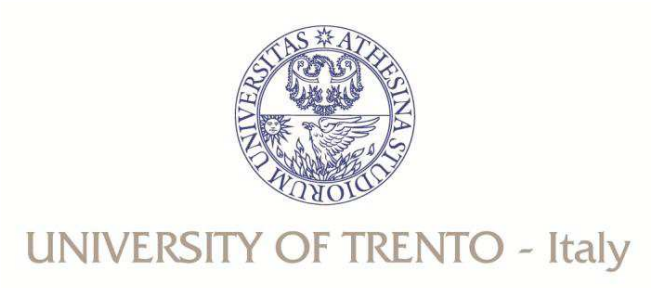

International Doctoral School in Biomolecular Sciences
XXVI Cycle

Fruit Polyphenols and their Fate in the Mammalian System after Ingestion

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Ph.D. Thesis of

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Ph.D. Thesis Abstract:

A range of different polyphenols can be ingested in a bowl of polyphenol-rich fruit, going from one-phenol hydroxybenzoic acid to more complex polymeric compounds. Epidemiologically, polyphenol consumption has been associated with a reduced risk of cancer and cardiovascular disease and neurological protection against brain ageing. However, only a small proportion of native polyphenols (5-10%) are absorbed and the remainder reach the colon, where they are extensively metabolised by the gut microbiota. The colonic microbiota produces a relatively small number of polyphenol microbial metabolites from a large number of different dietary polyphenols. During subsequent tissue distribution, the target organs and the effective concentration circulating remain largely unreported.

This Ph.D. thesis is divided into two parts: chemical analysis of food composition and *in vivo* bioavailability of polyphenol metabolites. Metabolomics offers an innovative approach that has recently been shown to be effective in both food chemistry and nutritional bioavailability studies.

Polyphenol composition in strawberries is studied in the first part of this Ph.D. thesis, with the aim of evaluating nutritionally significant amounts of polyphenols before ingestion. In this context, a targeted method for quantitative analysis of multiple classes of phenols was developed. A high sensitivity MRM-based method for 135 phenolics with a wide dynamic range was obtained, providing valuable insight and assisting with the analysis of complex matrices such as fruit, and more in general food.

Application of the method was tested in *Fragaria* spp., and along with another rapid method for the analysis of anthocyanins and ellagitannins, provided a general overview of polyphenol composition in strawberries. A total of 56 individual compounds were accurately identified and quantified, some of them for the first time, their concentration ranging from 1 ug/100 g for low abundant polyphenols to 40

mg/100 g of fresh fruit. Moreover the isolation of some ellagitannins and definition of their profile in *Fragaria* spp. was carried out during fruit ripening. Clarification of the main ellagitannin, agrimoniin, was obtained by isolation and it was ambiguously assigned as the main ellagitannin present in the diet.

In the second part of this Ph.D. thesis the *in vivo* bioavailability of a dose of polyphenol microbial metabolites reflecting dietary consumption of fruit was studied. The focus was on the metabolites of polyphenols which can be found in the bloodstream after gut microflora metabolism. Their distribution was explored in rats in different organs, in particular in the brain, considering their possible neuropreventive properties. Development of a specific quantitative method for the quantification of selected polyphenol microbial metabolites made it possible to analyse complex biological samples resulting from *in vivo* trials with rats treated with a nutritionally significant dose of polyphenol microbial metabolites, intravenously injected. A high-throughput, sensitive and reproducible method for 23 polyphenol metabolites in six different biological matrices was developed. A purification protocol made it possible to obtain cleaner and more concentrated samples, with low limits of quantification. Specific organ-tropism was observed, mainly hepatotropism.

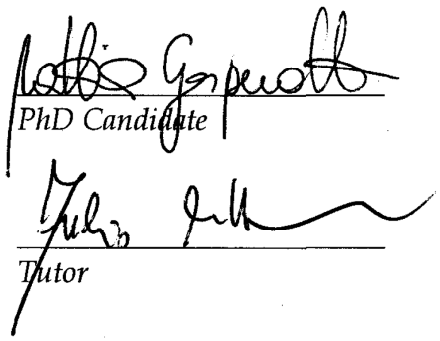
Remarkably, in this study the brain was reported to be one of the target organs for these molecules, already being present at basal level or increasing their concentration after treatment. Furthermore, the amount of 10 out of 23 compounds significantly increased with a nutritionally significant dose.

Der Mensch ist, was er ißt
(Man is what he eats)

Ludwig A. Feuerbach (1804-1872)

'Declaration

I, Mattia Gasperotti, confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.'



PhD Candidate

Tutor

Contents

Introduction and literature overview

Polyphenols, gut microflora metabolism and their effects on human health

This introduction has been adapted* and improved, including the relevant parts, from:

Up-regulating the Human Intestinal Microbiome Using Whole Plant Foods, Polyphenols, and/or Fiber Kieran M. Tuohy, Lorenza Conterno, **Mattia Gasperotti**, and Roberto Viola *Journal of Agricultural and Food Chemistry* **2012** 60 (36), 8776-8782

Attached as an original paper after this introduction.

Epidemiologically, fruits and vegetables protect against chronic diseases including cardiovascular disease, cancer, obesity, and diabetes. Plant secondary polyphenolic compounds are thought to contribute significantly to the underlying protective processes (1-3). Interestingly, polyphenols potentially interact with the gut microbiota: for many polyphenols microbial transformation modifies bioavailability and activity.

Plant polyphenols are a class of chemically diverse secondary metabolites that carry out many different biological activities both in plants and the animals which eat them (4). Mainly because of their presence in foods and medicinal plants, they have been studied at length, due to interaction with mammalian physiological processes that play a role in chronic human disease. As well as being antioxidants and possessing inherent free radical scavenging abilities, plant polyphenols have the potential to affect certain risk factors for cardiovascular disease, such as plasma lipid oxidation state, endothelial function and platelet aggregation; they protect against cancer by reducing DNA damage, cell proliferation, and metastasis; they modulate immune function; they inhibit bacterial pathogens and protect against neurological decline (see Figure 1). Most of the evidence relating to these effects on health stems from animal studies using either whole plant foods or plant polyphenol extracts. Consequently, both the active chemical moiety, and in many cases the underlying mechanism of action in humans, have yet to be determined (4).

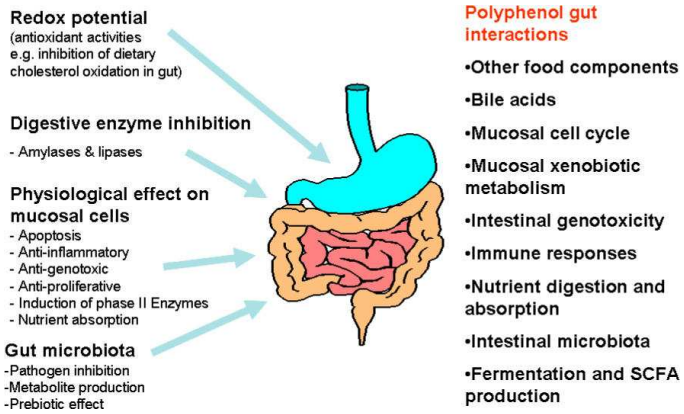


Figure 1 Diagram illustrating the direct intestinal effects of plant food polyphenols.

Human Gut Microbiota

The human gut microbiota is a diverse collection of microorganisms making up some 1000 species, with each individual having their own unique collection of species. These microorganisms play an important role in human health and disease, and inter-individual variation in microbiota makeup influences the profile of metabolites released from dietary components that reach the colon and may also affect an individual's risk of chronic disease. Recent studies have identified a limited number of "enterotypes" within the human gut microbiota, characterised by a predominance of Prevotella, Bacteroides and/or Ruminococcus (5,6).

Moreover, these enterotypes appear to be determined by long-term diet and could play an important role in an individual's risk of developing chronic diet-associated diseases such as obesity, metabolic syndrome and certain cancers (6). Different profiles of gut bacteria have also been characterised in populations with chronic immune or metabolic-related diseases, including inflammatory bowel disease (IBD), celiac disease, diabetes and obesity (7, 8). Typically, these conditions show a lower prevalence of beneficial butyrate-producing bacteria, such as *Faecalibacterium prausnitzii*, and the bifidobacteria, which appear to be indicative of a well-functioning, healthy saccharolytic type microbiota. These diseases are also often associated with a high prevalence of Enterobacteriaceae, a phylum that includes many important gastrointestinal pathogens including *Escherichia coli*, *Shigella*, *Salmonella*, *Campylobacter* and *Helicobacter*. These diseases are also often associated with increased intestinal permeability or "leaky gut", a process that appears to be regulated both by diet and by gut microbiota-associated characteristics.

Works on germ-free animals, animal models of obesity and the metabolic syndrome, and a limited number of human studies have shown that the gut microbiota of obese individuals differs from that of lean individuals. The obese appear to be typified by a gut microbiota with a reduced *Bacteroidetes*/*Firmicutes* ratio

and perturbations within important fibre-degrading saccharolytic populations (11). Indeed, this obese type microbiota has been shown to revert to a lean type profile with increased relative abundance of the *Bacteroidetes* when obese individuals lose weight on low-energy diets. Such microbiota compositional differences within the obese, and other disease states, are likely to have a dramatic impact on the colonic metabolic output and subsequent physiological processes in the host, including control of food intake, inflammation, energy storage and energy expenditure (see Figure 1) (11). Certain shifts or profiles for gut bacteria are associated with poor diet, diets low in whole plant foods such as fruits and vegetables. Similar profiles for gut bacteria are also observed in chronic diseases related to affluent Western lifestyles, such as obesity, metabolic syndrome, certain cancers and autoimmune diseases such as IBD.

Both the type and quantity of foods that escape digestion in the upper gut have an important impact on the composition and therefore, activity of the colonic microbiota. As much as 90% of plant polyphenols escape digestion and absorption in the upper gut and persist in the colon. Here they come into contact with the gut microbiota, acting as substrates for microbial production of small phenolic acids and SCFA, or indeed affecting species composition and their metabolic activity.

Polyphenols and the Human Gut Microbiota

As previously mentioned, after the consumption of food rich in polyphenols, more than 90 % of plant polyphenols escape digestion and absorption in the upper gut and reach the colon. Hydrolysis by the gut microbiota can increase the bioavailability and possibly the biological activity of polyphenols reaching the colon. The human intestinal microbiota has extensive hydrolytic activities and breaks down many complex polyphenols into smaller phenolic acids, which can be absorbed across the intestinal mucosa. These polyphenol catabolites are only then available to exert their biological activities systemically within the host. However, to date, with the

exception of a few compounds such as the phyto-estrogens equol, enterolactone and enterodiol, very little is known about the biological activities of phenolic acids deriving from microbiota polyphenol metabolism (2, 3). Table 1 presents some examples of parent plant polyphenols, microbial catabolites and their potential biological effect when known. Given the diversity of plant polyphenols, their possible microbial catabolites and the fact that they occur as mixtures in whole plant foods or plant extracts, only a small number of well-studied polyphenols are presented. Biological activities have not been described for most of the small phenolic acid catabolites, but common activities recognised so far include antibacterial activities, especially against Gram-negative species such as Enterobacteriaea, anti-inflammatory activities, anti-AGE formation, stimulation of xenobiotic degrading enzymes and detoxification processes and phyto-estrogenic activities.

Recently, Miene et al. (13) demonstrated that microbial metabolites of quercetin and chlorogenic acid/caffeic acid, 3,4-dihydroxyphenylacetic acid (ES), and 3-(3,4-dihydroxyphenyl) propionic acid (PS) respectively, could significantly up-regulate GSTT2 expression and decrease COX-2 expression, a modulation seen as protective against colon cancer, and at the same time reduce DNA damage using an intestinal cancer cell line. González-Sarrías et al. (14) showed that ellagic acid (EA) and its colonic metabolites, urolithin-A (3,8-dihydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one, Uro-A) and urolithin-B (3-hydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one, Uro-B), modulate the expression and activity of CYP1A1 and UGT1A10 and inhibit several sulfotransferases in colon cancer cell lines (Caco-2). These phase I and phase II detoxifying enzymes are important components of how our bodies deal with toxic and xenobiotic compounds, and increased expression is associated with a reduced risk of colon cancer in laboratory animals. However, these effects appeared to be critically affected by the food matrix in the rat colon. Nevertheless, urolithins from pomegranate have already been shown to reduce the growth of cancer cells in an animal model of prostate cancer (15).

3,4-dihydroxyphenylpropionic acid (3,4-DHPPA), 3-hydroxyphenylpropionic acid, and 3,4-dihydroxyphenylacetic acid (3,4-DHPAA), derived from colonic

catabolism of proanthocyanidins, have been shown to reduce the inflammatory response of human peripheral blood mononuclear cells stimulated with lipopolysaccharide (LPS), an inflammatory cell wall component from Gram-negative bacteria such as the Enterobacteriaceae. Secretion of IL-6, IL-1, and TNF-α was reduced, suggesting that microbial metabolites may be involved in dampening the inflammatory response to bacterial antigens, which may have implications for chronic inflammatory or autoimmune diseases such as IBD.(16) On screening 18 microbial catabolites of polyphenols for their anti-inflammatory potential *in vitro*, Larrosa et al. (17) found that hydrocaffeic (HCAF), dihydroxyphenylacetic (dOHPA) and hydroferulic acid (HFER) reduced prostaglandin E(2) production by at least 50% in CCD-18 colon fibroblast cells stimulated with IL-1β. The same compounds were also shown to reduce inflammation in rodents, and HCAF was also shown to reduce intestinal inflammation in the DSS-mouse model of ulcerative colitis with reduced mucosal expression of cytokines IL-1β, IL-8, and TNF-α, reduced malonyldialdehyde (MDA) levels, and decreased oxidative DNA damage (measured as 8-oxo-2′-deoxyguanosine levels). Radnai et al. (18) also showed that ferulaldehyde, a microbial catabolite of curcumin, has anti-inflammatory properties *in vivo* in an animal model of LSP-induced septic shock. Intraperitoneal injection of ferulaldehyde (6 mg/kg) reduced markers of inflammation and prolonged the lifespan of LPS-treated animals, indicating that microbial catabolites might, at least in part, account for the observed anti-inflammatory activity of certain herbal medicines and functional foods.

Microbial catabolites of plant polyphenols may also affect disease risk in metabolic syndrome, a cluster of biomarkers associated with obesity and increased risk of cardiovascular disease and diabetes. Verzelloni et al. (19) demonstrated that urolithins and pyrogallol, microbial ellagitannin-derived catabolites, are highly antiglycative compared to parent polyphenolic compounds in an *in vitro* model of protein glycation. Protein glycation plays an important pathological role in diabetes and pathologies associated with diabetes, including blindness. They also found that chlorogenic acid-microbially derived catabolites, dihydrocaffeic acid, dihydroferulic acid and feruloylglycine, were most effective at protecting cultured neural cells *in*

vitro, indicating that colonic catabolites of dietary polyphenols may play an important role in the improved cognitive function and protection from neuronal degeneration observed in animals fed polyphenol-rich foods such as certain berries.

Plant polyphenols have also been shown to have a direct effect on carbohydrate fermentation by the human gut microbiota *in vitro*. Using anaerobic faecal batch cultures, Bazzocca et al. (20) found that apple proanthocyanidins inhibited both metabolic degradation of short proanthocyanidins and SCFA production. This observation may apparently suggest that polyphenols might work against production of beneficial microbial metabolites within the colon. As mentioned above, in individuals on a Western style, low-fibre diet, the proximal colon is the main site of saccharolytic fermentation, with potentially damaging proteolytic fermentation increasing distally as the carbohydrate substrate becomes limiting. Retardation of carbohydrate fermentation in the proximal colon may extend SCFA production to the distal colon, thereby reducing the harmful effects of amino acid catabolites. However, this remains to be investigated either *in vivo* or using complex multistage continuous culture models of the colonic microbiota. Similarly, polyphenols have also been shown to directly affect the relative abundance of different bacteria within the gut microbiota, with tea polyphenols and their derivatives reducing numbers of potential pathogens including *Clostridium perfringens* and *C. difficile* and certain Gram-negative *Bacteroides* spp., with less inhibition toward beneficial clostridia, bifidobacteria, and lactobacilli (21).

Table 1 Examples of important plant polyphenols and their microbial catabolites[a]

plant polyphenol	microbial catabolite	possible health effects
(−)-epicatechin (29-31)	4-hydroxyphenylacetic acid	antimicrobial/antimycotic activity *in vitro*
	3-(3-hydroxyphenyl)propionic acid	antimicrobial activity against Gram-negative enterobacteria via outer membrane destabilization
	5-(3,4-dihydroxyphenyl)-γ-valeric acid	
	(−)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone	
(−)-epigallocatechin (31)	4-hydroxyphenylacetic acid	antimicrobial/antimycotic activity *in vitro*
	(−)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone	
(−)-epigallocatechin-3-*O*-	pyrocatechol	

plant polyphenol	microbial catabolite	possible health effects
gallate (29-34)	pyrogallol	antibacterial activity (especially against Gram-negative enterobacteria), acetylcholinesterase inhibition greater than gallic acid parent; inhibition of *Vibrio* spp. quorum sensing
	4-hydroxyphenylacetic acid	antimicrobial/antimycotic activity *in vitro*
	(−)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone	
chlorogenic acid (17)	hydrocaffeic acid dihydroxyphenyl acetic acid hydroferulic acid	reduced intestinal mucosal inflammation and oxidative damage in animal models of IBD
proanthocyanidins (16)	3,4-dihydroxyphenylpropionic acid 3-hydroxyphenylpropionic acid 3,4-dihydroxyphenylacetic acid	reduced inflammatory response from LPS-stimulated blood lymphocytes
curcumin (18)	ferulaldehyde	reduced inflammatory response from LPS-stimulated blood lymphocytes
daidzein (35-37)	equol	phytoestrogen important for heart and bone health and possible colon cancer protectants
daidzein (17, 33)	*O*-demethylangolensin	estrogenic and/or antiestrogenic activity
quercetin (33)	2-(3,4-dihydroxyphenyl)acetic acid 2,3-(3-hydroxyphenyl)acetic acid 3,4-dihydroxybenzoic acid phloroglucinol 3-(3,4-dihydroxyphenyl)propionic acid 3-(3-hydroxyphenyl)propionic acid	
kaempferol (33)	2-(4-hydroxyphenyl)acetic acid	
naringenin (33)	3-(4-hydroxyphenyl)propionic acid phloroglucinol	antimicrobial activity against Gram-negative enterobacteria via outer membrane destabilization
isoxanthohumol (33)	8-prenylnaringenin	
catechin and epicatechin (24, 33)	3-(3-hydroxyphenyl)propionic acid 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone 5-(3′-hydroxyphenyl)-γ-valerolactone 3-hydroxyhippuric acid pyrogallol 5-(3,4-dihydroxyphenyl)valeric acid 5-(3-hydroxyohenyl)valeric acid 3-(3,4-dihydroxyphenyl)propionic acid 5-(3-methoxyohenyl)valeric acid 3-(3,4-dihydroxyphenyl)propionic acid 5-(3-methoxyohenyl)valeric acid 2,3-dihydroxyphenoxyl 3-(3′,4′-dihydroxyphenyl)propionic acid	antimicrobial activity against Gram-negative enterobacteria via outer membrane destabilisation
ellagitannins/ellagic acid (3, 14, 17, 34)	urolithin-A	estrogenic and/or antiestrogenic activity, antimalarials, induction of detoxification enzymes
	urolithin-B	estrogenic and/or antiestrogenic activity, antimalarials, induction of detoxification enzymes
	urolithin-C	estrogenic and/or antiestrogenic activity
	urolithin-D	estrogenic and/or antiestrogenic activity

plant polyphenol	microbial catabolite	possible health effects
rutin (24, 35, 36)	3-hydroxyphenylacetic acid	rutin and catabolites inhibit AGE formation *in vitro*; antimicrobial activity against Gram-negative enterobacteria via outer membrane destabilisation
	3,4-dihydroxybenzoic acid	
	4-hydroxybenzoic acid	
	3-(3-hydroxyphenyl)propionic acid	
	3,4-dihydroxyphenylacetic acid	
lignans (28)	enterolactone	Phytoestrogen important for heart and bone health; possible colon cancer protectants
	enterodiol	Phytoestrogen important for heart and bone health; possible colon cancer protectants

[a] The putative health effects of selected catabolites are also presented.

Human data on the impact of polyphenols on the gut microbiota are scarce. One recent work from the University of Reading has shown that a high cocoa-flavanol (494 mg/day) drink can mediate prebiotic type modulation of the gut microbiota in a randomised intervention study compared to a low cocoa-flavanol (23 mg/day) drink. The high-flavanol drink resulted in significant increases in faecal bifidobacteria and lactobacilli and a reduction in clostridial counts and a concomitant reduction in plasma triglycerides and C-reactive protein, both important markers of metabolic disease (22). These results were mirrored by previous *in vitro* studies using faecal batch cultures by the same group (23).

Polyphenol Microbial Metabolites and the Brain

The biological properties of polyphenol microbial metabolites have been little explored and reported in the literature, mostly in relation to their neurological relevance and brain ageing prevention. Many experiments have been done on *in vitro* models, while evidences from *in vivo* systems are rare.

Dietary intervention and epidemiological studies on humans have concluded that polyphenols and their metabolites may have some involvement in neurological protection/prevention and may promote brain health (37). The phenolic structure could protect against oxidative stress and their activity could be exerted at local or

systemic levels (33). However, antioxidant protection does not seem to be enough in predicting and assessing the bioactivity of these metabolites against certain disorders affected by ageing, and as a consequence other mechanisms of actions could be involved in neurological protection. Other mechanisms of action resulting in the literature are: direct effects on signalling to promote neuronal communication, the ability to buffer against calcium, enhancement of neuroprotective stress shock proteins and reduction of any stress signals (38). Recently mitochondrial dysfunction has also received attention in the pathogenesis of neurodegenerative diseases and brain ageing. Mitochondria are often considered as both initiators and targets of oxidative stress, during which polyphenol metabolites could modulate mitochondrial functions (39).

However, it has not yet been clearly established whether these compounds reach the brain in a sufficient concentration and there are little information about the kind of interaction between polyphenols or their metabolites and the blood brain barrier (40). Moreover, reliable data on uptake into the brain are limited and with ambiguous protocols or data acquisition (41).

The blood brain barrier is made up of brain capillary endothelial cells and other different cell types, such as perycytes, astrocytes and neuronal cells. A mandatory requisite for any kind of activity in the brain is therefore that polyphenol metabolites and their conjugates are able to pass the blood brain barrier and enter the brain. Many aspects limit brain permeability, brain capillary and endothelial cells having tight junctions which prevent paracellurar transport of water-soluble compounds. Transcellular transport is also limited. Possibly for these reasons, most experiments reporting on biological activities and the targets of polyphenols or their metabolites have used *in vitro* experiments, which are prone to artefact or not in line with real complex situations involving the brain and the blood brain barrier (39).

The bioactivity of selected polyphenol metabolites *in vitro* has been tested at physiologically relevant doses against advanced glycation endproduct formation and the ability to counteract mild oxidative stress on human neuronal cells (19). All the catabolites investigated, divided into groups correlated with the consumption of

raspberries/pomegranates, coffee or berries/red wine have shown antiaglycane activity. Urolithin A and B, for example, from the microbial metabolism of ellagitannins, significantly reduced protein glycation at 1 μmol/L. In a vitro cell system with PC12 cells, protocatechuic acid has been shown to decrease mitochondrial dysfunction (42). Protocatechuic acid, the main microbial metabolite of anthocyanins, has been detected in human and animal blood, but at the moment there is no information available regarding brain uptake.

The presence of gallic acid in the brain *in vivo* was shown in one interesting paper involving rats after the administration of proanthocyanidins (43). Gallic acid and some other methylated metabolites were observed inside the brain, albeit in trace amounts.

Research on the effects of polyphenol metabolites in the future must involve *in vivo* models or artefact-free *in vitro* experiments and testing at physiological concentrations (39). In order to obtain more realistic observations, it is important to carry out experiments starting from the plasma concentration of these metabolites, which are in the range of sub-uM or nM in normal dietary consumption. Furthermore, the bioavailability of polyphenol microbial metabolites has to be carefully taken into account in nutrition-based studies and brain ageing experiments, along with the already investigated bioavailability of native polyphenols in plants.

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Review

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Up-regulating the Human Intestinal Microbiome Using Whole Plant Foods, Polyphenols, and/or Fiber

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ABSTRACT: Whole plant foods, including fruit, vegetables, and whole grain cereals, protect against chronic human diseases such as heart disease and cancer, with fiber and polyphenols thought to contribute significantly. These bioactive food components interact with the gut microbiota, with gut bacteria modifying polyphenol bioavailability and activity, and with fiber, constituting the main energy source for colonic fermentation. This paper discusses the consequences of increasing the consumption of whole plant foods on the gut microbiota and subsequent implications for human health. In humans, whole grain cereals can modify fecal bacterial profiles, increasing relative numbers of bifidobacteria and lactobacilli. Polyphenol-rich chocolate and certain fruits have also been shown to increase fecal bifidobacteria. The recent FLAVURS study provides novel information on the impact of high fruit and vegetable diets on the gut microbiota. Increasing whole plant food consumption appears to up-regulate beneficial commensal bacteria and may contribute toward the health effects of these foods.

KEYWORDS: *human gut microbiota, whole plant foods, polyphenols, fiber*

INTRODUCTION

Epidemiologically, fruits and vegetables protect against chronic diseases including cardiovascular disease, cancer, obesity, and diabetes. Fiber, composed of plant structural and storage polysaccharides, and plant secondary polyphenolic compounds are thought to contribute significantly to the underlying protective processes.[1−3] Interestingly, both potentially interact with the gut microbiota: for many polyphenols microbial transformation modifies bioavailability and activity, and fiber is the major energy source for fermentation in the colon, the dominant metabolic activity of the gut microbiota.

Plant polyphenols are a class of chemically diverse secondary metabolites that possess many different biological activities both within the plant and in the animals which eat these plants.[4] Mainly because of their presence in foods and medicinal plants, they have long been studied for their interactions with mammalian physiological processes that play a role in chronic human disease. As well as being antioxidants and possessing inherent free radical scavenging abilities, plant polyphenols have the potential to affect certain risk factors of cardiovascular disease such as plasma lipid oxidation state, endothelial function, and platelet aggregation; protect against cancer by reducing DNA damage, cell proliferation, and metastasis; modulate immune function; inhibit bacterial pathogens; and protect against neurological decline (see Figure 1). The majority of the evidence for such health effects stems from animal studies using either whole plant foods or plant polyphenol extracts. Consequently, both the active chemical moiety and, in many cases, the underlying mechanism of action in humans remain to be determined.[4] In this review, we will focus on interactions between polyphenols and fibers in whole plant foods with the human intestinal microbiota.

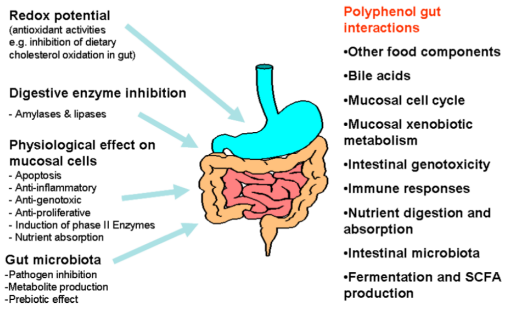

Figure 1. Schematic illustrating the direct intestinal effects of plant food polyphenols.

HUMAN GUT MICROBIOTA

The human gut microbiota is a diverse collection of microorganisms making up some 1000 species, with each individual presenting with their own unique collection of species. They play an important role in human health and disease, and interindividual variation in microbiota makeup influences the profile of metabolites released from dietary components that reach the colon and may also affect an individual's risk of chronic disease. Recent studies have identified a limited number of "enterotypes" within the human gut microbiota characterized by a predominance of *Prevotella*, *Bacteroides*, and/or *Ruminococcus*.[5,6] Moreover, these

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enterotypes appear to be determined by long-term diet and could play an important role in an individual's risk of developing chronic diet-associated diseases such as obesity, metabolic syndrome, and certain cancers.[6] Shotgun metagenomic sequencing has revealed differences in functional genes associated with these enterotypes, and these phenotypes appear to be linked to dietary fat, protein, fiber, and carbohydrate contents. Although no switching between enterotypes was observed during a small short-term dietary intervention with high-fat/low-carbohydrate or low-fat/high-carbohydrate diets, changes within the composition of the gut microbiota of the 10 subjects studied were observed after 24 h of dietary intervention. This indicates that even short-term dietary change can have a detectable impact on the makeup of the gut microbiome, in terms of both relative bacterial populations and the overall metabolic potential of the gut microbiota.[6] Different profiles of gut bacteria have also been characterized in populations with chronic immune or metabolic-related diseases including inflammatory bowel disease (IBD), celiac disease, diabetes, and obesity.[7,8] Typically, these conditions present with lower prevalence of beneficial butyrate-producing bacteria, such as *Faecalibacterium prausnitzii*, and the bifidobacteria, which appear to be indicative of a well-functioning, healthy saccharolytic type microbiota. These diseases are also often associated with high prevalence of Enterobacteriaceae, a phylum that includes many important gastrointestinal pathogens including *Escherichia coli*, *Shigella*, *Salmonella*, *Campylobacter*, and *Helicobacter*. These diseases too are often associated with increased intestinal permeability or "leaky gut", a process that appears to be regulated both by diet and by gut microbiota-associated characteristics.

Works in germ-free animals, animal models of obesity and the metabolic syndrome, and a limited number of human studies have shown that the gut microbiota of obese individuals differs from that of lean individuals (reviewed in refs 9 and 10). The obese appear to be typified by a gut microbiota with a reduced *Bacteroidetes/Firmicutes* ratio and perturbations within important fiber-degrading saccharolytic populations.[11] Indeed, this obese type microbiota has been shown to revert to a lean type profile with increased relative abundance of the *Bacteroidetes* when obese individuals lose weight on low-energy diets. Such microbiota compositional differences within the obese, and other disease states, are likely to have a dramatic impact on the colonic metabolic output and subsequent physiological processes in the host, including control of food intake, inflammation, energy storage, and energy expenditure (see Figure 1).[11] De Filippo et al.[12] recently characterized the gut microbiota composition of children living in rural Burkina Faso and urban Florence, Italy. The rural African children, following a traditional diet rich in whole plant foods, had a microbiota composition strikingly different from that of their European counterparts. Their microbiota was dominated by *Bacteroidetes*, notably the *Prevotella* and novel fiber-degrading species such as *Xylanibacter*, whereas the Italian children had a much lower ratio of *Bacteroidetes* to *Firmicutes*. The Italian children also had higher relative abundance of enterobacteria, including *E. coli*, *Shigella*, and *Salmonella*. In terms of microbiota metabolic activity, the African children had about 3-fold the concentration of short-chain fatty acids (SCFA) in their feces compared to the Italian urban children on a typical Western-style diet. Thus, the picture that is emerging is that although each of us carries our own unique collection of microorganisms, certain shifts or profiles of gut bacteria are associated with poor diet, diets low in whole plant foods such as fruits and vegetables. Similar profiles of gut bacteria are also observed in chronic diseases of affluent Western lifestyles, such as obesity, metabolic syndrome, certain cancers, and autoimmune diseases such as IBD. We have recently shown that the type and quantity of dietary carbohydrate and fat can have a significant impact on the human gut microbiota and its metabolic output in people with metabolic syndrome.[13] This highlights the real possibility of modulating the risk of metabolic disease by affecting interactions between diet and the gut microbiota.[7,8,13]

Both the type and quantity of foods that escape digestion in the upper gut have an important impact on the composition and, therefore, activity of the colonic microbiota. Dietary fiber is the main source of carbohydrate for the gut microbiota. Fiber, usually as a result of its chemical structure or inaccessibility in whole plant foods, is naturally resistant to digestion in the upper gut. However, recent studies have shown that food-processing methodologies, including grain particle size upon milling or cooking processes, can modify both the digestibility of complex polysaccharides in foods and their impact on the human gut microbiota.[14,15] Similarly, as much as 90% of plant polyphenols escape digestion and absorption in the upper gut and persist into the colon. Here they come into contact with the gut microbiota, acting as substrates for microbial production of small phenolic acids and SCFA or, indeed, affecting species composition and their metabolic activity.

FIBER AND THE GUT MICROBIOTA

In 2008 the European Commission presented a comprehensive definition of what constitutes dietary fiber. Commission Directive 2008/100/EC (28 October 2008) states that "fiber" means "carbohydrate polymers with three or more monomeric units, which are neither digested nor absorbed in the human small intestine and belong to the following categories: edible carbohydrate polymers naturally occurring in the food as consumed; edible carbohydrate polymers which have been obtained from food raw material by physical, enzymatic or chemical means and which have a beneficial physiological effect demonstrated by generally accepted scientific evidence; edible synthetic carbohydrate polymers which have a beneficial physiological effect demonstrated by generally accepted scientific evidence".

Importantly, this definition recognizes the role of dietary fiber in maintaining human health. Fiber is consistently found to be inversely associated with chronic human diseases such as cancer and cardiovascular disease in large-scale human epidemiological studies. Similarly, studies in laboratory animals have provided mechanistic data linking high dietary fiber (typically 10% w/w diet) and protection from these same diseases. This is particularly true for the prebiotic dietary fibers. The most recent definition of a prebiotic defines a dietary prebiotic as "... a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health".[16] Dietary prebiotics have been repeatedly shown in both animals and humans to modulate the gut microbiota using state of the art culture-independent microbiological techniques, and they typically increase the relative abundance of bifidobacteria. There is also strong animal data linking prebiotics with protection from metabolic syndrome, obesity, type 2 diabetes, colon cancer, and IBD and fortifying the gut microbiota against invading gastrointestinal pathogens.[8,17]

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Table 1. Examples of Important Plant Polyphenols and Their Microbial Catabolites[a]

plant polyphenol	microbial catabolite	possible health effects
(−)-epicatechin[37−39]	4-hydroxyphenylacetic acid	antimicrobial/antimycotic activity in vitro
	3-(3-hydroxyphenyl)propionic acid	antimicrobial activity against Gram-negative enterobacteria via outer membrane destabilization
	5-(3,4-dihydroxyphenyl)-γ-valeric acid	
	(−)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone	
(−)-epigallocatechin[39]	4-hydroxyphenylacetic acid	antimicrobial/antimycotic activity in vitro
	(−)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone	
(−)-epigallocatechin-3-O-gallate[37−42]	pyrocatechol	
	pyrogallol	antibacterial activity (especially against Gram-negative enterobacteria), acetylcholinesterase inhibition greater than gallic acid parent; inhibition of *Vibrio* spp. quorum sensing
	4-hydroxyphenylacetic acid	antimicrobial/antimycotic activity in vitro
	(−)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone	
chlorogenic acid[25]	hydrocaffeic acid	reduced intestinal mucosal inflammation and oxidative damage in animal models of IBD
	dihydroxyphenyl acetic acid	
	hydroferulic acid	
proanthocyanidins[24]	3,4-dihydroxyphenylpropionic acid	reduced inflammatory response from LPS-stimulated blood lymphocytes
	3-hydroxyphenylpropionic acid	
	3,4-dihydroxyphenylacetic acid	
curcumin[26]	ferulaldehyde	reduced inflammatory response from LPS-stimulated blood lymphocytes
daidzein[43−46]	equol	phytoestrogen important for heart and bone health and possible colon cancer protectants
daidzein[25,46]	*O*-demethylangolensin	estrogenic and/or antiestrogenic activity
quercetin[46]	2-(3,4-dihydroxyphenyl)acetic acid	
	2,3-(3-hydroxyphenyl)acetic acid	
	3,4-dihydroxybenzoic acid	
	phloroglucinol	
	3-(3,4-dihydroxyphenyl)propionic acid	
	3-(3-hydroxyphenyl)propionic acid	
kaempferol[46]	2-(4-hydroxyphenyl)acetic acid	
naringenin[46]	3-(4-hydroxyphenyl)propionic acid	antimicrobial activity against Gram-negative enterobacteria via outer membrane destabilization
	phloroglucinol	
isoxanthohumol[46]	8-prenylnaringenin	
catechin and epicatechin[37,46]	3-(3-hydroxyphenyl)propionic acid	
	5-(3′,4′-dihydroxyphenyl)-γ-valerolactone	
	5-(3′-hydroxyphenyl)-γ-valerolactone	
	3-hydroxyhippuric acid pyrogallol	
	5-(3,4-dihydroxyphenyl)valeric acid	
	5-(3-hydroxyohenyl)valeric acid	
	3-(3,4-dihydroxyphenyl)propionic acid	antimicrobial activity against Gram-negative enterobacteria via outer membrane destabilization
	5-(3-methoxyohenyl)valeric acid	
	3-(3,4-dihydroxyphenyl)propionic acid	
	5-(3-methoxyohenyl)valeric acid	
	2,3-dihydroxyphenoxyl 3-(3′,4′-dihydroxyphenyl)propionic acid	
ellagitannins/ellagic acid[3,22,25,47]	urolithin-A	estrogenic and/or antiestrogenic activity, antimalarials, induction of detoxification enzymes
	urolithin-B	estrogenic and/or antiestrogenic activity, antimalarials, induction of detoxification enzymes
	urolithin-C	estrogenic and/or antiestrogenic activity

Table 1. continued

plant polyphenol	microbial catabolite	possible health effects
	urolithin-D	estrogenic and/or antiestrogenic activity
rutin[37,48,49]	3-hydroxyphenylacetic acid	rutin and catabolites inhibit AGE formation in vitro; antimicrobial activity against Gram-negative enterobacteria via outer membrane destabilization
	3,4-dihydroxybenzoic acid	
	4-hydroxybenzoic acid	
	3-(3-hydroxyphenyl)propionic acid	
	3,4-dihydroxyphenylacetic acid	
lignans[45]	enterolactone	phytoestrogen important for heart and bone health; possible colon cancer protectants
	enterodiol	phytoestrogen important for heart and bone health; possible colon cancer protectants

[a]The putative health effects of selected catabolites are also presented.

However, few human studies have been conducted with levels of dietary fiber or prebiotic demonstrated in animals to protect against these chronic diseases.

The majority of plant complex polysaccharides fall within the EC definition of dietary fibers, and estimates of fiber intake for Western populations are in the region of 20 g per day compared to populations consuming traditional diets rich in fruits, vegetables, and grains and our ancestral hunter-gatherers, who consume or consumed between 70 and 120 g fiber per day.[1] As well as plant nonstarch structural polysaccharides, which by virtue of their chemical makeup resist the degradative activities of human digestive enzymes in the stomach and small intestine, plant storage carbohydrates such as starch can also reach the colon when rendered physically or chemically inaccessible to human digestive enzymes.[18] Food processing and food preparation processes such as cooking and cooling can affect greatly the proportion of dietary starch becoming resistant and reaching the colon. Similarly, other food components can affect the quantity of starch reaching the colon. For example, polyphenol-rich beverages when consumed at meal times can affect directly starch digestion by inhibiting starch-degrading enzymes in the upper gut and thus blunting postprandial glucose peaks. Similarly, the Maillard reaction in cooked foods can increase the recalcitrance of food macromolecules to digestion, leading to increased survival of both carbohydrates and proteins until the colon.[8] However, once these food compounds reach the colon, they become available to the fermentative activities of the human colonic microbiota. Recent metagenomic studies have highlighted that the gut microbiota is specifically evolved for the digestion of complex plant polysaccharides, possessing a range of polysaccharide- and glycan-degrading enzymes not present in the human genome.[6,19] In this way, the human gut microbiota can be viewed as a closely coevolved microbial partner to the human genome, extending host-encoded functions and allowing the host to derive energy and other biologically active compounds from food components that would otherwise remain inaccessible and be excreted as waste.

Carbohydrate fermentation is the chief energy source for the gut microbiota, and the proximal colon can be viewed as a saccharolytic environment, where the dominant fermentative activity is carbohydrate fermentation leading to the production of the short-chain fatty acids acetate, propionate, and butyrate. These small organic acids have diverse functions in the host, not just supplying energy to the intestinal mucosa, heart, brain, and muscle but also playing important roles in human cell differentiation, proliferation, and programmed cell death; regulation of immune function; thermogenesis; and lipid metabolism.[10] In individuals following a Western style diet at least, as dietary fiber and colonic carbohydrate are used up in the proximal colon, saccharolytic fermentation decreases along the transverse and distal colon as the concentration of substrate decreases. Microorganisms then switch to other energy sources including dietary- or host-derived proteins and amino acids. The end products of amino acid fermentation include SCFA but also branched-chain fatty acids, amines, indoles, sulfides, and phenols, some of which are potentially harmful, being variably genotoxic, cytotoxic, and carcinogenic. As described above, human populations with higher intakes of dietary fiber and whole plant foods tend to have higher concentrations of SCFA in their feces, suggesting that in these populations carbohydrate fermentation may be extended along the length of the colon. This may thus avoid buildup of toxic or harmful metabolites produced when bacteria switch their fermentation substrate from carbohydrate to amino acids. We have recently shown that a 3-fold increase in dietary fiber results in a proportional increase in SCFA production by the gut microbiota and extends saccharolytic fermentation into the transverse and distal colon using an in vitro three-stage model of the human colonic microbiota.[20] It would be very interesting to confirm in humans following a low-fiber Western style diet whether intervention with high levels of dietary fiber can modulate the health-promoting saccharoltyic activities of the gut microbiota to a similar degree in vivo.

POLYPHENOLS AND THE HUMAN GUT MICROBIOTA

Hydrolysis by the gut microbiota can increase the bioavailability and possibly, the biological activity of polyphenols reaching the colon. The human intestinal microbiota has extensive hydrolytic activities and breaks down many complex polyphenols into smaller phenolic acids, which can be absorbed across the intestinal mucosa. These polyphenol catabolites are only then available to exert their biological activities systemically within the host. However, to date, with the exception of a few compounds such as the phytoestrogens equol, enterolactone, and enterodiol, very little is known about the biological activities of phenolic acids derived from microbiota polyphenol metabolism.[2,3] Table 1 presents some examples of parent plant polyphenols, microbial catabolites, and their potential biological effect when known. Given the diversity of plant polyphenols, their possible microbial catabolites, and the fact that they occur as mixtures in whole plant foods or plant extracts, only a small number of well-studied polyphenols are presented. Biological activities have not been described for most of the small

phenolic acid catabolites, but common activities recognized so far include antibacterial activities especially against Gram-negative species, like the Enterobacteriaea, anti-inflammatory activities, anti-AGE formation, stimulation of xenobiotic degrading enzymes and detoxification processes, and phytoestrogenic activities.

Recently, Miene et al.[21] demonstrated that microbial metabolites of quercetin and chlorogenic acid/caffeic acid, 3,4-dihydroxyphenylacetic acid (ES), and 3-(3,4-dihydroxyphenyl)propionic acid (PS), respectively, could significantly up-regulate GSTT2 expression and decrease COX-2 expression, a modulation seen as protective against colon cancer, and at the same time reduce DNA damage using an intestinal cancer cell line. González-Sarrías et al.[22] showed that ellagic acid (EA) and its colonic metabolites, urolithin-A (3,8-dihydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one, Uro-A) and urolithin-B (3-hydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one, Uro-B), modulate the expression and activity of CYP1A1 and UGT1A10 and inhibit several sulfotransferases in colon cancer cell lines (Caco-2). These phase I and phase II detoxifying enzymes are important components of how our bodies deal with toxic and xenobiotic compounds, and increased expression is associated with a reduced risk of colon cancer in laboratory animals. However, these effects appeared to be critically affected by food matrix in the rat colon. Urolithins, from pomegranate, had, however, already been shown to reduce the growth of cancer cells in an animal model of prostate cancer.[23]

The 3,4-dihydroxyphenylpropionic acid (3,4-DHPPA), 3-hydroxyphenylpropionic acid, and 3,4-dihydroxyphenylacetic acid (3,4-DHPAA), derived from colonic catabolism of proanthocyanidins, have been shown to reduce the inflammatory response of human peripheral blood mononuclear cells stimulated with lipopolysaccharide (LPS), an inflammatory cell wall component from Gram-negative bacteria such as the Enterobacteriaceae. Secretion of IL-6, IL-1, and TNF-α was reduced, suggesting that microbial metabolites may be involved in dampening the inflammatory response to bacterial antigens, which may have implications for chronic inflammatory or autoimmune diseases such as IBD.[24] Upon screening 18 microbial catabolites of polyphenols for their anti-inflammatory potential in vitro, Larrosa et al.[25] found that hydrocaffeic (HCAF), dihydroxyphenylacetic (dOHPA), and hydroferulic acid (HFER) reduced prostaglandin E(2) production by at least 50% in CCD-18 colon fibroblast cells stimulated with IL-1β. The same compounds were also shown to reduce inflammation in rodents, and HCAF was also shown to reduce intestinal inflammation in the DSS-mouse model of ulcerative colitis with reduced mucosal expression of cytokines IL-1β, IL-8, and TNF-α, reduced malonyldialdehyde (MDA) levels, and decreased oxidative DNA damage (measured as 8-oxo-2′-deoxyguanosine levels). Radnai et al.[26] also showed that ferulaldehyde, a microbial catabolite of curcumin, has anti-inflammatory properties in vivo in an animal model of LSP-induced septic shock. Intraperitoneal injection of ferulaldehyde (6 mg/kg) reduced markers of inflammation and prolonged the lifespan of LPS-treated animals, indicating that microbial catabolites might, at least in part, account for the observed anti-inflammatory activity of certain herbal medicines and functional foods.

Microbial catabolites of plant polyphenols may also affect disease risk in metabolic syndrome, a cluster of biomarkers associated with obesity and increased risk of cardiovascular disease and diabetes. Verzelloni et al.[27] demonstrated that urolithins and pyrogallol, microbial ellagitannin-derived catabolites, are highly antiglycative compared to parent polyphenolic compounds in an in vitro model of protein glycation. Protein glycation plays an important pathological role in diabetes and the pathologies associated with diabetes including blindness. They also found that chlorogenic acid-microbially derived catabolites, dihydrocaffeic acid, dihydroferulic acid, and feruloylglycine, were most effective at protecting cultured neural cells in vitro, indicating that colonic catabolites of dietary polyphenols may play an important role in the improved cognitive function and protection from neuronal degeneration observed in animals fed polyphenol-rich foods such as certain berries.

Plant polyphenols have also been shown to have a direct effect on carbohydrate fermentation by the human gut microbiota in vitro. Using anaerobic fecal batch cultures, Bazzocca et al.[28] found that apple proanthocyanidins inhibited both metabolic degradation of short proanthocyanidins and SCFA production. On its face, such an observation may suggest that polyphenols might work against production of beneficial microbial metabolites within the colon. As discussed above, in individuals on a Western style, low-fiber diet, the proximal colon is the major site of saccharolytic fermentation, with potentially damaging proteolytic fermentation increasing distally as carbohydrate substrate becomes limiting. Retardation of carbohydrate fermentation in the proximal colon may extend SCFA production to the distal colon, thereby reducing the harmful effects of amino acid catabolites. However, this remains to be investigated either in vivo or using complex multistage continuous culture models of the colonic microbiota. Similarly, polyphenols have also been shown to directly affect the relative abundance of different bacteria within the gut microbiota with tea polyphenols and their derivatives reducing numbers of potential pathogens including *Clostridium perfringens* and *C. difficile* and certain Gram-negative *Bacteroides* spp., with less inhibition toward beneficial clostridia, bifidobacteria, and lactobacilli.[29]

Human data on the impact of polyphenols on the gut microbiota are scarce. One recent work from the University of Reading has shown that a high cocoa-flavanol (494 mg/day) drink can mediate a prebiotic type modulation of the gut microbiota in a randomized intervention study compared to a low cocoa-flavanol (23 mg/day) drink. The high-flavanol drink resulted in significant increases in fecal bifidobacteria and lactobacilli and a reduction in clostridial counts and a concomitant reduction in plasma triglycerides and C-reactive protein, both important markers of metabolic disease.[30] These results were mirrored by previous in vitro studies using fecal batch cultures by the same group.[31]

WHOLE PLANT FOODS AND THE GUT MICROBIOTA

Although much work has been conducted on the effect of isolated fibers on the composition and activity of the human gut microbiota, few studies have examined the impact of whole plant foods on gut microbial species composition and relative abundance. Recently, whole grain breakfast cereals have been shown to mediate a prebiotic modulation of the gut microbiota, giving significant increases in fecal bifidobacteria and/or lactobacilli without changing the relative abundance of other dominant members of the gut microbiota.[32,33] Costabile et al.[32] also showed that ingestion of either whole grain wheat or wheat bran breakfast cereal increased plasma and urine concentrations of ferulic acid, a polyphenol commonly complexed with dietary

fiber in whole grain cereals. In a small scale (n = 8) observational study, two apples per day for 2 weeks was also shown to increase numbers of fecal bifidobacteria, lactobacilli, and streptococci/enterococci while reducing numbers of enterobacteria and lecithinase-positive clostridia, including *C. perfringens* and *Pseudomonas* species.[34] However, the study did not use culture-independent microbiology techniques and suffered from the lack of a control group. Gill et al.[35] did not observe any discernible change in fecal bacterial populations in another small human feeding study (n = 10) with raspberry puree (200 g/day for 4 days) due to high intersubject variation in fecal bacteria but did report that microbial raspberry polyphenol metabolite profiles varied greatly between individuals, indicating that species makeup of the gut microbiota may affect catabolite profiles released by bacteria in the colon. In a recent large-scale dietary intervention, the FLAVURS group investigated the relative impact of increased fruit and vegetable intake of differing flavonoid content on markers of cardiovascular disease risk.[36] One hundred and fifty-four subjects at risk of cardiovascular disease were divided into three parallel groups consuming either their habitual diet, a high-flavonoid fruit and vegetable diet, or a low-flavonoid fruit and vegetable diet for 18 weeks. Subjects on the fruit and vegetable diets consumed two 80 g portions per day for 6 weeks, increasing to four portions and then six portions for the following two 6 week periods. The dietary objectives were broadly met by the subjects with the inclusion of complex and processed foods containing flavonoids, and significant increases in urinary total flavonoids were observed for the high-flavonoid group, whereas both fruit and vegetable diets gave dose-proportional increases in plasma vitamin C and other phytochemicals. Nonstarch polysaccharides, a measure of dietary fiber, also increased in both fruit and vegetable treatment groups as reported by diet diaries. Culture-independent analysis of fecal bacteria revealed that in the first cohort (n = 59), dietary intervention with either flavonoid-rich or flavonoid-poor fruits and vegetables significantly increased groups of commensal bacteria important for human health including *Bifidobacterium*, *Atopobium*, *Ruminococcus*, *Roseburia*, *Eubacterium*, and *Faecalibacterium prausnitzii*, whereas the flavonoid-poor diet also increased lactobacilli compared to the control diet. No changes in gut microbiota profile occurred in the group following the control diet and fecal SCFA did not change. Currently, at Fondazione Edmund Mach in Italy, we are conducting a full metabolomics profile of biofluids and gut metagenomic analysis of samples collected from these subjects to assess the impact of increased fruit and vegetable intake on the gut microbiota and how this may affect systemic metabolism via up-regulation of the metabolite flux from the colon.

■ DISCUSSION

There is growing evidence from animal and human studies that the two main classes of biologically active molecules in whole plant foods, dietary fibers and polyphenols, can have dramatic effects on the human gut microbiota. These compounds appear to modulate both species composition within the gut microbiota and the profile of metabolites absorbed from the colon. However, to date, few studies in humans have focused on the possible biological activities of the small phenolic catabolites produced by gut microibota transformation of plant polyphenols. Similarly, few studies have considered the possible synergistic activity of fiber and polyphenols present in whole plant foods. From the limited number of intervention studies with whole grain cereals that also measured gut microbiota effects, it appears that increasing consumption of whole plant foods might significantly up-regulate groups of commensal bacteria thought to be associated with human health. There is, however, a dearth of randomized, controlled, human intervention studies with whole plant foods that include measures of the gut microbiota or their metabolic output. This current reality is limiting full appreciation of how the closely coevolved relationship between whole plant foods and our gut microbiota regulates human health.

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Notes

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Aim and Overview
of the Ph.D. Project

The effects of fruit consumption on the mammalian system

Recently, numerous studies have suggested that a wide range of polyphenols and their metabolites may have neuroprotective effects both *in vitro* and *in vivo*. Berries and strawberries are certainly among the best examples of "superfruit" with proven positive healthy effects. In order to correctly evaluate the nutritional impact of fruit consumption it is first important to assess the precise composition of fruit polyphenols. In this study strawberries were chosen, as they are the most widely consumed type of soft fruit. Furthermore, it was necessary to prove whether these compounds can reach the brain in sufficient concentrations and in biologically active forms in order to exert beneficial effects.

The aim of my project was to study the effects of fruit consumption on the mammalian system and it was designed to cover two complementary fields of research: food chemistry and nutritional bioavailability. With the growing interest in the use of metabolomics, a wide range of biological targets and food applications related to nutrition and quality are rapidly emerging, offering the opportunity to gain deeper insight into several fields of research. A better knowledge of which types of polyphenols affect the mammalian metabolism can be achieved with the implementation of well-designed metabolomics experiments with different targets.

Accurate knowledge about the content of compounds important in the human diet in terms of polyphenols and their bioavailability is still not available, therefore the final goal of the study was to provide a substantial improvement in understanding the relevance of a diet rich in polyphenols to health.

The thesis, gathering together the main results published and manuscripts about to be published, is divided into these two fields of research. Each publication or manuscript is introduced by a brief overview of the literature and the scope and the main results obtained, along with a precise statement of my personal involvement in the work.

In the food chemistry section, *Chapter 1.1* presents a targeted metabolomics method for the detection of several classes of polyphenols in a short chromatographic run, while in *Chapter 1.2* the attention was focused on a precise class of polyphenols also present in the strawberry: ellagitannins. The objective was to identify and clarify the chemical structures of the main ellagitannins in the strawberry, followed by their isolation and precise characterisation. Moreover in a second publication, further abundant ellagitannins were isolated and overall ellagitannin profiling with high definition mass spectrometry was performed, along with their quantification. *Chapter 1.3* instead combines the results presented in the previous chapters with the precise polyphenolic composition of a bowl of strawberries, presented for the first time.

In the nutritional bioavailability section, Chapter 2.1 presents the development of a targeted metabolomics method for the detection of 23 polyphenol microbial metabolites in different organs of rats, i.e. blood, brain, liver, kidneys, heart and urine. Chapter 2.2 instead brings together the results of an *in vivo* experiment tracing the fate of 23 polyphenol microbial metabolites in rats, primarily in the brain but also in other organs, resulting from the putative consumption of a bowl of strawberries. The main goal was to prove whether these metabolites can reach the brain, supporting their ability to promote neuroprevention of brain ageing and neurological disease, as reported in epidemiological studies or *in vitro* experimentation.

Food Chemistry

Chapter 1.1

A targeted metabolomics method for the rapid quantification of multiple classes of phenolics

This chapter has been reprinted* from:

A Versatile Targeted Metabolomics Method for the Rapid Quantification of Multiple Classes of Phenolics in Fruits and Beverages Urska Vrhovsek, Domenico Masuero, **Mattia Gasperotti**, Pietro Franceschi, Lorenzo Caputi, Roberto Viola, Fulvio Mattivi *Journal of Agricultural and Food Chemistry* **2012** *60* (36), 8831-8840

Along with the widespread interest in phenolic compounds as food constituents, numerous separation and detection methods for their identification and quantification in different matrices have been developed in recent years (28*).

However, the number of compounds that have been quantified in a single run with existing methodologies is still relatively modest when compared to the potential coverage of metabolites that could be achieved using the powerful technologies available on the market for chromatography and mass spectrometry, which are widely used in other fields, such as multi-residue pesticide analysis in food (29) and hormones (30). Targeted metabolomics represents an attractive strategy for food analysis. This methodology aims to quantify a predefined set of metabolites, typically dozens or hundreds of known compounds, based on metabolite-specific signals (33, 34).

In particular, in targeted metabolomics approaches, using triple-quadrupole mass spectrometers, a precursor ion and a fragment of the precursor ion, producing a molecular weight and structure-specific measurement for a single metabolite, are used for the sensitive and accurate determination of the compound concentration over a wide dynamic range. Simultaneous analysis of multiple transitions results in multiple reaction monitoring (MRM).

The aim of this study was to develop a rapid and versatile targeted metabolomics method for the quantification of multiple classes of phenolics that could be used for high-throughput analysis of fruits and beverages.

A detailed introduction, description of the methods and discussion of the results are attached in the original publication.

An UPLC-MS/MS metabolite profiling method for food analysis, allowing rapid exploration of the presence of polyphenols in different food matrices, with

* References are reported in the same order and listed in the attached publication

converging opportunities for research applications in plant science and human nutrition was developed. The high sensitivity of MRM-based mass spectrometry and the wide dynamic range of triple-quadrupole spectrometers provide valuable insight for the analysis of complex matrices such as fruit, and more in general food, in which analyte concentrations span several orders of magnitude.

The method developed for the profiling of phenolic compounds is versatile and could be successfully applied to the analysis of a range of different matrices, not limited to those chosen for validation. The short duration of the analysis and straightforward sample preparation make the methodology suitable for high-throughput varietal screening studies and for use in assisting plant breeders to select specific chemotypes.

My personal contribution to this work mainly concerned the setting up of instrumental conditions, strategies for the execution of future routine analysis and validation processes. After automatic detection of the best transition for each compound in the method and separation during the chromatographic run, I was personally involved in optimising the parameters to construct the method, hence in the selection of the quantifier ion, qualifier ion, and their specific ratio (according to European pesticide guidelines (40)), all these results being gathered together in *Table 1*. This table represents the core of the method, giving a list of all the transitions, energies involved in the fragmentation and ratios, along with the retention times of all the 135 phenolic compounds in a total run of 11 minutes. Moreover, I was involved in the preparation of the standard solution for calibration, the rational design of the dilution and mixing of different compounds, processing of the curves with subsequent validation of the methods in terms of linearity and limits of quantification (*Figures 1 and 2, Supplementary Table S2*). Finally, I was involved in sample preparation and analysis of the preliminary samples for the initial application of the method adopting different fruits and beverages using UPLC-MS/MS. The quantitative results of this application are graphically presented in *Figure 6* with all the compounds identified in the matrices.

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A Versatile Targeted Metabolomics Method for the Rapid Quantification of Multiple Classes of Phenolics in Fruits and Beverages

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Supporting Information

ABSTRACT: Compelling evidence of the health benefits of phenolic compounds and their impact on food quality have stimulated the development of analytical methods for the identification and quantification of these compounds in different matrices in recent years. A targeted metabolomics method has been developed for the quantification of 135 phenolics, such as benzoates, phenylpropanoids, coumarins, stilbenes, dihydrochalcones, and flavonoids, in fruit and tea extracts and wine using UPLC/QqQ-MS/MS. Chromatography was optimized to achieve separation of the compounds over a period of 15 min, and MRM transitions were selected for accurate quantification. The method was validated by studying the detection and quantification limits, the linearity ranges, and the intraday and interday repeatability of the analysis. The validated method was applied to the analysis of apples, berries, green tea, and red wine, providing a valuable tool for food quality evaluation and breeding studies.

KEYWORDS: *metabolite profiling, polyphenols, food analysis, UPLC/QqQ-MS/MS, mass spectrometry*

INTRODUCTION

Phenolics represent one of the most chemically diverse and ubiquitous classes of secondary metabolites in plants. Phenolics can be classified into different classes on the basis of their chemical structure and can also occur in polymeric forms such as hydrolyzable tannins, condensed tannins, and lignins and in glycosylated and acylated forms.

Phenolics biosynthesis would seem to be an early acquisition of plants deriving from the primary metabolism during their adaptation to life on land.[1,2] It has been suggested that the ability to produce secondary metabolites with antimicrobial or UV-protection properties, such as those possessed by phenolic compounds, could have given plants some advantages in facing the numerous challenges associated with the water-to-land transition.[3]

Phenolics have been credited with a variety of key functions important for plant growth, development, and survival. Some compounds are common mediators of plant responses to biotic and abiotic stresses.[4,5] Some are part of complex species-specific bouquets emitted as floral attractants to pollinators[6] or contribute to the color of flowers and fruits.[7] Several phenolics act as inducers of plant−microbe symbioses,[8] whereas others exhibit broad-spectrum antimicrobial activity and are therefore thought to help plants fight microbial diseases.[9] They also possess phytoalexin properties, and their synthesis can be induced in response to wounding, feeding by herbivores,[10] or infection by pathogens.[11] Thanks to their structure, containing aromatic rings and hydroxyl groups, phenolic compounds are good protecting agents against UV radiation and potent antioxidants.[12,13]

In recent years, interest in phenolic compounds has been increasing due to compelling evidence of their health benefits and their impact on food quality. Indeed, due to their widespread presence in plants, phenolics enter the human diet from a variety of edible plants and plant products, such as fresh and cooked vegetables, fruit juices, tea, wine, and infusions. For instance, a comprehensive analysis of food composition data revealed that 502 polyphenols, including flavonoids, phenolic acids, lignans, and stilbenes, have been reported in 452 foods so far.[14] Many systematic molecular, in vitro, and epidemiological studies have confirmed their effect on various pathological situations, and their mechanisms of action are under investigation.[15] Recently published papers have reviewed the activity of food polyphenols in decreasing the risk of cancer[16,17] and in preventing allergic diseases,[18] atherosclerosis,[19] obesity,[20] bone resorption,[21] aging,[22] neurodegeneration and dementia,[23] hypertension,[24] and dental caries.[25]

Recently, a few specialist sources, such as Phenol Explorer (www.phenol-explorer.eu)[26] and the USDA database (http://www.ars.usda.gov/Services/docs.htm?docid=8964) have significantly improved the possibility of correctly estimating the nutritional intake of the main classes of phenolics in common

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food.[27] However, the presence of phenolics in most food databases worldwide is still limited, and we are convinced that there is a general need to improve coverage by inserting data on a larger number of phenolic compounds and food sources.

Recent advances in understanding the importance of the compositional quality of plant-derived food for human health are also changing the focus of crop producers and breeders from the traditional improvement of pest resistance and yield to their chemical composition and nutritional value.

As a consequence of this widespread interest in phenolic compounds as food constituents, numerous separation and detection methods for their identification and quantification in different matrices have been developed, these having been recently reviewed by Kalili and de Villiers.[28] However, the number of compounds that have been quantified in a single run with existing methodologies is still relatively modest when compared to the potential coverage of metabolites that could be achieved using the powerful technologies available on the market for chromatography and mass spectrometry, which are widely used in other fields, such as multiresidue pesticide analysis in food[29] and hormones.[30] For instance, a rapid LC-MS/MS method for the quantification of phenols and polyphenols in authentic wine samples, developed by Jaitz et al.[31] targeted only 11 compounds, and the method developed by Guillarme et al. for catechins quantification in tea extracts targeted only 8 metabolites.[32]

Targeted metabolomics represents an attractive strategy for food analysis. This methodology aims to quantify a predefined set of metabolites, typically dozens or hundreds of known compounds, based on metabolite-specific signals.[33,34] In particular, in targeted metabolomics approaches, using triple-quadrupole mass spectrometers, a precursor ion and a fragment of the precursor ion, producing a molecular weight and structure-specific measurement for a single metabolite (referred to as transition), are used for the sensitive and accurate determination of the compound concentration over a wide dynamic range. Simultaneous analysis of multiple transitions results in multiple reaction monitoring (MRM).

The aim of this study was to develop a rapid and versatile targeted metabolomics method for the quantification of multiple classes of phenolics that could be used for high-throughput analysis of fruits and beverages. This would have applications for food quality evaluation but could also assist plant breeders to select a chemical phenotype or "chemotype".

■ MATERIALS AND METHODS

Chemicals. Methanol and acetonitrile were of LC-MS grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chloroform anhydrous stabilized with 0.5−1% ethanol and formic acid were also purchased from Sigma-Aldrich. The majority of the chemical standards are commercially available and were obtained from different suppliers (Table S1 of the Supporting Information). Viniferins were isolated from leaves of a hybrid *Vitis vinifera* as described by Mattivi et al.[11] The hydroxycinnamoyltartaric acids (*trans*-caftaric acid, *trans*-coutaric acid, and *trans*-fertaric acid) were extracted and purified according to the method described by Vrhovsek.[35] *cis*-Resveratrol and *cis*-piceid were produced by photochemical isomerization of the *trans* forms, as described by Mattivi et al.[36] Milli-Q water was used for the chromatography.

Preparation of Standard Solutions. About 145 phenolic compounds were initially selected for the assay. The choice of the metabolites was mainly based on their importance and/or relevance for food quality, covering the major classes. In particular, benzoates, phenylpropanoids, coumarins, stilbenes, dihydrochalcones, and flavonoids commonly occurring in plants were included, together with metabolites specific to a single species or family. High molecular weight polymers such as tannins were not included in this study. Because good separation of the positively charged anthocyanidins requires chromatography to be carried out in particular conditions, that is, with a very low pH, they were not included in the assay.

The compound names, chemical formulas, and CAS Registry No. are listed in Table S1 of the Supporting Information. Furthermore, the METLIN ID of the compounds, which links the compounds to the Metabolite and Tandem MS Database (http://metlin.scripps.edu/), and the KEGG ID, which can help to visualize the metabolites on the pathways (KEGG Pathway Database, http://www.genome.jp/kegg/pathway.html), are provided.

Stock solutions of each individual standard were prepared in pure methanol with the following exceptions: salicin, 4-hydroxybenzoic acid, dihydroxybenzoic acids, and vanillic acid were prepared in methanol/water (1:1). These starting solutions were used to prepare 16 standard mixtures including 6−10 compounds each. Serial dilutions were prepared to obtain 24 lower concentrations (dilution factors of 1−60000) for linear dynamic range assessment. The composition of each mixture and the starting concentrations of the analytes are reported in Table S1 of the Supporting Information.

Sample Preparation. *V. vinifera* cv. Sangiovese grape berries (2009 harvest, Tuscany, Italy), *Malus domestica* cv. Golden Delicious apples, *Fragaria* × *ananassa* cv. Elsanta strawberries, *Rubus idaeus* cv. Tulameen raspberries, and *Prunus avium* cv. Kordia cherries, all purchased from a local market and grown in the Trentino region (Italy), were ground under liquid nitrogen using an IKA analytical mill (Staufen, Germany) to obtain a frozen powder. The protocol used for the extraction of the phenolic metabolites from these matrices was adapted from that of Theodoridis et al.[37] Briefly, 2 g of powder from each sample was extracted in sealed glass vials using 5 mL of a mixture of water/methanol/chloroform (20:40:40). After vortexing for 1 min, the samples were put in an orbital shaker for 15 min at room temperature. Samples were centrifuged at 1000*g* and 4 °C for 10 min, and the upper phases constituted of aqueous methanol extract were collected. Extraction was repeated by adding another 3 mL of water/methanol (1:2) to the pellet and chloroform fractions and shaking for 15 min. After centrifugation, the upper phases from the two extractions were combined, brought to 10 mL, and filtered through a 0.2 μm PTFE filter prior to analysis.

Ground dry green tea leaves (*Camellia sinensis*, Vivi Verde COOP, organic farming) purchased from a local store were extracted with aqueous 80% methanol in a ratio of 1 g to 10 mL of solvent (w/v) using a sonicator for 30 min.[38] After centrifugation at 1000*g* and 4 °C for 10 min, the supernatant was collected and filtered through a 0.2 μm PTFE filter prior to analysis.

Sangiovese wine (red table wine, Tavernello, traceability code LB0097WO, Caviro, Italy) was analyzed after filtration on 0.2 μm PTFE filters.

Liquid Chromatography. Ultraperformance liquid chromatography was performed on a Waters Acquity UPLC system (Milford, MA) consisting of a binary pump, an online vacuum degasser, an autosampler, and a column compartment. Separation of the phenolic compounds was achieved on a Waters Acquity HSS T3 column 1.8 μm, 100 mm × 2.1 mm (Milford, MA, USA), kept at 40 °C. Mobile phase A was water containing 0.1% formic acid; mobile phase B was acetonitrile containing 0.1% formic acid. The flow was 0.4 mL/min, and the gradient profile was 0 min, 5% B; from 0 to 3 min, linear gradient to 20% B; from 3 to 4.3 min, isocratic 20% B; from 4.3 to 9 min, linear gradient to 45% B; from 9 to 11 min, linear gradient to 100% B; from 11 to 13 min, wash at 100% B; from 13.01 to 15 min, back to the initial conditions of 5% B. The injection volume of both the standard solutions and the samples was 2 μL. After each injection, the needle was rinsed with 600 μL of weak wash solution (water/methanol, 90:10) and 200 μL of strong wash solution (methanol/water, 90:10). Samples were kept at 6 °C during the analysis.

Mass Spectrometry. Mass spectrometry detection was performed on a Waters Xevo TQMS (Milford, MA, USA) instrument equipped with an electrospray (ESI) source. Capillary voltage was 3.5 kV in positive mode and −2.5 kV in negative mode; the source was kept at 150 °C;

Table 1. MRM Parameters of the Selected Metabolites

				quantifier ion		qualifier ion 1			qualifier ion 2	
compound	RT (min)	ES	cone voltage (V)	Q1 *m/z*	collision energy (V)	Q2 *m/z*	collision energy (V)	Q2 *m/z*	expected % ratio ion 1/quantifier	collision energy (V)	Q2 *m/z*
Benzoic Acid Derivatives											
catechol	2.89	−	36	109	12	81	16	53	13		
benzoic acid	5.61	−	22	121	12	77					
3-hydroxybenzaldehyde	3.97	+	20	123	10	95	16	77	13		
salicylic acid	6.06	+	22	139	12	121					
4-hydroxybenzoic acid	2.84	+	20	139	18	77	26	65	9		
2,4-dihydroxybenzoic acid	3.31	+	24	153	14	109	16	65	34		
2,5-dihydroxybenzoic acid	2.88	+	26	153	16	109	16	81	5		
2,6-dihydroxybenzoic acid	3.61	+	26	153	14	109	18	135	11	20	91
3,4-dihydroxybenzoic acid	2.12	+	24	153	20	81	22	91	64		
3,5-dihydroxybenzoic acid	2.10	+	26	153	14	109	14	65	9		
gallic acid	1.41	+	28	169	22	79	22	97	42	18	78
anthranilic acid	3.92	+	14	138	26	65	34	92	11	30	39
vanillin	4.07	+	20	153	16	93	10	125	61	22	65
vanillic acid	3.23	+	18	169	14	93	18	110	25	22	95
methyl gallate	2.90	+	32	183	22	124	16	168	5	28	78
cinnamic acid	7.55	+	16	149	12	131	10	103	1		
acetovanillone	4.56	+	20	167	12	43	12	125	7	18	110
syringaldehyde	4.35	+	20	183	12	123	10	155	51	18	95
syringic acid	3.40	+	22	197	14	182					
salicin	2.21	+	38	309	16	185	18	147	67		
ellagic acid	4.38	−	52	301	34	145	30	185	15		
Coumarins											
4-hydroxycoumarin	6.06	+	42	163	18	91	20	69	99		
umbelliferone	4.40	+	32	163	20	107	20	91	34		
4-methylumbelliferone	5.87	+	34	177	20	121	18	105	84		
daphnetin	3.17	+	36	179	22	123	24	105	16		
esculetin	3.49	+	34	179	22	133	22	123	38		
esculin	2.41	+	24	341	18	179	40	133	9		
scopoletin	4.53	+	34	193	20	133	22	178	32		
fraxin	3.07	+	32	369	20	207	32	192	38		
Phenylpropanoids											
p-coumaric acid	4.04	+	16	165	24	91	26	119	21		
m-coumaric acid	4.76	+	18	165	12	147	16	119	8	22	91
o-coumaric acid	5.70	+	18	165	16	103	22	91	15		
caffeic acid	3.19	+	16	181	16	145	22	117	26		
ferulic acid	4.52	+	12	195	16	145	24	117	40		
sinapic acid	4.55	−	30	223	14	208	18	164	45	20	149
caftaric acid	2.31	−	18	311	10	149	14	179	62	28	135
neochlorogenic acid	2.18	−	26	353	18	191	18	179	52	34	135
cryptochlorogenic acid	2.87	−	34	353	14	173	16	179	19	30	135
chlorogenic acid	2.76	−	36	353	20	191					
1,3-dicaffeoylquinic acid	3.43	−	34	515	20	353	34	191	33	32	179
1,5-dicaffeoylquinic acid	5.20	−	26	515	28	191	16	353	11		
rosmarinic acid	6.04	−	30	359	18	161	18	197	14		
coniferyl aldehyde	5.70	−	22	179	14	147	18	119	23		
coniferyl alcohol	4.04	+	12	163	10	131	18	103	25		
sinapyl alcohol	4.07	−	14	193	10	161	16	133	31	20	105
fertaric acid	3.13	−	20	325	16	193					
trans-coutaric acid	2.85	−	20	295	12	163					
raspberry ketone	5.52	−	36	107	18	77					
Stilbenes											
4-hydroxystilbene	10.67	−	44	195	26	93					
trans-resveratrol	6.56	−	38	227	18	185	26	143	128		
cis-resveratrol	7.55	−	40	227	26	143	22	185	39	18	159
piceatannol	5.10	−	40	243	28	159	22	201	24	20	175
pterostilbene	10.58	−	40	255	18	240	32	197	44	38	169
trans-piceid	4.27	−	28	389	18	227	36	185	7		

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Table 1. continued

					quantifier ion		qualifier ion 1			qualifier ion 2	
compound	RT (min)	ES	cone voltage (V)	Q1 *m/z*	collision energy (V)	Q2 *m/z*	collision energy (V)	Q2 *m/z*	expected % ratio ion 1/quantifier	collision energy (V)	Q2 *m/z*
Stilbenes											
cis-piceid	5.86	−	28	389	20	227	38	185	7		
astringin	3.53	−	36	405	20	243	36	201	6		
isorhapontin	4.69	−	30	419	18	257	36	241	46		
cis-ε-viniferin	7.93	−	48	453	22	347	22	359	80	36	225
trans-ε-viniferin	8.11	−	52	453	22	347	20	359	27	34	225
cis-ω-viniferin	8.50	−	46	453	20	347	20	359	31	30	225
trans-ω-viniferin	8.62	−	48	453	22	347	22	359	21	30	225
caffeic acid + catechin condensation product	5.56	−	34	451	18	341	34	177	10		
pallidol	6.00	−	32	453	30	265	14	359	20		
ampelopsin D + quadrangularin A	6.65	−	42	453	18	359	26	343	47	28	289
α-viniferin	8.59	−	48	677	30	437	26	571	19		
E-cis-miyabenol	8.17	−	54	679	30	345	26	573	15		
Z-miyabenol C	8.85	−	54	679	36	345	24	573	21		
isohopeaphenol	7.57	−	48	905	38	359	30	811	8		
ampelopsin H + vaticanol C-like isomer	8.01	−	52	905	30	811	40	475	3	42	335
Dihydrochalcones											
phloretin	8.23	−	32	273	18	167	24	123	11		
phloridzin	6.22	−	32	435	16	273	30	167	17		
trilobatin	6.72	−	36	435	18	273	18	297	21	32	167
Isoflavones											
daidzein	6.98	+	30	255	24	199	26	137	132		
genistein	8.67	+	44	271	24	215					
Flavones											
6-methoxyflavone	10.62	+	16	253	38	108	26	238	44	40	153
chrysin	10.16	−	44	253	30	143	26	107	58	32	63
apigenin	8.28	−	42	269	34	117	20	151	1	24	148
apigenin-7-*O*-glucoside	5.71	+	24	433	20	271	52	153	6		
apiin	5.44	+	26	565	30	271	14	433	3		
sinensetin	9.84	+	8	373	28	343	26	312	88	48	153
morin	6.73	+	38	303	32	153	26	229	75	28	137
eupatorin-5-methylether	8.87	+	12	359	18	329	26	298	62	46	153
luteolin	7.37	+	52	287	32	153	30	135	40		
luteolin-7-*O*-glucoside	4.56	−	46	447	24	285	48	151	2		
luteolin-8-C-glucoside	3.84	+	36	449	30	329	22	413	25	34	299
baicalein	8.82	+	46	271	32	123	32	103	9		
hesperetin	8.60	+	32	303	26	153					
hesperidin	5.84	+	18	611	24	303	10	449			
galangin	6.09	+	48	271	32	153					
Flavanones											
naringenin	8.19	−	32	271	18	151	24	119	20	26	107
naringenin-7-*O*-glucoside	6.20	−	16	435	14	273					
sakuranetin	10.20	−	32	287	30	119	24	167	31	22	147
eriodictyol	7.18	−	30	289	24	153					
Flavan-3-ols											
catechin	2.80	−	32	289	20	203	32	123	33		
epicatechin	3.32	−	34	289	20	203	30	123	46	28	109
gallocatechin	1.89	−	32	305	26	125	18	179	28		
epigallocatechin	2.50	−	32	305	22	125	16	179	34		
catechin gallate	4.45	−	34	441	18	289	20	169	27	40	125
epicatechin gallate	4.36	−	34	441	18	289	20	169	26	38	125
gallocatechin gallate	3.56	−	26	457	20	169	18	305	6	38	125
epigallocatechin gallate	3.37	−	32	457	16	169	20	305	6	38	125
procyanidin A2	4.62	−	42	575	30	285	22	449	15	24	289
procyanidin B1	2.40	−	32	577	26	289	16	425	7	22	407
procyanidin B2 + B4	3.01	−	30	577	24	289	16	425	15	24	407

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Table 1. continued

compound	RT (min)	ES	cone voltage (V)	quantifier ion Q1 *m/z*	quantifier ion collision energy (V)	quantifier ion Q2 *m/z*	qualifier ion 1 collision energy (V)	qualifier ion 1 Q2 *m/z*	qualifier ion 1 expected % ratio ion 1/quantifier	qualifier ion 2 collision energy (V)	qualifier ion 2 Q2 *m/z*
Flavan-3-ols											
procyanidin B3	2.72	–	34	577	22	289					
Flavonols											
quercetin	8.40	+	50	303	34	153	28	229	81	30	137
quercetin-3-sulfate	4.50	-	24	381	18	301	28	179	3	30	151
quercetin-3-*O*-rhamnoside	5.55	+	16	449	10	303	14	129	1	22	85
quercetin-3-*O*-glucoside	4.50	+	18	465	12	303	48	229	5	52	153
quercetin-4′-*O*-glucoside	5.69	+	18	465	12	303	48	229	20	56	153
quercetin-3-*O*-galactoside	4.37	+	18	465	12	303	44	229	8	48	153
quercetin-3-*O*-glucose-6′-acetate	5.71	+	20	507	16	303	18	187	8	30	109
quercetin-3-*O*-glucuronide	4.45	+	20	479	18	303	48	229	6	50	153
rutin (quercetin-Glc-Rha)	4.18	+	18	611	22	303	12	465	8	34	85
quercetin-3-Glc-Ara	3.90	+	20	597	22	303	14	465	4	72	153
quercetin-3,4′-diglucoside	3.57	+	18	627	32	303	16	465	39	78	153
taxifolin	4.72	+	20	305	14	259	14	153	30	26	149
kaempferol	8.45	+	50	287	32	153	26	165	26	30	121
kaempferol-3-*O*-glucoside	5.45	+	16	449	14	287	48	153	5		
kaempferol-3-*O*-glucuronide	5.45	+	18	463	16	287	44	165	2	56	153
kaempferol-3-*O*-rutinoside	5.00	+	18	595	20	287	12	449	9	34	85
robinin	3.80	+	22	741	38	287	14	595	6	20	433
dihydrokaempferol	6.07	+	18	289	18	153	16	243	64	22	149
myricetin	6.07	+	44	319	32	153	28	245	23	26	165
myricitrin	4.30	+	14	465	10	319					
laricitrin	7.46	+	44	333	26	318	42	219	79	34	153
syringetin	8.58	+	40	347	28	153	24	287	60	30	258
syringetin-3-*O*-glucoside + syringetin-3-*O*-galactoside	5.71	+	16	509	14	347	40	287	6	42	153
rhamnetin	9.53	+	48	317	42	123	28	243	73	32	123
isorhamnetin	8.67	+	44	317	34	153	26	302	50	32	229
isorhamnetin-3-*O*-glucoside	5.69	+	16	479	14	317	30	302	13	50	153
isorhamnetin-3-rutinoside	5.30	+	18	625	20	317	12	479	7	52	302

desolvation temperature was 500 °C; cone gas flow, 50 L/h; and desolvation gas flow, 800 L/h. Unit resolution was applied to each quadrupole. Flow injections of each individual metabolite were used to optimize the MRM conditions. For the majority of the metabolites, this was done automatically by the Waters Intellistart software, whereas for some compounds the optimal cone voltages and collision energies were identified during collision-induced dissociation (CID) experiments and manually set. A dwell time of at least 25 ms was applied to each MRM transition.

Data Analysis. Data processing was done using Waters MassLynx 4.1 and TargetLynx software. Data visualization and annotation of high-resolution spectra were done using the R software suite (http://www.R-project.org),[39] with specific use of the Gplot library for heat map graphics.

Method Validation Study. Method validation was performed by studying the linear dynamic range, precision of the analysis, and limit of quantification (LOQ) for the standard compounds. The linear dynamic range was evaluated using standard solutions, prepared as described above, in a concentration range spanning >5 orders of magnitude for most of the compounds. The intraday (n = 5) and interday (n = 5) precision of the analyses was evaluated at two intermediate dilutions (100- and 1000-fold dilutions of the starting mixture). The limit of quantification (LOQ) for each compound was evaluated as the concentration at which the quantifier transition presented a signal-to-noise (S/N) ratio of >10.

Application to Fruit, Tea Extracts, and Wine. The method developed was applied to the analysis of several fruit extracts (apple, strawberry, raspberry, and cherry), tea leaves, and red wine. The precision of the instrumental analysis was evaluated by repeatedly injecting the samples (n = 5) and evaluating the RSD% of the peak areas and retention times of the detected metabolites.

RESULTS AND DISCUSSION

UPLC/QqQ-MS/MS Optimization. UPLC conditions were optimized to achieve good separation of the compounds in a short chromatographic run. Although acetonitrile and methanol were both found to perform well in terms of separation power, the choice of acetonitrile as mobile phase was mainly determined by the lower operational pressure on the column. Addition of 0.1% formic acid to the mobile phases improved chromatographic separation and ionization efficiency for most of the metabolites. Chromatography was performed on an Acquity HSS T3 column packed with a trifunctional C18 alkyl phase, which improves retention of the more polar metabolites. The gradient was optimized to provide separation of isomeric compounds, although in some cases this could not be achieved. For instance, the method allowed separation of all five dihydroxybenzoic acid isomers (2,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, and 3,4-dihydroxybenzoic acid). The *ortho*, *meta*, and *para* isomers of coumaric acid could also be separated, as could chlorogenic acid isomers, to mention just a few. Unfortunately, some isomeric forms could not be well separated and were quantified as a single compound. These compounds included glucoside and galactoside forms of

syringetin, procyanidins B2 and B4, ampelopsin H, and vaticanol C-like isomer. Under the optimized chromatographic conditions, all of the metabolites eluted within 11 min. The total run time was 15 min, including washing and conditioning steps.

MRM conditions were either manually or automatically optimized for each compound, in both positive and negative ESI ionization modes, during infusion studies. All tuning data acquired automatically through the IntelliStart software were manually examined to ensure proper selection of product ions and collision energy. In general, the MS conditions were first optimized in quadrupole 1 (Q1), which transmits only an ion of specific m/z. The ion selected for fragmentation was in most cases the protonated or deprotonated quasi-molecular ion. After CID studies, the conditions were adjusted for the third quadrupole (Q3) to provide optimal signals from the daughter ions. For a few metabolites extensive in-source fragmentation of the molecule was observed. Thus, the main fragment ion was selected as the parent ion. This was the case of coniferyl alcohol, sinapyl alcohol, and raspberry ketone. The two alcohols had in-source fragmentation, and the main ions in the MS spectra were m/z 163 and 193, respectively, generated by the loss of the hydroxyl group, whereas raspberry ketone gave an intense ion at m/z 107. Another particular case was salicin, or 2-(hydroxymethyl)phenyl-β-D-glucopyranoside, occurring mainly as the Na^+ adduct, for which the adduct was selected as parent ion.

During optimization of the methodology, nine compounds were excluded from the study because they displayed ionization problems resulting in an inconsistent response. These were cuminaldehyde, 2-methyl-4-vinylphenol, 1,3-dihydroxybenzene, 1,3,5-trihydroxybenzene, guaiacol, tyrosol, methyl salicylate, 1-phenylethyl acetate, and genistein-4,7-dimethyl ether. Shikimic acid was also excluded from the study because it was not well retained on the column in the chosen conditions.

In total, the MRM signals for 135 metabolites were optimized and a unique acquisition channel was created for each compound. The precursor and product ions, quantifier and qualifiers (when present), collision energies, and cone voltages for the single metabolites are listed in Table 1, as well as the expected ratio between the area of qualifier 1 and the quantifier. This ratio was computed as the average of the experimental values included in the calibration curve of each standard compound. Using the reference standard of each compound to obtain the RT, the quantifier and one or two qualifier ions are accepted as confirmation of the compound.[40] All compounds that achieved this requirement are presented in boldface in Tables S3 of the Supporting Information, whereas all others remain tentatively identified.

Method Validation. One of the challenges that must be faced in the analysis of complex matrices such as fruit extracts, tea, or wine is the fact that the variety of analytes to quantify can occur at very different concentration levels. The measurement sensitivity for each compound can also vary greatly in the assay. Therefore, in this study the linear dynamic range of the instrument for each metabolite was explored, in addition to limits of quantification (LOQs). Dilutions of the 16 mixtures of metabolites were injected to assess the linearity of the response over >4 orders of magnitude. The ranges of linearity, parameters of the curves, and LOQs are reported in Table S2 of the Supporting Information. The distribution of the linearity ranges for the phenolic compounds is shown in Figure 1. The response of the detector was linear over 3−4 orders of magnitude for all of the compounds included in the assay, with correlation coefficients (R^2) >0.990 for >88% of the metabolites. The instrumental LOQs were defined as the concentration at which the quantifier transition presented a signal-to-noise (S/N) ratio >10, and they were calculated using the peak-to-peak algorithm from the closest injected concentration. About 11% of the compounds displayed a LOQ between 0.5 and 5 pg; 58% had an LOQ in the 5−50 pg range, and 31% had an LOQ of >50 pg. The distribution of the LOQs for the metabolites included in the study is shown in Figure 2.

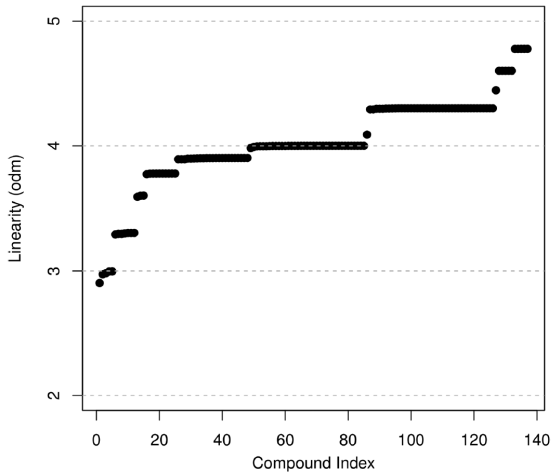

Figure 1. Distribution of the linearity ranges of the assayed metabolites. The y axis indicates the orders of magnitude.

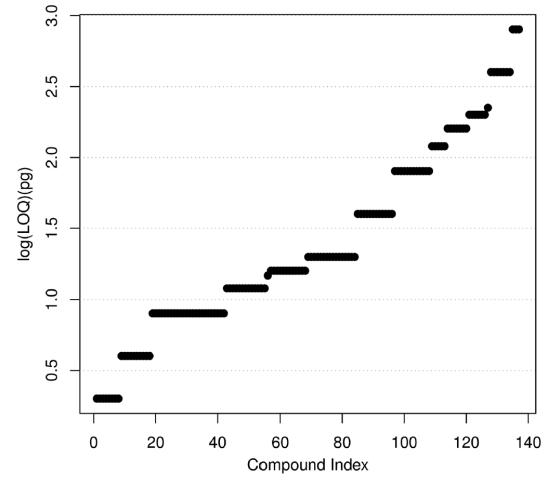

Figure 2. Distribution of the limits of quantification (LOQ) of the assayed metabolites. Note that the y axis sets on a log scale.

Instrumental precision was determined by injecting the 16 standard mixtures at two intermediate concentration values in the linear range, both intraday (n = 5) and interday (n = 5). The samples were analyzed by the same operator, and the relative standard deviation (RSD%) of the peak areas and retention time were calculated. In the intraday precision experiment, the majority of the metabolites (98.6% at the higher concentration and 88.5% at the lower concentration)

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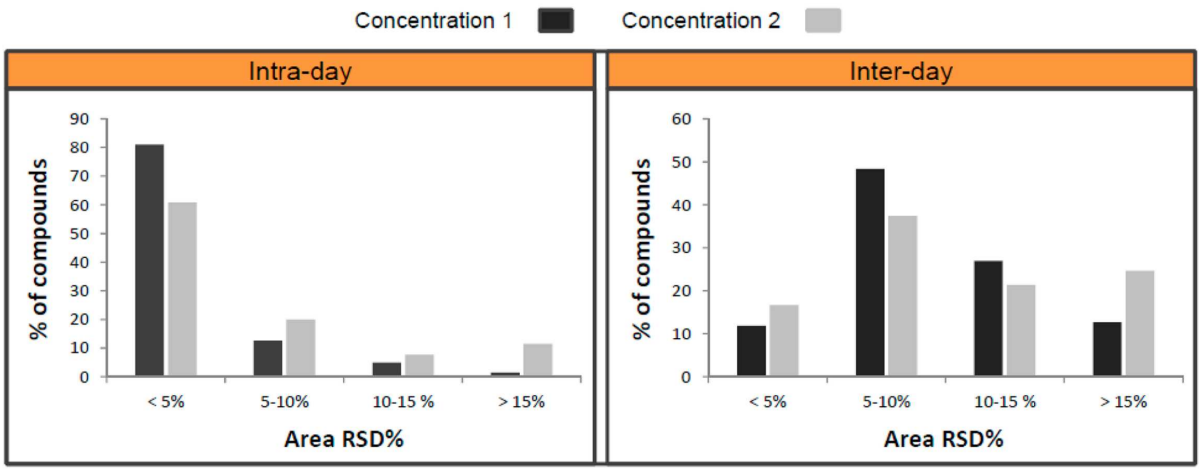

Figure 3. Inter- and intraday instrumental precision. The RSD of the areas of all the metabolites was measured at two different concentrations in the linearity range.

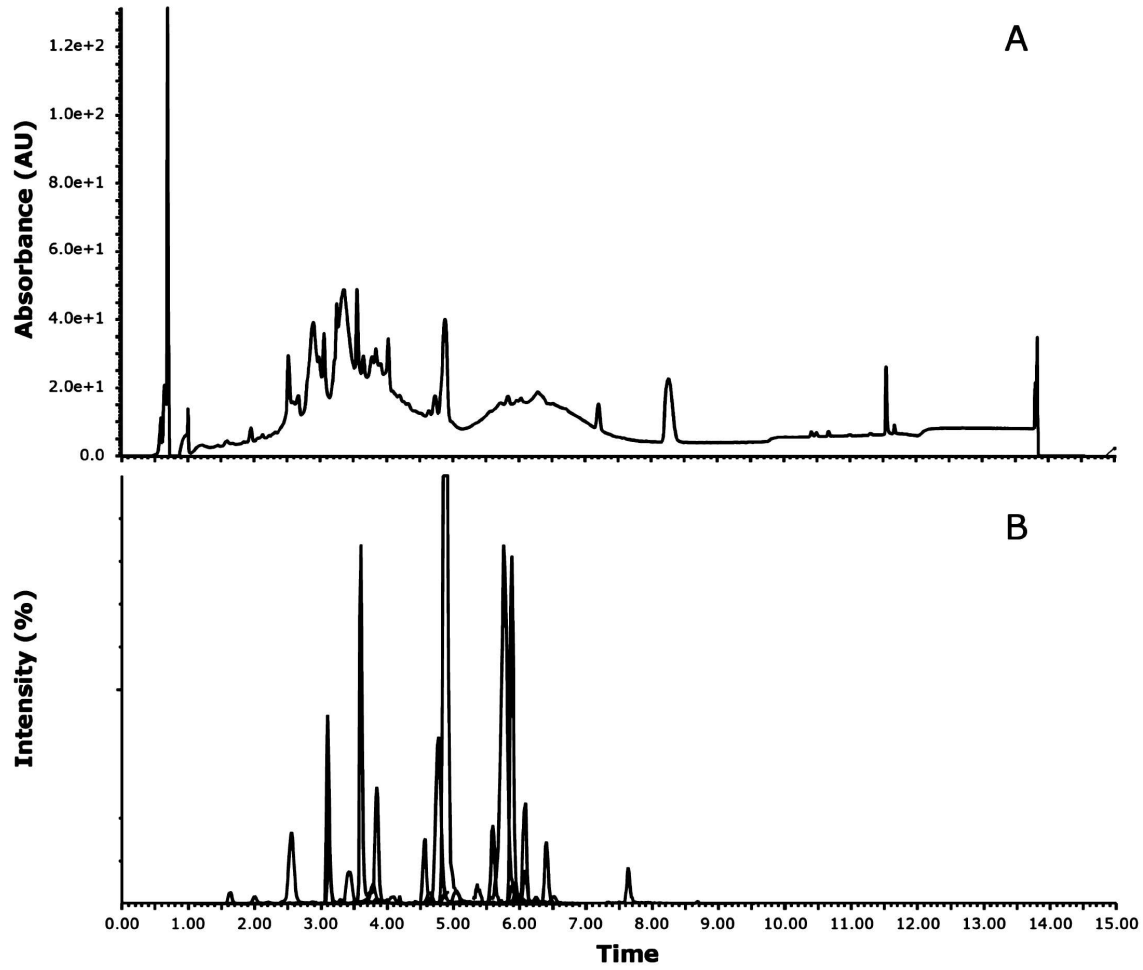

Figure 4. UPLC-DAD (A) and UPLC/QqQ-MS/MS (B) chromatograms obtained from grape extract. Whereas in the DAD chromatogram the separation is incomplete and does not allow quantification of the metabolites, the MRM ion chromatograms display clean peaks with baseline resolution that allow unambiguous quantification.

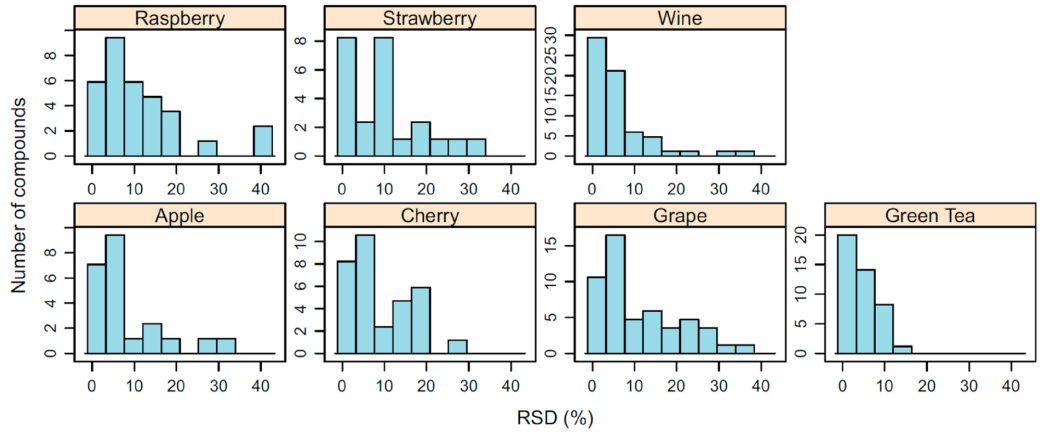

Figure 5. Instrumental precision of the matrix analysis. The RSD% of the areas of the metabolites identified in the different matrices is displayed as histograms.

displayed a RSD of the area lower than 15% (Figure 3). It is worth noting that 81% of the compounds displayed an area RSD lower than 5% at concentration 1 (100-fold dilution of the standard mixtures) and 60.8% at concentration 2 (1000-fold dilution). In the interday experiment the number of metabolites with a RSD% lower than 15% was 87.3% at concentration 1 and 75.4% at concentration 2, respectively, indicating that the instrumental response was stable over a period of 5 days (Figure 3). The retention time of the standard compounds was also very stable. In fact, with very few exceptions, its RDS was lower than 0.01 min (as displayed in Figure S1A of the Supporting Information).

Occurrence of Phenolic Compounds in Fruit, Green Tea, and Red Wine. The validated method was applied to quantitative analysis of several fruit extracts, green tea, and red wine. In particular, apples, grapes, strawberries, raspberries, and cherries were included in this preliminary study. The number of metabolites detected at a concentration above the LOQ varied in the different matrices: it was possible to quantify 17 compounds in apples, 49 in grapes, 22 in strawberries, 29 in raspberries, 33 in cherries, 58 in green tea, and 57 in red wine. Furthermore, some metabolites were detected in these samples at concentrations lower than the LOQ and could not be quantified. In total, 87 metabolites were identified and quantified in at least one extract, representing a detection rate of about 64%. Figure 4 shows the MRM traces of the phenolics present in the grape extract, as an example.

The precision of the analysis was evaluated after repeated injection of the samples (n = 5) by calculating the RSD of the detected peak area and the standard deviation of the retention time. The results showed that for the majority of the hits the RSD% was lower than 10, and values above 20 were exceptions indicating that the reproducibility of the analysis was good, as shown in Figure 5. The RSD of retention time of the detected compounds was found to be lower than 0.02 min for the majority of the compounds (Supporting Information, Figure S1B).

The results of the analysis are shown in the form of a heat map in which the concentration value logarithms are within a color scale (Figure 6). The concentrations of the detected metabolites (provided in Table S3 of the Supporting Information) spanned several orders of magnitude, from nanograms to milligrams per gram of extracted material. For about 90% of the 236 hits the concentration values occurred in the linearity range, whereas 25 measurements were above the upper limit. These data suggest that the method represents a promising approach for the quantification of phenolic compounds in a wide dynamic range, although in some cases sample dilution could be required.

Similarities between the samples analyzed in terms of metabolite content are shown, in the form of a dendrogram, in Figure 6. The phenolic profiles of strawberries, apples, and raspberries appeared to be closely related. This hierarchical chemotype clustering information partially reflects the phylogenetical classification of the Rosaceae family. Indeed, raspberries and strawberries belong to the Rosoideae subfamily, whereas cherries and apples belong to the Spiraeoideae subfamily.[41,42] The analysis also emphasized the similarities between grapes and wine in terms of metabolite composition, whereas green tea clustered separately from all of the other samples, as expected.

The high sensitivity offered by the method developed also provided some interesting information about the composition of the fruit extracts analyzed in this study, revealing the presence of compounds in fruits that had never previously been reported, probably due to their low concentration. One of the most interesting observations was the presence of the stilbenes *trans*- and *cis*-piceid (or *trans*- and *cis*-resveratrol-3-*O*-glucosides), which mainly occur in grapes, peanuts, and some berries, such as strawberries and lingonberries, in apples at concentrations above the LOQ (0.04 and 0.02 μg/g, respectively). Furthermore, the dihydrochalcone phloridzin (phloretin-3-*O*-glucoside), normally occurring in apples and plums, was found in grapes at a concentration of 0.08 μg/g.

Further experiments were carried out to validate these hits using a completely different approach. New extracts were prepared and analyzed using a longer chromatography (63 min) and a time-of-flight (TOF) detector according to the method of Theodoridis et al.[37] Peak matching and annotation were performed on the basis of mass values and retention times by comparison with a database developed in-house for plant secondary metabolites. The results fully confirmed the hits with a mass accuracy lower than 5 ppm and a retention time window of 20 s. High-resolution spectra of *trans*- and *cis*-piceid and phloridzin in apples and grapes are provided in Figure S1 of the Supporting Information. Although relatively unusual, these

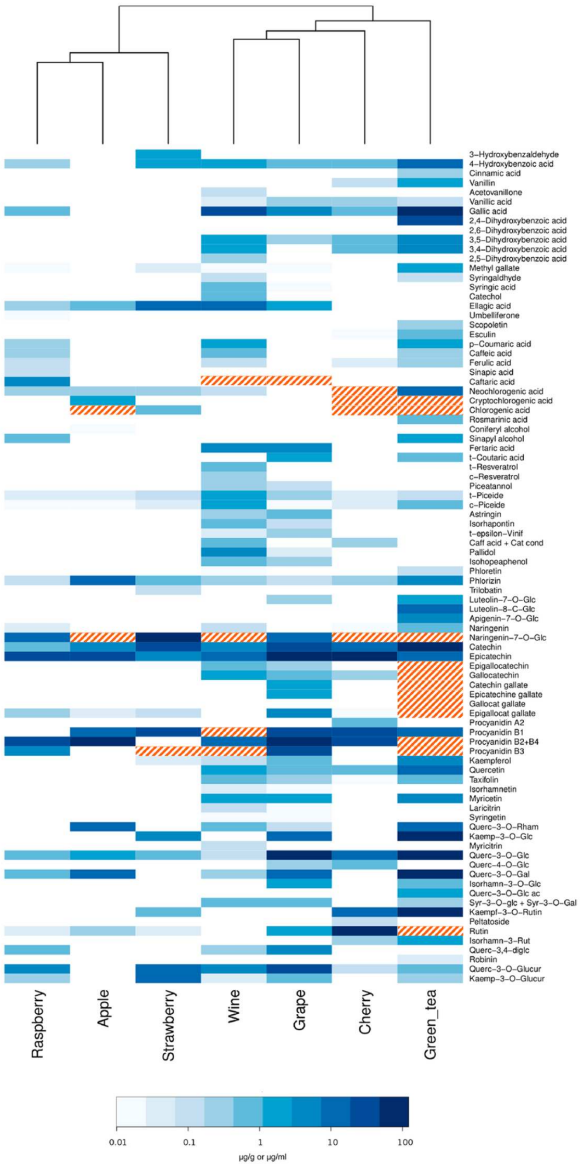

Figure 6. Concentrations of the metabolites in the different matrices visualized as a heat map. The dendrogram represents the hierarchical clustering of the samples. Diagonal slashes indicate compounds in saturation.

results are also supported by evidence that stilbene synthases developed from chalcone synthases during the course of their evolution and that their activity can be directed toward one or another product by substituting a few amino acids.[43] Moreover, minor cross-reactions have been observed, possibly due to conformational flexibility of their active sites.[44] It is therefore possible to speculate that the occurrence of minute amounts of stilbenes in apples and dihydrochalcones in grapes are the result of cross-reactions of the stilbenes and chalcone synthases responsible for their production.

In conclusion, we developed a UPLC/QqQ-MS/MS metabolite profiling method for food analysis that should allow rapid exploration of the presence of polyphenols in different food matrices, with converging opportunities for research applications in plant science and human nutrition. This work shows that targeted metabolomics using UPLC/QqQ-MS/MS represents an attractive and effective strategy for food analysis. Indeed, the high sensitivity of MRM-based mass spectrometry and the wide dynamic range of triple-quadrupole spectrometers provide a valuable tool for the analysis of complex matrices such as fruit, and more in general food, in which analyte concentrations span several orders of magnitude. The method we have developed for the profiling of phenolic compounds is versatile, and it could be successfully applied to the analysis of a range of different matrices, not limited to those chosen for validation. Its sensitivity revealed the presence of compounds that were not reported before in some matrices, suggesting that this approach can also play a role in redesigning metabolic networks, also exploring minor branches of the plant metabolism. It could be easily integrated with the insertion of additional groups of compounds, repeating the simple protocol of optimization here described. The short duration of the analysis and the straightforward sample preparation make the methodology suitable for high-throughput varietal screening studies and for use in assisting plant breeders to select specific chemotypes.

■ ASSOCIATED CONTENT

Supporting Information

Additional figures and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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Food Chemistry

Chapter 1.2

Ellagitannins in strawberries: Their isolation, structural elucidation and profiling during fruit ripening

This chapter has been reprinted* from:

1. *Clarifying the Identity of the Main Ellagitannin in the Fruit of the Strawberry, Fragaria vesca and Fragaria ananassa Duch.* Urska Vrhovsek, Graziano Guella, **Mattia Gasperotti**, Elisa Pojer, Mirella Zancato, Fulvio Mattivi, *Journal of Agricultural and Food Chemistry*, **2012**, 60, 10, 2507-2516

2. *Evolution of Ellagitannin Content and Profile during Fruit Ripening in Fragaria spp.* **Mattia Gasperotti**, Domenico Masuero, Graziano Guella, Luisa Palmieri, Paolo Martinatti, Elisa Pojer, Fulvio Mattivi, Urska Vrhovsek *Journal of Agricultural and Food Chemistry* **2013** 61 (36), 8597-8607

Ellagitannins are a complex family of hydrolysable tannins which have been found only in dicotyledoneous angiosperms (1[†]). Ellagitannins are present in the human diet, being contained in berries and strawberries in large amounts (2).

This class of natural polyphenols has recently received considerable attention in the light of experimental evidence regarding purported anticancer properties (3, 4), antiproliferative properties (5, 6), antibacterial activity in relation to intestinal pathogens (7), and very recently anti-inflammatory activity at gastric level (8).

There is little knowledge about the native forms of ellagitannins in strawberries and their biosynthetic behaviour during ripening (14-19). Their structural complexity is a major limiting step that prevents their study at molecular level. Due to the considerable diversity of ellagitannins, they still represent a challenge to food science and are a source of discussion in relation to their correct identification. Correct identification of the structure is clearly a prerequisite for understanding their bioavailability, bioactivity and metabolism.

The structural elucidation of ellagitannins is a difficult task, because they are made up of the same building blocks (including but not limited to glucose, ellagic and gallic acid, and hexahydroxydiphenoyl (HHDP) units. As a consequence, many structurally related ellagitannins display characteristic, but very similar or sometimes almost identical, mass spectra. This issue, together with the lack of commercially available standards, makes their accurate identification and quantification very demanding. In the case of strawberries, there is fundamental disagreement about the identification of the main ellagitannin in the fruit.

Due to the sheer complexity, the qualitative and quantitative composition of this class in strawberries has not yet been thoroughly resolved to date (14). This represents one of the major limitations in the study of health benefits and the human metabolism at molecular level. In order to assign the correct health properties to these compounds it is important to have specific knowledge about the chemical structure of

[†] References are reported in the same order and listed in the second publication attached

the native form of ellagitannins, their concentration, and the ellagitannin profiles present in fruits at different ripening stages and not only in ripe fruit.

In this context, the aim of these two publications was primarily elucidation and profiling of the main ellagitannins. In the first publication, identification of the most abundant ellagitannin, after isolation, MS and NMR analysis, was clearly defined. The scope was to solve a major open question, namely whether the main ellagitannin in the strawberry is agrimoniin or sanguiin H-6, two isomeric forms of dimeric ellagitannins

In the second publication, the scope was instead to isolate other ellagitannins and ellagic acid conjugates and their characterisation for a precise quantification. Moreover, with the use of high definition MS an exhaustive profile of all major ellagitannins was one of the aims of the study, also in order to explain the behaviour of this class of compounds during fruit ripening in different *Fragaria* spp. It is important to answer this question due to several areas of scientific interest. From the point of view of plant biochemistry it is important to understand the fate of these compounds throughout ripening of the fruit, while on the other hand producers and consumers are concerned about the healthy properties of food. They are interested in learning how these compounds change while the fruit is edible, and the variability of the different genotypes.

A detailed introduction, descriptions of the methods and discussion of the results are attached for both publications.

The first important result was the unequivocal identification of agrimoniin as the most abundant ellagitannin in strawberries. After many years of uncertainty this was the first time that agrimoniin has been isolated and its structure characterised in the fruit of *F. vesca* and its presence reported as the main ellagitannin in both *F. vesca* and *F. ananassa* D. fruit.

In the second publication the isolation and structural elucidation of other ellagitannins were reported, i.e. casuarictin and one ellagic acid conjugate, namely

methyl ellagic rhamnoside. Furthermore, MS profiling of 26 ellagitannins was performed in different *Fragaria* genotypes. Major qualitative and quantitative differences in the amount and profile of ellagitannins and ellagic acid conjugates were observed, both due to the variety and ripening stage.

The results of this study show that genotype is a major factor in defining ellagitannin concentration and pattern in strawberries. The concentration of ellagitannins drops during ripening in all varieties.

Both studies show that of fruit containing ellagitannins, strawberries represent the most important source of this class of compounds in the human diet, with agrimoniin probably being the most important ellagitannin consumed.

My involvement in the first publication concerned the isolation of agrimoniin from *Fragaria Vesca* with the use of preparatory HPLC (see *Figure 2*) and UV and high definition analysis, the results being reported in *Figure 6* and *Figure 8*. I also performed accurate quantification of agrimoniin with HPLC-DAD in different *Fragaria* spp. In the second publication I was again involved in isolation (*Figure 1*), UV analysis (*Table S6*) and MS characterisation. However the most challenging part of the research in which I was involved was high definition MS profiling of the 26 ellagitannins identified, based on their spectra in each *Fragaria* spp. considered (the results are reported not only in the text but also in *Figure 2*, *Table 1* and *Table S5*). After profiling and assignment of putative identification, I also performed quantification of the compound detected with a HPLC-DAD system, using the appropriate isolated ellagitannins (*Table 2*). A summary of the quantitative results for each cultivar and the woodland strawberry types are shown graphically in *Figure 4*, highlighting the drop in ellagitannins during fruit maturation. Moreover, as the first author I was responsible for writing the manuscript and managing the comments and improvements to the text of other co-authors.

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Clarifying the Identity of the Main Ellagitannin in the Fruit of the Strawberry, *Fragaria vesca* and *Fragaria ananassa* Duch.

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Supporting Information

ABSTRACT: Although the composition of strawberry fruit has been extensively studied, especially for the most abundant phenolic compounds, agrimoniin has never been univocally identified as one of the most abundant phenolic compounds in the fruit. In this study agrimoniin was isolated in the fruit of *Fragaria vesca* and its structure characterized. Furthermore, its presence was definitively established to be the main ellagitannin in both *F. vesca* and *Fragaria ananassa* D. fruit. The presence of sanguiin H-6 and lambertianin C as minor compounds was confirmed in both *F. vesca* and *F. ananassa* D. samples. For the first time here is reported the full NMR assignments for agrimoniin. These data should represent a point of reference for NMR analysis of this and other structurally related ellagitannins. Finally, the establishment of an HPLC protocol for separation provided information making it possible to avoid confusion with sanguiin H-6, the main ellagitannin in *Rubus* species, which is also present in strawberries but at a much lower concentration.

KEYWORDS: *Fragaria, strawberry, ellagitannins, agrimoniin, NMR, mass spectrometry, circular dichroism, UV*

INTRODUCTION

Of the most commonly consumed berries, strawberries (*Fragaria ananassa* Duch.) are the most popular choice with consumers, being eaten both fresh and frozen, as well as in different processed products such as desserts, juice, nectar, puree, jam, syrup, and wine. Strawberries are indeed one of the most important crops worldwide. The statistical database of the Food and Agriculture Organization of the United Nations[1] reports a global production area of 254 523 ha distributed over 77 countries on all of the continents in 2009, with estimated worldwide production of 4 178 152 tons. The major production areas are located in Europe (1 353 149 t), North America (1 289 882 t), eastern Asia (401 990 t), North Africa (332 239 t), and western Asia (326 548 t). With a rounded figure of 600 g per year theoretically available for each of the 7 billion inhabitants on our planet, strawberries are the most commonly consumed berries and one of the most important sources of polyphenols in the human diet, with a strong influence on human health.[2−6]

The nutritional quality of strawberries is correlated to the presence of soluble sugars, organic acids, amino acids, vitamins, and important secondary metabolites such as polyphenols.[7] Strawberries are a rich source of bioactive compounds, including proanthocyanidins, anthocyanins, flavonols, phenolic acids, and ellagitannins.[8] Their chemical composition and putative influence on the healthy properties of the fruit have been studied.[9] The three major classes of phenolic compounds in the fruit are, in decreasing order, proanthocyanidins, anthocyanins, and ellagitannins. Proanthocyanidins and flavan-3-ols are the main classes, and a recent paper reported the profiling of different oligomeric forms and the degree of polymerization in different strawberry cultivars.[8] Anthocyanins are another important group of polyphenols responsible for fruit pigmentation and which have also been suggested contribute to the inhibition of ethanol-induced ulcers in rats fed strawberry extracts.[6]

Ellagitannins are a class of compounds present only in some fruit and nuts (e.g., strawberries, raspberries, blackberries, pomegranates, muscadine grapes, and walnuts). The major sources of ellagitannins in the Western diet are strawberries, raspberries, and blackberries.[9] Their structural complexity is a major limiting step that prevents their study at the molecular level. Due to the considerable diversity of ellagitannins, they still represent a challenge to food science and are a source of discussion in relation to their correct identification.[10] Correct identification of the structure is clearly a prerequisite for understanding their bioavailability, bioactivity, and metabolism.

The structural elucidation of ellagitannins is a difficult task, because they are made up of the same building blocks (including but not limited to glucose, ellagic and gallic acid, and hexahydroxydiphenoyl (HHDP) units), which are organized into an impressive number of different but similar structures.[11] As a consequence, many structurally related ellagitannins display characteristic, but very similar or sometimes almost identical, mass spectra.[12] This issue, together with the lack of commercially available standards, makes their accurate identification and quantitation very demanding. In the case of

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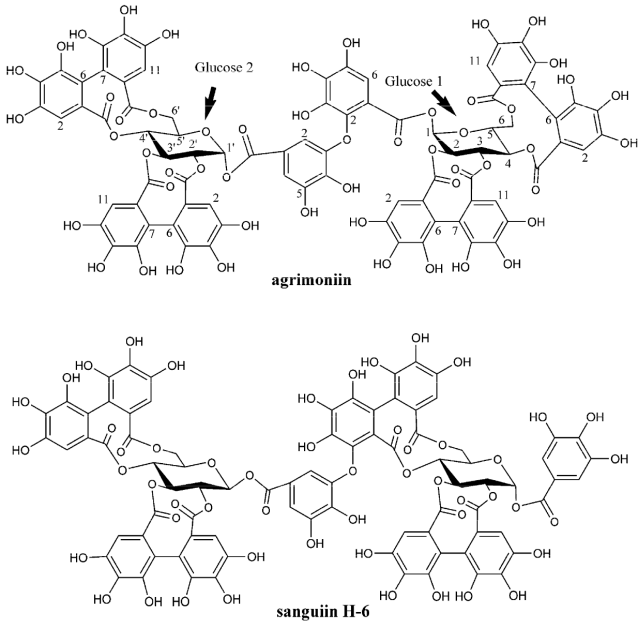

Figure 1. Molecular structures of agrimoniin (atom numbering used in NMR spectra assignments in Table 1) and sanguiin H-6.

strawberries, there is fundamental disagreement about the identification of the main ellagitannin in the fruit.

The ellagitannin composition of the fruit has not yet been reported, even in the most comprehensive food databases.[13] Reports on the presence of agrimoniin (Figure 1) in strawberry fruit are scarce and contradictory. In strawberry fruit the most abundant ellagitannins reported so far are lambertianin C, sanguiin H-6 (Figure 1), and galloyl-bis-HHDP-glucose.[8,14] The question of whether the major ellagitannin was agrimoniin or sanguiin H-6, because the fragmentation patterns matched both, was left open.[15] The ellagitannins from strawberries were reported to be structurally different from those of cloudberries and raspberries, with the predominance of casuarictin-like and/or potentillin-like (galloyl diHHDP glucose) structures.[16] The mass 1870 Da was observed, but the identity of the molecule was not completely assigned.[15] The presence of agrimoniin was described in green strawberry fruit.[5] In conclusion, most of the papers dealing with the identification of native ellagitannins in strawberries have reported sanguiin H-6 as the main ellagitannin, probably due to the lack of the reference standards and the similarity of the mass spectrum with agrimoniin.

Identification is more consistent in studies considering other parts of the strawberry, especially the leaves. Agrimoniin was reported to be the main soluble phenol in strawberry leaves, accounting for up to 40% of total soluble phenols[17] in strawberry leaves[18] and Fragariae folium extracts.[19] Three different isomers of agrimoniin were reported to be present in the leaves of *Fragaria* sp.[18,20,21] Agrimoniin was also found in different parts of strawberry flowers[12] and suggested to be present in both achenes and receptacles during fruit development.[22]

The purpose of this study was to isolate and characterize the structure of agrimoniin in woodland strawberries (*Fragaria vesca*) and also to confirm its presence in cultivated strawberries (*F. ananassa* D.).

MATERIALS AND METHODS

Standards and Solvents. All of the chromatographic solvents were of HPLC grade or LC-MS grade for the MS experiments. Acetonitrile, acetone, methanol, diethyl ether, hexane, and formic acid were purchased from Sigma-Aldrich (Milan, Italy). Hexane and formic acid were purchased from Carlo Erba (Milan, Italy). Ellagic acid standard (purity ≥ 96%) was purchased from Fluka (Steinheim, Germany). Sanguiin H-6 and lambertianin C were isolated as described previously.[23]

Plant Material. One kilogram of woodland strawberries (*F. vesca*) and 60 g of strawberries (*F. ananassa* D. cv. Darselect) were grown in an experimental field in Vigalzano (Trento, Italy). All of the plants were grown under the same conditions to minimize the effect of environmental and agronomic factors. Woodland strawberries and strawberries were harvested at maturity and were frozen at −20 °C and then transported to the laboratory for solvent extraction. The extraction of polyphenols was carried out as reported in Mattivi et al.[24] with an acetone/water mixture (70:30 v/v). Before extraction, the fruit and extraction solution were cooled to 4 °C to limit enzymatic and chemical reactions. Sixty grams of fresh fruit was homogenized in an 847-86 model Osterizer blender at speed 1, in 2 × 100 mL of a mixture of acetone/water (70:30 v/v) for 1 min and made up to 250 mL with the same solvent.

Isolation of Agrimoniin from Woodland Strawberries (*F. vesca*). Aqueous acetone strawberry extracts (1 kg of fruits extracted in 4 L of acetone/water mixture (70:30 v/v)) were evaporated until dryness in a pear-shaped flask, using rotary evaporation under reduced pressure at 37 °C. The sample was diluted to 1 L with methanol/water mixture (30:70 v/v) and filtered using a Durapore 0.45 μm filter (Millipore, Vimodrone, Italy). Isolation of agrimoniin was carried out in two consecutive steps using a preparative HPLC Shimadzu SCL-10 AVP equipped with a Shimadzu SPD-10 AVP UV−vis detector, 8A pumps and Class VP software (Shimadzu Corp., Kyoto, Japan). The UV signal was recorded at 260 nm.

Step 1. The first step in isolation was purification of the methanolic extract for the removal of anthocyanins from the sample. After this step, the sample for isolation was made up of only the ellagitannin fraction of the woodland strawberries. Purification was done using Sephadex LH-20 and carried out with a slightly modified version of our protocol.[23] In particular, the purification method was changed to

scale-up the size. A column cartridge (10 × 4 cm) was packed with Sephadex LH-20 resin, connected to a vacuum line to speed elution, prewashed with 50 mL of methanol, and equilibrated with 100 mL of methanol/water (30:70 v/v). An aliquot of 50 mL of the aqueous methanol berry extract was loaded, and anthocyanins were washed off with 500 mL of methanol/water (30:70 v/v). The yellowish fraction containing the ellagitannins was eluted from the cartridge using 350 mL of acetone/water (70:30 v/v). The ellagitannin fraction was combined together for each purification step and then dried using rotary evaporation under reduced pressure at 37 °C and reconstituted in 50 mL of methanol for the next isolation step.

Step 2. The chromatographic isolation of agrimoniin was performed using preparative HPLC with a 250 × 50 mm, 10 μm, Discovery HS C18 column (Supelco, Bellefonte, PA, USA). The column was protected by using a 2 μm PEEK filter (Gilson, Milano, Italy). The sample obtained from the purification step (50 mL in methanol) was evaporated using rotary evaporation under reduced pressure at 40 °C, reconstituted with water (2 L), and loaded into the column using a solenoid valve. The injection volume with the solenoid valve was 44 mL for each consecutive injection. The mobile phases were distilled water (solvent A) and acetonitrile (solvent B). The column was conditioned for 10 min with 10% B. Separation was achieved using a linear gradient from 10 to 30% B in 120 min, at a flow rate of 35 mL/min, and the elution was monitored in UV at 260 nm. The peaks of interest were manually collected in a separate flask, checking the chromatographic run and their retention times for each injection before the fractions were pooled. After separation, agrimoniin was dried using rotary evaporation under reduced pressure and then dissolved in the smallest possible volume of methanol, diluted with diethyl ether and hexane. The pure isolated compound (ca. 200 mg) was recovered by filtration and precipitation from *n*-hexane as an amorphous pale rose powder, which was further characterized by NMR and MS. When heated in a Kofler melting point microscope (model Reichert Thermovar, USA), it showed decomposition at temperatures >200 °C, leading eventually to a gummy dark material at 250 °C.

NMR. NMR spectra (^{1}H NMR, COSY, NOESY, HSQC, and HMBC) for agrimoniin were recorded in both hexadeuterated acetone (99.90% CD_3COCD_3) and tetradeuterated methanol (99.90% CD_3OD) at 298 K on a Bruker-Avance 400 MHz NMR spectrometer by using a 5 mm BBI probe with 90° proton pulse length of 9.1 μs at a transmission power of 0 db. The chemical shift scales (δ) were calibrated on the residual signal of protonated acetone at δ_H 2.040 and δ_c 29.80 for spectra taken in CD_3COCD_3 and on the residual protonated signal of methyl group at δ_H 3.310 and δ_c 49.00 for spectra taken in CD_3OD.

Molecular mechanics calculations were carried out by the computer program GMMX as implemented in PCMODEL 7.0 (PCMOD 7.0/GMMX version 1.5, Serena Software, Bloomington, IN, USA).

UV and Circular Dichroism (CD). The UV spectra of agrimoniin were recorded both in methanol and in ethanol, on a Hitachi U-2000 spectrometer (Tokyo, Japan). The following molar extinction coefficients were observed: in methanol, $\varepsilon_{260\ nm}$ = 58178 M^{-1} cm^{-1}; in ethanol, $\varepsilon_{260\ nm}$ = 67508 M^{-1} cm^{-1}.

The CD spectra of agrimoniin were recorded in methanol (1.8 × 10^{-6} M) on a Jasco J-40AS dichrograph. The following Cotton effects expressed in molar ellipticity Θ (mol^{-1} L cm^{-1}) at the corresponding wavelengths (λ) were observed: Θ = +4.9 × 10^5 (240 nm), Θ = −1.8 × 10^5 (264 nm), Θ = +1.3 x10^5 (284 nm), Θ = −4.5 x10^4 (310 nm).

Mass Spectrometry. Separation was carried out with a Waters Acquity UPLC system equipped with a UV−vis Waters PDA (Waters Corp., Milford, MA, USA) under the same conditions described for HPLC analysis. Detailed compound characterization was carried out using a Waters HDMS-QTOF Synapt mass spectrometer with electrospray ionization system (ESI) and MassLynx 4.1 software. HDMS analysis was performed in negative mode in the following conditions: capillary voltage, 3 kV; sampling cone, 40 V; extraction cone, 3 V; source temperature, 100 °C; desolvation temperature, 350 °C; cone gas flow (N_2), 50 L/h; desolvation gas flow (N_2), 800 L/h. The *m*/*z* range was 50−3000 Da.

The MS was calibrated using sodium formate, and leucine enkephalin was used as the lock mass.

RESULTS AND DISCUSSION

Isolation of Agrimoniin. Agrimoniin was the first dimeric hydrolyzable tannin isolated from a plant, *Agrimonia pilosa*.[25] In our study the isolation of agrimoniin was performed from *F. vesca* fruits. After purification of the extract on a Sephadex resin with the scope of eliminating anthocyanins, the compounds were isolated using preparative HPLC. Figure 2 shows good separation of agrimoniin from other compounds in a preparative chromatographic run. The retention time for agrimoniin was 65 min in these elution conditions. Good separation allowed relatively straightforward isolation of this compound. After chromatographic isolation, a good grade of precipitation was required for further characterization of the compounds isolated. The protocol used was the same as the one developed for the main *Rubus* ellagitannins.[23] The solution with the isolated compound was dried, dissolved in the smallest possible volume of methanol, and then diluted with diethyl ether. In the same way, hexane was then also added, but in much larger volumes. As a result of this and its insolubility in hexane, the solution quickly formed precipitate of the pure compound. The final agrimoniin yield obtained from 1 kg of *F. vesca* was ca. 200 mg, with a purity of 98%, as assessed by NMR.

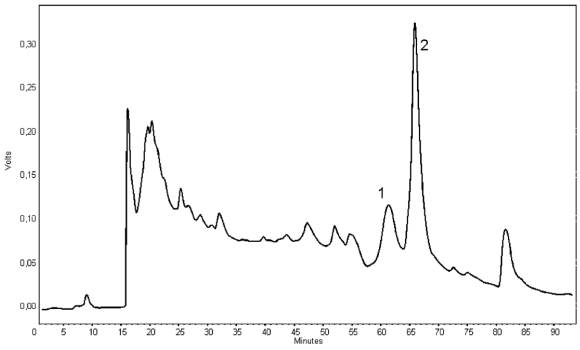

Figure 2. Chromatogram of preparative HPLC-DAD for isolation, detected at 260 nm. Peaks: 1, ellagic acid (Rt 60 min); 2, agrimoniin (Rt 65 min).

Structural Elucidation of Agrimoniin by NMR and CD. Heteronuclear, ^3J (H, C) optimized, direct (HSQC) and long-range correlation (HMBC)-2D NMR measurements allowed us to assign and to report here for the first time (see Table 1) all of the ^{1}H and ^{13}C NMR resonances of agrimoniin. Because only partial descriptions of the NMR agrimoniin spectra are present in the literature,[25,26] these data should represent a point of reference for NMR analysis of other structurally related ellagitannins.

Due to the structural resemblance of agrimoniin with sanguiin H-6[23] (Figure 1), the two structures share the same general NMR features, namely, the presence in their corresponding ^{1}H NMR spectra (Table 1; Figure 3) of sharp singlets attributable to protons on HHDP groups and meta-coupled doublets for protons on galloyls in the aromatic region, besides the presence, at higher field, of a series of sugar multiplets. On the other hand, distinctive NMR resonances and possibly different glucose−proton *J* coupling patterns were promptly detected, due to the specific structural differences

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Table 1. NMR (400 MHz, 298 K) Assignments for Agrimoniin in CD_3COCD_3

moiety	carbon no.	^{1}H NMR δ_H	^{13}C NMR δ_C
α-glucose 1	1	6.56 (d, $J_{1,2}$ = 3.9)	90.6 d
	2	5.35 (dd, $J_{1,2}$ = 3.9, $J_{3,2}$ = 9.5)	73.8 d
	3	5.48 (dd, $J_{3,2}$ = 9.5, $J_{3,4}$ = 10.2)	75.6 d
	4	5.15 (t, $J_{4,3} \approx J_{4,5}$ = 10.2)	68.7 d
	5	4.48 (dd, $J_{4,5}$ = 10.2, $J_{5,6}$ = 6.2)	71.0 d
	6	3.68 (d, J_{gem} = 13.2)	62.9 t
		5.23 (dd, J_{gem} = 13.2, $J_{5,6}$ = 6.2)	
α-glucose 2	1′	6.54 (d, $J_{1',2'}$ = 4.1)	90.8 d
	2′	5.36 (t, $J_{1',2'}$ = 4.1 $J_{3',2'}$ = 9.5)	73.7 d
	3′	5.54 (dd, $J_{3',2'}$ = 9.5, $J_{4',3'}$ = 10.2)	75.7 d
	4′	5.19 (t, $J_{4',3'} \approx J_{4',5'}$ = 10.2)	68.8 d
	5′	4.64 (dd, $J_{6'5'}$ = 6.5, $J_{4',5'}$ = 10.2)	70.7 d
	6′	3.78 (d, J_{gem} = 13.3)	62.9 t
		5.30 (d, J_{gem} = 13.3, $J_{6'5'}$ = 6.5)	
2-O,3,4,5-trihydroxybenzoate on O−C(1) of glucose 1	1		114.6 s
	2		137.5 s
	3		140.1 s
	4		140.9 s
	5		143.4 s
right part of DHDG	6	7.29 s	109.6 d
	1-OC═O		162.8 s
3-O,4,5-dihydroxybenzoate on O−C(1″) of glucose 2	1		125.4 s
	2	7.38 (d, $J_{2,6}$ = 2.0)	112.1 d
	3		146.5 s
	4		140.9 s
	5		148.1 s
	6	6.93 (d, $J_{2,6}$ = 2.0)	108.2 d
left part of DHDG	1′-OC═O		164.9 s
2,3 HHDP on glucose 1	1		126.6 s
	2	6.43 s	107.0 d
	3		145.1 s
	4		136.4 s
	5		144.9 s
	6		114.7 s
	7		114.2 s
	8		144.9 s
	9		136.1 s
	10		145.0 s
	11	6.33 s	107.1 d
	12		126.6 s
	2-OC═O		168.3 s
	3-OC═O		169.3s

moiety	carbon no.	^{1}H NMR	^{13}C NMR (HSQC and HMBC)
4,6 HHDP on glucose 1	1		125.8 s
	2	6.60 s	107.7 d
	3		145.1 s
	4		136.6 s
	5		144.4 s
	6		116.1 s
	7		115.7 s
	8		144.4 s
	9		136.3 s
	10		145.1 s
	11	6.65 s	108.1 d
	12		126.1 s
	4-OC═O		167.7 s

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Table 1. continued

moiety	carbon no.	^{1}H NMR	^{13}C NMR (HSQC and HMBC)
	6-OC═O		168.1 s
2′,3″ HHDP on glucose 2	1		126.6 s
	2	6.55 s	107.2 d
	3		145.1 s
	4		136.5 s
	5		144.1 s
	6		114.9 s
	7		114.2 s
	8		144.1 s
	9		136.1 s
	10		145.0 s
	11	6.34 s	107.1 d
	12		126.5 s
	2′-OC═O		168..3 s
	3′-OC═O		169.2 s
4′,6′ HHDP on glucose 2′	1		125.7 s
	2	6.59 s	107.7 d
	3		145.1 s
	4		136.6 s
	5		144.3 s
	6		116.1 s
	7		115.6 s
	8		144.3 s
	9		136.3 s
	10		145.1 s
	11	6.64 s	108.1 d
	12		126.1 s
	4′-OC═O		167.7 s
	6′-OC═O		168.0 s

between these two dimeric ellagitannins. First of all, both the glucose moieties in agrimoniin are involved in α relative configurations, whereas in sanguiin H-6 glucose 2 presents a β linkage to its acetal center. In the corresponding ^{1}H NMR spectra (Table 1), this is reflected by the presence of identical coupling constants ($J \sim 4$ Hz) for acetal protons at both the glucose units in agrimoniin and quite different J values for the corresponding protons in sanguiin H-6 (4 Hz for glucose 1 and 8 Hz for glucose 2). A significant solvent effect on these acetal protons is shown by the ^{1}H NMR spectrum of agrimoniin in methanol (Figure 4B) in comparison with acetone (Figure 4A) . Curiously, it shifts them in opposite directions, leading to an upfield effect on the H−C(1) of glucose 1 and a downfield effect on H−C(1′) of glucose 2. This outcome is worth noting, because it allows good spectroscopic resolution even for spectra obtained using low-field NMR instruments. It is important to note here that the assignment of acetal protons in ellagitannins is a *conditio sine qua non* for correctly assigning the relative configurations of the chiral centers. Another striking structural/biogenetic difference between agrimoniin and sanguiin H-6 is the different linking unity between monomers, this being a dehydrodigalloyl group (DHDG) in the former and a sanguinsorboyl group in the latter. As a consequence, the small NMR differences detected in agrimoniin at its corresponding structural sites are due only to the asymmetry imposed by the central DHDG.

Two sets of resonances for ester groups are present in agrimoniin, one set at δ_C about 167−169, attributable to −COO groups linked to HHDP moieties, and a second set at δ_C about 163−165 for −COO groups linked to DHDG linking groups. The shielded values for the latter can be explained by taking into account the fact that these two carboxyl groups should assume a conformation wherein they are almost coplanar with the corresponding aromatic rings (galloyl), thus allowing better conjugation and leading to an overall upfield effect. For carboxyl groups linked to HHDP units, the adoption of this conformation is strongly hindered by the conformational constraints imposed by the carbon−carbon bond linking the two aromatic rings in the HHDP moiety. As previously reported for sanguiin H-6,[23] molecular mechanics (MM) calculations carried out through extended geometry optimization of agrimoniin (Figure 5) confirm that the dihedral angle formed by −C═O with the HHDP aromatic rings in all the groups is about 50°, whereas it is much lower (∼20°) in both the galloyls embedded in the DHDG linking unit. More importantly, MM calculations strongly suggest that both 10-membered rings (defined by 2,3 junctions of HHDP) and 11-membered rings (defined by the 4,6 junction of HHDP) adopt a twisted chairlike conformation, within which the two ester carbonyl groups are in *anti* orientation (dihedral angle between the two ester O—C═O groups estimated to be ∼170°) with the diaryl units resulting heavily twisted (∼60°).

The absolute configuration of the four chiroptical HHDP groups on the ^{4}C$_1$ glucose core was unambiguously established to be *S* by the positive sign of the strong Cotton effect at λ 240 nm and the negative Cotton effect at λ 266 nm.[27]

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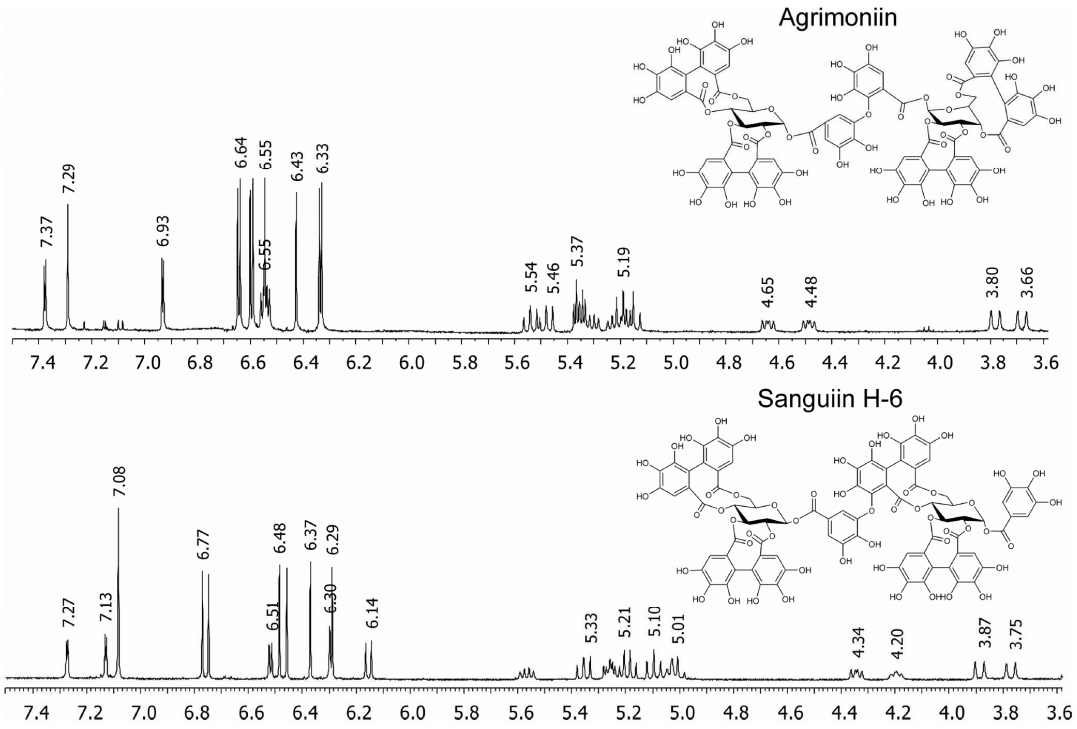

Figure 3. ^{1}H NMR spectra in acetone-d_6 of agrimoniin (top) and sanguiin H-6 (bottom).

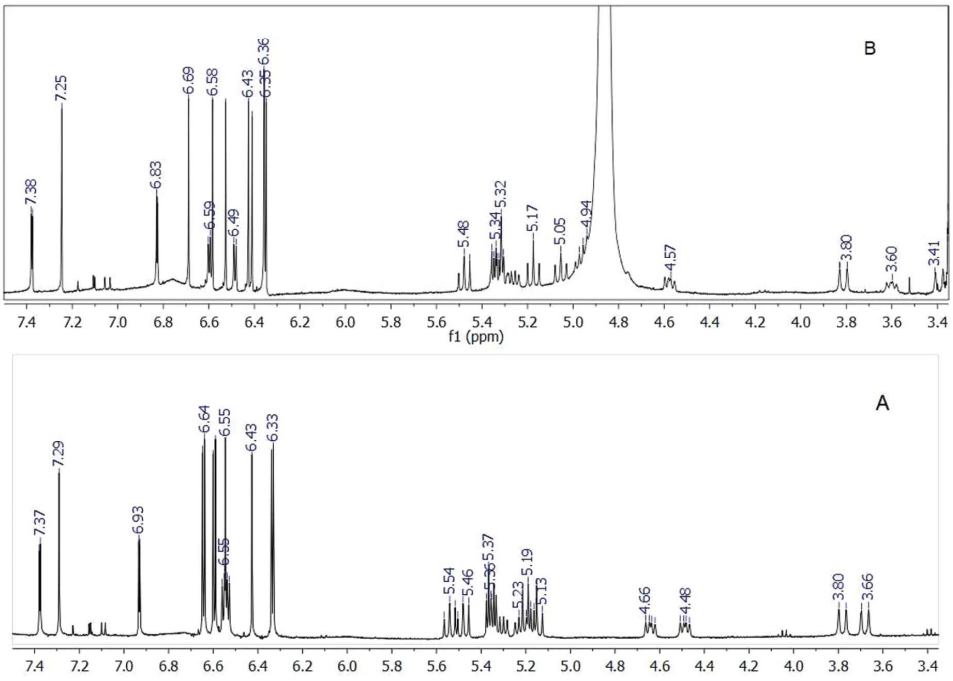

Figure 4. ^{1}H NMR spectra of agrimoniin in (A) acetone-d_6 and (B) methanol-d_4.

ESI(−) High-Resolution Mass Measurements and Mass Spectrum Interpretation. Sanguiin H-6 and agrimoniin are two isomers built out of the same monomeric units, galloyl-bis-HHDP-glucose (Figure 1), and consequently their negative ion mode ESI-MS spectra are very similar (Figure 6). In both spectra the parent ion $[M - H]^-$ can be observed, for agrimoniin at m/z 1869.146 and for sanguiin H-6 at m/z 1869.154 (theoretical monoisotopic mass of both isomeric

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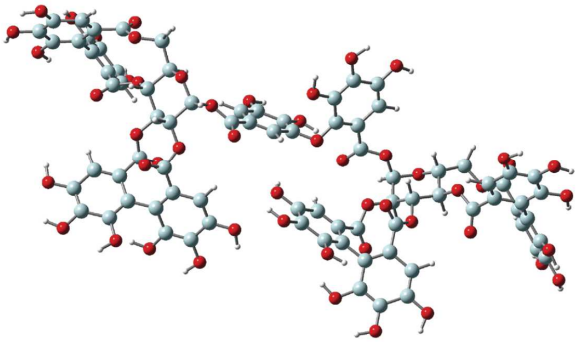

Figure 5. Energy-minimized structure of agrimoniin, as obtained from molecular mechanics calculations.

forms calculated for $C_{82}H_{53}O_{52}$ is 1869.151). Characteristic fragment ions (Figure 7) correspond to the loss of some common building blocks in ellagitannins (ellagic, HHDP, or HHDP-glucoside units). In particular, the low intensity ion at *m/z* 1567.151 (**1**, Figure 7) derives from the loss of one HHDP unit followed by 2H transfer from HHDP to glucose (calculated theoretical monoisotopic mass for $C_{68}H_{47}O_{44}$ of **1** is 1567.145), whereas the ion at *m/z* 935.079 (**3a**, Figure 7) must result from the break of the C−O bond linking the two monomers, thus affording the galloyl-bis-HHDP-glucose 1 negative ion (theoretical monoisotopic mass calculated for $C_{41}H_{27}O_{26}$ of **3a** is 935.079). After 2H transfer, the HHDP moiety is prone to give the free ellagic acid (**2**, Figure 7), which in ESI(−) ion mode presents itself as an anion at *m/z* 300.998 (theoretical monoisotopic mass calculated for $C_{14}H_5O_8$ of **2** is 300.999). Through the loss of an HHDP unit and 2H transfer from the HHDP toward glucose 1 unit, **3a** leads to the anion fragment **4a** at *m/z* 633.072 (theoretical monoisotopic mass calculated for $C_{27}H_{21}O_{18}$ is 633.073). Isobaric ion daughter ions (**3b** and **4b**, Figure 7) can, however, be generated along a different route through symmetrical bond breaking. Because the ESI(−)/MS fragmentation pattern of sanguiin H-6 is almost superimposable (Figure 6) to that of agrimoniin, there is a clear indication that the central diaryl ether function is the weakest

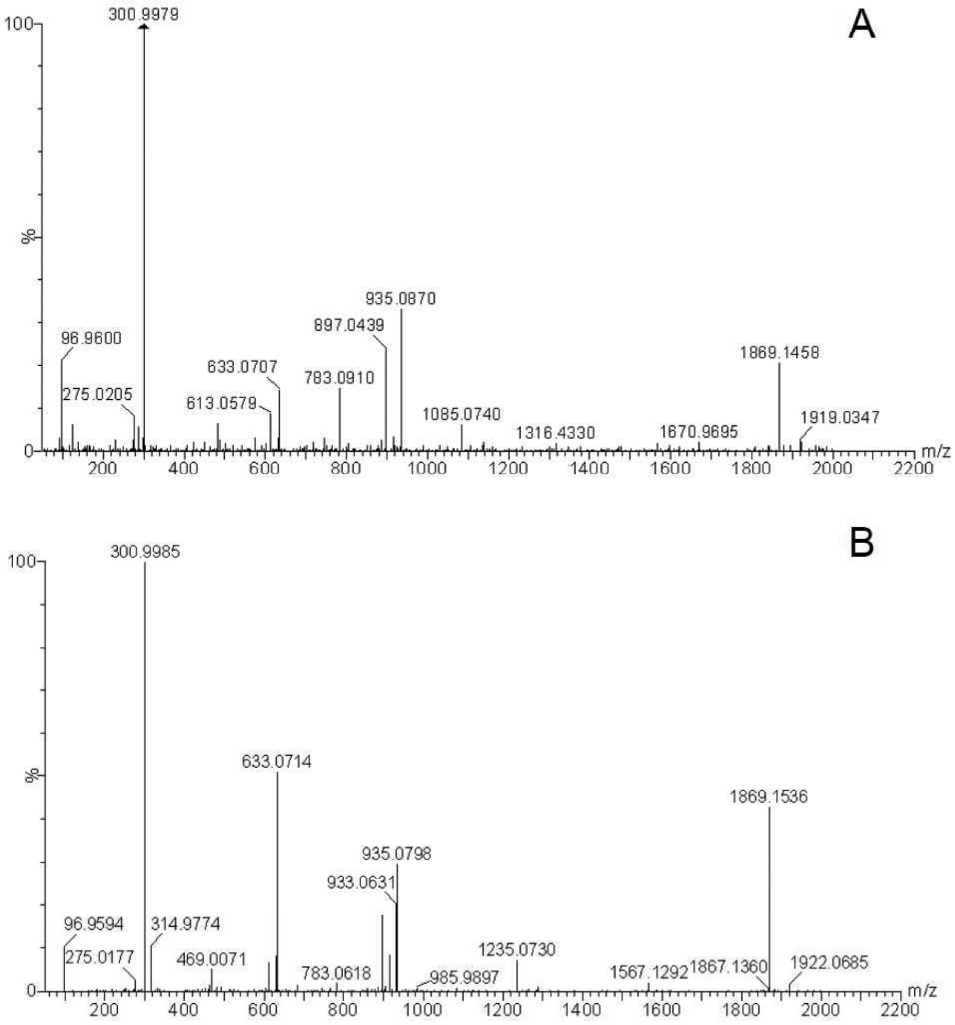

Figure 6. High-resolution ESI(−) mass spectra of agrimoniin (A) and sanguiin H-6 (B).

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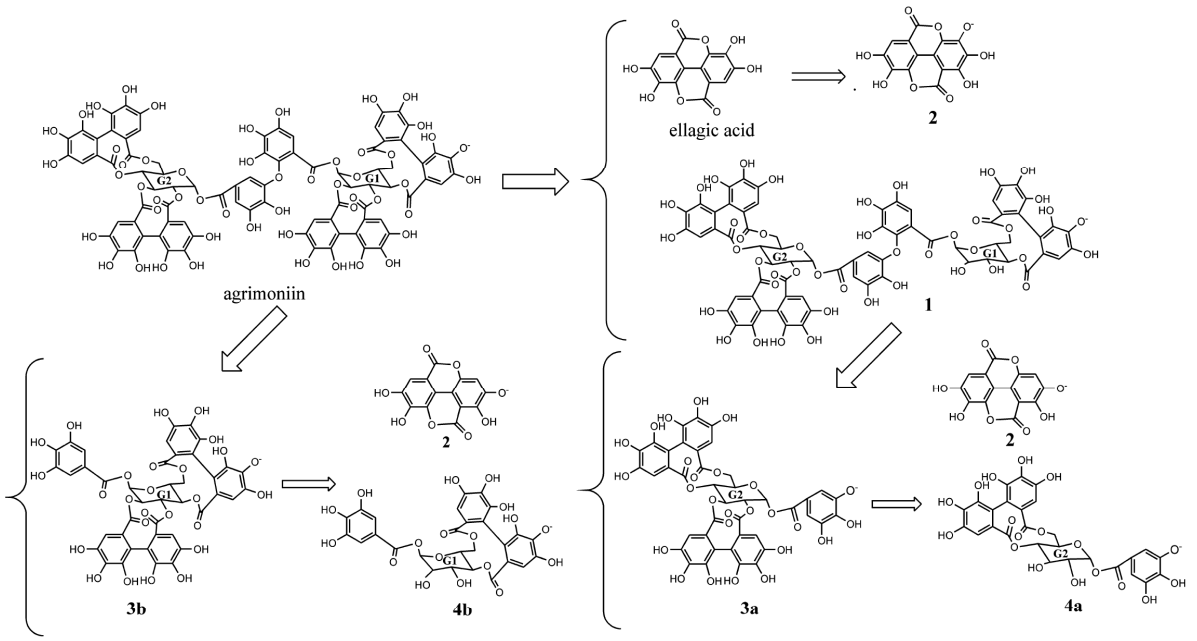

Figure 7. Fragmentation routes in the high-resolution ESI(−)-MS spectrum of agrimoniin.

bond in dimeric ellagitannins, thus undergoing significant dissociation in the ESI source.

By comparison of the chromatographic runs of *F. vesca*, *F. ananassa* Duch, and standard mix (Figure 8) it was demonstrated that the main ellagitannin in *Fragaria* has the same molecular mass as sanguiin H-6. However, on the basis of the retention time, it was possible to assign the main compound as agrimoniin, which elutes later during the gradient, because its polarity is considerably lower. With our separation method on a reversed phase C18 column, sanguiin H-6 elutes at 26.7 min and agrimoniin at 36.4 min, whereas the retention time of ellagic acid was 28.5 min (Figure 8). To properly and uniquely assign the identity to the compounds, the availability of true standards is crucial to compare the retention times of the two compounds. In the event of a lack of reference standards, the elution order of the compounds should be carefully evaluated to tentatively assign the main strawberry ellagitannins. We suggest that ellagic acid, widely available, should be taken as a reference on the C18 column, because sanguiin H-6 elutes before and agrimoniin after it (Figure 8).

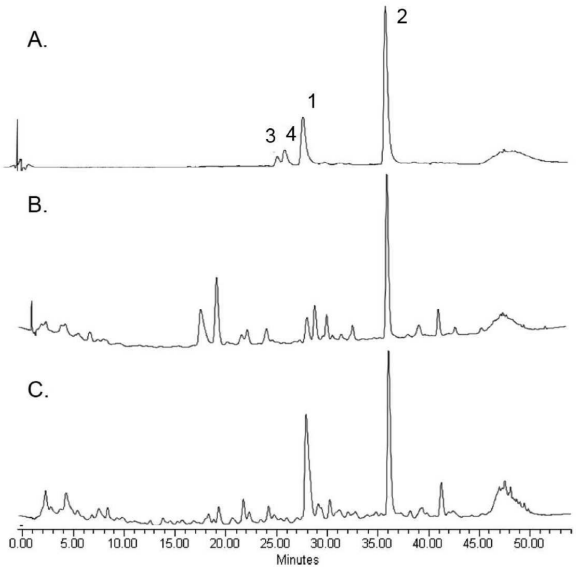

Figure 8. HPLC-DAD (260 nm) chromatograms: (A) ellagitannin standard mix; (B) woodland strawberry; (C) strawberry cv. Darselect. Peaks (in the standard mix): 3, lambertianin C; 4, sanguiin H-6; 1, ellagic acid; 2, agrimoniin.

After apples, strawberries were recently reported to be the fruit contributing most to polyphenol intake, estimated in the SU.VI.MAX French cohort.[28] Of fruit containing ellagitannins, strawberries are the most widely consumed, and agrimoniin is therefore expected to be one of the most widely present ellagitannins in the human diet. Due to the many healthy properties associated with ellagitannins, agrimoniin should play an important, yet still largely unexplored, role in the beneficial health effects associated with the consumption of strawberries by humans. It is known that agrimoniin has been used for treatment of diarrhea and hemorrhaging.[26,27,29] It has also been reported to have antitumor properties.[30−32]

Although the composition of strawberry fruit has been extensively studied, especially for the most abundant phenolic compounds, agrimoniin has never been unequivocally identified as one of the most abundant phenolic compounds in the fruit. To our knowledge, this is the first time that agrimoniin has been isolated and its structure characterized in the fruit of *F. vesca* and its presence reported as the main ellagitannin in both *F. vesca* and *F. ananassa* D. fruit. The presence of sanguiin H-6 and lambertianin C as minor compounds was confirmed in both *F. vesca* and *F. ananassa* D. samples.

■ ASSOCIATED CONTENT

Supporting Information

Figure SM1. HSQC spectrum of agrimoniin in acetone-d_6. Figure SM2. HMBC spectrum of agrimoniin in acetone-d_6. Figure SM3. COSY spectrum of agrimoniin in acetone-d_6. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ NOTE ADDED AFTER ASAP PUBLICATION

There was an error in Figure 7 of the version of this paper published March 1, 2012. The correct version published March 2, 2012.

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Article

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Evolution of Ellagitannin Content and Profile during Fruit Ripening in *Fragaria* spp.

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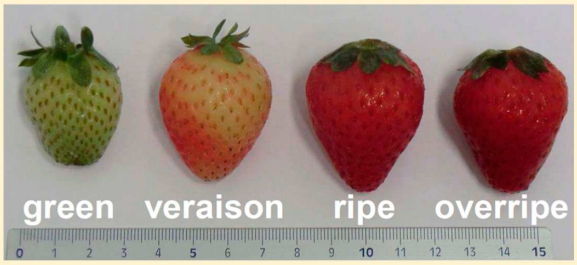
Supporting Information

ABSTRACT: Ellagitannins and ellagic acid conjugates are polyphenols present in the human diet, in particular strawberries (*Fragaria* spp.). The first aim of this study was isolation and structural characterization of casuarictin and 3-*O*-methyl ellagic acid 3′-*O*-α-rhamnopyranoside, which were found to be abundant in *Fragaria* spp., along with agrimoniin. The second aim was accurate profiling and quantification of 26 ellagitannins and ellagic acid conjugates in six *Fragaria* x *ananassa* cultivars and two *Fragaria vesca* species. The third aim was to describe the ellagitannins behavior during fruit ripening from the green stage to over-ripeness. It was shown that there are major qualitative and quantitative differences in the amount and profile of ellagitannins and ellagic acid conjugates between *Fragaria* spp. Genotype is a major factor in defining ellagitannin concentration and patterns between strawberries, and variable behavior of the genotypes was observed, in the context of a significant drop in ellagitannins during ripening.



KEYWORDS: *Fragaria, strawberry, fruit ripening, ellagitannins, casuarictin, methyl ellagic acid rhamnoside*

INTRODUCTION

Ellagitannins are a complex family of hydrolyzable tannins which have been found only in dicotyledoneous angiosperms.[1] Ellagitannins are present in the human diet, as they are contained in berries and a number of other sources, such as pomegranates, walnuts, muscadine grapes, and many medicinal plants.[2] This class of natural polyphenols has recently received considerable attention in the light of experimental evidence regarding purported anticancer properties,[3,4] antiproliferative properties,[5,6] antibacterial activity in relation to intestinal pathogens,[7] and very recently anti-inflammatory activity at gastric level.[8] Ellagitannins are present at a relatively high concentration in some berries, such as raspberries, blackberries, strawberries, boysenberries, cloudberries, rose hips, and sea buckthorn.[9−12] Due to the greater awareness of producers and consumers as regards health benefits, the selection of cultivars in the last years has also focused on factors influencing the content of bioactive compounds in fruit,[13] besides the usual agronomical and product characteristics.

There is little knowledge about the native forms of ellagitannins in strawberries and their biosynthetic behavior during ripening,[14−19] although they are probably the most important dietary sources of ellagitannins in the human diet.[15] Due to the sheer complexity, the qualitative and quantitative composition of this class in strawberries has not yet been thoroughly resolved to date.[14] This represents one of the major limitations in the study of health benefits and the human metabolism at the molecular level. In order to assign the correct health properties to these compounds it is important to have specific knowledge about the chemical structure of the native form of ellagitannins, their concentration, and the ellagitannin profiles present in fruits at different ripening stages and not only in ripe fruit.

It has been shown that there are both qualitative and quantitative differences in the concentrations of secondary metabolites in different strawberry cultivars.[16,19−22] Besides the cultivars, the chemical composition of fruit is also strongly influenced by the ripening stages, since both primary[23,24] and secondary metabolism change[23−25] while the fruit ripens. Specifically, the influence of ripening on polyphenol content, including ellagitannins, was recently investigated by Aaby et al.,[14] who reported that the changes observed in three strawberry cultivars during the stages considered suitable for consumption were doubtful.

From the point of view of plant biochemistry it is important to understand the fate of these compounds throughout ripening of the fruit (from green to red), while on the other hand it could be in the interest of producers and consumers concerned about the healthy properties of food to know how these

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compounds change while the fruit is edible. Strawberries are usually harvested prematurely in order to prevent postharvest losses, whereas consumers are likely to get fully ripe fruits. Fruit quality and metabolite synthesis are complex traits that are genetically controlled, but at the same time they depend on the plant's stage of development and of course on agronomical and other environmental factors. To better understand the role played by each factor, the variation in metabolite content is often correlated with data derived from genetics, transcriptomics, and environmental studies.[26] The results can be used to further investigate metabolite biosynthesis, which in the case of ellagitannins is still not well-defined, and to develop new molecular markers useful in marker-assisted selection.[27]

A previous work described the isolation and structural elucidation of agrimoniin, the main ellagitannin in *Fragaria* spp.[15] The purpose of this study was isolation of a further two ellagitannins which are particularly abundant in strawberries and woodland strawberries, along with agrimoniin. Furthermore, accurate quantification of the main ellagitannins and ellagic acid conjugates, using their respective standards in 6 different strawberry cultivars and 2 woodland strawberry types at four different ripening stages was carried out. Evaluation of the content and profile at different stages was used to understand the behavior of this important class of polyphenols during fruit ripening, from the green stage to overripeness.

MATERIALS AND METHODS

Chemicals and Reagents. All the chromatographic solvents were HPLC grade or LC−MS grade for the MS experiments. Acetonitrile, acetone, methanol, and diethyl ether were purchased from Sigma Aldrich (Milan, Italy). Hexane and formic acid were purchased from Carlo Erba (Milan, Italy). Ellagic acid standard (purity >96%) was purchased from Fluka (Steinheim, Germany). Sanguiin H6 and lambertianin C were isolated as described in Gasperotti et al.[10] and agrimoniin as described in Vrhovsek et al.[15]

Plant Material. For isolation of ellagitannins and ellagic acid conjugates, 1 kg of woodland strawberries (*Fragaria vesca*) and 5 kg of strawberries (*Fragaria* × *ananassa* D. cv. Darselect) were used.

For analysis of ellagitannins in different cultivars at different ripening stages, the experimental design consisted of 96 samples (6 cultivars and 2 accessions × 3 repetitions × 4 stages). Six *Fragaria* × *ananassa* strawberry cultivars (Alba, Clery, Eva, Elsanta, Darselect, Portola) and 2 accessions (one red and one white) of *Fragaria vesca* were grown in the same experimental field in Vigalzano (Trentino, Italy; 520 AMSL) during the 2011 season. All cultivars were produced under standardized conditions in order to minimize the effect of environmental and agronomic factors. The plants were cultivated using the soilless technique, in an adequate quantity to obtain three biological replicates for each cultivar. For each cultivar analyzed 3 repeats of 6 plants (18 plants in total) were considered. This meant that plants of the same cultivar were obtained vegetatively from the same mother. The agronomical system involved the use of plastic pots (50 × 25 × 11 cm) filled with an inert substratum (peat). The fertirrigation drip system consisted of a proportional liquid dispenser with a flow meter, where the fertilizers and the microelements were premixed. Soil acidification (pH 5.2 to 5.8) was carried out using nitric acid. The flux was regulated on the basis of the soil volume, in order to have 25 cm^3 of nutrient solution for each liter of pit. Irrigation took place daily and was manually timed for 12 h, on the basis of climate changes, with cycles of a few minutes each 0.5−1 h.

For each cultivar and each time stage 250 g of strawberries at 4 phenological stages (1, green; 2, veraison; 3, ripe; 4, overripe) were collected in triplicate (biological replicates) from different plants. Strawberries have staggered flowering, and this results in the evolution of nonsimultaneous formation of fruits on the plant. Moreover the fruit developing from primary inflorescence is bigger than the fruit developing from secondary, tertiary, or quaternary inflorescence. Consequently the number of achenes is determined by the flower order.[28] To avoid sampling variability, all fruits were sampled from any single plant belonging to the same lot of six plants in two different production periods. Then the fruits were divided according to their phenological stage into the 4 homogeneous groups mentioned above. Once collected the samples were stored in a freezer at −20 °C until extraction. The extraction of strawberry polyphenols was performed as reported in Mattivi et al.[29] with an acetone/water mixture (70/30 v/v), avoiding the addition of acids in order to prevent chemical hydrolysis.

Total Soluble Solids (TSS), Total Titratable Acidity (TTA), and pH. Samples (40 g) were homogenized using an Ultraturrax (Ika, Staufen, Germany) for 3 min at 13500 rpm and then centrifuged at 4 °C at 8000 rpm for 5 min. An aliquot was used to determine pH and TSS. TSS was expressed as ° Brix with a refractometer (RFM 81, Bellingham & Stanley Ltd., U.K.). TTA was measured following the AOAC Official Method 942.15[30] using an automatic titrator, Compact Tritator (Crison, Barcellona, Spain). Results were expressed as g of citric acid kg^{-1} of fresh weight.

Agronomical Parameters. The strawberry cultivars were evaluated by measuring the following descriptors in 10 fruits for each cultivar: color, firmness, weight, and size at ripening stage. Fruit color was measured using a Minolta spectrophotometer (CM-3600d) equipped with the light source D65 and observation angle of 10°. This instrument measures the following CIELAB variables:[31] L^*, a^*, b^*. Chroma (C^*ab) was calculated using the equation: $C^*ab = [(a^*)^2 + (b^*)^2]^{1/2}$. Flesh firmness was measured by using a digital fruit firmness tester (TR Turoni srl, Forlì, Italy) on the equatorial part of the fruit with a 6 mm probe. The weight was determined with a digital fruit firmness tester (KERN EW1500-2M) at ripening stage 3 for all varieties and at each ripening stage for Elsanta, for which the percentage of weight increase at the different stages was calculated. Fruit size was determined using a caliper (Sylvac model S 235 PAT), measuring the diameter at the equatorial part of the fruit and the height. The descriptors' mean value was calculated for each strawberry.

Isolation of Ellagitannins from *Fragaria vesca* and *Fragaria* × *ananassa* D. Isolation was performed as described in detail by Vrhovsek et al.[15] In short, for the isolation of methyl ellagic acid rhamnoside, 1 kg of red woodland strawberries, and for the isolation of galloyl-bis-HHDP glucose, 5 kg of Darselect cv. strawberries were used. Isolation of the two compounds was carried out with a preparative HPLC Shimadzu SCL-10 AVP equipped with a Shimadzu SPD-10 AVP UV/vis detector, 8A pumps, and Class VP Software (Shimadzu Corp., Kyoto, Japan). The UV signal was recorded at 260 nm. The first phase of isolation involved purification of the anthocyanins from the sample using Sephadex LH20. The second phase in the isolation of compounds was performed using preparative HPLC with a 250 × 50 mm 10 μm Discovery HS C18 column (Supelco, Bellefonte, PA, USA). The pure isolated compounds were recovered by precipitation from *n*-hexane as an amorphous pale rose powder, which was further characterized by NMR, UV, and MS.

NMR and Circular Dichroism (CD) Analysis. NMR spectra (^{1}H NMR, COSY, NOESY, HSQC, and HMBC) were recorded in tetradeuterated methanol (99.90% CD_3OD) or in hexadeuterated acetone (99.9% CD_3COCD_3) at 300 K on a Bruker-Avance 400 MHz NMR spectrometer by using a 5 mm BBI probe with 90° proton pulse length of 9.1 μs at a transmission power of 0 dB. The chemical shift scales (δ) were calibrated on the residual signal of methyl group of methanol at δ_H 3.310 ppm and δ_C 49.00 ppm for spectra acquired in CD_3OD solutions (casuarictin). The residual signal of methyl group of acetone at δ_H 2.04 ppm and δ_C 29.00 ppm was used for calibration for spectra acquired in CD_3COCD_3 solutions (methyl ellagic-rhamnoside).

The CD spectra of casuarictin (2.1×10^{-5} M) and ellagic derivative (3.0×10^{-5} M) were recorded in methanol (1.8×10^{-6} M) on a Jasco J-40AS dichrograph. The following Cotton effects expressed in molar ellipticity Θ (mol^{-1} L cm^{-1}) at the corresponding wavelengths (λ) were observed: for casuarictin, $\Theta = +1.8 \times 10^4$ (240 nm), $\Theta = -4.1 \times 10^3$ (268 nm), $\Theta = -1.8 \times 10^3$ (310 nm); for ellagic derivative, $\Theta = +3.5 \times 10^3$ (240 nm).

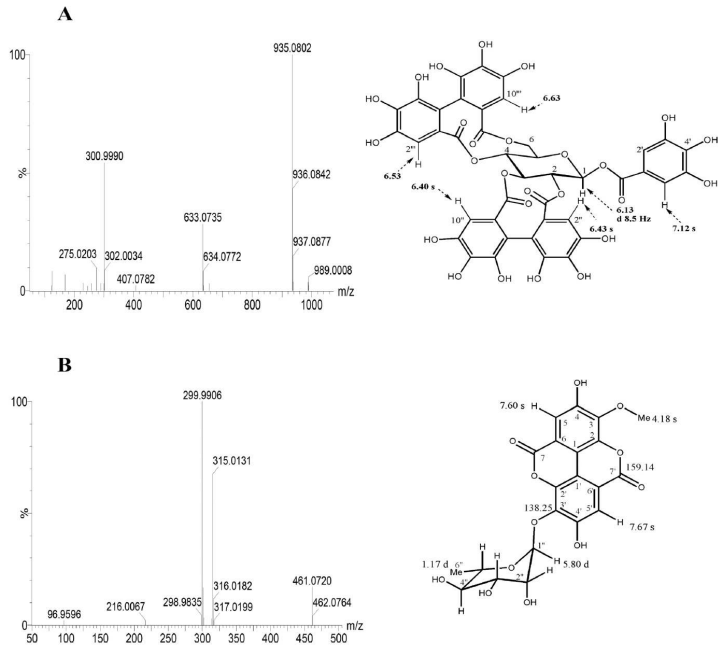

Figure 1. HRMS spectra and selected ^{1}H NMR resonances of casuarictin (A) and methyl ellagic acid rhamnoside (B) in CD3OD at 300 K.

UV Measurement. The UV spectra of methyl ellagic acid rhamnoside and casuarictin were recorded both in methanol and ethanol, on a Hitachi U-2000 spectrometer (Tokyo, Japan). The following molar extinction coefficients were observed. In methanol: methyl ellagic acid rhamnoside, ε_{260nm} = 15500 M^{-1} cm^{-1}; casuarictin, ε_{260nm} = 29200 M^{-1} cm^{-1}. In ethanol: methyl ellagic acid rhamnoside, ε_{260nm} = 15600 M^{-1} cm^{-1}; casuarictin: ε_{260nm} = 31800 M^{-1} cm^{-1}. The reported molar extinction coefficient for casuarictin[32] in methanol was ε_{260nm} = 38900.

Structural Elucidation Using UPLC-Q-TOF-HDMS Analysis. Chromatographic separation was carried out with a Waters Aquity UPLC system equipped with UV−vis Waters PDA (Waters Corp., Milford, MA) under the same conditions described for quantitative HPLC analysis. Detailed compound characterization was carried out using a Waters HDMS-QTOF Synapt (Waters Corp., Milford, MA) mass spectrometer with electrospray ionization system (ESI) and MassLynx Software 4.1 (Waters Corp., Milford, MA). HDMS analysis was performed in negative mode in the following conditions: capillary voltage 3 kV, sampling cone 40 V, extraction cone 3 V, source temperature 100 °C, desolvation temperature 350 °C, cone gas flow (N_2) 50 L/h, desolvation gas flow (N_2) 800 L/h. The m/z range was 50−3000 Da.

The MS was calibrated using sodium formate, and leucine enkephalin was used as the lock mass. The experimental m/z values reported were detected and accepted within ±7 ppm of the monoisotopic m/z for the exact theoretical structures.

Sample Preparation for Quantitative HPLC Analysis. Anthocyanins are the main source of interference in HPLC analysis of ellagitannins, and their elimination is essential for obtaining high quality MS spectra of minor ellagitannins. The purification of ellagitannins from anthocyanins using the Sephadex LH-20 was performed following the same protocol described in Gasperotti et al.[10] However, unlike the *Rubus* analysis reported in the paper, for the purification and subsequent quantification of strawberries, an aliquot of 40 mL of extract was used, due to the different concentration range of target compounds.

Quantitative Analysis Using HPLC-DAD. The purified extract was evaporated until dryness in a 100 mL pear-shaped flask using rotary evaporation under reduced pressure at 40 °C. Then the dry sample was diluted to 1 mL with methanol. HPLC analysis was carried out using a Waters 2690 HPLC system equipped with Waters 996 DAD (Waters Corp., Milford, MA), and Empower Software (Waters) as described in Gasperotti et al.[10] All the compounds were quantified using UV detection at 260 nm. Calibration curves such as ranges and limits of detection (LOD) and quantification (LOQ) for standard casuarictin, lambertianin C, sanguiin H-6, ellagic acid, agrimoniin, and methyl ellagic acid rhamnoside are reported in detail in Table S1 in the Supporting Information. Casuarictin, lambertianin C, sanguiin H-6, ellagic acid, agrimoniin, and methyl ellagic acid rhamnoside were quantified following calibration with their respective pure isolated standards and expressed as mg/kg FW. Other ellagitannins were quantified as equivalents of agrimonin and the ellagic acid glycosides as equivalent of ellagic acid, expressed as mg/kg of fresh fruit.

Statistical Analysis. Statistical analysis was carried out using the STATISTICA s/w data analysis software system (version 9, StatSoft, Tulsa, OK, USA). Cluster analysis of the results obtained for the 3 batches of fruits from the 6 cultivars of *Fragaria* × *ananassa* and two *Fragaria vesca*, based on 27 variables describing the individual concentration of the 26 different ellagitannins and ellagic acid conjugates plus their total content, was performed separately for each of the four stages, based on the single linkage, Euclidean distance. The combined effects of the two genotype and ripening stage factors were explored using multivariate repeated measure ANOVA analysis on the same 27 variables used for cluster analysis.

■ RESULTS AND DISCUSSION

Agronomical Parameters. Detailed knowledge of agronomical parameters is the starting point for better understanding the differences between the cultivars analyzed at the chemical level. Indeed, as previously reported,[33−35] most of these (color, size, sweetness, sourness, etc.) are correlated with polyphenol content.

The strawberry cultivars investigated were selected from those most widely cultivated in both Italian and European temperate zones (Belgium, The Netherlands, Germany, and English-speaking areas) and are listed in Table S2 in the Supporting Information. Morphological trait values such as color (chroma) and firmness were different for each selected

cultivar with increasing values from Elsanta to Portola (Table S2 in the Supporting Information). Glossiness ranged from 26.2 (Clery) to 35.0 L^* (Darselect) respectively. The weight, diameter, and fruit height size parameters ranged from 10.4 g (Clery) to 15.8 g (Portola), from 28.1 mm (Alba) to 33.2 mm (Darselect), and from 33.5 mm (Clery) to 40.3 mm (Darselect) respectively (Table S2 in the Supporting Information). For the Elsanta variety the fresh fruit mean weight increased by ~200% from the green stage to veraison and by only ~7% between veraison and ripeness (Table S3 in the Supporting Information). The pH value was relatively high at the green stage, decreased during veraison, and increased at the overripe stage for the *F. vesca* selections. The pH increment was inconsistent for Portola as compared to the other *Fragaria* × *ananassa* varieties. Citric acid content was highest in the Darselect cultivar but fell drastically during ripening. In the red *F. vesca* TTA was high at the green stage, increased at veraison, and decreased during the last two stages, whereas in the white selection the amount was low at the green stage but increased at veraison and the ripe stage, to then decrease at the overripe stage. Sugar content increased proportionally in all samples, except for Alba and Eva, which turned from green to red quite quickly, showing a small drop at veraison, and was significantly higher at the ripe and overripe stages in the white *vesca* (Table S4 in the Supporting Information).

These data, in conjunction with productivity (not reported), show some of the main agronomical characteristics behind the development and commercial use of these cultivars,[36] confirming the representativeness of the genotypes included in the study.

Structural Assignment of Isolated Compounds. *Methyl Ellagic Acid Rhamnoside.* In this study a high concentration of methyl ellagic acid rhamnoside was found in the red and white woodland strawberry for the first time. It had previously been found in the bark of *Eucalyptus globulus*,[37] in *Punica granatum* heartwood,[38] and in the fruit of *Caraipa densifolia* Mart.[39] To date, a few ellagic acid glycosides have been reported in strawberries, but without specific knowledge of the type of glycoside conjugated.[40,41] Using high resolution mass spectrometry (HRMS) analysis (Figure 1) the isolated compound (t_R 39 min) showed a $[M - H]^{-1}$ ion at m/z 461.0720, suggesting an anionic species with the molecular formula $C_{21}H_{17}O_{12}$, whose calculated molecular mass (461.0725) is in excellent agreement (Δm = 0.2 ppm) with experimental measurements. The two main daughter ions detected at 315.0131 and 299.9906 suggested species with the molecular formulas $C_{15}H_7O_8$ (calculated mass 315.0146) and $C_{14}H_4O_8$ (calculated mass 299.9912); the former derives from the loss of a deoxy-hexose moiety and the latter from further loss of a methyl group. All these findings can be accounted for by assuming a structure where the methyl ellagic acid is linked to a deoxy-hexose monosaccharide.

The nature of this deoxy-hexose moiety and the structural details of this compound were established using 1D and 2D NMR measurements carried out in CD_3COCD_3. In particular, its ^1H NMR spectrum showed resonances (doublet signal at δ_H 1.17 ppm coupled to an axial proton signal at δ_H 4.30 ppm) attributable to a 6-deoxy monosaccharide, whereas analysis of the homo- and heteronuclear correlations were in agreement with the structure of an α-rhamnose moiety. 2D NMR analysis indicated that the anomeric carbon of rhamnose was linked to the 3′-OH group of 3-*O*-methyl ellagic acid; thus, peak 25 (Figure 2) was unambiguously established to be 3-*O*-methyl ellagic acid 3′-*O*-α-rhamnopyranoside.

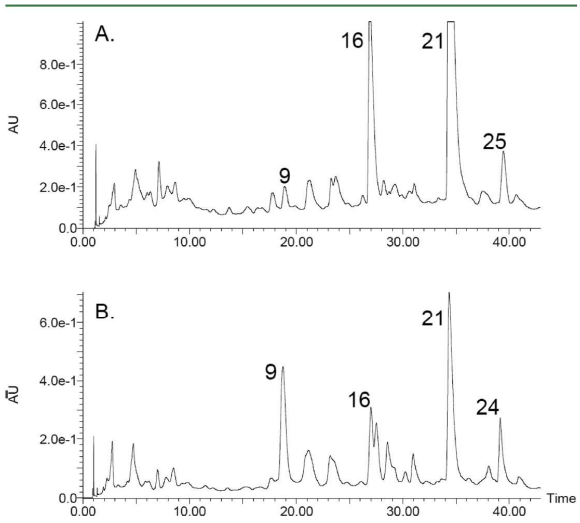

Figure 2. PDA chromatogram (260 nm) of ellagitannin profiling in woodland strawberries (A) and the strawberry cv. Clery (B) Peak numbers refer to casuarictin (**9**), ellagic acid (**16**), agrimoniin (**21**), lambertianin C like (**24**), and methyl ellagic acid rhamnoside (**25**).

Casuarictin (Galloyl-bis-HHDP Glucose). Casuarictin represents one of the monomers frequently found as constituents of the oligomeric ellagitannins found in *Rubus* spp., *Fragaria* spp., and other natural sources.[42] It is also known as galloyl-pedunculagin or casuarictin/potentillin, on the basis of the configuration of the glucose core. Specifically, if it is in α position, the monomer is potentillin, whereas it is casuarictin if in the beta position.[43] From the biosynthetic point of view, galloyl-bis-HHDP glucose comes from the oxidation of the vicinal galloyl units of pentagalloylglucose, leading to the formation of the 2 HHDP units.[44−46]

In HRMS structural analysis (Figure 1) the isolated compound (t_R 18.4 min) showed a $[M - H]^{-1}$ ion at m/z 935.0802, suggesting an anionic species with the molecular formula $C_{41}H_{27}O_{26}$ whose calculated molecular mass (935.0796) is in excellent agreement (Δm = 0.6 ppm) with the experimental measuremets. The two main daughter ions detected at 633.0735 and 300.9990 suggested single charged anionic species with the molecular formulas $C_{27}H_{21}O_{18}$ (calculated mass 633.07333) and $C_{14}H_5O_8$ (calculated mass 300.9990); the former comes from the loss of a HHDP unit followed by proton transfer and the latter by internal rearrangement of the HHDP itself, both being common fragmentation patterns of polymeric ellagitannins. Consequently, on the basis of the fragmentation pattern and the molecular ion, the compound was identified as the monomer galloyl-bis-HHDP glucose, with a monoisotopical mass of 936.0868 ($C_{41}H_{28}O_{26}$).

NMR analysis carried out in deuterated methanol using 1D and 2D techniques suggested the presence of a pentaacyl-substituted glucose. In particular, the characteristic ^{13}C sugar signals were found in the HSQC-NMR spectrum, coupled with a series of multiplets at δ_H 3.9−5.2 ppm, while the anomeric C1 (δ_C 91.9 ppm) was shown to be coupled with the deshielded H1 at δ_H 6.13 ppm. On the other hand, the strong ^3J(C,H)

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Table 1. Characterisation and Tentative Identification of Ellagitannin and Ellagic Acid Conjugates Found in Strawberries and Woodland Strawberries Using UPLC-Q-TOF

peak no.	t_R (min)	m/z ESI(−)	tentative structural assignment	MM[a]	Δ mass (ppm)[b]	cultivar
1	4.5	$[783.0685]^{-1}$, $[633.0741]^{-1}$, $[481.0617]^{-1}$, $[300.9992]^{-1}$	pedunculagin	784.0759	−0.6	strawberry, Elsanta cv.
2	6.1	$[951.0735]^{-1}$, $[783.0685]^{-1}$, $[631.0582]^{-1}$, $[481.0627]^{-1}$, $[300.9986]^{-1}$	unknown ellagitannin	952.0818	0.4	woodland strawberry, red type
3	8.0	$[633.0634]^{-1}$, $[481.0477]^{-1}$,$[331.0544]^{-1}$, $[300.9970]^{-1}$	strictinin	634.0806	−1.1	strawberry, Elsanta cv.
4	9.9	$[933.0657]^{-1}$, $[466.0246]^{-2}$, $[300.9982]^{-1}$	castalagin isomer	934.0712	−2.6	woodland strawberry, red type
5	13.4	$[933.0670]^{-1}$, $[466.0257]^{-2}$, $[301.0000]^{-1}$	castalagin isomer	934.0712	−4.0	woodland strawberry, red type
6	14.0	$[933.0671]^{-1}$, $[466.0253]^{-2}$, $[301.0002]^{-1}$	castalagin isomer	934.0712	−4.4	strawberry, Portola cv.
7	17.4	$[785.0861]^{-1}$, $[615.0650]^{-1}$, $[392.0355]^{-2}$, $[300.9984]^{-1}$, $[169.0141]^{-1}$	digalloyl-HHDP-glucose	786.0915	−3.2	woodland strawberry, red type
8	18.0	$[1103.0848]^{-1}$, $[951.0808]^{-1}$, $[933.0637]^{-1}$, $[783.0696]^{-1}$, $[633.0737]^{-1}$, $[300.9983]^{-1}$	sanguiin H-2	1104.0927	0.0	woodland strawberry, red type
9	18.4	$[935.0809]^{-1}$, $[633.0739]^{-1}$, $[463.0519]^{-1}$, $[301.0004]^{-1}$	casuarictin	936.0868	−2.1	strawberry, Elsanta cv.
10	19.5	$[1567.1527]^{-1}$, $[933.0659]^{-1}$, $[783.0694]^{-2}$, $[633.0746]^{-1}$, $[300.9983]^{-1}$	sanguiin H-10	1568.1518	−5.6	strawberry, Elsanta cv.
11	21.1	$[1235.0723]^{-1}$, $[933.0806]^{-1}$, $[633.0744]^{-1}$, $[617.0284]^{-2}$, $[300.9995]^{-1}$	unknown ellagitannin	1236.0775	−2.2	strawberry, Elsanta cv.
12	22.9	$[1869.1497]^{-1}$, $[1567.1431]^{-1}$, $[1235.0636]^{-1}$, $[934.0724]^{-2}$, $[933.0567]^{-1}$, $[783.0611]^{-1}$, $[633.0667]^{-1}$, $[300.9987]^{-1}$	sanguiin H-6 isomer	1870.1581	−0.3	strawberry, Elsanta cv.
13	23.3	$[1103.0811]^{-1}$, $[951.0764]^{-1}$, $[933.0662]^{-1}$, $[783.0716]^{-1}$, $[633.0734]^{-1}$, $[300.9982]^{-1}$	sanguiin H-2 isomer	1104.0927	3.4	woodland strawberry, red type
14	24.8	$[1401.1096]^{-2}$, $[1235.0768]^{-1}$, $[933.0657]^{-1}$, $[933.0567]^{-1}$, $[783.0702]^{-1}$, $[633.0757]^{-1}$, $[300.9984]^{-1}$	lambertianin C	2804.2293	2.1	woodland strawberry, red type
15	25.8	$[1869.1560]^{-1}$, $[1567.1537]^{-1}$, $[1235.0636]^{-1}$, $[934.0739]^{-2}$, $[933.0665]^{-1}$, $[783.0730]^{-1}$, $[633.0743]^{-1}$, $[300.9986]^{-1}$	sanguiin H-6	1870.1581	−3.0	woodland strawberry, red type
16	26.2	$[300.9984]^{-1}$, $[257.0085]^{-1}$, $[229.0139]^{-1}$	ellagic acid	302.0062	−0.3	woodland strawberry, red type
17	26.7	$[447.0563]^{-1}$, $[300.9990]^{-1}$, $[299.9914]^{-1}$	ellagic acid deoxyhexose	448.0641	−0.2	strawberry, Elsanta cv.
18	28.4	$[2037.1661]^{-1}$, $[1018.0735]^{-2}$, $[1567.1521]^{-1}$, $[1235.0841]^{-1}$ $[933.0655]^{-1}$, $[783.0684]^{-1}$, $[300.9997]^{-1}$	sanguiin H-6 with galloyl moiety	2038.1639	0.5	strawberry, Elsanta cv.
19	30.7	$[2501.2153]^{-1}$, $[1869.1633]^{-1}$, $[1567.1493]^{-1}$, $[1250.1063]^{-2}$, $[933.0693]^{-1}$, $[783.0734]^{-1}$, $[633.0746]^{-1}$, $[300.9993]^{-1}$	lambertianin C without ellagic moiety	2502.2230	−2.2	strawberry, Elsanta cv.
20	32.5	$[1085.0804]^{-1}$, $[542.0308]^{-2}$, $[301.0008]^{-1}$	galloyl-castalagin	1086.0822	−5.6	woodland strawberry, red type
21	33.8	$[1869.1555]^{-1}$, $[1567.1484]^{-1}$, $[1265.1418]^{-1}$, $[934.0709]^{-2}$, $[933.0670]^{-1}$, $[783.0685]^{-1}$, $[633.0732]^{-1}$, $[300.9985]^{-1}$	agrimoniin	1870.1581	−2.8	strawberry, Elsanta cv.
22	35.2	$[1085.0787]^{-1}$, $[542.0303]^{-2}$, $[300.9985]^{-1}$	galloyl-castalagin isomer	1086.0822	−4.1	woodland strawberry, red type
23	37.5	$[939.1123]^{-1}$, $[483.0210]^{-1}$, $[300.9996]^{-1}$, $[169.0130]^{-1}$	pentagalloyl glucose	940.1181	−2.2	woodland strawberry, red type
24	38.7	$[1401.1008]^{-2}$, $[933.0692]^{-1}$, $[783.0727]^{-1}$, $[633.0737]^{-1}$, $[481.0657]^{-1}$, $[300.9992]^{-1}$	lambertianin C like	2804.2293	4.2	strawberry, Elsanta cv.
25	39.0	$[461.0720]^{-1}$, $[315.0138]^{-1}$, $[299.9896]^{-1}$	methyl ellagic acid rhamnoside	462.0798	−0.2	woodland strawberry, red type
26	41.0	$[2019.1527]^{-1}$, $[1567.1472]^{-1}$, $[1235.0774]^{-1}$, $[1009.0682]^{-2}$, $[933.0688]^{-1}$, $[783.0714]^{-1}$, $[633.0747]^{-1}$, $[300.9991]^{-1}$	unknown ellagitannin	2020.1606		strawberry, Elsanta cv.

[a]MM: theoretical molecular monoisotopic mass of the putative metabolite. [b]Δ mass (ppm): deviation of the observed ion mass from the corresponding theoretical monoisotopic mass.

heterocorrelations of the carbonyl groups with the corresponding protons on the sugar moiety detected in the HMBC-NMR spectrum made it possible to clearly define the overall substitution pattern of the sugar moiety. Of the five resonances detected for these 5 ester groups, four at δ_C about 168−170 ppm are attributable to −COO groups linked to HHDP moieties, and one at δ_C 165.2 ppm to the −COO group linked to the galloyl group at the anomeric C-1 of the glucose. Moreover, the coupling pattern of the deshielded H1 proton (δ_H 6.13 ppm, ^3J(H1,H2) = 8.5 Hz) indicated that galloyl group was in the β position (see Figure 1 and Figure S1 in the Supporting Information). Therefore peak 11 (Figure 2) was unambiguously established to be casuarictin.[47] The absolute configuration of the four chiroptical HHDP groups on the $^4C_1$ glucose core was established to be *S* by the positive sign of the strong Cotton effect at λ 240 nm and the negative Cotton effect at λ 268 nm.

Identification of Ellagitannins and Ellagic Acid Glycosides in the Different Strawberry Cultivars. Use of electrospray ionization high resolution mass spectrometry (ESI-HRMS) has been shown to be a useful technique for profiling ellagitannins and their structural characterization in

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raspberries and blackberries.[10] In the same way, the profiles of ellagitannins in different strawberry cultivars and wild strawberry types were identified. ESI-HRMS analysis of different strawberry cultivars and wild strawberry types allowed characterization of 26 compounds and their tentative identification, with an accuracy of within 7 ppm in relation to the exact monoisotopical molecular mass. The compounds identified are given in Table 1 with their monoisotopical molecular ions, the most characteristic fragments, their tentative identification, and the ppm difference from the exact monoisotopical mass. The MS features of the ellagitannins and ellagic acid conjugates reported in this paper were verified in all the different cultivars investigated, and the exact molecular ions for each are given in Table S5 in the Supporting Information.

Ellagitannin Structural Profiling. The compounds were first identified using a UV−vis detector (Figure 2) and then analyzed with HRMS, checking their monoisotopical molecular ions and characteristic fragmentation pattern. The fragmentation pattern is often similar in different ellagitannins, due to their structural similarity.

Compound **1** (t_R 4.45 min) showed a $[M - H]^{-1}$ ion at m/z 783 and main fragments at m/z 481 (loss of HHDP) and 301 (ellagic acid). On the basis of these fragments and the difference in ppm in relation to molecular ion, this compound was identified as bis-HHDP-glucose or pedunculagin, 784 Da.[40] Compound **2** (t_R 6.1 min) showed a $[M - H]^{-1}$ ion at m/z 951 and fragments at m/z 783 (loss of galloyl fragment), 481, 301. This compound showed the same molecular ion already reported as an unknown ellagitannin.[40] The compound can be tentatively identified as sanguisorboyl-HHDP-glucose. Compound **3** (t_R 8 min) showed a $[M - H]^{-1}$ ion at m/z 633 and main fragments at m/z 481 (loss of gallic acid), 331 (loss of HHDP), 301. On the basis of these fragments and the molecular ion, compound **3** was identified as galloyl-HHDP glucose or strictinin, 634 Da. Compounds **4**, **5**, and **6** (t_R 9.9, 13.4, and 14 min respectively) showed the same $[M - H]^{-1}$ ions at m/z 933 and $[M - H]^{-2}$ at m/z 466. They also had the common fragment at m/z 301. These 3 compounds were in accordance with previous findings,[4,6] where they were identified as unknown ellagitannins. It was also suggested that these fragmentation patterns could be related to the presence of different isomeric forms of castalagin, 934 Da.[24] Compound **7** (t_R 17.4 min) showed a $[M - H]^{-1}$ ion at m/z 785 and $[M - H]^{-2}$ at m/z 392, fragments at m/z 615, 301, and 169 (gallic acid). This compound was tentatively identified as digalloyl-HHDP-glucose.[40] Compounds **8** and **13** (t_R 18 and 23.3 min) showed a $[M - H]^{-1}$ ion at m/z 1103. On the basis of these molecular ions and the fragmentation of 951, 933, 783, 633, and 301, the compounds were identified as sanguiin H-2 isomers, 1104 Da. Compound **9** (t_R 18.4 min) showed a $[M - H]^{-1}$ ion at m/z 935 and fragments at m/z 633, 301. On the basis of this information and the results of NMR analysis, after isolation of the peak, the compound was identified as the characteristic ellagitannin monomer casuarictin. Compound **10** (t_R 19.5) showed a $[M - H]^{-1}$ ion at m/z 1567 and $[M - H]^{-2}$ at m/z 783. The compound was identified as sanguiin H-10, 1568 Da, and the fragmentation patterns were made by the fragment at m/z 933, 783, 633, 301. Compound **11** (t_R 21.1 min) showed a $[M - H]^{-1}$ ion at m/z 1235 and $[M - H]^{-2}$ at m/z 617, giving a molecular mass of 1236, and has already been previously reported as unknown ellagitannin in the strawberry.[40] It has also been reported with the common name davuriicin M1.[48] Compound **12** (t_R 22.9 min) showed a $[M - H]^{-1}$ ion at m/z 1869 and $[M - H]^{-2}$ at m/z 934 with fragments at m/z 1567, 1235, 933, 783, 633, 301. On the basis of this information this compound was identified as an isomer of sanguiin H-6. This isomeric form of sanguiin H-6 had a different retention time as compared to sanguiin H-6. Compound **14** (t_R 24.8) was in accordance with the retention time of lambertianin C previously isolated in the raspberry[10] and showed $[M - H]^{-2}$ at m/z 1401, giving a molecular mass of 2804. In the light of this evidence, the same retention time, and the double charged signal, this compound was identified as lambertianin C, also due to the characteristic fragmentation pattern of the polymeric ellagitannins in *Rubus* (933, 783, 633, 301). Compound **15** (t_R 25.8) was in accordance with the retention time of sanguiin H-6 previously isolated in the raspberry[10] and showed $[M - H]^{-2}$ at m/z 934, giving a molecular mass of 1870, and also had the same characteristic fragmentation pattern. Compound **18** (t_R 28.4 min) showed a $[M - H]^{-1}$ ion at m/z 2037 and $[M - H]^{-2}$ at m/z 1018 with fragments at m/z 1567, 1235, 933, 783, 301. On the basis of this information and the literature[10,41,48] this compound was tentatively identified as sanguiin H-6 with galloyl moiety. Compound **19** (t_R 30.7 min) showed a $[M - H]^{-1}$ ion at m/z 2501 and $[M - H]^{-2}$ at m/z 1250 with fragments at m/z 1869, 1567, 933, 783, 633, 301. The compound was tentatively identified as lambertianin C without an ellagic moiety, 2502 Da.[10] Compounds **20** and **22** (t_R 32.5 and 35.2 min) showed a $[M - H]^{-1}$ ion at m/z 1085 and $[M - H]^{-2}$ at m/z 542. These two compounds had previously been reported in a paper[40] as unknown ellagitannins or as putative galloyl-castalagin, with a mass of 1086 Da, but had never previously been reported in the strawberry.[24] Compound **21** (33.8 min) was in accordance with the retention time of agrimoniin previously isolated and clarified in the strawberry[15] and showed $[M - H]^{-1}$ at m/z 1869 and a $[M - H]^{-2}$ at m/z 934, giving a molecular mass of 1870. Compound **23** (t_R 37.5 min) showed a $[M - H]^{-1}$ ion at m/z 939 and fragments at m/z 483 (loss or 3 galloyl moieties), 301, 169. On the basis of these observations, compound **23** was identified as pentagalloyl-glucose. Compound **24** (t_R 38.7 min) showed a $[M - H]^{-2}$ ion at m/z 1401 and fragments at m/z 933, 783, 633, 481, 301. This compound, with a monoisotopical mass of 2804, was identified as an isomer of lambertianin C on the basis of the double charged signal and may be formed by the polimerization of agrimoniin with another galloyl-bisHHDP-glucose. Compound **26** (t_R 41 min) showed a $[M - H]^{-1}$ ion at m/z 2019 and $[M - H]^{-2}$ at m/z 1009, giving a molecular mass of 2020 Da, with fragments at m/z 1567, 1235, 933, 783, 633, 301. This compound was identified as an unknown ellagitannin, as already reported.[41]

Ellagic Acid and Ellagic Acid Glycosides. Ellagic acid and related glycosides can be distinguished in UV−vis by their characteristic spectra at 260 and 360 nm as compared to ellagitannin spectra. In the literature, only a few ellagic acid glycosides have been reported in strawberries.[14,17,41]

Compound **16** (t_R 26.2) showed an intense $[M - H]^{-1}$ ion at m/z 301 and fragments at m/z 257 and 229, typical of ellagic acid. The compound was confirmed with the injection of the pure standard. Compound **17** (t_R 26.7) showed a $[M - H]^{-1}$ ion at m/z 447 and fragment at m/z 300. The compound was tentatively identified as an ellagic acid deoxyhexose.[14,40] Compound **25** (t_R 39 min) showed a $[M - H]^{-1}$ ion at m/z 461 and fragments at m/z 315 and 300. This compound was identified as methyl ellagic acid rhamnoside after isolation from

the woodland strawberry and structural elucidation carried out using HRMS and NMR.

Qualitative Variability in the Ellagitannin Profile. Variability in the composition was observed in different cultivars (Table S5 in the Supporting Information). The variability was clearly not due to analytical errors, since it was confirmed in all 12 individual analyses for each genotype.

The quantitative and qualitative differences between cultivars are shown in the results of cluster analysis of the composition of berries at the same stage of maturity. The results of cluster analysis were similar for stages 3 and 4, while the separation was limited using the data for unripe samples (stage 1). The example of cluster analysis at stage 3 (ripe) is reported in the hierarchical tree plot of Figure 3, while at stages 2 and 4 in

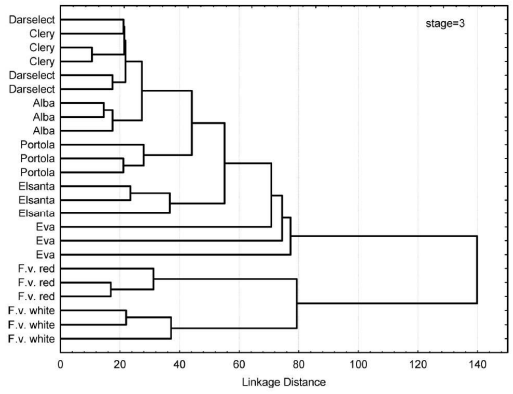

Figure 3. Cluster analysis based on the concentration of 26 different ellagitannins and ellagic acid conjugates plus their total content in strawberry cultivars and woodland strawberry types at stage 3 (ripe). The same figure at stages 2 and 4 is given as Supporting Information.

Figure S2 in the Supporting Information. The separation of the samples was due to the qualitative and quantitative differences in the presence and concentration of 26 different ellagitannins and ellagic acid conjugates, described below.

The two *Fragaria vesca* genotypes clustered apart both at the ripe and overripe stages, due to higher concentrations of ellagitannins, and the two genotypes were well separated, due to their different profile, while Darselect, Clery, Alba, and Portola appeared as nearest neighbors on the opposite site of the cluster. The Elsanta and Eva cultivars were intermediate, the latter in particular being closer to the *Fragaria vesca* samples, possibly in the light of a relatively high concentration of ellagitannins at full ripeness. Such grouping remained qualitatively comparable even at stage 2, with the peculiarity that at veraison the samples of Eva were the most distant among all the studied genotypes, including the *F. vesca*.

The two groups of *Fragaria* × *ananassa* cultivars and *Fragaria vesca* accessions were qualitatively different, since as many as five different ellagitannins (**4**, **7**, **10**, **13**, **20**) and methyl ellagic acid rhamnoside (**25**) were exclusively found in the two *Fragaria vesca* genotypes.

In the red woodland strawberry, 22 of the 26 compounds were present, with the exception of compounds **6** (castalagin isomer), **17** (ellagic acid deoxyhexose), **24** (lambertianin C like), and **26** (unknown ellagitannin). The ellagitannin profile was less complex in the white woodland strawberry. Indeed, when comparing the two types, red and white, four ellagitannins were absent in the white genotype: compounds **2** (sanguisorboyl-HHDP glucose), **5** (castalagin isomer), **8** (sanguiin H-2 isomer), and **22** (galloyl-castalagin isomer). The latter four compounds (**2**, **5**, **8**, **22**) were also not detected in the berries of all six *Fragaria* × *ananassa* cultivars.

In strawberries, ellagic acid and 10 ellagitannins (compounds **1**, **3**, **9**, **11**, **12**, **14**, **15**, **18**, **19**, **21**) were present in all the cultivars/accessions included in the study. Specifically, the main ellagitannins, casuarictin and agrimoniin, were present in all of them. It was more interesting to observe the variability in the cultivars, where some ellagitannins were either absent or present in only one of them. Ellagitannin **26** was present only in the Elsanta and Clery cvs. Compound **23** (pentagalloylglucose) was found only in the berries of the cv. Clery. Furthermore, compound **6**, a castalagin isomer, was detected in a single cultivar of *Fragaria* × *ananassa*, Portola; and compound **17** was present in all the *Fragaria* × *ananassa* cultivars with the exception of Alba, while it was not detected in the woodland strawberries. Only the 2 cultivars Darselect and Eva showed the same qualitative profile in terms of ellagitannins and ellagic acid glycosides.

Quantitative Variability in the Concentration of Ellagitannin and Ellagic Acid Conjugates. Quantification of ellagitannins as the equivalent of other commercially available compounds, such as gallic acid or ellagic acid, may reveal the inadequacy of the approach. Molar extinction factors of 10600[49] and 58200[15] M^{-1} cm^{-1}, respectively, at a 1 to 5 ratio were observed at 260 nm in methanol for gallic acid and agrimoniin. This ratio is related to the number of chromophores present in the molecules, and better quantification will be possible with an equivalent compound more similar to the analytes. The same can be observed in relation to the quantification of casuarictin, with a gallic acid ratio of 1 to 3. Consequently, correct quantification of ellagitannins can only take place with proper reference compounds. To support this conclusion in Table S6 in the Supporting Information we list other ellagitannins with a comparable number of chromophores (ellagic acid and gallic acid moieties) and their ratio between molar extinction factors and molecular weight.

The content of ellagitannins and ellagic acid conjugates in strawberries was found to be rather variable, ranging from 84.8 to 1636 mg/kg, with an average of 637 mg/kg, depending on the species, cultivar, and ripening stage (Table 2). It is worth taking a closer look at these values, since this is the first attempt to give an accurate quantitative estimate of their individual concentrations.

Considering the amounts at ripeness, the average results are substantially in accordance with one of the recent papers on strawberry and ellagitannin quantification in their native form, at least in terms of the order of magnitude,[50] although we would emphasize that it is not entirely accurate to compare quantitative data expressed as equivalent of ellagic acid for all the ellagitannins with our data, in which quantification was carried out using isolated ellagitannin standards.

The 11 ellagitannins and ellagic acid conjugates found in all strawberry samples (Table 2) showed a range of concentrations and average concentration in fruits respectively in the following decreasing order: agrimoniin, **21** (25.0−747 mg/kg; 223 mg/kg); casuarictin, **9** (19.0−386 mg/kg; 100 mg/kg); sanguiin H-6 isomer, **12** (1.5−133 mg/kg; 40.2 mg/kg); sanguiin H-6 with a galloyl mojety, **18** (6.4−95.2 mg/kg; 40.0 mg/kg); unknown ET, **11** (3.9−95.8 mg/kg; 30.4 mg/kg); lambertianin C without one ellagic acid mojety, **19** (2.2−82.6 mg/kg; 28.6 mg/kg); pedunculagin, **1** (5.7−108 mg/kg; 27.3 mg/kg); ellagic acid, **16**

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Table 2. Quantification of Ellagitannin and Ellagic Acid Conjugates at Different Ripening Stages in Different Strawberry Cultivars and Woodland Types[a]

	peak #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	
		pduncalagin	unknown ET	strictinin	castalagin	castalagin isomer	castalagin isomer	digalloyl-HHDP-glucose	sanguiin H-2	casuarictin	sanguiin H-10	unknown ET	sanguiin H-6 isomer	sanguiin H-2 isomer	lambertianin C	sanguiin H-6	ellagic acid	ellagic acid deoxyhexose	sanguiin H-6 with galloyl moiety	lambertianin C without ellagic moiety	galloyl-castalagin	agrimoniin	galloyl-castalagin isomer	pentagalloyl glucose	lambertianin C like	methyl ellagic acid rhamnoside	unknown ET	total amount
	t_R	4.6	6.1	8	9.9	13.4	14	17.4	18	18.4	19.5	21.1	22.9	23.3	24.8	25.8	26.2	26.7	28.4	30.7	32.5	33.8	35.2	37.5	38.7	39	40.7	
Strawberry cultivar																												
Darselect	green	29.7	nd	12.5	nd	nd	nd	nd	nd	266	nd	56.4	80.4	nd	11.9	17.7	15.9	21.3	70.2	66	nd	419.6	nd	nd	78.8	nd	nd	1146.5
	sd	*1.2*		*1.5*						*31*		*13.8*	*6.6*		*4.1*	*4.6*	*2.7*	*3.4*	*17*	*18.3*		*90.3*			*18.3*			*212.8*
	veraison	13.6	nd	4.9	nd	nd	nd	nd	nd	112.1	nd	22.6	40.1	nd	3.6	6	8.4	10	40.3	21.1	nd	156.2	nd	nd	23.6	nd	nd	462.7
	sd	*3.9*		*0.5*						*5.3*		*1.1*	*2.3*		*0.8*	*0.7*	*1*	*0.6*	*1.6*	*1.2*		*19.6*			*0.9*			*39.7*
	ripe	11.6	nd	4	nd	nd	nd	nd	nd	87.1	nd	17.9	24.1	nd	1.8	8.2	5.5	7	31.7	15	nd	136.4	nd	nd	18.8	nd	nd	369.3
	sd	*1.5*		*0.3*						*7.5*		*2*	*2.2*		*0.8*	*7.5*	*0.5*	*0.9*	*1.9*	*1.5*		*13.8*			*2.2*			*42.6*
	overripe	9.9	nd	4.3	nd	nd	nd	nd	nd	68.9	nd	14.9	20.6	nd	2.3	3.8	6.4	7.2	21.9	12.7	nd	111	nd	nd	13	nd	nd	296.9
	sd	*0.6*		*2.5*						*8.5*		*1.9*	*1.9*		*0.4*	*0.2*	*1.1*	*1*	*7.9*	*2.2*		*14.4*			*3.2*			*45.8*
Alba	green	20	nd	6.5	nd	nd	nd	nd	nd	84.5	nd	26.2	31.1	nd	5.9	5.7	18.7	nd	36.3	26.9	nd	132	nd	nd	35.7	nd	nd	429.5
	sd	*4.6*		*2.4*						*22.7*		*6.7*	*3.5*		*2.8*	*2.4*	*6.5*		*10.8*	*7.7*		*33.9*			*11.5*			*115.3*
	veraison	19.8	nd	4.6	nd	nd	nd	nd	nd	78.3	nd	32	33.8	nd	7.1	7.1	23.8	nd	47.8	29.6	nd	163	nd	nd	36.3	nd	nd	483.1
	sd	*4.2*		*1.9*						*15.3*		*7.7*	*7.1*		*1.4*	*1.3*	*4.6*		*9.1*	*6.4*		*32.9*			*9.8*			*101.7*
	ripe	16.1	nd	4.2	nd	nd	nd	nd	nd	52.9	nd	13.5	18.2	nd	3.4	3.9	17	nd	27.1	13.8	nd	78.2	nd	nd	20.3	nd	nd	268.8
	sd	*1.8*		*1.3*						*7.7*		*3.3*	*1.8*		*0.1*	*0.1*	*1.8*		*0.5*	*0.7*		*9.8*			*0.9*			*29.8*
	overripe	8.3	nd	2.2	nd	nd	nd	nd	nd	28.3	nd	5.4	4.6	nd	0.7	1.3	11.2	nd	12.1	5.4	nd	38.1	nd	nd	8	nd	nd	125.4
	sd	*2.1*		*0.3*						*6.2*		*1.3*	*2.6*		*0.1*	*0.4*	*1.3*		*5.4*	*2.8*		*12.5*			*5.4*			*40.6*
Portola	green	24.7	nd	10.2	nd	nd	3.6	nd	nd	242.2	nd	46.7	119.4	nd	13.5	12.9	17	15.5	38.3	52.5	nd	217.1	nd	nd	54.6	nd	nd	868.2
	sd	*2*		*1.3*			*0.8*			*24.6*		*8.3*	*12.3*		*1.3*	*1.9*	*2.4*	*1.4*	*6*	*9.3*		*42.9*			*6.9*			*121.6*
	veraison	18.1	nd	5.6	nd	nd	2.3	nd	nd	136.1	nd	31.9	63.8	nd	7.6	8.1	12.5	12.4	28.3	31.4	nd	132.7	nd	nd	28.7	nd	nd	519.4
	sd	*3.1*		*1*			*0.6*			*10.2*		*5.1*	*6.8*		*1.3*	*0.9*	*1.8*	*1*	*3.5*	*3.2*		*12.1*			*2.3*			*53*
	ripe	10.8	nd	4.6	nd	nd	1.8	nd	nd	102.2	nd	20.4	36.2	nd	4.6	5.3	10.8	8.8	17	14.5	nd	60.6	nd	nd	13.4	nd	nd	311
	sd	*2.4*		*2*			*0.6*			*18.3*		*4.8*	*8.3*		*2*	*1.7*	*1.3*	*2.1*	*3*	*1.9*		*5.6*			*2.9*			*56.9*
	overripe	8.4	nd	3.5	nd	nd	1.1	nd	nd	77	nd	14.2	26.1	nd	2.3	2.9	8.5	7.4	10.5	7	nd	42.6	nd	nd	7.5	nd	nd	219.1
	sd	*1.4*		*0.6*			*0.8*			*17.1*		*3*	*5.4*		*0.7*	*0.8*	*1.3*	*1.6*	*2*	*1.3*		*8.7*			*2*			*46.7*
Eva	green	25.9	nd	7.6	nd	nd	nd	nd	nd	322	nd	63.6	94.6	nd	12.9	11.5	15.3	19.2	42.6	48.7	nd	346	nd	nd	56.2	nd	nd	1066
	sd	*2.1*		*1.9*						*61.2*		*30.4*	*29*		*2.3*	*4.4*	*4.2*	*4.5*	*10.5*	*14.2*		*100*			*18.3*			*283*
	veraison	29.1	nd	9.7	nd	nd	nd	nd	nd	265.5	nd	55.3	80.9	nd	9	12.6	17.8	20.2	46.7	44	nd	286.7	nd	nd	45.8	nd	nd	923.4
	sd	*7.7*		*2.6*						*33.4*		*11*	*15.3*		*1.5*	*2.1*	*4*	*4.1*	*9.2*	*7.3*		*31*			*8.2*			*137.5*
	ripe	20.8	nd	6.4	nd	nd	nd	nd	nd	202.8	nd	38.3	58.7	nd	5.7	7.9	14.2	14.6	39	28.7	nd	190.8	nd	nd	41.1	nd	nd	668.7
	sd	*4*		*2.1*						*41.6*		*14.3*	*21.5*		*3*	*3.2*	*1*	*1.5*	*10.4*	*10.3*		*52.4*			*12.4*			*177.8*
	overripe	12.8	nd	2.9	nd	nd	nd	nd	nd	154.3	nd	26.3	41	nd	5.2	6	12.4	11.1	31.1	19.1	nd	138.3	nd	nd	31.1	nd	nd	491.6
	sd	*1.3*		*0.2*						*10.7*		*3.4*	*3.8*		*0.2*	*0.9*	*2.4*	*2.3*	*4.5*	*3.5*		*31.7*			*2.8*			*67.6*
Clery	green	38.6	nd	13.2	nd	nd	nd	nd	nd	210.4	nd	47.2	83.7	nd	10.3	10.8	16.3	19.4	66.6	51.1	nd	294.5	nd	28.4	84.7	nd	19.1	994.4
	sd	*6.4*		*1.9*						*32.2*		*8.2*	*16.3*		*1.9*	*2*	*2.6*	*3.9*	*11.1*	*10*		*70.9*		*5.9*	*19.5*		*6.8*	*199.7*
	veraison	20.3	nd	5.1	nd	nd	nd	nd	nd	112.3	nd	27.5	38.1	nd	5.4	6	13	12.3	38.9	25	nd	151.2	nd	13.2	36.2	nd	9.6	514.1
	sd	*0.6*		*0.3*						*14.8*		*1.4*	*3.6*		*1.4*	*0.8*	*2.4*	*1.1*	*3.4*	*2.7*		*22.1*		*1.7*	*5.3*		*1.9*	*63.6*
	ripe	16.1	nd	3.8	nd	nd	nd	nd	nd	81.5	nd	18.7	27.7	nd	3.9	4.7	12.9	10.3	29.6	15.1	nd	101.5	nd	7.4	21.1	nd	6.6	361
	sd	*1.9*		*0.5*						*8.2*		*0.5*	*1.6*		*0.3*	*0.5*	*3.4*	*0.7*	*2*	*2.6*		*10.4*		*0.9*	*1.8*		*1.2*	*36.4*
	overripe	12.3	nd	2.7	nd	nd	nd	nd	nd	66.7	nd	14.7	17.6	nd	2.8	3.4	11.6	10.8	26.6	12.4	nd	98.9	nd	6.3	15.6	nd	7.2	309.6
	sd	*4.8*		*0.9*						*20.1*		*7.3*	*10.4*		*1.8*	*1.8*	*1.8*	*2.5*	*7.9*	*6.8*		*25.6*		*3.4*	*6.9*		*3.1*	*105.2*
Elsanta	green	20.6	nd	6.8	nd	nd	nd	nd	nd	56.1	nd	33.3	49.2	nd	6.4	9.5	14.9	14	37.8	33.4	nd	406.1	nd	nd	36.9	nd	16.8	741.9
	sd	*6.4*		*2.5*						*12.3*		*6.4*	*13.8*		*1.2*	*1.3*	*2.3*	*2.9*	*6.6*	*5.7*		*82.9*			*12.7*		*5.2*	*162.2*
	veraison	13.4	nd	4.4	nd	nd	nd	nd	nd	40.3	nd	24.1	34.1	nd	4.8	7	12.8	10.8	27.7	22.6	nd	284.2	nd	nd	23.8	nd	10.1	520.1
	sd	*1.6*		*0.2*						*3.3*		*2*	*4.5*		*0.6*	*0.9*	*2.3*	*1.9*	*1.9*	*1.4*		*37.9*			*0.8*		*0.3*	*59.4*
	ripe	7.5	nd	2	nd	nd	nd	nd	nd	21.4	nd	12.2	14.9	nd	0.9	2.6	7.4	6	15.3	9.6	nd	146.6	nd	nd	10.8	nd	5.5	262.5
	sd	*1*		*0.4*						*3.1*		*2.1*	*3.2*		*0.5*	*0.3*	*1.8*	*0.8*	*2.7*	*2.5*		*29.4*			*2.5*		*1.2*	*51.5*
	overripe	6.4	nd	1.6	nd	nd	nd	nd	nd	21.1	nd	12.8	14.6	nd	2.1	3.4	7.4	5.9	15.3	10.4	nd	139.9	nd	nd	10.1	nd	5.4	256.2
	sd	*0.6*		*0.4*						*1.7*		*1.4*	*1.2*		*0.8*	*0.9*	*1.1*	*0.8*	*1.8*	*1.3*		*8.8*			*0.8*		*0.6*	*22.1*
Woodland strawberries																												
Red type	green	75.4	14	34.4	28.7	7.4	nd	14.5	11.1	53.6	14.1	59	35.2	52.6	16.1	20.8	66.7	nd	76.4	46.4	49.4	442.6	54.4	22.3	nd	86.7	nd	1281.9
	sd	*19.1*	*3.1*	*19.9*	*4.4*	*1.1*		*1.6*	*0.5*	*8.7*	*1.9*	*8.3*	*6.1*	*12.6*	*1.6*	*2*	*10*		*14.8*	*16*	*10.7*	*83.7*	*10.3*	*7.1*		*26.3*		*269.9*
	veraison	81.7	19.2	39.1	24	7.4	nd	15.1	11.3	47.7	13.7	45.9	30.4	35.7	13.1	16.7	58.3	nd	67.3	42.1	40.5	305.2	39	17.7	nd	91.4	nd	1062.4
	sd	*23.5*	*5.6*	*13.1*	*6.4*	*2*		*4.3*	*3.2*	*10.8*	*4.1*	*8.7*	*5.9*	*6.6*	*1.2*	*2.4*	*7.7*		*15.1*	*13*	*4.4*	*77.5*	*4.4*	*5.9*		*37.2*		*263*
	ripe	64.7	15.5	33.3	20.7	4.9	nd	13.5	7.6	33.7	11.7	33.3	24.2	23.7	8.7	13.5	52	nd	56.1	41.6	33.7	244.1	35.7	15.5	nd	71.6	nd	859.3
	sd	*5.3*	*2*	*5.5*	*2.1*	*1.6*		*4.2*	*3*	*1.6*	*0.6*	*1.4*	*0.5*	*3.5*	*1.8*	*2.8*	*3.1*		*5.4*	*4.4*	*4.1*	*15.4*	*2.2*	*2.3*		*3.5*		*76.1*
	overripe	46.5	13.4	29.5	19.4	3.8	nd	15.9	8.1	37.3	11.8	44.3	25.6	25	10.9	15.6	67.1	nd	59	41.8	43	264.9	43.7	18.9	nd	68.1	nd	913.7
	sd	*8*	*4*	*5.4*	*3.6*	*1.6*		*6.9*	*5.1*	*11.8*	*6.4*	*6*	*6.8*	*10*	*3.9*	*4.5*	*6.6*		*11.2*	*7.5*	*8.1*	*36.6*	*7.1*	*3*		*15.4*		*179.7*
White type	green	64.8	nd	33.1	23.6	nd	nd	9.7	nd	47.1	13.4	40.8	40.4	57.5	9.7	15.2	67.9	nd	72.6	44.7	39.9	625.9	nd	33.9	nd	55.3	nd	1295.5
	sd	*15.7*		*13.4*	*5.3*			*4.6*		*18.1*	*6.7*	*13.9*	*15.3*	*11.4*	*3.5*	*4.7*	*30.2*		*20.7*	*9.1*	*15*	*121.8*		*12.9*		*12.1*		*334.4*
	veraison	48.9	nd	37	21.1	nd	nd	7.2	nd	34.3	11.9	29.5	30.1	41.6	7.5	10	50.3	nd	62.5	29.8	31.1	408.8	nd	21.6	nd	46.9	nd	930.3
	sd	*9.1*		*14.2*	*3.2*			*2.6*		*7.1*	*3*	*3.4*	*2.4*	*9.1*	*1.4*	*4.9*	*5.5*		*2.9*	*2.4*	*2.2*	*16*		*0.8*		*15*		*105.1*
	ripe	42.2	nd	23	15.7	nd	nd	6.5	nd	28.8	11.1	22.8	24.4	27.1	7.2	6.6	41.7	nd	47.1	27.9	24.4	307.9	nd	13.9	nd	41.3	nd	719.5
	sd	*9.6*		*0.6*	*1*			*3.7*		*4.1*	*1.2*	*5.9*	*2.4*	*1.2*	*1*	*1.5*	*1.4*		*2.8*	*3.2*	*8.7*	*18.8*		*3.5*		*1.4*		*72.1*
	overripe	34.7	nd	19.6	13.5	nd	nd	*3.7*	nd	33.6	9.9	21.3	23.4	28.3	5.8	10.2	49.5	nd	41.6	24.1	19.7	279.7	nd	18.7	nd	55.3	nd	697.1
	sd	*7.5*		*2.6*	*1.8*			*3.7*		*13.7*	*1*	*3.3*	*1*	*2*	*1.5*	*2.8*	*1.4*		*4.4*	*1.8*	*2.8*	*16.5*		*0.2*		*4.6*		*69.6*

[a]The values reported are the mean value of the three biological replicates in mg/kg of fresh weight fruit and their respective standard deviations (sd).

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(4.9−97.8; 23.9 mg/kg) strictinin, **3** (1.2−54.2 mg/kg; 12.0 mg/kg); sanguiin H-6, **15** (0.8−23.0 mg/kg; 8.6 mg/kg); lambertianin C, **14** (0.3−17−9 mg/kg; 6.7 mg/kg).

In conclusion, agrimoniin was confirmed to be the main ellagitannin in strawberries, as suggested by Vrhovsek et al.,[15] and casuarictin, for the first time reported as a natural constituent of strawberries, was the second compound in terms of quantitative importance among ellagitannins in *Fragaria* × *ananassa* fruits alone, reaching a concentration as high as that of agrimoniin in some cultivars, such as Eva, Portola, and Darselect (Table 2).

Of the ellagitannins specific to *Fragaria* × *ananassa*, only the lambertianin C-like ellagitannin, **24**, was found to be among the main ellagitannins (range 2.3−98.8 mg/kg, average 31.3 mg/kg), while **6**, **17**, and **26**, when present, were minor constituents (Table 2).

The *Fragaria vesca* samples were in general richer in ellagitannins and ellagic acid conjugates (range 658−1636 mg/kg, average 970 mg/kg), once again having agrimoniin as the main ellagitannin, and with a significant presence and at similar levels as **25**, **18**, **1**, and **16** (Table 2). In particular, the compound 3-*O*-methyl ellagic acid 3′-*O*-α-rhamnopyranoside, **25** (33−132 mg/kg, average 64.6 mg/kg), was found to be the main ellagic acid conjugate in *Fragaria vesca* samples.

Concentration of Ellagitannins and Ellagic Acid Glycosides in Different Cultivars and at Different Stages. It is well-known from the literature that different strawberry cultivars differ in terms of their phenolic composition and concentration, but few papers have described the ellagitannin profile and content in detail.[6,14,20,50,51] Moreover the influence of fruit maturity on the content of ellagitannins in their native form in soft fruits is not particularly well documented. An interesting paper published by Aaby et al.[14] studied the influence of three stages of maturity within the phase considered suitable for consumption on the concentration of different polyphenol classes, including ellagitannins, in three *Fragaria* × *ananassa* cvs. The changes in ellagitannins observed in this study were ambiguous and marginal as compared to important changes in anthocyanins and hydroxycinnamates.

Our results for quantitative HPLC-DAD analysis of ellagitannins are reported in Table 2, which gives the means, with standard deviations, for each of the 26 ellagitannins in the 3 biological replicates. The variability observed is relatively reasonable, considering that these were biological (not technical) replicates, which were treated as separate individual samples from plant growth up to harvest, sample extraction, and analysis.

Changes in the Amount and Pattern of Ellagitannins During Fruit Ripening. The combined effects of the two genotype and ripening stage factors were analyzed using multivariate ANOVA analysis for repeated measures on 27 variables. These included the individual concentration of 26 different ellagitannins and ellagic acid conjugates in the 3 batches of fruit from the 6 *Fragaria* × *ananassa* cultivars and two *Fragaria vesca* analyzed at four ripening stages, for a total of 96 observations.

Both the genotype and stage factors and the interaction factor (genotype × stage) were found to be highly significant, as shown in Table S7 in the Supporting Information. It is important to note that the genotype had a bigger influence than the ripening stage.

Figure 4 highlights the general trend, namely, a huge drop in the mean total concentration of ellagitannins during the early

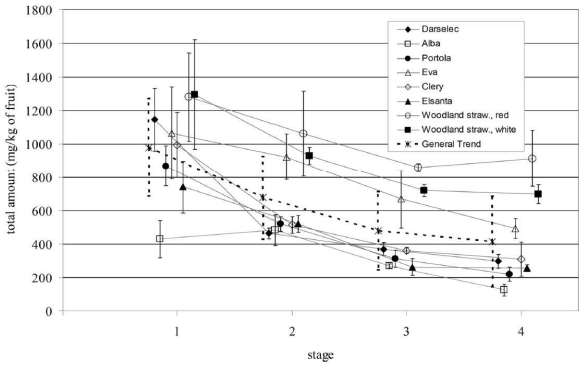

Figure 4. Behavior of total ellagitannins and ellagic acid conjugates during the ripening stages for strawberry cultivars and woodland strawberry types and general trend as an average of all samples, per fresh weight. Error bars refer to the standard deviation of the 3 biological replicates. The symbols are slightly split to avoid any overlapping of error bars. Stages are numbered accordingly to 1, green; 2, veraison; 3, ripe; 4, overripe.

phase of berry ripening (stages 1, 2, 3), common to all genotypes, with more limited changes during the two final stages (3, 4). The average total concentration of ellagitannins remaining in the berry at maturity (stage 3) was half that measured in green fruit (stage 1). The huge drop in ellagitannins is comparable with results reported in some other papers in which a strong decrease was evaluated using colorimetric assay[52] or ellagitannin hydrolysis.[53] However, the use of these two strategies meant that any molecular background or individual variability was lost. Only a few ellagitannins were reported and described individually during fruit development by Fait et al.,[24] but not quantified and expressed only in terms of relative peak response area.

Indeed, the overall effect was consequently a major drop in the concentration of ellagitannins. This was caused by a significant drop in most of the ellagitannins, including the major peaks in Figure 2 (casuarictin **9**, agrimoniin **21**, and the isomer of lambertianin C, **24**) as well as other ellagitannins with intermediate concentrations (peaks 1, 4, 11, 12, 14, 15, 17, 18, 19), which all showed similar behavior. However, this behavior was not general. Observing the univariate results (data not reported), some of the ellagitannins found only in *Fragaria vesca* (**2**, **7**, **8**, **10**), as well as ellagic acid **16** and methyl ellagic acid rhamnoside **25**, did not show any statistically significant changes in their concentration with the ripening stages.

Ellagitannins are generally synthesized early during fruit development and tend to decline in terms of fresh weight during the ripening stages, as described for the first time in the strawberry by Cheng and Breen,[54] considering the mechanism for regulating anthocyanin and phenolic production. Subsequently, the biosynthetic processes in strawberry development were also described by comparing the levels of gene transcript and enzyme activity, while a biosynthetic decline in terms of metabolite levels as the one observed in our study was reported so far only for ellagic acid.[51]

We observed a significant effect of the genotype × stage interaction factor, which was shown to be variable in terms of the drop in ellagitannins during fruit ripening. Figure 4 also shows the drop in total ellagitannin content for the different genotypes. Another paper observed a strong genotype variability in terms of bioactive compounds in strawberry but

comparing the environmental effect and not the fruit development.[55] The two *Fragaria vesca* genotypes showed a less pronounced drop, which was partly explained by a relatively large amount of ellagic acid and methyl ellagic acid rhamnoside. Of the *Fragaria* × *ananassa* cultivars, Eva retained a higher concentration of ellagitannins at ripening, being similar to Clery and Darselect at stage 1, but retaining a higher amount of ellagitannins at stages 3 and 4.

To conclude, we observed major qualitative and quantitative differences in the amount and profile of ellagitannins and ellagic acid conjugates in *Fragaria* × *ananassa* and *Fragaria vesca* species, as well as several qualitative differences in some minor ellagitannins in the *Fragaria* × *ananassa* cultivars. This information suggests that the ellagitannin profile could also be interesting for characterizing cultivars.

The working hypothesis derived from this study is therefore that genotype is a major factor in defining ellagitannin concentration and pattern in strawberries, and that in the context of a major drop in ellagitannins during ripening, variable behavior of the genotypes still exists, which could also be considered in order to retain the optimal concentration of ellagitannins in fruit at the ripening stages most suitable for consumption.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1: ^{1}H NMR spectrum in CD3OD of casuarictin. Figure S2: Cluster analysis based on the concentration of 26 different ellagitannins and ellagic acid conjugates plus their total content in strawberry cultivars and woodland strawberry types, respectively at stages 2 (veraison) and 4 (overripe). Table S1: Analytical parameters used for quantitative analysis. Table S2: Summary of variety kinship, provenance and agronomical parameters. Table S3: Average weight of Elsanta cultivar strawberries at different stages. Table S4: Summary of agronomical analysis. Table S5: Ellagitannin and ellagic acid conjugates profiling of different strawberry cultivars and woodland strawberry types using UPLC-Q-TOF. Table S6: Molar extinction factors reported in the literature for some ellagitannins, in relation to their molecular weight and characteristic number of chromophore residues. Table S7: Multivariate tests of significance for the multivariate repeated measures ANOVA aimed to evaluate the influence of the two genotype and ripening stage factors on the concentration of ellagitannins and ellagic acid conjugates in strawberries (sigma-restricted parametrization; effective hypothesis decomposition). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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Food chemistry

Chapter 1.3

Polyphenols in a bowl of strawberries: Comprehensive profiling for better correlation with nutritionally significant properties

This chapter will shortly be submitted for publication as a manuscript:

Overall dietary polyphenol intake in a bowl of strawberries: the influence of Fragaria spp. in nutritional studies **Mattia Gasperotti**, Domenico Masuero, Fulvio Mattivi, Urska Vrhovsek

In Europe, average strawberry consumption is 2.16 kg/year per person, including raw and processed fruit (2[‡]). On this basis, strawberries would clearly appear to be one of the most widely consumed berries. Furthermore strawberries are one of the most important sources of polyphenols in the human diet, being a rich source of these healthy phytochemical compounds (3,4).

The regular consumption of strawberries in the diet contributes significantly to the intake of different phenolic compounds. Several classes of polyphenols are present in a bowl of strawberries (5,6). Studies of different cultivars including profiling of polyphenols have already been performed in some cases, but without quantitative determination or detailed characterisation (7-11). The most abundant classes of polyphenols in the strawberry are proanthocyanidins and anthocyanins, followed by ellagitannins and flavonol glycosides (3,4,14).

Comprehensive quantification of polyphenols in food matrices is an essential step in assessing their biological mechanisms, nutritional properties and healthy effects. However due to the huge variety of polyphenol compounds, their precise characterisation in fruit or vegetables, even the most widely consumed, is still unclear. To study the nutritional quality of any fruit it is essential to start from phytochemical profiling of the native compounds in fruits and then move on to more complex bioavailability studies with *in vivo* trials.

Thus the aim of this study was to present a comprehensive picture of strawberry polyphenols, reflecting their intake in the human diet. To date a detailed overview has been lacking in the literature or the databases available.

A detailed introduction, description of the methods and discussion of the results have been included in the manuscript ready for the submission.

[‡] References are reported in the same order and listed in the attached manuscript

A total of 56 individual compounds were accurately identified and quantified with a triple-quadrupole mass spectrometer, their concentration ranging from 1 ug/100 g for low abundant polyphenols to 40 mg/100 g of fresh fruit. Furthermore, a spectrophotometric assay was carried out for precise estimation of high-molecular mass proanthocyanidins (HMWP).

Quantitative data at molecular level are provided for all 56 compounds and indirect quantification was applied to the complex family of HMWPs. Several compounds were identified and quantified for the first time. In *Fragaria Vesca,* taxifolin, which is one of the most concentrated polyphenols after anthocyanins and ellagitannins, was quantified in the strawberry for the the first time. This study represents the most comprehensive targeted profiling of strawberry polyphenols, in terms of the number of individual compounds identified and quantified.

My personal involvement in this manuscript regarded evaluation of the gap in knowledge relating to the precise composition of strawberry polyphenols in the literature. I performed quantitative analysis by applying the targeted method developed in Chapter 1.1 and I developed a new rapid UPLC-MS/MS method for anthocyanins and ellagitannins (see *Table 1*, *Figure 1*), with the isolated ellagitannins presented in Chapter 1.2. I produced a list of compounds with their precise quantifications in different *Fragaria* spp. (*Table 2* and *Figure 2*). Moreover, I performed the spectrophotometric assay and explorative analysis for the analysis of proanthocyanidins, using high definition MS (*Figure 3*). Furthermore, as the first author I was responsible for writing the manuscript and managing the comments and improvements to the text by other co-authors.

Overall dietary polyphenol intake in a bowl of strawberries: the influence of *Fragaria* spp. in nutritional studies

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Abstract

Strawberry (*Fragaria x Ananassa* Duch) represents one of the most important crops worldwide. Moreover strawberries are one of the most important sources of polyphenols in the human diet, being a rich source of these healthy phytochemical compounds.

Strawberries are now considered to be a functional food, as demonstrated by evidence regarding their antioxidant, anti-inflammatory, antihypertensive and antiproliferative properties. However which kinds of compounds are involved is not fully understood at the moment.

The aim of this study was to present a comprehensive picture of the amount of polyphenols present in the human diet. Attention was focused on the consumption of a standard bowl of different *Fragaria* spp. An already published method for the quantification of multiple classes of phenols and the development of a method for the simultaneous quantification of anthocyanins and ellagitannins was used for this purpose. Furthermore, spectrophotometric assay for precise estimation of high-molecular mass proanthocyanidins was applied.

A total of 56 individual compounds were accurately identified and quantified with a triple-quadrupole mass spectrometer their concentration was ranging from 1 ug/100 g, for low abundant polyphenols, to 40 mg/100 g of fresh fruit. Several compounds were identified or quantified for the first time in strawberry fruits.

Keywords strawberry, polyphenols, ellagitannins, anthocyanins, flavonoids

Introduction

According to the statistical database of the Food and Agriculture Organization of the United Nations (FAO) (1), the strawberry (*Fragaria x Ananassa* Duch) represents one of the most important crops worldwide, with worldwide production and overall global trading of 4.1 million tons. In terms of production, there was a global increase of 20% from 2005 to 2011, with an increase of 50% in Europe and the United States alone.

In the European market, average strawberry consumption is 2.16 kg/year per person, including raw fruit and processed food (2). On the basis of this figure, strawberries would clearly appear to be one of the most widely consumed berries. Furthermore strawberries are one of the most important sources of polyphenols in the human diet, being a rich source of these healthy phytochemical compounds (3, 4).

Regular consumption of strawberries in the diet contributes significantly to the intake of different phenolic compounds. Several classes of polyphenols are present in a bowl of strawberries (5, 6). Studies of different cultivars including profiling of polyphenols have already been performed in some cases, but without quantitative determination or without detailed characterisation (7–11). Furthermore the chemical composition varies with the genotype, as already reported, but also depending on agricultural practices, the degree of maturity and environmental factors (12,13). The most abundant polyphenols are proanthocyanidins and anthocyanins, followed by ellagitannins and flavonol glycosides (3,4, 14). Analysis of proanthocyanidins and ellagitannins, polymeric and oligomeric compounds, has so far been little studied, due to analytical problems and the complexity of their chemical characterisation. Only recently has been published a complete profile for ellagitannins, with identification and precise quantification of several molecules (15), while similarly detailed information is still not present in the literature in relation to

proanthocyanidins, although this class of compounds represents the main group of polyphenols in the strawberry (16).

Strawberries are now considered to be a functional food, with multiple health benefits over and beyond nutritional needs, as demonstrated by extensive evidence regarding their antioxidant, anti-inflammatory, antihypertensive and antiproliferative properties (17). Epidemiological studies have been shown an inverse correlation between the consumption of fruits rich in polyphenols and degenerative or proliferative disease, cardiovascular disease and chronic diseases (3, 17–19). The potential effects of the consumption of strawberries on health have been well described in the literature for decades, but the precise mechanism of polyphenols' healthy effect on the mammalian system are still generally unclear. Even which kinds of compounds are involved is not fully understood at the moment.

Comprehensive quantification of polyphenols in food matrices is an essential step in assessing their biological mechanism, nutritional properties and healthy effects. However due to the huge variety of polyphenol compounds, their precise characterisation in fruit and vegetables, even the most widely consumed, is still unclear. To study the nutritional quality of any fruit it is essential to start with phytochemical profiling of the native compounds and then move on to more sophisticated bioavailability studies. Moreover, in bioavailability studies today the role of gut microflora also has to be taken into account and can improve the variability of the phytochemical compounds present in the consumed fruits (20, 21).

The aim of this study was to present a comprehensive picture of the quantity of polyphenols present in the human diet. Attention was focused on the consumption of a standard bowl of different *Fragaria* spp. (100 g). Indeed, to assist long and expensive future nutritional studies on the effective healthy effects of the strawberry, it is necessary to carry out detailed profiling of its polyphenolic composition. Thus an already published method for the quantification of multiple classes of phenols and the development of a method for the simultaneous quantification of anthocyanins and ellagitannins was used for this purpose. Furthermore, spectrophotometric assay for precise estimation of high-molecular mass proanthocyanidins (HMWP) was used.

A total of 56 individual compounds were accurately identified and quantified with a triple-quadrupole mass spectrometer, ranging from 1 ug/100 g, for low abundant polyphenols, to 40 mg/100 g of fresh fruit.

To our knowledge this is the first time that such a comprehensive targeted profile has been performed on strawberry polyphenols, in terms of the number of individual compounds identified and quantified at the same time with proper standard references.

Materials and Methods

Chemicals and Reagents. All the chromatographic solvents were HPLC grade or LC-MS grade for the MS experiments. Acetonitrile, acetone, methanol and formic acid were purchased from Sigma Aldrich (Milan, Italy). Detailed information regarding the standard references used and vendors are reported in Vrhovsek et al. (22) Sanguiin H6 was isolated as described in Gasperotti et al. (23), agrimoniin as described in Vrhovsek et al. (24), casuarictin and methyl ellagic acid rhamnoside as described in Gasperotti et al. (15).

Plant Material. Different *Fragaria* spp. 6 *Fragaria* x *ananassa* cultivars (Alba, Clery, Eva, Elsanta, Darselect, and Portola) and 2 accessions (one red and one white) of *Fragaria vesca* were used. The plants were grown in the same experimental field in Vigalzano (Trentino, Italy; 520 AMSL) during the 2011 season. All the cultivars were grown in standardised conditions in order to minimise the effect of environmental and agronomic factors. For each *Fragaria* spp. analysed, 3 repeats of 6 plants (18 plants in total) were considered, in order to obtain 3 biological replicates. The plants were cultivated with the soilless technique. Plants of the same cultivar were obtained vegetatively. For each cultivar, 250 g of strawberries were harvested at commercial ripeness. Detailed information regarding the agronomical conditions is reported in

Gasperotti et al. (15). The strawberries were harvested at commercial ripeness. Once collected the samples were stored in a freezer at -20 ° C until extraction.

Extraction of Phenolic Compounds. Extraction was performed as reported in Mattivi et al. (25) with an acetone/water mixture (70/30 v/v), avoiding the addition of acids in order to prevent any possible chemical hydrolysis. Briefly, 60 g of fresh fruit was homogenised in a 847-86 model Osterizer blender at speed one, in 2 x 100 mL of mixture acetone/water (70/30 v/v) for 1 min and made up to 250 ml with the same solvent. The centrifuged extracts were stored at –20 °C until analysis.

Sample Preparation for UPLC-MS/MS Analysis. An aliquot of the extract, 1.5 ml, was completely dried under a stream of nitrogen. Then, 150 µL of methanol/water (50:50), with 1mg/L of rosmarinic acid as internal standard, was added to the dry sample and filtered (0.45 µm) into an HPLC vial. The samples, 10 times concentrated, were now ready for further UPLC-MS/MS analysis. The internal standard was added to monitor the analytical performance of the instrument.

UPLC-MS/MS Method for Anthocyanins and the Main Ellagitannins. An UPLC-MS/MS method was developed by combining anthocyanins, reported in the literature (26), and the main ellagitannins detected (15) in the strawberry. The analysis was performed with an ultra performance LC (UPLC) system coupled with a triple quadrupole (TQ) mass spectrometer. The UPLC system used was a Waters Acquity UPLC system (Milford, Massachusetts, USA). Separation was performed with a Waters Acquity UPLC column (Milford, Massachusetts, USA), BEH C18 (150 mm x 2.1 mm, 1.7 µm) equipped with the proper guard column, maintained at 60°C. The injection volume was 2 µL and solvent flow was 0.45 mL/min. Mobile phases of 2.5% formic acid in Milli-Q water (A) and acid in acetonitrile (B) were used. Chromatographic separation was performed using a gradient as follows: 0 min, 5% B; 0-10 min, 5%-25% B; 10-10.5 min; 25-90% B; 10.5-12.50 min, 90% B, 12.60-16.60 min, 5% as equilibration time. The TQ mass spectrometer used was a Waters Xevo TQ

(Milford, Massachusetts, USA) coupled with an electrospray interface. Direct injections of each individual compound were used to optimise the MRM conditions. This was done automatically by the Waters IntelliStart software, and then manually checked for selection of the quantifier and qualifier ions. The MRMs for each compound are listed in Table 1 with cone voltages and collision energies. Calibration curves were performed for each individual compound for precise quantification, using commercially available anthocyanins and the previously isolated ellagitannins.(15, 23, 24) Calibration curves were prepared ranging from 0.0005 mg/L to 20 mg/L.

UPLC-MS/MS Method for Multiple Classes of Phenolics. Quantitative analysis for the rest of the polyphenols, excluding anthocyanins and ellagitannins, was carried out with a previously validated method for the rapid quantification of multiple classes of phenolics (22). Briefly, the analysis was performed with the same ultra performance LC (UPLC) system, coupled with the triple quadrupole (TQ) mass spectrometer used in the previous method. Separation was performed with a Waters Acquity UPLC column (Milford, Massachusetts, USA), HSS T3 (100 mm x 2.1 mm, 1.8 µm) equipped with the proper guard column, maintained at 40°C. The injection volume was 2 µL, with a flow of 0.4 mL/min. Mobile phases of 0.1% formic acid in Milli-Q water (A) and 0.1% formic acid in acetonitrile (B) were used. Chromatographic separation was performed using a gradient as follows: 0 min, 5% B; 0-3 min, 5%-20% B; 3-4.30 min; 20% B; 4.30-9 min, 20%-45% B, 9-11 min, 45%-100% B, 11-14 min, 100%; and 14.01-17 min, 5% as equilibration time (22). Further information regarding the UPLC-MS/MS method and MRM quantification are described in Vrhovsek et al. (22). A total of 44 polyphenols were identified among the samples and proper calibration curves were obtained for each individual compound for precise quantification. Calibration curves were prepared, with appropriate standard references, ranging from 0.0005 mg/L to 20 mg/L.

Analysis of Proanthocyanidins. The assay to determine the quantity of proanthocyanidins was based on their transformation into anthocyanidins, in a warm, acid environment (27). An aliquot of the initial extract, 5 mL, was evaporated using a rotavapor in order to eliminate the acetone fraction and dissolved in a 5 mL flask with 0.05 M H2SO4. One mL of this solution was loaded onto a conditioned Sep-Pak C18 (Waters, Milford, MA, USA), which was previously washed with 2 mL of 5 mM H2SO4. Proanthocyanidins were eluted with 3 mL of MeOH and collected in a 50 mL flask, shielded from light with aluminium foil, containing 9.5 mL absolute EtOH. 12.5 mL of FeSO4 was added to the mixture in concentrated HCl (300 mg/L) and then immediately placed in a boiling water bath and refluxed for 50 min. After 10 min, the spectrum from 380 to 700 nm was recorded in a 10 mm cell, against a blank (water). The tangent from the minimum (450 nm) was drawn, and the absorbance between the maximum (550 nm) and the tangent was measured. To subtract natural anthocyanins present in the sample, which can interfere with the assay, one mL of the latter solution dissolved in 0.05 M H2SO4 was prepared in the same conditions. However, in this case the reaction was carried out in an ice bath and the absorbance obtained was then subtracted to obtain the net absorbance value. The proanthocyanidins concentration (mg/L) can be conventionally expressed as the cyanidin formed. Further information is present in the literature (27–29).

Explorative Profiling of Proanthocyanidins. An aliquot of the acetone extract was concentrated 4 times and dissolved in methanol/water (50:50) for explorative analysis using high definition mass spectrometry (HDMS), a Synapt Waters, coupled with UPLC and injected with a previously 60 min. chromatographic method(23). The molecular ions of the main oligomeric form were selected and extracted from the HDMS spectrum.

Data Analysis and Statistical Analysis. Data processing was carried out using Waters MassLynx 4.1 and TargetLynx software. All samples were in biological

triplicates. The data collected were presented as average values and standard deviations.

Results and Discussion

Variety. The varieties chosen for the dietary intake of polyphenols included 2 wild accessions of *Fragaria Vesca*, the red and white types, whereas the 6 main representative cultivars of *Fragaria x Ananassa* were chosen: Portola, Eva, Elsanta, Clery, Darselect and Alba. The chosen cultivars were selected from those most widely consumed in both Italy and Europe. All the samples were grown and picked in Trentino, Italy in the same agronomical conditions and harvested at commercial maturity to avoid any environmental and agronomical effects on the polyphenolic composition (30). Due to the difference in the varieties, cultivars and accessions investigated, it was expected that the amount of polyphenols would make it possible to obtain a clear estimate of the intake of strawberry polyphenols in both Italy and Europe associated with the consumption of a bowl of strawberries, 100 g, of the most widely used cultivars or wild species.

Targeted Profiling of Strawberry Polyphenols. The polyphenols in the fruit of the different *Fragaria* spp. considered were identified and quantified precisely with the use of a TQ mass spectrometer. With the use of the triple quadrupole mass spectrometer several compounds were found in traces, leading to quantification of some polyphenols never previously reported in the strawberry. Each compound was identified and confirmed in the samples by checking whether both transitions (MRMs), qualifier and quantifier ions prepared for the method of quantification with the appropriate standard references, were present in the chromatograms. With the use of the two complementary UPLC-MS/MS methods (Figure 1), a total of 56 compounds were separated, identified and quantified with standard references in the

samples. The list of all the compounds identified is presented in Table 1, together with the amount recovered for each sample. The values are reported in ug/100 g, as the average value of the 3 replicates (and their standard deviation). The amounts found ranged from a few ug/100 g to many mg/100 g of fresh fruit.

As the main outcome, a list of polyphenols was produced for the creation of databases reporting on polyphenol composition in fruit and vegetables (5, 6) providing more information also as compared to similar studies involving targeted profiling of strawberry polyphenols (7, 8, 10, 14, 31, 32). Moreover, in comparison to untargeted profiling studies on the strawberry (8, 11, 33) a similar number of polyphenolic compounds was reported but with a major improvement in identification/quantification and not only with tentative identification on the basis of fragmentation patterns. The total amounts and the relative percentages of the main classes or groups of classes are shown graphically in Figure 2. In order to simplify the pie charts, 5 groups were highlighted: proanthocyanidins (HMWP), ellagitannins, anthocyanins, flavonoids (adding together flavones, flavanones, flavan-3-ols and flavonols) and other polyphenols (adding together benzoic acid derivatives, phenylpropanoids, stilbenes, phenol glycosides and dihydrochalcones). From the pie charts it is clear that proanthocyanidins are the most abundant polyphenols in the strawberry (16). The other two main groups are anthocyanins and ellagitannins, followed by flavonoids and traces of other polyphenols. Considering the total amounts in the strawberry, the highest values were found for the two accessions of *Fragaria Vesca*, 324 and 448 mg/100 g respectively for the red and white types. The amounts in the cultivars were lower, falling between 204 and 288 mg/100 g respectively for the Clery cv and the Darselect cv. The data on total polyphenol content are in accordance with the average values calculated in a different manner, i.e. Follin assay for total polyphenol content, and present in databases, but of course with more detailed characterisation of the individual amounts (5, 6).

Classes of Polyphenols. Using 3 rapid methods, 2 instrumental and one spectrophotometric assay, it was possible to identify the main classes of polyphenols

present in the strawberry, but it was also possible to detect many minor polyphenols present in traces. A total of 56 polyphenols are listed in Table 1, all identified and quantified with the proper standard references, with the exclusion of proanthocyanidins. In this way it was possible to build up a comprehensive picture of the presence and type of different classes of polyphenols in a bowl of strawberries, along with the quantity of proanthocyanidins, not described in detail at molecular level, that may be consumed in the human diet.

Proanthocyanidins. Precise quantification and characterisation of these is still an open problem in terms of polyphenol analysis, due to their complexity, given that they are compounds with a high molecular weight. A number of strategies have been reported for the study of high molecular weight proanthocyanidins (HMWPs), including the use spectrophotometric assay (28), normal phase separation coupled with fluorescent detector (34), phloroglucinol adducts or the thiolysis method (35). Interestingly, in terms of estimation, good correlation with the accepted Bate-Smith assay was shown as compared to more laborious instrumental methods (i.e phloroglucinol adducts or thiolysis) (29). For this reason Bate-Smith assay was used for the analysis of HMWPs, given that other more precise methods are not reliable for their characterisation and quantification. However, as an exploratory example, the HDMS spectrum of a sample of the red type *Fragaria Vesca* showed extracted ions of the main tentative proanthocyanidins reported in the strawberry (16) (Figure 3). HDMS analysis confirmed the presence of some peaks at m/z 577 (dimers), 865 (trimers), 1153 (tetramers) and 720 (bicharged pentamers) corresponding to (epi)catechin-based proanthocyanidins.

Representing the most abundant polyphenols in the strawberry (Figure 2), the amount consumed in a bowl of strawberries is between 135 mg/100 g and 205 mg/100 g respectively for the Clery cv and the Darselect cv, while the amount is higher in the red type of wild strawberry, namely 270 mg/100 g. As a major class of strawberry polyphenols, they represent between 54.8% and the 77.4% of polyphenolic compounds. The cultivar with the highest relative value is the Portola cv.

Anthocyanins. The red colour of strawberries comes from the presence of anthocyanins. In strawberries, anthocyanins are made up of a mixture of cyanidin, pelargonidin and peonidin glycisides, mainly glucosides, galactosides and rutinosides (26). On the contrary, anthocyanins are not present in the white type of *Fragaria Vesca*, with the exception of some traces of cyanidin glucoside and galactoside, and as a consequence the fruit is white/green. The main anthocyanin in the red strawberry is pelargonidin-3-glucoside (26, 36) (up to 43 mg/100 g in the Darselect cv), followed by cyanidin-3-glucoside (up to 12 mg/100 g in the red type of *Fragaria Vesca* and up to 0.4 mg/100 g in the cultivars) and cyanidin-3-galctoside. Cyanidin-3-rutinoside, pelargonidin-3-rutinoside and peonidin-3-galactoside were also found in the cultivars, while they were not present at all in *Fragaria Vesca*.

A certain variability in terms of the compounds identified and the amounts can be observed in the cultivars. The Elsanta cv had the lowest number of different anthocyanins, although the total amount recovered was nearly 35 mg/100 g, while the Portola cv, with the largest number of different anthocyanins, was the cultivar with the lowest total amount, 24 mg/100 g.

Ellagitannins. In the strawberry ellagitannins are the third most concentrated class of polyphenols, the main ellagitannins having recently been isolated for their clear identification and precise quantification (15, 24). From previous publications, only the 3 main ellagitannins – agrimoniin, casuarictin and sanguiin H-6 – and ellagic acid with the methyl ellagic acid rhamnoside, isolated and characterised using NMR (15, 23, 24), were considered for the purpose of having a picture of the overall intake of polyphenols present in a bowl of strawberries. More in-depth profiling with many other ellagitannins has already been proposed for the same cultivars (15).

As already shown, agrimoniin was the main ellagitannin in the strawberry (from 84 to 9 mg/100 g), followed by casuarictin (from 19 to 5 mg/100 g) and then sanguiin H-6 (from 2.2 to 0.2 mg/100 g. The concentration of ellagic acid was from 7 to 0.3 mg/100 g.

Methyl ellagic acid rhamnoside was present in large amounts in *Fragaria Vesca*, with a concentration of 28 and 35 mg/100 g respectively for the white and red types.

Interestingly, traces of these ellagic conjugates were also found in the cultivars (11-2.8 ug/100 g) while in previous publications they were not reported (15).

Flavonoids. The fourth most characteristic group of polyphenols (see Figure 2) in the strawberry is the flavonoid family (without considering the anthocyanins described above as an independent class of polyphenols). The flavonoids found in strawberry were flavones, flavanones (naringenin alone), flavan-3-ols and flavonols and dihydroflavonols.

In the literature considered for this publication, no information regarding the survey of flavanones, naringenin or flavones, namely luteolin or hesperidin, was found. However, some traces of luteolin, luteolin-7-glucoside, hesperidin and naringenin were detected in the samples chosen.

The main flavan-3-ol detected in the strawberry was catechin (5.9-1.8 mg/100 g), while epicatechin was about 10 times lower in all the *Fragaria spp*. The dimers of the flavan-3-ols identified were instead procyanidin B1 and B3, in accordance with the literature.(8) In this case procyanidin B3 was about one grade of magnitude higher then procyanidin B1 (2.8-1 as compared to 0.58-0.17 mg/100 g). These flavan-3-ols are not listed in the HMWP group, since they do not respond so well to the Bate-Smith assay, being more selective for high molecules (29) and due to the fact that there are standard references available for quantification of these molecules.

The content of overall flavonols, i.e. quercetin, unbound kaempferol and glycosides and isorhamnetin glycosides, varied in the samples from 0.9 mg/100 g (Portola) to 11 mg/100 g (*F. Vesca*, red type). Of the glycosides, quercetin-3-glucuronide was the most abundant in all the cultivars and wild types, in accordance with the literature for the cultivars (10, 16) but not reported in relation to the wild types (37). Quercetin-3-glucuronide was followed by kaempferol-3-glucuronide, which was the second most abundant (from 0.28 to 0.66 mg/100 g) and kaempferol-3-glucoside (from 0.09 to 0.26 mg/100 g) in the cultivars selected. In the wild type quercetin-3-glucuronide was followed by quercetin-3-glucoside and kaempferol-3-glucoside, in accordance with the literature (37).

Interestingly, five different adducts made with rutinose are present in almost all the cultivars considered, but have never been reported in the literature (10, 16, 31, 37, 38), with the exception of isorhamnetin rutinoside (11) and quercetin rutinoside (rutin) (7, 33), but with no information regarding their concentration. Another rutinoside adduct clearly found for the first time is kaempferol-3-rutinoside, not present in any of the articles considered.

Likewise, the free forms of flavonols, i.e. quercetin and kaempferol, have never been reported in the literature, or in untargeted profiling studies of the strawberry (33), and some traces in the samples were detected.

The dihydroflavonol family (added to flavonols in Table 2), i.e dihydroquercetin (taxifolin) and dihydrokaempferol in the *Fragaria spp*. considered, was present. Quantitative amounts are proposed for the first time in this publication, since their presence was only tentatively reported in an untargeted study (39). Remarkably, taxifolin appears to be the most abundant flavonoid, after anthocyanins, with a concentration of 9.5 and 8.5 mg/100 g respectively in the red and white types of wild strawberry.

Other Polyphenols. In this work it was possible to significantly increase the number of compounds detected with the use of a TQ mass spectrometer and also to characterise in detail many other polyphenols which are not as abundant as the previous polyphenols discussed above. These minor polyphenols were included in the category of other polyphenols (incorporating benzoic acid derivatives, phenylpropanoids, stilbenes, phenol glycosides and dihydrochalcones) in Figure 2 and they represent less than 0.1 % of the total amount of polyphenols that may be consumed in a bowl of strawberries. The individual concentration was no more than 100 ug/100 g in any of the samples.

Compounds deriving from the benzoic acid, i.e. *p*-hydroxybenzoic acid, cinnamic acid, vanillin, vanillic acid, 2-6-dihydroxybenzoic acid methyl gallate or cathecol, were present together, with a concentration spanning from 30 ug/100 g to 112 ug/100 g. They have already been identified as putative metabolites but with no information about their concentration (39).

Caffeic acid, ferulic acid, *p*-coumaric acid and chlorogenic acid – classified as phenylpropanoids – have already been reported in the literature but only confirmed with the photo diode array system (38), while chlorogenic acid was detected in untargeted data (11) as a putative metabolite with no information about concentration, although chlorogenic acid was the most abundant among the latter compounds (75 ug/100 g in cv. Elsanta). Traces of *trans*-coutaric acid were also found and have never been reported in previous studies.

Some stilbenes were also detected, in particular *trans*-piceid and *cis*-piceid, which have never been detected in the strawberry, due to their extremely low concentrations. The total concentration of stilbenes was from 3.4 to 7 ug/100 g in the Portola cv and white type of *F. Vesca* respectively.

Phloretin and phlorizin, belonging to the dihydrochalcones family, were reported for the first time by Hilt et al. (40), but no evidence was found in other publications. The presence of another dihydrochalcone, namely trilobatin, detected in traces in the samples considered, has also never been reported in the literature.

Conclusion

This work offers some important new aspects and improvements in the study of strawberry polyphenols, which may also apply to the study of other polyphenols-based food. The 56 polyphenols listed in this publication certainly do not represent a complete list of polyphenols present in the strawberry, but to our knowledge, at the moment they offer the most up-to-date list of clearly identified compounds, without any tentative identification, since they were compared with their reference compounds. Furthermore, quantitative data are provided for all the compounds and estimation only for the complex HMWP family. Several compounds were identified or quantified for the first time, not only because present in traces. Indeed, taxifolin

was quantified for the first time in *Fragaria Vesca,* representing one of the most concentrated polyphenols after anthocyanins and ellagitannins.

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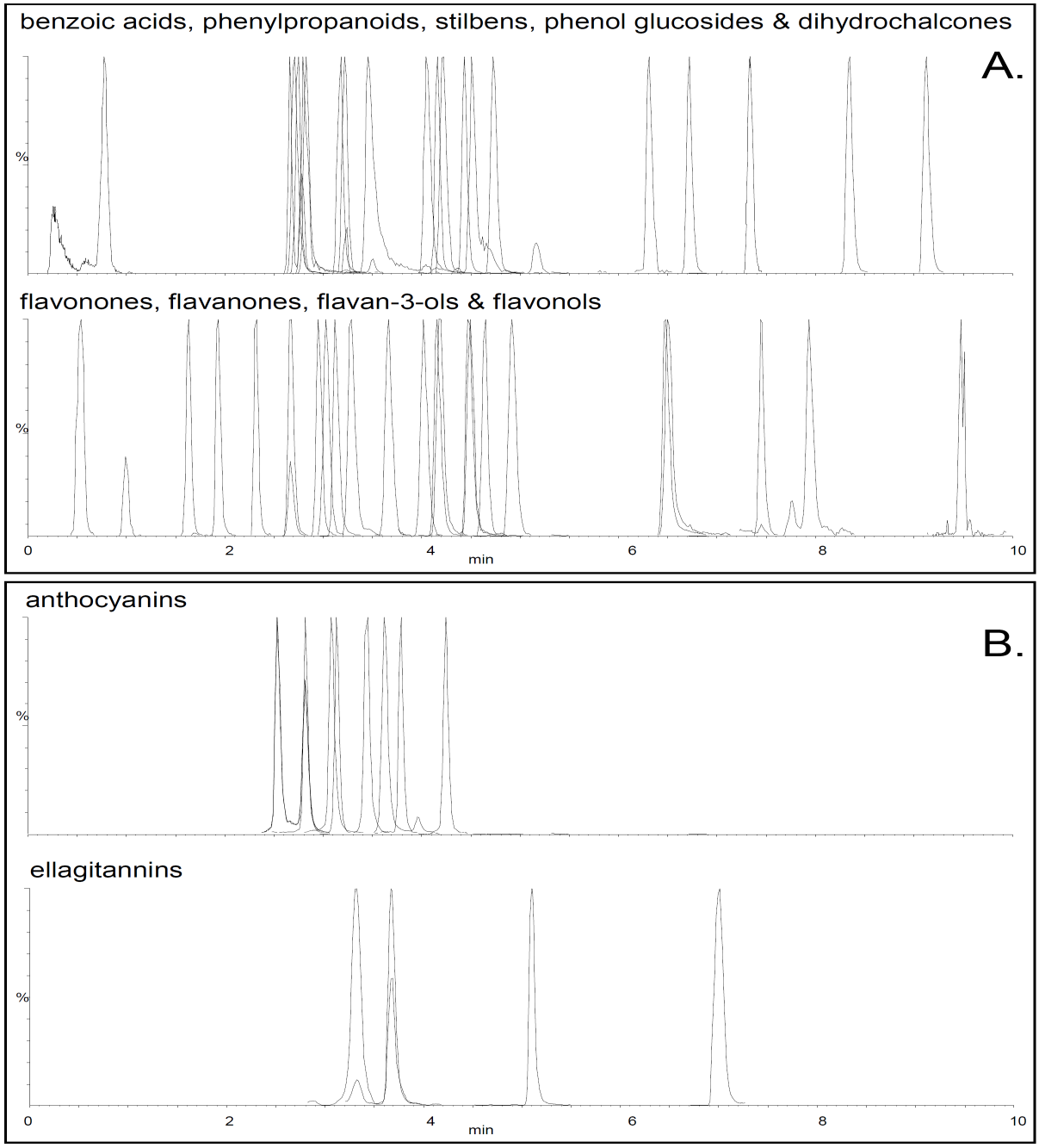

Figure 1 UPLC profile of the polyphenols detected in strawberry

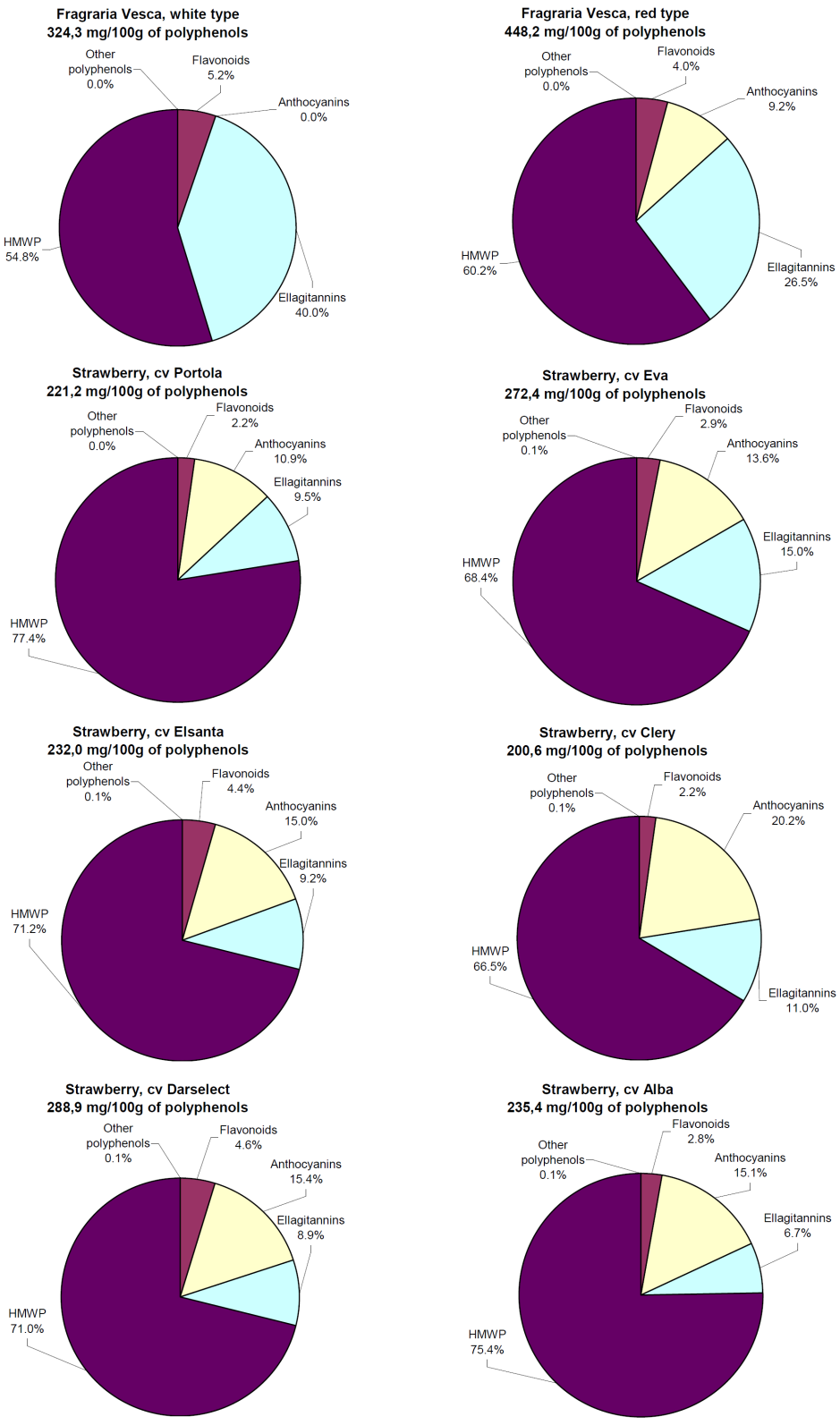

Figure 2 Total amount of polyphenols and their percentage in *Fragaria* spp., grouped accordingly to Figure 1, with the addition of proanthocyanidins.

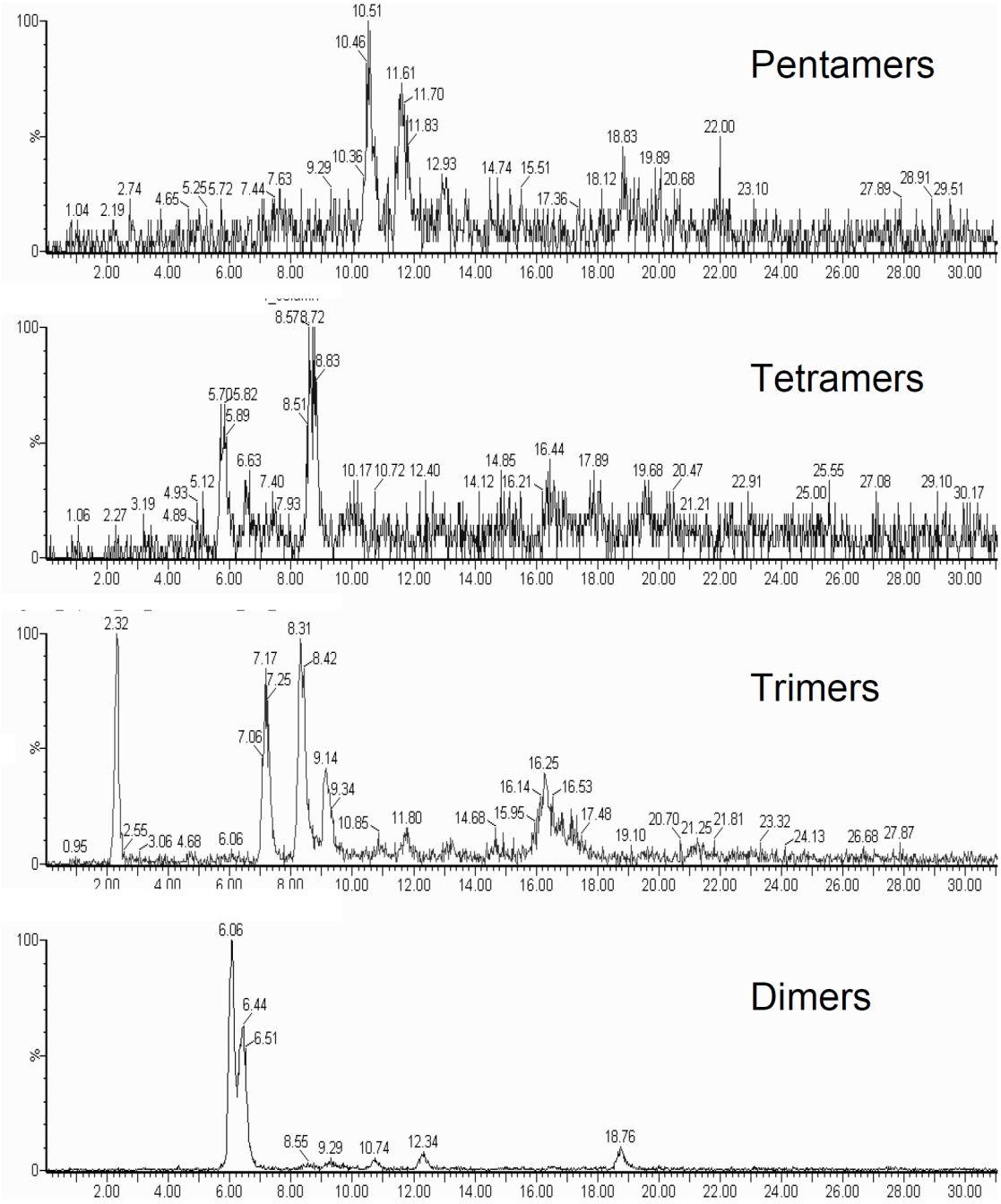

Figure 3 Preliminary High Definition MS analysis of proanthocyanidins.

Table 1 MRM Parameters of anthocyanins and ellagitannins

					quantifier ion		qualifier ion	
compound	**Rt min.**	**ES**	**Cone Voltage (V)**	**Q1** *m/z*	**Collision Energy (V)**	**Q2** *m/z*	**Collision Energy (V)**	**Q2** *m/z*
anthocyanins								
Cyanidin-3-galactoside	2.54	+	26	449.3	22	287.2	66	137.2
Cyanidin-3-sambioside	2.81	+	30	581.3	26	287.2	72	137.2
Cyanidin-3-glucoside	2.83	+	26	449.3	28	287.2	58	137.2
Cyanidin-3-arabinoside	3.09	+	24	419.3	24	287.2	56	137.2
Cyanidin-3-rutinoside	3.15	+	34	595.4	38	287.2	66	137.2
Pelargonidin-3-glucoside	3.45	+	24	433.3	24	271.2	56	121.1
Peonidin-3-galactoside	3.63	+	24	463.3	24	301.1	42	286.1
Pelargonidin-3-rutinoside	3.94	+	30	579.2	20	433.1	30	271
Peonidin-3-rutinoside	4.37	+	30	609.2	30	301.7	56	286.1
ellagitannins								
Casuarictin	3.27	-	66	934.7	26	632.7	46	300.8
Sanguiin H6	3.63	-	34	934.1	22	632.8	38	300.8
Agrimoniin	5.06	-	32	934.1	22	632.8	46	300.8
Methyl ellagic acid rhamnoside	6.97	-	30	460.8	18	314.8	20	299.8

Table 2 Quantitative results of all the polyphenols detected in *Fragaria* spp. Data are the average of the three replicates and their standard deviation in italics.

	ug/100g of fresh fruit	F. Vesca, white type		F. Vesca, red type		Strawberry, cv Portola		Strawberry, cv Eva		Strawberry, cv Elsanta		Strawberry, cv Clery		Strawberry, cv Darselect		Strawberry, cv Alba	
1	anthranilic acid	1.3	0	5.5	0	n.d.	0	0.1	0	1.7	1	0.1	0	0.1	0	n.d.	0
2	p-hydroxybenzoic acid	18.5	5	22.8	4	16.6	2	17.5	2	26.9	8	19.5	2	32.8	4	24.3	5
3	cinnamic acid	29.5	6	59.9	9	2.1	1	19.4	12	63.7	5	18.5	4	44.5	24	27.4	30
4	vanillin	7.3	1	5.6	0	1.6	0	1.3	0	1.5	0	1.1	0	2.3	1	2.0	0
5	vanillic acid	3.6	0	3.7	0	0.8	0	1.4	0	0.4	0	1.0	0	0.5	0	0.6	0
6	2,6-dihydroxybenzoic acid	1.2	1	5.0	1	3.8	1	6.9	2	9.4	5	16.3	3	7.0	1	6.7	1
7	methyl gallate	1.1	0	0.8	0	1.1	0	1.6	1	1.2	0	1.5	1	0.9	0	1.3	0
8	catechol	13.1	4	7.6	5	4.0	3	8.2	1	7.7	1	1.8	0	13.9	4	5.2	3
	total benzoic acid derivatives	**75.6**		**110.8**		**30.0**		**56.4**		**112.6**		**59.6**		**101.9**		**67.5**	
9	p-coumaric acid	2.2	0	2.0	0	3.8	1	18.9	5	11.0	1	13.3	4	9.7	2	11.9	7
10	caffeic acid	1.7	1	1.5	0	1.2	0	2.4	0	1.6	1	3.4	2	0.8	0	3.9	3
11	ferulic acid	20.1	0	7.1	1	0.9	0	0.8	0	1.0	0	0.9	0	1.3	0	1.0	0
12	chlorogenic acid	9.5	1	13.9	0	13.8	4	23.3	3	75.4	10	32.0	3	12.8	5	30.2	5
13	trans-coutaric acid	2.3	1	2.4	0	0.5	0	0.5	0	1.3	0	0.6	0	0.6	0	0.3	0
	total phenylpropanoids	**35.8**		**27.0**		**20.3**		**45.8**		**90.2**		**50.2**		**25.1**		**47.3**	
14	trans-piceide	1.6	0	1.6	0	0.5	0	2.0	0	1.4	1	1.6	0	2.2	1	1.6	0
14	cis-piceide	5.4	1	3.6	1	2.9	0	3.2	0	3.4	0	2.5	1	4.1	1	2.8	0
	total stilbens	**7.0**		**5.3**		**3.4**		**5.2**		**4.8**		**4.2**		**6.2**		**4.4**	
16	arbutin	2.2	1	1.8	0	2.6	1	3.3	1	3.6	0	1.6	1	3.5	0	1.1	0
	total phenol glycosides	**2.2**		**1.8**		**2.6**		**3.3**		**3.6**		**1.6**		**3.5**		**1.1**	
17	phloretin	0.1	0	0.1	0	0.5	0	0.2	0	1.5	1	0.4	0	1.0	0	0.3	0
18	phlorizin	2.9	1	7.1	1	46.5	5	40.7	1	80.1	7	76.1	9	98.2	21	83.0	21
19	trilobatin	0.0	0	1.9	1	5.8	1	1.9	0	14.1	3	2.7	1	12.0	2	4.5	1
	total dihydrochalcones	**3.0**		**9.1**		**52.7**		**42.8**		**95.7**		**79.3**		**111.2**		**87.8**	
20	luteolin	15.8	2	11.8	2	2.0	0	1.7	0	5.5	1	2.8	1	2.0	0	1.8	1
21	luteolin-7-O-Glc	0.8	0	0.9	0	1.7	0	1.3	0	2.3	1	1.0	0	1.5	0	1.3	0
22	hesperidin	0.6	0	1.2	1	0.6	0	0.5	0	0.5	0	0.3	0	0.4	0	0.5	0
	total flavones	**17.3**		**13.9**		**4.2**		**3.5**		**8.2**		**4.1**		**3.9**		**3.6**	
23	naringenin	2.2	0	2.1	0	0.2	0	0.2	0	0.2	0	0.2	0	0.3	0	0.2	0
	total flavanones	**2.2**		**2.1**		**0.2**		**0.2**		**0.2**		**0.2**		**0.3**		**0.2**	
24	catechin	4515.6	976	3810.5	572	1810.3	906	4245.8	788	5991.8	438	2069.9	139	8065.7	1502	3659.7	936
25	epicatechin	48.1	11	43.4	5	19.2	3	35.6	9	53.3	8	18.4	5	86.4	18	22.4	7
26	procyanidin B1	381.5	54	298.4	60	203.1	37	432.2	92	409.3	32	174.7	13	581.6	171	179.9	44
27	procyanidin B3	2468.6	529	2565.2	186	1233.6	151	1633.1	435	2837.5	442	1094.6	91	2883.6	726	1341.5	226
	total flavan-3-ols	**7413.8**		**6717.5**		**3266.3**		**6346.7**		**9291.9**		**3357.5**		**11617.3**		**5203.5**	
28	kaempferol	1.9	0	5.3	0	5.8	1	13.9	1	22.0	7	14.3	1	19.7	5	19.7	2
29	quercetin	6.4	1	17.6	1	3.4	0	3.1	0	13.2	2	2.5	1	6.0	1	2.6	1
30	taxifolin	8497.3	810	9678.7	1224	2.3	1	0.9	0	2.1	1	0.9	0	1.1	0	3.7	0
31	kaempferol-3-glucoside	194.1	30	195.0	20	97.8	4	265.3	46	160.1	12	100.8	31	227.5	35	103.0	15
32	quercetin-3-glucoside	154.3	62	406.9	3	47.8	8	37.7	9	46.7	9	40.9	19	32.6	8	73.8	14
33	isorhamnetin-3-glucoside	71.9	14	178.7	13	2.9	0	4.5	1	8.8	2	4.1	2	4.7	1	11.2	2
34	quercetin-3-acetylglucoside	35.9	14	92.2	5	1.5	1	7.0	2	n.d.	0	n.d.	0	4.6	1	11.9	3
35	kaempferol-3-rutinoside	n.d.	0	n.d.	0	15.8	2	63.7	9	0.1	0	24.8	13	63.6	14	37.0	10
36	quercetin-3-glucosilarabinoside	35.7	17	75.5	9	2.1	1	1.4	1	1.4	1	1.2	1	0.7	0	1.3	0
37	rutin	n.d.	0	n.d.	0	8.4	2	3.3	1	0.2	0	6.1	5	2.8	1	16.4	6
38	isorhamnetin-3-rutinoside	n.d.	0	0.3	0	1.1	0	1.4	1	n.d.	0	1.8	1	0.6	0	6.5	3
38	quercetin-3,4-diglucoside	3.5	1	5.8	2	1.3	0	1.5	1	1.0	0	1.1	0	1.6	1	1.7	0
40	dihydrokaempferol	3.0	1	20.4	1	5.2	1	11.3	4	21.1	11	9.6	1	11.9	3	16.5	0
41	quercetin-3-glucuronide	296.7	78	540.1	36	928.7	181	604.2	149	420.8	147	507.4	180	580.3	130	772.3	261
42	kaempferol-3-glucuronide	56.7	31	78.3	14	384.7	18	484.7	43	280.7	73	480.3	125	667.2	110	301.2	65
	total flavonols	**9357.3**		**11294.7**		**1508.8**		**1503.8**		**978.2**		**1195.9**		**1624.9**		**1378.8**	
43	cyanidin-3-arabinoside	n.d.	0	1.3	0	n.d.	0	n.d.	0	n.d.	0	n.d.	0	n.d.	0	n.d.	0
44	cyanidin-3-glucoside	3.5	2	12793.1	1381	414.1	51	234.9	28	182.3	40	162.2	1	193.9	56	621.0	43
45	cyanidin-3-galactoside	1.6	1	567.5	25	34.3	2	23.1	3	17.8	4	15.1	0	19.3	4	56.4	4
46	cyanidin-3-sambioside	n.d.	0	21.4	3	0.0	0	0.6	0	n.d.	0	n.d.	0	n.d.	0	n.d.	0
47	cyanidin-3-rutinoside	n.d.	0	n.d.	0	9.7	2	2.2	0	n.d.	0	2.8	0	3.5	1	27.9	4
48	pelargonidin-3-glucoside	n.d.	0	28006.9	4952	23226.6	927	36466.1	1554	34614.8	6642	36021.7	5209	43612.0	2288	34045.3	5058
49	pelargonidin-3-rutinoside	n.d.	0	n.d.	0	431.9	29	362.4	53	0.4	0	365.7	42	600.1	62	663.3	86
50	peonidin-3-galactoside	n.d.	0	n.d.	0	17.8	3	22.3	7	31.1	14	16.2	4	45.5	20	35.3	13
51	peonidin-3-rutinoside	n.d.	0	n.d.	0	0.2	0	0.4	0	n.d.	0	0.8	0	0.4	0	4.5	1
	total anthocyanins	**5.1**		**41390.2**		**24134.7**		**37112.2**		**34846.3**		**36584.5**		**44474.6**		**35453.7**	
52	ellagic acid	5529.4	570	7772.6	1232	814.0	260	711.9	226	316.7	276	692.3	174	551.0	74	1132.0	185
53	casuarictin	10091.2	686	11022.3	1974	11360.8	2946	19926.9	2970	3038.1	850	9063.2	1700	9375.3	1692	5046.0	1094
54	sanguiin H-6	1505.2	177	2202.5	62	1144.1	127	1086.3	103	209.1	24	446.1	45	263.6	4	244.5	7
55	agrimoniin	84329.1	5145	61627.6	6469	7635.6	554	19094.5	2874	17760.1	1388	13334.8	2626	15510.8	861	9271.7	1490
56	methyl ellagic acid rhamnoside	28349.6	2276	35969.6	3659	10.9	3	2.8	0	9.8	2	8.7	1	11.5	1	3.2	0
	total ellagitannins	**129804.4**		**118594.6**		**20965.4**		**40822.5**		**21333.7**		**23545.1**		**25712.3**		**15697.5**	
	proanthocyanidins (HMWP)	177537	10003	270012	13853	171307	6105	186431	7819	165253	12214	135706	11656	205187	34189	177470	16583
	total proanthocyanidins	**177537**		**270012**		**171307**		**186431**		**165253**		**135706**		**205187**		**177470**	
	total polyphenols (mg/100g)	**324.3**		**448.2**		**221.3**		**272.4**		**232.0**		**200.6**		**288.9**		**235.4**	

Nutrient bioavailability

Chapter 2.1

Targeted profiling method for twenty-three metabolites related to polyphenol gut microbial metabolism in biological samples

This chapter has been reprinted* from:

Development of a targeted method for twenty-three metabolites related to polyphenol gut microbial metabolism in biological samples, using SPE and UHPLC-ESI-MS/MS **Mattia Gasperotti**, Domenico Masuero, Graziano Guella, Fulvio Mattivi, Urska Vrhovsek, *Talanta*, **2014** 120, 221-230

By identifying biologically active molecules and their mechanisms, interaction or dietary response in individuals, modern nutrition tries to understand how the biological system can be affected or influenced by dietary intervention.

Polyphenol bioactivity has been correlated to bioavailability and catabolism, but their biological effects cannot be attributed only to the native forms, as found in food sources, but also and above all to their metabolites (4-7[§]). The factor most influencing their fate after consumption is the microbial polyphenol metabolism, made up of the gut microbiota, which represents all the microorganisms present in the gastrointestinal tract (8). The majority of those ingested polyphenol reach the colon without any degradation, after passing thorough the oral cavity or the stomach. The colonic microflora has been confirmed as the major responsible for the polyphenol metabolism, from the release of the aglycone flavonoids to the small phenolic acids. The microbial polyphenol metabolism follows a general pattern, in which this extremely diverse group of plant polyphenols is converted to a relatively small number of common metabolites. For this reason, the compounds that can reach cells, tissues or target organs are chemically and biologically different from the original dietary polyphenols (11).

In nutritional metabolomics, targeted based metabolomics or targeted profiling is aimed at quantitative analysis of a predefined metabolite group associated with a predefined class of compounds or pathway (19). Several pathways related to the microbial catabolism of polyphenols have been reported in the literature (23-26). However, there is a need for rapid and sensitive analytical methods that can quantify such metabolites for a large number of samples, as in the case of clinical studies or long term dietary intervention, in different matrices, with rapid and sensitive targeted metabolomics analysis (19).

For these reasons the aim of the this part of the project was the development of a sensitive targeted metabolomics method for analysis of 23 polyphenol microbial

[§] References are reported in the same order and listed in the attached accepted manuscript

metabolites, suitable for application to rat organs and biofluids, carefully considering all aspects of sample preparation for such complex matrices and the expected low amount to be detected, in relation to the consumption of fruit rich in polyphenols. This part of the work was developed in order to analytically support *in vivo* experiments using a physiologically relevant dose of polyphenol microbial metabolites (reflecting the consumption of a putative dose of strawberries) or to eventually be translated to human studies.

A detailed introduction, description of methods and discussion of the results are attached in the manuscript ready for the submission.

A high-throughput, sensitive and reproducible method for targeted metabolomics for quantitative analysis of 23 polyphenol metabolites in six different biological matrices was developed. The development of a purification procedure made it possible to obtain cleaner samples and more concentrated samples, with low LOQs. By analysing different biological samples, such as blood, urine, liver, kidney, heart and brain spiked with target metabolites, it was possible to test metabolite detection in the matrix and validate the overall recovery of the method, from purification to quantification.

Considering the variety of matrices which can be treated in the same conditions with a single general quantitative analytical protocol for targeted based metabolomics, this method can be considered a general method for the simultaneous analysis of several chemically different compounds in a complex matrix.

Consequently, this method can be used for nutritional studies and in particular with the expected amounts of polyphenol metabolites reported by Manach et al. (4), in which the expected total plasma concentration of polyphenol metabolites ranges from 0-4 μmol/L, with an intake of 50 mg of polyphenol aglycone equivalent.

My personal involvement in this research started with the experimental design for the method development and selection of the metabolites included in the study. Moreover, I carried out the synthesis of two of the metabolites, urolithin A and

urolithin B, which are not commercially available. Along with the synthesis, I also carried out purification of the raw products of the synthesis, their precipitation and initial characterisation using MS. Precise identification and purity were then assigned using NMR, kindly performed by Prof. Graziano Guella. As regards method development, I personally performed all the experiments, protocols and validation presented in the manuscript. As first author I was responsible for writing the manuscript and managing the comments and improvements to the text by the other co-authors.

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Development of a targeted method for twenty-three metabolites related to polyphenol gut microbial metabolism in biological samples, using SPE and UHPLC–ESI-MS/MS

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ABSTRACT

An increasing number of studies have concerned the profiling of polyphenol microbial metabolites, especially in urine or plasma, but only a few have regarded their accurate quantification. This study reports on a new ultra-performance liquid chromatography tandem mass spectrometry method with electrospray ionisation (UHPLC–ESI-MS/MS) using a simple clean-up step with solid phase extraction (SPE) and validation on different biological matrices. The method was tested with spiked samples of liver, heart, kidneys, brain, blood and urine.

The purification procedure, after the evaluation of three different cartridges, makes it possible to obtain cleaner samples and better quantification of putative trace metabolites, especially related to dietary studies, with concentrations below ng/g in tissue and for urine and blood, starting from ng/ml. Limits of detection and linear range were also assessed using mixed polyphenol metabolite standards.

Short chromatographic separation was carried out for 23 target compounds related to the polyphenol microbial metabolism, coupled with a triple quadrupole mass spectrometer for their accurate quantification. By analysing different spiked biological samples we were able to test metabolite detection in the matrix and validate the overall recovery of the method, from purification to quantification.

The method developed can be successfully applied and is suitable for high-throughput targeted metabolomics analysis related to nutritional intervention, or the study of the metabolic mechanism in response to a polyphenol-rich diet.

1. Introduction

Nutritional research today deals with health promotion, disease prevention and protection through functional food. By identifying biologically active molecules and their mechanisms, interaction or dietary response in individuals, modern nutrition tries to understand how the biological system can be affected by dietary intervention [1]. The nutritional relevance of polyphenols has been reported in several studies and the role of these natural compounds in a polyphenol-rich diet has been associated with many healthy effects in humans [2], not only related to their antioxidant activity. This wide and heterogeneous class of secondary plant metabolites is distributed in several foods, in particular in fruits and vegetables [3]. Of the most widely consumed fruit, berry fruits represent an unique source of polyphenols, due to their high concentration and variability in terms of the different classes of polyphenols present [2].

Although polyphenol bioactivity has been correlated to bioavailability and catabolism, their biological effects cannot be attributed only to the native forms, as found in food sources, but also and above all to their metabolites [4–7]. The factor most influencing their fate after consumption is the microbial polyphenol metabolism, made up of the gut microbiota, which represents all the microorganisms present in the gastrointestinal tract [8]. The microbial polyphenol metabolism follows a general pattern, in which this extremely diverse group of plant polyphenols is converted to a relatively small number of common metabolites. Hence a relatively small number of metabolites are biotransformed in the colon from a wide group of natural polyphenols [9,10]. For this reason, the compounds that can reach cells, tissues or target organs are chemically and biologically different from the original dietary polyphenols [11]. In recent years, considerable effort has

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been devoted to understanding the fate of polyphenols, their metabolism, the way that polyphenols can be modified by the gut microbiota and what type of lower molecular mass metabolites are produced from each class of polyphenols and released into the cardiovascular system [2,5,12–14]. As a consequence, an increasing number of studies have regarded the determination of polyphenol microbial metabolites, especially in urine or plasma, but only a few have been dedicated to their accurate quantification in different biofluids and tissues [15–19].

Metabolomics makes it possible to characterise the effects of nutrients or diet, analyse many metabolites in a given biological sample at the same time and explore the metabolic effects of nutrients in a global way [20]. Related to this type of study and in nutritional metabolomics in general, target based metabolomics or targeted profiling is aimed at quantitative analysis of a predefined metabolite group associated with a predefined class of compounds or pathway [20]. Targeted metabolomics provides accurate quantitative data and is regarded as a data-driven method [21]. For this purpose different analytical strategies are adopted, but the best choice for this kind of targeted metabolomics analysis is a triple quadrupole mass spectrometer, coupled with a liquid or a gas chromatographic system [22].

New metabolomics or nutrimetabolomics strategies related to intake and health benefits have been proposed for assessing nutritional status, food composition, the consequence of nutritional intervention or the study of the metabolic mechanism in response to diet [21,23].

Several pathways related to the microbial catabolism have been reported in the literature, in vivo with humans and through in vitro digestion with the faecal fermentation system [24–27]. There is therefore a need for rapid and sensitive analytical methods that can quantify such metabolites for a large number of samples, as in the case of clinical studies or long term dietary intervention, in different matrices, with rapid and sensitive targeted metabolomics analysis [20].

The metabolites investigated and used for method development in this work were chosen from the most representative and commercially available, on the basis of a literature survey. The selection was made considering metabolites from different metabolic pathways related to the polyphenol microbial metabolism reported in the literature [5,25,26]. Synthesis was performed for urolithin A and B, as they are specific markers of ellagitannin consumption and are not commercially available as standard references [28].

The main purpose was to develop a unique method, suitable for the analysis of polyphenol microbial metabolites in blood, urine, brain, liver, kidneys and heart, in contrast to the literature, in which specific methods have been developed especially for blood and urine, without considering other organs. As the purpose is to develop a general method for several matrices and chemically diverse metabolites, this could rise to some issues in terms of the optimisation of the SPE procedure, quantification and general recovery in all the samples. General optimisation of the method was performed taking these issues into account and obtaining the best analytical conditions in a holistic manner.

This study reports on a new method using a simple clean-up step for a complex biological matrix, with short chromatographic separation and quantification, using a triple quadrupole mass spectrometer for 23 target metabolites related to the consumption of polyphenols and their microbial metabolism.

2. Materials and methods

2.1. Chemicals and standards

Phloroglucinol (> 99%), pyrogallol (> 98%), gallic acid (> 99%), protocatechuic acid (> 97%), 3,4-dihydroxyphenylacetic acid (> 98%), 4-hydroxyhippuric acid (> 98%), 4-hydroxybenzoic acid (> 99%), pyrocatechol (> 99%), caffeic acid (> 98%), vanillic acid (> 97%), 3-hydroxyphenylacetic acid (> 99%), homovanillic acid (> 99%), 3-(4-hydroxyphenyl)propionic acid (> 98%), 3-(3-hydroxyphenyl)propanoic acid (> 98%), hydroferulic acid (> 96%), *trans*-ferulic acid (> 99%), *trans*-isoferulic acid (> 98%), sinapic acid (> 98%), *m*-coumaric acid (> 99%), *o*-coumaric acid (> 97%) and *p*-coumaric acid (> 98%) were purchased from Sigma-Aldrich (Saint Luis, Missouri, USA). Isotopically labelled compounds, *trans*-cinnamic acid-*d*5 (IS1) and butyric acid-*d*7 (IS2), were used as internal standards and purchased from C/D/N Isotopes Inc. (Quebec, Canada). Urolithin A and urolithin B were synthesised following a published protocol [28] and characterised using NMR for structure confirmation and purity (> 99%). LC/MS formic acid, Chromasolv LC/MS methanol and acetonitrile, were purchased from Sigma-Aldrich (Saint Luis, Missouri, USA).

2.2. Solutions

Standard stock solutions of 1000 mg/L were prepared in methanol for all compounds. Several further dilutions were prepared in methanol for the different steps involved in the method. All solutions were kept in dark vials at −20 °C.

2.3. Biological samples and extraction

This method was developed for different biological samples (liver, kidneys, heart and brain) and biofluids (blood and urine). Biological samples were obtained from previously sacrificed animals, in another experiment already approved by the Ethics Committee and published [29]. Tissue samples were ground with a CryoMill (Retsch, Germany) grinder using liquid nitrogen to ensure the quality of the sample and avoid any melting or degradation reactions. Deep frozen powders were kept at −80 °C before extraction. An aliquot of ground tissue, 1 g, was extracted with 9 mL of methanol 95%. An internal standard (0.1 µg/mL), *trans*-cinnamic acid-d_5 (IS1), was added to the extraction solvent to monitor the extraction procedures and further steps in sample preparation. The extract was then shaken at room temperature for 15 min in an orbital shaker and centrifuged for 5 min at 4 °C (5000 g) with a SIGMA 3–30 K centrifuge (Sigma Laborzentrifugen, Osterode am Harz, Germany). The supernatant was transferred into a 10 mL calibrated flask and the volume was adjusted. For blood and urine, the same ratio between volume sample and solvent, 1:9 was maintained and then adjusted after centrifugation to 10 mL. After solvent extraction the samples were ready for the clean-up protocols before injection into the UHPLC–MS/MS system. Further protocol details regarding blood and tissue extraction are reported in Vanzo et al., 2013 [29].

2.4. Solid phase extraction (SPE) and purification

For the clean-up phase and purification of the biological matrix, comparison of three different SPE cartridges for sample purification was performed. Waters Sep-Pak C18 cartridge, 1 g (Milford, Massachusetts, USA) is a silica-based bonded phase with strong hydrophobicity. It can be used, as reported by the manufacturer, to adsorb analytes of even weak hydrophobicity from aqueous solutions, including drugs and their metabolites in serum, plasma or urine or organic acids in beverages. Biotage Isolute ENV+, 1 g (Uppsala Sweden) is a hydroxylated polystyrene-divinylbenzene copolymer for non-polar metabolites in aqueous matrix. It can be a good alternative for the extraction of very polar drugs and metabolites that are not retained by C18 cartridges [30]. Phenomenex Strata-X, 1 g (Torrance, California, USA) is based on a polymeric sorbent that contains N-vinylpyrrolidone for a wide range of

metabolites and is suitable for the removal of phospholipids from the biological matrix. Due to the presence of the phenyl ring in the pyrrolidone ligand, the Strata-X cartridge is suitable for compounds with aromatic structures.

In general, the SPE protocol was applied following a procedure developed by Passamonti et al. [31], with some modifications. After solvent extraction with methanol 95%, an aliquot of samples (5 mL) was evaporated and reconstituted with 10 mL of H_2SO_4 0.01 N in water. The conditioning of the cartridges was done with 20 mL of methanol and 20 mL of H_2SO_4 0.01 N in water. After loading the sample, the cartridges were washed with 10 mL of Milli-Q water, dried under a stream of nitrogen and eluted with 20 mL of methanol. Eluates were evaporated to dryness with a rotavapor, and the samples were dissolved in 500 μL of methanol/water (50:50 v/v). The second internal standard, butyric acid-d7 (IS2), was dissolved in methanol/water (50:50 v/v) at a concentration of 1 μg/mL and added to the sample to monitor quantitative recovery during sample reconstitution. The sample was filtered with a 0.22 μm filter and injected into the UPLC–MS/MS system.

SPE recovery efficiency for the three cartridges was calculated for each metabolite by comparing recovery after spiking an aqueous solution with H_2SO_4 0.01 N with the mixed polyphenol metabolite standards at a final concentration of 0.05 and 0.25 μg/mL, after the SPE protocol.

2.5. UHPLC–MS/MS conditions

The ultra performance LC system used was a Waters Acquity UPLC (Milford, Massachusetts, USA) equipped with binary pump, autosampler, column compartment and Acquity PDA eλ detector. Separation of the 23 targeted metabolites and 2 deuterated internal standards was performed with a Waters Acquity UPLC column (Milford, Massachusetts, USA), HSS T3 (100 mm × 2.1 mm, 1.8 μm) equipped with the proper guard column. Mobile phases of 0.1% formic acid in Milli-Q water (A) and 0.1% formic acid in acetonitrile (B) were used and chromatographic separation was performed using the linear gradient reported in Vrhovsek et al., 2012 [32]. The injection volume was 10 μL. All the metabolites analysed eluted in 11 min, with a total run time and column equilibration of 17 min. The MS system used was a Waters Xevo TQ (Milford, Massachusetts, USA) triple quadrupole mass spectrometer, coupled with an electrospray interface and polarity switching option during acquisition. To optimise detection, each metabolite was directly infused in the MS system in combined mode with 50/50 v/v of solvents A and B. Characteristic MS conditions were automatically optimised using a Waters Acquity IntelliStart (Milford, Massachusetts, USA), optimising ionisation mode, cone volt energy and collision energy. The two most abundant fragments were selected for each metabolite to establish a MRM (multiple reaction monitoring) quantitative method. The first transition, corresponding to the most abundant fragment, was used as quantifier ion, and the second as qualifier ion. MS parameters for the MRM method and retention time are summarised in Table 1.

2.6. Confirmation of the targeted metabolites detected

For each metabolite, the most intense fragment was used for quantification analysis and confirmed on the basis of the second most intense fragment, which was used as the qualifier ion. The presence of the detected metabolites was considered to be confirmed when the conditions agreed with EC document no. SANCO/12495/2011, used for the validation of pesticide analysis [33]. According to this EC document, confirmation is achieved for samples containing one of the targeted metabolites if the

Table 1
UHPLC–ESI-MS/MS conditions for quantification and confirmation of polyphenol metabolites and internal standards (IS 1 & 2).

Compound	RT ($_{min}$)	ESI mode	Precursor ion (m/z)	Cone voltage (V)	Quantifier		Qualifier	
					Product ion (m/z)	Collision energy (V)	Product ion (m/z)	Collision energy (V)
Phloroglucinol	1.26	+	127	12	53	20	99	16
Gallic acid	1.39	+	171	20	109	14	81	22
Pyrogallol	1.62	+	127	22	81	22		
		–	125	34			79	16
Protocatechuic acid	2.10	+	155	20	65	20	93	14
3,4-Dihydroxyphenylacetic acid	2.33	+	168	18			77	26
		–	167	14	95	18		
4-Hydroxyhippuric acid	2.33	+	196	6	105	10		
		–	194	18			73	8
4-Hydroxybenzoic acid	2.83	+	139	20	77	18	65	24
Butyric acid-d7 (IS2)	2.85	+	96	24	50	12	46	16
Pyrocatechol	2.86	–	109	36	81	12	53	14
Caffeic acid	3.18	+	181	10	145	16	117	22
Vanillic acid	3.22	+	169	10	93	14	65	22
3-Hydroxyphenylacetic acid	3.40	–	151	18	65	20	79	20
Homovanillic acid	3.40	+	183	14	137	14	122	26
3-(4-Hydroxyphenyl)propionic acid	3.81	+	167	12	107	10		
		–	165	26			93	12
p-Coumaric	4.01	+	165	8	91	22	65	30
Hydroferulic acid	4.20	–	195	28	136	16	121	24
3-(3-Hydroxyphenyl)propanoic acid	4.28	+	167	8	121	12	107	22
trans-Ferulic acid	4.49	+	195	6	145	16	117	22
Sinapic acid	4.54	–	223	22	208	14	164	16
m-Coumaric acid	4.72	+	165	14	91	22	65	30
trans-Isoferulic acid	4.80	+	195	8	145	16	117	22
o-Coumaric acid	5.67	+	165	6	123	12	103	16
Urolithin A	6.93	+	229	10	157	22	128	34
trans-Cinnamic acid-d5 (IS1)	7.47	+	154	6	107	18	135	4
Urolithin B	8.90	–	211	42	139	28	117	28

precursor ion and both quantifier and qualifier ions are present with a signal-to noise ratio greater than 3. Confirmation was achieved when the qualifier/quantifier ratios, based on their peak area in samples as compared to the standard, did not differ by more than the fixed percentage reported in the document [33] and related to each individual qualifier/quantifier ratio.

2.7. Method validation

The parameters established for validation of the method were performed fully for blood samples, as the most available and commonly used biological samples. The parameters established for blood were selectivity, limit of detection, limit of quantification, linearity, matrix effect, accuracy and precision. In contrast, validation of the purification protocol was performed for all biological matrices, leading to validation of recovery for the SPE protocol.

2.7.1. Selectivity

Selectivity is the ability to differentiate the analytes in the complex mixture of components present in any biological matrix. To ensure selectivity of the method, blank blood samples and spiked blood samples were analysed. With the chromatographic conditions and MRM transition used, all the analytes were resolved without interference from the matrix at the retention time and both mass transitions of the analytes, also as compared to the standards analysed in solvent.

2.7.2. Limit of detection, limit of quantification, matrix effects, linearity and calibration curves

Calibration curves were established using blood for matrix-match calibration and pure solvent (methanol: water, 50:50 v/v) to check the absence of any matrix effects. Calibration curves were performed using a mixture of all the standards spanning from 0.00001 μg/mL to 10 μg/mL. Calibration curves were built using linear regression and not forced to pass through zero. Furthermore, a 1/x statistical weight was applied to obtain the most reliable calibration curves for all the metabolites. The weighting factor typically ensures the best fit of the plot, as determined by visual inspection and the correlation coefficient for both matrix-match and solvent calibration curves. The effective range of calibration curves was obtained on the basis of the linearity of the responses for each individual metabolite. Acceptable linearity was tested using the coefficient of determination (R^2) and the p-value of the *lack-of-fit* test. The limits of quantification (LOQ) and limits of detection (LOD) were evaluated at the concentration in which the quantifier transition presented a signal-to-noise (S/N) ratio of >10 and >3 respectively.

2.7.3. Accuracy and precision

Accuracy and precision were ensured by analysing replicated spiked blood samples at low, middle and high concentration levels. The levels were set at 0.05, 0.25 and 1 μg/mL for each analytes, for the low, middle and high concentration levels respectively. Precision was reported as the relative standard deviation (RSD) between the replicate measurements in spiked blood samples, while accuracy was reported as the relative error (RE), which was calculated as the difference between the measured value and the theoretical value, divided by the theoretical value and expressed as a percentage.

2.7.4. Recovery

In contrast to previous validation parameters, recovery was assessed in all the different biological blank matrices considered (blood, urine, liver, kidneys, heart and brain). Blank samples were spiked with 0.02 ug/mL of each metabolite, close to the LOQ at the low level, and analysed after the SPE clean-up procedure. Recovery was calculated for 5 or 10 replicates, depending on sample availability. The recovery trials were carried out by comparing the peak areas of the spiked samples with the peak areas of the respective pure standards, the ratio of the areas then being expressed as a percentage, and the standard deviation (sd) of the replicate was considered. In some cases, when endogenous amounts of some metabolites were present in the matrix, the known concentration of the endogenous amount was subtracted from the total peak area, thus revealing the concentration of the spiked metabolite. Furthermore, analysis without the purification step was performed and compared with analysis after the clean-up phase.

2.8. Statistical analysis

Data processing was done using Waters MassLynx 4.1 and TargetLynx software. Data visualisation and calibration curve processing were done using R software.

3. Results and discussion

3.1. UHPLC–MS/MS—analytical performance

The UHPLC separation method was developed based on a previously published method optimised for the rapid quantification of 130 polyphenols in fruits [32]. The injection volume was increased from 2 to 10 μL, considering the low amount to be expected in biological samples. The solvent for sample reconstitution before UHPLC injection was changed from methanol to methanol/water (50/50 v/v), with the scope of improving the peak shape and to comply with the retention capacity of the column. All the metabolites were well retained under these conditions, despite the fact that polyphenol metabolites are small, polar molecules compared to polyphenols in their intact form (Fig. 1). Detection of the transitions was performed using IntelliStart software and then manually corrected, although some molecules have a low molecular mass and this can be critical for choosing the transitions (see Table 1). A total of 50 MRM transitions were chosen, covering metabolites and internal standards. Some peak overlap inevitably occurred, due to the shortness of the chromatographic method. Because of this, scheduled MRMs were checked to ensure that there were sufficient data points in each peak. Close retention times between some metabolites were not a problem because of the use of selective MRM transitions for each metabolite. Application of the mass spectrometry rules commonly accepted for the analysis of pesticides in food and feed in the European Union [33] and in particular the strategies used for this method with two MRM transitions for each metabolite made it possible to obtain solid quantitative data. Compared to other methods, in which only one transition is used for identification and quantitative analysis of microbial polyphenol metabolites, the method offered better confirmation of the analytes [15].

A general quantification study covering a variety of different small molecules in different biological matrices and with a long sample preparation would be problematic without the use of internal standards for monitoring the quality of the manual execution and the clean-up reproducibility [20]. For this reason cinnamic acid-d_5 was used and added directly to the extraction solvent. Furthermore, a large instrumental variation in analytic response caused by the interference between the ESI source and the matrix can occur in long sequences, leading in most cases to a reduction of the signals. For the monitoring of any signal variation during the analysis, a second isotopically labelled internal standard butyric acid-d_7 was added to the sample just before filtration.

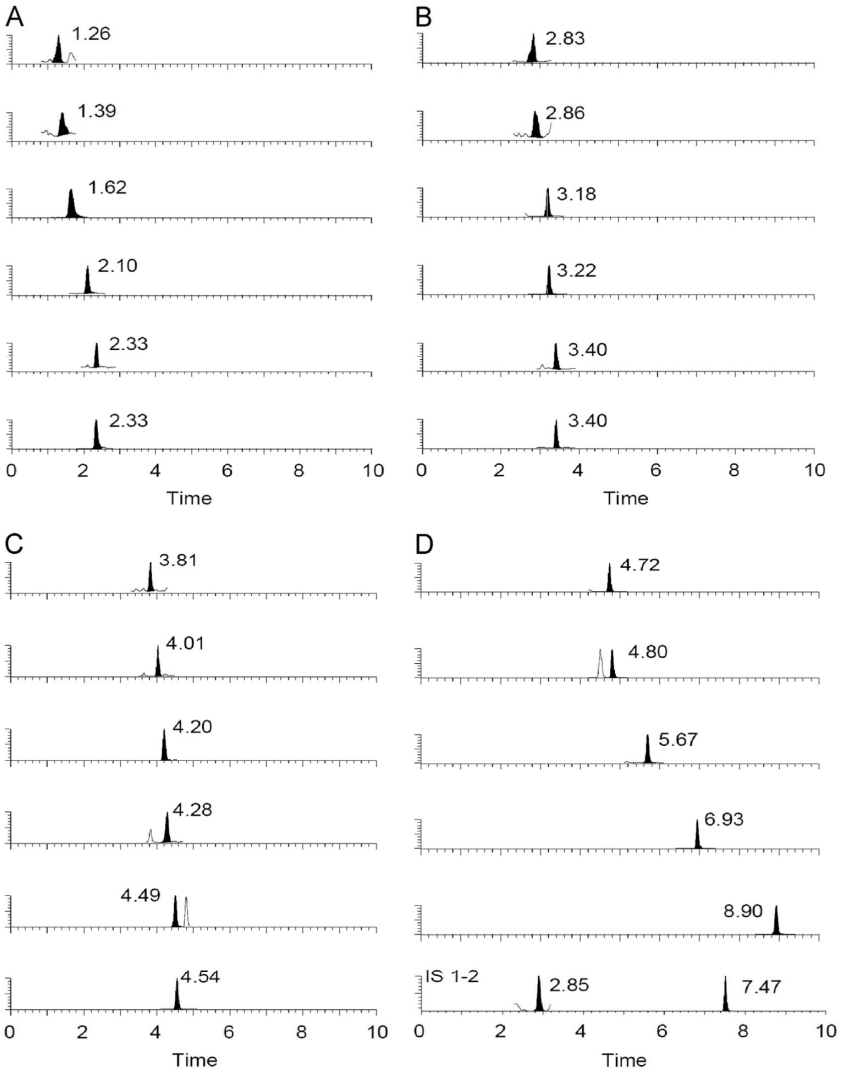

Fig. 1. UHPLC–ESI-MS/MS chromatograms of the quantifier MRM transition for the 23 polyphenol metabolites spiked in blood blank matrix at 0.01 ug/mL, listed accordingly to their retention time. *Panel A*: phloroglucinol, gallic acid, pyrogallol, protocatechuic acid, 3,4-dihydroxyphenyl acetic acid, 4-hydroxyhippuric acid; *Panel B*: 4-hydroxybenzoic acid, pyrocatechol, caffeic acid, vanillic acid, 3-hydroxyphenyl acetic acid, homovanillic acid; *Panel C*: 3–(4-hydroxyphenyl)propionic acid, *p*-coumaric acid, hydroferulic acid, 3-(3-hydroxyphenyl)propanoic acid, *trans*-ferulic acid, sinapic acid; *Panel D*: *m*-coumaric acid, *trans*-isoferulic acid, *o*-coumaric acid, urolithin A, urolithin B, butyric acid-d_7 and (IS2) *trans*-cinnamic acid-d_5 (IS1).

This also made it possible to check the regular injection of each sample. With the use of isotopically labelled internal standards, quantitative data can be monitored when the initial concentration is known.

3.2. Enzymatic hydrolysis

An additional step for those interested in the phase II metabolite deriving from the polyphenol microbial metabolite (glucuronides and sulphates forms) could be the enzymatic hydrolysis of the samples, due to the rare availability of this kind of conjugated metabolite. Protocols for hydrolysis are reported in the literature, especially for the analysis of urine and blood [15,34] using β-glucuronidase and sulphatase. However, these approaches provide very indirect information on the metabolites produced and inaccurate quantitative estimates, since there is very little data available on enzymatic hydrolysis efficiency [7].

3.3. Sample extraction and solid phase extraction (SPE) to clean up biological samples.

The solvent used for sample extraction was methanol:water (95:5), with a sample-solvent ratio of 1:9, being a rational choice for the extraction of polar and semi-non polar compounds such as the analytes from biological tissue and biofluids investigated. Moreover methanol: water (95:5) is an efficient solvent for protein precipitation and enzyme deactivation for rapid sample quenching [35–37] and the percentage of water can help in the extraction of very polar phenolic acids [38]. Remarkably, when analysing different extraction mixtures for different analytes in different

animal tissue such as liver, kidneys, muscles and brain, it has been reported that methanolic extraction can be the most suitable [39].

After selection of the solvent extraction, the three SPE cartridges selected (Sep-Pak C18, Isolute ENV+, Strata-X), widely used for the clean-up phase and purification of the biological matrix, were tested for the clean up protocols. SPE cartridges are suitable for a wide range of metabolites or drugs, especially for small polar molecules, acids and aromatic compounds, such as the metabolites covered by this method. The selected SPE cartridges have already been used for the purification of polyphenols or polyphenol metabolites from biological samples [31,40–42]. The conditions for the elution of polyphenol and phenolic acid are relatively consistent [38]. The solvents are slightly acidified in order to prevent ionisation of phenolics, which could reduce compound retention [38].

An aqueous solution with H_2SO_4 0.01 N was spiked with the mixed polyphenol metabolite standards and then subjected to the SPE protocol with the different cartridges. However, as shown graphically in Fig. 2, they showed different recovery results for the metabolites investigated, due to their specific chemistry. In Fig. 2, the results of recovery efficiency (%) for the three different cartridges are presented as a box-plot, at two different final spiked concentrations of 0.25 and 0.05 μg/mL, showing the distribution and variability of the individual results for each metabolite. In the case of Strata-X, the overall recovery results for the two spikes were not comparable and were different for the two final concentrations. Indeed with Strata-X, which had a final concentration spike of 0.25 μg/mL most of the metabolites were below 30%, with higher overall recovery for the final concentration spike of 0.05 μg/mL (Fig. 2).

The results on Sep-Pak C18 showed no retention for 4 metabolites at a final concentration of 0.05 μg/mL: phloroglucinol, pyrocathecol, pyrogallol and 3-hydroxyphenylacetic acid. Three of the metabolites not retained were very similar, simple benzenes with different phenol groups which can also be related to their similar negative behaviour with Sep-Pak C18. Phloroglucinol was also not retained with Strata-X.

However, the overall recovery for Sep-Pak C18 was comparable with the different concentrations of the standard mix and the majority of metabolites were within the optimum range of 70–120% for recovery trials [33]. The best results in terms of metabolite retention and recovery for the two concentration levels were achieved with Isolute ENV+, 1 g, as shown in Fig. 2. All the metabolites were retained, with acceptable recoveries. Indeed most of the metabolites, considering both spiked concentrations, were within the optimum range of 70–120% for recovery trials.

In the trials the two last cartridges, Sep-Pak C18 and ENV+, showed a relatively similar affinity to the analytes, as already observed [42]. Moreover, ENV+ has already been observed to have the best recovery at pH 2.0 as compared to the C18 cartridge, obtained in the present protocol with an aqueous solution with H_2SO_4 0.01 N, while the elution of the phenolic fraction with pure methanol is a common procedure for both C18 and ENV+ cartridges [42]. On the basis of these results, Isolute ENV+ cartridges (1 g) were chosen for the clean-up phase and for further validation of the targeted metabolomics method for polyphenol metabolites in the matrix.

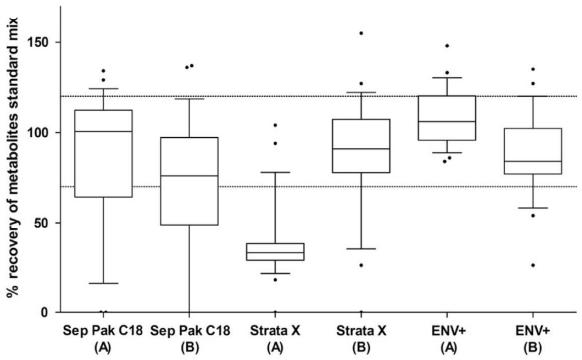

Fig. 2. Distribution and variability of the recovery (%) of polyphenol metabolite standard mix dissolved in aqueous solution with H_2SO_4 0.01 N, for three different SPE cartridges at a final spiked concentration of 0.25 (A) and 0.05 μg/mL (B).

3.4. Method validation

3.4.1. Selectivity

Blank blood samples were extracted using the SPE protocol described above. After injection and MS analysis, the MRM chromatograms obtained were checked for the presence of interference at the metabolite retention time. The blank blood samples were then spiked with mixture of polyphenol microbial metabolites at low concentration to prove the selectivity of the method at the low limit of quantification (Fig. 1). The method was shown to be selective and discriminative in the chromatographic conditions and with the MRM transitions used, with the presence of polyphenol microbial metabolites at low concentration. No interference from the matrix composition al the MRM transition and retention time of the analytes was observed in blood, also in comparison to the standard references injected in solvent (Fig. 1).

3.4.2. Linearity, matrix effects, limit of detection and limit of quantification.

Calibration curves were performed both in matrix, using blank blood samples, and in solvent with a mixture of all the 23 polyphenol metabolites, at different concentration levels, ranging from 0.00001–10 μg/mL.

Linearity was assessed by studying the level of the calibration curves constructed both in solvent and in blood (matrix-matched). The overall response (see Table 2), in both types of calibration, was characterised by high linearity and a linear dynamic range (LDR) of 3–4 orders of magnitude with a coefficient of determination (R2) of >0.99. Moreover, the linearity of the calibration curves was confirmed by the *p*-values of the *lack-of*-fit test. All the resulting *p*-values were below 0.01, meaning a significant lack of fit. These parameters indicate good linearity within the stated ranges for calibration curves.

The slopes resulting from the matrix-match calibrations and the solvent calibrations in the linear range defined were used to evaluate the percentage of matrix effects for each analyte. The slope ratios were determined as (1-slope in solvent/slope in matrix), expressed as a percentage [43,44]. The percentages of matrix effects (%ME) are listed in Table 2. The %ME values were from -17% to 1%. %ME in the range of $\pm 20\%$ can be considered not to be relevant, because the variability is close to repeatability values [44]. The overall matrix effects in blood showed a slight suppression. This slight suppression was probably the result and main advantage of using a SPE protocol for sample purification. Indeed, comparison of the %ME and the calibration curve graphs (Supplementary Fig. 1) shows that the two different types of calibration curve, in solvent and matrix-match, are very similar. These %ME results obtained in blood should also be similar for the other matrices considered, as the SPE procedure is most responsible for this low ion suppression. This observation agrees with already published data, in which low limits of detection and quantification were reported in polyphenol microbial metabolites analysis, as well and most importantly a reduction in the matrix effect when SPE was used as the sample purification protocol [45].

Table 2
Calibration parameters, linear dynamic range (LDR), coefficient of determination (R^2) limit of quantification (LOQ) and limit of detection (LOD) obtained by using polyphenol metabolite standards in solvent and in blood after SPE procedure, with evaluation of the matrix effects (ME).

	Matrix-match calibration (blood)						Solvent calibration (methanol: water, 1:1)						ME
	LDR	LOD	LOQ	Curve			LDR	LOD	LOQ	Curve			
	μg/mL	ng/mL	ng/mL	*a* (slope)	*b* (offset)	R^2	μg/mL	ng/mL	ng/mL	*a* (slope)	*b* (offset)	R^2	%
Phloroglucinol	0.01–1	3.0	10.0	2730	112	0.991	0.01–1	3.0	10.0	2810	8	0.999	−3
Gallic acid	0.01–1	3.0	10.0	44,072	475	0.997	0.01–1	3.0	10.0	43,810	−197	0.998	1
Pyrogallol	0.01–5	3.0	10.0	9708	−5534	0.986	0.01–5	3.0	10.0	11,228	−362	0.996	−16
Protocatechuic acid	0.005–1	1.5	5.0	127,704	33	0.998	0.005–1	1.5	5.0	142,585	312	0.999	−12
3,4-Dihydroxyphenyl acetic acid	0.001–1	0.3	1.0	11,742	−115	0.996	0.001–1	0.3	1.0	13,408	10	0.998	−14
4-Hydroxyhippuric acid	0.00025–5	0.1	0.3	91,714	24	0.999	0.00025–5	0.1	0.3	95,567	−30	0.999	−4
4-Hydroxybenzoic acid	0.001–0.5	0.3	1.0	67,773	82	0.990	0.001–0.5	0.3	1.0	78,060	65	0.997	−15
Pyrocatechol	0.01–2.5	3.0	10.0	2243	4	0.997	0.005–2.5	1.5	5.0	2360	5	0.994	−5
Caffeic acid	0.0025–1	0.8	2.5	68,262	−24	0.999	0.001–1	0.3	1.0	79,599	338	0.997	−17
Vanillic acid	0.001–1	0.3	1.0	199,368	152	0.998	0.001–1	0.3	1.0	219,717	553	0.995	−10
3-Hydroxyphenyl acetic acid	0.01–5	3.0	10.0	636	1	0.991	0.01–5	3.0	10.0	734	−12	0.992	−16
Homovanillic acid	0.0005–1	0.2	0.5	190,238	150	0.999	0.0005–1	0.2	0.5	210,340	145	0.998	−11
3-(4-Hydroxyphenyl) propionic acid	0.0025–0.5	0.8	2.5	116,145	125	0.995	0.0025–0.5	0.8	2.5	134,124	187	0.998	−15
p-Coumaric acid	0.001–0.5	0.3	1.0	115,839	40	0.997	0.001–0.5	0.3	1.0	132,438	35	0.999	−14
Hydroferulic acid	0.0005–1	0.2	0.5	32,778	1	0.998	0.0005–1	0.2	0.5	35,969	−11	0.998	−10
3-(3-Hydroxyphenyl) propanoic acid	0.001–1	0.3	1.0	86,380	194	0.999	0.001–1	0.3	1.0	92,948	97	0.998	−8
trans-Ferulic acid	0.00025–5	0.1	0.3	192,183	−7	0.999	0.00025–5	0.1	0.3	205,149	78	0.997	−7
Sinapic acid	0.01–5	3.0	10.0	13,093	−107	0.998	0.002–5	0.6	2.0	14,983	−5	0.999	−14
m-Coumaric acid	0.001–0.5	0.3	1.0	213,837	−2	0.999	0.001–0.5	0.3	1.0	241,138	27	0.999	−13
trans-Isoferulic acid	0.0005–1	0.2	0.5	145,338	−24	0.998	0.0005–1	0.2	0.5	151,884	18	0.999	−5
o-Coumaric acid	0.001–0.5	0.3	1.0	162,635	92	0.999	0.001–0.5	0.3	1.0	176,940	131	0.999	−9
Urolithin A	0.001–0.5	0.3	1.0	264,624	−90	0.999	0.0001–0.5	0.0	0.1	284,039	73	0.998	−7
Urolithin B	0.0005–0.5	0.1	0.5	27,976	−4	0.998	0.00025–0.5	0.1	0.3	30,794	18	0.996	−10

Therefore, calibration curves in solvent can be used for analyte quantification in these conditions without the need for any matrix match calibration (Table 2). The calibration curves obtained in solvent up to this point were then used for metabolite quantification in the different matrices, for the recovery and comparison of samples with or without SPE purification.

The LODs and LOQs for solvent calibration are listed in Table 2. The metabolite values differed. However as already observed for the matrix effects, LODs and LOQs were comparable between solvent and blood being the SPE procedure an important factor in the reduction of the matrix effect and as consequence also influencing the LODs and LOQs. The lowest LOD and LOQ values in solvent were for *trans*-ferulic acid, 4-hydroxyhippuric acid and urolithin B, with values of 0.1 ng/mL and 0.3 ng/mL respectively. The highest values were for pyrogallol, gallic acid, phloroglucinol and 3-hydroxyphenyl acetic acid with the LOD and LOQ being 3 ng/mL and 10 ng/mL respectively. The LODs and LOQs calculated using this method were below or within the concentration range used with a similar instrumental platform, but with a higher number of microbial polyphenol metabolites as compared to the previously published method [18]. The LODs and LOQs obtained using this method were in agreement or even lower than the results of previous publications [19,46]. Moreover, the concentration of these metabolites in humans is expected to be higher than the LOQs calculated for this method [4], thus showing calibration curves were built using linear regression and not forced to pass through zero. Furthermore, a 1/x statistical weight was applied to obtain the most reliable calibration curves for all the metabolites.

3.4.3. Accuracy and precision

Accuracy and precision were evaluated by spiking blank blood samples with 3 different known concentrations: 0.05 μg/mL (low), 0,25 μg/mL (medium) and 1 μg/mL (high) in triplicates. For most of the compounds, accuracy, expressed as RE, was within ± 15% for the three concentration levels. Only pyrogallol and sinapic acid were slightly higher, while precision, expressed as the RSD of the mean concentration, was below 12% for all three concentration levels in all the analytes. The results obtained for accuracy and precision are summarised in Table 3. The results showed that the proposed method is sufficiently accurate and precise to be applied to real samples.

3.4.4. Evaluation of analytes recovery

The recovery of the purification protocol was studied by spiking the samples with standard mix solutions containing metabolites at a concentration close to the LOQ, 0.02 μg/mL. In this case, all the matrices proposed for the method development were tested, i.e. liver, kidneys, heart, brain, blood and urine of rats, these being representative matrices used in nutritional studies and bioavailability studies of bioactive compounds for in vivo experiments with mammals. Recovery experiments were performed 10 times, except in the case of heart and urine (5 times). Overall recovery validation was done considering not only the matrix effect but also the SPE clean-up step and instrumental variations. The overall method was checked for recovery, from extraction to quantitative data.

Along with the recovery evaluation of each metabolite, the recovery of *trans*-cinnamic acid-d5 (IS1) was also checked in the different matrices. The results ranged from 80% to 113% in the different matrices and reflected mainly the extraction efficiency and the overall execution of the purification protocol, while the recovery of butyric acid-d7 (IS2) was not evaluated for the SPE procedure, since this was added after purification to monitor analytical performance.

The results of the recovery trial are shown in Table 3 and graphically in the first part of Fig. 3. In the matrix-dependent recovery trials a slightly variability was observed in the results. Tissues are subject to more complex mixture interference, which can significantly affect recovery [47]. However the SPE procedure, reducing the matrix complexity [45] and purifying the analytes, could lead, and as observed in the present method, to obtain very similar results in terms of recovery between the different biological samples considered with few exceptions.

Table 3
Recovery (%, ± sd), in different biological matrices, precision (%RSD) and accuracy (%RE) in blood after ENV+ (1 g) for method validation.

	Recovery												Precision			Accuracy		
	Blood		*Brain*		*Heart*		*Liver*		*Kidneys*		*Urine*							*Blood*
	%	± sd	%	± sd	%	± sd	%	± sd	%	± sd	%	± sd	L %RSD	M %RSD	H %RSD	L %RE	M %RE	H %RE
Phloroglucinol	11.0	*3.9*	76.5	*6.4*	9.0	*1.9*	21.8	*4.6*	95.4	*5.8*	61.7	*12.6*	10	3	4	0	5	−13
Gallic acid	53.9	*5.6*	52.4	*20.0*	72.2	*6.4*	53.4	*2.6*	65.5	*4.3*	19.2	*2.0*	2	1	0	8	2	0
Pyrogallol	42.0	*2.9*	50.7	*5.5*	48.9	*6.8*	54.9	*2.3*	82.0	*4.7*	31.0	*3.5*	4	10	6	13	−39	−37
Protocatechuic acid	80.1	*7.9*	79.5	*13.1*	94.6	*5.4*	72.4	*2.7*	78.4	*4.0*	35.0	*4.9*	3	1	1	8	2	0
3,4-Dihydroxyphenyl acetic acid	49.1	*4.7*	68.7	*25.2*	74.7	*16.2*	65.7	*4.8*	78.5	*5.3*	60.9	*7.0*	3	5	2	−16	−6	−6
4-Hydroxyhippuric acid	20.9	*3.0*	63.9	*20.6*	88.6	*4.4*	79.5	*3.5*	91.3	*6.1*	63.5	*2.4*	1	1	0	7	−3	1
4-Hydroxybenzoic acid	93.6	*6.8*	86.0	*6.1*	119.9	*11.3*	90.5	*1.9*	94.8	*5.9*	66.6	*17.9*	7	1	3	0	−4	−7
Pyrocatechol	64.7	*9.5*	62.0	*11.3*	52.2	*5.1*	65.9	*9.7*	57.6	*6.6*	19.8	*3.4*	9	7	7	0	−17	−16
Caffeic acid	73.4	*5.1*	73..9	*14.9*	82.6	*14.1*	79.4	*2.6*	62.5	*3.5*	16.3	*2.0*	4	1	1	−13	−11	−11
Vanillic acid	90.6	*6.3*	88.4	*6.2*	86.9	*8.1*	82.9	*2.7*	79.9	*3.5*	35.3	*6.2*	1	1	2	1	−1	−2
3-Hydroxyphenyl acetic acid	66.8	*15.0*	52.7	*16.8*	72.8	*16.8*	87.7	*20.3*	94.3	*15.3*	–	–	7	7	7	15	−8	−4
Homovanillic acid	85.9	*8.0*	110.7	*8.4*	83.4	*7.8*	84.0	*1.6*	95.1	*5.9*	103.5	*3.5*	3	1	1	−9	−4	−6
3-(4-Hydroxyphenyl) propionic acid	78.7	*6.1*	75.6	*7.7*	79.1	*9.9*	74.3	*3.4*	97.6	*5.2*	–	–	1	2	1	6	−2	−5
p-Coumaric acid	88.6	*5.4*	85.1	*4.6*	48.1	*9.0*	88.4	*2.3*	94.2	*8.5*	67.4	*31.4*	6	3	1	4	−1	−7
Hydroferulic acid	83.0	*5.8*	79.6	*4.4*	91.8	*3.1*	80.3	*3.0*	76.4	*5.1*	39.0	*3.8*	7	1	4	−10	−5	−5
3-(3-Hydroxyphenyl) propanoic acid	122.5	*7.8*	88.4	*8.1*	77.6	*4.2*	89.0	*5.7*	153.5	*16.5*	–	–	11	2	0	19	4	5
trans-Ferulic acid	90.8	*6.3*	91.6	*5.4*	68.3	*3.4*	88.9	*2.7*	86.9	*9.7*	59.4	*5.5*	4	2	3	1	1	−2
Sinapic acid	87.9	*5.9*	82.4	*5.4*	78.4	*6.6*	75.3	*12.5*	74.6	*10.2*	54.4	*2.5*	10	12	7	−29	−26	−11
m-Coumaric acid	86.9	*5.9*	84.8	*6.2*	106.8	*5.5*	89.3	*3.1*	89.8	*6.1*	84.6	*2.9*	2	1	1	3	2	−4
trans-Isoferulic acid	90.6	*6.4*	93.0	*5.7*	80.1	*9.5*	94.3	*3.0*	88.3	*9.9*	70.6	*5.4*	3	4	2	2	3	0
o-Coumaric acid	89.1	*6.0*	83.2	*7.2*	95.7	*3.0*	89.7	*3.1*	87.9	*5.7*	56.6	*2.3*	2	2	2	2	1	−3
Urolithin A	40.7	*5.8*	42.5	*9.8*	44.6	*3.7*	47.1	*4.5*	20.9	*5.3*	35.7	*4.0*	3	2	2	−1	−4	−12
Urolithin B	38.1	*2.7*	29.1	*11.9*	2.1	*1.5*	30.4	*3.0*	0.8	*0.5*	29.1	*1.6*	9	2	2	6	2	−11

The variability related to the type of biological matrix was in few cases very extensive, as in the case of phloroglucinol or 4-hydroxyhippuric acid, ranging from 10 to 95%. These extremely widespread recovery values are nevertheless correlated with acceptable standard deviation of the purification protocol for the repetitions considered in the trials. Only one metabolite, 3-(3-hydroxyphenyl)propanoic acid, had a higher recovery value of 153% in kidneys and 122% in blood, while in the other matrices it was around 77–89%. The weakest recovery data were for urolithin B, with a recovery of 1–2% obtained, again for heart and kidneys. In this case, future data for the analysis of urolithin B clearly cannot be accurate but may at least be semi-quantitative.

With regards to urine samples, the recovery of three metabolites, respectively 3-hydroxyphenylacetic acid, 3-(4-hydroxyphenyl)propionic acid and 3-(3-hydroxyphenyl)propanoic acid did not take place in the experiment because the ratio between the endogenous level of the metabolites in urine and the amount of spiked metabolite was too high. These three metabolites were found in high concentrations endogenously. The endogenous concentrations were estimated at 0.043, 0.034 and 0.131 μg/mL respectively for 3-hydroxyphenylacetic acid, 3-(4-hydroxyphenyl) propionic acid and 3-(3-hydroxyphenyl)propanoic acid.

On average, recovery for the other metabolites in the different matrices was in the range of 40–120% in most cases (Table 3). Considering all the metabolites in the different matrices (23 metabolites in 6 matrices, $n=138$), 58% of them were within the recovery range of 70–120%, 5% of them showed over 120% recovery, 25% of them were within the range 40–70% and 13% of them were below 40% recovery.

Considering the standard deviation of recovery for the SPE protocol for all the metabolites in the different matrices (23 metabolites in 6 matrices, $n=138$), 85,5% of them had a standard deviation below < 10%, while the rest of them were within 10 and 20%. This variability in terms of standard deviation associated with the use of ENV+ has already been observed [42]. In terms of overall recovery, this method would again seem to be suitable as a general protocol, with fast sample preparation.

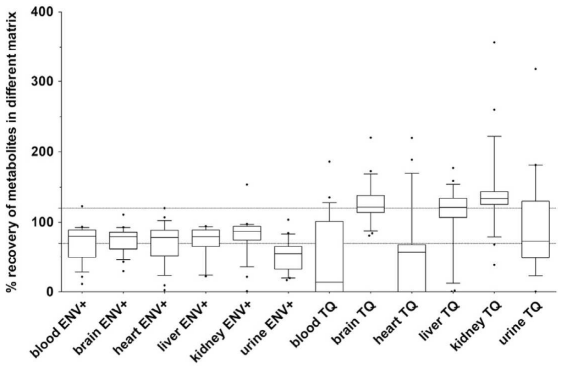

Fig. 3. Distribution and variability of the recovery (%) of polyphenol metabolites standard mix in blood, brain, heart, liver, kidney and urine with SPE (ENV+) and without (TQ) at a spiked concentration of 0.02 μg/mL.

3.5. Comparison of recovery with and without matrix purification

In addition to the recovery validation described above, the same sample set was also checked without the SPE clean-up step. The comparison is shown in Fig. 3, in which the first half refers to samples prepared with ENV+ purification, while the right-hand side of Fig. 3 refers to the aliquot of samples with no purification (TQ). The data are expressed as % of recovery after spiking the samples with standard mix solutions containing metabolites at 0.02 μg/mL.

Comparison of the two approaches for the analysis of biological matrices shows clearly that quantitative analysis can be different with and without a sample purification step before injection into

the MS system. This comparison offers further support for validation of the purification protocol in terms of recovery and further supports the need to use an SPE step before MS analysis. In samples without SPE purification, the metabolites were extremely spread out along the recovery axis, meaning that in this case recovery went from 0 to 300%, while most of the metabolites were outside the ideal range of expected recovery (70–120%). Moreover many metabolites were not detected, especially in blood, heart and liver. In blood 8 metabolites were not detected (phloroglucinol, gallic acid, pyrogallol, protocatechuic acid, 3,4-dihydroxyphenylacetic acid, caffeic acid, *trans*-ferulic acid, urolithin A), while in heart 6 metabolites (phloroglucinol, gallic acid, 3,4-dihydroxyphenylacetic acid, pyrocatechol, caffeic acid, 3-(4-hydroxyphenyl)propionic acid) were not detected. An explanation of these results relates to suppression of the signal in the ESI source, due to matrix complexity. The ESI source is highly susceptible to matrix composition and as a result it is possible to observe ion suppression or ion enhancement, due to the by now well-known matrix effect. In addition to ion suppression and signal reduction, in which the worst case scenario is that target compounds are not detected, strong enhancement of the signals of some of the metabolites can be observed (Fig. 3). The strongest ion enhancement was observed in kidneys, especially for 3-hydroxyphenylacetic acid and 3-(3-hydroxyphenyl)propanoic acid, with recovery of 356% and 259%, respectively.

A good strategy for avoiding this kind of problem and reducing the complexity of matrix composition is the use of a SPE step before quantitative analysis. The improvement in the recovery results is evident on looking at Fig. 3. Indeed, recovery seems to be the most important parameter and is clearly most susceptible to matrix compositions and most affected by them observing the extreme variability in the data-point for the samples without SPE purification. With the use of SPE purification the results are more reliable and closer to the real values. Another positive point in the use of "cleaner" samples is the possibility of concentrating samples, 10-fold for this purification protocol. The final concentration detectable is sometimes a major issue, especially for experiments with physiological levels of bioactive compounds. With this preparation protocol, the samples are concentrated 10-fold without affecting the analytical performance for the type of matrix considered, since the negative effect of the matrix was resolved by the clean-up step.

4. Conclusion

A high-throughput, sensitive and reproducible method for targeted metabolomics for the quantitative analysis of 23 polyphenol metabolites in six different biological matrices was developed. In contrast to previous quantification methods, which are optimised and developed for a few metabolites and for a specific matrix, the method developed allows simultaneous quantification of many polyphenol metabolites, with a general protocol for different matrices commonly considered in nutritional and bioavailability studies.

The purification procedure made it possible to obtain cleaner and more concentrated samples, with low LOQs and better quantification of possible trace metabolites, especially related to dietary studies with concentrations below ng/g in tissue, and for urine and blood, starting from ng/mL. As compared to the samples without the clean up step, the use of SPE for the samples also serves to concentrate them 10-fold.

Method sensitivity and linear range were assessed using mixed polyphenol metabolite standards. By analysing different biological samples, such as blood, urine, liver, kidneys, heart and brain spiked with target metabolites, we were able to test metabolite detection in the matrix and validate the overall recovery of the method, from purification to quantification. No significant interferences were detected in the different biological matrices. Considering the variety of matrices which can be treated in the same conditions with a single general quantitative analytical protocol for targeted based metabolomics, this method can be considered very flexible and may be widely applied.

Consequently, this method can be used for nutritional studies. in particular with the expected amounts of polyphenol metabolites reported by Manach et al. [4], in which the total plasma concentration of polyphenol metabolites ranges from 0–4 μmol/L, with an intake of 50 mg of polyphenol aglycone equivalent. We can conclude that the method can be applied to targeted based metabolomics analysis of different biological matrices and related to the consumption of polyphenols, also considering the recovery values for adjustment of the quantitative data, if such data are needed.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.04.058.

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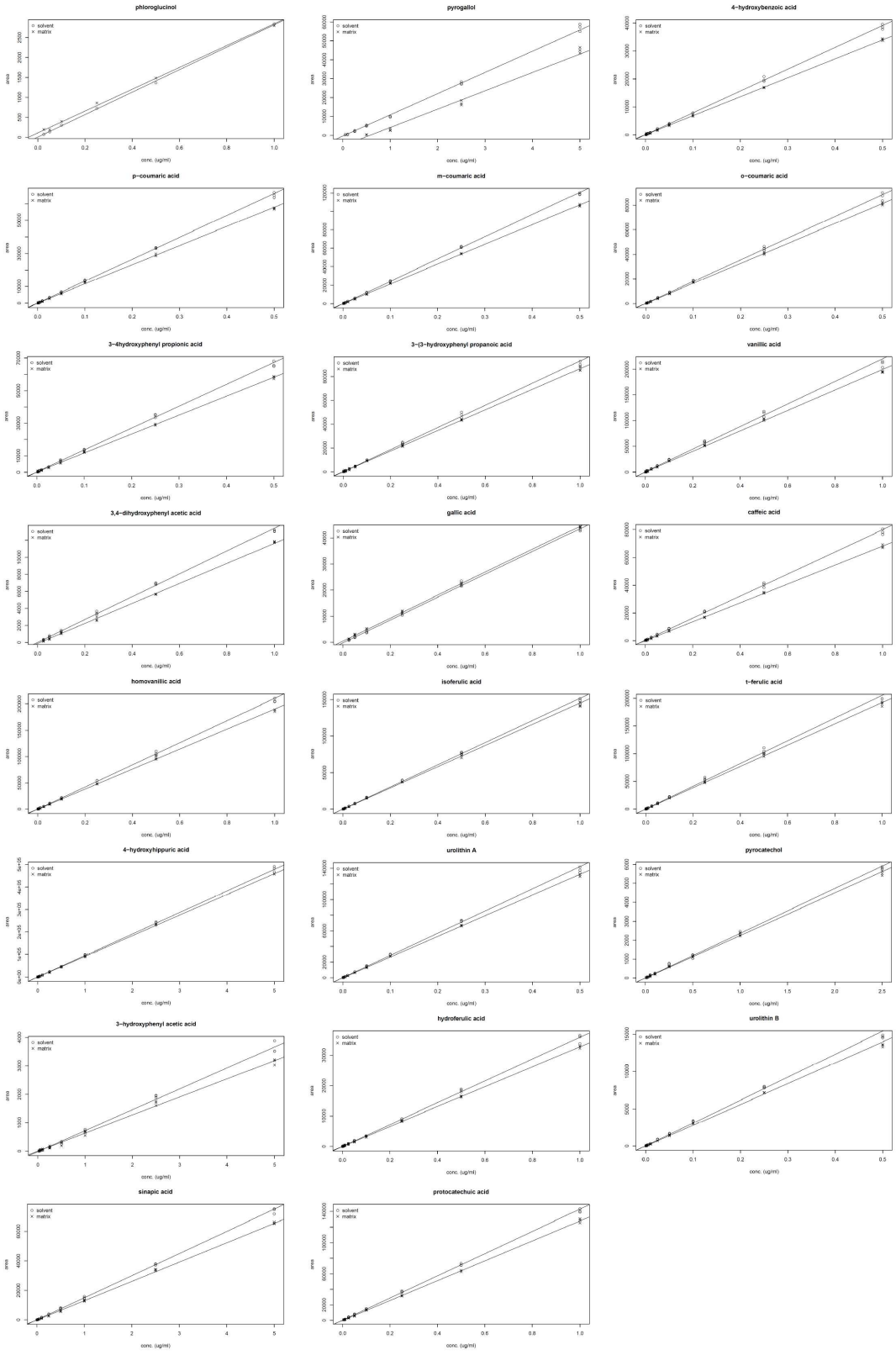
Supplementary Fig. 1.

Matrix-match calibration and solvent calibration curves and graphic displays



Nutrient bioavailability

Chapter 2.2

Fate of microbial metabolites of dietary polyphenols in rats: Is the brain their target destination?

This chapter will be submitted shortly for publication as a manuscript:

Fate of microbial metabolites of dietary polyphenols in rats: is the brain their target destination? **Mattia Gasperotti**, Sabina Passamonti, Federica Tramer, Domenico Masuero, Graziano Guella, Fulvio Mattivi, Urska Vrhovsek.

As already explained, the colonic microbiota produces a relatively small number of polyphenol microbial metabolites (PMMs) from a large number of different dietary polyphenols. These metabolites, being more absorbable than their polyphenolic precursors, are regarded as responsible for the healthy effects associated with the consumption of polyphenols (3, 19[**]).

The biological activity of polyphenols has been explored *in vivo* on animal models and also in some clinical trials (8). Epidemiological studies have associated polyphenol consumption with a reduced risk of cancer and cardiovascular disease (20, 21). Research on the neuroprotective effects and prevention of brain ageing resulting from the use of dietary polyphenols has also been significantly developed in the last few years (21–27).

However, PMMs may also have some important bioactivities, resulting in an impact on general health (19). Current information concerning their absorption and tissue distribution is still scarce (8, 28) and not reliable enough in order to give sound dietary recommendations. Additional studies are therefore needed to support the hypothesis that PMMs are also bioactive in the mammal organism.

To respond to the need for knowledge about the absorption and tissue distribution of PPMs, focusing the attention especially on the brain, a nutrikinetics experiment with rats was set up. The main question was whether these metabolites are able to reach the brain in nutritional conditions. Thus, the *in vivo* trials were performed by injecting a nutritionally relevant dose to mimic the plasma circulating level of PPMs after the consumption of a bowl of strawberries.

A detailed introduction, description of methods and discussion of the results are included in the manuscript ready for submission.

[**] References are reported in the same order and listed in the attached manuscript

Interestingly, the final results obtained show for the first time the simultaneous quantitative profile of some polyphenol microbial metabolites in rats, proving their distribution in the brain and the main excretory organs, i.e. the liver and kidneys, and in biofluids (urine and blood).

The question of whether the brain was a targeted organ for this class of molecules was resolved by first proving that some of the metabolites injected were already present in the brain, supporting the idea that these molecules are able to pass through the blood brain barrier. Moreover, in experimental conditions, with a nutritional amount of injected compounds (typically a sub-acute dosage), 10 out of 23 compounds were present in significantly increased amounts.

My personal involvement in this last part of the project was in the initial set up of the entire *in vivo* experiment, from evaluation of already published protocols for *in vivo* experiments and their evolution to the protocols presented, improving the reliability of the surgical and analytical aspects. Moreover, I was present in the surgery room at the Animal Facilities at the University of Trieste during the experiment for the management of all the procedures and the collection of the samples, in collaboration with Prof. Sabina Passamonti, called on to perform the surgical protocols. After sample collection I performed all the steps for the sample preparation, acquisition, processing and critical evaluation of quantitative data. As the first author I was responsible for writing the manuscript and managing the comments and improvements to the text by the other co-authors.

Fate of microbial metabolites of dietary polyphenols in rats: Is the brain their target destination?

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Abstract

On consuming a serving of fruits rich in polyphenols a range of different polyphenols can be ingested, spanning from one-phenol hydroxybenzoic acid to more complex polymeric compounds. Only a minor part of the polyphenols (5-10%) is absorbed and the remainder of the reaches the colon, where they are extensively metabolized by gut microbiota to small molecular weight metabolites. Then the subsequent tissue distribution, the target organs remain largely undefined along to the effective circulating concentration.

Here we report for the first time a nutrikinetics experiment testing simultaneously 23 polyphenol microbial metabolites following a fast time line (within 15 min.) in rat's organs and biofluids, with a total intravenous injected dose of 2.7 µmol. Some of the metabolites were already find as basal level in low amount, also in the brain, coming from endogenous metabolism or from nutritional intake. In blood a pseudo-first order decay was observed with the simultaneous tissue distribution and excretion in the urine. Specific organ-tropisms were observed, mainly hepatotropism.

Remarkably brain was reported in this study as one of the target organs of such molecules, being already present at basal level or increasing their concentration after the treatment. Ten out of 23 polyphenol metabolites were increased their concentration during the observation time in significant amount, while their concentration in blood was vanishing.

Together, these results suggest a significant interplay within the gut-brain axis in which the polyphenol metabolites might be responsible in the alteration endogenous equilibrium in relationship with the nutritional intake of polyphenols.

Keywords polyphenols, gut microbiota, target metabolomics, mass spectrometry, brain, fruit consumption

Introduction

A diet rich in fruits and vegetables is regarded an important factor for protecting the human population from chronic diseases (1). Dietary recommendations require sound knowledge about food composition, identification of bioactive food components, and characterisation of their bioavailability, tissue distribution and elimination patterns, as well as their specific effects on the cellular homeostasis.

Polyphenols, which are a wide and heterogeneous group of bioactive compounds found in plant-based foods (2), are regarded as responsible for protecting the human organisms from oxidative stress-related chronic diseases. However, polyphenols bioactivity may be much wider. Among vegetables and fruits, berries are the major sources of dietary polyphenols (3).

After ingestion, only a minor part of the polyphenols (5-10%) is absorbed in the small intestine. The remainder reaches the colon, where they are extensively metabolized by gut microbiota to small molecular weight compounds (4). Gut microbiota consists of all the microorganisms in the intestinal tract, which is mainly populated by bacteria with many trillions of microbial cells (5). The metabolic activities of this heterogeneous bacterial population are gaining strong interest, because they modulate human metabolic phenotypes (6-8) and so playing an essential role in human health.

The microbiota break down plant polyphenols through the actions of glucosidases, esterases, demethylases, dehydroxylases and decarboxylases (5,9-18), obtaining smaller common metabolites, such as phenolic acids and short chain-fatty acids (9), collectively known as polyphenol microbial metabolites (PMMs). They are absorbed by the colonic epithelium and found in the blood, with patterns that correlate with one's diet and microbial composition of one's microbiota (19).

PMMs are regarded as responsible for the healthy effects correlated with regular consumption of berry fruits (4,20). Epidemiological studies have associated polyphenol consumption with a reduced cancer and cardiovascular disease risk (21,22). Research on the neuroprotective effects and prevention of brain aging from the use of dietary polyphenols has moreover been greatly developed in the last years (22-28). The literature reports that diets rich in polyphenols attenuate neuropathology indicators and cognitive decline (25). Some studies report on a direct effect of polyphenols on intracellular targets, e.g. three members of the secretase family, known to be involved amyloidal aggregation connected with the onset and progression of Alzheimer's disease (29). However, the question whether these compounds are able to pass the blood-brain barrier and attain there a pharmacologically relevant concentration is still unanswered.

The importance of the microbiota for the brain development function has been pointed out by numerous studies in experimental animals (30-32), and these observations can be translated to human brain disorders. For instance, autism, a defect of brain development leading to impaired social interactions, is now being understood as linked to abnormal microbiota population and poor intake of fruits and vegetables (33). It might be speculated that PMMs are involved in normal neural development and function. Indeed, they are chemically identical or similar to amino acid and neurotransmitter catabolites.

However, it seems that PMMs may also have some yet uncharacterised bioactivity, resulting in an impact on the general health status (20). Current information concerning the absorption and distribution of PMMS in mammalian tissues is still poor (9,34) and not enough to support the hypothesis that PMMs are the bioactive agents of our diet. Consequently, it's not possible to work out dietary recommendations in favour of prevention of brain dysfunctions.

Given this background, this study set out to characterize the time-dependent tissue distribution of a mixture of PMMs, following a single intravenous injection in anaesthetized rats. The focus of this study was on characterizing their ability to arrive into the brain, relative to the main excretory organs, i.e. the liver and the kidneys.

The data obtained show for the first time the simultaneous quantitative profile of several PMMs in a mammalian system, proving their distribution into the brain, into the main excretory organs, i.e. the liver and the kidneys, and in biofluids. For some PMMs, specific tropisms were also observed, highlighting for instance the target organs for future experimentations. Remarkably for the support of the neurological relevance of fruits rich in polyphenols, the data obtained show that at least 10 different PMMs incrementally appear in the brain within 15 min, while they simultaneously disappear from the blood and/or reach other organs.

Materials and Methods

Chemicals. Phloroglucinol, pyrogallol, gallic acid, protocatechuic acid, 3,4-dihydroxyphenylacetic acid, 4-hydroxyhippuric acid, 4-hydroxybenzoic acid, pyrocatechol, caffeic acid, vanillic acid, 3-hydroxyphenylacetic acid, homovanillic acid, 3-(4-hydroxyphenyl)propionic acid, 3-(3-hydroxyphenyl)propanoic acid, hydroferulic acid, trans-ferulic acid, trans-isoferulic acid, sinapic acid, m-coumaric acid, o-coumaric acid and p-coumaric were purchased from Sigma-Aldrich (Saint Luis, Missouri, USA). Isotopically labelled compounds, butyric acid-d7 and cinnamic acid-d5, were used as internal standard and purchased from C/D/N Isotopes Inc. (Quebec, Canada). Urolithin A and urolithin B were synthesized following an already published protocol (35) and characterized by NMR for the structure confirmation and purity. LC/MS formic acid, Chromasolv LC/MS methanol and acetonitrile, were purchased from Sigma-Aldrich (Saint Luis, Missouri, USA).

Study Design. The experiment was designed as a multi-component pharmacokinetic study with quantitative analysis of organs (liver, kidney, heart and brain) and biofluids (blood and urine). The purpose was for the exploration of the fate of polyphenol microbial metabolites mixture in a mammalian system. The

experiment was divided into four different time point: 15 seconds, 2 minutes, 5 minutes and 15 minutes (Figure 1). Each time point was represented by 4 replicates, 4 rats received the dose with the polyphenol microbial metabolites, and one blank rat, as control, without polyphenol metabolites mixture but with identical experimental procedure.

Twenty male rats (*Rattus norvegicus*, Wistar, Harlan Italy S.r.l.) were bred at the animal facility of the University of Trieste. The experiment was approved by the bioethical committee of the University of Trieste (doc. 865PAS12). The experiments on the animals were carried out in compliance with the provisions of the European Community Council Directive. Rats (n=20) at the same age (12 weeks) and weight (288 ± 20 g) were maintained in temperature-controlled rooms at 22-24 °C, 50-60% humidity and 12 hours light/dark cycles. They were fed until the night before the experiment with standard laboratory chow.

During the *in vivo* experiment the rats were kept under quiet conditions. The cages were covered with cloth and so they were either sleeping before the anaesthetic injections or were awake but without little apparent nervous behaviour. After the anaesthetic the animals were left alone to fall asleep in another cage, also covered. In all cases the animals' conditions were monitored.

Polyphenol Microbial Metabolites Mixture for Intravenous (I.V.) Administration. The polyphenol metabolites mixture was made up of 23 different metabolites selected from among the product of polyphenol metabolism by gut microflora after the consumption of berry fruits. The metabolites were selected by the literature and the dosage for each metabolite was chosen while seeking to maintain the same levels and ratios as reported in the literature. Each metabolite was dissolved in methanol and then mixed together accordingly to the diverse selected amounts. Detailed information regarding the polyphenol metabolites mixture dosages is given in Figure 2.

Treatment of Rats. The rats were divided into two groups for a two-day experiment, with 2 pharmacokinetic time point per day. The first day, time points 15 min. and 2 min. were performed. Time points 5 min. and 15 sec. were performed in the second day. The night before the experiment, the rats (10 animals) were starved overnight, but provided with water *ad libitum*. They were divided into 5 different cages for the experiment.

The rats were anaesthetised with intra-peritoneal administration of Tiletamine/Zolazepam (1:1, 25 mg/kg body weight) and Xylazine (10mg/kg body weight). The Rat received an i.v. administration of 0.3 mL PBS (phosphate buffer solution), with 2.7 µmol of polyphenol metabolite mixture, dissolved in 30 ul of methanol. The blank animals, used as controls for each time point, received an i.v. injection of 0.3 ml of PBS with 30 ul of methanol to maintain the same volume and same condition as the treated animals.

During anaesthesia (10 min in all cases), the heart and ventilation rate were controlled. The rats were placed on their backs; the penis was extruded by sliding the prepuce downwards. With the use of a nipper the glans penis was held at the tip. The dorsal penis vein was then seen and exactly 10 min. after anaesthesia 0.3 ml PBS with (treated) or without (blank) 2.7 µmol of polyphenol metabolite mixture was injected using an insulin syringe. Then the injection site was pressed for a few seconds, and the glans was retracted to prevent bleeding (36). After the PBS injection one min. before the sacrifice of the rat - at the end of each time point - sodium heparin (0.1 ml, 500 IU) was injected again into the dorsal penis vein, which was exposed in the same way. For the time point 15 sec. the procedure was slightly different, after 10 min of anaesthesia 0.2 ml PBS and 0.1 ml sodium heparin with (treated) or without (blank) 2.7 µmol of polyphenol metabolite mixture was injected at the same moment using an insulin syringe.

Exactly 10 min after anaesthesia and the corresponding time point after i.v. administration (15 sec., 2 min., 5 min., and 15 min.), the rats were sacrificed by decapitation. Blood was drained from the body. Urine was collected through the urinary bladder with a syringe. Liver, kidney, heart and brain were excised from the

body, washed with MilliQ water and immediately frozen in liquid nitrogen and stored at -80 °C.

Organs Collection and Extracts Preparation. *Biofluids.* After sampling, an aliquot of blood (5 ml) was transferred into aqueous methanol (95:5, v/v) in a ratio of 1:9 (v/v). Cinnamic acid-d_5, as internal standard, was dissolved in aqueous methanol at a concentration of 0.1 mg/L for the monitoring of the extraction protocol. The sample was shacked and extracted for 10 min. The extract was then centrifuged for 5 min at 3600 rpm at 4 °C, blood extract with the buoyant (non-cellular) portion was transferred to 50 ml dark glass vessels and stored at -80 °C (37).

In the same way an aliquot of urine collected with a syringe was extracted with aqueous methanol (95:5, v/v). The urine was weight and the amount of solvent for extraction was adjusted to the ratio 1:9 (w/v). The urine was centrifuged and then transferred to 10 ml dark glass vessels and stored at -80 °C. Biofluid samples after solvent extraction were ready for the subsequent clean-up purification protocol for the injection in the UPLC-MS/MS system.

Tissues. Organs, frozen in liquid nitrogen immediately after excision, were stored at -80°C. Frozen liver, kidney, heart and brain were grounded under cryogenic conditions, using liquid nitrogen, with a CryoMill (Retsch, Germany) using a single 25 mm i.d. steel ball (30 sec. with a frequency of 25/sec.). Tissue powder (1 g) was transferred (without thawing) into aqueous methanol (5:95 v/v) at a ratio of 1:9 (w/v). Cinnamic acid-d_5, as internal standard, was dissolved in the aqueous methanol at a concentration of 0.1 mg/l for the monitoring of the extraction protocol. The samples were extracted with an orbital shaker for 10 min., centrifuged and decanted, as described for blood (37). The final volume was then adjusted to 10 ml to balance any possible variation in the amount of water among the organs and stored at -80 °C. Tissue samples after solvent extraction were ready for the clean-up purification protocol before injection in the UPLC-MS/MS system.

Solid Phase Extraction (SPE) Purification and Sample Preparation. SPE protocol was applied following a procedure developed by Passamonti et al. (38), but with some modifications and as validated by Gasperotti et al. (Chapter 2.1). The cartridges used for the SPE purification were Biotage Isolute ENV+, 1 g (Uppsala Sweden).

An aliquot of extracted sample (5 mL), was evaporated and reconstituted with 10 ml of H_2SO_4 0.01 N in water. The conditioning of cartridges was carried out using 20 ml of methanol and 20 ml of H_2SO_4 0.01 N in water. After loading the sample, the cartridges were washed with 10 ml of Milli-Q water, dried under a nitrogen stream and eluted with 20 ml of methanol. The eluates were evaporated to dryness and the samples were dissolved in 500 µL of methanol/water (50:50 v/v). Butyric acid-d_7, as internal standard, was dissolved in the methanol/water (50:50 v/v) at a concentration of 1 mg/l and added to the sample for the monitoring of quantitative recovery during sample reconstitution. Samples were filtrated with a 0.22 µm filter and injected in the UPLC-MS/MS system.

Targeted Metabolomics Analysis. Targeted metabolomics analysis was performed with an ultra performance LC (UPLC) system coupled to a triple quadrupole (TQ) mass spectrometer. The UPLC system used was a Waters Acquity UPLC system (Milford, Massachusetts, USA). Separation of the 23 target metabolites, and 2 deuterated internal standards was performed with a Waters Acquity UPLC column (Milford, Massachusetts, USA), HSS T3 (100mm x 2.1mm, 1.8 µm) equipped with the proper guard column. The injection volume was 10 µL. Mobile phases of 0.1% formic acid in Milli-Q water (A) and 0.1% formic acid in acetonitrile (B) were used. Chromatographic separation was performed using a gradient as follows: 0 min, 5% B; 0-3 min, 5%-20% B; 3-4.30 min; 20% B; 4.30-9 min, 20%-45% B, 9-11 min, 45%-100% B, 11-14 min, 100%; and 14.01-17 min, 5% as equilibration time. The TQ mass spectrometer used was a Waters Xevo TQ (Milford, Massachusetts, USA) coupled with an electrospray interface. The two most abundant fragments were selected for each metabolite to establish a MRM (multiple reaction monitoring) quantitative

method. The first transition, corresponding to the most abundant fragment, was used as quantifier ion, and the second as qualifier ion. Detailed information regarding the UPLC-MS/MS method and quantification are described in Gasperotti et al. (Chapter 2.1).

For calibration, a standard mixture of polyphenol metabolites was serially diluted in aqueous methanol (50:50), in a concentration range 0.01 µg/L - 100 mg/L. The range of calibration curves was obtained on the basis of the linearity of the responses. Acceptable linearity was achieved when the coefficient of calibration curves (R^2) was at least 0.99. Quantitative data were processed with Targetlynx software (Masslynx, Waters). Quality control acquisition, recovery and data variability information are reported in the supplementary material.

Blood Residual Subtraction for the Brain Samples. The concentrations in the rat brain may be significantly influenced by the quantities of metabolites in the residual blood. The correction was made by estimating the amount of metabolite in the intravascular blood present in the brain, assuming that the volume of blood brain is 47.7 µL/g (39) and then subtracting this from the total amount found in the brain. The results of this subtraction are presented in Figure 4 compared also with the data of the brain before the blood residual subtraction.

Calculation of Pharmacokinetics Parameters. Pharmacokinetics parameters were calculated using the GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) with the function one phase decay suitable for I.V. injection experiments. For each metabolites detected and quantified in blood, the extrapolated dose at time 0 (t_0), the dose at the steady state (t_∞) were obtained from the plotted curves. The area under the curve ($AUC_{0 \rightarrow 15\ min.}$) and the elimination rate (K_e) were also obtained from the plotted curves.

Statistical Analysis. The quantitative data for the PMM levels are presented as mean values ± standard error (n = 4) for all the graphs. Difference between time

points in the brain, compared with the control were evaluated by the Student's test, with $p < 0.1$ considered statistically significant.

Results and discussion

The Tested Mixture of Polyphenol Microbial Metabolites: a Rational Choice. The strategy was to simultaneously inject 23 metabolites, composing a well-defined mixture (figure 1), in a single animal. The metabolites were chosen for being products of the gut-microflora metabolism of polyphenols occurring in berries (11–13, 17, 40). Their individual concentrations in the mixture were chosen to mimic their average plasma concentration, after a putative intake of a regular berry fruit serving (41). All individual molecules are commercially available, except for urolithin A and B. The latter are regarded as specific biomarkers of gut microbial metabolism of dietary ellagitannins. They were synthesized in house (35).

An analytical method, based on UPLC-MS/MS, enabled to simultaneously measure this set of compounds in biological fluids and tissue extracts from a single rat, achieving a remarkable reduction of experimental animals, as recommended by regulatory bodies (42).

The *in vivo* injection of a multi-component mixture could give rise to certain problems. Some metabolic pathways could transform some metabolites into other compounds already present in the mixture, so altering the chemical composition of the injected mixture. Moreover, the simultaneous injection of different metabolites could affect the rate of membrane transporters, enzyme pathways or cellular regulatory mechanisms, resulting in competitive interactions, rapid states of saturation or cooperative responses, just to mention a few cases. Nevertheless, the injection of a multi-component mixture offered the possibility to simultaneously assess the fate of each compound, so closely simulating a nutritionally relevant

scenario in blood, even if in the presence of other congeners or further metabolites not observed. This was the main goal of the study.

It should be therefore appreciated that this mixture was designed to represent what results from two principal biochemical processes of the mammalian digestive tract after the dietary intake of fruits rich in polyphenols (Figure 2, panel A.). The first one is the transformation of a raw mixture of dietary plant polyphenols by the gut microflora, yielding a digested extract. In this scenario the gut microflora represents the connection or the mediator between plant and mammalian systems. The second one consists in the selective transport of the digested extract across the intestinal epithelial barrier, which can be regarded as a filter, into the blood, and the subsequent tissue distribution.

Dosage, Route of Administration and Time-line: a Strategy for an Accurate Tissue Distribution Analysis. The most accurate approach to study the tissue distribution of PMMs is to administer the PMMs by intravenous injection, so to avoid the main factors of inter-individual variability of gut metabolism and absorption (6). Any other downstream process, such as distribution into tissues and eventual metabolism is comparatively more homogenous among individuals (43) allowing to attain a satisfying variation coefficient in all measured parameters using reduced numbers of animals (n=4).

The injected dose was 2.7 μmol, resulting from the sum of 23 individual amounts of PMMs (Figure 1), spanning from 0.9 μmol (gallic acid) to 0.0005 μmol (sinapic acid). Considering that the rats (body mass=288±20 g) had an estimated blood volume of 16 ml (44) and assuming that all the mixture components has free access to the internal volume of blood cells, the initial blood concentration was 168 μM, spanning from 56.25 μM (gallic acid) to 0.31 μM (sinapic acid). These values are within the concentration range observed in humans following consumption of a standard serving of berry fruits (45).

An overview of the time-line and the analytical procedure, from sample preparation to instrumental analysis, for the data acquisition of the experiment is

graphically presented in Figure 1, panel B. The experiment was designed on the basis of previous data showing the exceptionally fast uptake and metabolism of a dietary flavonoid, i.e. cyanidin 3-glucoside, an ubiquitous pigment in red fruits (43). It was assumed that the plasma disappearance kinetics of low-molecular weight metabolites of flavonoids and other dietary polyphenols should follow a quite similar pattern.

The site for the intravenous injection of the PMMs mix was the dorsal penis vein, easily accessible through a thin epidermal layer and afferent to the general circulation (36). The minimal manipulation of an anaesthetized animal is seen as a crucial factor, since any inflammation mediator locally released following a surgical trauma might affect the permeability of the blood-brain barrier and/or the basal functions determining distribution (i.e. membrane transporters), metabolism (i.e. enzymes) and excretion (again, membrane transporters).

The choice of the time points seems appropriate for a reasonable description of the time course of both disappearances of the injected molecules from the blood and their presence into the organs and the urine. Under the chosen conditions, the animals experienced minimal duration of anaesthesia and of physical stress. Overall, this experiment is a refinement of previous protocols adopted by us (37, 43).

The Presence of Basal Levels of the Mixture Components in Control Animals. Many of the mixture components arise not only from the microbial metabolism of dietary polyphenols, but also from mammalian catabolism of endogenous substrates (41). Thus, some are expected to occur in the rat organs and fluids at basal levels in control animals. Compounds that are exclusive products of microbial metabolism, for instance, are the urolithins, the ferulic acids, gallic acid, coumaric acids and protocatechuic acid (41). However, also these might be expected to occur in control animals, being markers of a normal nutritional status, like, for instance, ferulic acid. The basal levels of the 23 mixture components were in general very low. Exceptions are mentioned case by case below.

Tracing Polyphenol Metabolites in Blood. The blood of control rats contained 11 out of the 23 injected molecules. Of these endogenous compounds, see Table 1, six were less than 1% of the amount occurring in the injected mixture, other 3 were less than 2%. The 2 outliers were p-coumaric acid (3.3%) and phloroglucinol (17.8%). Thus, the selected mixture represented a valid challenge for a pharmacokinetics test, because of these very low basal levels. This test seemed to reliably simulate what happens in the organism after consumption of a typical berry fruit serving, after a short period of "wash out".

Most injected compounds disappeared following apparent pseudo-first order kinetics, as shown in Figure 3. The calculated parameters, i.e. the extrapolated amounts at time zero (t_0) and at steady state (t_∞), and the disappearance rate constant (k_e) are listed in Table 1 according to the value of k_e, which spanned from 2.73 to 0.38 min^{-1} ($t/2$=0.25 to 1.84 min), pointing to a quite rapid process. There were a few exceptions.

One compound, i.e. pyrogallol, could never be detected, though its recovery and analysis posed no specific issues; the mechanisms whereby it underwent disappearance cannot be guessed. Two compounds, i.e. pyrocathecol and 3,4-dihydroxyphenyl acetic acid, were detected only at 15 sec. Urolithin B, detected at very low levels at both 15 sec and 2 min, rose thereafter by an order of magnitude. Phloroglucinol occurred at a similar level during the entire experiment.

With the above-mentioned few exceptions, steady state values were attained even earlier than 5 min, so assuring that the duration of the experiment was appropriate. In most cases, the compounds were essentially disappeared at 15 min.

The calculated amounts at t_0, and the amounts at any time points, were always lower that the sum of the injected dose and the endogenously present compound in control samples, if any. Expressed as percent of the injected amounts in Table 1, the calculated t_0 ranged from 43.5 % (m-coumaric acid) to 1% (gallic acid). This is no longer surprising; having already seen how fast is the uptake and excretion of one of the precursors of these metabolites, i.e. the fruit pigment cyanidin 3-glucoside (43).

An explanation of the loss of part of the injected dose and of the extremely rapid blood disappearance may be a quick uptake and distribution in organs. Only some major organs were collected, thus an accurate recovery assessment cannot be provided. The compounds might have also distributed in large tissues, such as the adipose tissue, the vascular endothelium, or the connective tissue. Moreover, the administered compounds could be metabolically transformed, but these were not analysed in this experiment, which is the first attempt of a simultaneous kinetic characterization of 23 polyphenol metabolites.

Polyphenol Microbial Metabolites in the Brain. The main findings concerned the detection of PMMs in the brain, at times when their blood concentrations were vanishingly low (Figure 4). Each plot shows 2 curves, representing the total amounts measured in whole brains (dot line) and those corrected for blood residual (solid line), as recommended (39, 46–48). Indeed, brains were not perfused to wash out the blood, since this procedure would have altered the chemical equilibrium between the vascular compartment and the whole brain.

The more so since 13 out of 23 compounds of the mixture were found in the control brains, as endogenous metabolites (Table 2). Disregarding the time of observation, 10 compounds out of 23 where found in the brain at a significantly increased amount with respect to the control (Table 2). In most cases, their appearance in the brain was biphasic, with an early wave at 2 min, with 4 compounds, and a second one starting at 5 min; at 15 min, 9 compounds were detected. Gallic acid, which was the most abundant compound in the mixture (Fig. 2) best showed this bi-phasic accumulation trend. Noteworthy, it accumulated in the brain by about 11 times above the basal concentration measured in the control as early as 15 sec. Similarly, caffeic acid had two peaks, and accumulated by ca. 20 and 34 times at 2 min and 15 min, respectively (Table 2).

Importantly, two compounds were neither detected in the brain as endogenous metabolites nor ever appeared in the brain, i.e. *trans*-isoferulic acid and o-coumaric acid, in spite of their relatively low plasma elimination rate constant, so

proving that the blood-brain barrier was intact during the experiment. Other endogenous compounds never increased their concentration in the brain, i.e. protocatechuic acid and phloroglucinol.

Those compounds that accumulated in the brain should be active, because in all cases, except for urolithin B, they were also endogenous metabolites already present in the control brains. Their most predictable effect is unbalancing a pre-existent metabolic equilibrium. Ferulic acid, accumulated at 15 sec in the experiment, is reported to be able to reduce oxidative damage and amyloid pathology in Alzheimer's disease (49). Some PMMs are identical to metabolites of neurotransmitters. For instance, vanillic acid and homovanillic acid are catecholamine catabolites, found in the in brain and in cerebrospinal fluid (50). Moreover, 3,4 dihydroxyphenyl acetic acid is neuronal metabolite of dopamine and involved with the dopamine catabolism in the pathogenesis of Parkinson's disease (51). Another mechanism of action is by affecting enzyme kinetics. For instance, caffeic acid may bind to tyrosine ammonia lyase, thus altering serotonin homeostasis (52), or by inhibiting acetylcholinesterase and butyrylcholinesterase activities and as consequence preventing oxidative stress-induced neurodegeneration (53). Gallic acid has also demonstrated activities on key enzyme activities in the brain (26, 54) and able to accumulate in the brain after repeated dose (55).

Polyphenol microbial metabolites in other organs. The analyses of PMMs in the heart, the liver, the kidneys, and the urine are shown in Fig. 5 (and in Supplementary Figures 1-4). Eighteen out of 22 compounds attained quite high concentrations in the urine (Figure 5, Supplementary Figure 1), with the exception of pyrocatechol and urolithin B, which were not found in the urine, urolithin A, which concentration slightly increased, and phloroglucinol, which did not change with respect to the value found in control animals. Thus, most PMMs can be defined as nephrotropic. Indeed, 20 out of 22 compounds dramatically increased their parenchymal concentrations from basal values of zero or close to zero up to values higher by at least 2 orders of magnitude (Figure 5, Supplementary Figure 2). For this

extraordinary velocity, the apparent rate of accumulation in the kidneys could not be calculated. Accumulation lasted no more than 2 min and often less, after which time their concentration started decaying. By contrast, the appearance in the urine was slower, with a lag phase lasting 15 sec before the onset of urinary excretion, seen in most cases (20 out of 22).

Only a few compounds were found to be rather hepato- than nephrotropic, such as phloroglucinol, urolithin A and 4-hydroxybenzoic acid (Figure 5, Supplementary Figure 3). One compound (sinapic acid) could not be detected in the liver for 3 consecutive times, at which it was however present in the blood. Thus, the amount of blood retained in the organ was absolutely negligible. Similarly, the basal levels of 3-(3-hydroxyphenyl)propanoic acid did not change in the liver, in spite of its presence in the blood at concentrations higher by 2 orders of magnitude.

Only 3 compounds were found to be cardiotropic, i.e. vanillic acid and both urolithins (Figure 5, Supplementary Figure 4). They accumulated to a maximum concentration at 15 sec, but then vanillic acid and urolithin A disappeared, whereas urolithin B started to increase again at 5 min. Three compounds (phloroglucinol, 3,4-dihydroxyphenyl acetic acid and pyrocatechol), though present in the blood, could not be detected in the heart, showing the negligible blood contamination.

The low apparent hepatotropism of most PMMs leads to conclude that PMMs absorbed from the colon under normal conditions undergo limited first-pass metabolism into the liver, so that they can distribute to the other organs readily after absorption. Then, they are eliminated in the urine after prior accumulation in the kidneys. Only one compound, i.e. 4-hydroxybenzoic acid, appeared in the urine at 15 sec, likely by glomerular filtration.

The Case of Urolithins. Urolithins are the largest molecules among the PMMs (Figure 1), arising from microbial metabolism of ellagitannins and ellagic acid conjugates (56). Urolithin A differs from urolithin B for the one additional hydroxyl group. No other mammalian enzyme pathway is known to produce such end-products from more complex precursors. In facts, none of them was detected in the

organs of control animals. Urolithin A and B were administered at different doses, i.e. 55 and 25 nmol, respectively. Nevertheless, their concentrations in the blood differed by an order of magnitude at 15 sec (332.6 and 20.3 pmol/ml, respectively). Thus, urolithin B was sequestered (and/or metabolized) more rapidly then the other in the tissues.

Both of them displayed a unique pattern of blood concentration, tissue distribution and urinary excretion. Indeed, though urolithin A had a normal mono-exponential disappearance from the blood, urolithin B, increased in the blood after 2 min from a very low level (see above). It seems, therefore, that urolithin A underwent a so unusual as mechanistically unexplained de-hydroxylation reaction. Only bacterial de-hydroxylases are known.

Both of them were negligibly excreted in the urine, unique cases among the PMMs. They were presumably excreted as glucuronyl derivatives (11), which were not analysed. Rapid uptake of urolithin A into the kidneys was observed, whereas urolithin B appeared in the kidneys only later than 2 min, i.e. when it was also present in the blood. The liver slowly took up urolithin A up to 5 min, but no urolithin B could be detected there for 15 min. This shows that the liver could neither convert urolithin A to urolithin B, nor take the latter up from the blood.

The organ that displayed the highest capacity in this sense was the heart, where both urolithin A and B were found at 15 sec, at about 300 and 2000 pmol/g, respectively. It seems therefore that the following happened: i) urolithin A was rapidly (by 15 sec) taken up into the heart and de-hydroxylated to urolithin B by unknown enzyme(s); in the heart, both urolithins could be converted to other compounds, that were not followed; ii) urolithin B was released from the heart into the blood, where indeed it raised from 2 min to 5 min; iii) from 5 min on, the tissue concentrations of urolithin B increased at higher levels than in the blood.

Urolithins have a demonstrated activity in reducing glycation of proteins (57) in neuronal cell, and possibly even in the heart. In facts, the advanced glycation endproducts, deriving from the glycation reaction in heart and vascular tissue are

responsible of the reduction of artery and heart elasticity and have a role in the progression of cardiovascular complication associated with diabetes (58).

Is the Brain a Target of Polyphenol Metabolites? The findings would tell so. First, not less than 13 PMMs were found at basal levels in control brains and this is the first report, to our knowledge. As a consequence, the possibility of their passage through the blood-brain barrier must be assumed a priori, as a process to ensure their exchange with the blood compartment.

Ten out of 23 were found significantly increased after the injection of the PMMs mixture in the treated animals. Some of them might have an exquisite dietary origin, such as gallic acid or *t*-ferulic acid. Others could instead be catabolites of endogenous compounds, such as catecholamine, dopamine, aminoacids and others. Fig. 6 highlights the concentrations of gallic acid attained in all the organs considered at various times. The brain shows a clear capacity to extract gallic acid from the blood, even higher than the liver. On the contrary, the tightness of the blood-brain barrier is demonstrated by its absolute impermeability to o-coumaric acid, in spite of its higher hydrophobic index (logP: 2.45, while gallic acid logP is 0.91). Yet, both gallic acid and o-coumaric acid occurred in the blood at similar concentrations.
These data provide further health implications of the brain-gut microbiota axis (53), highlighting how PMMs could be one of the putative connections/messengers between microflora and brain. These bidirectional interactions, for instance was already explored highlighting the importance of the gut microbiota for brain development and behaviour (54), or for the influence on anxiety and depression (55). Being the PMMs the product of the dietary intake of polyphenols and the metabolism of the gut microflora, it is reasonable to assume that those compounds might be able to regulate the human health and disease state (56). Moreover the role of the gut microbiota in the alteration of mammalian blood metabolite levels was already demonstrated combining data from germ free and conventional animals (57). Their action might be in the reduction of oxidative stress, but more likely in the modulation of biochemical and physiological processes, changing the level of endogenous

compounds also in the brain, along with other organs as observed in the present study.

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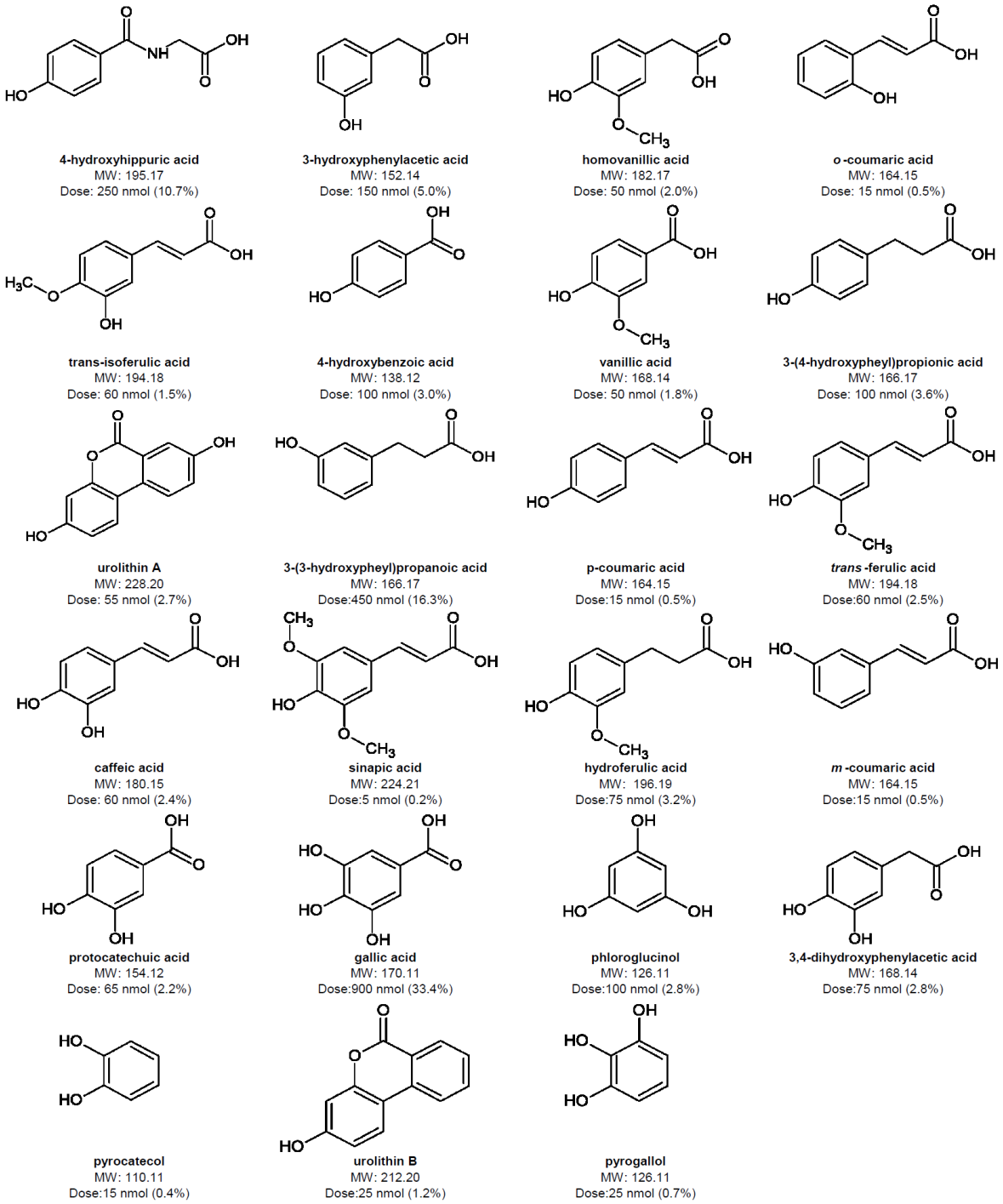


Figure 1 Mixture of polyphenol microbial metabolites used for the intravenous injection, their chemical formula, molecular weight (MW), dose and relative percentage on the total amount injected, 2690 nmol (dosage:168.13 µM)

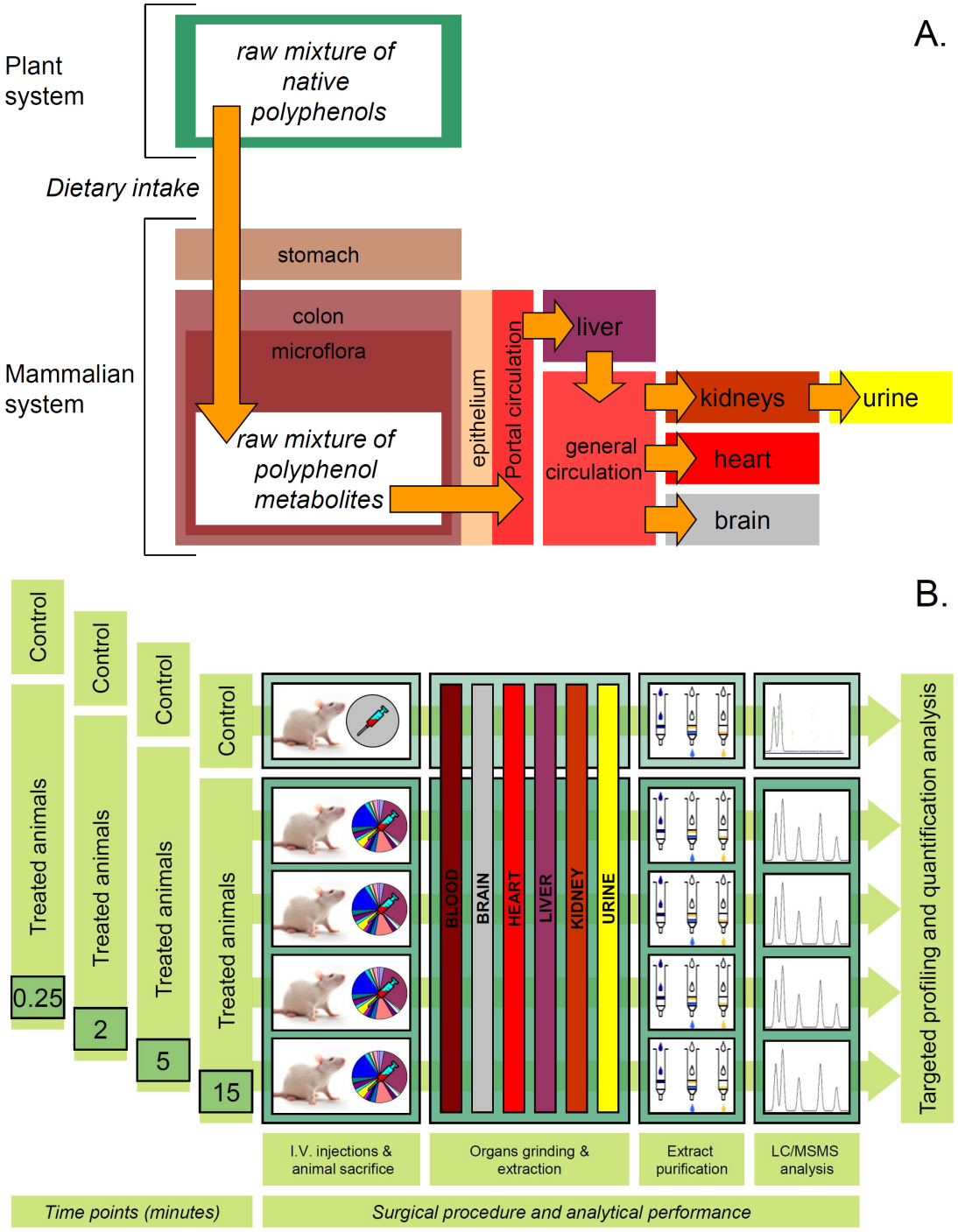


Figure 2 *Panel A.* represents the main biochemical processes of the mammalian digestive tract, from the transformation of a raw mixture of dietary plant polyphenols by the gut microflora, yielding a raw mixture of polyphenol metabolites and their distribution, after the passage through the intestinal epithelial barrier. *Panel B.* represents the time points observed for the kinetics and the main surgical and analytical steps for the acquisition of the quantitative information.

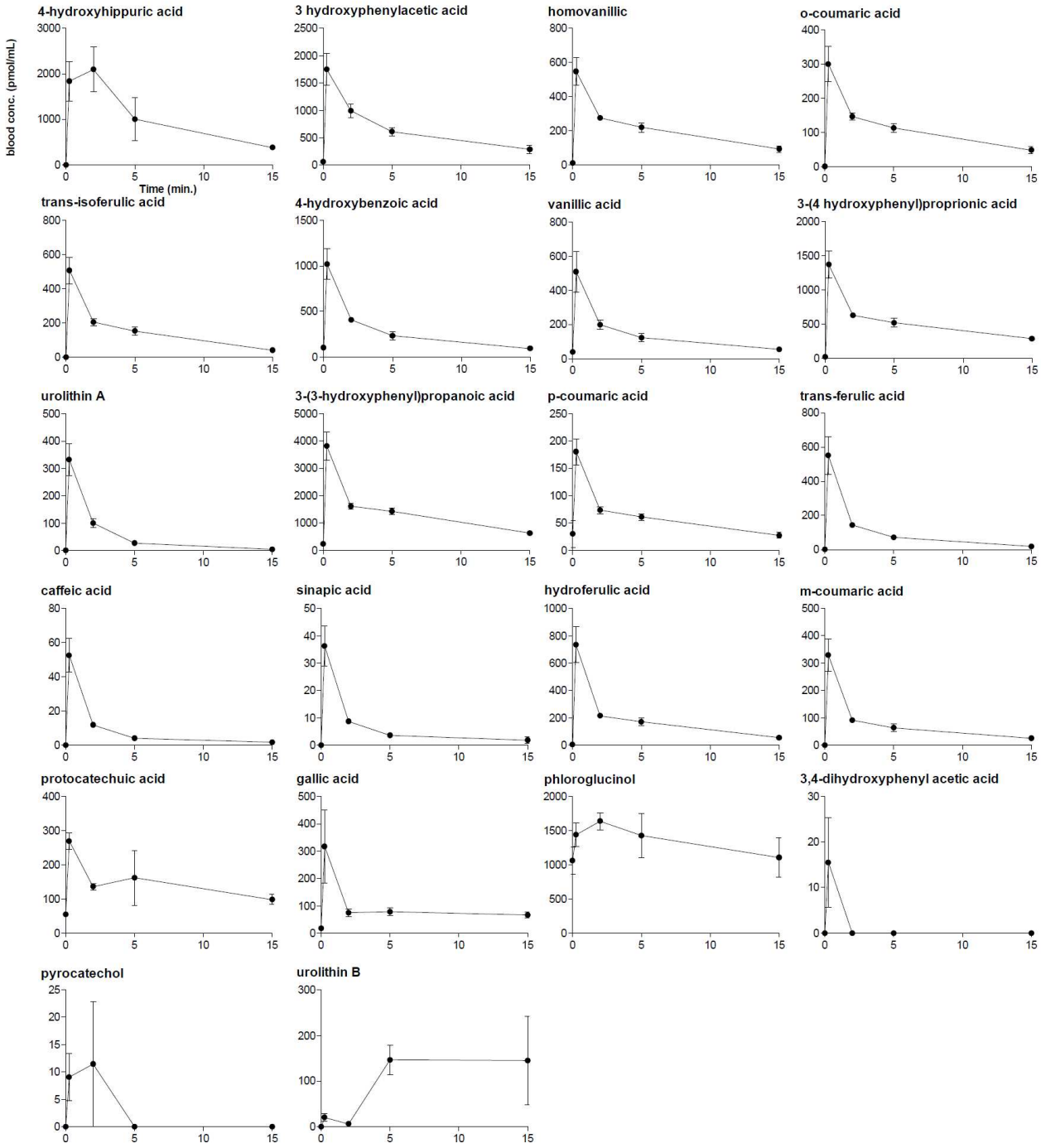


Figure 3 Polyphenol microbial metabolites in blood and their pharmacokinetic profiles

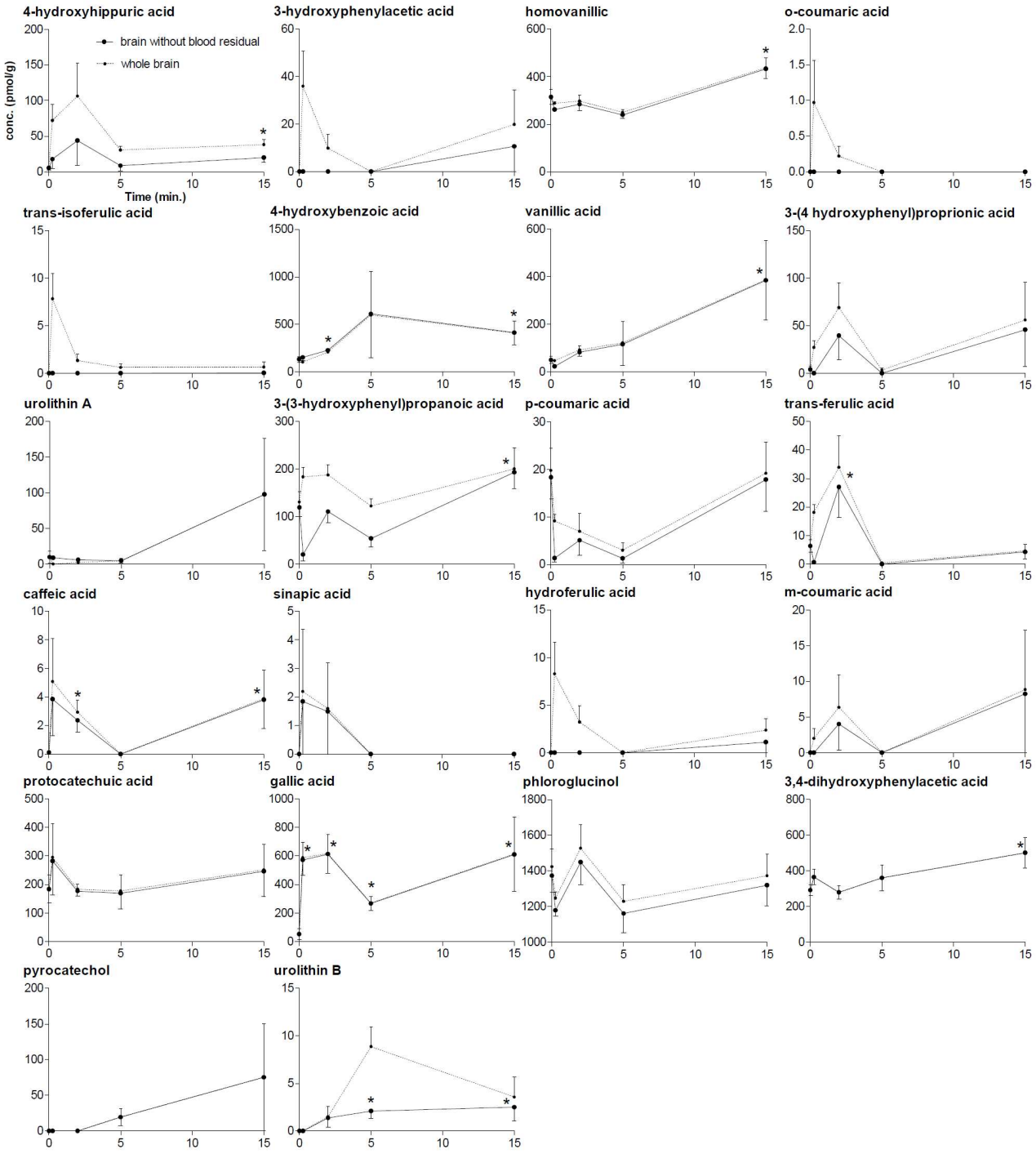


Figure 4 Polyphenol microbial metabolites in brain and their profiles. Dotted lines are referred to the amount found in the whole brain, while continuous lines are the data after the subtraction of the blood residual in the brain. Data with the asterisk are significantly increased from the control samples ($p < 0.1$).

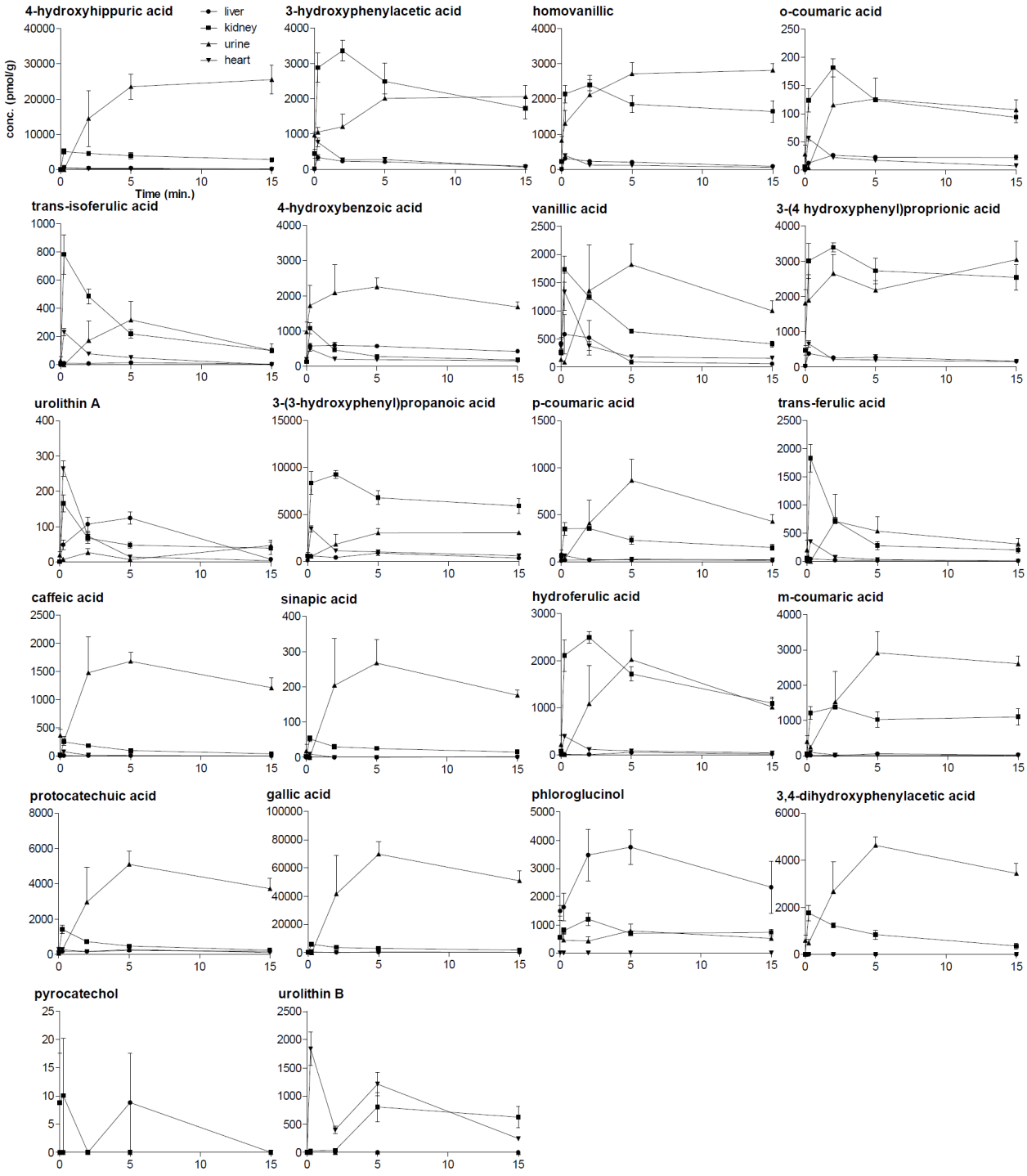


Figure 5 Polyphenol microbial metabolites in liver, kidney, urine and heart and their profiles.

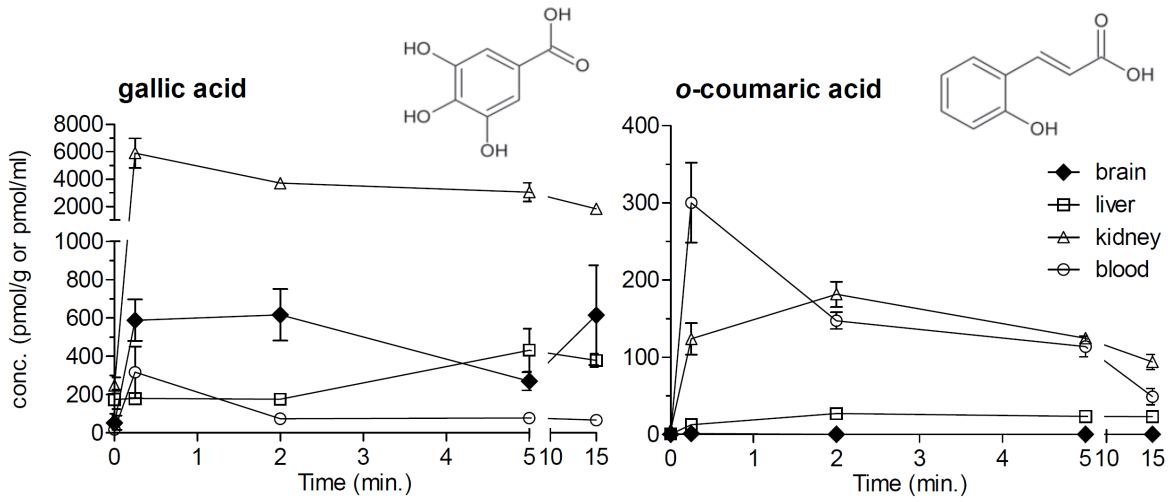


Figure 6 Profile of gallic acid and o-coumaric acid in brain, liver, kidneys and blood.

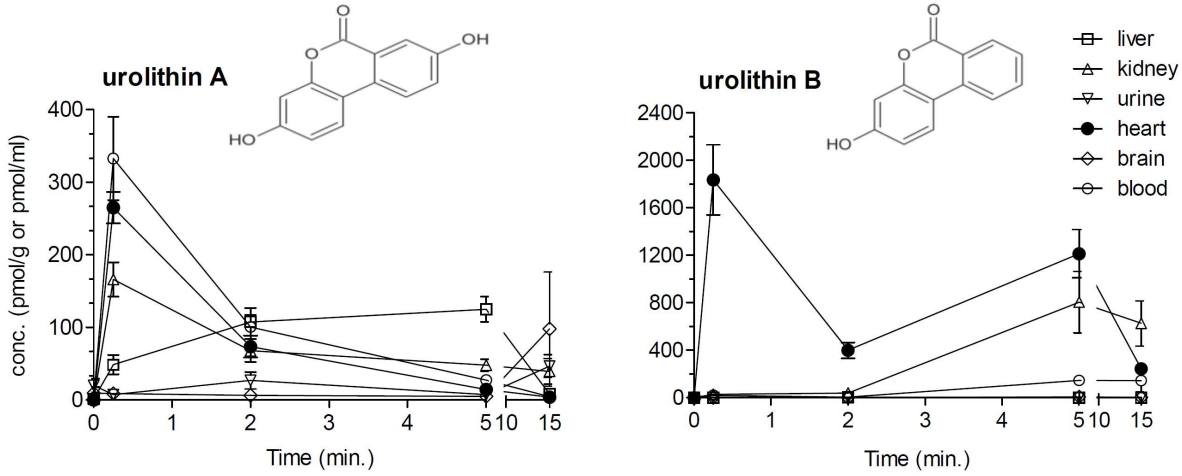


Figure 7 Profile of urolithin A and B in all the organs considered for the experiment.

Table 1 Percentage of recovery in blood compared to initial injected dose and pharmacokinetic analysis of polyphenol microbial metabolites. For the recovery the values given are the percentages of dose found in rats after the i.v. injection at four different time points ($t_{0.25}$, t_2 ,t_5, $t_{15\ min.}$) and control, sd is the standard deviation of 4 replicates. For t_0 the percentage reported is an extrapolation based upon the pharmacokinetics curves associate to a first order exponential decay for an i.v. injection.

			Time Points (min.)										Pharmacokinetics		
	dose	**t_0 ***	**$t_{0.25}$**		**t_2**		**t_5**		**t_{15}**		***Control***		**Steady state $_{t=\infty}$**	**AUC $_{(0\rightarrow15)}$**	**k_e**
	nmol	**%**	**average recovery % ± st. dev.**										**pmol/ml**	**pmol*min/ml**	**min^{-1}**
4-hydroxyhippuric acid	250	13.3	11.7	*5.5*	13.4	*6.2*	6.4	*6.1*	2.4	*0.6*	0.0	*0.0*	0.00	15017	0.09
3-hydroxyphenylacetic acid	150	19.9	18.6	*6.2*	10.5	*2.7*	6.5	*1.5*	3.0	*1.6*	0.6	*0.2*	304.50	9238	0.38
homovanillic acid	50	18.7	17.5	*5.2*	8.8	*0.8*	7.0	*1.7*	3.0	*1.2*	0.4	*0.3*	118.40	3022	0.45
o-coumaric acid	15	34.6	32.0	*11.1*	15.7	*2.3*	12.1	*2.8*	5.2	*2.3*	0.1	*0.1*	62.70	1597	0.47
trans-isoferulic acid	35	25.9	23.2	*7.1*	9.3	*1.8*	7.0	*2.3*	1.9	*1.0*	0.0	*0.0*	73.40	2125	0.58
4-hydroxybenzoic acid	100	18.5	16.3	*5.3*	6.5	*0.6*	3.8	*1.5*	1.5	*0.4*	1.7	*0.9*	131.50	3864	0.62
vanillic acid	50	18.7	16.3	*7.6*	6.4	*1.8*	3.9	*1.5*	1.8	*0.4*	1.3	*0.5*	75.70	1992	0.67
3-(4-hydroxyphenyl)propionic acid	100	24.8	21.9	*6.4*	10.1	*1.0*	8.4	*2.2*	4.6	*1.1*	0.4	*0.5*	371.50	7538	0.69
urolithin A	55	11.5	9.7	*3.3*	2.9	*0.9*	0.8	*0.1*	0.1	*0.1*	0.0	*0.0*	9.80	733	0.71
3-(3-hydroxyphenyl)propanoic acid	450	15.5	13.5	*3.6*	5.7	*0.8*	5.1	*0.8*	2.2	*0.6*	0.8	*0.6*	929.30	19569	0.72
p-coumaric acid	15	22.2	19.3	*5.0*	7.9	*1.3*	6.6	*1.3*	3.0	*1.1*	3.3	*5.4*	40.50	872	0.74
trans-ferulic acid	60	18.0	14.7	*5.9*	3.8	*0.5*	1.9	*0.7*	0.5	*0.5*	0.0	*0.1*	39.30	1387	0.88
caffeic acid	60	1.8	1.4	*0.5*	0.3	*0.0*	0.1	*0.1*	0.0	*0.1*	0.0	*0.0*	2.53	108	0.95
sinapic acid	5	14.5	11.6	*4.8*	2.8	*0.3*	1.2	*0.1*	0.6	*0.7*	0.0	*0.0*	2.40	84	0.95
hydroferulic acid	75	19.3	15.7	*5.7*	4.6	*0.4*	3.6	*1.2*	1.2	*0.7*	0.1	*0.1*	105.30	2535	0.96
m-coumaric acid	15	43.5	35.1	*12.6*	9.8	*1.5*	6.8	*2.9*	2.7	*1.8*	0.0	*0.0*	41.60	1044	0.97
protocatechuic acid	65	8.5	6.6	*1.2*	3.3	*0.5*	4.0	*3.9*	2.4	*0.7*	1.4	*0.1*	129.70	2101	1.72
gallic acid	900	1.0	0.6	*0.5*	0.1	*0.0*	0.1	*0.1*	0.1	*0.0*	0.0	*0.0*	72.40	1296	2.73
phloroglucinol	100	26.3	23.0	*5.8*	26.2	*4.1*	22.8	*10.8*	17.7	*9.8*	17.0	*6.7*	§	19951	§
3,4-dihydroxyphenyl acetic acid	75	§	0.3	*0.4*	0.0	*0.0*	0.0	*0.0*	0.0	*0.0*	0.0	*0.0*	§	13	§
pyrocatechol	15	§	1.0	*0.9*	1.2	*2.4*	0.0	*0.0*	0.0	*0.0*	0.0	*0.0*	§	35	§
urolithin B	25	§	1.3	*1.0*	0.4	*0.2*	9.4	*4.2*	9.3	*12.4*	0.0	*0.0*	§	1721	§

* extrapolated value from the pharmacokinetic curves for the dose at time zero.
§ no available extrapolated data due to the non fitting with the proper pharmacokinetic curves associated to an i.v. injection.

Table 2 Endogenous amounts of polyphenol metabolites in control brain and polyphenol metabolites significantly increased compared to control brain at any time points ($p<0.1$).

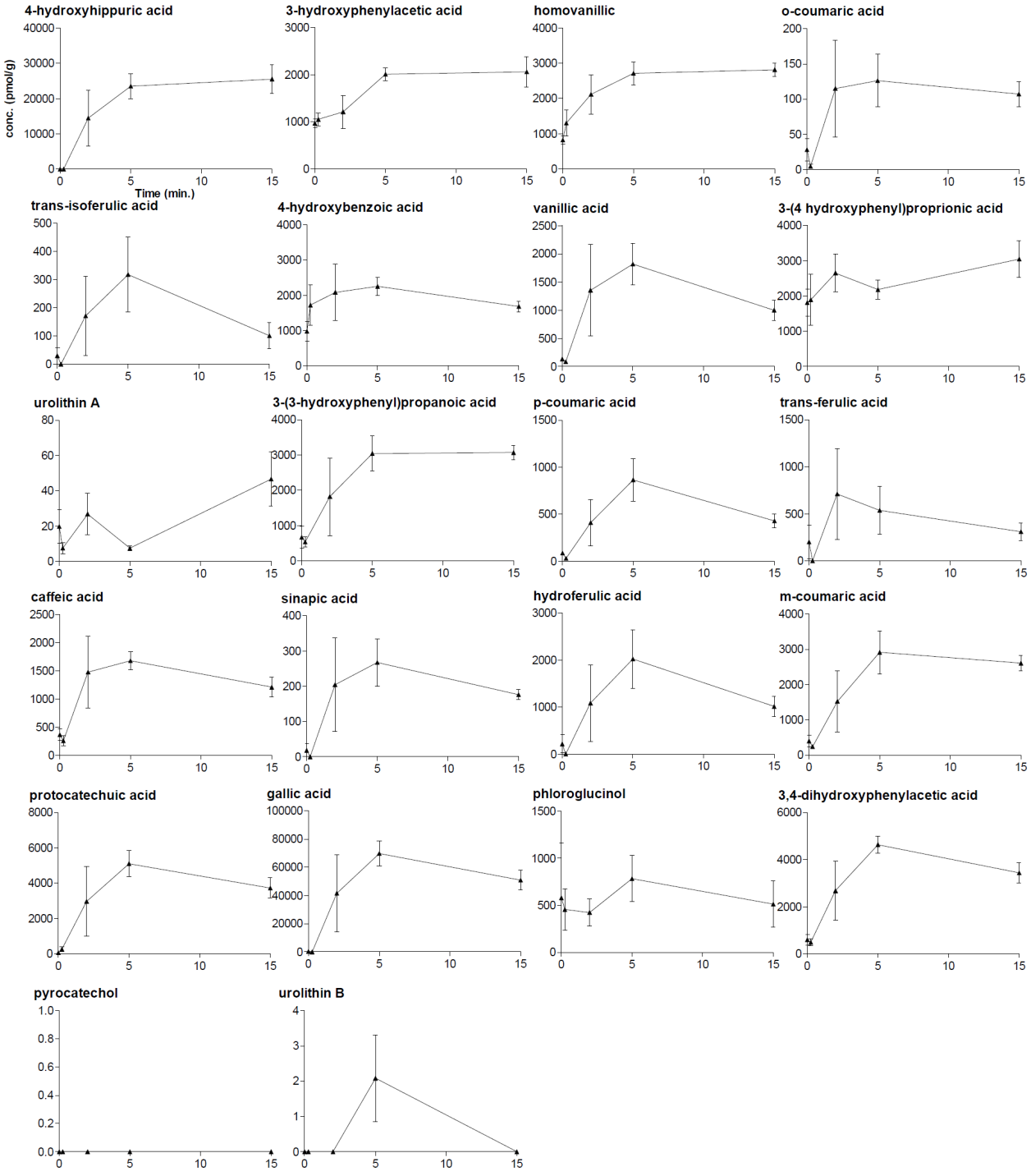
	dose	control	$t_{0.25}$	t_2	t_5	t_{15}	fold of change t_2/control	fold of change t_{15}/control
	nmol	*pmol/g*						
4-hydroxyhippuric acid	250	5.52				20.06		2.63
3-hydroxyphenylacetic acid	150	n.d.						
homovanillic acid	50	314.81				433.16		0.38
o-coumaric acid	15	n.d.						
isoferulic acid	35	n.d.						
4-hydroxybenzoic acid	100	126.63		206.97		407.92	0.63	2.22
vanillic acid	50	99.71				384.71		2.86
3-(4-hydroxyphenyl)propionic acid	100	3.88						
urolithin A	55	9.72						
3-(3-hydroxyphenyl)propanoic acid	450	119.01				192.81		0.62
p-coumaric acid	15	18.34						
trans-ferulic acid	60	6.34		27.01			3.26	
caffeic acid	60	0.11		2.36		3.81	20.45	33.64
sinapic acid	5	n.d.						
hydroferulic acid	75	n.d.						
m-coumaric acid	15	n.d.						
protocatechuic acid	65	183.40						
gallic acid	900	52.17	573.00	612.72	265.97	610.82	10.74	10.71
phloroglucinol	100	1373.36						
3,4-dihydroxyphenyl acetic acid	75	291.82				501.19		0.72
pyrocatechol	15	n.d.						
urolithin B	25	n.d.			2.09	2.50		> 2.50
compounds increased at any time ($p<0.1$)			1	4	2	9		

Supplementary Material

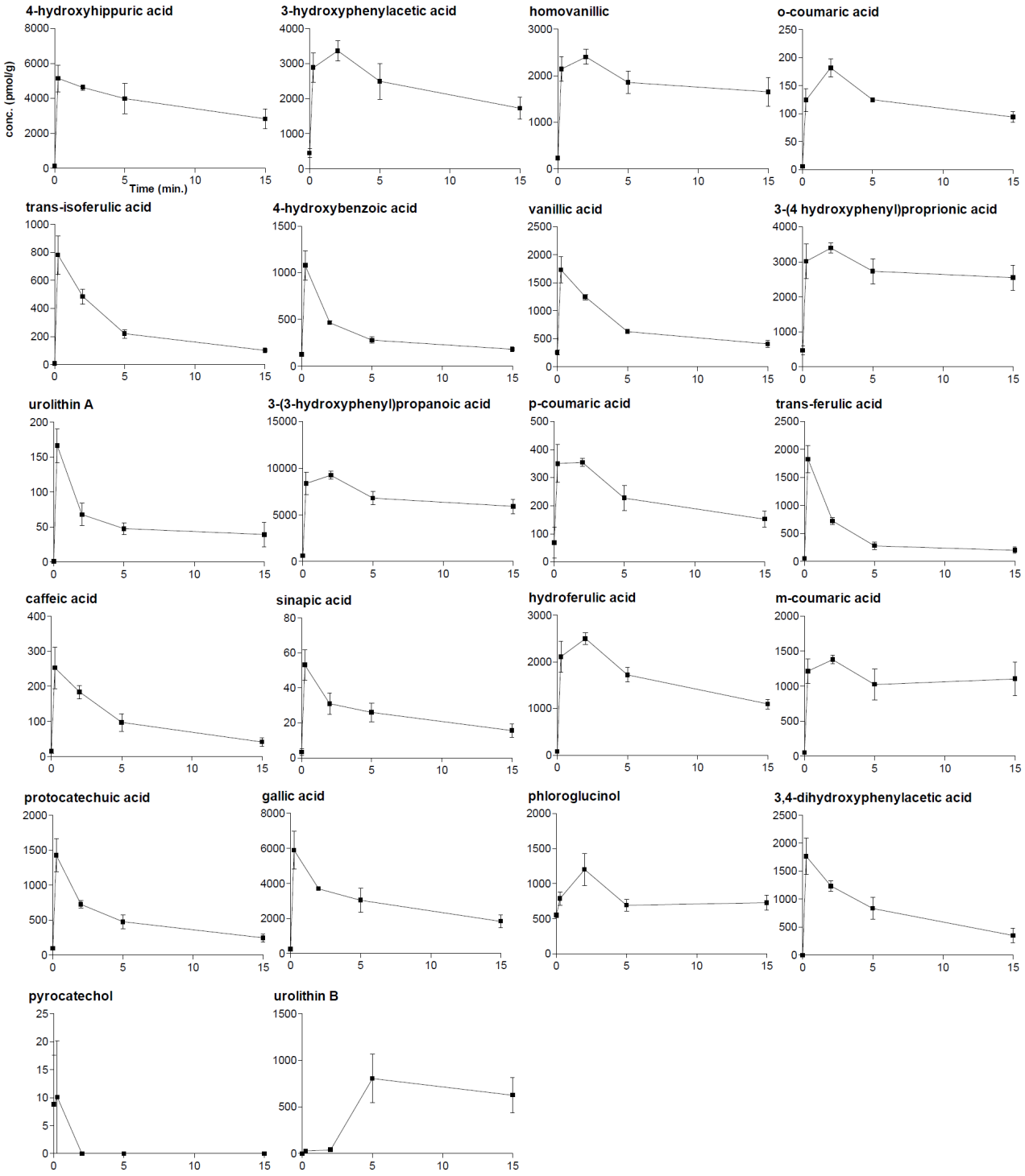
Quality Control Acquisition, Recovery and Data Variability. For each individual sample, the quality of sample preparation (extraction and purification) and analytical performance (acquisition) were constantly monitored through the quantitative results of two internal standards and polyphenol metabolite mix injected every 10 biological samples during instrumental analysis. The relative standard deviation (RSD %) for the entire batch sequence in all samples (n=126) as regards the two internal standards was 15 % and 14 %, respectively for butyric acid-d_5 and cinnamic acid-d_7. In the case of the standard mix injections (n=14), with every 10 samples, there was a relative standard deviation below 16 % for all polyphenol metabolites. Detailed information about quality control acquisition are reported in Supplementary Table 1.

The instrumental method used for the detection and quantification of the polyphenol metabolites was already validated and published by Gasperotti et al. and the recovery in all matrixes was validated (Chapter 2.1). Quantitative data for each metabolite was thus normalized with the corresponding recovery value in the different validated biological matrixes.

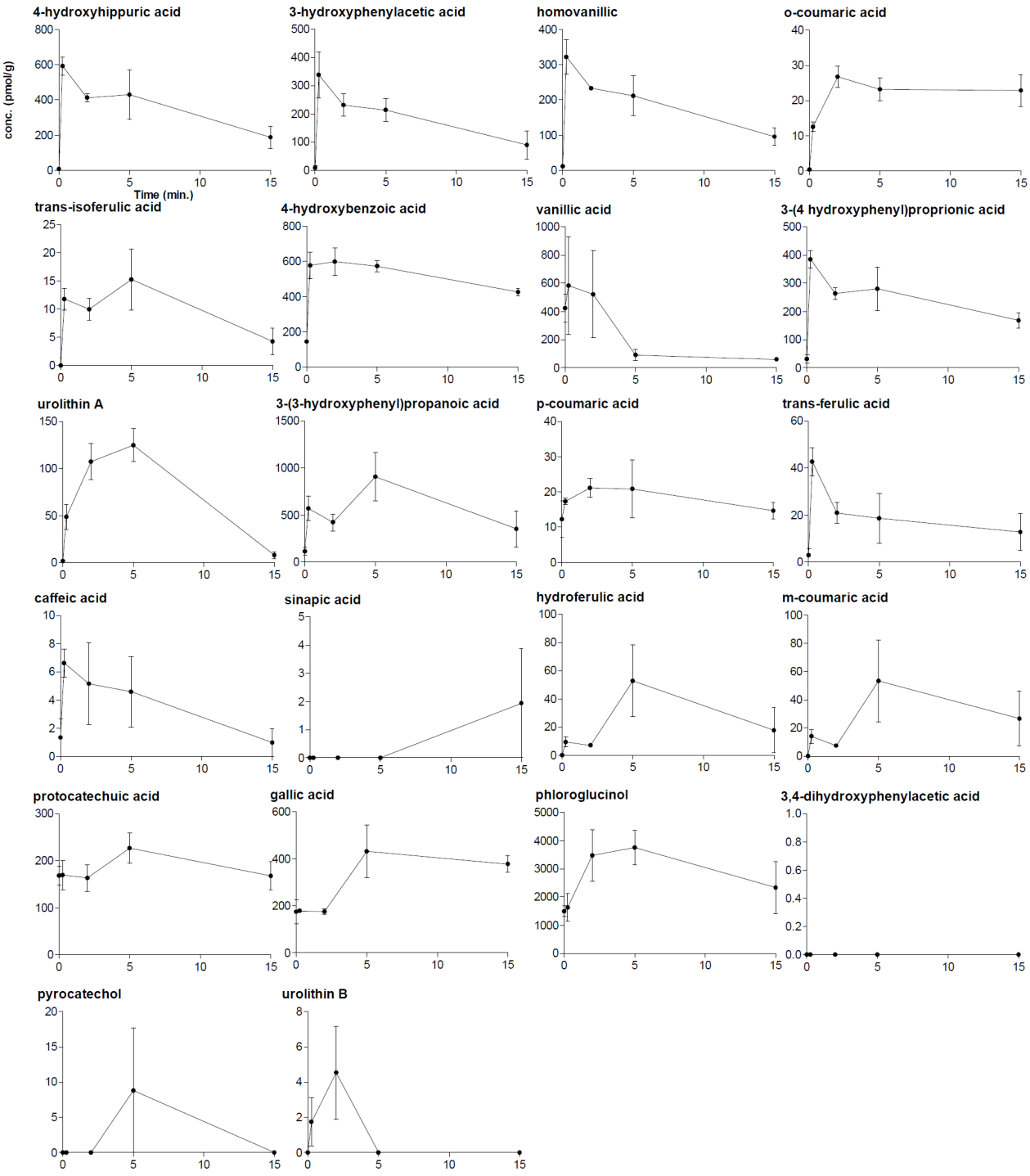
The control animals not injected with the polyphenol metabolites mix can be considered 4 control replicates. The short kinetics trials time did not affect the results of the control animals in terms of already present endogenous metabolites.



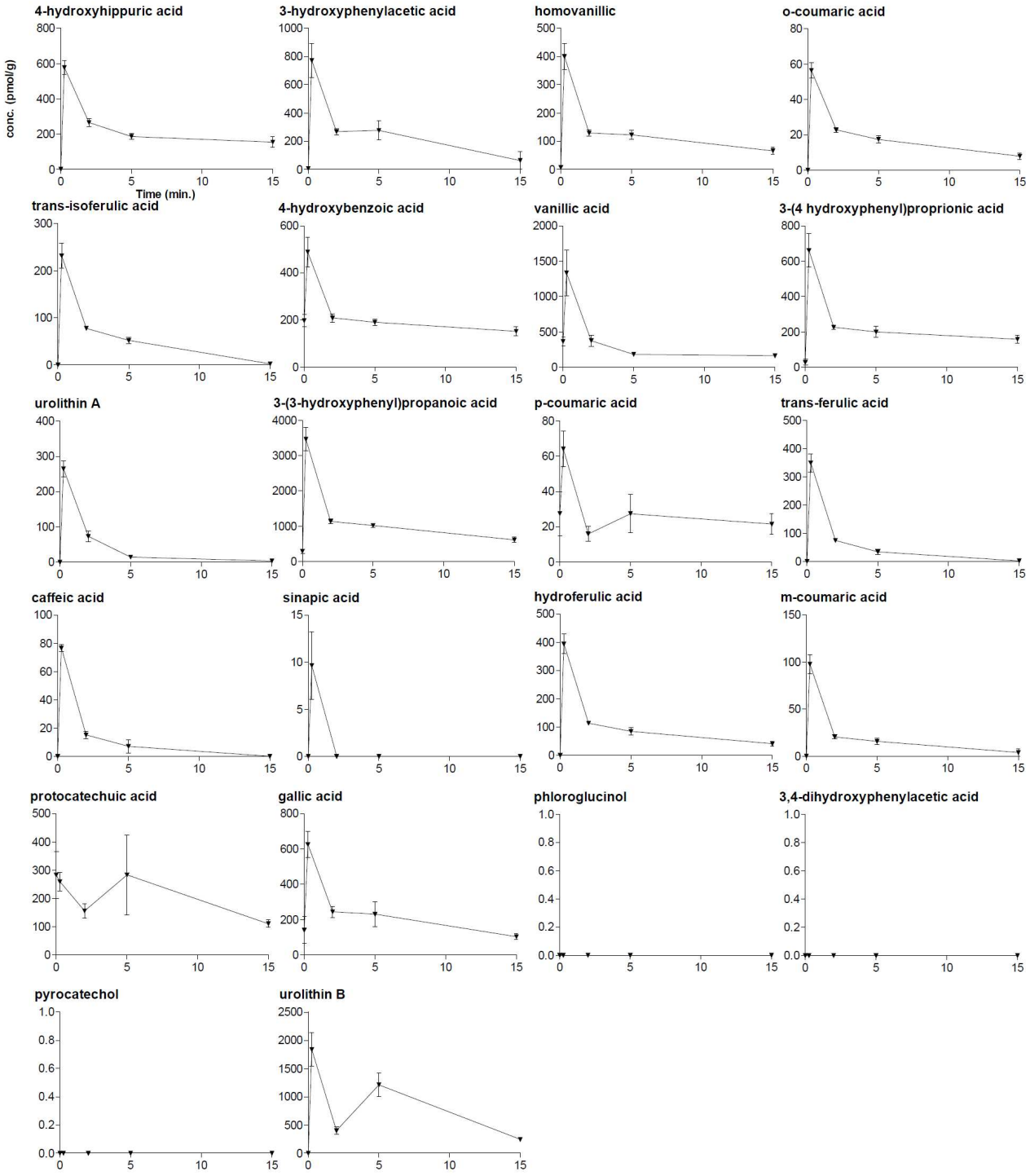
Supplementary Figure 1 Polyphenol microbial metabolites in urine and their profiles.



Supplementary Figure 2 Polyphenol microbial metabolites in kidneys and their profiles.



Supplementary Figure 3 Polyphenol microbial metabolites in liver and their profiles.



Supplementary Figure 4 Polyphenol microbial metabolites in heart and their profiles.

Supplementary Table 1 Quality control acquisition

	standards mix (n=14)			internal standards (n=126)		
	mean (mg/L)	**s.d.**	**CV%**	**mean (mg/L)**	**s.d.**	**CV%**
t-cinnamic acid d5 - IS				0.78	0.12	15
butyric acid d7 - IS				1.06	0.15	14
phloroglucinol	1.03	0.04	4			
gallic acid	1.01	0.06	6			
pyrogallol	0.96	0.04	4			
protocatechuic acid	0.94	0.06	6			
3.4-dihydroxyphenyl acetic acid	0.99	0.03	3			
4-hydroxyhippuric acid	0.95	0.07	8			
4-hydroxybenzoic acid	0.96	0.03	4			
pyrocatechol	0.97	0.07	7			
caffeic acid	0.92	0.06	7			
vanillic acid	0.94	0.05	5			
3-hydroxyphenylacetic acid	0.86	0.10	11			
homovanillic acid	0.98	0.04	4			
3-(4-hydroxyphenyl)propionic acid	0.99	0.06	6			
p-coumaric	0.98	0.04	4			
hydroferulic acid	0.86	0.07	8			
3-(3-hydroxyphenyl)propanoic acid	0.96	0.06	6			
trans-ferulic acid	0.98	0.04	4			
sinapic acid	0.78	0.12	16			
m-coumaric acid	0.98	0.03	3			
trans-isoferulic acid	0.98	0.06	6			
o-coumaric acid	0.94	0.05	5			
urolithin A	0.97	0.02	3			
urolithin B	0.80	0.06	8			

Conclusion

The main aim of this Ph.D. project was on the one hand to study a plant system, with molecular characterisation of polyphenol composition, and on the other hand bioavailability in the mammalian system. The link between these two systems, or between the two plant and mammalian metabolomes, is represented by the microbiota. Indeed the gut microbiota could be considered as an intermediary, being responsible for the alteration/improvement of the mammalian metabolome via the plant metabolome after nutritional intake and involved in shaping the variety of exogenous metabolites that interact with the mammalian metabolome.

The central biological question in the Ph.D. project was to understand whether and to what extent polyphenol microbial metabolites are able to pass through the blood brain barrier after the consumption of strawberries, since according to the literature and *in vitro* experiments they should exert some neurological protection. Moreover, in addition to their accessibility in the brain, it was planned to explore tissue distribution in other organs in order to get a clearer picture of general tissue distribution. Before *in vivo* trials of the nutritional bioavailability of polyphenol microbial metabolites, it was necessary to better characterise the native composition of strawberry polyphenols in order to obtain more precise data for dietary recommendations and nutritional studies.

In the first part of the thesis dealing with food chemistry an essential step in the study of the biological relevance of polyphenols was to develop a targeted metabolomics method for analysis of several classes of phenolic compounds, allowing better characterisation of the composition of fruits rich in polyphenols at molecular level, with a significant improvement in terms of compounds and time-consuming analysis.

The targeted method developed makes it possible to simultaneously identify and quantify more than 150 phenolic compounds in a short chromatographic run, with the aim of profiling several foods and beverages using simple sample

preparation. The high sensitivity of MRM-based mass spectrometry and the wide dynamic range of triple-quadrupole spectrometers provide valuable insight into the analysis of complex matrices, in which concentrations span several orders of magnitude. Furthermore, it helps to determine the type and amount of dietary compounds consumed and the eventual correlation of these with modifications in the host system.

The application of the targeted method for polyphenols was tested on different *Fragaria* spp., implementing the method with another short run for anthocyanins and ellagitannins. Quantitative data at molecular level were provided for 56 compounds for the first time. Some of the compounds were identified and quantified for the first time in the strawberry. This study represents the most comprehensive targeted profiling of strawberry polyphenols, in terms of the number of individual compounds identified and quantified.

In addition to a general method for analysis of several classes of polyphenols, it is also important to focus attention on a specific class of compounds and to provide detailed structural identification and quantification. This was done for the class of ellagitannins in the strawberry, obtaining the chemical structure of the native form of ellagitannins, their concentration, and the ellagitannin profiles during fruit ripening. In this part of the project, clarification of the main ellagitannin was achieved for the first time after several erroneous identifications, with its isolation and subsequent structural characterisation. The most notable results were obtained in the field of ellagitannin profiling, with 26 compounds being identified in different *Fragaria* spp., providing structural and quantitative information in the most accurate manner. The individual concentrations decreased during ripening in all varieties.

In the nutritional bioavailability section on the other hand, several limitations in terms of knowledge were encountered during the planning of the *in vivo* part, since polyphenol microbial metabolites have only recently gained attention in polyphenol-based food and nutritional studies, together with the huge growing of interest

regarding microbiota. Indeed, limited data were available regarding specific plasma or blood concentrations associated with nutritionally relevant consumption.

First of all a method for the detection of polyphenol microbial metabolites was required. For this purpose a sensitive targeted metabolomics method for the analysis of 23 polyphenol microbial metabolites, suitable for application to rat organs (brain, heart, liver and kidneys) and biofluids (blood and urine) was developed. The development of a purification procedure made it possible to obtain cleaner and more concentrated samples, with low limits of detection corresponding to the consumption of fruit rich in polyphenols. Considering the variety of matrices which can be treated with the same purification and quantitative analysis sample protocol, this method can be considered a general method for the simultaneous analysis of several chemically different compounds in a complex matrix.

Finally, a clear scenario for tissue distribution was obtained in the *in vivo* trials, with the use of a nutritionally relevant dose of metabolites in order to reproduce the blood circulating level of these metabolites *in vivo*. The treatment was carried out by intravenous injection, with the aim of bypassing the digestion and gut microflora metabolism with its inter-individual variability. Interestingly, the results obtained in animals show the simultaneous quantitative profile of some polyphenol microbial metabolites for the first time, proving their distribution in the brain and the main excretory organs, i.e. the liver and kidneys, and in biofluids (urine and blood).

The remarkable results obtained *in vivo* showed that the brain is one of the targets of certain polyphenol microbial metabolites, along with some more specific organ-tropism. With effective surgical and analytical protocols and with a nutritionally relevant dose injected, notably 10 out of 23 compounds were present in significantly increased amounts.

This shows an extraordinary improvement in the biological relevance of a diet rich in polyphenols and their possible role in neurological protection and the prevention of brain ageing. However, on the basis of this data more biological experiments now need to prove the real effect of these polyphenol microbial metabolites on the brain system in the conditions tested. The results obtained in the

brain were surprising, not only because some metabolites were able to pass through the blood-brain barrier and arrive in the brain or were already present as basal level, but more importantly because such low amounts of dietary metabolites can affect homeostasis in such a complex organ and alter its basal level.

To conclude, these data provide further health implications for the brain-gut microbiota axis, showing how polyphenol microbial metabolites could be one of the putative connections/messengers between microflora and the brain. Furthermore, polyphenol microbial metabolites being the result of the action of the microbiota on the dietary intake of polyphenols, it is feasible to consider that these compounds may be relevant in the regulation of human health and disease. Their action may lead to a reduction in oxidative stress, but more probably to alteration of biochemical and physiological processes, also changing the level of endogenous compounds in the brain, along with other organs.

The main follow-up for this Ph.D. project will regard the study of how microbial metabolites impact on the primary metabolism of the mammalian system. This means studying possible alterations in the homeostasis of different organs. The data concerned regard specific biomarkers affected by the intervention (by shifting their basal level in a significant manner) and will be directly correlated with the presence of polyphenol microbial metabolites, mainly using untargeted metabolomics analysis and with major involvement of bioinformatics tools.

The future prospects for development of this thesis, adopting a multidisciplinary approach, would involve using the detailed information acquired from the strawberry polyphenol profile to perform *in vitro* digestion with faecal water, scaling the system and using an amount comparable to the nutritional intake and following the production of microbial metabolites. The biological samples of fermented faecal water should then be analysed to determine the polyphenol content, using the same method developed for analysis of the biological matrix, as already tested in preliminary trials. Alternatively, a complete profile of the variety of microbial metabolites could be achieved with untargeted metabolomics analysis.

Another interesting future perspective could be to study in detail the plasma circulating level of this metabolite after the consumption of strawberries in humans and then to directly correlate this with the native composition of polyphenols. From the point of view of cell biology and the results obtained from the *in vivo* study, it would be interesting to test whether the concentration used and found in the brain would be enough to exert any neuroprotective effect with neuronal cells, glia cells or astrocytes against oxidative stress first of all, but also against the more complex mechanisms reported in the literature. Of course all these different aspects will require the involvement of different skills and make it necessary to set up a multidisciplinary team to better address these issues.

Other Publications

1.

Identification of intermediates involved in the biosynthetic pathway of 3-mercaptohexan-1-ol conjugates in yellow passion fruit (Passiflora edulis f. flavicarpa) Bruno Fedrizzi, Graziano Guella, Daniele Perenzoni, **Mattia Gasperotti**, Domenico Masuero, Urska Vrhovsek, Fulvio Mattivi, *Phytochemistry*, **2012**, 77, 287-293.

2.

Metabonomic investigation of rat tissues following intravenous administration of cyanidin 3-glucoside at a physiologically relevant dose Andreja Vanzo, Matthias Scholz, **Mattia Gasperotti**, Federica Tramer, Sabina Passamonti, Urska Vrhovsek, Fulvio Mattivi, *Metabolomics*, **2013**, 9, 88-100.

3.

Ellagitannins from Rubus Berries for the Control of Gastric Inflammation: In Vitro and In Vivo Studies. Enrico Sangiovanni, Urska Vrhovsek, Giuseppe Rossoni, Elisa Colombo, Cecilia Brunelli, Laura Brembati, Silvio Trivulzio, **Mattia Gasperotti**, Fulvio Mattivi, Enrica Bosisio, Mario Dell'Agli *PLoS ONE*, **2013**, *8*, e71762.

Acknowledgment

This PhD project was carried out in the Metabolomics Platform of Department of Food Quality and Nutrition at the Fondazione E. Mach (FEM - San Michele all'Adige, IT) in collaboration with the Center for Integrative Biology (CIBIO) at the University of Trento (UNITN). Financial support for the work was provided by the International Ph.D. Programme in Genomics and Molecular Physiology of Fruit (GMPF) at Fondazione E. Mach and the International Doctoral School in Biomolecular Sciences at University of Trento.

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In the end I wish to dedicate my thesis to my grandmothers, Fabiola e Eleonora, being always present in my life since when I was a child, and transmitting the attitude to be quiet but even stubborn with my personal ideals and objectives, for being proud of the life and open to the others. With all my love I dedicate this thesis to you.

Trento, 2014

Mattia Gasperotti