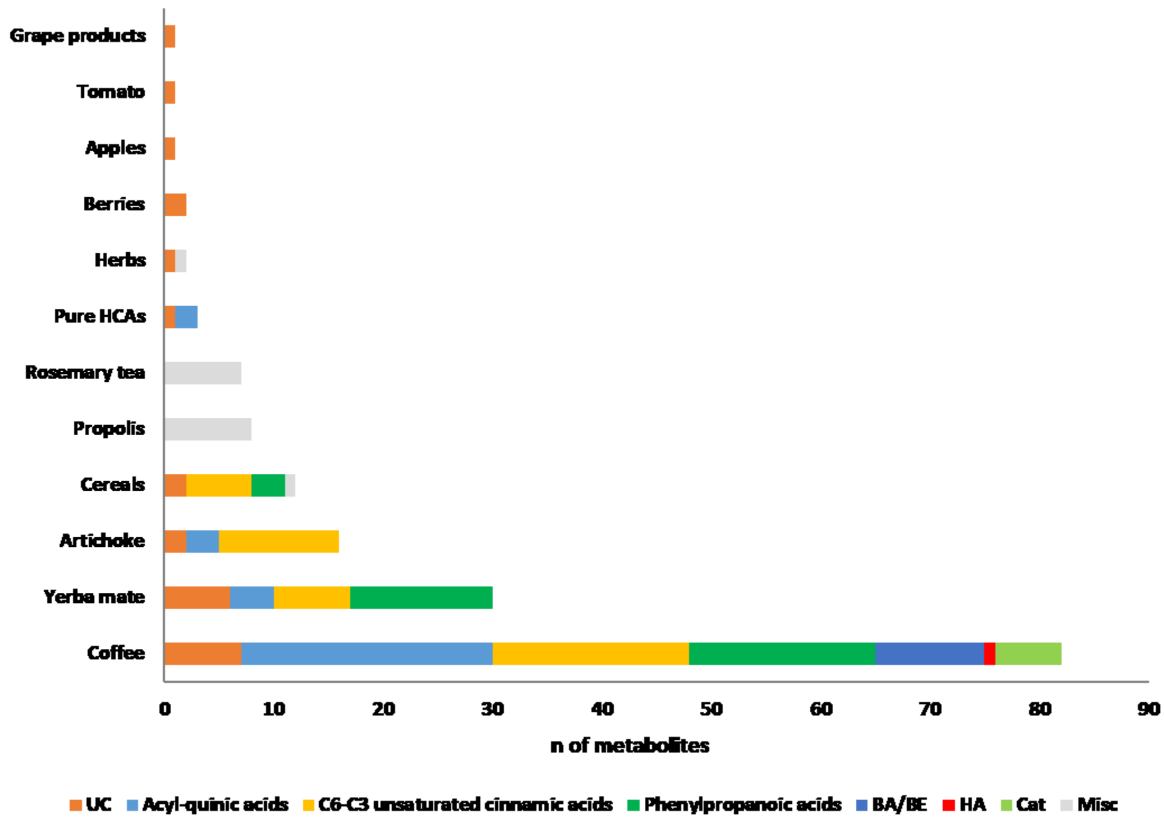


Figure 2. Number of circulating compounds quantified in blood/urine samples following intake of the different HCA sources by healthy humans. UC: unchanged acyl-quinic and C_6-C_3 unsaturated cinnamic acids; BA/BE: benzoic acids and benzaldehyde derivatives; HA: hippuric acids; Cat: catechols; Misc: miscellaneous. Acyl-quinic acids include caffeoylquinic acids, feruloylquinic acids, and coumaroylquinic acids. C_6-C_3 unsaturated cinnamic acids include derivatives of 3′,4′-dihydroxycinnamic acid (*aka* caffeic acid), 4′-hydroxy-3′-methoxycinnamic acid (*aka* ferulic acid), 3′-hydroxy-4′-methoxycinnamic acid (*aka* isoFerulic acid), 3′,5′-dimethoxy-4′-hydroxycinnamic acid (*aka* sinapic acid), and hydroxycinnamic acid (*aka* coumaric acid) and cinnamic acid. Phenylpropanoic acids include derivatives of 3-(3′,4′-dihydroxyphenyl)propanoic acid (*aka* dihydrocaffeic acid), 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid (*aka*

dihydroferulic acid), 3-(3′-hydroxy-4′-methoxyphenyl)propanoic acid (*aka* dihydroisoferulic acid), and 3-(hydroxyphenyl)propanoic acid (*aka* dihydrocoumaric acid). Misc class include data for unknown forms of methoxycinnamic acid sulfate and hydroxymethoxycinnamic acid, derivatives of rosmarinic acid, artepillin C, drupanin, capillartemisin A, 2,2-dimethylchromene-6-propenoic acid, 3,4-dihydroxy-5-prenyl cinnamic acid and culifolin.

Some C_6-C_3 unsaturated cinnamic acids [including derivatives of 3′,4′-dihydroxycinnamic acid, 4′-hydroxy-3′-methoxycinnamic acid, 3′-hydroxy-4′-methoxycinnamic acid, 3′,5′-dimethoxy-4′-hydroxycinnamic acid, hydroxycinnamic acid, cinnamic acid] were also found metabolites after consumption of other phytochemicals from berries (n of metabolites=18), grape products (11), orange (10), tomato (10), rosemary tea (9), olive oil (6), nuts (3), pure compounds (2), herb (1), propolis (1), and cereals (1) (Table 1). More chemical data for each metabolite described in Table 1 is reported in the database PhytoHub (www.phytohub.eu) . Circulating metabolites were grouped based on their metabolic pathway and chemical structure in up to 16 classes, namely unchanged acyl-quinic acids and C_6-C_3 unsaturated cinnamic acids, aglycones and phase 2 conjugates of acyl-quinic acids (n=3 classes) [i.e. (i) caffeoylquinic acids, (ii) feruloylquinic acids, (iii) coumaroylquinic acids], C_6-C_3 unsaturated cinnamic acids (n=5 classes) [i.e. derivatives of (i) 3′,4′-dihydroxycinnamic acid, (ii) 4′-hydroxy-3′-methoxycinnamic acid, (iii) 3′-hydroxy-4′-methoxycinnamic acid, (iv) 3′,5′-dimethoxy-4′-hydroxycinnamic acid, (v) hydroxycinnamic acid and cinnamic acid], phenylpropanoic acids (n=4 classes) [i.e. derivatives of (i) 3-(3′,4′-dihydroxyphenyl)propanoic acid, (ii) 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid, (iii) 3-(3′-hydroxy-4′-methoxyphenyl)propanoic acid, (iv) 3-(hydroxyphenyl)propanoic acid], benzoic acids and benzaldehydes and catechols. Miscellaneous included unchanged and phase 2 conjugates of rosmarinic acid, artepillin C, drupanin, capillartemisin A, 2,2-dimethylchromene-6-propenoic acid, 3,4-dihydroxy-5-prenyl cinnamic acid, culifolin, methoxycinnamic acid sulfate and hydroxymethoxycinnamic acid.

2.4 Nutrikinetics and urinary excretion of circulating compounds

2.4.1 Nutrikinetic parameters and urinary excretion of the different classes of metabolites

T_{max} and C_{max} values for circulating compounds, grouped by classes, are presented in **Table 2** and **Figure 3**. Derivatives of 3′-hydroxy-4′-methoxycinnamic acid had the highest C_{max} [648 ± 1591 (mean ± SD) and 70 (19-390) nmol/L (median (25th-75th percentile)) at 3.8 ± 3.5 and 1.9 (1.0-6.3) h (T_{max})], followed by derivatives of 4′-hydroxy-3′-methoxycinnamic acid [500 ± 1155 and 83 (30-310) nmol/L at 3.2 ± 2.8 and 1.6 (1.0-4.8) h], miscellaneous [396 ± 675 and 106 (46-306) nmol/L at 2.3 ± 3.0 and 1.7 (1.4-2.1) h], catechols [353 ± 654 and 110 (61-355) nmol/L at 3.3 ± 2.1 and 4.0 (0.8-5.0) h], derivatives of 3′,4′-dihydroxycinnamic acid [285 ± 905 and 37 (6-86) nmol/L at 2.7 ± 3.1 and 1.0 (1.0-3.3) h], and derivatives of 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid [206 ± 200 and 112 (89-358) nmol/L at 6.5 ± 1.4 and 6.3 (6.0-7.7) h].

Table 2. Nutrikinetic parameters and urinary excretion (% of intake) data for acyl-quinic acids, C_6-C_3 unsaturated cinnamic acids and their metabolites, grouped by classes based on their metabolic pathway and chemical structure, quantified in blood/urine samples following HCA intake by healthy humans. C_6-C_3 unsaturated cinnamic acids include compounds quantified in biofluids after consumption of other phytochemicals. Data are reported as mean ± SD (n indicates the number of biological values collected from literature for each parameter for the classes of circulating compounds). See table 1 for identity of unchanged acyl-quinic acids and C_6-C_3 unsaturated cinnamic acids. Box plot for C_{max} and T_{max} for the classes are reported in figure 3. Single values of urinary excretion (% of intake) for each class are described in figure 4.

Classes of circulating compounds	C_{max} (nmol/L)	C_{max} normalized ((nmol/L)/total µmol of ingested parental compounds)	T_{max} (h)	AUC (nmol/L*h)	AUC normalized ((nmol/L*h)/ total µmol of ingested parental compounds)	C_{avg} ((nmol/L*h)/n hours)	C_{avg} normalized ((nmol/L*h)/ total µmol of ingested parental compounds /n hours)	$t_{1/2}$ (h)	Urinary excretion (% of intake)
Unchanged acyl-quinic acids and C_6-C_3 unsaturated cinnamic acids	38.1 ± 114.2 (n=79)	5.5 ± 29.3 (n=79)	1.7 ± 1.9 (n=81)	70.0 ± 141.2 (n=60)	1.3 ± 7.6 (n=60)	3.7 ± 6.8 (n=60)	0.1 ± 0.3 (n=60)	3.0 ± 8.7 (n=24)	0.8 ± 1.5 (n=56)
Acyl-quinic acids									
CQAs*	16.6 ± 17.9 (n=15)	0.0 ± 0.0 (n=15)	1.0 ± 0.8 (n=15)	35.0 ± 22.2 (n=8)	0.1 ± 0.0 (n=8)	1.5 ± 0.9 (n=8)	0.0 ± 0.0 (n=8)	0.4 ± 0.1 (n=8)	0.8 ± 1.3 (n=23)
FQAs*	17.7 ± 20.2 (n=16)	0.0 ± 0.0 (n=16)	3.7 ± 3.3 (n=17)	122.3 ± 175.9 (n=8)	0.1 ± 0.2 (n=8)	6.0 ± 8.0 (n=8)	0.0 ± 0.0 (n=8)	3.8 (n=1)	0.2 ± 0.3 (n=7)
CoQAs*	56.6 ± 47.5 (n=6)	0.2 ± 0.2 (n=6)	7.4 ± 1.8 (n=7)	548.5 ± 631.0 (n=6)	1.7 ± 2.0 (n=6)	22.9 ± 26.3 (n=6)	0.1 ± 0.1 (n=6)	-	3.6 ± 4.6 (n=7)
C_6-C_3 unsaturated cinnamic acids									
CA*	284.6 ± 905.0 (n=65)	12.1 ± 52.0 (n=65)	2.7 ± 3.1 (n=61)	1462.5 ± 4481.1 (n=48)	50.6 ± 191.0 (n=48)	190.1 ± 627.8 (n=48)	10.0 ± 38.2 (n=48)	1.3 ± 0.4 (n=4)	1.9 ± 4.9 (n=59)
FA*	499.8 ± 1154.5 (n=118)	8.9 ± 49.7 (n=118)	3.2 ± 2.8 (n=112)	2043.4 ± 4312.9 (n=93)	34.5 ± 163.8 (n=93)	162.5 ± 546.1 (n=93)	6.0 ± 32.5 (n=93)	8.8 ± 8.3 (n=14)	8.4 ± 17.4 (n=87)
isoFA*	648.0 ± 1590.6 (n=52)	1.1 ± 2.5 (n=52)	3.8 ± 3.5 (n=52)	11249.3 ± 29164.0 (n=39)	8.8 ± 24.8 (n=39)	479.7 ± 1212.4 (n=39)	0.4 ± 1.0 (n=39)	-	0.6 ± 0.8 (n=48)
Sin*	41.6 ± 38.1 (n=8)	0.0 ± 0.0 (n=8)	4.2 ± 1.7 (n=8)	269.7 ± 240.8 (n=8)	0.2 ± 0.1 (n=8)	11.2 ± 10.0 (n=8)	0.0 ± 0.0 (n=8)	-	0.4 ± 0.3 (n=11)
Cou/Cinn*	163.2 ± 349.8 (n=34)	4.3 ± 17.4 (n=34)	3.5 ± 2.9 (n=33)	852.3 ± 1212.4 (n=29)	17.8 ± 65.8 (n=29)	80.4 ± 209.2 (n=29)	3.5 ± 13.2 (n=29)	2.1 ± 2.0 (n=2)	1.5 ± 2.8 (n=26)
Phenylpropanoic acids									
Di-CA*	167.4 ± 172.4 (n=22)	1.0 ± 1.3 (n=22)	6.6 ± 1.1 (n=22)	783.8 ± 1174.9 (n=22)	4.2 ± 6.4 (n=22)	36.3 ± 49.6 (n=22)	0.2 ± 0.4 (n=22)	2.2 ± 0.9 (n=8)	2.4 ± 3.4 (n=25)
Di-FA*	206.2 ± 199.6 (n=30)	1.6 ± 2.6 (n=30)	6.5 ± 1.4 (n=26)	1025.7 ± 1230.1 (n=26)	8.1± 12.0 (n=26)	51.0 ± 55.8 (n=26)	0.5 ± 0.8 (n=26)	2.8 ± 1.1 (n=8)	1.6 ± 0.8 (n=32)
Di-isoFA*	79.1 ± 85.7 (n=15)	1.2 ± 2.0 (n=15)	7.7 ± 1.3 (n=15)	313.6 ± 372.0 (n=15)	5.1 ± 7.9 (n=15)	18.9 ± 24.5 (n=15)	0.3 ± 0.5 (n=15)	-	0.4 ± 0.2 (n=11)
Di-Cou*	33.1 ± 17.1 (n=6)	0.1 ± 0.1 (n=6)	5.5 ± 1.4 (n=6)	772.5 ± 1007.0 (n=12)	13.5 ± 22.2 (n=12)	12.2 ± 9.0 (n=6)	0.0 ± 0.0 (n=6)	-	0.7 ± 0.7 (n=18)
BA/BE*	39.9 ± 53.8 (n=12)	0.1 ± 0.2 (n=12)	5.0 ± 1.8 (n=12)	318.3 ± 455.2 (n=12)	1.0 ± 1.5 (n=12)	13.3 ± 19.0 (n=12)	0.0 ± 0.1 (n=12)	-	3.9 ± 4.0 (n=22)
Cat*	352.6 ± 653.7 (n=13)	0.8 ± 1.3 (n=13)	3.3 ± 2.1 (n=13)	1276.7 ± 1308.9 (n=9)	3.9 ± 4.3 (n=9)	53.8 ± 54.1 (n=9)	0.2 ± 0.2 (n=9)	-	11.0 ± 18.2 (n=12)
Misc‡	395.8 ± 674.7 (n=16)	0.3 ± 0.6 (n=16)	2.3 ± 3.0 (n=16)	1400.1 ± 2304.0 (n=16)	1.2 ± 1.9 (n=16)	59.8 ± 96.4 (n=16)	0.1 ± 0.1 (n=16)	20.7 ± 34.5 (n=7)	0.3 ± 0.9 (n=11)

Unchanged acyl-quinic and C_6-C_3 unsaturated cinnamic acids include compounds n 3, 4, 5, 16, 20, 21, 22, 30, 33, 36, 37, 38; CQAs: Caffeoylquinic acids; FQAs: Feruloylquinic acids; CoQAs: Coumaroylquinic acids; CA: derivatives of 3′,4′-dihydroxycinnamic acid (*aka* caffeic acid); FA: derivatives of 4′-hydroxy-3′-methoxycinnamic acid (*aka* ferulic acid); isoFA: derivatives of 3′-hydroxy-4′-methoxycinnamic acid (*aka* isoFerulic acid); Sin: derivatives of 3′,5′-dimethoxy-4′-hydroxycinnamic acid (*aka* sinapic acid); Cou/Cinn: derivatives of hydroxycinnamic acid (*aka* coumaric acid) and cinnamic acid; Di-CA: derivatives of 3-(3′,4′-dihydroxyphenyl)propanoic acid (*aka* dihydrocaffeic acid); Di-FA: derivatives of 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid (*aka* dihydroferulic acid); Di-isoFA: derivatives of 3-(3′-hydroxy-4′-methoxyphenyl)propanoic acid (*aka* dihydroisoferulic acid); Di-Cou: derivatives of 3-(hydroxyphenyl)propanoic acid (*aka* dihydrocoumaric acid); BA/BE: derivatives of benzoic acid and benzaldehyde; Cat: catechols; Misc: miscellaneous. C_{max}: maximum plasma concentration; T_{max}: time to reach C_{max}; AUC: area under the curve; C_{avg}: average concentration; $t_{1/2}$: half elimination time; *symbol: when the class includes data derived from both aglycones and their phase 2 conjugates; - means any data was collected for that pharmacokinetic parameter; ‡sybmol: this class include data for unchanged and phase 2 conjugates of unknown forms of methoxycinnamic acid sulfate and hydroxymethoxycinnamic acid, derivatives of rosmarinic acid, artepillin C, drupanin, capillartemisin A, 2,2-dimethylchromene-6-propenoic acid, 3,4-dihydroxy-5-prenyl cinnamic acid and culifolin.

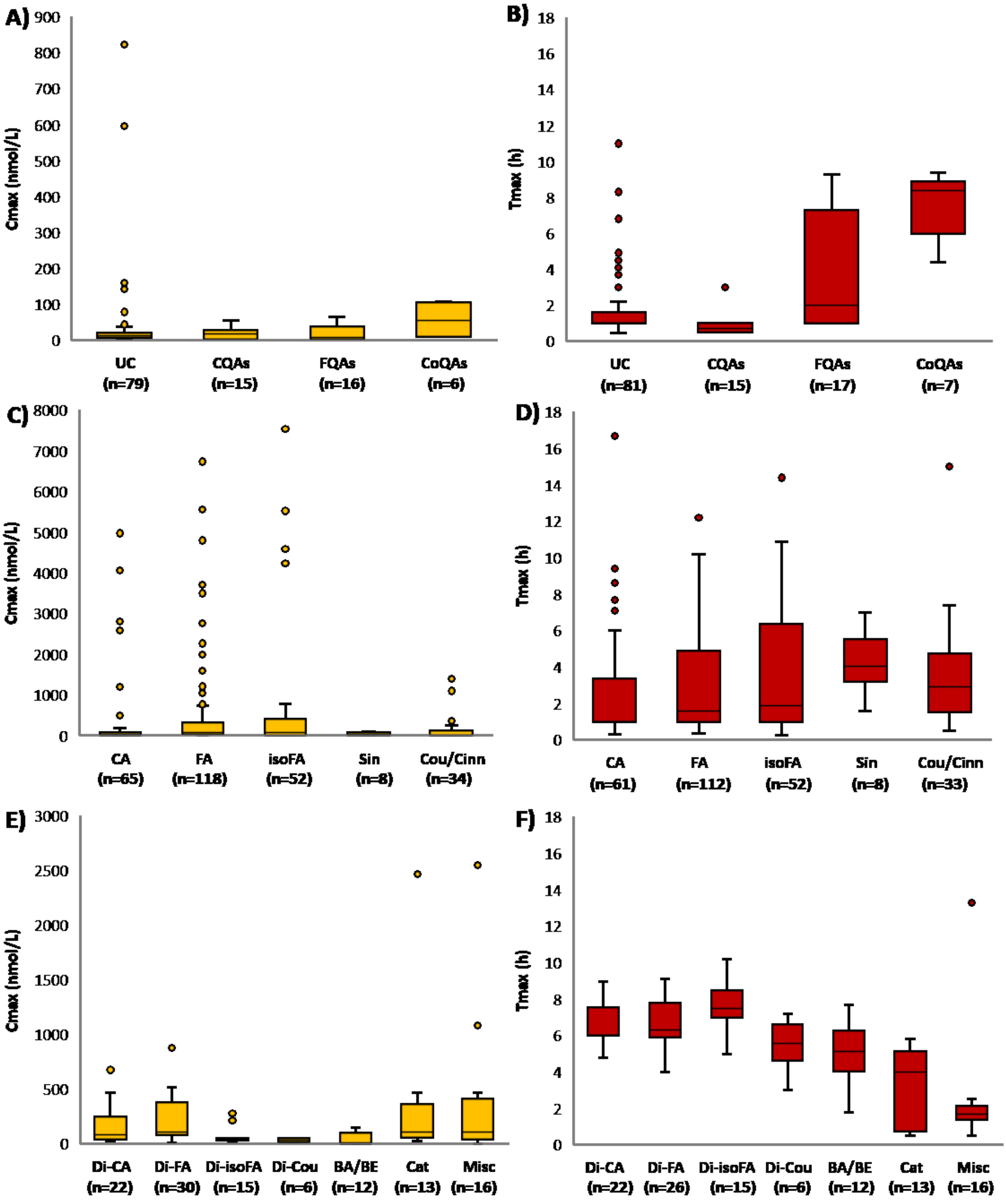


Figure 3. Box plot for C_{max} (nmol/L) (**A**, **C**, **E**) and T_{max} (h) (**B**, **D**, **F**) of unchanged acyl-quinic and C_6-C_3 unsaturated cinnamic acids (UC), caffeoylquinic acids (CQAs), feruloylquinic acids (FQAs), coumaroylquinic acids (CoQAs), derivatives of 3′,4′-dihydroxycinnamic acid (aka caffeic acid) (CA), derivatives of 4′-hydroxy-3′-methoxycinnamic acid (*aka* ferulic acid) (FA), derivatives of 3′-hydroxy-4′-methoxycinnamic acid (*aka* isoFerulic acid) (isoFA), derivatives of 3′,5′-dimethoxy-4′-hydroxycinnamic acid (*aka* sinapic acid) (Sin), derivatives of hydroxycinnamic acid (*aka* coumaric acid) and cinnamic acid (Cou/Cinn), derivatives of 3-(3′,4′-dihydroxyphenyl)propanoic acid (*aka* dihydrocaffeic acid) (Di-CA), derivatives of 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid (*aka* dihydroferulic acid) (Di-FA), derivatives of 3-(3′-hydroxy-4′-methoxyphenyl)propanoic acid (*aka* dihydroisoferulic acid) (Di-isoFA), derivatives of 3-

(hydroxyphenyl)propanoic acid (*aka* dihydrocoumaric acid) (Di-Cou), derivatives of benzoic acid and benzaldehyde (BA/BE), catechols (Cat), miscellaneous (Misc). C_6-C_3 unsaturated cinnamic acids include compounds quantified in biofluids after consumption of other phytochemicals. Apart for UC, classes of CQAs, FQAs, CoQAs, C_6-C_3 unsaturated cinnamic acids, phenylpropanoic acids, BA/BE and Cat. include data derived from both aglycones and their phase 2 conjugates. Misc class include data for unchanged and phase 2 conjugates of methoxycinnamic acid sulfate and hydroxymethoxycinnamic acid, derivatives of rosmarinic acid, artepillin C, drupanin, capillartemisin A, 2,2-dimethylchromene-6-propenoic acid, 3,4-dihydroxy-5-prenyl cinnamic acid and culifolin. C_{max}: maximum plasma concentration; T_{max}: time to reach C_{max}; n indicates the number of biological replicates collected for the same class of HCA metabolites and for the same nutrikinetic parameter.

Pooling C_{max} and T_{max} values of all the compounds belonging to each class of C_6-C_3 unsaturated cinnamic acids, phenylpropanoic acids and acyl-quinic acids, C_6-C_3 unsaturated cinnamic acids reached a C_{max} of 423 ± 1125 (mean ± SD) and 63 (15-183) (median (25th-75th percentile)) nmol/L at 3.3 ± 3.0 and 1.7 (1.0-4.9) h , followed by phenylpropanoic acids [154 ± 172 and 88 (42-220) nmol/L at 6.7 ± 1.4 and 6.6 (6.0-7.8) h] and acyl-quinic acids [24 ± 29 and 17 (2-27) nmol/L at 3.3 ± 3.3 and 1.2 (1.0-5.9) h] (Table 2 and Figure 3).

Derivatives of 3′-hydroxy-4′-methoxycinnamic acid had the highest C_{avg} [480 ± 1212 (mean ± SD) and 25 (2-100) (median (25th-75th percentile)) nmol/L], followed by derivatives of 3′,4′-dihydroxycinnamic acid [190 ± 628 and 4 (1-15) nmol/L] and 4′-hydroxy-3′-methoxycinnamic acid [163 ± 546 and 15 (1-81) nmol/L]. Pooled data of C_{avg} for C_6-C_3 unsaturated cinnamic acids, phenylpropanoic acids and acyl-quinic acids confirmed the same trend previously observed for C_{max}: the C_{avg} of C_6-C_3 unsaturated cinnamic acids was 209 ± 704 (mean ± SD) and 13 (1-54) (median (25th-75th percentile)) nmol/L, followed by phenylpropanoic acids [36 ± 47 and 17 (3-50) nmol/L] and acyl-quinic acids [9 ± 16 and 1 (0-8) nmol/L] (Table 2). $T_{1/2}$ values ranged from 0.4 ± 0.1 (mean ± SD) and 0.5 (0.4-0.5) (median (25th-75th percentile)) h to 20.7 ± 34.5 and 3.8 (1.8-20.0) h for caffeoylquinic acids and miscellaneous, respectively (Table 2). C_{max}, AUC and C_{avg} values normalized for the ingested dose of parent compounds for each class of metabolites are reported in Table 2. Overall, normalized C_{max} values revealed the importance of considering derivatives of 3′,4′-dihydroxycinnamic acid and 4′-hydroxy-3′-methoxycinnamic acid, together with unchanged acyl-quinic acids and C_6-C_3 unsaturated cinnamic acids, important contributors of the concentration levels that circulating compounds reached following HCA intake. The urinary excretion data for circulating metabolites, grouped by classes, are presented in Table 2. Catechols and derivatives of 4′-hydroxy-3′-methoxycinnamic acid were excreted at the highest amounts when compared to the other classes of metabolites, equal to 11 ± 18 (mean ± SD) [2 (1-8) (median (25th-75th percentile))] and 8 ± 17 [2 (1-5)] % of intake for catechols and derivatives of 4′-hydroxy-3′-methoxycinnamic acid, respectively (Table 1). Overall, all the compounds belonging to C_6-C_3 unsaturated cinnamic acid classes were excreted in an amount equal to 4 ± 11 (mean ± SD) [0 (0-2) (median (25th-75th percentile)] % of intake, followed by all the phenylpropanoic acids [1 ± 2 and 1 (0-2) % of intake] and all the acyl-quinic acids [1 ± 2 and 0 (0-1) % of intake]. Single values of urinary excretion for each class of metabolites highlighted that derivatives of 4′-hydroxy-3′-methoxycinnamic acid were extensively excreted in urine with respect to feruloylquinic acids, derivatives of 3′,5′-dimethoxy-4′-hydroxycinnamic acid and miscellaneous (Figure 4 and Table 2).

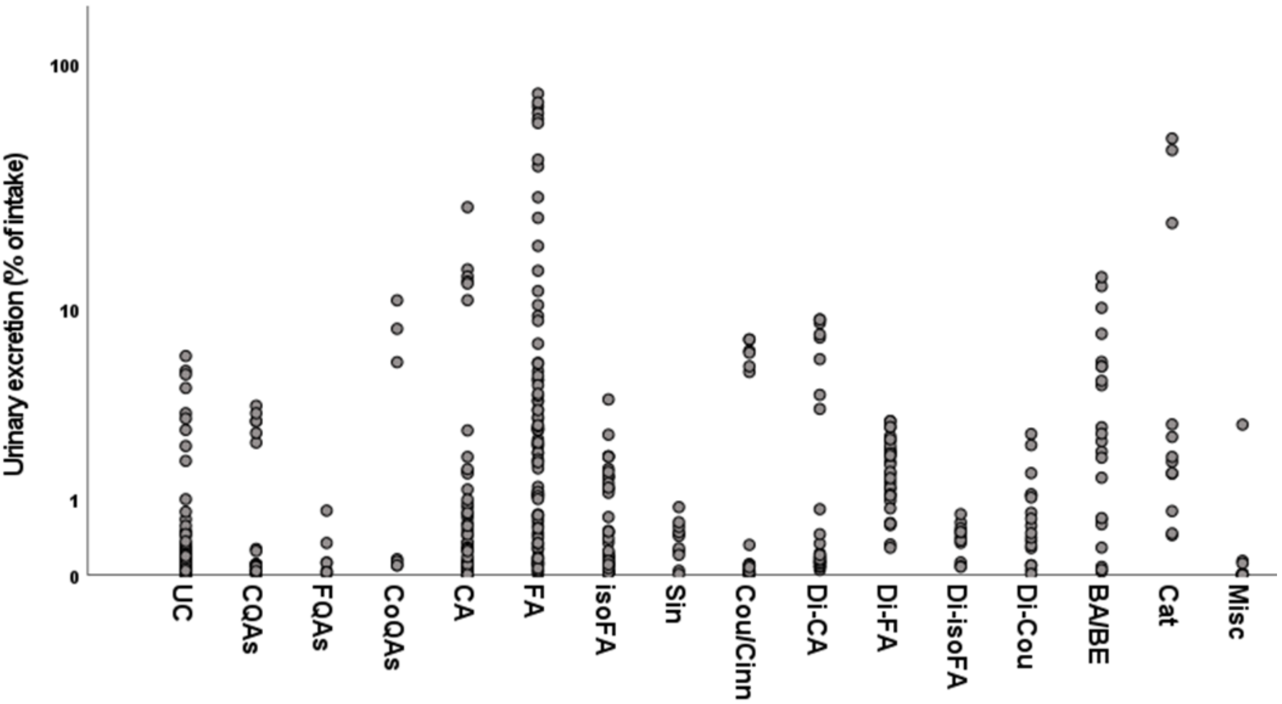


Figure 4. Single values of urinary excretion (% of intake) for unchanged acyl-quinic and C_6-C_3 unsaturated cinnamic acids (UC), caffeoylquinic acids (CQAs), feruloylquinic acids (FQAs), coumaroylquinic acids (CoQAs), derivatives of 3′,4′-dihydroxycinnamic acid (*aka* caffeic acid) (CA), derivatives of 4′-hydroxy-3′-methoxycinnamic acid (*aka* ferulic acid) (FA), derivatives of 3′-hydroxy-4′-methoxycinnamic acid (*aka* isoFerulic acid) (isoFA), derivatives of 3′,5′-dimethoxy-4′-hydroxycinnamic acid (*aka* sinapic acid) (Sin), derivatives of hydroxycinnamic acid (*aka* coumaric acid) and cinnamic acid (Cou/Cinn), derivatives of 3-(3′,4′-dihydroxyphenyl)propanoic acid (*aka* dihydrocaffeic acid) (Di-CA), derivatives of 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid (*aka* dihydroferulic acid) (Di-FA), derivatives of 3-(3′-hydroxy-4′-methoxyphenyl)propanoic acid (*aka* dihydroisoferulic acid) (Di-isoFA), derivatives of 3-(hydroxyphenyl)propanoic acid (*aka* dihydrocoumaric acid) (Di-Cou), derivatives of benzoic acid and benzaldehyde (BA/BE), catechols (Cat), miscellaneous (Misc). C_6-C_3 unsaturated cinnamic acids include compounds quantified in biofluids after consumption of other phytochemicals. Apart for UC, classes of CQAs, FQAs, CoQAs, C_6-C_3 unsaturated cinnamic acids, phenylpropanoic acids, BA/BE and Cat. include data derived from both aglycones and their phase 2 conjugates. Misc class include data for unchanged and phase 2 conjugates of methoxycinnamic acid sulfate and hydroxymethoxycinnamic acid, derivatives of rosmarinic acid, artepillin C, drupanin, capillartemisin A, 2,2-dimethylchromene-6-propenoic acid, 3,4-dihydroxy-5-prenyl cinnamic acid and culifolin.

2.4.2 Nutrikinetic parameters of the main blood metabolites

Based on the 83 normalized C_{max} mean values calculated for all the metabolites quantified in blood fractions (serum/plasma) (Supplementary excel file), up to 18 compounds including 10 C_6-C_3 unsaturated cinnamic acids [3′,4′-dihydroxycinnamic acid, 4′-hydroxycinnamic acid-3′-sulfate (*aka* caffeic acid-3′-sulfate), 3′,4′-dimethoxycinnamic acid (*aka* dimethylcaffeic acid), 4′-hydroxy-3′-methoxycinnamic acid, 3′-methoxycinnamic acid-4′-sulfate, 3′-methoxycinnamic acid-4′-glucuronide (*aka* ferulic acid-4′-glucuronide), 3′-hydroxy-4′-methoxycinnamic acid, 4′-methoxycinnamic acid-3′-glucuronide (*aka* isoFerulic acid-3′-glucuronide), cinnamic acid, and cinnamic acid-4′-sulfate (*aka* coumaric acid-4′-sulfate)], 7 phenylpropanoic acids [3-(3′,4′-dihydroxyphenyl)propanoic acid, 3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate (*aka* dihydrocaffeic acid-3′-sulfate), 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid, 3-(3′-methoxyphenyl)propanoic acid-4′-sulfate (*aka* dihydroferulic acid-4′-sulfate), 3-(3′-methoxyphenyl)propanoic acid-4′-glucuronide (*aka* dihydroferulic acid-4′-glucuronide), 3-(3′-hydroxy-4′-methoxyphenyl)propanoic acid, 3-(4′-methoxyphenyl)propanoic acid-3′-glucuronide (*aka* dihydroisoferulic acid-3′-glucuronide)] and one catechol, namely hydroxybenzene-sulfate (*aka* catechol-sulfate, unknown form) were established as the most abundant blood metabolites of HCAs. The nutrikinetic data for the main blood metabolites, including their normalized values for C_{max}, AUC and C_{avg}, are presented in Supplementary Table S3. Box plots for C_{max} and T_{max} for 4 out of 10 main blood C_6-C_3 unsaturated cinnamic acids and 4 out of 8 among the main phenylpropanoic acids and hydroxybenzene-sulfate are reported in Figure 5 and Figure 6, respectively.

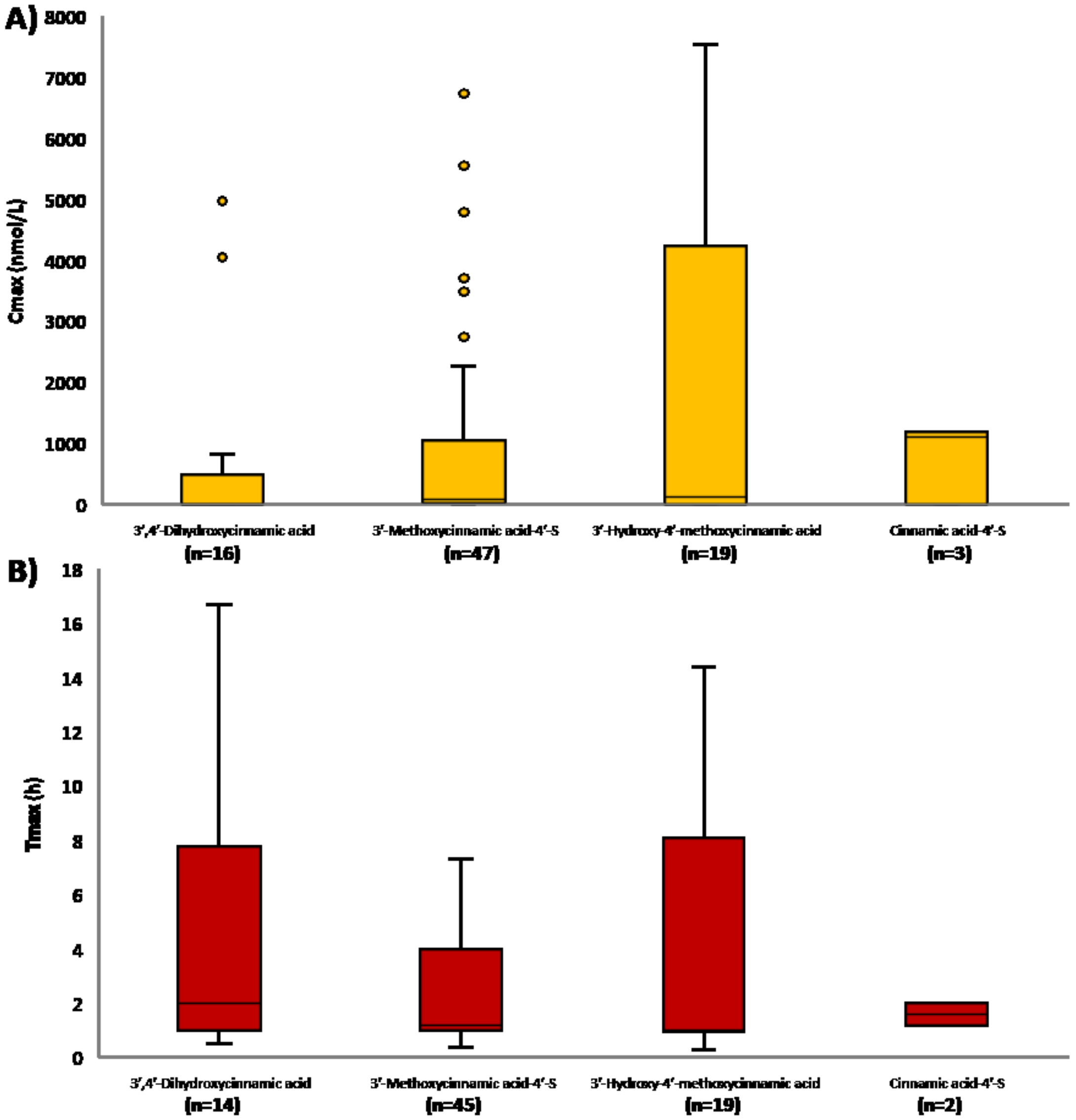


Figure 5. Box plot for C_{max} (nmol/L) (**A**) and T_{max} (h) (**B**) for 4 out of 10 main C_6-C_3 unsaturated cinnamic acids quantified in blood sample after intake of HCAs and/or other phytochemicals (see Supplementary Table 3 for the complete list of the main 18 plasma HCA metabolites). Sulfate (S). The main plasma circulating compounds were selected based on a normalized C_{max} value ≥ 0.4 nmol/L, calculated using at least 3 biological replicates deriving from at least 2 manuscripts. C_{max}: maximum plasma concentration; T_{max}: time to reach C_{max}; n indicates the number of biological replicates collected for the same HCA metabolite and for the same nutrikinetic parameter. Metabolites are named according to (Kay et al., 2020).

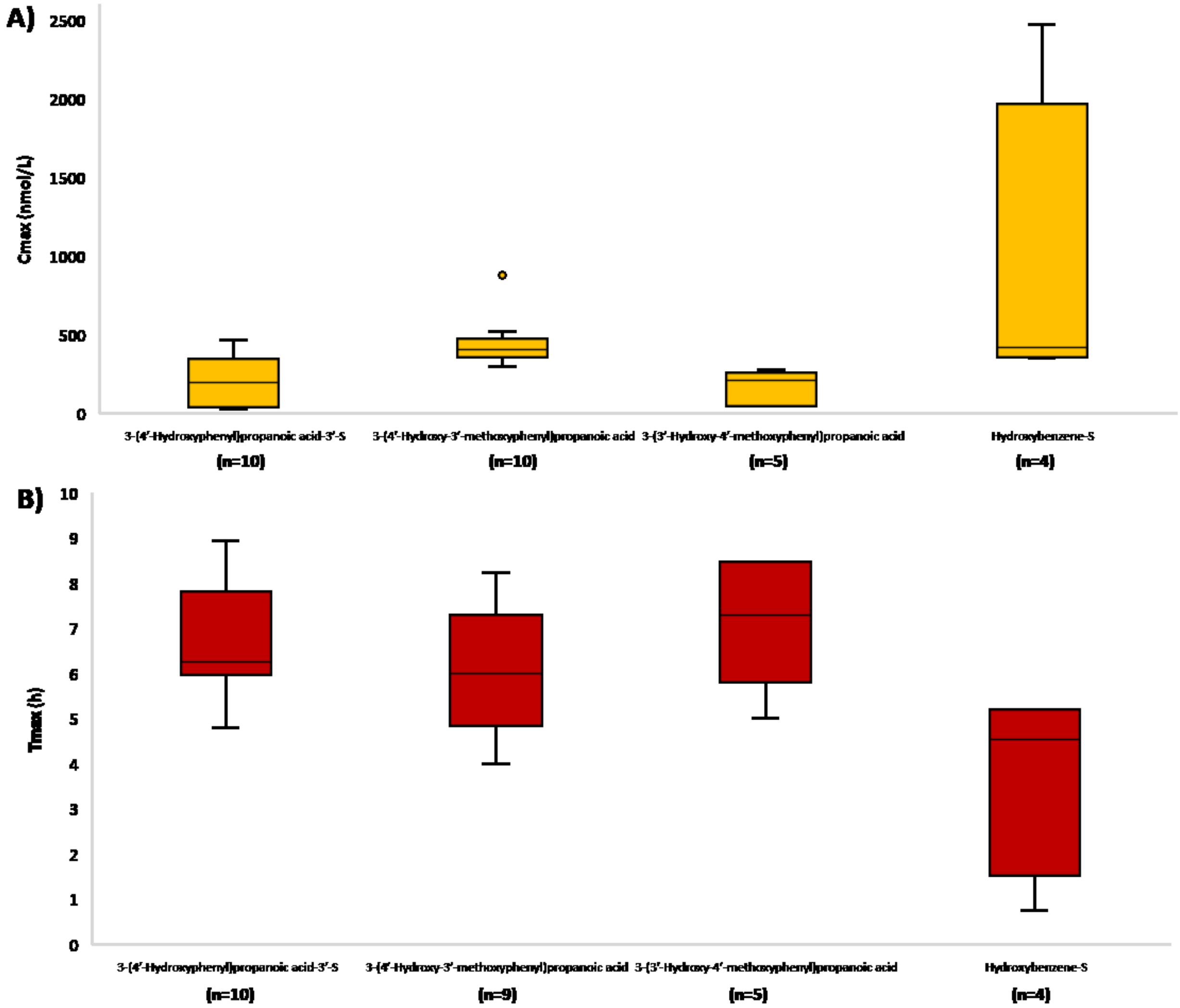


Figure 6. Box plot for C_{max} (nmol/L) (**A**) and T_{max} (h) (**B**) for 4 out of 8 main phenylpropanoic acids and catechols quantified in blood sample after intake of HCAs (see Supplementary Table 3 for the complete list of the main 18 plasma HCA metabolites). Sulfate (S). The main plasma circulating compounds were selected based on a normalized C_{max} value ≥ 0.4 nmol/L, calculated using at least 3 biological replicates deriving from at least 2 manuscripts. C_{max}: maximum plasma concentration; T_{max}: time to reach C_{max}; n indicates the number of biological replicates collected for the same HCA metabolite and for the same nutrikinetic parameter. Metabolites are named according to (Kay et al., 2020).

3′-Hydroxy-4′-methoxycinnamic acid reached the highest C_{max} value [1494 ± 2429 (mean ± SD) and 119 (20-2503) nmol/L (median (25th-75th percentile)) at 4.3 ± 4.5 and 1.0 (1.0-8.0) h (T_{max})], followed by 3′-methoxycinnamic acid-4′-sulfate [966 ± 1707 and 82 (38-975) nmol/L at 2.1 ± 1.8 and 1.2 (1.0-4.0) h], hydroxybenzene-sulfate [915 ± 1037 and 418 (363-970) nmol/L at 3.8 ± 2.1 and 4.6 (3.1-5.2) h] and cinnamic acid-4′-sulfate [768 ± 654 and 1100 (558-1145) nmol/L at 1.6 ± 0.6 and 1.6 (1.4-1.8) h] (Supplementary Table S3, Figure 5, Figure 6). The C_{max} of the main blood C_6-C_3 unsaturated cinnamic acids was higher than all the main blood phenylpropanoic acids [main blood C_6-C_3 unsaturated cinnamic acids: 553 ± 1301 (mean ± SD) and 83 (29-310) nmol/L (median (25th-75th percentile) (C_{max}) at 3.1 ± 3.2 and 1.4 (1.0-4.6) h (T_{max}); main blood phenylpropanoic acids: 159 ± 169 and 92 (45-231) nmol/L at 6.9 ± 1.4 and 6.6 (6.0-8.0) h]. 3′-Hydroxy-4′-methoxycinnamic acid reached the highest C_{avg} value [1232 ± 1826 (mean ± SD) [32 (1-2409) (median (25th-75th percentile))] nmol/L] with respect to the other main blood metabolites (Supplementary Table S3). The C_{avg} of data pooled for all the main blood C_6-C_3 unsaturated cinnamic acids was higher than those of the main blood phenylpropanoic acids [280 ± 829 and 17 (1-82) nmol/L and 41 ± 50 and 21 (5-59) nmol/L for C_6-C_3 unsaturated cinnamic acids and phenylpropanoic acids, respectively]. $T_{1/2}$ values ranged from 1.3 ± 0.4 (mean ± SD) [1.2 (1.1-1.4) (median (25th-75th percentile))] to 32.5 ± 15.7 [32.5 (27.0-38.1)] h for 4′-hydroxycinnamic acid-3′-sulfate and 4′-hydroxy-3′-methoxycinnamic acid, respectively (Supplementary Table S3).

2.4.3 Urinary excretion and stoichiometry of the main urinary metabolites

Based on the 76 urinary excretion (% of intake) mean values calculated for all the metabolites quantified in urine fraction (Supplementary excel file), up to 16 compounds including 3 acyl-quinic acids [3-caffeoylquinic lactone-sulfate (unknown form), 4-caffeoylquinic lactone-sulfate (unknown form), 3-feruloylquinic acid], 8 C_6-C_3 unsaturated cinnamic acids [3′,4′-dihydroxycinnamic acid, unknown isoform of hydroxycinnamic acid-glucuronide (*aka* caffeic acid-glucuronide), 4′-hydroxy-3′-methoxycinnamic acid, 3′-methoxycinnamic acid-4′-sulfate, 3′-methoxycinnamic acid-4′-glucuronide, 3′-methoxy-4′-hydroxycinnamoyl-glycine, 4′-hydroxycinnamic acid (*aka p*-coumaric acid), cinnamic acid-4′-glucuronide (*aka p*-coumaric acid-4′-glucuronide)], and 5 phenylpropanoic acids [3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate, 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid, 3-(3′-methoxyphenyl)propanoic acid-4′-sulfate, 3-(3′-methoxyphenyl)propanoic acid-4′-glucuronide, 3-(phenyl)propanoic acid-4′-sulfate (*aka* dihydrocoumaric acid-sulfate)] were established as the main urinary metabolites of HCAs. 3′-Methoxycinnamic acid-4′-glucuronide was excreted at the highest level [17 ± 28 (mean ± SD) and 1 (0-23) (median (25th-75th percentile)) % of intake], followed by 4′-hydroxy-3′-methoxycinnamic acid [7 ± 12 and 0 (0-7) % of intake], hydroxycinnamic acid-glucuronide [6 ± 10 and 1 (0-13) % of intake], and 3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate [5 ± 4 and 6 (2-8) % of intake] (Supplementary Table S4 and Figure 7). Pooling data from the main urinary metabolites accordingly to their class, we found that the main urinary C_6-C_3 unsaturated cinnamic acids were excreted in amounts equal to 7 ± 15 (mean ± SD) and 1 (0-5) (median (25th-75th percentile)) % of intake, while acyl-quinic acids and phenylpropanoic acids in % of intake equal to 2 ± 2 [1 (0-3)] and 2 ± 2 [2 (1-3)] for acyl-quinic acids and phenylpropanoic acids, respectively. Stoichiometric balances for the main urinary compounds are described in Supplementary Table S5. Molar mass recovery varied from lower than 0%, for 4′-hydroxycinnamic acid and cinnamic acid-4′-glucuronide, up to 4.4 and 5.3% for 3′-methoxy-4′-hydroxycinnamoyl-glycine and 3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate, respectively. In parallel, the ingestion of about 19 and 23 μmol of HCAs would be needed to reach 1 μmol of urinary 3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate and 3′-methoxy-4′-hydroxycinnamoyl-glycine, respectively. Stoichiometric balances increased to more than 4000 μmol of HCAs that would be ingested to potentially result in excretion of 1 μmol 4′-hydroxycinnamic acid and cinnamic acid-4′-glucuronide (Supplementary Table S5).

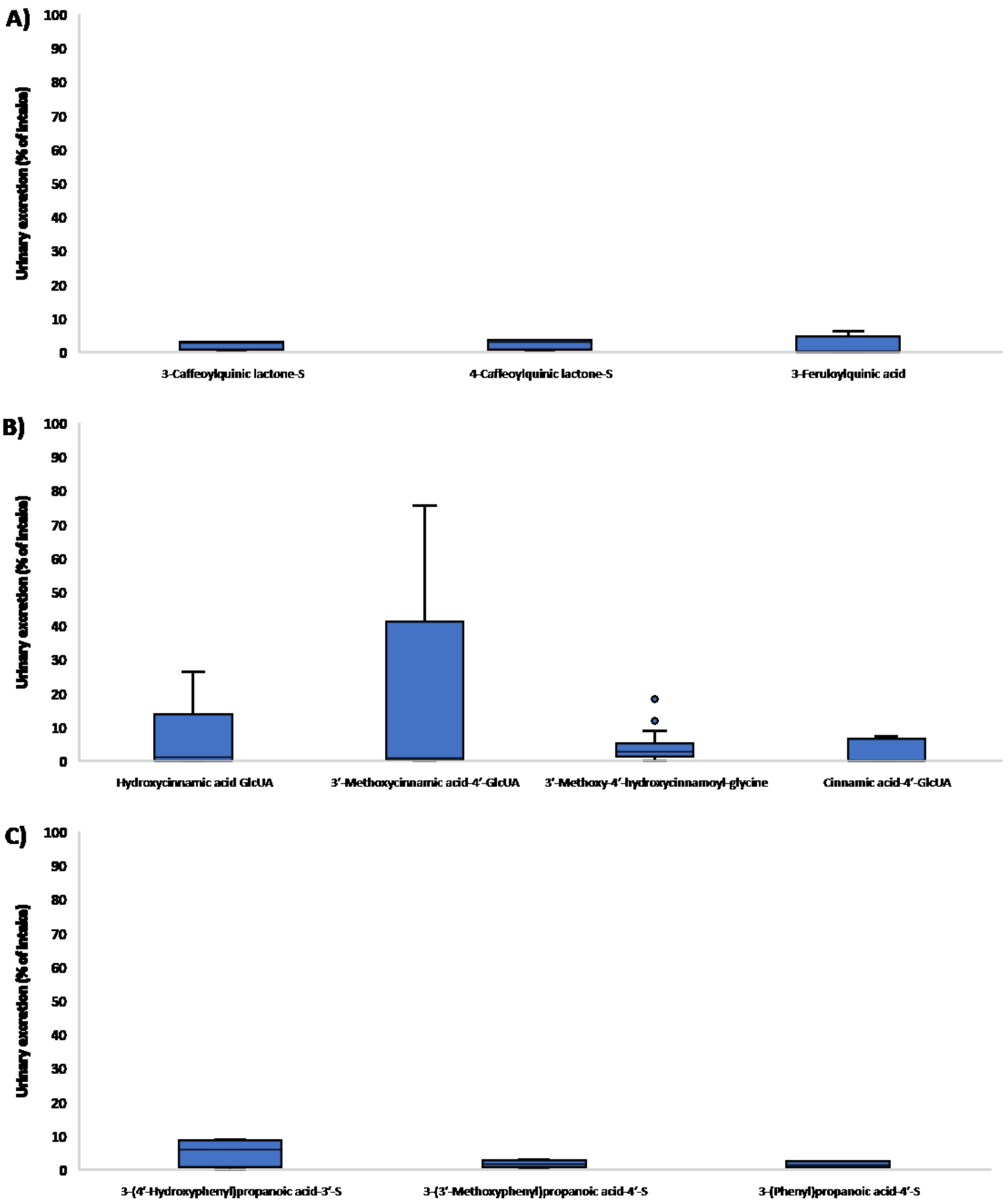


Figure 7. Box plot of urinary excretion (% of intake) for (**A**) the main urine acyl-quinic acids, (**B**) 4 out of 8 main urine C_6-C_3 unsaturated cinnamic acids, (**C**) 3 out of 5 main urine phenylpropanoic acids. See Supplementary Table 4 for the complete list of the main 16 urine HCA metabolites. C_6-C_3 unsaturated cinnamic acids include compounds quantified in biofluids after consumption of other phytochemicals. Glucuronide (GlcUA); sulfate (S); n indicates the number of biological replicates collected for the same HCA metabolite and for the same nutrikinetic parameter. The main urine compounds were selected based on a urinary excretion value, expressed as percentage of intake ≥ 1.5 %, calculated using at least 3 biological replicates deriving from at least 2 manuscripts. Metabolites are named according to (Kay et al., 2020).

2.5 Bioavailability of hydroxycinnamic acids

The 17 values of HCA bioavailability (%) collected from literature and/or estimated from urinary excretion data employed to calculate the value for HCA bioavailability are described in Supplementary Table S6. The mean bioavailability of HCAs was 25 ± 19 % [median 25th-75th percentile: 22 (13-28) %] (Figure 8).

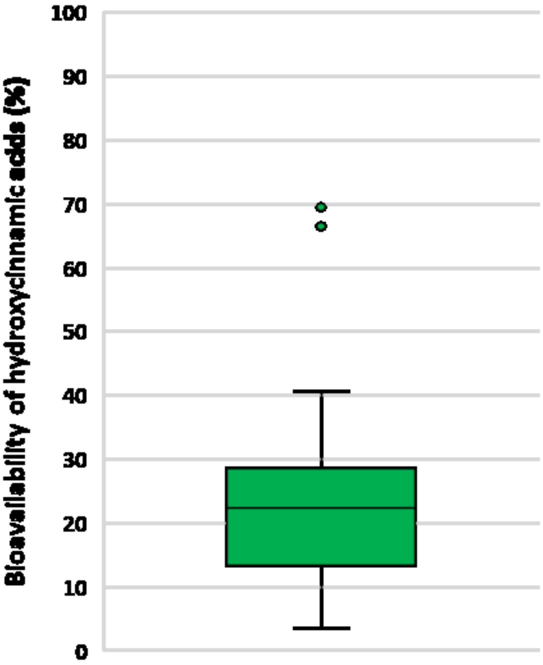


Figure 8. Box plot for bioavailability (%) of HCAs calculated taking into account all the values of HCA bioavailability collected from literature and/or estimated from urinary excretion data derived from studies analyzed (n of values of HCA bioavailability (%)=17). Details on HCA bioavailability (%) values employed to calculate the value for bioavailability of HCAs are reported in Supplementary Table 6.

Bioavailability values were compared source by source with the ingested amount (µmol) of total parent compounds deriving from each study (Figure 9A), and they were averaged to estimate the mean bioavailability of HCAs for each source employed in the analyzed studies (Figure 9B). Bioavailability of HCAs from coffee was 31 % (number -n- of HCA bioavailability values collected/estimated for each source=11), followed by cereals (16 %) (n=4), yerba mate (13 %) (n=1), and artichoke (4 %) (n=1) (Figure 9B). Regardless of the ingested dose of HCAs, derivatives of 4′-hydroxy-3′-methoxycinnamic acid and coumaroylquinic acids contributed to the bioavailability of HCAs for about 7 and 5 % for 4′-hydroxy-3′-methoxycinnamic acid and coumaroylquinic acids, respectively, followed by unchanged acyl-quinic and C_6-C_3 unsaturated cinnamic acids (4 %) and caffeoylquinic acids (3 %). Considering specific colonic metabolites of HCAs, namely phenylpropanoic acids, derivatives of 3′,4′-dihydroxycinnamic acid and 4′-hydroxy-3′-methoxycinnamic acid contributed both to 4 % of HCA bioavailability (Supplementary Figure S3). Finally, considering late products of HCA metabolism, benzoic acid and benzaldehyde accounted for 21 %, followed by catechols (5 %). A similar trend was also found when only studies evaluating HCA bioavailability from coffee, the most investigated food source, were taken into account (Supplementary Figure S4).

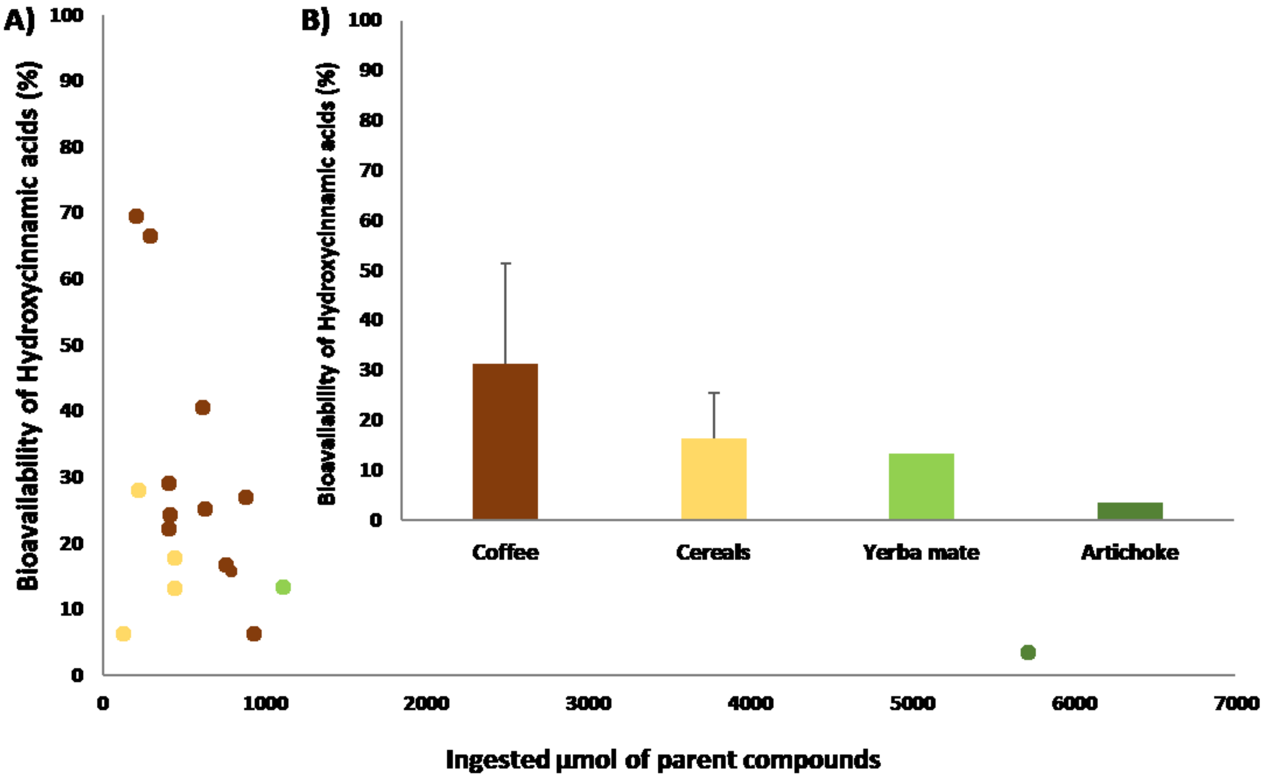


Figure 9. (**A**) Values of bioavailability (%) for HCAs, collected from literature and/or estimated from urinary excretion data, and ingested µmol of the different HCA sources. Each bullet indicates the bioavailability (%) value for HCAs, obtained for every single study, and related to each dose of consumed HCAs in the study. (**B**) Bioavailability of HCAs calculated for the different food sources employed in the human studies that underwent data analyses. Data are expressed as mean and SD. HCA source (n of values of HCA bioavailability (%)): coffee (11), cereals (i.e. wheat, oat) (4), yerba mate (1), and artichoke (1).

3. Discussion

In this systematic review, a workflow already applied from our group to unravel the ADME of another important class of (poly)phenols, namely flavan-3-ols (Di Pede et al., 2022), was used to assess the extent with which HCAs are metabolized in humans. Highlighting the ADME of bioactive (poly)phenols represents a key point for correlating their intake to the multitude of potential beneficial effects observed in human studies (Carregosa et al., 2022, 2019; Guerreiro et al., 2022; Williamson, 2017).

A relevant intra- class and metabolite variability emerged, as shown from the SD values presented (Table 2, Supplementary Table S3 and Supplementary Table S4). For this reason, nutrikinetic and urinary recovery data of metabolites produced after HCA intake were also expressed as median (25th-75th percentile). Our work pointed out that after their intake, unchanged acyl-quinic and C_6-C_3 unsaturated cinnamic acids are rapidly absorbed (T_{max} about 1.7 h) (Table 2 and Figure 3B), providing that they cross the gastric and/or intestinal epithelium. Nevertheless, some unchanged mono-acyl quinic acids (i.e. 5-caffeoylquinic acid, 3-caffeoylquinic acid, 5-feruloylquinic acid, 4-feruloylquinic acid) and 1,5-dicaffeoylquinic acid attained T_{max} values higher than 3 h (Feliciano et al., 2017; Liu et al., 2010; Mena et al., 2021), suggesting their absorption at colonic level. Differences in absorption rates for acyl-quinic acids, presumably correlated to their chemical-structural features (i.e. n of acyl quinic moieties, hydrophobicity) were previously demonstrated *in vitro* (Farrell et al., 2011).

Aglycones and phase 2 conjugates of acyl-quinic acids were grouped into three categories based on their C_6-C_3 trans-hydroxycinnamic acid skeleton (Table 2). Caffeoylquinic acids appeared in blood and urine fractions only as sulfate conjugates (Table 1), to be readily absorbed at gastric and/or small intestine level (T_{max} about 1 h) and quickly removed to the circulatory system ($t_{½}$ about 0.4 h). T_{max} value three times higher than for caffeoylquinic acids was observed for feruloylquinic acids (T_{max} about 3.7 h, ranging from 1 to over 9 h), since this category included products of both phase 2 conjugation and hydrogenation (Table 1) (Figure 3B). These observations suggested that the metabolism of feruloylquinic acids might occur at both upper and lower gastrointestinal tract level. Coumaroylquinic acids were absorbed very slowly (T_{max} about 7.4 h) (Table 2 and Figure 3B), and they were found circulating as glucuronide conjugates and dihydrocoumaroylquinic acids after coffee and yerba mate intake (Gómez-juaristi et al., 2018; Gómez-Juaristi et al., 2018; Mena et al., 2021). Late dehydroxylation and demethoxylation steps on feruloylquinic and/or caffeoylquinic acid skeletons through colon microbiota action might be involved in the production of coumaroylquinic acids. We found that coumaroylquinic acids reached C_{max} value three times higher than caffeoylquinic and feruloylquinic acids, with values somewhat comparable (about 16 nmol/L) (Table 2), proving that coumaroylquinic acids circulate in blood at higher concentration with respects to their hydroxylated and methylated derivatives (Figure 3A). This finding is also supported by C_{avg} and normalized values for C_{max} and C_{avg}, and the same trend was also found in analyzing values of urinary excretion for these classes of acyl-quinic acids (Table 2 and Figure 4). Low blood circulating levels and limited urinary recovery for acyl-quinic acids suggested that after their intake, acyl-quinic acids are highly susceptible to hydrolysis by epithelial esterase, to be more prone to producing mammalian and gut microbiota metabolites.

Partial or total removal of acyl-quinic acid moiety may occur at gastric, small intestine and/or colonic levels through mammalian and bacterial esterase activity (Andreasen et al., 2001; Buchanan et al., 1996; Erk et al., 2014; Guy et al., 2009; Ludwig et al., 2013a; Xie et al., 2016), resulting into C_6-C_3 unsaturated cinnamic acids and, to a less extent into acyl-quinic acids. C_6-C_3 unsaturated cinnamic acids were grouped into five categories (Table 2). Overall, it seems that 3′,4′-dihydroxycinnamic acid derivatives are absorbed more rapidly than their methylated and dehydroxylated derivatives (Table 2 and Figure 3D), in line with the lower T_{max} value observed for caffeoylquinic acids with respect to feruloylquinic and coumaroylquinic acids (Table 2). Free 3′,4′-dihydroxycinnamic acid might arise from its direct absorption and/or release through hydrolysis of ingested caffeoylquinic acids, to be further subjected to phase 2 conjugation steps (Lafay et al., 2006; Ludwig et al., 2013b; Stalmach et al., 2009). 3′-Hydroxy-4′-methoxycinnamic acid is considered the most prominent methylated product of 3′,4′-dihydroxycinnamic acid with respect to 4′-hydroxy-3′-methoxycinnamic acid, to be both further conjugated by mammalian enzymes (Clifford et al., 2017), even if this dogma was not fully supported by the previous work of (Rubió et al., 2021). Nevertheless, free 4′-hydroxy-3′-methoxycinnamic acid might also derive from its direct absorption and/or post-absorption hydrolysis of feruloylquinic acids (Gómez-Juaristi et al., 2018; Ludwig et al., 2013b; Poquet et al., 2008). Even if 3′,4′-dihydroxycinnamic acid might be considered a source of mono- and di-methylated products, it has been demonstrated that 4′-hydroxy-3′-methoxycinnamic acid metabolites mainly derive from hydrolysis of the ingested feruloylquinic acids *in vivo* (Clifford et al., 2017; Stalmach et al., 2010, 2009). 3′,5′-Dimethoxy-4′-hydroxycinnamic acid is a metabolic product of 4′-hydroxy-3′-methoxycinnamic acid (Farah et al., 2008; Kern et al., 2003), while cinnamic derivatives result from dehydroxylation and demethoxylation steps catalysed on C_6-C_3 unsaturated skeleton (Baba et al., 2004; Choudhury et al., 1999; Farah et al., 2008). 4′-Methylated products of 3′,4′-dihydroxycinnamic acid (3′-hydroxy-4′-methoxycinnamic acid derivatives) circulated in blood at higher concentrations compared to 4′-hydroxy-3′-methoxycinnamic and 3′,5′-dimethoxy-4′-hydroxycinnamic acid derivatives (Table 2 and Figure 3C). On the other hand, mono-methylated C_6-C_3 unsaturated metabolites circulated in blood at higher levels than their di-methylated derivatives, and some differences between 4′-hydroxy-3′-methoxycinnamic and 3′-hydroxy-4′-methoxycinnamic acid derivatives in their concentration levels might occur *in vivo*. Taking into account the main blood circulating C_6-C_3 unsaturated cinnamic acids, 3′-hydroxy-4′-methoxycinnamic acid reached the highest C_{max} (over 1400 nmol/L), followed by 3′-methoxycinnamic acid-4′-sulfate (about 966 nmol/L), in line with data on the classes. This study showed that aglycones of 3′,4′-dihydroxycinnamic acid and 3′-hydroxy-4′-methoxycinnamic acid reached higher blood concentrations than their phase 2 conjugated derivatives. Surprisingly, an opposite behavior was found for 4′-hydroxy-3′-methoxycinnamic and cinnamic acids (Figure 5 and Supplementary Table S3). The variability observed in T_{max} values for both classes and main blood circulating C_6-C_3 unsaturated cinnamic acids might be explained by their biphasic profiles due to enterohepatic recycling and/or colonic absorption (Del Rio et al., 2013; Rodriguez-Mateos et al., 2014) (Table 2, Figure 3D, Figure 5 and Supplementary Table S3). Derivatives of 4′-hydroxy-3′-methoxycinnamic acid were extensively excreted in urine, reaching over 8 % of intake, with 3′,4′-dihydroxycinnamic acid and C_6-C_1 unsaturated derivatives having values rather comparable (about 1.7 % of intake) (Table 2 and Figure 4). Kidneys might not represent the preferred target organ for 3′-hydroxy-4′-methoxycinnamic acid derivatives, as suggested by their low urinary excretion levels (about 0.6 %). Based on blood and urine data, we suggest that 4′-hydroxy-3′-methoxycinnamic acid derivatives might have the highest metabolic efficiency with respect to the other classes *in vivo*. Eight main C_6-C_3 unsaturated cinnamic acids were found being excreted in urine in amounts ranging from 2.3 up to 17.0 % of intake for 4′-hydroxycinnamic acid and 3′-methoxycinnamic acid-4′-glucuronide, respectively (Figure 7 and Supplementary Table S4). Interestingly, four compounds, namely 3′-methoxy-4′-hydroxycinnamoyl-glycine, an unknown isoform of hydroxycinnamic acid glucuronide, 4′-hydroxycinnamic acid and cinnamic acid-4′-glucuronide were found being interesting metabolites in urine (in terms of urinary recovery), but not in blood. 3′-Methoxy-4′-hydroxycinnamoyl-glycine has been suggested as biomarker of intake of acyl-quinic acids at a relatively small ingested amount (Clifford et al., 2017; Rothwell et al., 2018). The unknown isoform of hydroxycinnamic acid glucuronide might correspond to 3′-hydroxycinnamic acid-4′-glucuronide which is widely quantified in biofluids than the 3’-glucuronide one (Domínguez-Fernández et al., 2022; Feliciano et al., 2017, 2016; Heiss et al., 2022; Mena et al., 2021, 2019; Mills et al., 2017; Rodriguez-Mateos et al., 2016a). Overall, our work pointed out that sulfates may represent the main blood HCA metabolites and that glucuronidation is catalysed to a less extent, supporting the large body of evidence in this field (Clifford et al., 2020, 2017), even if some glucuronide conjugates were also found being excreted in urine in moderate amounts (Supplementary Table S4).

About 70 % of unabsorbed acyl-quinic acids and/or C_6-C_3 unsaturated cinnamic acids reach the colon, where they will be subjected to the gut microbiota activity (Clifford et al., 2017). Specific metabolites produced from catabolic activities of HCAs occurring at colonic level were grouped into three categories (Table 2). Phenylpropanoic acids result through the hydrogenation step on the side chain of C_6-C_3 unsaturated cinnamic acids catalyzed by both colonic and mammalian enzymes (Clifford et al., 2017; Williamson and Clifford, 2017), as shown from their T_{max} values ranging from 5.5 to 7.7 h (Table 2 and Figure 3F). The highest blood circulating levels for derivatives of 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid (C_{max} and C_{avg} values of 206 and 51 nmol/L, respectively) (Table 2 and Figure 3E) suggest that 4′-hydroxy-3′-methoxycinnamic acid might be highly susceptible to enzymatic hydrogenation. Seven phenylpropanoic acids were found as the main blood HCA metabolites. In line with data on phenylpropanoic acid classes (Table 2), 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid attained higher C_{max} and C_{avg} values than the other main phenylpropanoic acids (Figure 6 and Supplementary Table S3). Urine data showed that phenylpropanoic acids are excreted in urine at a lower % than their C_6-C_3 unsaturated precursors. Unexpectedly, derivatives of 3-(3′,4′-

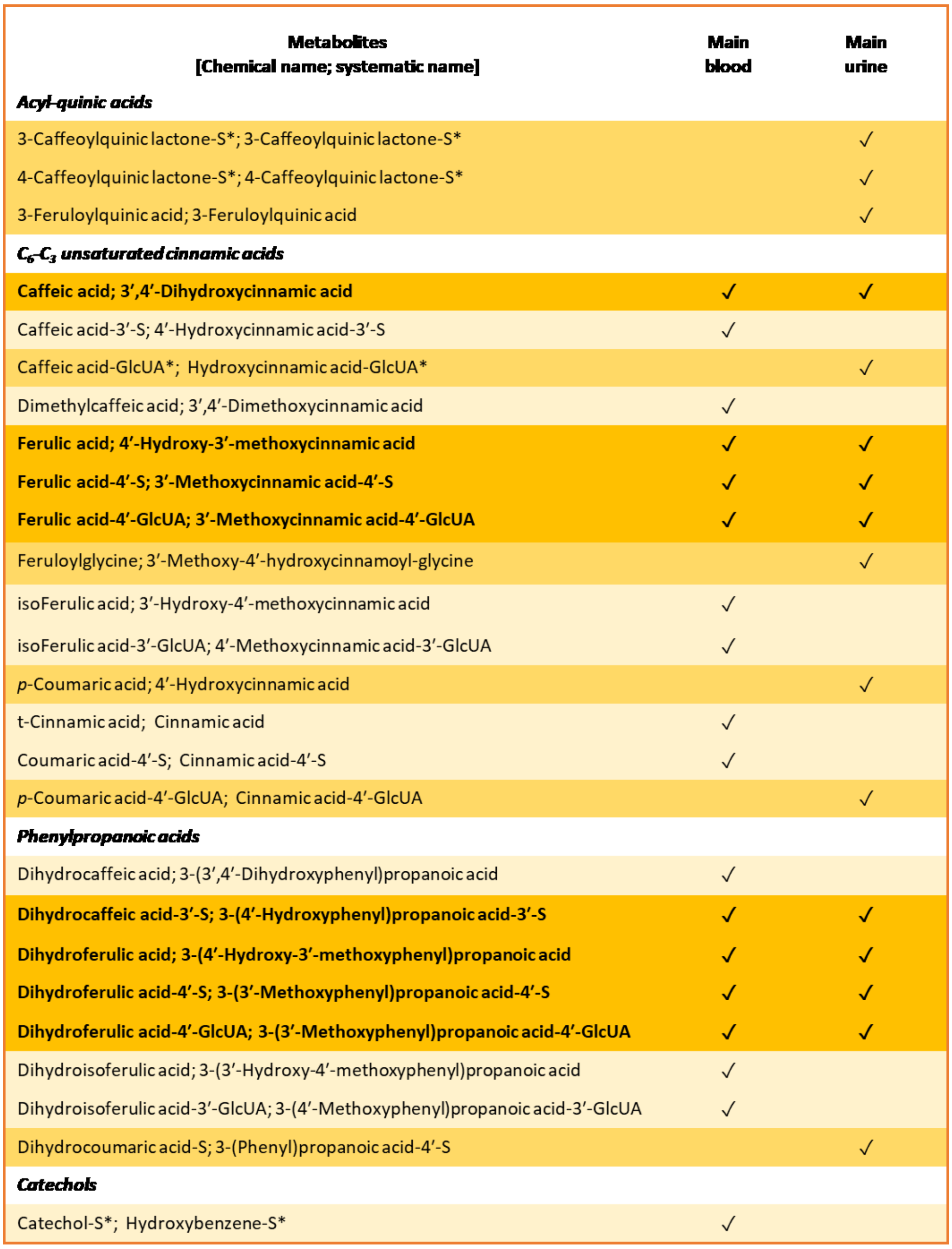
dihydroxyphenyl)propanoic acid were highly excreted in urine (about 2.4 % of intake) than the other phenylpropanoic acid classes (Table 2 and Figure 4). In parallel, among the main five urinary phenylpropanoic acids, 3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate reached over 5 % of urinary excretion (Figure 7 and Supplementary Table S4).

Phenylpropanoic acids are converted by two α-oxidation steps to benzoic acids by microbiota and/or mammalian enzyme activity (Clifford et al., 2017). Data on benzoic acids were pooled together with benzaldehydes to maximize the data harmonisation due to the low number of biological replicates for these classes (Table 2). Finally, benzoic acids are further decarboxylated in the colon, yielding the corresponding catechols (Williamson and Clifford, 2017) (Table 2). T_{max} values of benzoic acids and catechols suggest their absorption at colonic level (Table 2 and Figure 3F), even if it seems that catechols would be more easily absorbed than their C_6-C_1 precursors, as shown from T_{max} values of catechols ranging from 0.5 to 5.8 h after coffee intake (Lang et al., 2013; Mena et al., 2021). Blood and urine data pointed out that catechols contributed more to ADME of HCAs than benzoic acids (Table 2, Figure 3E and Figure 4).

This work demonstrated that HCAs have a moderate bioavailability (about 25 %) in humans (Figure 8). Even if data on the bioavailability of HCAs for each ingested source (Figure 9B) must be considered preliminary, due to the variable number of biological replicates collected from the analysed studies, the inter-study and intra/inter-source variability (Figure 9A), the low yield of HCA metabolites after artichoke intake highlighted that food matrix might play a role in affecting the ADME of these dietary phytochemicals.

4. Conclusions

HCAs are extensively metabolized along the human gastrointestinal tract, with up to 105 compounds recovered in blood and urine fractions after intake. This might be considered the first work that systematically harmonized the enormous amount of data published in the literature on the ADME of this relevant class of dietary phenolic acids. After HCA intake, C_6-C_3 unsaturated cinnamic acids attained the highest blood concentration levels (C_{max} and C_{avg} about 423 and 209 nmol/L, respectively), with T_{max} values ranging from 2.7 to 4.2 h. C_6-C_3 unsaturated cinnamic acids were excreted in higher amounts than their phenylpropanoic acid derivatives (4 and 1 % for C_6-C_3 unsaturated cinnamic acids and phenylpropanoic acids, respectively), but both in a lower percentage than catechols (11 %). Taking into account all the metabolites produced after HCA intake, and both blood and urinary data, it is possible to assume that derivatives of 4′-hydroxy-3′-methoxycinnamic acid might have the most interesting profile *in vivo*. Nutrikinetic and urinary recovery data revealed the presence of 18 and 16 main metabolites of HCAs, respectively. Nevertheless, 3′,4′-dihydroxycinnamic acid, 4′-hydroxy-3′-methoxycinnamic acid, 3′-methoxycinnamic acid-4′-sulfate, 3′-methoxycinnamic acid-4′-glucuronide, 3-(4′-hydroxyphenyl)propanoic acid-3′-sulfate, 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid, 3-(3′-methoxyphenyl)propanoic acid-4′-sulfate and 3-(3′-methoxyphenyl)propanoic acid-4′-glucuronide were found reaching the highest blood concentration levels, and at the same time being excreted in urine in moderate amount. These compounds might be considered as ‘golden metabolites’ of HCAs (Figure 10) to which attention should be paid in i) bioavailability studies when the ADME of dietary HCAs would be assessed, and ii) *in vivo* and *in vitro* models aiming to investigate their bioactivity at physiological concentration levels. Finally, the lack of clarity on HCA bioavailability for each ingested source lays the basis for designing a comprehensive human study assessing the ADME of HCAs for all their most consumed dietary sources. To our knowledge, data on ADME of HCAs from some plant-based foods such as potatoes, cereals, and artichoke were absent or inconsistent.



Metabolites **[Chemical name; systematic name]**	**Main blood**	**Main urine**
Acyl-quinic acids		
3-Caffeoylquinic lactone-S*; 3-Caffeoylquinic lactone-S*		✓
4-Caffeoylquinic lactone-S*; 4-Caffeoylquinic lactone-S*		✓
3-Feruloylquinic acid; 3-Feruloylquinic acid		✓
C_6-C_3 unsaturated cinnamic acids		
Caffeic acid; 3′,4′-Dihydroxycinnamic acid	✓	✓
Caffeic acid-3′-S; 4′-Hydroxycinnamic acid-3′-S	✓	
Caffeic acid-GlcUA*; Hydroxycinnamic acid-GlcUA*		✓
Dimethylcaffeic acid; 3′,4′-Dimethoxycinnamic acid	✓	
Ferulic acid; 4′-Hydroxy-3′-methoxycinnamic acid	✓	✓
Ferulic acid-4′-S; 3′-Methoxycinnamic acid-4′-S	✓	✓
Ferulic acid-4′-GlcUA; 3′-Methoxycinnamic acid-4′-GlcUA	✓	✓
Feruloylglycine; 3′-Methoxy-4′-hydroxycinnamoyl-glycine		✓
isoFerulic acid; 3′-Hydroxy-4′-methoxycinnamic acid	✓	
isoFerulic acid-3′-GlcUA; 4′-Methoxycinnamic acid-3′-GlcUA	✓	
p-Coumaric acid; 4′-Hydroxycinnamic acid		✓
t-Cinnamic acid; Cinnamic acid	✓	
Coumaric acid-4′-S; Cinnamic acid-4′-S	✓	
p-Coumaric acid-4′-GlcUA; Cinnamic acid-4′-GlcUA		✓
Phenylpropanoic acids		
Dihydrocaffeic acid; 3-(3′,4′-Dihydroxyphenyl)propanoic acid	✓	
Dihydrocaffeic acid-3′-S; 3-(4′-Hydroxyphenyl)propanoic acid-3′-S	✓	✓
Dihydroferulic acid; 3-(4′-Hydroxy-3′-methoxyphenyl)propanoic acid	✓	✓
Dihydroferulic acid-4′-S; 3-(3′-Methoxyphenyl)propanoic acid-4′-S	✓	✓
Dihydroferulic acid-4′-GlcUA; 3-(3′-Methoxyphenyl)propanoic acid-4′-GlcUA	✓	✓
Dihydroisoferulic acid; 3-(3′-Hydroxy-4′-methoxyphenyl)propanoic acid	✓	
Dihydroisoferulic acid-3′-GlcUA; 3-(4′-Methoxyphenyl)propanoic acid-3′-GlcUA	✓	
Dihydrocoumaric acid-S; 3-(Phenyl)propanoic acid-4′-S		✓
Catechols		
Catechol-S*; Hydroxybenzene-S*	✓	

Figure 10. Overview of the main metabolites quantified in blood/urine samples following HCA intake by healthy humans. C_6-C_3 unsaturated cinnamic acids include compounds quantified in biofluids after consumption of other phytochemicals. Different yellow scales indicate the belonging of metabolite at each category (main blood, main urine, both main blood and urine).

5. Methods

5.1 Search strategy and study selection

This systematic review was reported in line with the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement guidelines (Moher et al., 2009; Page et al., 2021). The systematic literature search was conducted using PubMed, Scopus, and the Web of Science databases in April 2022, using the syntaxes reported in Supplementary Table S1. Any temporal or spatial filters were applied to the search. Studies were included in the present systematic review provided (i) they were human studies investigating the ADME of HCAs in healthy subjects, (ii) volunteers consumed single or repeated (multiple) dose(s) of HCAs through a dietary source, an extract or a pure compound, (iii) they provided a quantitative characterization of the total content of ingested precursor compounds, (iv) native HCAs and their derived metabolites were quantified in plasma, serum, and/or urine samples without applying a hydrolysis step to remove phase-2 conjugating moieties, and (v) at least one nutrikinetic parameter is reported, namely peak plasma concentration (C_{max}), time to reach C_{max} (T_{max}), area under the curve (AUC), elimination half-life ($t_{½}$), total cumulative urinary excretion, or urinary excretion (expressed as % of intake), for native HCAs and their circulating metabolites. Exclusion criteria included (i) the consumption of HCAs through a mixture of different HCA sources, and (ii) studies reported in a non-European language. No restrictions for the characteristics of study participants for age, sex and ethnicity were applied.

5.2 Data extraction

Two author pairs independently assessed the studies for their inclusion. Disagreement between authors was resolved through consultation with a third author. Data were extracted from each identified study using a standardized form and the following information was collected: first author name; publication year; type of study (intervention or observational); characteristics of the circulating compound (i.e. chemical name, molecular weight, PhytoHub ID (https://phytohub.eu/)) and type of biofluid(s) (i.e. plasma, serum, urine) in which it was quantified; origin of HCA metabolite [unchanged (when the native HCA did not undergo any metabolic step following its ingestion), host metabolism (when the compound derived from a biotransformation by small intestine, hepatic or renal phase 1 or phase 2 enzymes), gut microbiota metabolism (when the compound derived from HCA metabolism through gut microbiota activity), host and gut microbiota metabolism (when the compound derived from HCA metabolism through gut microbiota activity and further conjugation by a phase 2 enzyme); chemical name of the precursor compound(s) of the metabolite [as i) single compound when it was clearly a precursor of that metabolite, or ii) class when various compounds belonging at HCA and/or other phytochemical classes were putative precursors of the same metabolite]; classification (i.e. food, pure compound, extract) and description of the ingested HCA source; type of ingested dose(s) (i.e. single or repeated (multiple)); intervention duration (for studies in which multiple doses were ingested); ingested amount (µmol) of total precursor compounds (for multiple dose studies, the total daily dose was provided); description of the study population (i.e. number of subjects, sex, age, body mass index (BMI), and ethnicity, if available); and published values (i.e. mean, concentration unit, dispersion parameter type, dispersion parameter value, and time covered for AUC) for nutrikinetic parameters (i.e. T_{max}, C_{max}, AUC, and $t_{1/2}$) and urinary excretion data (expressed as cumulative excreted amount and/or % of intake) of the circulating compounds. Data on circulating compounds presented as mean and/or sum of metabolites belonging to different chemical species but grouped based on their chemical structure were excluded. On the other hand, data on simple phenolic acids that were not strictly related to HCA intake, due to their putative production through the metabolism of other dietary compounds (Del Rio et al., 2013; Rodriguez-Mateos et al., 2014; Selma et al., 2009), were not collected. Finally, only data on unconjugated and phase-2 conjugated forms of C_6-C_3 unsaturated cinnamic acids were collected.

5.3 Data analysis

Data were analyzed according to (Di Pede et al., 2022) with minor modifications. Chemical names of circulating metabolites were standardized following the recommendations of Kay and colleagues (Kay et al., 2020). If the total amount (µmol) of ingested precursor compounds was not reported in the manuscript, it was calculated by summing the amount ingested of individual compounds, ignoring those which accounted for less than 5 % of the total consumed precursors. Nutrikinetic parameters and urinary excretion data for each metabolite were processed to obtain the following parameters (using harmonized units): (i) C_{max} (nmol/L); (ii) T_{max} (h); (iii) AUC (nmol/L*h); (iv) $t_{1/2}$ (h); (v) urinary excretion expressed as cumulative excreted amount (µmol), calculated by summing the excreted amounts over different time intervals when it was not reported; (vi) % of intake, calculated as the ratio between the cumulative urinary excretion (µmol) of the metabolite and the total intake (µmol) of ingested precursor compounds when no directly reported [urinary excretion data (expressed as % of intake) > 100%, possibly due to underestimations of the ingested dose of precursor compounds or to overestimations of the excreted amount occurring when metabolites were quantified without the proper reference standards (Ottaviani et al., 2018), were excluded]; and (vii) average concentration (C_{avg}) (nmol/L) as the ratio between AUC $(\text{nmol/L*h})_{(0\text{-t})}$ and the total number of hours considered for AUC calculation (Mena et al., 2021) (when the time interval employed for AUC calculation was equal to 0-inf, it was considered as 24 h). When a circulating compound in a publication had a C_{avg} value exceeding its C_{max} value, C_{avg} value was excluded due to its low physiological relevance. C_{avg} values that could not be compared with their respective C_{max} values due to the absence of published C_{max} values were excluded. C_{max}, AUC and C_{avg} values for each circulating compound were also normalized by dividing their value by the dose (µmol) of ingested parent compounds (Mullen et al., 2009; Di Pede et al., 2022); in the case of multiple-dose studies, values of C_{max}, AUC and C_{avg} were normalized by using the total daily amount (µmol) of consumed native compounds. Normalized C_{max} values [C_{max} (nmol/L)/ingested µmol of parent compounds] were used for comparisons among studies to determine the main circulating blood metabolites of HCAs, thus avoiding any bias related to the dose-response relationship existing in the production of phenolic metabolites (Favari et al., 2020; Feliciano et al., 2017; Rodriguez-Mateos et al., 2016a, 2016b). Mean normalized C_{max} value ≥ 0.4 nmol/L was selected as the threshold value to define the main circulating forms of blood HCA metabolites. This value was established by ranking the metabolites according to their normalized C_{max} values and considering C_{max} values reached in the context of regular HCA dietary intake (Farah et al., 2008; Gómez-Juaristi et al., 2018; Lang et al., 2013; Stalmach et al., 2014, 2012, 2009). Mean urinary excretion value ≥ 1.5 % of intake was selected as the threshold value to define the main urinary HCA metabolites. Finally, to ensure data robustness, the main blood and urinary metabolites of HCAs were selected when their mean normalized C_{max} and urinary excretion (% of intake) values were calculated using at least three biological replicates deriving from at least two manuscripts. In accordance with (Ou et al., 2014; Pede et al., 2022; Stoupi et al., 2009; Di Pede et al., 2022), molar mass recoveries in the production of the main urinary HCA metabolites were calculated comparing the mean value of ingested HCAs (µmol) with the mean cumulative urinary excretion for each metabolite (µmol) expressing data as percentage (%). Stoichiometric balances in the production of the main urinary HCA metabolites were estimated through molar mass recoveries assuming the production of each compound from 1 µmol of ingested parent HCAs. When data on HCA bioavailability (%) was not reported in the manuscript, it was calculated by computing the ratio between the total HCA metabolite urinary excretion (µmol) and the total intake (µmol) of parent HCAs for each ingested source. Values for HCA bioavailability (published and/or estimated) deriving from each study were averaged to provide a mean bioavailability value, while excluding bioavailability data if they were i) < 1 and/or > 100%, or ii) calculated by excluding an exhaustive panel of host gut microbiota metabolites produced after HCA intake. Finally, to unravel the contribution of each metabolite class to the overall bioavailability of HCAs, for each study and each ingested source of HCAs, the bioavailability of the classes of HCA metabolites was calculated by computing the ratio between the total excreted µmol of each metabolite class and the ingested µmol of HCAs and thus bioavailability values for each metabolite class were averaged. Data on blood and urinary metabolites and on the bioavailability of HCAs were expressed as mean ± standard deviation (SD) and median (25th-75th percentile).

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

GDP: conceptualization (equal); data curation and formal analysis (equal); visualization (equal); writing-original draft preparation (lead). PM: conceptualization (equal); data curation and formal analysis (equal); visualization (equal); writing—review & editing (lead). LB: writing—review & editing (equal). MA: writing—review & editing (equal). RL-R: writing—review & editing (equal). RE: writing—review & editing (equal). RL: writing—review & editing (equal). SK: writing—review & editing (equal). DW: writing—review & editing (equal). ARM: writing—review & editing (equal). MC: writing—review & editing (equal). AC: writing—review & editing (equal). CM: writing—review & editing (equal). DDR: conceptualization (equal); writing—review & editing (lead).

Conflict of Interest statement

The authors declare no conflict of interest.

Funding statement

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Supplementary Material

Supplementary Table S1; Supplementary Table S2; Supplementary Table S3; Supplementary Table S4; Supplementary Table S5; Supplementary Table S6; Supplementary Figure S1; Supplementary Figure S2; Supplementary Figure S3; Supplementary Figure S4; Supplementary excel file.

References

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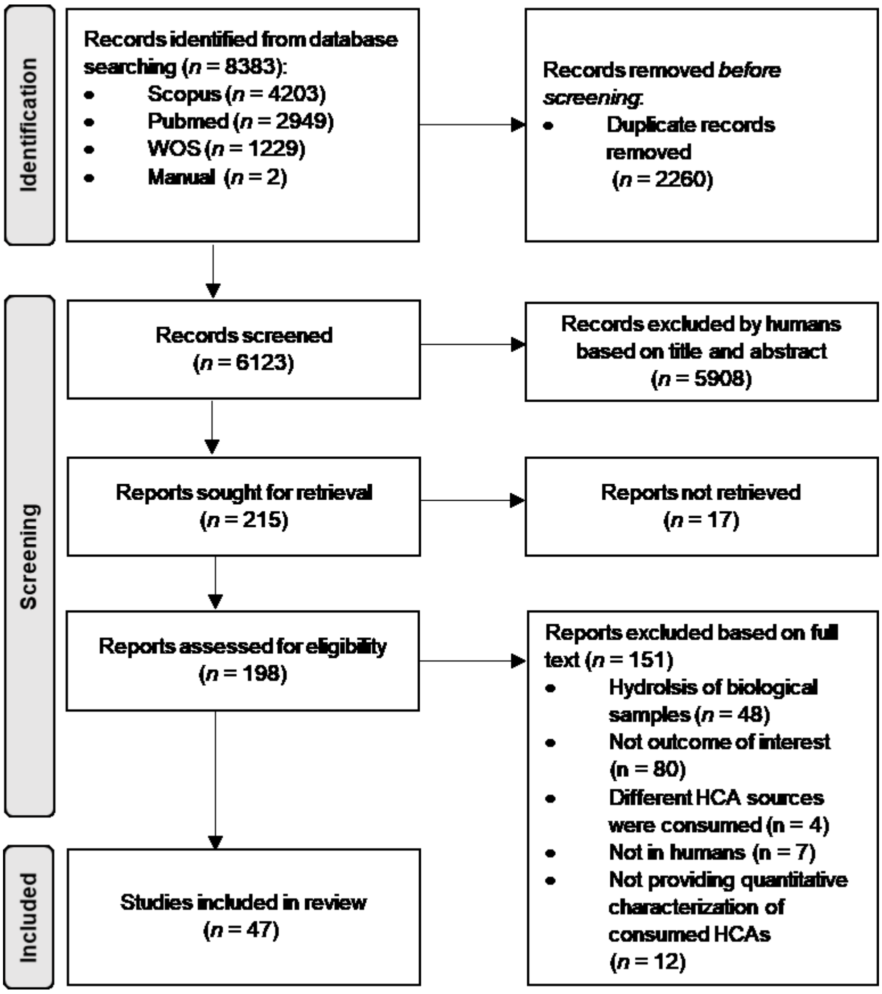
Supplementary Material

Supplementary Table S1. Syntaxes used to perform the literature search.

DATABASE	***SYNTAX***
PubMed	(cataboli*[TIAB] OR absor*[TIAB] OR absorption[MeSH] OR excret*[TIAB] OR “Drug Elimination Routes”[MeSH] OR biotransform*[TIAB] OR biotransformation[MeSH] OR bioavailab*[TIAB] OR “Biological Availability”[MeSH] OR *kinetic*[TIAB] OR pharmacokinetics[MeSH] OR ADME[TIAB] OR conjugat*[TIAB] OR glucuroni*[TIAB] OR sulfate[TIAB] OR sulphate[TIAB] OR (microbi*[TIAB] AND metabolit*[TIAB])) AND (humans[MeSH] OR subjects[TIAB] OR men[TIAB] OR male[MeSH] OR women[TIAB] OR female[MeSH] OR male[TIAB] OR female[TIAB] OR patient*[TIAB] OR volunteer*[TIAB] OR participant*[TIAB] OR population[TIAB] OR cohort [TIAB]) AND (“acyl-quinic acid”[TIAB] OR *cinnam*[TIAB] OR "cinnamates"[Mesh] OR *caffeoylquinic*[TIAB] OR *feruloylquinic*[TIAB] OR *coumaroylquinic*[TIAB] OR *chlorogenic*[TIAB] OR "chlorogenic acid"[Mesh] OR caffeic*[TIAB] OR "caffeic acids"[Mesh] OR ferulic*[TIAB] OR coumaric*[TIAB] OR "coumaric acids"[Mesh] OR chicoric*[TIAB] OR “sinapic acid” [TIAB])
Web of Science	(cataboli* OR absor* OR excret* OR biotransform* OR bioavailab* OR *kinetic* OR adme OR conjugat* OR glucuroni* OR sulfate OR sulphate OR (microbi* AND metabolit*)) AND (men OR women OR patient* OR volunteer* OR participant* OR subject* OR male OR female OR population OR cohort) AND (“acyl-quinic acid” OR *cinnam* OR *caffeoylquinic* OR *feruloylquinic* OR *coumaroylquinic* OR *chlorogenic* OR caffeic* OR ferulic* OR coumaric* OR chicoric* OR “sinapic acid”)
Scopus	(cataboli* OR absor* OR excret* OR biotransform* OR bioavailab* OR *kinetic* OR adme OR conjugat* OR glucuroni* OR sulfate OR sulphate OR (microbi* AND metaboli*)) AND (men OR women OR patient* OR volunteer* OR participant* OR subject* OR male OR female OR population OR cohort) AND (“acyl-quinic acid” OR *cinnam* OR *caffeoylquinic* OR *feruloylquinic* OR *coumaroylquinic* OR *chlorogenic* OR caffeic* OR ferulic* OR coumaric* OR chicoric* OR “sinapic acid”)



Supplementary Figure S1. Flowchart of the study selection process. Legend: WOS: Web of Science.

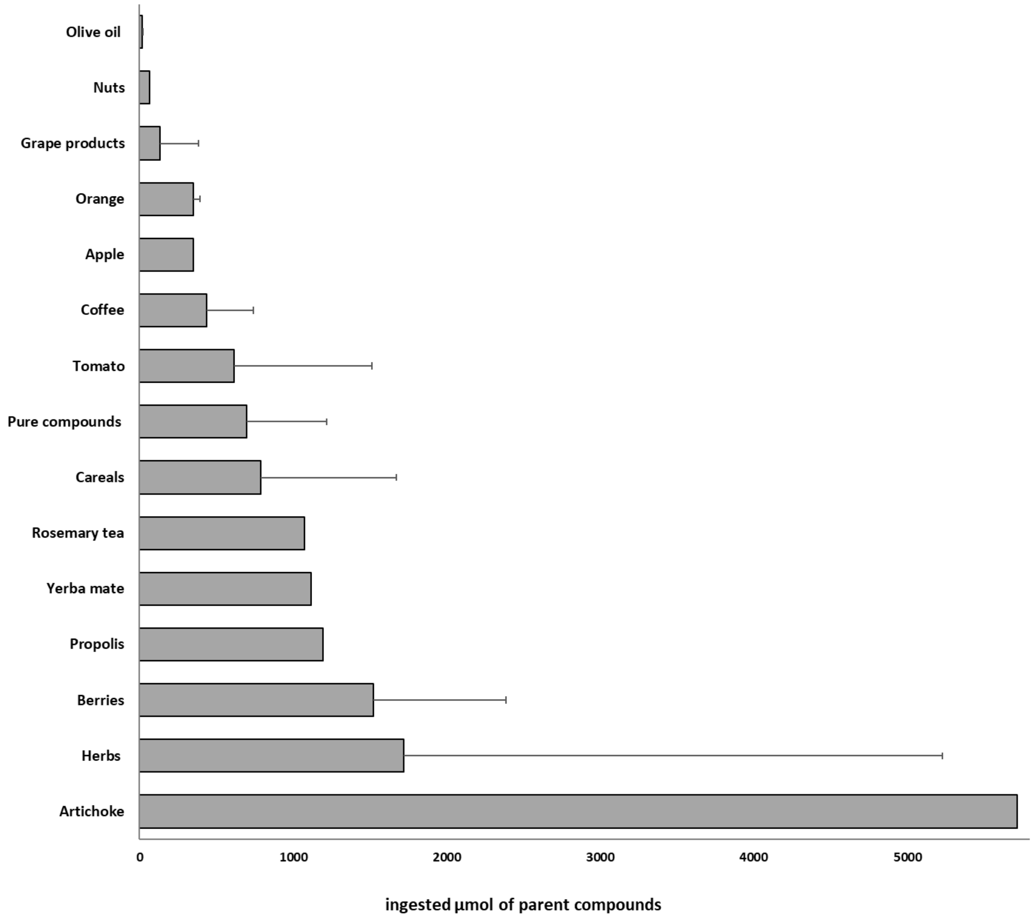
Supplementary Table S2. Characteristics of the human intervention studies that met inclusion criteria and underwent data analyses.

Dietary source classification	Dietary source description	Total ingested parent compounds (µmol)	Dose type	Study duration	Sample size	Description of population	Ref.
food	200 mL of coffee (3.4 g of instant powder)	412	S	-	11	8 M; 3 F; Age=19-35 y; BMI=24.3±2.3 kg/m^2	(Stalmach et al., 2009)
food	160 g of bran-enriched bars	891	S	-	16	8 M; 8 F; Age=26.6±7.5 y; BMI=21.9±2.1 kg/m^2	(Gamel et al., 2019)
food	120 g of bran-enriched crackers	669					
food	4.91 g of yerba mate in 250 mL water	1118	S	-	12	7 M (Age=27.86±3.48 y; BMI=23.42±2.52 kg/m^2); 5 F (Age=28.88±3.56 y; BMI=22.43±3.33 kg/m^2)	(Gómez-Juaristi et al., 2018)
food	350 mL of coffee (18.66 g of coffee)	519	S	-	13	6 F (Age=24–28 y); 7 M (Age=27–30 y)	(Lang et al., 2013)
extract	480 mg of green coffee bean extract	291 131 123	S	-	15	M	(Morton et al., 2018)
food	500 g of tomato 250 g of tomato sauce 250 g of tomato sauce + refined olive oil (5%)	20 23 23	S	-	40	19 M; 21 F; Age=28±11 y; BMI=23.3±3.8 kg/m^2	(Martínez-Huélamo et al., 2016)
food	200 mL of instant coffee beverage 200 mL of coffee + 2 bread rolls + honey 200 mL of coffee + 1 bread roll + peanut butter	48	S	-	14	7 M; 7 F; Age=29.36±8.06 y; BMI=22.95±1.72 kg/m^2; Caucasians	(Scherbl et al., 2017)
extract	Coffee tablets	933	M	4 weeks (3 doses daily)	11	9 F; 2 M; Age=28±6 y; BMI=21.8±1.6 kg/m^2	(Mena et al., 2019)
food	200 mL of instant coffee beverage	412 635 795	S	-	11	8 M; 3 F; Age=19-35 y; BMI=24.3±2.3 kg/m^2	(Stalmach et al., 2014)
food	250 mL of coffee (3.5 g of green-roasted coffee)	761	S	-	12	7 M (Age=27.86±3.48 y; BMI=23.42±2.52 kg/m^2); 5 F (Age=28.88±3.56 y; BMI=22.43±3.33 kg/m^2)	(Gómez-Juaristi et al., 2018)
food	100 mL of water + 10 g of dried rosemary leaves	1074	S	-	12	8 F (Age=36±10 y; BMI=24±2 kg/m^2); 4 M (Age=24±3 y; BMI=23±2 kg/m^2)	(Achour et al., 2021)
extract	250 mL of red grape pomace aqueous extract	3709	S	-	10	10 M; Age=26±2 y; BMI=27±3 kg/m^2	(Castello et al., 2018)
food	300 g of homogenized raspberries	292	S	-	9	5 F; 4 M. Age=22-44 y. BMI=24.6±5.0 kg/m^2	(Ludwig et al., 2015)
food	wild blueberry beverage	962	S	-	12	6 M; 6 F. Age=29±5 y; BMI=28.8±2.2 kg/m^2. 1 Hispanic/african/american mixed; 2 caucasians; 2 hispanic; 2 asian; 5 african/american mixed.	(Zhong et al., 2017)
food	100g of tomato sauce without oil 100g of tomato sauce with 5% virgin olive oil 100g of tomato sauce with 5% refined oil	2049 1665 1695	S	-	5	M; Age=25-36 y; BMI=25±1.2 kg/m^2	(Tulipani et al., 2012)
food	450 mL of cranberry juice	1233	S	-	10	M; Age=18-35 y	(Feliciano et al., 2016)
food	94 g of wheat bread enriched in aleurone	222	S	-	15	8 M; 7 F; Age=26±4 y; BMI=21±3 kg/m^2	(Bresciani et al., 2016)

	fraction (6% w/w of aleurone)						
	190 g of bread enriched in aleurone fraction (6% w/w of aleurone)	448					
	94 g of wholegrain bread	448					
food	238 mL of hazelnut skin infusion	66	S	-	39	10 M; 29 F; Age=40.2±14.2 y; BMI=22.7±2 kg/m^2	(Mocciaro et al., 2019)
food	450 mL of cranberry drink	641 1234 1940 2404 2994	S	-	10	Age=18-35 y	(Feliciano et al., 2017)
food	100g bread + 1 g free curcumin 100g bread + 1 g encapsulated curcumin 100g bread + 1 g encapsulated curcumin + polyphenols	2703	S	-	10	Age=31±2 y; BMI=23.5±1.2 kg/m^2	(Vitaglione et al., 2012)
pure compound	500 mg of gelatin capsules of ^{13}C-labelled-cyanidin-3-glucoside	1033	S	-	7	na	(Ferrars et al., 2014)
food	350 mL of 100% Concord grape juice	155	S	-	8	3 M; 5 F. Age=20–40 y; BMI=20.7–26.1 kg/m^2	(Stalmach et al., 2012)
food	1 cup of coffee	205	M	1 month (1 daily dose)	21	11 F; 10 M; Age=22.9 ± 0.5; BMI: 22.3 ± 1.7 kg/m^2	(Mena et al., 2021)
	3 cups of coffee	615	M	1 month (3 dose daily)			
	1 cup of coffee + 2 cocoa products containing coffee	296	M	1 month (2 dose daily)			
food	200 g of sous-vide artichokes	5715	S	-	8	5 F; 3 M; Age=26.4±2.8; BMI: 22.8±2.5 kg/m^2	(Domínguez-Fernández et al., 2022)
food	500 mL of orange juice	329	S	-	9	7 F, 2 M; Age: 21-55 y; BMI: 21.1 ± 2.4 kg/m^2	(Castello et al., 2020)
	500 mL of fermented orange juice	418					
food	400 mL of istant coffee	900	S	-	9	4 M, 5 F; Age: 27-41 y	(Farrell et al., 2012)
pure compound	300 mg of pure 1,5-Dicaffeoylquinic acid	580	S	-	10	Chinese; 5 M; 5 F;	(Liu et al., 2010)
food	Coffee (200 mL) containing 3.4 g of powdered instant coffee	412	S	-	11	8 M; 3 F; age=19-35 y; BMI= 24.3±2.3 kg/m^2	(Wong et al., 2010)
food	3.6 g of ground coffee + 50 mL water	778 214	S	-	15	Age=26.3 ± 1.6 y; BMI=23.5 ± 0.5 kg/m^2	(Mills et al., 2017)
extract	2 capsules of Guizhi Fuling	2	S	-	10	F	(Zhong et al., 2016)
	3 capsules of Guizhi Fuling	8					
extract	Melissa Officinalis capsules	694 1389 1389	S	-	6	na	(Noguchi-shinohara et al., 2015)
food	4 g "Green Blend" instant Coffee	882	S	-	36	na	(Kerimi et al., 2020)
food	100 mL of red wine	5	S	-	5	M; Age=35±6 y; BMI=24.1±1.4 kg/m^2	(Simonetti et al., 2001)
	200 mL of red wine	10					
	300 mL of red wine	15					
pure compound	600 mg of 1,5-Dicaffeoylquinic acid	1163	S	-	5	na	(Gu et al., 2007)

extract	9.6 g of Gumiganghwal-tang tablets + 240 mL water	0.4	S	-	12	Korean; M; Age=21-32 y	(Jeong et al., 2021)
food	60 mL of guaco syrup	10274	S	-	5	2 F; 3 M	(Gasparetto et al., 2015)
extract	7.2 g of Socheongryong-tang tablets + 240 mL water	5	S	-	13	Korean; M; Age=20-55 y;	(Jeong et al., 2018)
extract	2.4 g of Shuanghua Baihe tablets	10	S M	- 3 daily doses (5 days)	12	Chinese; 6 M; 6 F	(Pan et al., 2016)
extract	3 capsules containing 360 mg of brazilian green propolis	1196	S	-	12	6 M; 6 F; Age F= 30.0 ± 6.5 y; age M=34.8 ± 9.5 y; BMI F=20.3 ± 0.9 kg/m^2; BMI M= 21.6 ± 1.4 kg/m^2	(Yamaga et al., 2021)
food	500 g of raw tomatoes 250 g of raw tomato sauce 250 g of refined olive oil-enriched tomato sauce	14 19 20	S	-	8	4 F; Age=27-46 y; BMI= 23 ± 0.93 kg/m^2	(Martínez-Huélamo et al., 2015)
food	700 mL of Brettacher apple juice	352	S	-	10	5 M; 5 F; Age=24-30 y	(Bitsch et al., 2001)
food	60 g oat bran porridge+200 mL semi-skimmed milk+100 mL water	131	S	-	7	M; Age=25-62 y	(Schär et al., 2018)
food	500 mL of orange juice	330	S	-	10	M; BMI= 21.7±0.6 kg/m^2	(Pereira-Caro et al., 2017)
food	500 mL of orange juice	330	S	-	10	M; BMI= 21.7±0.6 kg/m^2	(Pereira-Caro et al., 2020)
food	30 mL of virgin olive oil 30 mL of enriched virgin olive oil	13 20	S	-	13	7 M; 6 F; Age= 25-69 y; BMI men=26.7±1.9 kg/m^2; BMI women=24.9±1.5 kg/m^2	(Suárez et al., 2011)
food	450 mL of cranberry juice	641 1233 1940 2404 2993	S	-	10	M; Age=18-32 y	(Rodriguez-Mateos et al., 2016)
food	9 g of cranberry powder + 500 mL water	951	S M	- 1 month (1 dose daily)	45	M; Age=18-45 y	(Heiss et al., 2022)

Y: years; BMI: body mass index; S: single; Mu: multiple; na: not available; M: male; F: female.



Supplementary Figure S2. Administered doses (µmol) of total parent compounds through the intervention studies that evaluated ADME of HCA. Data are mean and SD. Parent compounds sources (n of doses; minimum and maximum values for ingested µmol of total parent compounds): artichoke (1; 5715 µmol); herbs (8; 0.4-10274.0 µmol); berries (15; 292.0-2993.6 µmol); propolis (1; 1196.0 µmol); yerba mate (1; 1118.0 µmol); rosemary tea (1; 1074.3 µmol); cereals (8; 131.4-2703.0 µmol); pure compounds (4; 10.3- 1162.8 µmol); tomato (9; 14.1-2048.9 µmol); coffee (21; 48.0-933.0 µmol); apple (1; 351.7 µmol); orange (4; 329.0-418.0 µmol); grape products (5; 5.0-3709.2 µmol); nuts (1; 66.2 µmol); olive oil (2; 13-20 µmol).

Supplementary Table S3. Pharmacokinetic parameter data for the main blood circulating C_6-C_3 unsaturated cinnamic acids and their metabolites, quantified following HCA intake by healthy subjects. C_6-C_3 unsaturated cinnamic acids were also quantified after consumption of other phytochemicals. Data are reported as mean ± SD (n indicates the number of biological values collected from literature for each parameter for the same compound). The main plasma circulating compounds were selected based on a normalized C_{max} value ≥ 0.4 nmol/L, calculated using at least 3 biological replicates deriving from at least 2 manuscripts. Metabolites are reported both with their chemical name and systematic name according to (Kay et al., 2020). Box plot for C_{max} and T_{max} of compounds highlighted in blue are reported in Figures 5 and 6.

Metabolites [Chemical name; systematic name]	C_{max} (nmol/L)	C_{max} normalized ((nmol/L)/total µmol of ingested parental compounds)	T_{max} (h)	AUC (nmol/L*h)	AUC normalized ((nmol/L*h)/ total µmol of ingested parental compounds)	C_{avg} ((nmol/L*h)/n hours)	C_{avg} normalized ((nmol/L*h)/ total µmol of ingested parental compounds /n hours)	$t_{1/2}$ (h)
C_6-C_3 unsaturated cinnamic acids								
Caffeic acid; 3′,4′-Dihydroxycinnamic acid	670.3 ± 1531.0 (n=16)	56.5 ± 107.3 (n=16)	4.4 ± 4.7 (n=14)	2562.7 ± 5683.0 (n=12)	151.9 ± 355.5 (n=12)	493.2 ± 1143.3 (n=12)	30.2 ± 71.2 (n=12)	1.6 ± 0.2 (n=2)
Caffeic acid-3′-S; 4′-Hydroxycinnamic acid-3′-S	288.3 ± 792.4 (n=12)	0.4 ± 0.4 (n=12)	1.0 ± 0.4 (n=12)	2506.2 ± 7437.4 (n=10)	1.1 ± 1.3 (n=10)	105.0 ± 309.7 (n=10)	0.1 ± 0.1 (n=10)	1.3 ± 0.4 (n=4)
Dimethylcaffeic acid; 3′,4′-Dimethoxycinnamic acid	123.3 ± 168.1 (n=7)	0.6 ± 0.5 (n=7)	0.8 ± 0.3 (n=7)	169.9 ± 115.0 (n=4)	3.5 ± 2.4 (n=4)	11.3 ± 7.7 (n=4)	0.2 ± 0.2 (n=4)	-
Ferulic acid; 4′-Hydroxy-3′-methoxycinnamic acid	140.8 ± 216.9 (n=23)	3.7 ± 11.4 (n=23)	3.7 ± 3.5 (n=22)	2017.9 ± 4813.9 (n=16)	9.5 ± 24.0 (n=16)	83.5 ± 139.5 (n=16)	1.7 ± 4.8 (n=16)	32.5 ± 15.7 (n=2)
Ferulic acid-4′-S; 3′-Methoxycinnamic acid-4′-S	965.8 ± 1707.3 (n=47)	17.1 ± 78.0 (n=47)	2.1 ± 1.8 (n=45)	3551.9 ± 6049.3 (n=33)	69.0 ± 268.1 (n=33)	329.3 ± 884.2 (n=33)	13.5 ± 53.7 (n=33)	6.3 ± 6.0 (n=11)
Ferulic acid-4′-GlcUA; 3′-Methoxycinnamic acid-4′-GlcUA	204.4 ± 203.4 (n=42)	4.9 ± 9.7 (n=42)	3.5 ± 3.0 (n=40)	979.9 ± 1154.4 (n=37)	20.9 ± 51.5 (n=37)	67.3 ± 125.6 (n=37)	2.2 ± 7.1 (n=37)	5.7 (n=1)
isoFerulic acid; 3′-Hydroxy-4′-methoxycinnamic acid	1493.9 ± 2429.5 (n=19)	2.6 ± 3.6 (n=19)	4.3 ± 4.5 (n=19)	29510.2 ± 43861.0 (n=14)	21.6 ± 38.9 (n=14)	1232.3 ± 1825.6 (n=14)	0.9 ± 1.6 (n=14)	-
isoFerulic acid-3′-GlcUA; 4′-Methoxycinnamic acid-3′-GlcUA	199.1 ± 200.6 (n=18)	0.4 ± 0.4 (n=18)	2.9 ± 2.4 (n=18)	1349.9 ± 1165.1 (n=14)	2.5 ± 2.0 (n=14)	69.3 ± 74.9 (n=14)	0.1 ± 0.1 (n=14)	-
t-Cinnamic acid; Cinnamic acid	99.8 ± 41.0 (n=7)	4.4 ± 11.5 (n=7)	5.1 ± 4.8 (n=7)	1345.5 ± 796.3 (n=7)	9.3 ± 21.8 (n=7)	56.1 ± 33.2 (n=7)	0.4 ± 0.9 (n=7)	2.8 (n=1)
Coumaric acid-4′-S; Cinnamic acid-4′-S	768.3 ± 654.0 (n=3)	47.8 ± 43.3 (n=3)	1.6 ± 0.6 (n=2)	2756.9 ± 2376.1 (n=3)	169.1 ± 149.5 (n=3)	548.5 ± 480.1 (n=3)	33.8 ± 29.9 (n=3)	-
Phenylpropanoic acids								
Dihydrocaffeic acid; 3-(3′,4′-Dihydroxyphenyl)propanoic acid	53.1 ± 19.8 (n=9)	0.5 ± 0.7 (n=9)	6.4 ± 1.0 (n=9)	237.1 ± 222.8 (n=9)	2.0 ± 2.9 (n=9)	12.0 ± 10.7 (n=9)	0.1 ± 0.2 (n=9)	1.9 ± 1.2 (n=4)
Dihydrocaffeic acid-3′-S; 3-(4′-Hydroxyphenyl)propanoic acid-3′-S	207.8 ± 155.5 (n=10)	1.5 ± 1.8 (n=10)	6.8 ± 1.3 (n=10)	1507.8 ± 1447.9 (n=10)	7.3 ± 8.1 (n=10)	68.8 ± 58.8 (n=10)	0.4 ± 0.6 (n=10)	2.6 ± 0.4 (n=4)
Dihydroferulic acid; 3-(4′-Hydroxy-3′-methoxyphenyl)propanoic acid	448.9 ± 164.9 (n=10)	3.2 ± 3.9 (n=10)	6.1 ± 1.4 (n=9)	2128.7 ± 1512.1 (n=9)	15.6 ± 17.6 (n=9)	104.4 ± 64.0 (n=9)	1.0 ± 1.2 (n=9)	1.9 ± 0.3 (n=4)
Dihydroferulic acid-4′-S; 3-(3′-Methoxyphenyl)propanoic acid-4′-S	81.6 ± 43.6 (n=13)	0.6 ± 0.9 (n=13)	6.4 ± 1.2 (n=12)	489.9 ± 462.0 (n=12)	3.0 ± 3.8 (n=12)	23.2 ± 19.5 (n=12)	0.2 ± 0.3 (n=12)	3.6 ± 0.8 (n=4)
Dihydroferulic acid-4′-GlcUA; 3-(3′-Methoxyphenyl)propanoic acid-4′-GlcUA	91.0 ± 27.3 (n=7)	1.1 ± 1.2 (n=7)	7.7 ± 1.1 (n=5)	326.1 ± 294.5 (n=5)	6.8 ± 6.2 (n=5)	21.8 ± 19.6 (n=5)	0.5 ± 0.4 (n=5)	-
Dihydroisoferulic acid; 3-(3′-Hydroxy-4′-methoxyphenyl)propanoic acid	165.6 ± 107.5 (n=5)	3.0 ± 2.8 (n=5)	7.2 ± 1.4 (n=5)	579.1 ± 527.2 (n=5)	12.0 ± 11.0 (n=5)	38.6 ± 35.1 (n=5)	0.8 ± 0.7 (n=5)	-
Dihydroisoferulic acid-3′- GlcUA; 3-(4′-Methoxyphenyl)propanoic acid-3′-GlcUA	37.2 ± 8.5 (n=8)	0.4 ± 0.4 (n=8)	8.3 ± 1.1 (n=8)	225.5 ± 176.1 (n=8)	2.0 ± 1.7 (n=8)	11.2 ± 7.3 (n=8)	0.1 ± 0.1 (n=8)	-
Catechols								
Catechol-S*; Hydroxybenzene-S*	914.9 ± 1037.4 (n=4)	2.2 ± 1.8 (n=4)	3.8 ± 2.1 (n=4)	2903.6 ± 907.0 (n=3)	9.0 ± 4.0 (n=3)	121.0 ± 37.8 (n=3)	0.4 ± 0.2 (n=3)	-

GlcUA: glucuronide; S: sulfate; C_{max}: maximum plasma concentration; T_{max}: time to reach C_{max}; AUC: area under the curve; C_{avg}: average concentration; $t_{1/2}$: half elimination time; - means any data was collected for that pharmacokinetic parameter; *symbol: when the position of the conjugation is unknown,

Supplementary Table S4. Urinary excretion (% of intake) data for the main urine acyl-quinic acids, C_6-C_3 unsaturated cinnamic acids and their metabolites. C_6-C_3 unsaturated cinnamic acids were also quantified after consumption of other phytochemicals. The main urine compounds were selected based on a urinary excretion value, expressed as percentage of intake ≥ 1.5 %, calculated using at least 3 biological replicates deriving from at least 2 manuscripts. Data are reported as mean ± SD (*n* indicates the number of biological values collected from literature for each parameter for the same compound). Metabolites are reported both with their chemical name and systematic name according to (Kay et al., 2020). Box plot for urinary excretion (% of intake) of compounds highlighted in blue are reported in Figure 7.

Metabolites **[Chemical name; systematic name]**	**Urinary excretion (% of intake)**
Acyl-quinic acids	
3-Caffeoylquinic lactone-S*; 3-Caffeoylquinic lactone-S*	2.1 ± 1.3 (n=4)
4-Caffeoylquinic lactone-S*; 4-Caffeoylquinic lactone-S*	2.4 ± 1.5 (n=4)
3-Feruloylquinic acid; 3-Feruloylquinic acid	1.7 ± 2.5 (n=10)
C_6-C_3 unsaturated cinnamic acids	
Caffeic acid; 3′,4′-Dihydroxycinnamic acid	2.7 ± 4.9 (n=16)
Caffeic acid-GlcUA*; Hydroxycinnamic acid-GlcUA*	6.4 ± 9.5 (n=9)
Ferulic acid; 4′-Hydroxy-3′-methoxycinnamic acid	6.9 ± 11.8 (n=18)
Ferulic acid-4′-S; 3′-Methoxycinnamic acid-4′-S	2.6 ± 2.4 (n=27)
Ferulic acid-4′-GlcUA; 3′-Methoxycinnamic acid-4′-GlcUA	17.0 ± 27.7 (n=27)
Feruloylglycine; 3′-Methoxy-4′-hydroxycinnamoyl-glycine	4.3 ± 4.6 (n=18)
p-Coumaric acid; 4′-Hydroxycinnamic acid	2.3 ± 3.2 (n=8)
p-Coumaric acid-4′-GlcUA; Cinnamic acid-4′-GlcUA	2.9 ± 3.6 (n=7)
Phenylpropanoic acids	
Dihydrocaffeic acid-3′-S; 3-(4′-Hydroxyphenyl)propanoic acid-3′-S	5.2 ± 3.5 (n=11)
Dihydroferulic acid; 3-(4′-Hydroxy-3′-methoxyphenyl)propanoic acid	1.7 ± 0.6 (n=8)
Dihydroferulic acid-4′-S; 3-(3′-Methoxyphenyl)propanoic acid-4′-S	1.7 ± 1.0 (n=14)
Dihydroferulic acid-4′-GlcUA; 3-(3′-Methoxyphenyl)propanoic acid-4′-GlcUA	1.5 ± 0.6 (n=10)
Dihydrocoumaric acid-S; 3-(Phenyl)propanoic acid-4′-S	1.6 ± 0.8 (n=5)

GlcUA: glucuronide; S: sulfate; *symbol: when the position of the conjugation is unknown.

Supplementary Table S5. Stoichiometric balances for the main urine acyl-quinic acids, C_6-C_3 unsaturated cinnamic acids and their metabolites. The main urine compounds were selected based on a urinary excretion value, expressed as percentage of intake ≥ 1.5 %, calculated using at least 3 biological replicates deriving from at least 2 manuscripts. Metabolites are reported both with their chemical name and systematic name according to (Kay et al., 2020).

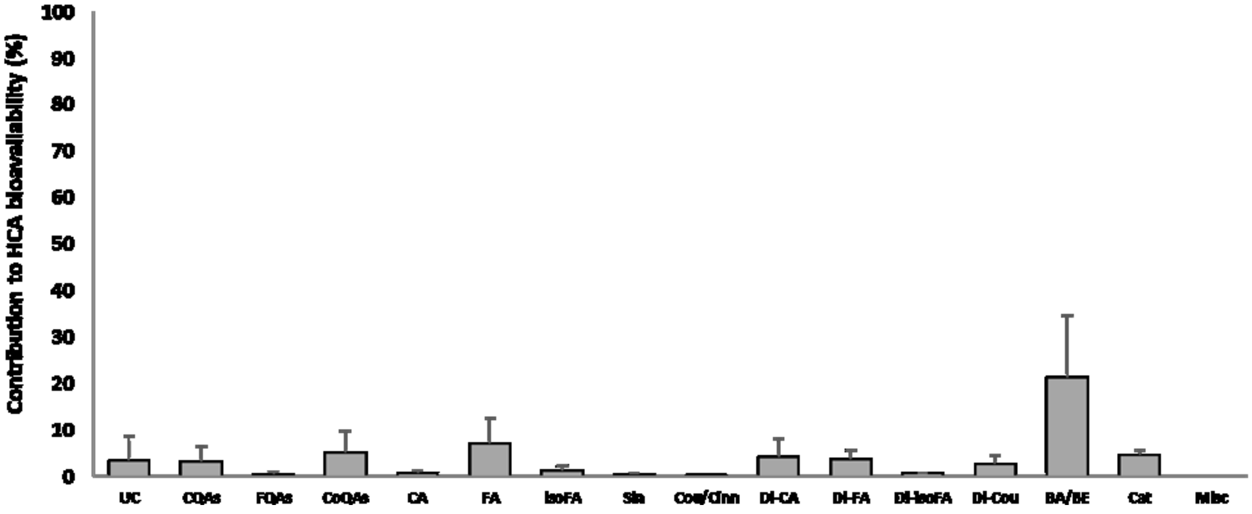
Metabolites **[Chemical name; systematic name]**	**n**	**Molar mass recovery (%)**	**Quantity of parent HCAs to achieve 1 µmol of metabolite in urine (µmol)**
Acyl-quinic acids			
3-Caffeoylquinic lactone-S*; 3-Caffeoylquinic lactone-S*	4	0.2	593
4-Caffeoylquinic lactone-S*; 4-Caffeoylquinic lactone-S*	4	0.1	727
3-Feruloylquinic acid; 3-Feruloylquinic acid	10	0.1	859
C_6-C_3 unsaturated cinnamic acids			
Caffeic acid; 3′,4′-Dihydroxycinnamic acid	3	0.1	1947
Caffeic acid-GlcUA*; Hydroxycinnamic acid GlcUA*	-	-	-
Ferulic acid; 4′-Hydroxy-3′-methoxycinnamic acid	4	0.9	117
Ferulic acid-4′-S; 3′-Methoxycinnamic acid-4′-S	16	1.4	69
Ferulic acid-4′-GlcUA; 3′-Methoxycinnamic acid-4′-GlcUA	7	0.8	119
Feruloylglycine; 3′-Methoxy-4′-hydroxycinnamoyl-glycine	15	4.4	23
p-Coumaric acid; 4′-Hydroxycinnamic acid	1	0.0	4531
p-Coumaric acid-4′-GlcUA; Cinnamic acid-4′-GlcUA	2	0.0	4474
Phenylpropanoic acids			
Dihydrocaffeic acid-3′-S; 3-(4′-Hydroxyphenyl)propanoic acid-3′-S	11	5.3	19
Dihydroferulic acid; 3-(4′-Hydroxy-3′-methoxyphenyl)propanoic acid	8	1.5	67
Dihydroferulic acid-4′-S; 3-(3′-Methoxyphenyl)propanoic acid-4′-S	14	1.8	55
Dihydroferulic acid-4′-GlcUA; 3-(3′-Methoxyphenyl)propanoic acid-4′-GlcUA	10	1.2	81
Dihydrocoumaric acid-S; 3-(Phenyl)propanoic acid-4′-S	5	1.3	80

n indicates the number of biological values collected from literature for the excreted µmol of each metabolite from which the molar masss recovery was calculated; molar mass recovery is calculated assuming the production of the metabolite from 1 µmol of ingested parent HCAs. GlcUA: glucuronide; S: sulfate; *symbol: when the position of the conjugation is unknown; -symbol: when the metabolite was quantified after intake of various phytochemical classes and molar mass recovery for it was not calculated.

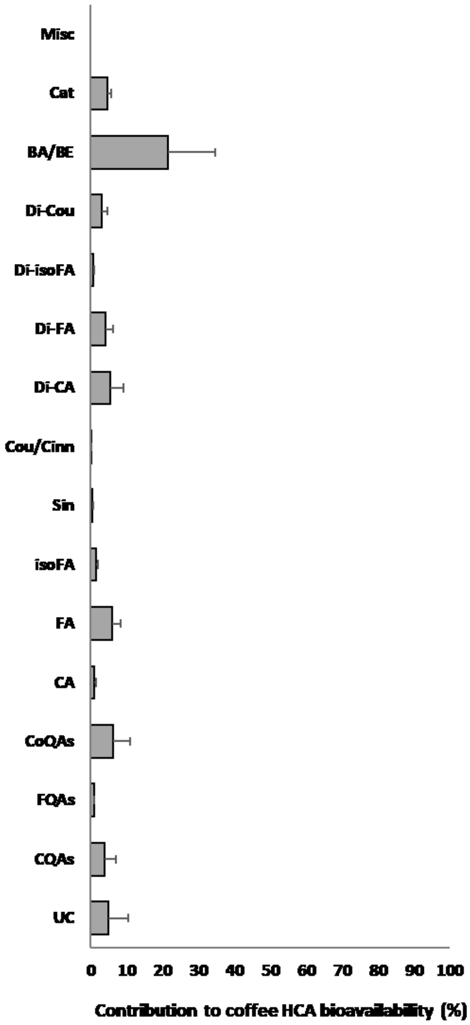
Supplementary Table S6. HCA bioavailability (%) values collected from literature and/or estimated from urinary excretion data employed to calculate the value for HCA bioavailability (%).

Bioavailability of HCAs (%)	HCA source	Ref
29	Coffee	(Stalmach et al., 2009)‡
13	Yerba mate	(Gómez-juaristi et al., 2018)‡
6	Coffee	(Mena et al., 2019)~*
24	Coffee	(Stalmach et al., 2014)‡
25	Coffee	(Stalmach et al., 2014)‡
16	Coffee	(Stalmach et al., 2014)‡
17	Coffee	(Gómez-Juaristi et al., 2018)‡
28	Wheat	(Bresciani et al., 2016)‡
18	Wheat	(Bresciani et al., 2016)‡
13	Wheat	(Bresciani et al., 2016)‡
70	Coffee	(Mena et al., 2021)‡
41	Coffee	(Mena et al., 2021)‡
67	Coffee	(Mena et al., 2021)‡
22	Coffee	(Wong et al., 2010)~
27	Coffee	(Kerimi et al., 2020)~
4	Artichoke	(Domínguez-Fernández et al., 2022)*~
6	Oat	(Schär et al., 2018)*~

‡symbol: indicates value for HCA bioavailability (%) collected from literature; ~ symbol: indicates values for HCA bioavailability (%) estimated from urinary excretion data; * symbol: bioavailability value was calculated taking into account the excreted µmol of HCAs (without considering the production of phenylpropanoic acids, phenylacetic acids, benzoic and hippuric acids) and the intake (µmol) of total (poly)phenols containing at least ≥ 80% of chlorogenic acids.



Supplementary Figure S3. Contribution (%) of each metabolite class to the overall bioavailability of HCAs. Values were calculated considering studies (n=17) employed to calculate the final value of HCA bioavailability. Unchanged acyl-quinic and C_6-C_3 unsaturated cinnamic acids (UC) (n, number of bioavailability values calculated for each class=11), caffeoylquinic acids (CQAs) (n=6), feruloylquinic acids (FQAs) (n=3), coumaroylquinic acids (CoQAs) (n=5), derivatives of 3′,4′-dihydroxycinnamic acid (*aka* caffeic acid) (CA) (n=13), derivatives of 4′-hydroxy-3′-methoxycinnamic acid (*aka* ferulic acid) (FA) (n=17), derivatives of 3′-hydroxy-4′-methoxycinnamic acid (*aka* isoFerulic acid) (isoFA) (n=13), derivatives of 3′,5′-dimethoxy-4′-hydroxycinnamic acid (*aka* sinapic acid) (Sin) (n=7), derivatives of hydroxycinnamic acid (*aka* coumaric acid) and cinnamic acid (Cou/Cinn) (n=5), derivatives of 3-(3′,4′-dihydroxyphenyl)propanoic acid (*aka* dihydrocaffeic acid) (Di-CA) (n=14), derivatives of 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid (*aka* dihydroferulic acid) (Di-FA) (n=14), derivatives of 3-(3′-hydroxy-4′-methoxyphenyl)propanoic acid (*aka* dihydroisoferulic acid) (Di-isoFA) (n=6), derivatives of 3-(hydroxyphenyl)propanoic acid (*aka* dihydrocoumaric acid) (Di-Cou) (n=5), derivatives of benzoic acid and benzaldehyde (BA/BE) (n=4), catechols (Cat) (n=3), miscellaneous (Misc) (n=0). Apart for UC, classes of CQAs, FQAs, CoQAs, C_6-C_3 unsaturated cinnamic acids, phenylpropanoic acids, BA/BE and Cat. include data derived from both aglycones and their phase 2 conjugates. Data on Misc are not reported due to the absence of bioavailability values. Data are reported as mean and SD.



Supplementary Figure S4. Contribution (%) of each metabolite class to coffee HCA bioavailability. Values were obtained from studies that calculated coffee HCA bioavailability (n=11). Unchanged acyl-quinic and C_6-C_3 unsaturated cinnamic acids (UC) (n, number of bioavailability values calculated for each class=8), caffeoylquinic acids (CQAs) (n=5), feruloylquinic acids (FQAs) (n=1), coumaroylquinic acids (CoQAs) (n=4), derivatives of 3′,4′-dihydroxycinnamic acid (*aka* caffeic acid) (CA) (n=10), derivatives of 4′-hydroxy-3′-methoxycinnamic acid (*aka* ferulic acid) (FA) (n=11), derivatives of 3′-hydroxy-4′-methoxycinnamic acid (*aka* isoFerulic acid) (isoFA) (n=10), derivatives of 3′,5′-dimethoxy-4′-hydroxycinnamic acid (*aka* sinapic acid) (Sin) (n=3), derivatives of hydroxycinnamic acid (*aka* coumaric acid) and cinnamic acid (Cou/Cinn) (n=4), derivatives of 3-(3′,4′-dihydroxyphenyl)propanoic acid (*aka* dihydrocaffeic acid) (Di-CA) (n=10), derivatives of 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid (*aka* dihydroferulic acid) (Di-FA) (n=10), derivatives of 3-(3′-hydroxy-4′-methoxyphenyl)propanoic acid (*aka* dihydroisoferulic acid) (Di-isoFA) (n=5), derivatives of 3-(hydroxyphenyl)propanoic acid (*aka* dihydrocoumaric acid) (Di-Cou) (n=4), derivatives of benzoic acid and benzaldehyde (BA/BE) (n=4), catechols (Cat) (n=3), miscellaneous (Misc) (n=0). Apart for UC, classes of CQAs, FQAs, CoQAs, C6-C3 unsaturated cinnamic acids, phenylpropanoic acids, BA/BE and Cat. include data derived from both aglycones and their phase 2 conjugates. Data on Misc are not reported due to the absence of bioavailability values. Data are reported as mean and SD.

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General conclusions

Since the ADME of (poly)phenols and their derived metabolites are the true "actors" in the framework of the preventive and beneficial effects of (poly)phenol-rich foods, this Doctoral Thesis studied the interaction of the main dietary (poly)phenols with human gut microbiota, comprehensively assessed the blood nutrikinetics and urinary excretion profiles of (poly)phenol-derived metabolites, also by paying attention at the metabolic efficiency (*aka* stoichiometry) in their production. Finally, "the cherry on the cake" was determining the bioavailability of the main classes of dietary flavonoids and phenolic acids, namely flavan-3-ols and hydroxycinnamic acids.

The main activities presented in this work included:

- ***In Vitro* Faecal Fermentation of Monomeric and Oligomeric Flavan-3-ols: Catabolic Pathways and Stoichiometry.** This study investigated for the first time the behavior of 12 pure monomeric flavan-3-ols and proanthocyanidins in an *in vitro* human colonic environment, with the aim of evaluating the influence of flavan-3-ol structure on the production of phenolic catabolites. 32 colonic catabolites out of 105 compounds monitored were quantified after colonic catabolism of parent compounds, with new catabolic routes resulting in procyanidin fission catabolites that were established. While monomers had the highest molar mass recoveries for total phenyl-γ-valerolactones and phenylvaleric acids, no differences were found in their production from A-type dimer and B-type procyanidins-despite the different degrees of polymerization. The stoichiometric balances calculated in this study could potentially support the experimental design of *in vivo* and *in vitro* models aiming at evaluating the catabolism of flavan-3-ols in bioavailability and dose-response studies, cell models.
- **The human Microbial Metabolism of Quercetin in Different Formulations: An *In Vitro* Evaluation**. In this study, quercetin derived from unformulated and phytosome-formulated quercetin was bioaccessible to colonic microbiota *in vitro*, resulting in the production of phenylpropanoic acid, phenylacetic acid and benzoic acid derivatives probably involved in the systemic effect associated to dietary (poly)phenol intake. 3 out of 4 microbial metabolites were quantified at higher concentrations after phytosome-formulated extract with respect to the unformulated one after 5 h. Overall, the extent of the microbial metabolism of quercetin was greater in the unformulated ingredient, and varied in a time-dependent manner. This was the first study providing data on the interaction between quercetin derived from different formulations and the human microbiota.
- ***In vitro* (poly)phenol catabolism of unformulated- and phytosome-formulated cranberry (*Vaccinium macrocarpon*) extracts**. 24 out of 44 microbial metabolites were quantified after the *in vitro* fermentation of cranberry (poly)phenols from unformulated- and phytosome-formulated extracts, after standardizing the concentration of incubated proanthocyanidins. Although some differences between extracts appeared in the production of phenylpropanoic and benzoic acid derivatives, the study demonstrated that the different formulation did not affect the profile of total phenyl-γ-valerolactones and phenylvaleric acids produced during the interaction of cranberry flavan-3-ols with the gut microbiota. These findings showed that cranberry (poly)phenols could produce a wide array of gut microbiota catabolites which are potentially involved in the beneficial effects associated to berry intake.
- **Revisiting the bioavailability of flavan-3-ols in humans: A systematic review and comprehensive data analysis.** Data analysis from 49 studies systematically demonstrated that flavan-3-ols are metabolized in up to 180 metabolites in humans, mainly as phase 2 conjugates of microbial catabolites (i.e. phenyl-γ-valerolactones). Flavan-3-ols resulted moderately bioavailable (31%). This work supported the importance in using authentic reference compounds to improve the accuracy in assessing the bioavailability of (poly)phenols. The stoichiometric balances calculated in the production of phenyl-γ-valerolactones and phenylvaleric acids from

cumulative urinary data were in line with those obtained from *in vitro* colonic fermentation of flavan-3-ol monomers and B-type dimers.

- **A systematic review and comprehensive nutrikinetics, urinary excretion and bioavailability of hydroxycinnamic acids**. The study assessed nutrikinetics and excretion profiles of 105 metabolites resulting after hydroxycinnamic acid intake, with C_6-C_3 unsaturated cinnamic acids reaching the highest blood concentration level. Stoichiometric balances calculated from urinary data suggested that phenylpropanoic acids have the highest metabolic yield. The bioavailability of hydroxycinnamic acids was moderate (25%). The work supported the need to conduct a comprehensive study to investigate the ADME of this (poly)phenol class, and opened new perspectives for assessing the potential of 8 metabolites in bioavailability studies and bioactivity models.

In conclusion, the findings of this Doctoral Thesis represent a starting point for improving and harmonising the available knowledge on the interaction of the most consumed dietary (poly)phenol classes with gut microbiota and their nutrikinetics profiles in humans. The results of this work could strongly support in understanding the metabolic fate and influencing factors of ingested (poly)phenols in humans, besides meta- and catabolic pathways associated with the production of gut microbiota and phase-2 derived (poly)phenol metabolites, and their circulating levels in biofluids, pivotal steps for underlying the beneficial effects observed after a higher adherence to plant-based dietary pattern.

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- EAT-Lancet Commission Brief for Everyone - EAT https://eatforum.org/lancet-commission/eatinghealthyandsustainable/ (accessed 10.5.22).
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Contribution for each included paper

- ***In Vitro* Faecal Fermentation of Monomeric and Oligomeric Flavan-3-ols: Catabolic Pathways and Stoichiometry**

Design and conduction of the study, sample analysis, data analyses and interpretation, manuscript writing

- **The human Microbial Metabolism of Quercetin in Different Formulations: An *In Vitro* Evaluation**

Methodology, investigation, writing and original draft preparation

- ***In vitro* (poly)phenol catabolism of unformulated- and phytosome-formulated cranberry (*Vaccinium macrocarpon*) extracts**

Writing and original draft, writing - review and editing

- **Revisiting the bioavailability of flavan-3-ols in humans: A systematic review and comprehensive data analysis**

Conceptualization; data curation and formal analysis; visualization; writing and original draft preparation

- **A systematic review and comprehensive nutrikinetics, urinary excretion and bioavailability of hydroxycinnamic acids**

Conceptualization; data curation and formal analysis; visualization; writing and original draft preparation