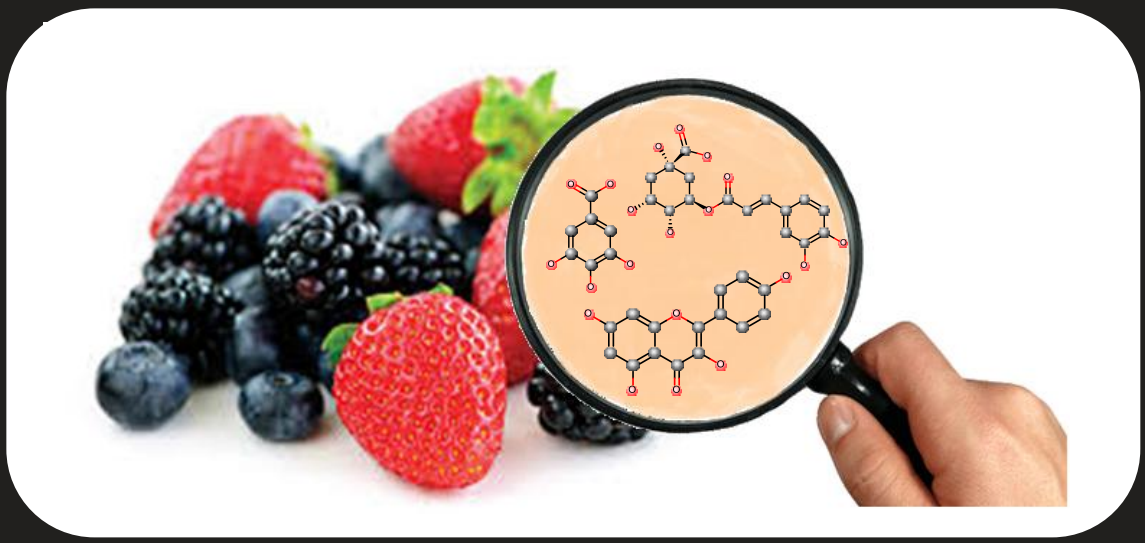
Exploring the bioavailability of (poly)phenols from berries and their potential activities in humans

Rui Carlos Soares Pimpão



Dissertation presented to obtain the Ph.D degree in Biochemistry

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
12th September,
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Knowledge Creation

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Abstract

(Poly)phenols are the most widely distributed secondary metabolites, in plants, and, therefore, are regular constituents of human food products. The regular ingestion of (poly)phenol-containing foods has been associated with a reduced risk of acquiring chronic diseases and many studies are currently trying to corroborate this theory. However, the precise contribution of (poly)phenols to disease prevention is still unknown. For understanding how (poly)phenols act on human health it is essential to acknowledge their bioavailability. After ingestion, compounds undergo several transformations in the gastrointestinal tract and also after absorption into the blood. Additionally, a great proportion of non-absorbed compounds reach the colon where they are catabolised by the colon microflora, resulting in simple phenolics which can also be absorbed and metabolized. However, these events are not fully understood and many questions can be posed during the entire process and many metabolites remain to be discovered.
In this study, we aimed to identify some of the most abundant bioavailable metabolites after the ingestion of dietary (poly)phenols from a mixture of berries and to predict their importance for human health.

The first goal of this work was to identify and quantify the compounds present in selected varieties of commercial blueberries, raspberries and blackberries, and two wild berries, strawberry tree fruit and Portuguese crowberry, since the latter hasnnever been analyzed. A methodology consisting on the hydrolysis of glycosides and esters of quinic acid and analysis of the resulting aglycones was developed by combination of enzymes obtained from *Aspergillus niger*. The selected berries were demonstrated to be a good source of some classes of (poly)phenols. These fruits, therefore, were chemically diverse and abundant in certain (poly)phenols, being ideal to be used in an intervention study.

In the second part of the work, the analysed berries were ground in the form of a puree and administered for breakfast to healthy human volunteers in a bioavailability study. Bioavailability studies are vital to understand the events that (poly)phenols undergo in the gastrointestinal tract and determine the metabolites that circulate in the blood stream. Based on the existing literature, an in silico library of potential bioavailable metabolites was constructed and used as reference for a preliminary search in urine collected from the volunteers using a LC-Orbitrap mass spectrometer. The metabolites preliminarily identified on the basis of their exact mass were then confirmed in urine by comparison with chemically or enzymatically synthesized standard compounds using LC-MS/MS. Several metabolites were positively confirmed in urine samples, mainly in conjugated form, as methyl, glucuronides and sulfates, some being identified in human samples for the first time.

For the third part of the work, the (poly)phenol metabolites previously identified in urine were quantified in plasma and their provenance from the ingested (poly)phenols was verified through to a crossover intervention with ingestion of fruit puree and a standard (poly)phenol-free meal by the volunteers. Standard compounds of sulfated phenolics were chemically synthesized for quantification purposes. Our results confirmed the presence of several phenolic sulfated and methylated-metabolites in plasma resulting from the ingestion of a (poly)phenol-rich berry fruit puree by humans and particularly emphasized the importance of the colonic catabolism. Nevertheless, some of the metabolites previously identified in urine did not fully result from the ingestion of (poly)phenols.

After identifying the phenolic metabolites circulating in the blood and determining their physiological concentration, it becomes possible to investigate their role in the human body. One of the relevant areas of (poly)phenols activity in humans is related with their effects in the brain and particularly at the level of

the blood-brain barrier, which controls the entrance of substances in the brain. In the final part of this work, confluent monolayers of brain microvascular endothelial cells (HBMEC) were used as an *in vitro* model of the blood-brain barrier for testing the effects of the sulfated-metabolites previously synthesized. The metabolites were tested for toxicity in HBMEC at physiological concentrations, and subsequently their ability to cross the cells was assessed suggesting the possibility of entering the brain. Nevertheless, the compounds were also assayed for a protective effect on HBMEC against injury induced by H_2O_2, suggesting a possible protective role at the level of the endothelial cells constituting the blood-brain barrier and without requiring entering the brain. Finally, no effect on the expression and activity of the efflux transporter P-glycoprotein (P-gp) in HBMEC was observed.

Resumo

Os (poli)fenóis são o grupo de metabolitos secundários com maior dispersão nas plantas e são, portanto, constituintes normais da alimentação humana. A ingestão regular de alimentos contendo (poli)fenóis tem sido associada a um menor risco de desenvolver doenças crónicas e muitos estudos estão a tentar corroborar esta teoria. No entanto, a contribuição exata dos (poli)fenóis para a prevenção de doenças ainda é desconhecida. Para melhor compreender como atuam os (poli)fenóis na saúde humana é essencial compreende a sua biodisponibilidade. Depois da ingestão, os compostos sofrem diversas transformações no trato gastrointestinal, bem como depois da sua absorção para a corrente sanguínea. Além disso, uma grande quantidade de compostos que não são absorvidos chegam ao cólon onde são catabolizados pela microbiota aí presente, resultando em fenóis mais simples que ainda podem ser absorvidos e metabolizados. No entanto, estes eventos não estão totalmente compreendidos e várias questões podem ser colocadas acerca todo o processo, bem como existem diversos metabolitos que ainda não foram descobertos.

Neste estudo pretendemos identificar os metabolitos biodisponíveis mais abundantes após a ingestão de (poli)fenóis dos alimentos e prever a sua importância para a saúde humana.

O primeiro objetivo deste trabalho foi identificar e quantificar os compostos presentes em variedades selecionadas de mirtilos, amoras e framboesas comerciais bem como de dois frutos selvagens, o medronho e a camarinha, sendo que este último nunca foi anteriormente analisado. Foi

desenvolvida uma metodologia consistindo na hidrólise dos glicósidos e ésteres de ácido quínico, através da combinação de enzimas obtidas de *Aspergillus niger*, com posterior análise das aglíconas resultantes dos (poli)fenóis. Estes pequenos frutos demonstraram ser uma boa fontes de algumas classes de (poli)fenóis. Por serem quimicamente diversos e abundantes em (poli)fenóis, estes frutos são ideais para serem usados num estudo de intervenção.

Na segunda parte do trabalho, os pequenos frutos analisados foram moídos na forma de um puré e administrados ao pequeno almoço a voluntários humanos saudáveis num estudo de biodisponibilidade. Os estudos de biodisponibilidade são vitais para compreender os eventos pelos quais os (poli)fenóis passam no trato digestivo e determinar os metabolitos que circulam na corrente sanguínea. Com base na literatura existente, foi construída um biblioteca *in silico* dos potenciais metabolitos biodisponíveis e usada como referência para uma procura preliminar em amostras de urina colhidas dos voluntários. As amostras foram analisadas por cromatografia líquida associada a um espectrómetro de massa Orbitrap. Os metabolitos preliminarmente identificados com base na sua massa exata foram então confirmados na urina por comparação com compostos padrão sintetizados química ou enzimaticamente, por intermédio de LC-MS/MS. Vários metabolitos foram positivamente confirmados nas amostras de urina, muitos deles na forma conjugada com grupos metilo, ácido glucurónico e sulfato, sendo que alguns foram mesmo identificados em amostras humanas pela primeira vez.

Na terceira parte do trabalho, os metabolitos de (poli)fenóis anteriormente identificados nas amostras de urina foram quantificados no plasma e a sua origem a partir dos (poli)fenóis ingeridos foi verificada através de um estudo de intervenção cruzado, em que os voluntários humanos alternaram entre a ingestão do puré de pequenos frutos e um pequeno almoço livre de polifenóis. Compostos padrão de fenóis-sulfatados foram sintetizados quimicamente para auxiliar na quantificação dos metabolitos. Os resultados confirmaram a presença no plasma de diversos metabolitos fenólicos conjugados com grupos sulfato e metilo, após a ingestão de um puré de pequenos frutos ricos em (poli)fenóis e enfatizou particularmente a importância do catabolismo no cólon. No entanto, houve alguns metabolitos previamente identificados na urina que não resultaram da ingestão de (poli)fenóis.

Após identificar os metabolitos fenólicos presentes na corrente sanguínea e determinar a sua concentração fisiológica, torna-se assim possível investigar o seu papel no corpo humano. Uma das área mais importantes no estudo da atividade dos (poli)fenóis em humanos está relacionada com os seus efeitos no cérebro e particularmente ao nível da barreira hematoencefálica, que controla o fluxo de substâncias para dentro e fora do cérebro. Na última parte deste trabalho, células endoteliais microvasculares de cérebro humano (HBMEC) foram utilizadas como modelo in *vitro* da barreira hematoencefálica para testar os efeitos dos metabolitos sulfatados, sintetizados anteriormente. A toxicidade destes

metabolitos foi testada nestas células em concentrações determinadas como fisiológicas. De seguida, a sua capacidade para atravessar estas células foi também determinada como uma indicação do seu potencial para penetrar a barreira hematoencefálica e entrar no cérebro. Adicionalmente, o efeito protetor destes compostos contra o dano causado por H_2O_2 nestas células foi também determinado, de forma a verificar um papel protetor ao nível do endotélio que compõe a barreira hematoencefálica ainda antes de entrar no cérebro. Por fim, também foi testado o seu efeito na expressão e atividade do transportador de efluxo P-glicoproteína nas HBMEC, que se verificou negativo.

Thesis Outline

This Dissertation is divided in six chapters.

Chapter one consists of a general introduction concerning (poly)phenols. It briefly describes the chemical diversity of compounds and their abundance in human foods. The functions of (poly)phenols in plants and their effects in animals are also addressed, particularly some of the proposed beneficial effects in humans. This chapter also focus on what is currently known about the bioavailability of (poly)phenols in humans.
This content of chapter is planned to be included in a review publication.

Chapter two focuses on the identification and quantification of (poly)phenols in berry fruits. From this work resulted a publication in *Journal of Agricultural and Food Chemistry* in which the author of this dissertation was the first author.

Chapter three describes the work on the identification of the bioavailable (poly)phenol-metabolites in urine of human volunteers after an intervention study where a puree made of berries was ingested. From this work several metabolites were identified and some of them were even identified in humans and associated with the consumption of (poly)phenols for the first time. From this work resulted a publication in *Molecular Nutrition & Food Research* in which the author of this dissertation was first author.

Chapter four consists in the confirmation and quantification in plasma of the previously identified bioavailable (poly)phenol-metabolites. The physiological concentration of the metabolites in the blood was therefore obtained. From this work resulted a paper submitted to *The British Journal of Nutrition* which is under revision and where the author of this dissertation will be first author.

In chapter five, the potential effects of the bioavailable (poly)phenol-metabolites on human brain microvascular endothelial cells were addressed *in vitro*. The results suggest the possibility of these metabolites to cross the blood-brain barrier as well as a protective effect against chemical insult at the level of the endothelial cells that compose this barrier. These results will be used in a future publication.

To finalize, chapter six consists of an integrated discussion of the global results obtained during this dissertation, focusing on the novelties achieved in this work and includes future perspectives.

Abbreviations List

Abbreviation	**Full form**
(Poly)phenols	Flavonoids, phenolic acids and their phenolic metabolites
4-MeCat	4-Methylcatechol
4-MeGA	4-Methylgallic acid
ACN	Acetonitrile
BBB	Blood-brain barrier
BHT	Butylated hydroxytoluene
CA	Caffeic acid
Cat	Catechol
CBG	Cytosolic β-glucosidase
cGMP	Cyclic guanosine mono phosphate
COMT	Catechol O-methyltransferase
CYP450	Cytochrome C P450
DAD	Diode array detection
DAPI	4',6-Diamidino-2-phenylindole
DHCA	Dihydrocaffeic acid
DHFA	Dihydroferulic acid
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FA	Ferulic acid

FBS	Fetal bovine serum
Fig.	Figure
GA	Gallic acid
GAE	Gallic acid equivalents
Glcrnd	Glucuronide
GLUTs	Glucose transporters
Glycsd	Glycoside
HBMEC	Brain microvascular endothelial cells
HPLC	High performance liquid chromatography
IS	Internal standard
LC-MS	Liquid Chromatography-mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantitation
LPH	Lactase phlorizin hydrolase
MEM	Minimum essential medium
MePyr	Methylpyrogallol
MRM	Multiple reaction monitoring
NEAA	Non-essential amino acids
NMR	Nuclear magnetic resonance
NO	Nitric oxide
ORAC	Oxygen Radical Absorbance Capacity
PA	Protocatechuic acid

PAPS	3'-Phosphoadenosine-5'-phosphosulfate
Pe	Permeability coefficient
P-gp	P-glycoprotein
PTFE	Polytetrafluoroethylene
Pyr	Pyrogallol
R123	Rhodamine 123
RPMI	Basal medium Roswell Park Memorial Institute
SGLT1	Sodium dependent glucose transporter 1
SGLts	Sodium-glucose transporters
SLC	Solute carry family
SSc	Soluble solids content
Sulf	Sulfate
SULTs	Sulfotransferases
TAc	Titratable acidity
TE	Trolox equivalents
TEER	Transendothelial electrical resistance
UDPGA	Uridine 5′-diphosphoglucuronic acid trisodium salt
UGTs	Uridine diphospho-glucuronosyltransferases
VA	Vanillic acid

Chapter 1

INTRODUCTION

RP wrote this chapter based on the referred bibliography.

Contents

(POLY)PHENOLS – STRUCTURE AND CHEMICAL CLASSES

Plants produce a great variety of secondary metabolites which can be classified according to their structural characteristics and biosynthetic pathways. Phenolic compounds, generically called (poly)phenols, are the most widely distributed secondary metabolites, ubiquitously present in plant kingdom [1]. They are a group of secondary metabolites characterized by the presence of at least one hydroxyl group attached to an aromatic ring. Their biosynthetic pathways are particularly complex with, in many cases, multiple alternative metabolic fates [2]. All plant phenolics are synthesized from the shikimate/phenylpropanoid pathway, which directly provides phenylpropanoids, or the "polyketide" acetate/malonate pathway, which can produce both monomeric and polymeric structures [3]. (Poly)phenols constitute an extremely diversified group not only in terms of chemical structure but also biological roles. They usually occur in conjugated form with sugars, carboxylic and organic acids, amines, lipids and other phenols and can be classified into different groups according to the number of phenol rings and on the basis of structural elements that bind these rings [4]. The main classes are represented in Fig. 1.

Simple phenolics are substituted phenols, where one or more hydroxyl groups are introduced in *ortho*, *meta* and *para* positions in relation to the benzene ring [5]. They include compounds such as catechol, resorcinol, pyrogallol or phloroglucinol.

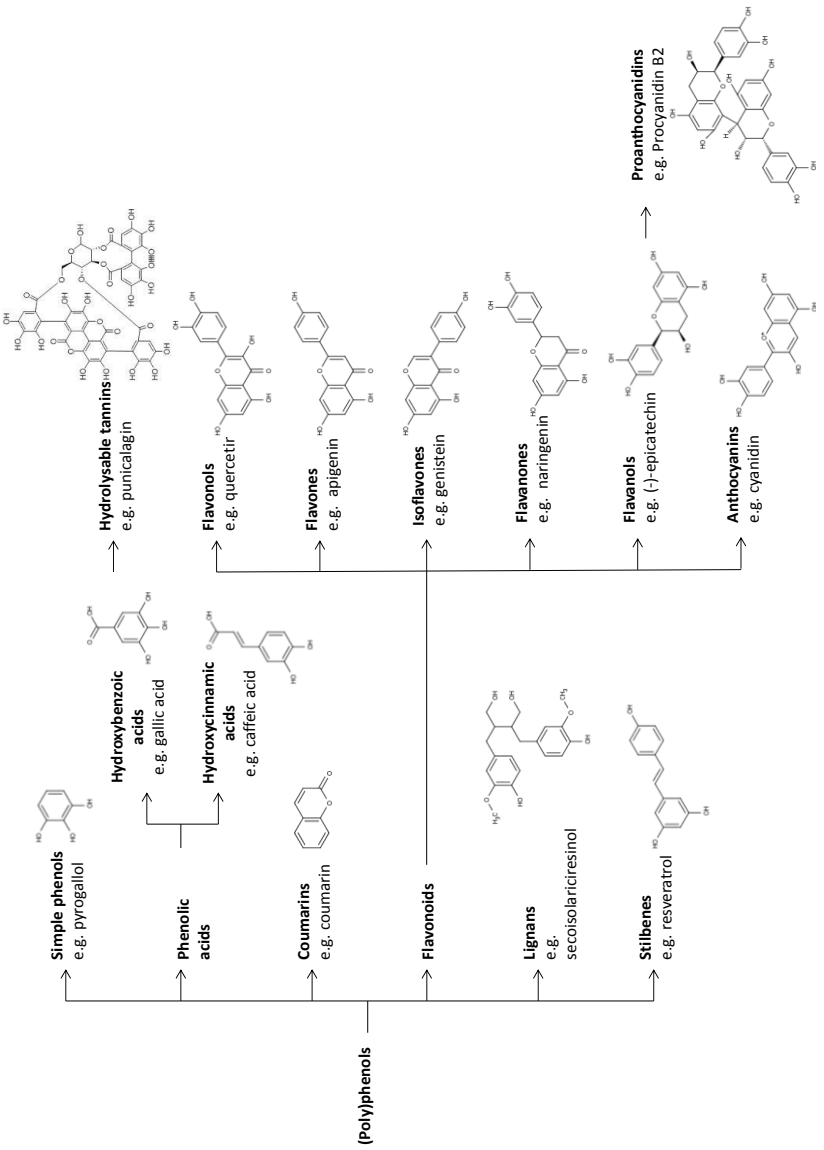

Figure 1. Classification and chemical structures of the main dietary (poly)phenol classes

Phenolic acids include two different groups, hydroxybenzoic acids and hydroxycinnamic acids. Hydroxybenzoic acids can be found both free and esterified in plants. They include gallic acid, which is present in fruits, herbs, tea and wine, and it is also the base unit of more complex compounds, such as ellagic acid and hydrolysable tannins, including gallotannins and ellagitannins, found in rubus fruits, pomegranate and cereal grains [6, 7]. Hydroxycinnamic acids consist mainly of *p*-coumaric, caffeic, ferulic and sinapic acids which are usually found glycosylated or conjugated with quinic, shikimic and tartaric acids [4, 6]. Chlorogenic acid, an ester of caffeic and quinic acids, can be found in several fruits and vegetables and is highly abundant in coffee. Ferulic acid is abundant in cereal grains [6].

Coumarins, with a C6-C3 skeleton, derive from the fusion of benzene and α-pyrone rings. They can be found in free form or conjugated with sugars and although they are distributed throughout all parts of the plant, including essential oils, coumarins occur at the highest levels in the fruits [1, 8].

Flavonoids constitute the biggest group of phenolic compounds in plants [1]. Structurally, they are based on a fifteen-carbon skeleton consisting of two benzene rings (A and B rings, Fig. 2) linked via a heterocyclic pyrane ring (C ring). They can be divided into several classes according to the level of oxidation and pattern of substitution of the C ring, while individual compounds within a class differ in the pattern of substitution of A and B rings [9]. The main classes of flavonoids are flavonols, flavones, flavanones, isoflavones, flavanols and anthocyanins.

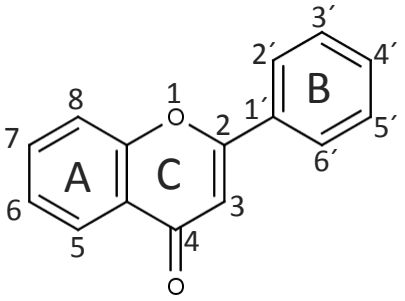

Figure 2. Representation of the general structure of flavonoids.

Flavonols are highly distributed in superior plants, although usually present at relatively low concentrations and in glycosilated form. Kaempferol and quercetin are the main representatives of this class, being found in fruits of the *Rosacea*e family, onions, leek, broccoli, etc. [2, 6, 10]. Flavones such as apigenin are much less common in fruits and vegetables. The main edible sources of flavones are oregano, parsley and celery and some cereals such as millet and wheat. Large quantities of polymethoxylated flavones have been found on the skin of citrus fruits [6, 11]. Flavanones such as hesperetin and naringenin are generally found glycosylated by a disaccharide in position 7, either a neohesperidose, which confers bitter taste, or a rutinoside, which is flavourless. They can be found in high concentrations in citrus fruits and in smaller concentrations in tomatoes and aromatic plants such as mint [6]. Isoflavones, having a similar molecular conformation to mammalian estrogens, are considered phytoestrogens. Worldwide, soybeans and its processed products are the almost exclusive dietary source of isoflavones, with genistein, daidzein and glycitein being the main aglycones [12]. Flavanols share in common with anthocyanins the lack of an oxygen

group at the 4-position of the heterocyclic C-ring (Fig. 1) [13]. Monomers of flavanols (catechins), such as (+)-catechin and (-)-epicatechin, can be found relatively abundantly in fruits, wine, chocolate and green tea [6, 13]. Unlike other (poly)phenols they are found as aglycones in plants [2]. Dimers, oligomers and polymers of catechins constitute the proanthocyanidins (also known as condensed tannins) which can be found in fruits such as apples and grapes, in drinks such as wine, cider, tea and beer, and also in cocoa, being responsible for their astringency [14]. Anthocyanins are glycosylated pigments located in the vacuole of cells, responsible for the shiny orange, pink, red, violet and blue colours in the flowers and fruits of some plants. They can also be present in vegetative tissues. Glycosides of cyanidin, delphinidin and pelargonidin are the most common in nature [15].

Lignans are diphenolic compounds formed by 2 phenylpropane units [4, 6]. Several lignans, such as secoisolariciresinol, are considered phytoestrogens [4]. They can be found in fruits, cereals, grains and certain vegetables although the main dietary source is linseed [6].

Stilbenes contain two phenyl moieties connected by a two-carbon methylene bridge [4]. They are commonly found in the roots, barks, rhizomes and leaves. However, the stilbenes are often in plants that are not routinely consumed for food, or in the non-edible tissue. One exception is resveratrol, found in grapes and in red wine in low concentrations [16].

(POLY)PHENOLS CONTENT IN FOOD AND DIETARY INTAKE

(Poly)phenols are present in virtually all parts of plants but the quantitative distribution varies between different organs of the plant and within different populations of the same plant species. These differences can be attributed to many factors including genetics, maturity, climate, agricultural practices, etc [2]. However, these differences make it difficult to standardize the amount of (poly)phenols present in food.

Quantification of (poly)phenols in food products has been done mainly by two approaches: the total amount of (poly)phenols in a certain food is usually estimated by colorimetric redox assays, based on the reducing properties of the phenolic groups, the Folin method, developed by Singleton and Rossi [17], being the most common one [18]; and chromatographic techniques, most commonly HPLC linked with several possible detection systems, are used not only for total estimates of (poly)phenols, but also for determining individual compounds in samples with great sensitivity [19].

The *in vitro* measurement of the antioxidant activity of (poly)phenols was initially considered as indicative of the potential biological activity of compounds, and for a while, (poly)phenols were regarded as a very important source of exogenous antioxidant defences [20]. However, recently this view has lost some credibility among the scientific community [21] and the measurement of the antioxidant activity can be considered nowadays more as a simple but rough alternative quantification method of (poly)phenols in food samples.

There are several sources of the (poly)phenolic content of foods and beverages, and the main ones are the United States Department of Agriculture (USDA) and the Phenol-explorer database (Table 1) [18]. These databases are very useful for research studies and for calculating the amount of ingested (poly)phenols by populations, required for predicting the health effects of compounds studied in epidemiology. It is thus important to calculate the dietary intake of (poly)phenols.

Estimation of the dietary intake of (poly)phenols has been, however, a very difficult task for several reasons: there are numerous food products where phenolic compounds have not yet been quantified; there are many (poly)phenols not yet identified in foods products due to many factors, such as the great variety of compounds in plants (and the lack of standards for their correct identification), which are obviously unaccounted for in quantification; and the intake is usually determined by diet histories, food frequency questionnaires or diet diaries, which are self-reporting and where accuracy is often uncertain [19]. Nonetheless, food frequency questionnaires are a good way of measuring long-term intake of food, being relatively inexpensive, substantially less time-consuming than other methods and can be self-administered. Due to reduced cost and time saving, online nutritional interventions and electronic methodologies for collecting dietary intake data are also being explored nowadays [22].

Although the richest sources of (poly)phenols are usually seasonings (Table 1), they are generally consumed in very low amounts. When considering the highest sources of (poly)phenols in our diet taking into consideration how much of that food we regularly ingest, the most

relevant sources are black and green tea, coffee, dark chocolate, walnuts, sesame oil and fruits such as blueberries, orange and grapefruit (Table 2). Berry fruits are a particularly rich source of (poly)phenols and their importance to the total consumption of (poly)phenols can be considerable. They are some of the richest sources in anthocyanins, flavonols and phenolic acids. In a recent study it was demonstrated that berry fruits have a low consumption by the Portuguese population in comparison to what is described for northern countries populations. Nonetheless, although berries account for only 9% of the total ingestion of fruits, they are responsible for 14% of the ingestion of (poly)phenols attributed to fruits and are the main dietary source of anthocyanins [22].

Table 1. **(**Poly)phenols content in the top thirty richest foods (mg per 100g)

Rank	Food	Food type	(Poly)phenols content
1	Cloves	Seasonings	15 188
2	Peppermint, dried	Seasonings	11 960
3	Star anise	Seasonings	5460
4	Cocoa powder	Cocoa products	3448
5	Mexican oregano, dried	Seasonings	2319
6	Celery seed	Seasonings	2094
7	Black chokeberry	Fruits	1756
8	Dark chocolate	Cocoa products	1664
9	Flaxseed meal	Seeds	1528
10	Black elderberry	Fruits	1359
11	Chestnut	Seeds	1215
12	Common sage, dried	Seasonings	1207
13	Rosemary, dried	Seasonings	1018
14	Spearmint, dried	Seasonings	956
15	Common thyme, dried	Seasonings	878
16	Lowbush blueberry	Fruits	836
17	Blackcurrant	Fruits	758
18	Capers	Seasonings	654
19	Black olive	Vegetables	569
20	Highbush blueberry	Fruits	560
21	Hazelnut	Seeds	495
22	Pecan nut	Seeds	493
23	Soy flour	Seeds	466
24	Plum	Fruits	377
25	Green olive	Vegetables	346
26	Sweet basil, dried	Seasonings	322
27	Curry, powder	Seasonings	285
28	Sweet cherry	Fruits	274
29	Globe artichoke heads	Vegetables	260
30	Blackberry	Fruits	260

Adapted from Pérez-Jiménez [18]

Table 2. (Poly)phenols content in foods

(Poly)phenol class	(Poly)phenol subclass	Example compounds	Dietary source (mg/serving)			
			<25	25-50	50-100	>100
Phenolic acids	Hydroxybenzoic acids; Hydroxycinnamic acids	Gallic acid, ellagic acid, vanillic acid; caffeic acid, ferulic acid, chlorogenic acid	Grape juice, Red wine, Rosemary, Grapefruit, Dark chocolate, White wine, Oregano, Rolled oats	Flaxseed, Black tea, Green olives, Black beans,	Plum, Kiwi, Chicory	Coffee, Walnut, Blueberry, Cherry, Aubergine
Flavonoids	Flavonols	Quercetin, Kaempferol, myricetin	Black tea, Walnuts, Black beans, Dark chocolate, Red wine, Almonds, White wine	Spinach, Plum, Onion, Blueberry	Capers, Curly Kale	
	Flavanones	Naringenin, hespretin	Red wine	Lemon juice	Grapefruit, Orange	
	Flavones	Apigenin, luteolin	Oregano			
	Isoflavones	Daidzein, genistein		Tofu, Tempeh	Soy beans, Soy flour	
	Flavanols	Catechin, epicatechin, epigallocatechin gallate, procyanidin	Grape juice, Plum, White wine, Almonds, Blueberry	Red wine, Cocoa	Dark chocolate, Beans	Black tea, Green tea
	Anthocyanins	Cyanidin, Delphinidin, Pelargonidin	Red wine	Black beans, Plum, Red cabbage	Strawberry	Blueberry, Blackberry, Blackcurrant, Aubergine, Cherry, Rhubarb, Black grape
Lignans		Laricіresinol, secoisolariciresinol			Flaxseed	Sesame oil
Stilbenes		Resveratrol	Red wine, White wine			
Other (poly)phenols	Tyrosols		Olive oil, Red wine, White wine		Green olives	
	Curcuminoids				Tumeric	

Adapted from Manach *et al.* [6] and Tangney *et al.* [11]

(Poly)phenols functions

As non-movable organisms, plants had to develop mechanisms for surviving in the different ecological niches on Earth. Evolution and development of the phenolic biosynthetic pathways was a key event for the adaptation of plants to terrestrial environment. Simple phenolics are structural units for the formation of lignin which provide mechanical support but also of cutin and suberin which are essential in transport and storage of water [23]. As secondary metabolites, phenolic compounds have defensive roles (against pathogens, herbivorous predators and other competitor plants), they act as signalling molecules, and protect against UV radiation [24, 25] or oxidative stress [26, 27].

(Poly)phenols were described to affect communication between plants, a phenomenon called allelopathy which can be detrimental or beneficial to the receiving plant, and accumulation in soils can influence the availability of soil nutrients and rates of nutrient cycling [28, 29]. (Poly)phenols also act as attractants of pollinators and seed dispersers. Red colours provided by anthocyanins are attractants of birds while yellow flavonoids contribute to insect attraction. Non-pigmented phenolics may also act as attractants, being detected by soil bacteria and fungi which can either be beneficial or prejudicial [23, 28].

Phenolic compounds, among other plant secondary metabolites, have also an important function in the defence system of plants against the attack of herbivores or pathogens such as fungi and bacteria. Defensive metabolites can be either produced constitutively and stored as inactive forms (phytoanticipins) or induced in response to the attackers (phytoalexins) [30].

Phytoanticipins are mainly activated by β-glucosidases during attack, which removes the glycosides and releases the biocidal aglycone metabolites [30]. Examples of phytoalexins are vitiferins, stilbenic polyphenols, dimers, trimers and tetramers of resveratrol which are produced in *Vitaceae* when infected by fungal pathogens. These compounds are thought to interfere with the activity of the fungi to prevent the disease process [31].

(Poly)phenols, as other classes of plant secondary metabolites ingested by animals, are xenobiotics which can exert beneficial or adverse reactions depending on their structure and concentrations in foodstuffs [32]. Human dietary (poly)phenols are usually non-toxic, at least in the normally ingested amounts, but there are thousands of toxic phytochemicals which taken together can affect virtually any function in a living organism [33]. Knowledge of their effects in animals can provide important information on their possible effects in humans.

With the function of deterring predator herbivores, (poly)phenols can act in many different ways. (Poly)phenols are substrates of peroxidases and polyphenol oxidases, enzymes present in plants, which generate semi-quinone free radicals and quinones using plant phenolics when there is herbivore or pathogen-damaging of foliage. These radicals can react with proteins and amino acids in plant cells, thus reducing their nutritional quality [34, 35]. Semi-quinone free radicals can also be generated by plant peroxidases in the mid-guts of larvae predators, decreasing larval performance [35]. The ability of plant compounds to influence the behaviour and fitness of predator herbivores has been well documented. Insect

larvae exposed to salycilates from *Salix* species grow slowly and consume less plant material. On the other hand, insects can use phenolics to increase their fitness. Sequestering of dietary flavonoids by larvae of *Polyommatus icarus* results in more attractive butterflies for the opposite sex [36].

To reduce foraging, plants can produce bitter compounds to make them unpalatable. Humans and other animals tend to reject foods excessively bitter, an instinct that for long has been useful since many of these bitter plants possess toxic and possibly fatal compounds. Several (poly)phenol compounds are responsible for the bitter taste of plants. In general, low-molecular-weight phenolics tend to be bitter whereas high-molecular-weight polymers tend to be more astringent. The flavanones tangeretin and nobiletin, the flavonol quercetin and flavanone naringin, the isoflavones genistein and daidzein and monomers and polymers of flavanols are responsible for bitter taste in foods [37]. Although humans have selected plants varieties with reduced bitterness, there are still several food products with distinctive bitterness such as red wine, tea, beer and chocolate [38]. On the other hand, changes in the structure of flavonoids can produce differences in palatability ranging from bitter to tasteless to extremely sweet. As was observed for flavanones, a rutinoside or a neohesperidoside attached to the position 7 in naringenin can produce, respectively tasteless and bitter compounds, whereas the chalcone and dihydrochalcone corresponding to naringenin-7-neohesperidoside (naringin) are extremely sweet [39].

Some compounds designated by phytoestrogens have structures similar to mammalian estrogens and exhibit estrogenic or anti-estrogenic activities [40]. Phytoestrogens have relatively weak activity compared to animal estrogens; however, exposure to high dietary levels may result in biological responses in humans and animals, with favourable or unfavourable consequences [40]. These include compounds such as isoflavones, coumestans and lignans. Lignans such as enterolactone and enterodiol occur widely in *Leguminosae* and are found in woody portion of plants, in seeds and cereal grains, being particularly high in flaxseed [16, 40]. Coumestans such as coumestrol and isoflavones such as genistein, daidzein and glycitein can also be found in legumes. Alfalfa, white clover and *Medicago* spp. contain basal reduced levels of coumestan phytoestrogens, which increase when attacked by aphids and fungal pathogens. Isoflavones are present in subterranean clover, red clover and soy, important in animal feeds [41]. In cattle, phytoestrogens were shown to cause infertility accompanied by visible signs of estrogenism including mammary development, swelling of the vulva, cystic ovaries and behaviour abnormalities. In sheep, infertility was also observed and in ewes infertility may be temporary and accompanied or not by visible signs. However, infertility can become permanent upon prolonged exposures to phytoestrogens [41]. In humans, soy and soy derived products are the main sources of pytoestrogens; no adverse effects have been observed by soy consumption and, in contrast, consumption of soy isoflavones may be advantageous for humans, as they were described to relieve menopausal symptoms among other health effects [40].

Tannins are very abundant (poly)phenols in plants, commonly ranging from 5 to 10% dry weight of leaves. They have the ability to bind proteins, and are thought to decrease the protein utilization by mammalian herbivores. Binding to plants cellular proteins is thought to reduce their digestibility and limit nitrogen supply and binding to salivary proteins would also decrease digestion efficiency [42, 43]. It is the ability of tannins to bind to proteins that transmit the feeling of astringency and possibly the cause of tannins acting as feeding deterrents [44]. Additionally, they can inhibit the growth and activity of ruminal microflora [43]. A toxic effect in some herbivorous insects has been observed upon ingestion of tannins which being oxidised cause lesions in the gut [42].

IMPORTANCE OF POLYPHENOLS FOR HUMAN NUTRITION

Although dietary (poly)phenols do not usually present any risk at normal intake, they are also not classically essential for humans nor even considered nutrients. However, (poly)phenols may have a positive impact on human nutrition. Their wide dispersion in plant products and abundance in certain foods makes them very good candidates as being responsible for the protective effects associated with vegetable and fruit-rich diets. In an attempt to verify this idea, several epidemiological studies have been conducted to evaluate the effects to human health of a long term ingestion of (poly)phenol-containing foods. And although some positive correlations were found in some studies, some of the results are controversial and contradictory [45, 46]. Most of the studies only regarded some of flavonoid sub-classes, neglecting others. Although many studies

do not actually observe no positive correlation between ingestion of (poly)phenols and prevention of diseases or reduced mortality, they cannot be comparable due to variability in the assessed flavonoid classes, demographic characteristics of the populations, outcomes assessed, and length of follow-up [46]., Additionally, although some studies show positive results and others show no effect, the studies suggesting a negative effect of (poly)phenols are almost non-existing. Some epidemiological studies fail in indicating which components of fruit and vegetables may be responsible for the effects and that is also related with an insufficient characterization and variable quantification of (poly)phenols contents in food [23]. Positive results from epidemiological studies should also be confirmed by clinical trials which can be very expensive. Therefore, experimental studies with animals and *in vitro* tests are important to search for evidence for the effects of (poly)phenols in humans and to verify their mechanisms of action. Nonetheless, all these different studies contribute with pieces of evidence which support that a continuous and prolonged intake of (poly)phenol-rich foods may help in prevention of several degenerative pathologies such as diabetes, cardiovascular diseases, neurodegenerative diseases and cancer, and to prevent symptoms associated to aging and menopause [47, 48].

Prevention of diabetes

Consumption of (poly)phenol-containing foods has been negatively correlated with glycemic index and therefore associated with prevention of developing type 2 diabetes and its metabolic disorders. Diabetes-

associated hyperglycemia is caused by insulin resistance and impaired carbohydrate metabolism, characterized by altered digestion and absorption of carbohydrates, depletion of glycogen storage, increased gluconeogenesis and over output of hepatic glucose [49]. Epidemiological studies have proposed that (poly)phenols can have an effect in reducing the risk of type 2 diabetes, and instead of being attributed to a single compound this effect would be correlated with the ingestion of (poly)phenol–rich foods [50, 51]. There are several possible mechanisms of action of (poly)phenols in reducing the risk of developing diabetes [50]. It is thought that they can inhibit α-glucosidase and α-amylase, important enzymes for digestion of carbohydrates into glucose [52]. There is also evidence that (poly)phenols can inhibit the absorption of sugars by interacting with sugar transporters from GLUTs (glucose transporters) and SGLTs (sodium-glucose transporters) families [53]. Additionally, it was shown that some flavonoids (anthocyanins) can modulate post-prandial glycaemia by decreasing GLUT expression in human intestinal Caco-2 cells [54]. Moreover, ingestion of (poly)phenols such as isoflavones can stimulate insulin production by pancreatic β-cells [55] and increase insulin sensitivity in peripheral tissues, thus requiring smaller amounts of insulin [56].

Prevention of cardiovascular diseases

There are several epidemiological studies associating the consumption of (poly)phenol-containing foods with a reduced risk of hypertension, stroke, coronary heart disease and myocardial infarction [57-60]. Although a

substantial amount of research has been dedicated to the subject, it is still not clear which (poly)phenols are more active against cardiovascular disorders and what are the mechanisms of action. It was proposed that soy and cocoa flavonoids have the most beneficial effect on reducing cardiovascular risk, based on clinical trials [61]. There are several possible mechanisms by which (poly)phenols might act in prevention of cardiovascular diseases. They can act via a vasodilatory effect, mediated by nitric oxide (NO) production and increase of cyclic guanosine monophosphate (cGMP) in vascular smooth muscle [62, 63]; anti-inflamatory effects are also described most likely through impacts on transcriptional networks or signalling cascades that modulate gene expression, promoting anti-inflammatory mediators and NO production [11]; inhibition of platelet aggregation was also demonstrated for the ingestion of (poly)phenol-containing foods such as cocoa, purple grape juice, coffee and tea [64-67]; and (poly)phenols such as monomeric and polymeric catechins were shown to have an impact on lipid digestion and absorption [68, 69], helping in prevention of lipid accumulation in arterial wall and development of atherosclerosis [70].

Hormone replacement therapy substitutes

Menopause is associated with hormonal alterations which translate into uncomfortable symptoms and can increase the risk of developing several pathologies. (Poly)phenols characterized as phytoestrogens are thought to be a natural alternative to hormone replacement therapy used to minimize the physiological alterations associated with menopause [71]. However, the

studies contained numerous deficiencies and results have been inconclusive and conflicting [72]. Although isoflavones were shown to prevent bone loss in animal models [73], clinical trials in humans did not confirm it [74]. Epidemiological studies also suggest a possible role of isoflavones in prevention of menopause associated cancers such as breast cancer [75, 76]. However, a 6-month intervention of mixed soy isoflavones did not reduce breast epithelial proliferation in healthy western women, suggesting a lack of efficacy for breast cancer prevention [77]. Nevertheless, there is evidence that isoflavones have potential to treat premenstrual symptoms. In a study where women consumed soy isoflavones in comparison with a placebo, it was observed that there was an improvement of somatic symptoms of swelling, headache, aches and breast tenderness [78].

Prevention of neurodegenerative diseases and aging

Almost two decades ago, James Joseph and its team observed that a diet supplemented with (poly)phenol-enriched extracts from strawberry and spinach, also characterized as rich in "antioxidants", prevented age related decreases in motor and cognitive behaviour as well as brain function in Fisher 344 rats [79]. Afterwards, they focused on the effects of these supplementations (where blueberry was included) in reversing age-related deficits in brain and behavioural function in aged rats. Their findings revealed that supplementation improved cognitive behaviour and particularly blueberries also improved motor performance, carbachol-stimulated GTPase activity and oxotremorine-enhanced dopamine

release [80]. Since these observations, research on the neuroprotective abilities of (poly)phenols has expanded and many studies have been conducted to demonstrate neuroprotection from other plant-based foods or beverages. Positive results against the degenerative effects of aging were observed for coffee [81], concord grape juice [82, 83] and extra virgin olive oil [84]. A substantial amount of research has also been conducted on the neuroprotective abilities of (poly)phenols against diseases such as Alzheimer, Parkinson, Huntington and multiple sclerosis [85]. Evidence on the neuroprotective effects of (poly)phenols has been obtained from *in vitro*, *in vivo* and epidemiological studies. Although clinical studies are more reliable, these are scarce and no clear positive results have been reported so far. Nevertheless, epidemiological studies suggest that regular consumption of (poly)phenol-rich foods is associated with a lower risk of developing neurodegenerative disorders [86-88]. *In vitro* and *in vivo* research has been very important in unravelling the molecular mechanisms behind the neuroprotective effects. (Poly)phenols were shown to interfere with signalling pathways such as protein kinase and lipid kinase signalling cascades causing direct effects on cell survival, neuronal differentiation and suppression of neuroinflammation [89-91].

BIOAVAILABILITY OF (POLY)PHENOLS

Absorption, metabolism and excretion of (poly)phenols is important for understanding their effects in human health and to find strategies to potentiate these effects. It is thus necessary to study their bioavailability. For this aim, many different models can be used, ranging from *in vitro*

enzymatic activities, cellular models, animal models or human subjects. Although many differences can occur between human and other animals digestion, animals have been essential for the knowledge that we currently have on (poly)phenols bioavailability and effects.

The (poly)phenols most commonly ingested in the human diet are not necessarily the most active within the body either to a low intrinsic activity or because they are poorly absorbed, extensively metabolized and rapidly excreted [6]. During digestion and metabolism, (poly)phenols undergo several structural modifications and the resulting metabolites found in blood and tissues may differ from the native compounds in terms of biological activity.

One of the major difficulties in studying the bioavailability of (poly)phenols is their structural differences, resulting in different metabolic fates between compounds. Although common mechanisms exist between them, several differences in the metabolism can occur between classes or different compounds within the same class of (poly)phenols.

Metabolism in the proximal gastrointestinal tract

The gastrointestinal tract is covered by the mucosa, which functions as physical barrier, determining bioavailability of xenobiotics. This function is mediated by physical walls, metabolism and active transport mechanisms [92]. For a compound to enter the body, it must traverse the mucosa, composed of several layers. Absorption of most food products occurs mainly at the duodenum and the proximal half of the jejunum where modifications in the mucosa such as folds (plica circularis),

depressions (crypts) and finger-like projections (villi) increase the absorption area (Fig. 3).

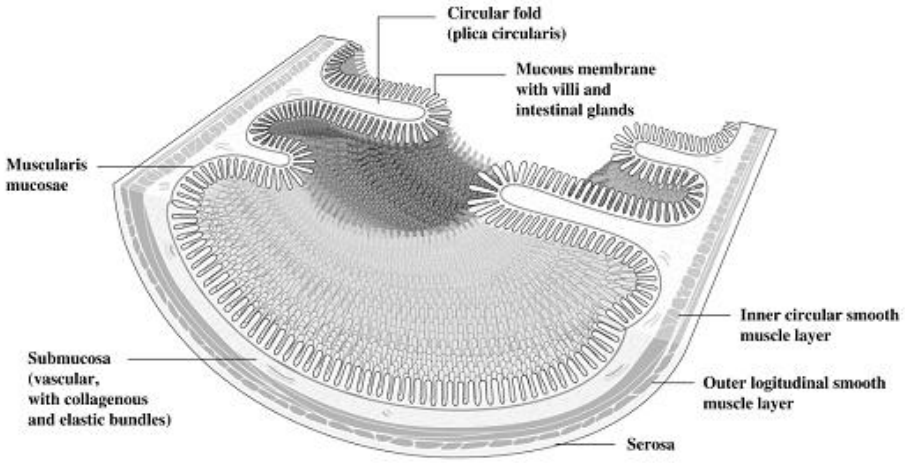

Figure 3. Cross-section of human proximal small intestine. Adapted from DeSesso and Jacobson [93].

Additionally, enterocytes, the predominant cells and the main ones responsible for absorption, contain in their apical cell membranes microvilli which increase the surface area of absorption [93]. Being lipophilic compounds, most flavonoid aglycones permeate intestinal cells by passive diffusion as assessed on Caco-2 cell model [94]. The same was demonstrated for hydroxycinnamic acids, being able to cross cells by transcellular route [95]. Passive intestinal permeability depends on several factors, including lipophilicity, hydrogen bonding capacity, molecule size, etc. However, in plants (poly)phenols are usually present in the form of esters, glycosides or polymers. All these forms are usually not absorbed directly

and they probably resist acid hydrolysis in the stomach, being able to reach the duodenum [6].

For glycosylated compounds, absorption is usually preceded by hydrolysis of the glycoside and release of the aglycone (Fig. 4). This is performed by the enzyme lactase phloridzin hydrolase (LPH) in the brush-border of the small intestine epithelial cells. After hydrolysis, the free aglycone can then enter the enterocytes by passive diffusion [96]. Alternatively, glycosylated compounds are thought to enter enterocytes by the active sodium-dependent glucose transporter SGLT1 and be hydrolysed by the cytosolic β-glucosidase (CBG) [97].

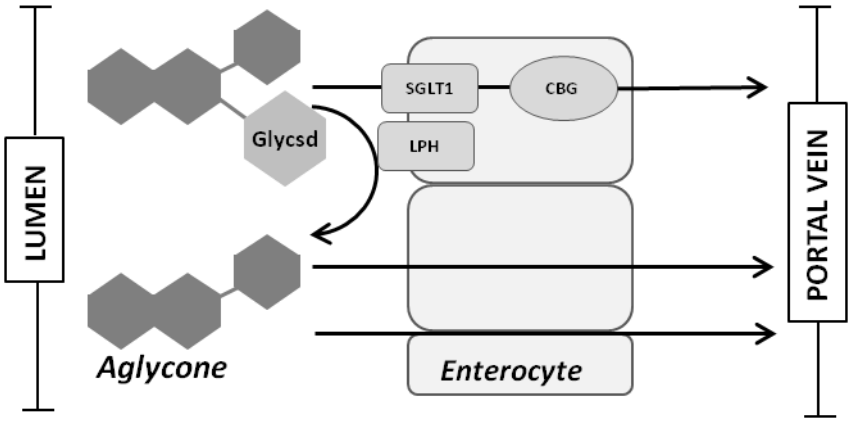

Figure 4. Mechanisms for hydrolysis of phenolic-glycosides and absorption of the resulting aglycone in enterocytes. Glycsd, glycoside; SGLT1, sodium-dependent glucose transporter; CBG, cytosolic β-glucosidase; LPH, lactase phloridzin hydrolase. Adapted from Del Rio *et al.*[47].

However, conjugation with different glycosides does not retrieve the same bioavailability for the aglycone, as it was observed that different glycosides are differentially hydrolysed in the small intestine [98]. Aglycones

conjugated with rutinoside are poorly hydrolysed in the small intestine and are absorbed in the colon after hydrolysis of the glycoside by the colon microflora [99, 100].

In the case of anthocyanins, research suggests that hydrolysis of the glycoside causes instability of the aglycone structure and induces a spontaneous degradation into phenolic acids and aldehydes [101-103]. This may be an essential point for the low bioavailability of anthocyanins, previously reported as being near 0.1% of intake [104].

For compounds that exist in plants in the aglycone form, such as flavanol monomers, their bioavailability has been reported to be higher, near 30%, with a fast absorption and excretion, typical of a small intestine absorption [47]. In contrast, oligomeric and polymeric flavanols, although having a high level of stability under gastric and duodenal digestion conditions, are thought to exhibit a very low extent of absorption [105]. An *in vitro* study with Caco-2 cells revealed their capacity to cross the cells is very low, decreasing with an increase in the molecular weight. Additionally, they are P-glycoprotein (P-gp) substrates, leading to their excretion by efflux pumps to the apical side [106]. The same results are observed *in vivo* with most proanthocyanidins being very poorly absorbed [105]. A slight exception was proanthocyanidin B2, which was partially absorbed intact after oral administration [107, 108].

Metabolism in the colon

Compounds not absorbed in the proximal gastrointestinal tract travel to the large intestine where absorption can also occur. Studies conducted

with subjects with an ileostomy, having had their colons removed surgically, have been very important for answering several questions. Information on the site of absorption of compounds is provided by their presence in urine in comparison with healthy subjects. Compounds absent in the urine of ileostomized patients should have originated from and be absorbed in the colon. Information about the metabolism in the lumen of small intestine is also provided in these studies, and collection of ileal fluid allows estimation of the amount of compounds that can pass from the small to the large intestine [109]. In this way, reports using ileostomized subjects revealed that substantial amounts of the initial (poly)phenols passed from the small to the large intestine, with the conjugated group (for example different glycosides) and the structure of the aglycone being key determinants [110, 111]. On studies with caffeoylquinic acids, it was observed that variation in the food matrixes and the composition of (poly)phenols and their amount on food also influences the intestinal absorption and the percentage of compounds reaching the colon [112, 113]. Analysis of subjects' ileal fluid after green tea ingestion revealed the presence of 70% of the ingested flavan-3-ols, 33% being in the form of parent compounds [114].

However, after reaching the colon, (poly)phenols can suffer the action of colonic microbiota which are able to degrade them into low molecular weight phenolics and aromatic acids [109].

Although bacteria are present in the entire human body, bacterial density increases in the distal small intestine, and particularly in the large intestine where it is estimated that there are 10^{11}–10^{12} bacteria per gram of

colonic content, with a large number of different species, ranging from 300 to 500 [115]. Of these, only a minority has been identified, with *Bacteroides* being the most common and constituting about 30% of all species. They are followed by *Clostridium*, *Prevotella*, *Eubacterium*, *Ruminococcus*, *Fusobacterium*, *Peptococcus*,and *Bifidobacterium*. *Escherichia* and *Lactobacillus* are also present, although to a lesser extent [116]. The colonic microbiota are able to induce several transformations in the (poly)phenol structure including *O*- and *C*- deglycosylation, hydrolysis of esters and amides, and deglucuronidation. The aromatic ring of the aglycones can be ruptured and they can undergo dehydroxylation, demethoxylation, demethylation and hydrogenation. Generally, colonic activity has the ability of converting complex (poly)phenols into simpler phenols and aromatic acids, but a great portion is also converted into oxaloacetate and ultimately CO_2 [117].

In vitro studies using faecal suspensions incubated with polyphenolic samples revealed the diversity of compounds generated by the colonic microbiota enzymatic activity. Due to the action of faecal microbiota, anthocyanins were deglycosylated, reconjugated with other groups and underwent breakdown of the anthocyanidin heterocyclic ring resulting in phenolic acids and other metabolites [102, 118]. Miocrobiota-induced break down of caffeoylquinic acids into caffeic and quinic acids and conversion into other phenolic acids was also demonstrated [119].

Cerdá and co-workers reported that after ingestion of several ellagitannins and ellagic acid containing foods, neither ellagitannins nor ellagic acid were detected in the urine of the subjects. However, the metabolite

urolithin B was suggested to have originated from the microbial degradation of the parent compounds in colon. Its variable amounts between subjects were suggested to be caused by differences in the colonic microbiota amongst the subjects [120]. Other urolithins and their metabolites were subsequently identified and their origin from colonic microbiota was confirmed *in vitro* [118, 121-123].

Catabolism of flavanols monomers but also proanthocyanidins oligomers and polymers by the colonic microbiota also resulted in formation of several simple phenols, phenolic acids and the characteristic valerolactones [124, 125]

Although a great portion of the colonic metabolites are excreted in faeces, they can still be absorbed and enter the blood flow. Their importance resides on a possible physiological effect or their use as biomarkers of polyphenol intake [6]. And because all individuals have their own unique signature of intestinal microbiota, the metabolites resulting from its metabolism will vary and have a different impact on the health of the host.

Biotransformation reactions of (poly)phenols

As commonly occurs with xenobiotics, which enter human or other animals body, (poly)phenols can undergo enzymatic reactions known as 'biotransformations'. The resulting compounds are usually less lipophilic so they can be more easily excreted by the body. These reactions include phase I and phase II reactions [32].

Phase I reactions, considered functionalization reactions due to the introduction or uncovering of a functional group in the xenobiotics molecule, allowing it to become more easily conjugated with endogenous substrates. These include oxidative and reductive reactions, where cytochrome c P450 (CYP450) and NADPH cytochrome c reductase have important roles; and hydrolytic reactions conducted by esterases and aminases [32]. The liver is considered the primary site for the catalytic activity of CYP450 enzymes but significant enzyme levels can be found in all cells lining the gastro-intestinal tract [33]. Although several studies characterized the ability of (poly)phenols to inhibit or activate phase I enzymes, or even to influence their gene expression, very little information is available about the occurrence of phase I reactions towards (poly)phenols [126-129].

Phase II reactions are conjugation reactions characterized by the introduction of an endogenous group, such as glucuronic acid, sulfate, methyl, acyl, glycine or reduced glutathione, onto the xenobiotic structure. The conjugates, being more water soluble, are usually rapidly excreted through bile or urine [32]. In the metabolism of (poly)phenols in human body, glucuronidation, sulfation and methylation are the most frequent phase II reactions. For example, the aglycone caffeic acid can be subjected to conjugation reactions, resulting in caffeic acid-*O*-sulfate, caffeic acid-*O*-glucuronide or its methylated form, ferulic acid (Fig. 5).

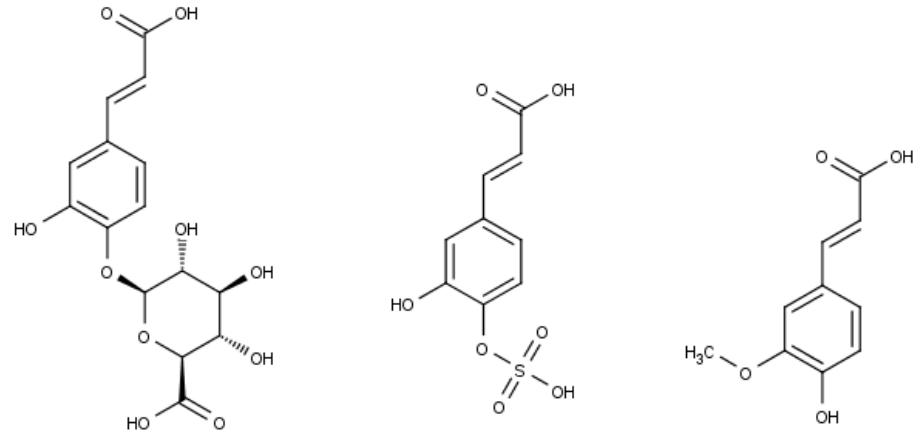

Figure 5 – Structures of caffeic acid in sulfated, glucuronidated and methylated form.

Glucuronidation of (poly)phenols is carried out by UDP-glucuronosyltransferases (UGTs) which catalyze the transfer of glucuronic acid from the cofactor UDP-glucuronic acid (UDPGA) to a suitable nucleophilic functional group such as hydroxyl, amine or carboxylic acid [130]. UGTs are predominantly transmembrane proteins located in the smooth endoplasmic reticulum and nuclear compartment of cells [130, 131]. Although the liver is the primary site of glucuronidation in the body, the kidney and the gastrointestinal tract are considered the most important sites of extra-hepatic metabolism [131]. The versatility of UGTs resides on the fact that this enzyme exists as an enzyme 'super family', with individual enzyme forms that exhibit distinct, but overlapping, substrate and inhibitor selectivities [131]. Although glucuronidation occurs to some extent in all mammalian species, significant variation in enzyme substrate selectivity and rates of glucuronidation between

species is common and well documented. For this reason, glucuronidation studies carried on laboratory animals not always correlate with humans [131].

Sulfation is conducted by sulfotransferases (SULTs), which are a group of enzymes localized in the soluble fraction of cells, capable of catalyzing the transfer of a sulfonate group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS), to a hydroxyl or amino group of substrate compounds. Sulfation is not only important for the biotransformation of xenobiotics, but also of endogenous catecholamines, steroid/thyroid hormones, cholesterol, and bile acids [132]. The ability of human SULTs to sulfate a broad range of substrates arises from the presence of multiple isoforms. Therefore, SULTs have been divided into several gene families and subfamilies based on their amino acid sequence identity and catalytic properties [133]. The SULT1 family is responsible for catalyzing the sulfation of many endogenous and exogenous phenols. Within this family, SULT1A has been demonstrated to have greater catalytic activity towards the sulfation of small planar phenolic compounds and flavonoids, whereas SULT1E1 was more active towards estrogen conjugation [134].

Methylation is mediated by the catechol *O*-methyltransferase (COMT). The catalytic activity of this enzyme has been described toward compounds containing a catechol group or a vicinal dihydroxyphenyl structure, by the transfer of a methyl group from *S*-adenosyl-L-methionine (AdoMet) to one of the two vicinal hydroxyl groups [132, 135]. Two forms of COMT have been described in humans, the cytoplasmic form (S-COMT) and the membrane-bound form (MB-COMT) located in

the rough endoplasmic reticulum [136]. Although COMT has an important function in the *O*-methylation of endogenous catecholamines (epinephrine, norepinephrine and dopamine) and catechol estrogens, it also catalyzes the *O*-methylation of catechol-containing xenobiotics [137]. Kuhnle demonstrated that *O*-methylation of the flavanols catechin and epicatechin occurs in intestinal epithelial cells and can precede other conjugation reactions [138]. When quercetin was intraperitoneally administered to rats, a great percentage was detected in urine in methylated form, a process that was proposed to inactivate the compound [139]. Other compounds have been described as substrates of COMT, either being simple phenols, phenolic acids or more complex (poly)phenols [135, 137].

Most (poly)phenols circulate in plasma in conjugated form. While for some compounds such as quercetin there is no free form in plasma, others such as epigallocatechin gallate are present in plasma mainly in unconjugated form [140]. Flavonols such as kaempferol and quercetin are mainly present as glucuronides after oral administration, however, sulfated (poly)phenols can also be very abundant [133].

Conjugation of (poly)phenols is therefore a very important phenomenon in the human body after absorption, occurring in many different locations, although the liver is described as the primary site.

Phase III – elimination of (poly)phenols

The final step in the elimination and/or detoxification of xenobiotics or exogenous compounds introduced into the body are the phase III transporters. These are mainly constituted by the ABC protein family,

including P-gp and multidrug resistance-associated protein (MRP2, ABCC2) and by solute carry family (SLC) transporters [92, 141]. In polarized cells that maintain barrier properties in tissues between lumen and the blood (i.e. intestinal mucosa cells, hepatocytes, kidney tubule cells, brain endothelial cells, etc), drug transporters have the task of eliminating the undesired compounds which are sent into the faeces, urine and bile [142]. The majority of conjugated-(poly)phenols are excreted in urine. However, a possible excretion back into the intestinal lumen by intestinal cells or to bile by liver parenchymal cells should also be regarded (Fig. 6).

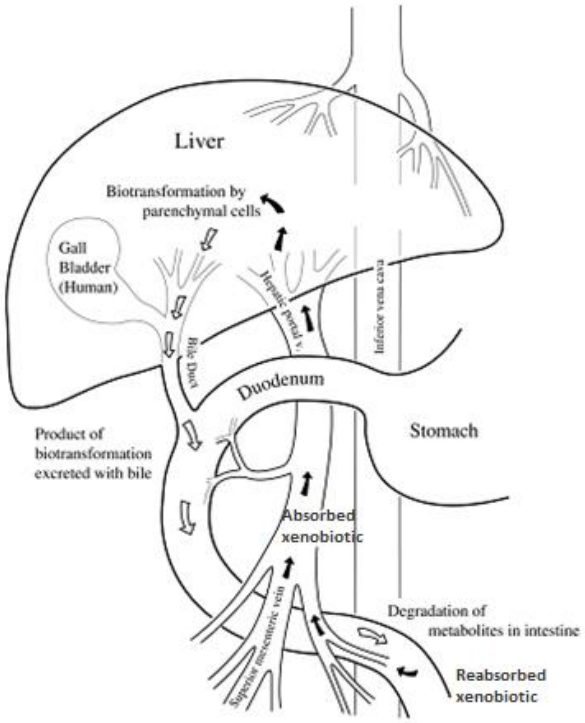

Figure 6. Enterohepatic recirculation. After absorption in the small intestine, xenobiotics are transported into the liver where they are biotransformed. The metabolites are transported into the bile and secreted back to the duodenum. Degradation of the metabolites in the intestine generates catabolites available for reabsorption. Adapted from DeSesso *et al.* [93].

In studies performed with rats, the extent of biliary and intestinal excretion between genistein, hesperetin and ferulic acid were shown to vary between the compounds causing considerable differences in their bioavailability [143]. Also, *in vitro* studies using Caco-2 cells have demonstrated the importance of intestinal apical efflux transporters in the bioavailability of (poly)phenols by excreting conjugated –phenolics [144-146]. Compounds excreted into bile are eventually emptied in the duodenum. Generally these conjugated substances are not absorbed in the small intestine, reaching the colon. In the colon these substances can either be excreted into the faeces or be degradaded by the colon microbiota and be reabsorbed [93].

Bioavailability of (poly)phenols is thus a multi-stage process comprising de-conjugation and possible catabolism, absorption, conjugation and excretion (Fig. 7). However, sequestration of compounds in body tissues has been usually undetermined and may contribute to underestimation of the bioavailability of (poly)phenols.

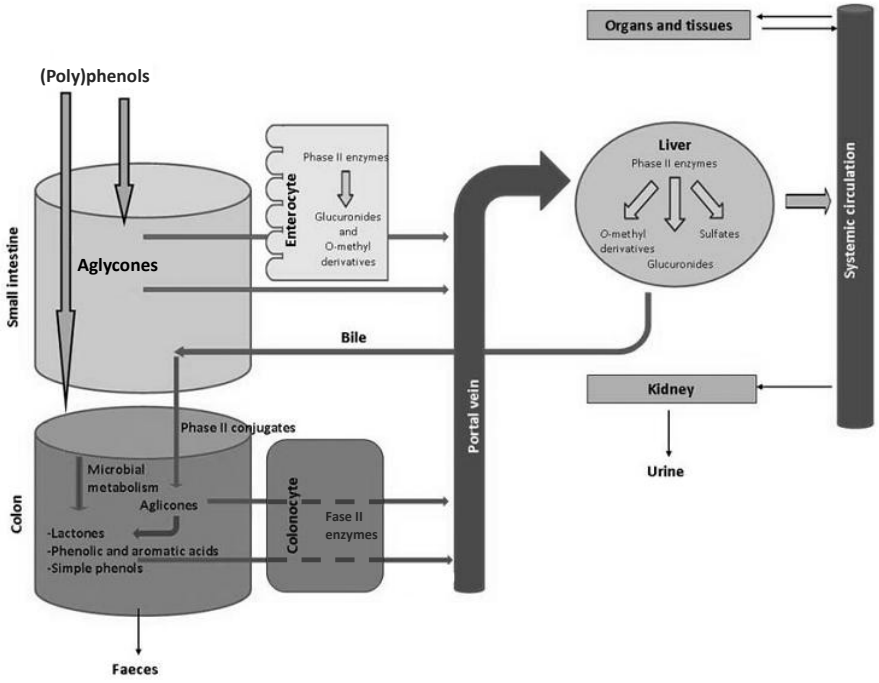

Figure 7. General diagram of absorption, biotransformation and excretion of (poly)phenols in the human body. Adapted from Monagas *et al*. [125]

AIMS AND SCOPE OF THE THESIS

This PhD thesis aims to identify the main bioavailable metabolites in humans derived from the ingestion of (poly)phenols present in berries fruits, particularly focusing on novel metabolites. For this five critical steps were undertaken:

i) Selection and characterization of fruits – The selection of the fruits to be used was based on their content in (poly)phenols. Of the chosen fruits, two are wild and native of the Portuguese territory (strawberry tree fruits and Portuguese crowberry), while the other three are agriculturally selected fruits (raspberry, blueberry and blackberry), having several varieties available and allowing selection of the most enriched in

(poly)phenols. Characterization of these fruits and identification and quantification of (poly)phenols was mainly performed by HPLC.

ii) Execution of an intervention study with human volunteers and collection of samples – ethical approval was obtained by the Faculdade de Farmácia da Universidade de Lisboa and healthy volunteers were recruited. Urine and plasma samples were collected from the volunteers after ingestion of an acute dose of the berries puree for breakfast. A two stage study was also conducted to exclude metabolites from the endogenous metabolism, revealed by the ingestion of a standards breakfast without (poly)phenols.

iii) Identification of the main bioavailable metabolites – identification of the most abundant bioavailable metabolites was performed in urine samples from the volunteers by recurring to an LC-Orbitrap MS which detects compounds by their exact mass. For this, an extensive library of possible bioavailable metabolites was constructed based on the metabolism previously described for (poly)phenols and the exact mass of compounds was calculated.

iv) Synthesis of compounds to be used as standards – standard compounds allow unequivocal identification of metabolites and also allow a much finer quantification. Since many of the compounds identified in this work did not have any commercially available standards, these had to be synthesized. Either with use of conjugative enzymes naturally present in animals (liver) or by laboratorial chemical synthesis, standard compounds were obtained.

v) Confirmation and quantification of metabolites in biological samples – metabolites were confirmed in urine and plasma samples by LC-MS/MS and quantified.

Identification and synthesis of novel metabolites represent a new opportunity for unravelling the mechanisms of action for the reported health effects associated with the consume of (poly)phenols-rich foods. Therefore, we aim to disclosing the protective effect of some of the identified and synthesized compounds against an oxidative stimulus on brain endothelial cells (HBMEC), which constitute the brain protective barrier. Integrity of this barrier is of great importance for prevention of neurodegenerative disorders. Additionally, we further tested the capacity of the compounds to cross the brain endothelial cells (HBMEC), simulating the entrance in the brain via blood-brain-barrier.

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Chapter 2

Analysis of (poly)phenols in berries

This chapter is based on the following manuscript:

Analysis of phenolic compounds in Portuguese wild and commercial berries after multi-enzyme hydrolysis.

Rui C. Pimpao; Tristan Dew; Pedro B. Oliveira; Gary Williamson;, Ricardo B. Ferreira; Cláudia N. Santos. *Journal of Agricultural and Food Chemistry* **2013,** 61, 4053-4062.

This chapter contains published data in which the author of this dissertation executed all the experimental work and wrote the manuscript.

Contents

ABSTRACT

Berry fruits are a good source of phenolic compounds, and thus potentially beneficial to health. Phenolic compounds are mainly present as a variety of conjugated forms, either with sugars via *O*-glycosidic bonds or with other polyols as esters. This chemodiversity makes characterization and identification highly demanding. Selected varieties of commercial blueberries, raspberries and blackberries, and two wild berries, Portuguese crowberry and strawberry tree fruit, were characterized for individual phenolic content by Liquid Chromatography – Diode Array Detection and mass spectrometry (HPLC-DAD-MS) after hydrolysis by a novel combination of the fungal glycosidases, hesperidinase and cellulase. This approach is shown to be a simple alternative to other existing methods for analysis of plant phenolic compound aglycones. The hydrolysis of glycosides and organic acid esters is efficient, and less aggressive than acid and alkaline hydrolysis. This method is able to disclose new sources of dietary phenolic compounds and the potential usefulness of Portuguese crowberry and strawberry tree fruit is herein demonstrated.

INTRODUCTION

Experimental and epidemiological evidence suggests that insufficient intake of fruit and vegetables may predispose the human body to a range of chronic health disorders. Consumption of fruit and vegetables has been associated with a decreased risk for certain degenerative diseases, such as coronary heart disease, stroke, cancer, diabetes mellitus and osteoporosis [1-6]. These effects have been associated with the ingestion of (poly)phenols, a class of plant secondary metabolites, which although not being classically "essential", are described as having beneficial health effects such as anti-inflammatory, antioxidant, neuroprotective and promoters of healthy ageing [7-14].

Berry fruits are particularly rich in a diverse range of (poly)phenols [1] from several different classes. The most abundant classes in berries are phenolic acids, tannins, and flavonoids, especially anthocyanins, responsible for the colored pigmentation [10]. Most phenolic compounds are present in plants as a variety of conjugated forms, either with sugars via *O*-glycosidic bonds or with other polyols as esters [15]. Numerous points of conjugation per aglycone can be possible (depending on class), leading to a diverse range of potential forms, differing in their bioavailabilities and bioactivities. This complexity also makes characterization and identification challenging. Following ingestion, whilst some (poly)phenols may be absorbed intact, typically absorption in the digestive tract requires hydrolysis of glycoside conjugates by small-intestinal lactase phlorizin hydrolase or cytosolic β-glucosidase [16, 17]. Aglycones are then subjected to phase II metabolism, and can be sulfated, methylated and

glucuronidated [18]. Compounds that escape from absorption in the small intestine can reach the colon and undergo biotransformation by the colonic microflora resulting in simpler catabolites which may also be absorbed and metabolized [19].

Commercial exploitation of a wider range of edible fruits could facilitate increased consumer intake, and characterization of (poly)phenols within these novel species may yield useful hints as to their potential bioactive capacity. Portuguese crowberry, *Corema album* L., is a low trailing shrub endemic to the Iberian Peninsula, belonging to the *Empetraceae* family and grows mainly in coastal habitats, particularly in sand dunes. It produces edible quasi-spherical drupes, approximately 1 cm in diameter, which are white when ripe and have a sugary and water-rich pulp [20]. The berries take months to ripen and peak of ripening happens in August and early September. Portuguese crowberries are edible and although they are not currently commercially exploited, are a potentially important source of nutrients and phytochemicals. Recently, they were described as a good source of phenolic acids and flavonols [21].

Strawberry tree, *Arbutus unedo* L., belongs to the *Ericaceae* family and is a typical shrub of the Mediterranean region. It produces edible spherical red berries when ripe, which are mostly processed to manufacture alcoholic beverages, jams and marmalades [22]. They are a good source of sugars, minerals, vitamins, carotenoids, organic acids and phenolic compounds such as proanthocyanidins and flavonoids and phenolic acids, which as expected are mostly glycosylated or esterified with organic acids [23-26].

In addition to these native berries, the specific climatic conditions in Portugal grant to producers the possibility of off-season production of

high quality berry fruits that are already commercially exploited, such as blueberries, raspberries and blackberries. Several commercial varieties of these berries are being tested in the Experimental Field of Fataca for perfecting production technologies and to enhance fruit quality and phytochemical value.

The aim of this study was to perform a novel combination of enzymes for hydrolysis of phenolics glycosides and quantification of aglycones by HPLC-diode array detection (DAD) with confirmation by mass spectrometry. The usefulness of this method was tested in five different berries, Portuguese crowberry, strawberry tree fruits, blueberry, raspberry and blackberry.

MATERIAL AND METHODS

Chemicals and standards

Organic solvents were purchased from Fischer Scientific and were of HPLC grade. Formic acid was purchased from the same company. Phloroglucinaldehyde, *p*-coumaric acid, ferulic acid, caffeic acid, punicalagin and avicularin were from Sigma Aldrich (St. Louis, MO, USA). All other reagents otherwise stated were purchased from Sigma Aldrich. Folin-Ciocalteau reagent and vanillic acid were from Fluka (Buchs, Switzerland). 5-Caffeoylquinic acid, 3-caffeoylquinic acid and protocatechuic acid were from Acros Organics (Geel, Belgium). 3-Methylgallic acid was from Apin Chemicals Ltd. (Abingdon, UK) and syringic acid from Alfa Aesar (Haverhill, USA). Gallic acid, daidzein, cyanidin-3-*O*-glucoside, (+)-catechin, (-)-epicatechin, ellagic acid,

myricetin, quercetin, kaempferol, myricitrin, hyperoside, quercitrin, rutin, isoquercitrin, astragalin and nicotiflorine were purchased from Extrasynthese (Lyon, France). Cellulase (EC. 3.2.1.4) and hesperidinase (EC. 3.2.1.40) from *Aspergillus niger* were purchased from Sigma Aldrich (St. Louis, MO, USA).

Plant material

Blueberry (*Vaccinum* spp.) fruits of six cultivars (Biloxi, Sharpblue, Misty, Jubilee, Star, Georgia Gem), Raspberry (*Rubus idaeus*) fruits of three varieties (Glen Lyon, Polka and Himbo Top) and Blackberry (*Rubus L.* subgenus Rubus Watson) of five varieties (Arapaho, Loch Tay, Olallie, Oros and Karaka Black) were grown in the Fataca experimental field in Odemira, Portugal. Wild Portuguese crowberry fruits (*Corema album* L.) were collected in the Comporta region and wild strawberry tree fruits (*Arbutus unedo* L.) were collected from the Alentejo region of Portugal and grouped. Fruits were hand-harvested at a mature stage determined by their colour and were stored until analysis at -20 °C for no more than 2 months.

Soluble solids content (SSc) and total titratable acidity (TAc)

Samples of the fruits were pooled to obtain a composite sample and analysed for SSc using a hand refractometer Milwaukee model MR32ATC, with a scale range of Brix degrees (°Brix) from 0% to 32.0%.

TAc was determined by titrating a known volume of fruit juice with 0.1 N NaOH until the pH reached 7. The pH was measured with a Crison micro-

pH 2002 pH meter. The volume of NaOH required was used to calculate TAc, which is expressed as g(tartaric acid)/L.

Extraction of phytochemicals

Fruits were blended using a domestic food processor until they were homogeneous, freeze-dried and were then stored at -80 °C. For total phenolics, anthocyanins and Oxygen Radical Absorbance Capacity (ORAC) analysis, phytochemicals were extracted as previously described [23]. To each gram of lyophilized powder, 12 mL of ethanol-water (1:1) solvent were added and the mixture was shaken for 30 min, at room temperature in the dark. The mixture was then centrifuged at 12,400 *g* during 10 min at room temperature. The supernatant was filtered through 0.20 μm cellulose acetate membrane filters. The resulting extracts were stored frozen at –80 °C.

Total phenolic measurement

Determination of total phenolic compounds was performed by the Folin-Ciocalteau method [27] adapted for microplates as described elsewhere [28]. Gallic acid was used as standard and the results were expressed in mg of gallic acid equivalents per g of dry weight of plant material (mg GAE/g dw).

Anthocyanin content

The total anthocyanin content of the fruit extracts was determined using a pH differential absorbance method as described elsewhere [29]. Absorbance readings were related to anthocyanin content using the molar extinction

coefficient of 26900 (L mol^{-1} cm^{-1}) for cyanidin-3-*O*-glucoside [30]. Results were expressed as mg of cyanidin-3-*O*-glucoside equivalents per 100 g of dry weight of plant material (mg cy-3-gluc/g dw).

Peroxyl Radical Scavenging Capacity Assay

Peroxyl radical scavenging capacity was determined by the ORAC method [31, 32] adapted to microplate as described elsewhere [28]. The final results were calculated using the area differences under the fluorescence decay curves between the blank and the sample, and were expressed as mM Trolox equivalents per 100 g of dry weight of plant material (mM TE/100 g dw).

Vitamin C determination

To quantify the total amount of vitamin C (ascorbic acid plus dehydroascorbic acid), 50 g of fruits were homogenised with 50 mL of phosphoric acid. The homogenate was then centrifuged at 13,000 *g* for 20 min at 4 °C and the supernatant filtered using 0.20 μm cellulose acetate membrane filters. To 1 mL of the filtrate was added 0.2 mL dithiothreitol (20 mg/mL) to reduce dehydroascorbic acid, and allowed to stand in darkness for 2 h [33]. The solution was then passed through a Millipore 0.45 μm PTFE filter and injected into the HPLC system. Ascorbic acid quantification was conducted on a Hitachi HPLC instrument (VWR) equipped with EZChrom Elite software (Agilent), a model L-2130 pump system, a model L-2200 autosampler, a model L2300 column oven and a model L2455 DAD system. A sample volume of 10 μL was injected and separations were achieved on an Inertsil ODS-3V column (250 × 4.6 mm, 5

μm), operated at 30 °C. The column was held at 30°C and ascorbic acid was eluted isocratically using a 0.7 mL/min flow of 0.01 M H_2SO_4 (pH 2.5). Solutions of L-ascorbic acid prepared in water with concentration values between 5 and 500 mg/L were used for the calibration curve and detection was made at 262 nm.

Enzymatic hydrolysis of glycosides

To perform enzymatic hydrolysis of glycosides, phytochemicals were extracted by homogenizing 0.3 g of blended fruits with 2.7 mL of ethanol-water (1:1) containing 1 mM of butylated hydroxytoluene (BHT) and 0.1 mM of daidzein as internal standard. Samples were vortexed (10 min), sonicated (10 min) then vortexed again (10 min), then centrifuged at 3000 *g* for 20 min at 4 °C. After collecting the supernatant, the pellet was re-extracted by the same procedure with 0.9 mL of the extraction solution. Supernatants were combined, centrifuged as described and filtered through a 0.2 μm PTFE filter. One part of the extract was used directly for HPLC analysis of total anthocyanins and caffeoylquinic acids. Another part of the extract was dried and reconstituted in water with 1 mM ascorbic acid, to protect samples from oxidation. Samples for aglycone analysis were adjusted to pH 3.8 with acetate buffer 0.2 M and incubated for 16 h at 40 °C with 0.02 U/mL hesperidinase (one unit corresponds to 333 mg of protein and is defined as the amount required to liberate 1.0 μmol of glucose from hesperidin per minute at pH 3.8 at 40 °C). The pH was then increased to 5.0 with sodium acetate and cellulase was added to a final concentration of 20 U/mL (one unit corresponds to 0.885 mg of protein and is defined as the amount required to liberate 1.0 μmol of

glucose from cellulose in 1 h at pH 5.0 at 37 °C). Samples were incubated for a further 4 h at 37 °C and then extracted 3 times with ethyl acetate. The ethyl acetate phase (containing phenolic aglycones) was separated, evaporated and samples were reconstituted with 2 mL of 0.1% (w/v) ascorbic acid solution in ethanol-water (1:1), and filtered through a 0.2 μm PTFE filter prior to analysis. The procedure was performed in triplicate for each fruit. Hydrolysis conditions were optimized by testing the hydrolysis efficiency of fruit extracts by testing individual enzymes incubated for 4 and 18 h and combined (hesperidinase for 18 h followed by cellulase for 4 h and cellulase for 18 h followed by hesperidinase for 4 h). Efficiency of hydrolysis was also tested by incubating different commercial standards of flavonol-glycosides (isoquercitin, hyperoside, rutin, quercitrin, avicularin, myricitrin, astragalin and nicotiflorine), 5-caffeoylquinic acid and 3-caffeoylquinic acid and punicalagin. Standards were dissolved to a concentration of 500 μM and were incubated with enzymes as described previously. Efficiency of hydrolysis was evaluated by substrate disappearance. Recoveries of the identified aglycones were calculated for each compound by spiking each fruit with the identified aglycones (table 5). Recoveries were used to correct the quantification values of aglycones by dividing the determined value by the percentage of the recovery.

HPLC analysis of (poly)phenols

HPLC analysis was conducted on a series 1200 HPLC instrument (Agilent Technologies, Manchester, UK) equipped with ChemStation software (B.03.01), a model G1379B degasser, a model G1312B binary gradient pump, a model G1367C temperature controlled autosampler, a model

G1316B column oven, and a model G1315C DAD. A sample volume of 5 μL was injected and separations were achieved on a Zorbax Eclipse XDB-C18 (4.6x50 mm; 1.8 μm), operated at 30 °C, with a 0.2 μm stainless steel in-line filter. The method was performed as described by Pandino and co-workers [34] with the exception of the mobile phase consisting in 0.1% (v/v) formic acid in water (solvent A) and 0.1% (v/v) formic acid in acetonitrile (solvent B), used at a flow rate of 0.5 mL/min. The gradient started with 5% B to reach 10% B at 5 min, 40% B at 20 min, 90% B at 25 min, 90% B at 29 min.

Chromatograms were recorded at 272, 280, 302, 324, 370 and 520 nm from diode array data collected between 200 and 600 nm. All standards were dissolved in ethanol or ethanol-water (1:1) and used as stock solutions. Calibration curves of peak area against concentration showed a good linear correlation with $r^2 > 0.99$. Limits of detection and quantification as well as the wavelengths use to detect each compound are represented in Table 1.

Statistical analysis

The results reported in this work are the averages of at least three independent experiments and are represented as the mean ± SD. Differences among treatments were detected by analysis of variance with Tukey HSD (Honest Significant Difference) multiple comparison test (α = 0.05) using SigmaStat 3.10 (Systat).

Table 1. Chromatography parameters of standard compounds used for peak identification

Standards	Retention Time (min)	Wavelength (nm)	LOD (mg/mL)	LOQ (mg/mL)	Calibration range (mg/mL)
Gallic acid	2.59	272	0.25	0.5	0.5-100
Protocatechuic acid	5.26	272	0.09	0.2	0.5-100
3-Methylgallic acid	6.28	272	0.09	0.2	0.5-100
3-Caffeoylquinic acid	6.03	324	0.5	0.75	0.75-100
Cyanidin-3-*O*-glucoside	8.07	520	0.75	2.5	2.5-100
5-Caffeoylquinic acid	8.40	324	0.25	0.75	0.75-100
(+)-Catechin	8.60	280	0.55	1	2.5-100
Vanillic acid	9.41	272	0.09	0.2	0.5-100
Caffeic acid	9.84	324	0.04	0.1	0.5-100
Syringic acid	10.06	272	0.05	0.2	0.25-100
(-)-Epicatechin	10.54	280	0.3	0.55	1-100
Phloroglucinaldehyde	12.00	280	0.05	0.1	0.1-100
***p*-Coumaric**	12.11	302	0.04	0.09	0.1-100
Ellagic acid	12.97	370	0.1	0.4	0.75-100
Ferulic acid	13.16	324	0.05	0.15	0.25-100
Myricetin	15.50	370	0.1	0.25	0.25-100
Daidzein	17.20	302	0.1	0.25	0.25-100
Quercetin	18.30	370	0.1	0.25	0.25-100
Kaempferol	20.83	370	0.1	0.25	0.25-100

RESULTS AND DISCUSSION

Portugal has good climatic conditions to facilitate production of different types of berries, and several different varieties have already been introduced in experimental fields. In order to select fruits to be further analyzed with a diverse chemical composition, an initial screening of chemical parameters for available commercial varieties and two wild species was performed.

Soluble solid content and titratable acidity

Fruit quality parameters, such as soluble solids content (SSc) and titratable acidity (TAc), important as a quality indexes for the consumer, were determined for the berries and their varieties (Table 2). Strawberry tree fruit showed the highest SSc, far above the other analyzed berries, in contrast to Portuguese crowberry, which exhibited the lowest value. Some variability between the varieties of each commercial berry was observed, although in general blueberry varieties possessed the highest SSc and raspberries the highest TAc values, whilst blueberries were lowest. The ratio between SSc and TAc was particularly low for raspberry fruits, Portuguese crowberry and blackberry variety Oros, suggesting that acidity would be obvious to consumers. The opposite was observed for blueberry varieties and strawberry tree fruit where a sweet flavour would be more prevalent. The high sugar content of strawberry tree fruit translates to a proclivity towards fermentation, making its chief utilization as a processed product rather than a fresh fruit. On the other hand the

high acidity of Portuguese crowberry fruits may contribute to the lack of demand by Portuguese consumers.

Table 2. Analysis of fruits for soluble solids content (SSc) and titratable acidity (TAc)

Fruit	**Variety**	**SSc (ºBrix)**	**TAc [g(tartaric acid)/L]**	**SSc/TAc**
Blueberry	Biloxi	12.2 ± 0.8^{a}	3.5	3.4
	Sharp Blue	12.0 ± 1.3^{a}	5.6	2.1
	Misty	11.3 ± 0.8^{a}	5.5	2.1
	Jubilee	12.7 ± 2.3^{a}	5.4	2.3
	Star	14.2 ± 2.1^{a}	2.9	4.8
	Georgia Gem	15.3 ± 1.5^{a}	2.6	5.8
Raspberry	Glen Lyon	9.2 ± 1.0^{a}	14.6	0.6
	Polka	8.7 ± 1.5^{a}	9.1	1.0
	Himbo Top	9.0 ± 0^{a}	13.3	0.7
Blackberry	Loch Tay	13.0 ± 2.1^{ab}	5.1	2.5
	Olallie	7.4 ± 0.9^{c}	4.5	1.7
	Arapaho	9.8 ± 1.0^{bc}	7.1	1.4
	Oros	7.1 ± 0.9^{c}	8.9	0.8
	Karaka Black	13.7 ± 2.0^{a}	6.2	2.2
Portuguese crowberry		6.8 ± 0	8.7	0.8
Strawberry tree fruit		27.8 ± 0.1	7.4	3.7

A, b, c represent the significance levels between varieties of the same fruit.

Phytochemical analysis

Fruit extracts were quantified for total phenols, anthocyanins and antioxidant capacity (Fig. 1). These methods, although having some limitations and interferences, were selected as preliminary assays for quantification of phenolic compounds, in order to choose one variety of

each for subsequent studies. Blackberries and blueberries were the fruits with higher content in total phenolic compounds and antioxidant capacity, which can be attributed to their high content of anthocyanins. The values of total phenolic compounds and antioxidant capacity of both wild berries were at a similar level to raspberries although their content of anthocyanins was very low. Whilst strawberry tree fruit have a red peel, their anthocyanin level is low. In terms of varietal diversity, for blueberries, Georgia Gem contained the highest values for total phenolic compounds and anthocyanins. For raspberries, Himbo Top contained the highest values of total phenolic compounds, anthocyanins and antioxidant capacity. All blackberry varieties possessed high values for anthocyanins and antioxidant capacity, and although Karaka Black contains slightly lower values for both parameters, it was the highest for total phenolic compounds content.

Based on these results, the blueberry variety Georgia Gem, the raspberry variety Himbo Top and the blackberry variety Karaka Black were selected together with strawberry tree and Portuguese crowberry fruits for a more detailed analysis of phenolic compounds and free aglycones for the proposed method.

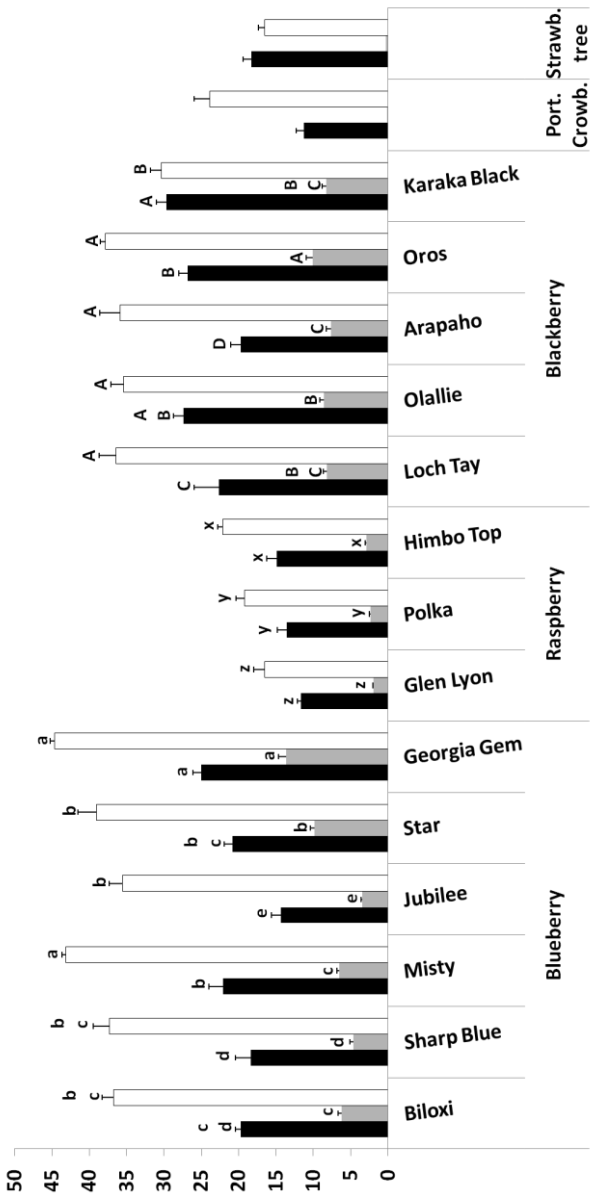

Figure 1. Phytochemical analysis of commercial varieties and wild fruits. Measurement of total phenols (■ - mg GAE/g dw), anthocyanins (■ - mg cy-3-glu/g dw) and antioxidant capacity (□ - mmol TE/100 g dw) in fruit hydroethanolic extracts. Statistical comparisons are made between the varieties of each fruit. The letters a, b, c, d, e; x, y, z; A, B, C, D represent the levels of significance between differences for each analyzed parameter independently.

HPLC analysis

Analysis of intact, non-hydrolyzed fruit extracts by HPLC-DAD revealed a great complexity and diversity of compounds hampering individual identification of compounds. Only 3-caffeoylquinic and 5-caffeoylquinic acids were identified and quantified based on their retention time and absorption spectra in comparison with standards (Table 3). Both caffeoylquinic acids were found to be the major compounds in Portuguese crowberry fruits, and 5-caffeoylquinic acid was also abundant in blueberry. In a recent study, 5-caffeoylquinic acid was identified as the most abundant compound in Portuguese crowberry fruits, at higher levels than observed in our study, although reported levels of 3-caffeoylquinic acid were lower [21]. The values of 5-caffeoylquinic acid are slightly lower than described previously for blueberry (47 mg/100 g fw cf published values of 64.6 - 208 mg/100 g fw [35, 36]. In the other analyzed berries, if present, these compounds were too low to be detected or were obscured by other peaks. Anthocyanins were also analyzed by HPLC with detection at 520 nm and expressed as cyanidin-3-*O*-glucoside equivalents. Blueberry expressed the highest values, whereas anthocyanins were not detected in Portuguese crowberry fruits. Despite the fact that strawberry tree fruit has a red exocarp (when mature), they contained only 9.7 mg/100 g fw of anthocyanins, but even this is higher than the value of 0.51 mg/100 g fw previously reported [37]. The anthocyanins present in strawberry tree fruits are expected to be cyanidin and delphinidin both conjugated with galactose and glucose, and the former also conjugated with arabinose [24, 37].

Table 3. Quantification[a] of total anthocyanins, 5-caffeoylquinic acid, 3-caffeoylquinic acid and total vitamin c in fruits by HPLC-DAD

Compounds	**Fruits**				
	Blueberry Var. Georgia Gem	**Raspberry Var. Himbo Top**	**Blackberry Var. Karaka Black**	**Portuguese crowberry**	**Strawberry tree**
Anthocyanins	433.2±28.8	61±1	160.6±3.7	n.d.	9.72±0.30
3-caffeoylquinic acid	n.d.	n.d.	n.d.	37.1±3.7	n.d.
5-caffeoylquinic acid	47.10±0.51	n.d.	n.d.	33.2±2.6	n.d.
Total vitamin C[b]	0.97±0.12	12.52±0.09	4.69±0.08	5.43±0.06	89.1±1.7

[a]Contents are expressed as means of mg/100 g fw of fruits. Antocyanins are quantified as means of cyanidin-3-*O*-glucoside equivalents. nd, not detected. [b]Total vitamin C was quantified as mg (L-ascorbc acid equivalents)/100 g fw of fruits.

Total vitamin C was determined for the berries (Table 3). Strawberry tree fruit vitamin C content was far above that of the other fruits and exceeded values reported previously by Pallauf and co-workers [24], but in agreement with Alarcão-E-Silva and co-workers, who found a similarly high ascorbate content compared to other common fruits [22]. Values for vitamin C in Portuguese crowberries were similar to blackberry while blueberries contained the smallest amount (Table 3).

Analysis of aglycones after enzyme hydrolysis of glycosides

To overcome the complexity of (poly)phenol analysis, the extracts were incubated with enzymes from *Aspergillus niger*: hesperidinase containing α-L-rhamnosidase and β-D-glucosidase activities, and cellulase containing endo-1,4-β-D-glycosidic activity. Other enzymes from *A. niger* have previously been used to release phenolic acids from carbohydrates [38-40].

Optimization of hydrolysis conditions and incubation time by using this novel combination of enzymes was performed. Fruit extracts and flavonol glycoside standards were used to test hydrolysis efficiency. Blueberry, due its phytochemical complexity, was selected for this optimization process, and the results extrapolated to the other fruits. First, individual and a combination of enzymes were incubated with fruit extracts and production of aglycones was compared. A combination of enzymes proved to be more efficient to obtain a higher degree of aglycones than individual enzymes. Hesperidinase hydrolysis was time-dependent whereas cellulase had similar efficiency in flavonol-glycoside hydrolysis for both 4 and 18 h incubation periods. On the basis of these results, incubation of fruit extracts with both enzymes, hesperidinase for 18 h and

cellulase for 4 h, allowed complementary hydrolysis optimized the reaction to obtain maximum yield of aglycones.

Further tests were performed to determine the hydrolytic efficiency of flavonol glycosides, by using standard compounds (Table 4). Incubation with the above enzyme combination enabled efficient hydrolysis of glucosides, rutinosides, and galactosides. The hydrolysis of arabinoside bonds was less efficient, as was hydrolysis of the rhamnoside bond in myricitrin and quercitrin. The enzymes also hydrolyzed the ester bond between caffeic and quinic acids of 3- and 5-caffeoylquinic acids, although hydrolysis of 3-caffeoylquinic acid was not complete (Table 4).

Table 4. Hydrolysis efficiency of standard compounds incubated with hesperidinase for 18 h, followed by cellulase for 4 h.

Aglycone	**Conjugated sugar**	**Compound Name**	**Efficiency of hydrolysis**
Quercetin	Glucoside	Isoquercitrin	Complete
	Galactoside	Hyperoside	Complete
	Rutinoside	Rutin	Complete
	Rhamnoside	Quercitrin	Incomplete (18%)
	Arabinoside	Avicularin	Incomplete (56%)
Myricetin	Rhamnoside	Myricitrin	Incomplete (17%)
Kaempferol	Glucoside	Astragalin	Complete
	Rutinoside	Nicotiflorine	Complete
		5-caffeoylquinic acid	Complete
		3-caffeoylquinic acid	Incomplete (61%)
		Punicalagin	Incomplete (73%)

The combination of enzymes was observed to hydrolyze punicalagin, an ellagitannin, although not completely, resulting in smaller compounds such as ellagic (Table 4). This additional hydrolysis (of non-glycosidic bonds) was probably due to the presence of other enzyme activities such as esterases in commercial enzymes which are only partially purified.

For each fruit extract, recovery of aglycones was calculated after spiking with the reference standard and the values were used for correction of quantification (Table 5). Values for most compounds in all extracts were satisfactory, with the exception of gallic acid. However, the (low) efficiency of extraction of gallic acid was reproducible, implying that the limitation was in the incomplete partitioning and not due to instability.

As expected, HPLC-DAD chromatograms of fruit extracts were far less complex following hydrolysis and the respective aglycones present in these berries were revealed (Fig. 2-6).

Table 5. Recovery values (%) for each compound calculated by HPLC-DAD after spiking of fruit extracts.

		Fruits				
Peak number	Compound	Blueberry Var. Georgia Gem	Raspberry Var. Himbo Top	Blackberry Var. Karaka Black	Portuguese crowberry	Strawberry tree
1	Gallic acid	23.8±1.0	47.3±3.1	28.6±1.6	34.2±4.9	31.6±3.7
2	Protocatechuic acid	47.3±4.0	86.6±4.0	66.8±5.1	74.6±5.7	65.7±2.8
3	3-Methylgallic acid	46.8±2.0	n.d.	n.d.	n.d.	n.d.
4	(+)-Catechin	n.d.	n.d.	n.d.	n.d.	77.4±7.4
5	Vanillic acid	80.8±1.5	n.d.	n.d.	82.4±5.0	n.d.
6	Caffeic acid	100.4±10.5	93.7±6.6	n.d.	89.6±16.3	n.d.
7	Syringic acid	59.5±5.5	n.d.	n.d.	n.d.	n.d.
8	(-)-Epicatechin	62.7±4.9	74.7±4.3	78.5±4.2	69.2±3.0	n.d.
9	Phloroglucinaldeyde	78.8±5.8	88.4±4.5	74.4±9.9	n.d.	71.9±8.8
10	p-Coumaric acid	99.0±5.8	n.d.	n.d.	92.5±6.7	n.d.
11	Ellagic acid	96.9±10.5	61.1±16.4	95.9±14.0	n.d.	50.0±9.1
12	Ferulic acid	97.1±7.3	95.8±6.8	96.7±5.6	90.7±4.2	n.d.
13	Myricetin	78.9±5.4	n.d.	n.d.	87.7±7.4	85.0±2.0
15	Quercetin	85.2±5.9	79.6±5.7	79.9±6.8	97.8±8.5	80.2±10.9
16	Kaempferol	73.2±3.9	n.d.	75.2±2.0	92.7±5.4	n.d.

n.d. – not determined (since it was not detected after hydrolysis)

Table 6. Quantification of phenolics in fruit extracts after enzyme hydrolysis using HPLC-DAD.

Peak number	Compound	Fruits				
		Blueberry Var. Georgia Gem	**Raspberry Var. Himbo Top**	**Blackberry Var. Karaka Black**	**Portuguese crowberry**	**Strawberry tree**
1	Gallic acid	16.32±3.10	1.44±0.06	6.08±0.25	1.12±0.15	117.2±2.4
2	Protocatechuic acid	1.95±0.03	3.88±0.30	12.98±0.97	1.32±0.05	2.27±0.12
3	3-Methylgallic acid	5.28±0.10	u.d.	u.d.	u.d.	u.d.
4	(+)-Catechin	u.d.	u.d.	u.d.	u.d.	25.46±0.02
5	Vanillic acid	1.03±0.05	u.d.	u.d.	0.63±0.03	u.d.
6	Caffeic acid	31.94±0.54	4.08±0.23	u.d.	39.42±2.15	u.d.
7	Syringic acid	5.08±0.12	u.d.	u.d.	u.d.	u.d.
8	(-)-Epicatechin	5.24±0.43	3.31±0.19	4.18±0.06	1.83±0.33	u.d.
9	Phloroglucinaldeyde	1.21±0.17	3.63±0.12	12.19±0.40	u.d.	0.41±0.03
10	*p*-Coumaric acid	1.89±0.16	u.d.	u.d.	0.89±0.02	u.d.
11	Ellagic acid	2.9±0.05	12.45±0.77	8.62±0.58	u.d.	7.43±0.12
12	Ferulic acid	3.06±0.07	1.98±0.09	0.62±0.02	0.56±0	u.d.
13	Myricetin	9.20±0.13	u.d.	u.d.	1.39±0	1.13±0.05
15	Quercetin	11.30±0.33	2.14±0.14	3.04±0.05	3.92±0.18	3.16±0.10
16	Kaempferol	1.01±0.01	u.d.	0.36±0.01	0.27±0	u.d.

Contents are expressed as means of mg/100 g dw of fruits. u.d. – under limit of detection

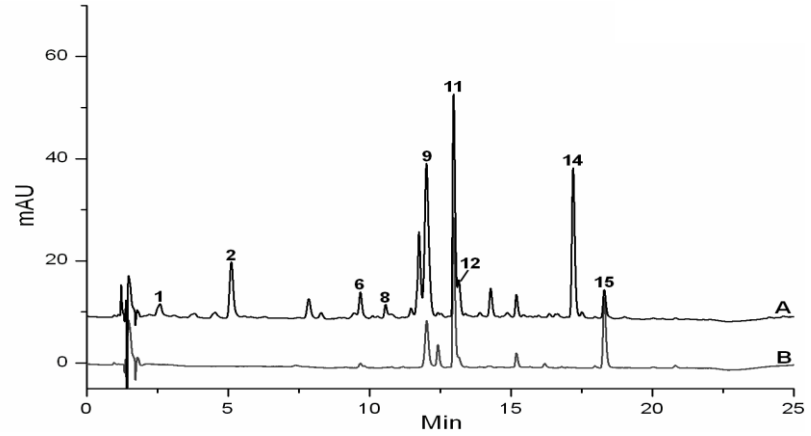

Figure 2. HPLC-DAD chromatograms of aglycones present in blueberry var. Georgia Gem extracts after enzyme hydrolysis: (**A)** recorded at 270 nm; (**B)** recorded at 370 nm. 1- Gallic acid, 2 - protocatechuic acid, 3 - 3-methylgallic acid, 5 - vanillic acid, 6 - caffeic acid, 7 - syringic acid, 8 - (-)-epicatechin, 9 - phloroglucinaldehyde, 10 - *p*-coumaric acid, 11 - ellagic acid, 12 - ferulic acid, 13 - myricetin, 14 - daidzein (IS), 15 - quercetin, 16 - kaempferol.

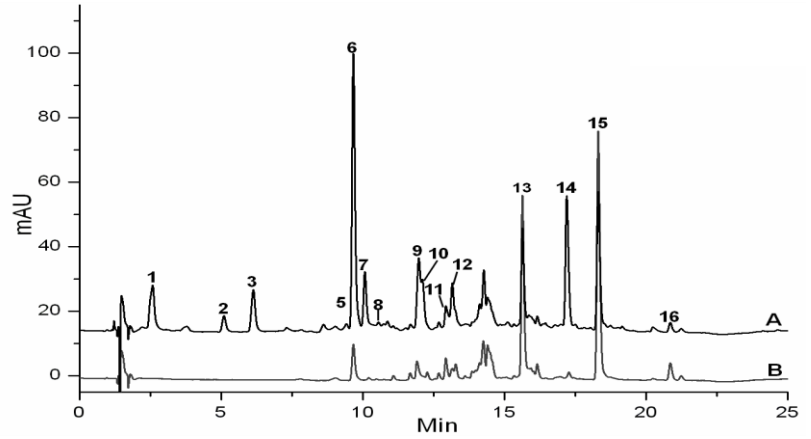

Figure 3. HPLC-DAD chromatograms of aglycones present in raspberry var. Himbo Top extracts after enzyme hydrolysis: (A) recorded at 270 nm; (B) recorded at 370 nm. 1- Gallic acid, 2 - protocatechuic acid, 6 - caffeic acid, 8 - (-)-epicatechin, 9 - phloroglucinaldehyde, 11 - ellagic acid, 12 - ferulic acid, 14 - daidzein (IS), 15 - quercetin.

Identification of the aglycones in the extracts was performed by use of LC-MS data and confirmed with reference standards. LC-MS data is shown as supplementary information (supplementary tables 1-5). Blueberry analysis revealed the presence of a high diversity of aglycones (Fig. 2, Table 5 and 6), with caffeic and gallic acids as the most abundant compounds. The majority of caffeic acid resulted from hydrolysis of 5-caffeoylquinic acid (as initially quantified) but a proportion is also likely derived from the hydrolysis of caffeic-4-*O*-glucoside, a minor compound previously reported in these berries [36]. The flavonols myricetin, quercetin and kaempferol were also present in blueberries, and are present in glycosylated form with values comparable to those reported here [35, 41]. Protocatechuic, gallic, *p*-coumaric and ferulic acids present in hydrolyzed extracts were previously found conjugated with glucose [36]. The same study also reported the presence of ferulic, *p*-coumaric and caffeic acids esterified with quinic acid. Of the flavanols, only (-)-epicatechin was detected in blueberries. This compound was found in previous studies in concentrations between 0.7 and 1.1 mg/100g fresh fruit [15, 42]. However, in another study, no (-)-epicatechin was observed in 4 varieties of blueberries whilst in three of these varieties, (+)-catechin and Procyanidin B2 were identified, neither of which were found in our study [43]. This clearly reflects some chemical diversity of the minor compounds within blueberry varieties.

In the hydrolysed extracts, 3-methylgallic acid, syringic acid, vanillic acid and phloroglucinaldeyde were also present, which might be derived from hydrolysis or catabolism of compounds initially present in berries.

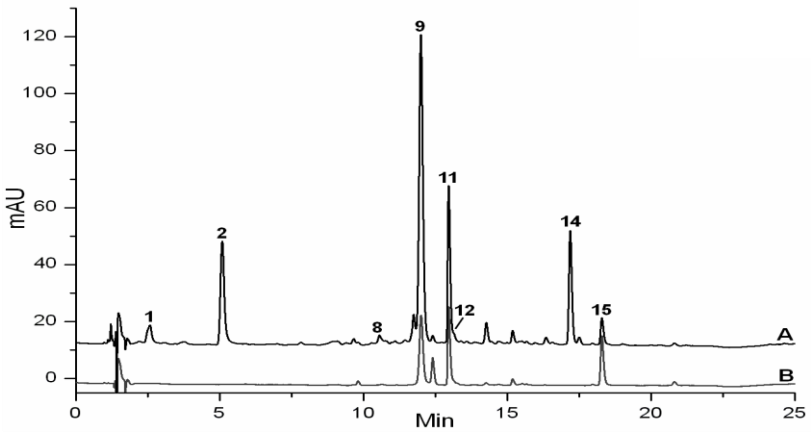

Figure 4. HPLC-DAD chromatograms of aglycones present in blackberry var. Karaka Black extracts after enzyme hydrolysis: (**A)** recorded at 270 nm; (**B)** recorded at 370 nm. 1- Gallic acid, 2 - protocatechuic acid, 8 - (-)-epicatechin, 9 - phloroglucinaldehyde, 11 - ellagic acid, 12 - ferulic acid, 14 - daidzein (IS), 15 - quercetin, 16 - kaempferol.

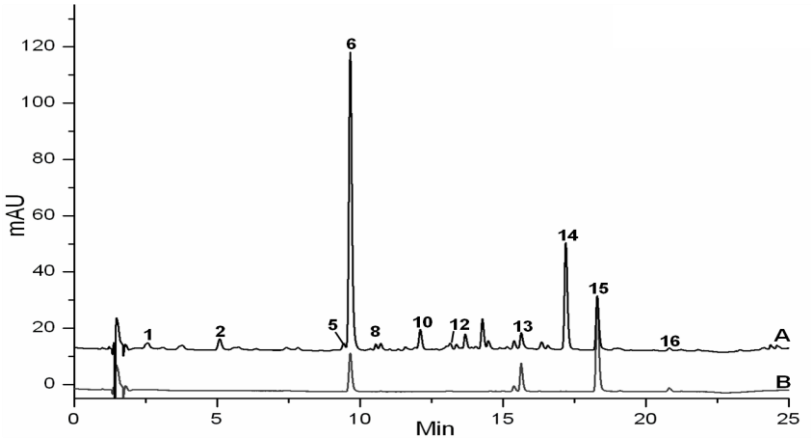

Figure 5. HPLC-DAD chromatograms of aglycones present in Portuguese crowberry fruit extracts after enzyme hydrolysis: (**A)** recorded at 270 nm; (**B)** recorded at 370 nm. 1- Gallic acid, 2 - protocatechuic acid, 5 - vanillic acid, 6 - caffeic acid, 8 - (-)-epicatechin, 10 - *p*-coumaric acid, 12 - ferulic acid, 13 - myricetin, 14 - daidzein (IS), 15 - quercetin, 16 - kaempferol.

For raspberries (Fig. 3, Table 5 and 6), the most abundant compound was ellagic acid. Whilst this compound has been previously quantified in the free form or attached to glycosides, it is mainly found esterified in ellagitanins such as sanguiin-H6 and lambertianin C [44, 45]. Of the flavanols, only quercetin and (-)-epicatechin were detected. The presence of other flavanols such as (+)-catechin and procyanidin B2 is inconsistent between studies and appears to vary between varieties [42, 46, 47]. The same appears to happen for kaempferol which was not detected in this work and also in some previous studies [46, 47] but was found in other studies [44, 45]. Caffeic acid was detected and may be derived from the 5-caffeoylquinic acid content previously reported in raspberries [48]. Mullen and co-workers reported a similar method for quantification of phenolics in raspberry, performing an hydrolysis of the extracts with β-glucosidase [45], but of the phenolic acids, only the presence of *p*-coumaric, caffeic and ellagic acids were reported. In this work, we also found gallic, protocatechuic and ferulic acids, and phloroglucinaldeyde in the hydrolyzed extracts (Fig. 3, Table 6).

In blackberries (Fig. 4, Table 5 and 6), the most abundant compounds were protocatechuic acid, phloroglucinaldeyde and ellagic acid. Ellagic acid was previously found in the free form in blackberries but also esterified as ellagitannins [49, 50] whilst protocatechuic acid was previously found in low amounts conjugated with glucose [36]. As in blueberry and raspberry, of flavanols, only (-)-epicatechin was detected in blackberries following hydrolysis. However, in previous studies, (+)-catechin was also found in low concentrations (6.6 mg/kg) [42, 51] as well as other flavanols (0.06 and 0.83 mg/100g) [51]. Therefore, the presence of some flavanols might be

variety dependent, sometimes under the limit of detection. Quercetin was the most abundant flavonol in blackberries, with kaempferol in lower amounts in contrast with its absence in previous studies [41, 52]. Gallic and ferulic acids were also found in hydrolysed blackberry extracts, both found previously conjugated with glucose, and ferulic acid with quinic acid [36, 49]. To be noted is the absence of caffeic acid, which was previously described by Schuster in blackberries as conjugated with glucose and quinic acid [36]. Differences in the presence or absence of some compounds in comparison with previous studies might be the result of differences in cultivar between fruits and growing conditions.

Portuguese crowberry extracts also contained a great diversity of phenolic aglycones after enzyme hydrolysis (Fig. 5, Table 5 and 6). The most abundant compound was caffeic acid, derived mainly from the hydrolysis of 3-caffeoylquinic and 5-caffeoylquinic acids, which were abundant in the non-hydrolyzed extracts (Table 3). Other hydroxycinnamates such as ferulic and *p*-coumaric acids were found at lower levels. The flavonols quercetin, kaempferol and myricetin were present in the hydrolyzed extracts. The same compounds were also detected conjugated with glycosides in a previous study by HPLC-DAD-MS/MS [21]. (-)-Epicatechin, however, was not found in the same study. Gallic, protocatechuic and vanillic acids were also found in Portuguese crowberry fruits (Table 6). These compounds had been found previously by GC-MS but not by HPLC [21], demonstrating the utility of the proposed hydrolysis method.

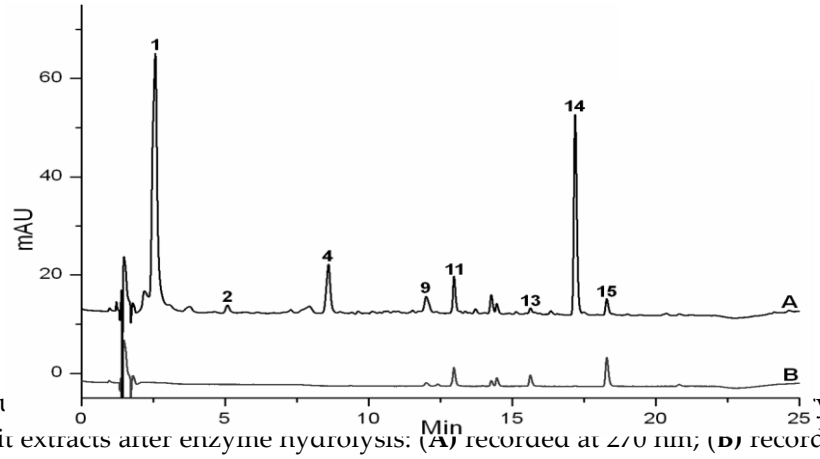

Figure 6 – HPLC-DAD chromatograms of strawberry tree fruit extracts after enzyme hydrolysis: (**A**) recorded at 270 nm; (**B**) recorded at 370 nm. 1- Gallic acid, 2 - protocatechuic acid, 4 - (+)-catechin, 9 - phloroglucinaldehyde, 11 - ellagic acid, 13 - myricetin, 14 - daidzein (IS), 15 - quercetin.

In strawberry tree fruit extracts (Fig. 6, Table 5 and 6), gallic acid appears as the most abundant compound, at a much higher level than the other berries. The abundance of gallic acid is supported by previous studies where gallic acid is in the free form or conjugated either with quinic acid, shikimic acid or glucose [23, 37, 53]. The second most abundant compound was (+)-catechin, a compound previously reported in this fruit [24]. However, the (+)-catechin content of strawberry tree fruits reported here is similar to the total content in flavanols reported previously [24]. It is possible that this higher content in catechin comes from the breakdown of proanthocyanidins during enzyme incubation. Ellagic acid was the third most abundant compound, and had been previously found conjugated in this fruit with glycosides [23, 24]. Myricetin, quercetin, and ellagic acid were also detected in hydrolyzed extracts (Table 6) and were previously found in these berries conjugated with glycosides [24]. Protocatechuic acid and phloroglucinaldehyde were also detected, and are believed to come mainly from hydrolysis and thermal degradation of anthocyanins, even at

acidic pH [54]. Anthocyanin degradation is the probable source of 3-methylgallic acid observed in hydrolyzed blueberry extracts [19]. Kay and co-workers have detected absorption and phase II metabolites of protocatechuic acid and phloroglucinaldehyde in a Caco-2 cell model after spontaneous degradation of anthocyanins, and suggest that these catabolites may be at least partially responsible for the biological activity of anthocyanins *in vivo* [55].

The use of this method in five different fruit matrices reveals its versatility. Moreover the method is very efficient for hydrolysis of glucosides, galactosides and rutinosides, although rhamnosides are less efficiently hydrolyzed. Overall, it is a simple and rapid method for identification and quantification of phenolic aglycones. Identification and quantification of phenolics in plant tissues can be sometimes difficult due to a high number and diversity of compounds. Therefore, hydrolysis of glycosides and analysis of the resulting aglycones is a usual methodology to overcome this problem. Traditional methods based on acid hydrolysis of glycoside-bonds require relatively high concentrations (1–2 M) of mineral acids at temperatures close to 100 ^{o}C [56, 57]. However, this methodology can be too harsh for some compounds which may undergo partial degradation, as observed for catechins and flavonols such as myricetin, kaempferol and quercetin [58]. It was observed that it is hard to reach a compromise as an increase in incubation time can increase degradation, but a reduced time can result in underestimation of the phenolic content [56, 58]. Alkaline hydrolysis can also lead to significant losses in hydroxycinnamic acid derivatives as reported by Krygier and co-workers [59].

Methods employing commercial enzymes to release and identify phenolic aglycones have also been previously used and have been reported to be adequate for phenolic acid quantification in cereals [60-62]. Hesperidinase has been used for hydrolysis of flavonol-glycosides and analysis of aglycones [63]. Fungal glycosidases can be very useful either for analytical or for other purposes. Previously, rhamnosidases were used to produce functional beverages by converting flavonoid-rhamnosides to glucosides, and thus increasing bioavailability [64, 65].

Here, we report for the first time, combined use of hesperidinase and cellulase as an efficient method for releasing flavonols and phenolic acids aglycones from glycosidic bonds for analytical purposes. Other methods have been used for this purpose, but each has advantages and limitations, and so should be chosen according to the aim of the determination. This method is a simple alternative to analyse complex samples, and its usefulness has been demonstrated by application to two non-commercial fruits. Blueberry, raspberry and blackberry fruits were also analysed by this method and results, with minor exceptions, are generally in agreement with published data.

The results highlight wild berries, Portuguese crowberry and strawberry tree, as good source of phenolic compounds. Strawberry tree fruit is mostly characterized by the presence of gallic acid derivatives, while Portuguese crowberry fruit contains high amounts of caffeic acid derivatives. Therefore, they constitute a good alternative to the other berry fruits traditionally consumed to increase and diversify of the ingestion of phenolic compounds in our diet.

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SUPPLEMENTARY MATERIAL

Table 1. LC-MS of blueberry fruit extracts after enzyme hydrolysis

Peak nr.	Retention Time (min)	Wavelength (nm)	m/z [M-H]	Compounds
1	2.59	272	**169**	Gallic acid
2	5.26	272	**153**, 109	Protocatechuic acid
3	6.28	272	**183**, 124	3-Methylgallic acid
5	9.41	272	**165**	Vanillic acid
6	9.84	324	**179**, 135	Caffeic acid
7	10.06	272	**197.1**	Syringic acid
8	10.54	280	**289**	(-)-Epicatechin
9	12.00	280	**153**	Phloroglucinaldehyde
10	12.11	302	**163**	*p*-Coumaric
11	12.97	370	**363**, 333	Ellagic acid
12	13.16	324	**193**, 355	Ferulic acid
13	15.50	370	**317**	Myricetin
15	18.30	370	**301**, 331	Quercetin
16	20.83	370	**345**	Kaempferol

Figures in bold are the main m/z. Peaks identification was performed with LC-MS data and confirmed with reference standards.
Total ion scan of fruit extracts was performed as described [34].

Table 2. LC-MS of raspberry fruit extracts after enzyme hydrolysis

Peak nr.	Retention Time (min)	Wavelength (nm)	m/z [M-H]	Compounds
1	2.59	272	**169**	Gallic acid
2	5.26	272	**153**, 109	Protocatechuic acid
6	9.84	324	**577**, 179	Caffeic acid
8	10.54	280	**289**	(-)-Epicatechin
9	12.00	280	**153**	Phloroglucinaldehyde
11	12.97	370	**301**	Ellagic acid
12	13.16	324	**301**, 193	Ferulic acid
15	18.30	370	**361**, 207, **178**, 301	Quercetin

Figures in bold are the main m/z. Peaks identification was performed with LC-MS data and confirmed with reference standards.
Total ion scan of fruit extracts was performed as described [34].

Table 3. LC-MS of blackberry fruit extracts after enzyme hydrolysis

Peak nr.	Retention Time (min)	Wavelength (nm)	m/z [M-H]	Compounds
1	2.59	272	**169**, 125	Gallic acid
2	5.26	272	**153**, 109	Protocatechuic acid
8	10.54	280	**289**, 319	(-)-Epicatechin
9	12.00	280	**153**	Phloroglucinaldehyde
11	12.97	370	**301**	Ellagic acid
12	13.16	324	**300.9, 223**	Ferulic acid
15	18.30	370	**301**	Quercetin

Figures in bold are the main m/z. Peaks identification was performed with LC-MS data and confirmed with reference standards.
Total ion scan of fruit extracts was performed as described [34].

Table 4. LC-MS of Portuguese crowberry fruit extracts after enzyme hydrolysis

Peak nr.	Retention Time (min)	Wavelength (nm)	m/z [M-H]	Compounds
1	2.59	272	**169**	Gallic acid
2	5.26	272	**153**, 109	Protocatechuic acid
6	9.84	324	**179**, 135	Caffeic acid
8	10.54	280	**289**, 381	(-)-Epicatechin
10	12.11	302	**163**, 119.1	*p*-Coumaric
12	13.16	324	**193**, 301	Ferulic acid
13	15.50	370	**317**	Myricetin
15	18.30	370	**331**	Quercetin
16	20.83	370	206.8, 178.9, 284.9	Kaempferol

Figures in bold are the main m/z. Peaks identification was performed with LC-MS data and confirmed with reference standards.
Total ion scan of fruit extracts was performed as described [34].

Table 5. LC-MS of strawberry tree fruit extracts after enzyme hydrolysis

Peak nr.	Retention Time (min)	Wavelength (nm)	m/z [M-H]	Compounds
1	2.59	272	**169**, 125	Gallic acid
2	5.26	272	**153**, 109	Protocatechuic acid
4	8.60	280	**289**	(+)-Catechin
9	12.00	280	197, 163, 206.9, 152.9	Phloroglucinaldehyde
11	12.97	370	**223.1, 301**	Ellagic acid
13	15.50	370	**317**	Myricetin
15	18.30	370	**206.9,** 301	Quercetin

Figures in bold are the main m/z. Peaks identification was performed with LC-MS data and confirmed with reference standards.
Total ion scan of fruit extracts was performed as described [34].

Chapter 3

Bioavailable (poly)phenol-metabolites in urine of human volunteers after ingestion of a mixed berry fruit puree

This chapter is based on the following manuscript:

Urinary metabolite profiling identifies novel colonic metabolites and conjugates of phenolics in healthy volunteers.

Rui C. Pimpao; Tristan Dew; Maria E. Figueira; Gordon J. McDougall; Derek Stewart; Ricardo B. Ferreira;, Cláudia N. Santos; Gary Williamson. *Molecular Nutrition & Food Research* **2014**, published online, DOI: 10.1002/mnfr.201300822.

This chapter contains published data in which the author of this dissertation had a major contribution in the planning of the experimental work, performed all the experiments and wrote the manuscript.

Contents

ABSTRACT

The colonic metabolism of dietary (poly)phenols is complex and many metabolites and conjugates have not yet been unambiguously identified in humans.

Urine samples from nine healthy human volunteers obtained after the ingestion of a puree of five (poly)phenol-rich berry fruits were analysed using LC-Orbitrap mass spectrometry to provide a preliminary indication of possible metabolites based on exact mass. In most cases, the identity of compounds was confirmed using standards produced either chemically or enzymically followed by analysis using LC-triple quadrupole mass spectrometry. Sulfated, glucuronidated and methylated forms of catechol, pyrogallol and protocatechuic acid mostly appeared in urine after 8 hours, suggesting colonic metabolism. Gallic acid and (-)-epicatechin conjugates appeared mainly before 4 hours, indicative of absorption from the small intestine. Conjugates of ferulic, caffeic, and vanillic acid appeared at intermediate times.

We have positively identified metabolites and conjugates, some novel, in the urine of healthy volunteers after intake of multiple phenolics from a mixed puree from berry fruits, with each being excreted at specific and signature times. Some of these compounds could potentially be used as biomarkers of fruit intake. The possible biological activities of these colonic metabolites require further assessment.

INTRODUCTION

The association between consumption of fruits and vegetables and the decrease in risk of suffering from degenerative diseases has been well established. Several studies have provided evidence for the effects of dietary fruit in reducing the risk of cardiovascular/inflammatory diseases [1, 2] as well as of Alzheimer's disease and other neuropathies [3]. These effects have been at least partially attributed to the presence of (poly)phenols, present in plant foods. Berry fruits are particularly rich in some classes of these compounds, such as phenolic acids, tannins, and flavonoids [4]. However, when trying to correlate the beneficial health effects described for (poly)phenols present in fruits and their bioavailability in humans, it is often observed that some of these compounds are poorly absorbed and may undergo several metabolic modification steps. Additionally, metabolism by the colonic microbiota may result in several catabolites which can be absorbed and contribute to an increased bioavailability [5, 6].

Thus, it is unlikely that the compounds initially present in fruits, or even their aglycones, are responsible solely for the biological effects *in vivo*. Nonetheless, current literature on bioavailability of (poly)phenol metabolites and their degradation pathways is still limited and therefore more studies are necessary to fill this gap. Recently, blueberries, raspberries, blackberries, Portuguese crowberry and strawberry tree fruits were characterized for phenolics [7], and these fruits provide a diverse range of (poly)phenols in the diet, ideal for an intervention study examining the metabolism of a broad mixture of (poly)phenols. In this work, healthy subjects ingested a standardised puree of these fruits and

the urinary excretion of phenolic compounds was assessed. The main urinary metabolites in healthy volunteers were initially assessed by using exact mass scanning with a LC-Orbitrap MS, and subsequently confirmed by comparison with chemically and enzymically synthesized standards and then analysed using LC-triple quadrupole MS.

MATERIALS AND METHODS

Preparation of fruit puree

The consumed fruit puree consisted of 100 g of each of five fruits: blueberries (*Vaccinum* spp. variety Georgia Gem), blackberries (*Rubus* L. subgenus Rubus Watson variety Karaka Black) and raspberries (*Rubus idaeus* L. variety Himbo Top) were harvested at the Fataca experimental field in Odemira, Portugal; strawberry tree fruits (*Arbutus unedo* L.) were harvested in the Alentejo region, Portugal; and Portuguese crowberries (*Corema album* L.) were harvested in the Comporta region, Portugal. Fruits were blended together using a domestic food processor, for 1 min at room temperature. The puree was prepared on the day of the study and was passed through a sieve to remove seeds before being given to the volunteers. A sample of the puree was also freeze-dried and stored at -80 °C until analysis.

Subjects and study design

This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the ethical committee of the Faculty of Pharmacy,

University of Lisbon, Portugal (02/CEECFFUL/2012). The protocol was explained to each volunteer and written informed consent was given before the study. Nine individuals (six females and three males) aged between 23 and 54 years with a body mass index of 23.2 ± 3.5 were recruited. Individuals were all considered healthy by a medical questionnaire and standard blood tests where the levels of glucose, urea, creatinine, total cholesterol, high-density lipoprotein cholesterol, triglycerides, C-reactive protein, and the activity of aspartate aminotransferase, alanine aminotransferase, gamma glutamyl transferase, were assessed. Volunteers did not have any history of cardiovascular diseases or any other medical illnesses, were non-smoking, and were not receiving medication or taking vitamins that could interfere with the study.

Volunteers followed a (poly)phenol-free diet for 2 days before the study and throughout the day of the study, and the compliance with this restriction was confirmed through a questionnaire. After an overnight fast, volunteers ingested 500 mL of the above fruit puree with a standard breakfast containing no additional (poly)phenols, consisting of bread, with ham or cheese, yogurt and biscuits.

Urine samples were collected into a container (containing 0.5 g of ascorbic acid) before the ingestion of the puree, and in the periods of 0-2 h, 2-4 h, 4-8 h and 8-24 h, and the volumes recorded. Sodium azide (0.1% w/v) was added to urine samples which were kept at -20 °C until analysis.

Determination of phenolic compounds in the prepared fruit puree

For determination of (poly)phenols, the dried puree was extracted with ethanol-water (1:1), containing 1 mM butylated hydroxytoluene (BHT) and 0.1 mM daidzein as internal standard. One part of the extract was used directly for HPLC-DAD analysis of total anthocyanins and caffeoylquinic acids. Another part of the extract was incubated with cellulase (EC. 3.2.1.4, Sigma Aldrich, St. Louis, MO, USA) and hesperidinase (EC. 3.2.1.40, Sigma Aldrich, St. Louis, MO, USA) from *Aspergillus niger* and analysed by HPLC-DAD, as described previously [7].

Extraction of phenolics from urine

A 2 mL aliquot of urine was spiked with 10 μL of 1 mg/mL rutin as internal standard and was added to 10 mL of acetonitrile. Samples were centrifuged at 3000 *g* for 15 min. The supernatant was collected, dried under vacuum and reconstituted in 500 μL of 50% (v/v) ethanol with 0.1% (w/v) ascorbic acid and 20 μg/mL of taxifolin as second internal standard for further analysis.

Prediction of metabolites in human samples

A list of metabolites and conjugates potentially present in human urine after ingestion of the fruits was drawn up based on prediction of metabolic pathways. The structures of 205 compounds were drawn and their exact mass calculated by using MarvinSketch software 5.7.0 from Chemaxon.

Urine analysis using LC-Orbitrap MS

A small proportion of each aliquot of urine from each time point from all volunteers were pooled together just for the LC-Orbitrap MS analysis. Phenolics were extracted as described above. Samples were separated on a HPLC Accela 600 HPLC system (Thermo Scientific) using a C18 Synergi Hydro RP18 column (Phenomenex, Macclesfield, UK) 4 μm particle size and dimensions 2 mm ID x 150 mm. The column was fitted with a Security GuardTM guard system containing an Aqua 10 μm C18 Guard Cartridge (2 mm ID x 4 mm) (Phenomenex, Macclesfield, UK) and eluted over a gradient of 100% solvent A (95% H_2O, 5% ACN with 0.1% (v/v) formic acid) to reach 15% B (95% ACN, 5% H_2O with 0.1% (v/v) formic acid) at 10 min, 25% B at 30 min, 60% B at 60 min, 100% B at 63 min, 100% B at 68 min at a flow rate of 0.26 mL/min. Analysis was done on a LTQ Orbitrap™ XL hybrid mass spectrometer (Thermo Scientific, Bremen, Germany).

MS analysis was performed using data dependent N^{th} order double play analysis comprising full scan mass range 80-2000 amu , 30000 resolution, Data type Centroid and data dependent MS/MS (60 seconds of exclusion duration) on the top 3 most intense ions detected above threshold automatically in the independent scan event. Electrospray ionization settings were as follows: source voltage, 3.4 kV; the capillary temperature was 300 °C with a sheath gas at 40 psi and auxiliary gas at 5 psi.

MS data handling software (Xcalibur QualBrowser software, Thermo Electron Corp.) was used to search for predicted metabolites by their

appropriate *m*/*z* value. All peaks were checked for *m*/*z* value and fragmentation products.

Preparation of liver cytosolic fractions

For preparation of liver cytosolic fractions, ox and pig liver were rinsed with 250 mM sucrose solution and a sample of 13 g was weighed. Samples were minced and homogenized with 50 mL Tris-HCl buffer 50 mM pH 7.5 using a polytron for 2 min on ice. Samples were centrifuged for 10 min at 17400 *g* at 6 °C. The supernatant was recovered and centrifuged for 90 min at 3500 *g* and 6 °C. The supernatant was recovered and stored at -80 °C until use.

Preparation of liver microsomes

For preparation of microsomes, pig liver was rinsed with 250 mM sucrose solution and a sample of 13 g was weighed. Samples were minced and homogenized with 50 mL of the sucrose solution using a polytron for 2 min on ice. Samples were centrifuged for 10 min at 17400 *g* at 6 °C, supernatant was recovered and 0.2 mL of 88 mM $CaCl_2$ was added to 20 mL of supernatant. The solute was left to stand on ice for 5 min with occasional swirling. The mixture was centrifuged at 27000 *g* for 15 min at 6 °C, and after discarding the supernatant the pellet was resuspended in 2.5 mL of 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM EDTA and 20% (v/v) glycerol. Microsomal fractions were stored at -80 °C until use.

Sulfation of phenolic substrates

Individual phenolic compounds used as substrates were incubated at a concentration of 300 μM with 35 μM adenosine 3′-phosphate 5′-phosphosulfate (PAPS) and 5.1 mM *p*-nitrophenyl sulfate (pNPS) as co-factors, 1 mM dithiothreitol, 10 mM sodium sulfite and 100 μM ascorbic acid in 100 mM potassium phosphate buffer, pH 7.4. To this solution, 25 μL of liver cytosolic fraction was added and the reaction was incubated at 37 °C for 4 h. The reaction was stopped with addition of 200 μL of ice cold, ACN containing 500 mM HCl. Samples were centrifuged twice for 15 min at 17000 *g* and the supernatant was stored at -20 °C until analysis.

Glucuronidation of phenolic substrates

The protocol is based on the modification of the method followed by Dueñas *et al*. [8]. Individual compounds used as substrates were incubated at a concentration of 800 μM with 25 mM HEPES buffer, pH 7.2, containing 1 $mg.mL^{-1}$ alamethicin, 2 mM uridine 5′-diphosphoglucuronic acid trisodium salt (UDPGA), and 1 mM saccharolactone in a final volume of 110 μL. The reaction was started with the addition of 5 μL of pig liver microsomes and incubation was performed at 37 °C for 4 h. Reaction was stopped by the addition of 200 μL of methanol containing 1.6 mM ascorbic acid. Samples were centrifuged twice for 15 min at 17000 g, and the supernatant was stored at -20 °C until analysis.

Chemical synthesis of hydroxycinnamate metabolites

The hydroxycinnamate and dihydroxycinnamate-sulfates and glucuronides used as standards were chemically synthesized and characterized as described previously [9] and were kindly provided by Prof. Denis Barron, NIHS, Lausanne, Switzerland.

LC-MS/MS analysis

Individual urine samples were analysed by LC-MS/MS. The HPLC system comprised an Agilent 1200 series micro degasser, SL binary pump, SL autosampler with chilled sample compartment (8 °C), column oven (30 °C), and diode array detector (Agilent Technologies, Cheadle, UK). The system was controlled and data processed by Agilent MassHunter software (version B.01.03). An Atlantis T3 Column, 100 Å, 3 μm, 2.1 mm ID x 100 mm HPLC column (Waters, Hertfordshire, UK) was used for chromatographic separations at a flow rate of 0.26 mL/min over a gradient of 100% solvent A (95% H_2O, 5% ACN with 0.5% (v/v) formic acid) for 10 min, reaching 15% B (95% ACN, 5% H_2O with 0.5% (v/v) formic acid) from 10 to 20 min. Solvent B increased to 25% at 40 min and to 100% B at 43 min where it was maintained for 5 min returning to 0% in 2 min. MS analysis was performed with an Agilent 6410a triple-quadrupole LC-MS-MS with electrospray source at 350 °C, a source voltage of 4 kV , and N_2 drying gas flow rate of 11 L/min at a pressure of 30 psi (Peak Scientific, NM30LA, Inchinnan, UK). The analysis was performed in negative MRM mode and optimisation for ion transmission was achieved by repeated injections of individual standards.

Relative amounts of compounds were estimated after normalizing to internal standard (rutin) and corrected by urinary volume.

Statistical analysis

Pharmacokinetic excretion profile of urine metabolites was constructed using GraphPad Prism 5. This package was also used for statistical analysis. Box-and-Whiskers plots for minimum and maximum values were produced. Comparisons in relation to the baseline were performed with two tailed Wilcoxon matched pairs test with a confidence level of 95%.

RESULTS

Identification and quantification of phenolic compounds in fruit puree

The puree containing blueberries, raspberries, blackberries, Portuguese crowberry and strawberry tree fruits was characterized by HPLC-DAD for the major compounds (Caffeoylquinic acids and anthocyanins, Table 1) and the aglycones were also quantified after multi-enzyme hydrolysis of the glycosides (Table 2). The most abundant compounds were anthocyanins, chlorogenic acids and gallic acid. Caffeic acid was abundant after hydrolysis and possibly resulted from esterase activity on caffeoylquinic acids plus glycosidase activity towards glycosides of caffeic acid.

Table 1. Quantification of total anthocyanins, 5-*O*-caffeoylquinic acid, and 3-*O*-caffeoylquinic acid [mg/500 mL] in fruit puree ingested by volunteers determined by HPLC-DAD (details see materials and methods section)

Compounds	Concentration (mg/500 mL fruit puree)
Total anthocyanins[1]	636 ±19
5-Caffeoylquinic acid	130 ± 2
3-Caffeoylquinic acid	5.9 ± 0.1

[1] Anthocyanin peaks were summed and quantified as cyanidin-3-*O*-glucoside equivalents

Table 2. Quantification of phenolic compounds in fruit juice, consumed by volunteers, after hydrolysis using hesperidinase and cellulase using HPLC-DAD relative to authentic standards.

Compound	Concentration (mg/500 mL fruit puree)
Gallic acid	425.9 ± 14.0
Protocatechuic acid	16.7 ± 0.2
3-Methylgallic acid	12.9 ± 0.1
(+)-Catechin	17.6 ± 3.9
Vanillic acid	2.9 ± 0.5
Caffeic acid	140.4 ± 2.2
Phloroglucinaldehyde	28.8 ± 0.3
Syringic acid	13.2 ± 0.6
(-)-Epicatechin	12.2 ± 1.6
Ellagic acid	24.8 ± 1.3
Ferulic acid	6.9 ± 0.1
Myricetin	15.6 ± 0.2
Quercetin	15.5 ± 0.4
Kaempferol	2.4 ± 0.0

Initial determination of metabolites in urine using exact mass data

Compounds were identified in pooled urine samples for each time point by comparing their exact mass and fragmentation patterns (when available) obtained using an LC- Orbitrap MS with predicted metabolites. By comparing chromatograms of urine samples before ingestion and after ingestion of the fruit puree, it was observed that several metabolites appeared after ingestion. Only metabolites which increased in abundance after ingestion of the fruit puree were selected for confirmation to exclude compounds that could result from endogenous metabolism (supplementary Tables 1 to 5). Anthocyanins, which were very abundant in the fruit puree, were not evident by LC-Orbitrap MS analysis. After this initial analysis, the most abundant compounds were selected for confirmation by comparison with enzymically or chemically synthesised standards in individual samples using the LC-triple quadrupole MS.

Enzymatic synthesis of phenolic compounds

For production of conjugated compounds, (poly)phenol aglycones were incubated *in vitro* with pig liver microsomes and UDP-GA to generate glucuronide conjugates, and with an ox liver cytosolic fraction in the presence of the co-factors PAPS and *p*-NPS to generate the formation of mono-sulfated compounds. Ox liver cytosolic fraction was used instead of pig liver as it was observed to be more efficient in generating sulfated conjugates. The resulting conjugates were identified by their mass and fragmentation pattern (multiple reaction monitoring - MRM transitions), and are shown in Table 3.

Table 3. Summary of HPLC- triple quadrupole MS/MS detection properties for commercial or synthesized standards and indication of their presence in urine. A = aglycone, M = metabolite, RT = retention time.

Metabolite code	RT	[M-H]$^-$ (m/z)		Metabolite identity	Presence in urine	Standards Origin
		Precursor	Fragment			
A1	8.1	109.1		Catechol	X	C
M1	7.12	189.1	109.1	Catechol-*O*-sulfate	✓	S
M2	11.7	285.1	109.1	Catechol-*O*-glcrnd	✓	S
A2	19.3	123.1		4-Methylcatechol	X	C
M3	18.7	203.1	123.1	4-Methylcatechol-*O*-sulfate *	✓	S
M4	20.9	299.1	123.1	4-Methylcatechol-*O*-glcrnd *	✓	S
A3	3.1	125.1		Pyrogallol	✓	C
M5	3.5	205.1	125.1	Pyrogallol-*O*-sulfate *	✓	S
M6	6.2	205.1	125.1	Pyrogallol-*O*-sulfate *	✓	S
M7	3.7	301.1	125.1	Pyrogallol-*O*-glcrnd *	✓	S
M8	9. 7	301.1	125.1	Pyrogallol-*O*-glcrnd *	✓	S
A4	13.1	139.1		1-*O*-Methylpyrogallol	X	C
M9	10.5	219.1	139.1	1-*O*-Methylpyrogallol-*O*-sulfate *	✓	S
M10	11.2	219.1	139.1	1-*O*-Methylpyrogallol-*O*-sulfate *	X	S
A5	9.0	139.1		2-*O*-Methylpyrogallol	X	C
M11	6.0	219.1	139.1	2-*O*-Methylpyrogallol-1-*O*-sulfate	✓	S
A6	6.5	153.1	108.1	Protocatechuic acid	✓	C
M12	5.9	233.1	153.1	Protocatechuic acid-*O*-sulfate *	✓	S
A7	16.6	167.1	151.8	Vanillic acid	✓	C
M13	7.8	247.1	167.1	Vanillic acid-4-*O*-sulfate	✓	S
A8	18.5	167.1	151.8	Isovanillic acid	X	C
M14	10.5	247.1	167.1	Isovanillic acid-3-*O*-sulfate	X	S
M15	5.0	301.1	167.1	Vanillic acid-*O*- glcrnd *	✓	S
M16	8.9	301.1	167.1	Vanillic acid-*O*- glcrnd *	✓	S
A9	2.9	169.1	124.9	Gallic acid	✓	C
M17	1.3	345.1	169.1	Gallic-acid-*O*- glcrnd *	X	S
M18	2.4	345.1	169.1	Gallic-acid-*O*- glcrnd *	X	S
M19	4.6	345.1	169.1	Gallic-acid-*O*- glcrnd *	✓	S
A10	10.0	183.1	167.8	4-*O*-Methylgallic acid	✓	C
M20	7.6	263.1	183.1	4-*O*-Methylgallic acid-3-*O*-sulfate	✓	S
A11	24.1	193	134, 178	Ferulic acid	X	C
A12	25.1	193	134, 178	Isoferulic acid	X	C
A13	18.2	174	135	Caffeic acid	✓	C
A14	15.0	181	137	Dihydrocaffeic acid	✓	C
A15	22.7	195	136	Dihydroferulic acid	✓	C
M21	18.5	369	193, 113	Ferulic acid-4-*O*-glcrnd	✓	P
M22	22.3	369	193, 113	Isoferulic acid-3-*O*-glcrnd	✓	P
M23	17.4	357	181, 137	DHC acid-4-*O*-glcrnd	X	P
M24	18.3	357	181, 137	DHC acid-3-*O*-glcrnd	✓	P
M25	20.3	371	195, 113	DHF acid-4-*O*- glcrnd	✓	P
M26	21.5	273	193, 178	Ferulic acid-4-*O*-sulfate	✓	P
M27	22.7	273	193, 178	Isoferulic acid-3-*O*-sulfate	✓	P
M28	19.2	259	179, 135	Caffeic acid-4-*O*-sulfate	✓	P
M29	20.1	259	179, 135	Caffeic acid-3-*O*-sulfate	✓	P
M30	17.4	261	181, 137	DHC acid-4-*O*-sulfate	✓	P
M31	17.6	261	181, 137	DHC acid-3-*O*-sulfate	✓	P
M32	20.3	275	195, 136	DHF acid-4-*O*-sulfate	✓	P
A16	21.0	289.1	244.9	(-)-Epicatechin	X	C
M33	17.2	465.1	289	Epicatechin-*O*-glcrnd *	✓	S
M34	17.8	465.1	289	Epicatechin-*O*-glcrnd *	X	S
M35	18.3	465.1	289	Epicatechin-*O*-glcrnd *	X	S
M36	18.9	465.1	289	Epicatechin-*O*-glcrnd *	X	S
M37	20.8	465.1	289	Epicatechin-*O*-glcrnd *	✓	S
M38	17.6	369.1	289	Epicatechin-*O*-sulfate *	✓	S
M39	20.1	369.1	289	Epicatechin-*O*-sulfate *	X	S
M40	21.9	369.1	289	Epicatechin-*O*-sulfate *	✓	S

* - Conjugated compounds which were not possible to distinguish between isomers due to more than one available position for conjugation.

Legend: ✓ – present in urine, X – absent in urine, Glcrnd – Glucuronide, C – Commercially available (HPLC grade), S – Enzymically synthesized, P – Provided by Prof. Denis Barron

When multiple conjugates resulted from the reaction owing to the presence of multiple available hydroxyl groups, it was not possible to distinguish the exact position of the conjugation, with exception for the compounds synthesized chemically.

Gallic acid (GA), although theoretically having only two *different* hydroxyl groups for conjugation, yielded three glucuronidated metabolites (Fig. 1 A). Also, vanillic acid (VA), possessing a single hydroxyl group available for conjugation, originated one sulfate as expected, but apparently two glucuronides Fig. 1 B). The one sulfated metabolite of 4-*O*-methylgallic acid (4-MeGA) was produced as expected; however, protocatechuic acid (PA) yielded only one peak (Fig. 1 C), even though two hydroxyl groups are available for conjugation. These events suggest a positional specificity of sulfotransferases for these compounds if only one metabolite is being produced, or alternatively overlapping of two metabolites in one peak.

(-)-Epicatechin yielded three sulfates, although five hydroxyl groups are theoretically available for sulfation or glucuronidation. However, glucuronidation of epicatechin yielded five peaks (Fig. 1 D) but with large differences in efficiency of conjugation for the different positions.

Conjugates of catechol (Cat), 4-methylcatechol (4-MeCat), pyrogallol (Pyr) and its *O*-methylated forms, 1-*O*-methylpyrogallol (1-MePyr) and 2-*O*-methylpyrogallol (2-MePyr), are summarized in Table 3 and occurred as expected. The only exception was 4-MeCat which, although having two available hydroxyl groups, yielded only one glucuronide and one sulfate.

Therefore, enzymic synthesis of sulfated compounds is not strictly regio-selective for most compounds, but reveals a preference for certain

positions, as revealed by differences in peak areas for synthesized isomers of the same parent compound.

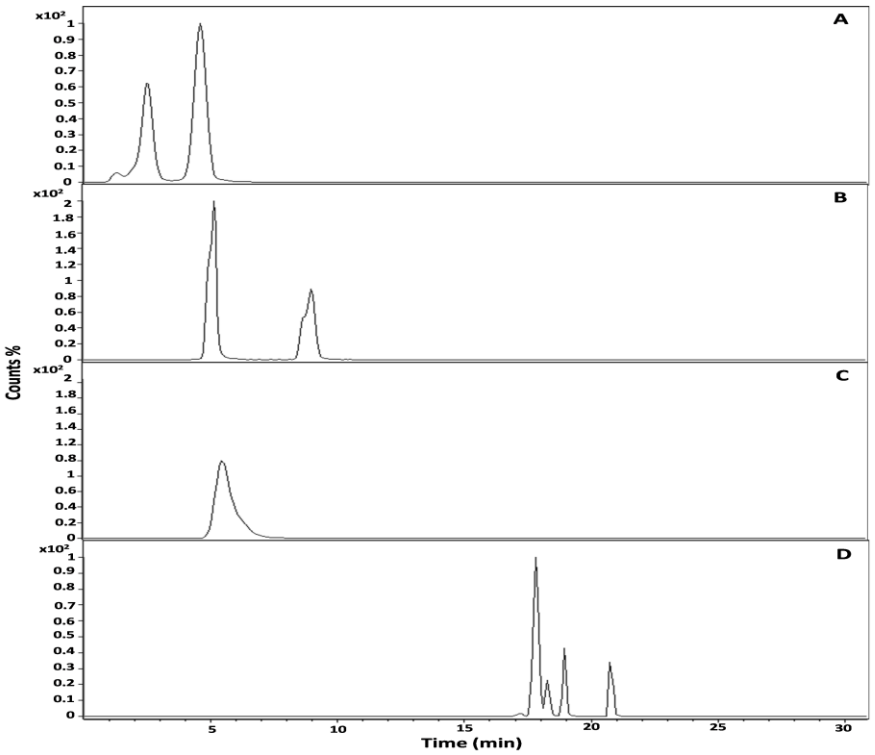

Figure 1. LC-MS chromatograms of enzymically produced compounds *in vitro*. The $[M-H]^-$ ion chromatograms were selected for: (A) m/z 345.1, GA-*O*-glucuronides; (B) m/z 301.1, VA-*O*-glucuronides; (C) m/z 233.1, PA-*O*-sulfate; (D) m/z 465.1, epicatechin-*O*-glucuronides.

Confirmation and kinetics of metabolites in human urine

Individual urine samples from each volunteer and each time point were analysed using the triple quadrupole LC-MS in MRM mode, using conditions optimized for the standard compounds, to confirm if compounds initially identified by exact mass scanning matched the synthesized standards. The presence of aglycones and conjugates in urine

is indicated in Table 3. The aglycones PA and dihydrocaffeic (DHC) acids, and GA-*O*-glucuronide, were not detected by the LC Orbitrap-MS due to low levels of the compounds combined with the lower sensitivity of this method, but were, however, detected with LC-triple quadrupole MS. On the other hand, some aglycones (such as Cat, 4-MeCat, ferulic and isoferulic acids) were tentatively identified using the Orbitrap-MS but were not found by the MRM method. This might result from technical limitations such as misleading identification of the peak due to other compounds with the same exact mass, or due to non-intentional in-source fragmentation of the conjugated compounds resulting in the detection of a "ghost" aglycone.

Therefore, the presence of sulfated and glucuronidated conjugates was confirmed in individual urine samples (Table 3). The levels of compounds which increased in urine after consumption of the fruit puree are shown in Figs. 2 to 8.

GA, found in the free form, appeared in urine at early time points, reaching a maximum between 0 and 4 h post ingestion (Fig. 2A). Phase II metabolites of GA (glucuronide, methyl and methyl-sulphate) reached a maximum excretion in urine between 2 to 4 h, slightly later than GA (Table 3, Fig. 2 B, C and D). *O*-Glucuronidated metabolites of GA were not observed with the initial screening using the LC-Orbitrap MS, but were found using the triple quadrupole LC-MS. One *O*-sulfated conjugate of PA was also found in urine samples (Table 3) and matched the single conjugate synthesized *in vitro*.

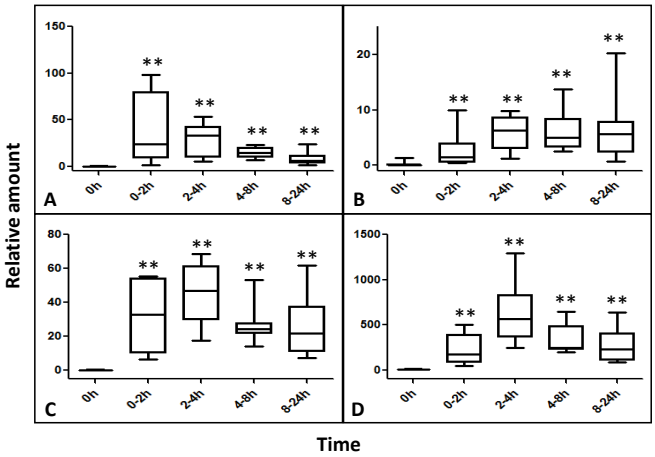

Figure 2. Box plot of relative quantification based on peak area of conjugated metabolites of GA found in urine at different collection times in human volunteers. (A) GA (A9), (B) GA-*O*-glucuronide (M19), (C) 4-MeGA (A10), (D) 4-MeGA-3-*O*-sulfate (M20). No star, not significant; *, $p < 0.05$; **, $p < 0.01$ relative to time zero.

PA-*O*-sulfate and phase II metabolites of VA had an intermediate time of excretion, peaking between 4 and 8 h (Fig. 3).

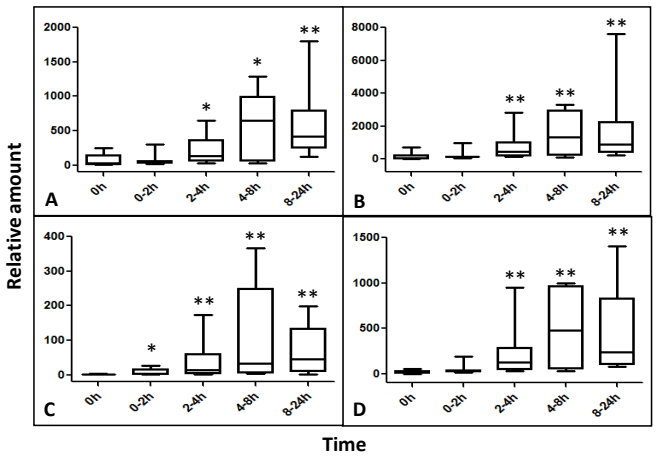

Figure 3. Box plot of relative quantification of conjugated metabolites of protocatechuic acid and vanillic acid found in urine at different collection times in human volunteers. (PA-*O*-sulfate (M12), (B) VA-4-*O*-sulfate (M13), (C) VA-*O*-glucuronide (M15), (D) VA-*O*-glucuronide (M16). No star, not significant; *, $p < 0.05$; **, $p < 0.01$ relative to time zero.

The excretion profiles of phase II metabolites of hydroxycinnamic acids (Table 3) are shown in Figs. 4 and 5.

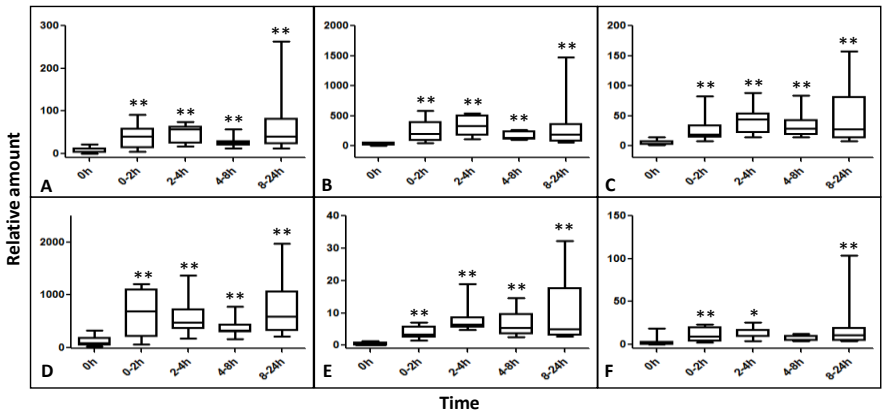

Figure 4. Box plot of relative quantification of conjugated metabolites of caffeic acid, ferulic acid and isoferulic acid found in urine at different collection times in human volunteers. (A) Caffeic acid-4-*O*-sulfate (M28), (B) caffeic acid-3-*O*-sulfate (M29), (C) ferulic acid-4-*O*-glucuronide (M21), (D) ferulic acid-4-*O*-sulfate(M26), (E) isoferulic acid-3-*O*-glucuronide (M22), (F) isoferulic acid-3-*O*-sulfate (M27). No star, not significant; *, $p < 0.05$; **, $p < 0.01$ relative to time zero.

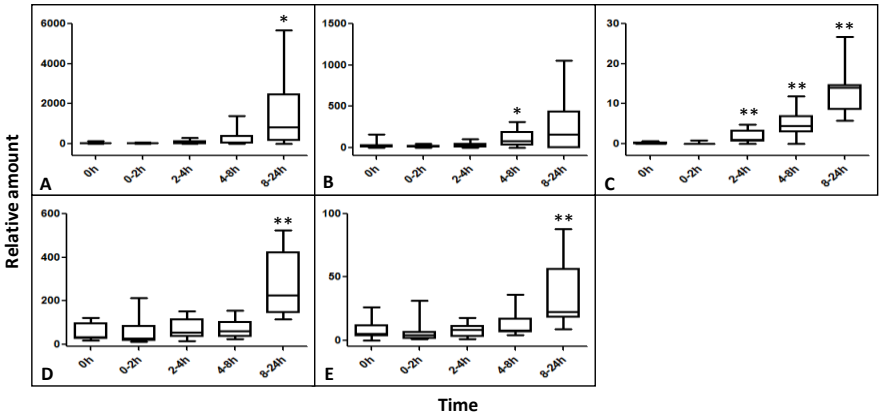

Figure 5. Box plot of relative quantification of conjugated metabolites of DHC acid and DHF acid found in urine at different collection times in human volunteers. (A) DHC acid-4-*O*-sulfate (M30), (B) DHC acid-3-*O*-sulfate (M31), (C) DHC acid-3-*O*-glucuronide (M24), (D) DHF acid-4-*O*-sulfate (M32), (E) DHF acid-4-*O*-glucuronide (M25). No star, not significant; *, $p < 0.05$; **, $p < 0.01$ relative to time zero.

Several (-)-epicatechin conjugates were enzymically produced *in vitro*, but only two *O*-sulfated and two *O*-glucuronidated metabolites were found in urine (Table 3), thus suggesting a more selective mechanism of conjugation *in vivo* by phase II enzymes. The time course in urine of (-)-epicatechin conjugates is consistent with a rapid excretion (Fig. 6).

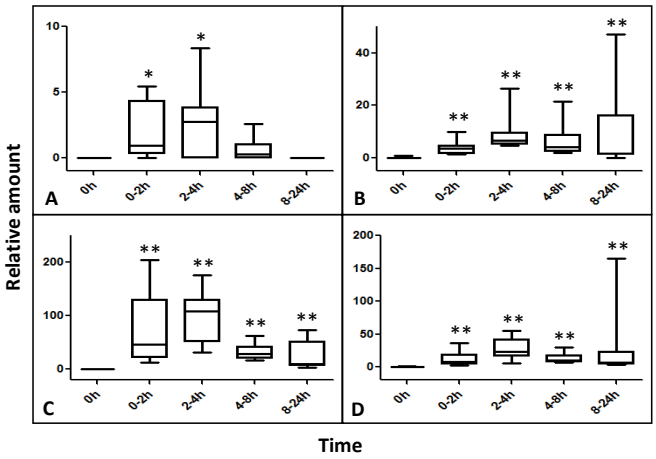

Figure 6. Box plot of relative quantification of conjugated metabolites of (-)-epicatechin found in urine at different collection times in human volunteers. (A) (-)-Epicatechin-*O*-glucuronide (M33), (B) (-)-epicatechin-*O*-glucuronide (M37), (C) (-)-epicatechin-*O*-sulfate (M38), (D) (-)-epicatechin-*O*-sulfate (M40). No star, not significant; *, $p < 0.05$; **, $p < 0.01$ relative to time zero.

Both *O*-glucuronides and *O*-sulfates of Cat and 4-MeCat were detected and had very similar profiles of appearance in urine (Fig. 7), starting from low levels at initial time points, and increasing especially after 4 h. The same excretion profile was observed for phase II metabolites of Pyr (Fig. 8). Only one sulfated metabolite of 1-MePyr was identified, although two metabolites were obtained *in vitro*.

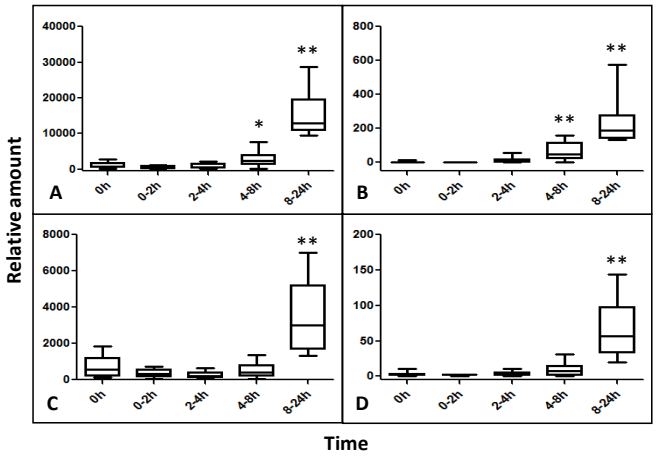

Figure 7. Box plot of relative quantification of conjugated metabolites from catechol and 4-methylcatechol found in urine at different collection times in human volunteers. (A) Catechol-*O*-sulfate (M1), (B) catechol-*O*-glucuronide (M2), (C) 4-methylcatechol-*O*-sulfate (M3), (D) 4-methylcatechol-*O*-glucuronide (M4). No star, not significant; *, $p < 0.05$; **, $p < 0.01$ relative to time zero.

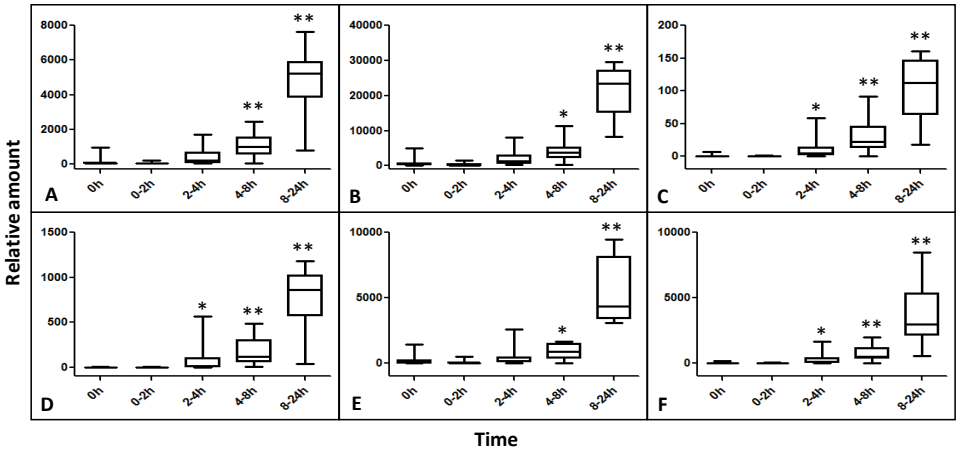

Figure 8. Box plot of relative quantification of conjugated metabolites of pyrogallol, 1-*O*-methylpyrogallol and 2-*O*-methylpyrogallol found in urine at different collection times in human volunteers. (A) pyrogallol-*O*-sulfate (M5), (B) pyrogallol-*O*-sulfate (M6), (C) pyrogallol-*O*-glucuronide (M7), (D) pyrogallol-*O*-glucuronide (M8), (E) 1-*O*-methylpyrogallol-*O*-sulfate (M9), (F) 2-*O*-methylpyrogallol-1-*O*-sulfate (M11). No star, not significant; *, $p < 0.05$; **, $p < 0.01$ relative to time zero.

DISCUSSION

Berries contribute significantly to the dietary intake of (poly)phenols [10] but many of the metabolites and conjugates produced after consumption are not known. This study focused on the identification of urinary metabolites and their conjugates after ingestion of a berry-rich fruit puree containing a high level of different (poly)phenols. Multi-enzyme hydrolysis was used for identification and quantification of aglycone equivalents in the fruit puree, as previously described for each individual fruit [7]. This hydrolysis also mimics to a certain extent the deglycosylation known to occur *in vivo*, either in small intestine or in colon, prior to absorption [6, 11].

Our results show that, as expected, excretion generally follows phase II conjugation. GA appears to be an exception, as it was detected in the urine of all volunteers in relatively high amounts in its unconjugated form, although it was also the most abundant aglycone in the fruit puree. This compound was also found in urine after phase II metabolism, i.e. in glucuronidated, methylated and sulfated forms. GA and 4-MeGA have been reported in humans after tea intake [12] and, in addition to these compounds, 4-MeGA-3-*O*-sulfate was reported in rats after ingestion of GA [13]. In humans, this metabolite was only tentatively identified after ingestion of (poly)phenol-rich juice [14] and red wine [15]. An *O*-glucuronidated conjugate of GA, although not detected with LC-Orbitrap MS, was detected in the urine of volunteers with LC-triple quadrupole MS, due to its very low levels. This compound was confirmed using the standard that we prepared, and, to our knowledge, this is the first report of this metabolite in mammals. Absorption of GA appears to be fast and

elimination of this compound and its phase II metabolites in urine peaked 2 to 4 h after ingestion (Fig. 1), even though conjugates are still found in urine between 8 and 24 h. This suggests that GA is still being produced and absorbed several hours after ingestion of the fruit puree, possibly due to degradation by the colonic microbiota of compounds such as esters of GA, although they may also arise as breakdown products of anthocyanins such as malvidin or delphinidin [16-18].

Anthocyanins were very abundant in the puree, mainly glycosides of cyanidin from raspberries and blackberries [19-21], but also glycosides of malvidin, delphinidin, petunidin, cyanidin and peonidin from blueberry [20, 21]. However, their presence in urine was not demonstrated in LC-Orbitrap MS, due to their very low levels. Recent evidence suggests that anthocyanins are found in plasma and urine in very low amounts; nevertheless, the products of their degradation, colonic catabolism and metabolism are much more abundant [16, 22-24]. In a recent study, Czank and co-workers [25] administered isotopically labelled cyanidin-3-*O*-glucoside to human subjects, and revealed that apparent bioavailability of anthocyanin-derived products was far higher than expected; metabolites present in urine and plasma included degradation products of cyanidin such as PA and phloroglucinaldehyde. These reactions are consistent with *in vitro* data [24, 26]. Additionally phenylacetic acids, phenylpropenoic acids, hippuric acid and phase II conjugates of PA as metabolites derived from labelled cyanidin-3-*O*-glucoside were also detected in humans [25].

In our study, phloroglucinaldehyde was not evident in the urine of volunteers by LC-Orbitrap MS analysis. However, PA, at least partly

resulting from the degradation of cyanidin, was found in urine essentially as one *O*-sulfated conjugate. Although two hydroxyl groups are available for sulfation in the PA molecule, only one peak was generated both *in vitro* and *in vivo*. However, two isomers have been described in humans for PA-*O*-sulfate [25]. VA, possibly resulting from *O*-methylation of PA, was also found in urine, although its isomer isovanillic acid was not. In fact, VA was found conjugated with sulfate and glucuronic acid, and VA-*O*-sulfate has been previously identified in humans [25, 27]. Phase II metabolites of PA appeared in urine, particularly between 4 and 8 h (Fig. 2), suggesting a colonic origin. The appearance of one more *O*-glucuronidated metabolites than the number of available hydroxyl groups in the VA molecule might be due to peak dissociation caused by the chromatography conditions, or possibly glucuronidation of the carboxylic group which has been observed before [27]. The same situation might occur with GA as we detected three glucuronidated metabolites after *in vitro* enzymic synthesis.

Caffeic acid (CA), also found in high amounts in the fruit puree mostly conjugated with quinic acid, was found in urine in sulfated forms. However, other conjugated metabolites were also found, possibly resulting from metabolism of CA in the digestive tract since CA generates ferulic and isoferulic acids (FA and IA, respectively) by methylation, DHC acid by reduction and dihydroferulic (DHF) acid by both events [9]. Although FA was also initially present in the fruit puree, it is possible that it also results from methylation of CA, as phase II metabolites of IA were found in urine and IA was not present in the fruit puree.

CA-*O*-sulfates were observed in two temporal phases (Fig. 3 A, B). The first phase (peaking between 2 and 4 h) is consistent with the hydrolysis of 5-*O*-caffeoylquinic acid by esterases in the small intestine [28] but CA might also be derived from glycosidase action in the small intestine on CA glycosides. The second phase (peaking between 8 and 24 h) might result from the action of esterases from colonic microbiota on caffeoylquinic acids. Additionally, the late urinary peaks of DHC and DHF acids conjugates (Fig. 4 A to E) could also be explained by the action of the colonic microbiota on CA, as suggested previously [28]. In fact, studies made with ileostomist patients reveal that a proportion of the ingested caffeoylquinic acids can be recovered in the ileal fluid of humans, and thus reach the colon (between 26 and 78%, depending on the food matrix) [29, 30].

Although not very abundant in fruit puree, the presence of phase II metabolites of (-)-epicatechin in urine of volunteers was observed. Two *O*-sulfates and two *O*-glucuronides of epicatechin were found in urine, although the position of the conjugation was not confirmed. These metabolites reached a maximum in urine between 2 and 4 h (Fig. 5), indicating early absorption and excretion. Previous work has also indicated that epicatechin metabolites reached a maximum amount in plasma generally before 4 h after ingestion [14, 31]. Previously, three *O*-glucuronidated conjugates of epicatechin were found in plasma and urine of human subjects, identified as epicatechin-3'-*O*-β-D-glucuronide, picatechin-4'-*O*-β-D-glucuronide and epicatechin-7-*O*-β-D-glucuronide [31]. The same study reported the presence of two *O*-sulfated conjugates of

epicatechin characterized as epicatechin-3'-*O*-sulfate and (-)-epicatechin-4'-*O*-sulfate.

Although not found in fruit puree, phase II metabolites of Pyr and Cat were relatively abundant in urine samples. Pyr and Cat in their free forms had been previously found as products of metabolism when Concord grape juice was incubated *in vitro* with faecal slurries [17]. They were also found in the urine of volunteers after ingestion of Concord grape juice especially at later time points, but neither of these compounds were observed in the urine of ileostomist patients [32], which strongly suggests a colonic origin based on degradation of other (poly)phenols. Pyr has also been suggested to be generated from colonic degradation of malvidin-3-*O*-glucoside [16]. In our study, Pyr in the free form was detected in low amounts but Cat was not detected. However, methylated, sulfated and glucuronidated metabolites of these compounds increased significantly at later time points in urine (Fig. 6 and 7). Cat-*O*-sulfate and Cat-*O*-glucuronide were previously found in urine of rats in which Cat had been injected into the renal portal circulation [33] or after administration of a *Glechoma longituba* extract [34]. However, to our knowledge, the presence of Cat-*O*-glucuronide in the urine of humans has never been described, and Cat-*O*-sulfate, although previously detected in human urine, was not related to the consumption of (poly)phenols [35]. Moreover, this is also the first report of *O*-sulfated and *O*-glucuronidated metabolites of 4-MeCat in human urine. Regarding Pyr-*O*-glucuronide, it was previously identified in rat urine after ingestion of gallic acid [13]. In the same study, 2-MePyr was found as the aglycone and also in a glucuronidated form [13]. Pyr-*O*-

glucuronide and Pyr-*O*-sulfate were also previously identified in volunteers after green and black tea consumption [27, 36, 37]. Isomers of MePyr-*O*-sulfate were also found after green and black tea consumption [27]. Phase II metabolites of both Pyr and Cat, therefore, appear to be generated from further catabolism of (poly)phenol metabolites by colonic microbiota.

In conclusion, this study focused particularly on detection and identification of phase II metabolites of phenolic acids after ingestion of a (poly)phenol-rich fruit puree. Either directly absorbed or produced via degradation of more complex (poly)phenols, these metabolites showed a significant and substantial increase in urine of volunteers after ingestion of the puree, and represent possible biomarkers for (poly)phenol intake. Several metabolites were for the first time reported and confirmed in human urine after ingestion of (poly)phenols, including 4-*O*-methylgallic acid-3-*O*-sulfate, gallic acid-*O*-glucuronide, catechol-*O*-sulfate and -*O*-glucuronide, and 4-methylcatechol-*O*-sulfate and -*O*-glucuronide. Additionally, when considering the effect of certain dietary (poly)phenols on human health, these metabolites and conjugates should be considered for their possible biological activities.

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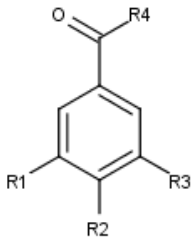
SUPPLEMENTARY MATERIAL

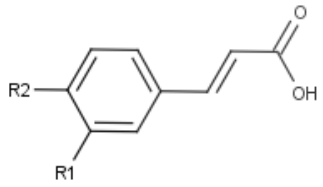
Table 1. Structure of metabolites of protocatechuic, vanillic and gallic acids, and presence in human urine using the Orbitrap LC-MS.



Metabolite	**R1**	**R2**	**R3**	**R4**	**Exact mass**	**Detected in urine**
Protocatechuic acid	OH	OH	OH	OH	154.0266	X
Protocatechuic acid-3-*O*-sulfate	OSO_3	OH	H	OH	233.9834	✓
Protocatechuic acid-4-*O*-sulfate	OH	OSO_3	H	OH		
Vanillic acid	OCH_3	OH	H	OH	168.0423	✓
Isovanillic acid	OH	OCH_3	H	OH		
Vanillic acid-4-*O*-sulfate	OCH_3	OSO_3	H	OH	247.9991	✓
Isovanillic acid-3-*O*-sulfate	OSO_3	OCH_3	H	OH		
Vanillic acid-4-*O*-glcrnd	OCH_3	OGlcrnd	H	OH	344.0743	✓
Isovanillic acid-3-*O*-glcrnd	OGlcrnd	OCH_3	H	OH		
Gallic acid	OH	OH	OH	OH	170.0215	✓
Gallic acid-1-*O*-glcrnd	OGlcrnd	OH	OH	OH	346.0536	X
Gallic acid-2-*O*-glcrnd	OH	OGlcrnd	OH	OH		
4-*O*-Methylgallic acid	OH	OCH_3	OH	OH	184.0372	✓
4-*O*-Methylgallic acid-3-*O*-sulfate	OSO_3	OCH_3	OH	OH	263.9940	✓

Legend: Glcrnd - Glucuronide

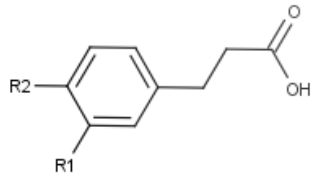
Table 2. Structure of metabolites of caffeic and ferulic acids, and presence in human urine using the Orbitrap LC-MS.



Metabolites	**R1**	**R2**	**Exact mass**	**Detected in urine**
Caffeic acid	OH	OH	180.0423	✓
Caffeic acid-3-*O*-sulfate	OSO_3	OH	259.9991	✓
Caffeic acid-4-*O*-sulfate	OH	OSO_3		
Caffeic acid-3-*O*-glcrnd	OGlcrnd	OH	356.0743	✓
Caffeic acid-4-*O*-glcrnd	OH	OGlcrnd		
Ferulic acid	OCH_3	OH	194.0579	✓
Ferulic acid-4-*O*-sulfate	OCH_3	OSO_3	274.0147	✓
Ferulic acid-4-*O*-glcrnd	OCH_3	OGlcrnd	370.0900	✓
Isoferulic acid	OH	OCH_3	194.0579	✓
Isoferulic acid-3-*O*-sulfate	OSO_3	OCH_3	274.0147	✓
Isoferulic acid-3-*O*-glcrnd	OGlcrnd	OCH_3	370.0900	✓

Legend: Glcrnd - Glucuronide

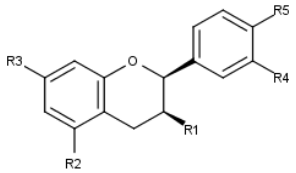
Table 3. Structure of metabolites of dihydrocaffeic acid and dihydroferulic acid, and presence in human urine using the Orbitrap LC-MS.



Metabolites	**R1**	**R2**	**Exact mass**	**Detected in urine**
DHC acid	OH	OH	182.0579	X
DHC acid-3-*O*-sulfate	OSO_3	OH	262.0147	✓
DHC acid-4-*O*-sulfate	OH	OSO_3		
DHC acid-3-*O*-glcrnd	OGlcrnd	OH	358.0900	X
DHC acid-4-*O*-glcrnd	OH	OGlcrnd		
DHF acid	OCH_3	OH	196.0736	X
DHF acid-4-*O*-sulfate	OCH_3	OSO_3	276.0304	✓
DHF acid-4-*O*-glcrnd	OCH_3	OGlcrnd	372.1056	✓

Legend: Glcrnd - Glucuronide

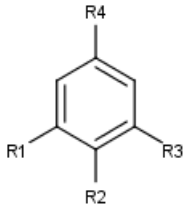
Table 4. Structure of metabolites of (-)-epicatechin and presence in human urine using the Orbitrap LC-MS.



Metabolites	**R1**	**R2**	**R3**	**R4**	**R5**	**Exact mass**	**Detected in urine**
(-)-Epicatechin	OH	OH	OH	OH	OH	290.0790	✓
(-)-Epicatechin-3-*O*-sulfate	OSO_3	OH	OH	OH	OH	370.0359	✓
(-)-Epicatechin-5-*O*-sulfate	OH	OSO_3	OH	OH	OH		
(-)-Epicat´Lechin-7-*O*-sulfate	OH	OH	OSO_3	OH	OH		
(-)-Epicatechin-3'-*O*-sulfate	OH	OH	OH	OSO_3	OH		
(-)-Epicatechin-4'-*O*-sulfate	OH	OH	OH	OH	OSO_3		
(-)-Epicatechin-3-*O*-glcrnd	Oglcrnd	OH	OH	OH	OH	466.1111	✓
(-)-Epicatechin-5-*O*-glcrnd	OH	Oglcrnd	OH	OH	OH		
(-)-Epicatechin-7-*O*-glcrnd	OH	OH	Oglcrnd	OH	OH		
(-)-Epicatechin-3'-*O*-glcrnd	OH	OH	OH	Oglcrnd	OH		
(-)-Epicatechin-4'-*O*-glcrnd	OH	OH	OH	OH	Oglcrnd		

Legend: Glcrnd - Glucuronide

Table 6. Structure of metabolites of catechol and pyrogallol, and presence in human urine using the Orbitrap LC-MS.



Metabolite	R1	R2	R3	R4	Exact mass	Detected in urine
Catechol	OH	OH	H	H	110.0368	✓
Catechol-1-*O*-sulfate	OSO_3	OH	H	H	189.9936	✓
Catechol-1-*O*-glcrnd	OGlcrnd	OH	H	CH3	286.0689	✓
4-Methylcatechol	OH	OH	H	CH3	124.0524	✓
4-Methylcatechol-1-*O*-sulfate	OSO_3	OH	H	CH3	204.0092	✓
4-Methylcatechol-2-*O*-sulfate	OH	OSO_3	H	CH3		
4-Methylcatechol-1-*O*-glucrnd	OGlcrnd	OH	H	CH3	300.0845	✓
4-Methylcatechol-2-*O*-glucrnd	OH	OGlcrnd	H	CH3		
Pyrogallol	OH	OH	OH	H	126.0317	✓
Pyrogallol-1-*O*-sulfate	OSO_3	OH	OH	H	205.9885	✓
Pyrogallol-2-*O*-sulfate	OH	OSO_3	OH	H		
Pyrogallol-1-*O*-Glcrnd	OGlcrnd	OH	OH	H	302.0638	✓
Pyrogallol-2-*O*-Glcrnd	OH	OGlcrnd	OH	H		✓
1-*O*-Methylpyrogallol	OCH_3	OH	OH	H	140.0473	X
2-*O*-Methylpyrogallol	OH	OCH_3	OH	H		
1-*O*-Methylpyrogallol-3-*O*-sulfate	OCH_3	OH	OSO_3	H	220.0042	✓
1-*O*-Methylpyrogallol-2-*O*-sulfate	OCH_3	OSO_3	OH	H		
2-*O*-Methylpyrogallol-1-*O*-sulfate	OSO_3	OCH_3	OH	H		
1-*O*-Methylpyrogallol-3-*O*-glcrnd	OCH_3	OH	OGlcrnd	H	330.0587	X
1-*O*-Methylpyrogallol-2-*O*-glcrnd	OCH_3	OGlcrnd	OH	H		
2-*O*-Methylpyrogallol-1-*O*-glcrnd	OGlcrnd	OCH_3	OH	H		

Legend: Glcrnd - Glucuronide

Chapter 4

Confirmation and quantification of phenolic-metabolites in plasma of human volunteers after ingestion of a mixed berry fruit puree

This chapter is based on the manuscript to be published as:

Quantification of phenolic-sulfates as colonic metabolites in plasma of human volunteers after ingestion of a mixed berry fruit puree.

Rui C. Pimpão, M. Rita Ventura, Ricardo B. Ferreira, Gary Williamson, Claudia N. Santos. Submitted to *British Journal of Nutrition* and is under revision.

This chapter contains unpublished data in which the author of this dissertation performed all the experimental work and wrote the manuscript.

Contents

ABSTRACT

Bioavailability studies are vital to assess the potential impact of bioactive compounds on human health. Although conjugated phenolics from colonic metabolism have been identified in urine, the quantification and appearance of these compounds in plasma is less well studied, but important when assessing potential activity *in vivo*. To address this gap, a crossover intervention with ingestion of fruit puree and a standard (poly)phenol-free meal by thirteen volunteers was conducted, and plasma metabolites identified by HPLC-MS/MS. Sulfated-standards were chemically synthesized to facilitate quantification. There was a fast absorption of gallic and caffeic acid-conjugates, reaching a maximum concentration between 1 and 2 h. Sulfated-metabolites resulting from colonic degradation of more complex (poly)phenols started to increase in plasma from 4 h, and reached 5-20 μM at 6 h for pyrogallol-sulfate and catechol-sulfate. In conclusion, phenolic-sulfates were quantified in plasma and reached much higher concentrations than their parent compounds. They have potential use as biomarkers of (poly)phenol intake and their biological activities need to be considered.

INTRODUCTION

Berry fruits assume great importance for daily consumption of (poly)phenols, due to the high contents of compounds such as phenolic acids and flavonoids, particularly anthocyanins [1, 2]. Even in populations where there is only a small consumption of berries, the contribution of these fruits to the overall ingestion of (poly)phenols can be high, in comparison to other food sources [3]. Epidemiological and experimental studies suggest that (poly)phenols reduce the risk of developing several pathological conditions [4-6]. *In vitro* studies are important to elucidate the mechanism of action of (poly)phenols in this regard. However, for the credibility of these studies, it is essential to use compounds that can actually be found in the human body and use them in physiologically-relevant concentrations. The role of intervention studies in calculating bioavailability and understanding the metabolic fate of the ingested compounds is very important [7, 8].

Recently, the idea has emerged that quantification of (poly)phenols in biological samples collected from humans and animals might be underestimated, since many metabolites formed from catabolism of (poly)phenols by colon microbiota can still be absorbed into the blood, and are usually not accounted for [9, 10]. Many of these compounds can undergo further metabolism and be conjugated by phase II enzymes, to form sulfated, glucuronidated and methylated compounds [11]. We previously identified several phenolics in conjugated form in the urine of healthy human volunteers, after ingestion of a puree composed of five different berry fruits [12]. Some of these compounds were indentified in

humans and associated with the consumption of (poly)phenols for the first time. It is now important to calculate the plasma appearance of these metabolites and assess the physiological concentration they can reach, in order to account for total and combined (poly)phenol bioavailability.

In this study, the sulfated compounds previously identified were chemically synthesized and used as standards for quantification in plasma of human subjects, after ingestion of the berry puree. A crossover study in which participants ingested either the fruit puree or a standard (poly)phenol-free meal was also conducted to assess any endogenous or non-(poly)phenol dietary origin of the studied metabolites.

MATERIAL AND METHODS

Reagents and reference materials

All chemicals used in this investigation were purchased from Sigma-Aldrich, unless stated otherwise and purchased reference standards were all HPLC grade (>95%). ACN (LS-MS grade) was purchased from Fisher Scientific Ltd. (Leicestershire, UK). LC-MS grade water was produced by an Elix/MilliQ purification system (Millipore, Waterford, UK). 4-Methylgallic acid and 2-methylpyrogallol were obtained from Apin chemicals (Oxon, UK). Taxifolin was obtained from Extrasynthese. Hydroxycinnamic acid-conjugates were chemically synthesized and characterized as described previously [13] and were kindly provided by Prof. Denis Barron, NIHS, Lausanne, Switzerland; protocatechuic acid-3-*O*-sulfate and protocatechuic acid-4-*O*-sulfate were kindly provided by Dr. Paul Needs, IFR, Norwich, UK.

Fruit puree

The fruit puree was prepared on the day of the study and its composition has been described previously [12]. Briefly, 100 g of each of five fruits were used: blueberries (*Vaccinum* spp. variety Georgia Gem), blackberries (*Rubus* L. subgenus Rubus Watson variety Karaka Black) and raspberries (*Rubus idaeus* L. variety Himbo Top) were harvested at the Fataca experimental field in Odemira, Portugal; strawberry tree fruits (*Arbutus unedo* L.) were harvested in the Alentejo region, Portugal; and Portuguese crowberries (*Corema album* L.) were harvested in the Comporta region, Portugal. Fruits were blended on a domestic food processor, for 1 min at room temperature.

Subjects and study design

Twenty two volunteers (4 of them male), aged between 22 and 54, with an average BMI of 22.6 kg/m^2 were recruited for this study. Individuals were all considered healthy by a medical questionnaire and standard blood tests. Volunteers did not have any history of cardiovascular diseases or any other medical illnesses, were non-smoking, and were not receiving medication or taking vitamins that could interfere with the study.

The study was divided in two groups. In the first group, nine volunteers followed a (poly)phenol-free diet for 2 days before the study and throughout the day of the study. After an overnight fast, volunteers ingested 500 mL of the above fruit puree with a standard breakfast containing no additional (poly)phenols, consisting of bread, with ham or cheese, yogurt and biscuits. Blood samples were collected into EDTA containing tubes before ingestion of the puree and after at 0.5, 1, 2, 4 and 6

h. The second group, containing thirteen volunteers, participated in a cross-over controlled trial. The procedure was similar as the first group, however the two stages, which occurred two weeks apart, varied with ingestion of the puree or a standard breakfast (poly)phenols free, where the puree was substituted by plain yogurt. Blood samples were collected into EDTA containing tubes before ingestion of the fruit puree and at 2 and 4 h after ingestion.

The compliance with the food restriction was confirmed through a questionnaire.

The study protocol was in accordance with the Declaration of Helsinki of 1975, as revised in 1983, and all procedures involving human subjects were approved by the ethical committee of the Faculty of Pharmacy, University of Lisbon, Portugal (02/CEECFFUL/2012). The protocol was explained to each volunteer and written informed consent was given before the study.

Sample treatment

After collection, blood samples were immediately centrifuged at 2200 g for 15 min, and 1 mL plasma samples were stored in cryotubes with addition of 30 μL of formic acid (50% v/v). Samples were kept at -80 °C prior to analysis.

Protein precipitation was based on a modification of a previously described method [14]. Briefly, to 380 μL of plasma were added 20 uL ascorbic acid 4 mg/mL (final concentration of 1 mM) and taxifolin as internal standard to a final concentration of 250 nM. Sample was combined with 1 mL hexane, homogenized and centrifuged for 10 min at

17 000 *g*. The aqueous phase was recovered and added dropwise to 1200 µL of ACN. The mixture was vortexed for 2 min and centrifuged for 10 min at 17 000 *g*. Supernatant was recovered and pellet was reconstituted with 400 µL ACN and after centrifugation, ACN supernatants were combined and dried on a centrifugal evaporator. Samples were reconstituted in 100 µL of water containing 1 µM of sinapic acid as second internal standard. Samples were centrifuged at 17 000 *g* for 10 min and the supernatants were filtered prior to HPLC-MS/MS analysis.

HPLC-MS/MS analysis

Plasma sample analysis was conducted on an Agilent HPLC 1200 series comprising a micro degasser, SL binary pump, SL autosampler with chilled sample compartment (8 °C), column oven (30 °C), and diode array detector (Agilent Technologies, Cheadle, UK) connected with a 6410a triple-quadrupole LC-MS-MS. Separation was achieved on an Atlantis T3 Column, 100 Å, 3 µm, 2.1 mm ID x 100 mm HPLC column (Waters, Hertfordshire, UK) at a flow rate of 0.26 mL/min over a gradient of 100% solvent A (95% H_2O, 5% ACN with 0.5% (v/v) formic acid) for 10 min, reaching 15% B (95% ACN, 5% H_2O with 0.5% (v/v) formic acid) from 10 to 20 min. Solvent B increased to 25% at 40 min and to 100% B at 43 min where it was maintained for 5 min returning to 0% in 2 min.

MS interface was set at 350 °C, a source voltage of 4kV, and N2 drying gas flow rate of 11 L/min at a pressure of 30 psi (Peak Scientific, NM30LA, Inchinnan, UK). The analysis was performed in negative Multiple Reaction Monitoring (MRM) mode optimized using the reference standards (see Table 1). The system was controlled and data processed by

Agilent MassHunter software (version B.01.03). The m/z transitions optimized for each standard were previously optimized [12]. Quantification of metabolites in plasma was obtained using calibration curves of the available standards. At least 8 concentrations, ranging from 0.05 to 20 μM, were constructed from analytical standards and each point was injected in triplicate. Standard curves were all linear within the concentration range and linearity was ensured as R^2=0.997-1.000 (Table 1).

Statistical analysis

Pharmacokinetic profile of plasma metabolites was constructed using GraphPad Prism 5. This package was also used for statistical analysis. Box-and-Whiskers plots for minimum and maximum values were produced. Comparisons in relation to the baseline were performed with two tailed Wilcoxon matched pairs test with a confidence level of 99%. For the cross-over study comparisons were made between consumption of fruit puree and the control breakfast for each time point and the same test was used.

Synthesis of sulfated compounds

The products were synthesized by a treatment of the initial aglycones (500 mg) with sulfor trioxide pyridine [pyridine:SO_3]. The aglycones (500 mg) and pyridine:SO_3 (1 equivalent for all compounds with exception of vanillic acid where 2 equivalents were used) were dissolved in 10 mL of anhydrous pyridine and kept at 65 °C with constant stirring for 24 h. Reaction was stopped by addition of water. Solvents were dried in *vacuo* and the residue was dissolved in water. The unreacted aglycones were

separated with ethyl acetate and the product in the water phase was purified with a Dowex 50W-X8 ion-exchange column loaded with Na^+. Purified compounds were then dried in *vacuo* and characterized by ^1H and ^{13}C NMR. NMR chemical shifts are reported using the residual solvent peak as reference. Peak assignments were based on COSY and HMQC experiments.

Catechol-*O*-sulfate [(2-hydroxyphenyl)-oxidanesulfonic acid] : 674 mg, 66% yield; ^1H NMR (400 MHz, DMSO-d_6): δ 8.85 (s, 1H, OH), 7.12 (dd, 1H, J 1.6, 7.96 Hz), 6.96 (ddd, 1H, J1.64, 7.96, 9.26 Hz), 6.83 (dd, 1H, J 1.6, 8 Hz), 6.75 (ddd, 1H, J 1.68, 7.92, 9.24 Hz); ^{13}C NMR (100 MHz, DMSO-d_6): δ 149.21, 140.92, 124.88, 123.13, 119.25, 117.24.

Pyrogallol-*O*-sulfate: 677 mg, 75% yield; mixture of two compounds in approximate similar proportion; pyrogallol-2-O-sulfate [(2,6-dihydroxyphenyl)-oxidanesulfonic acid]:^1H NMR (400MHz, D_2O): δ 6.97 (t, 1H, J 8.24 Hz, H-4), 6.51 (d, 2H, J 8.28 Hz, H-3 + H-5) and pyrogallol-O-sulfate [(2,3-dihydroxyphenyl)oxidanesulfonic acid]: ^1H NMR (400MHz, D_2O): δ 6.90 – 6.86 (m, 1H, H-5), 6.79 – 6.74 (m, 2H, H-4 + H-6); ^{13}C NMR (100 MHz, D_2O): δ 149.84, 145.42, 139.76, 127.19, 120.34, 114.31, 113,80, 108.93.

2-Methylpyrogallol-O-sulfate [(3-hydroxy-2-methoxyphenyl)-oxidanesulfonic acid]: 450 mg, 44% yield, having 11% contamination with the disulfated byproduct. ^1H NMR (400MHz, DMSO-d_6): δ 8.97 (s, 1H, OH), 6.97 (dd, J 1.52, 8.32 Hz, H-6), 6.74 (t, 1H, J 8.2 Hz, H-5), 6.51 (dd, 1H,

J 1.52, 8.08 Hz, H-4), 3.34 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ 150.57, 147.20, 139.10, 122.33, 112.30, 110.89, 60.09.

1-Methylpyrogallol-*O*-sulfate: 500 mg, 58% yield; (2-hydroxy-6-methoxyphenyl)-oxidanesulfonic acid and (2-hydroxy-3-methoxyphenyl)-oxidanesulfonic acid, mixture of both compounds in approximate equal amounts (56%:44%). ^1H NMR (400MHz, DMSO-d_6): δ 9.20 (s, 1H, OH), 8.52 (s, 1H, OH), 6.92 (t, 1H, J 8.60 Hz), 6.77-6.68 (m, 3H), 6,52-6.44 (dd, 2H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 153.39, 150.93, 149.17, 141.35, 139.07, 124.86, 118.07, 115.65, 110.08, 108.64, 104.11, 55.87, 55.72

4-Methylcatechol-*O*-sulfate: 604 mg, 66% yield. Mixture of the two compounds, (2-hydroxy-4-methylphenyl)-oxidanesulfonic acid and 2-hydroxy-5-methylphenyl)-oxidanesulfonic acid, not being possible to distinguish them, One is present at 64%, ^1H NMR (400MHz, DMSO-d_6): δ 8.76 (s, 1H, OH), 6.96 (d, 1H, J 8.08 Hz), 6.64 (d, 1H, J 1.76 Hz), 6.55 (dd, 1H, J 2.04, 8.6 Hz), 2.20 (s, 3H, CH_3). The other being 36% of the sample, ^1H NMR (400MHz, DMSO-d_6): δ 8.62 (s, 1H, OH), 6.93 (d, 1H, J 1.84 Hz), 6.77 (dd, 1H, J 1.56, 8.04 Hz), 6.71 (d, 1H, J 8.12 Hz), 2.19 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ 148.87, 146.80, 140.70, 138.80, 134.20, 128.14, 125.27, 123.58, 122.94, 119.87, 117.77, 116.96, 20.06, 20.47.

4-Methylgallic-3-*O*-sulfate [3-hydroxy-4-methoxy-5-(sulfooxy)benzoic acid]: 485 mg, 57% yield, having a contamination of 13% of the disulfate and 6% of the starting material;^1H NMR (400MHz, D_2O): δ 7.46 (d, 1H, J

2.04 Hz, H-2), 7.32 (d, 1H, J 2 Hz, H-6), 3.88 (s, 3H, CH_3) ; ^{13}C NMR (100 MHz, D_2O):δ 172.07, 149.19, 143.99, 142.85, 129.61, 115.42, 114.84, 61.29.

Vanillic acid-4-*O*-sulfate [3-methoxy-4-(sulfooxy)benzoic acid]: 924 mg, quantitative yield. ^1H NMR (400MHz, DMSO-d_6): δ 7.57 (d, 1H, J 8.16 Hz, H-2), 7.50-7.47 (m, 2H, H-5+H-6), 3.79 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ 167.39, 149.51, 148.4, 146.41, 121.96, 119.33, 112.71, 55.48.

RESULTS

Composition of fruit puree

Detailed identification and quantification of (poly)phenols in the fruit puree have been described previously [12]. The fruit puree (500 mL) contained anthocyanins (636 ±19 mg) and caffeoylquinic acids (5-caffeoylquinic and 3-caffeoylquinic acids, 135.9 ± 2.1 mg). Aglycones were quantified after hydrolysis by the use of gycosidases from *Aspergillus niger* as previously published [15]. Gallic acid (GA) was the most abundant aglycone (425.9 ± 14.0 mg/500 mL fruit puree). Caffeic acid (CA) was also abundant (140.4 ± 2.2 mg/500 mL fruit puree), resulting from hydrolysis of the caffeoylquinic acids and conjugated glycosides. After hydrolysis, several other phenolic acids were detected and quantified including the flavanols (-)-epicatechin and (+)-catechin, and the flavonols quercetin, myricetin and kaempferol. The presence of vanillic (VA), ferulic (FA) and protocatechuic (PA) acids were also observed in the hydrolyzed extract.

Chemical synthesis of sulfated phenolics

(Poly)phenol metabolites were identified previously in urine samples from human volunteers after ingestion of the fruit puree [12]. In this work, we focused on quantification of sulfated-metabolites in plasma. Some were chemically synthesized for this purpose (Table 1). Synthesized compounds were mostly pure as determined by NMR, except for 2-methylpyrogallol-*O*-sulfate (89% pure due to a double-sulfate subsituted compound) and 4-methylgallic acid-3-*O*-sulfate (81% pure due to the presence of some aglycone and a double-sulfate substituted derivative). The LC-MS/MS chromatographic profile of the synthesized standards is shown in Table 1, with most compounds resulting in single peaks. However, for the compounds 4-methylcatechol (4-MeCat) and 1-methylpyrogallol (1-MePyr), two different sulfated metabolites were obtained after synthesis as can be observed by NMR, although they co-eluted as only one chromatographic peak. Protocatechuic acid-3-*O*-sulfate (PA-3-sulf) and protocatechuic acid-4-*O*-sulfate (PA-4-sulf) also co-eluted when run together on HPLC-MS/MS.

Table 1- HPLC-MS/MS parameter for the quantification of phenolic metabolites in human plasma after ingestion of 500 mL of fruit puree

Compound name	**Compound abbreviation**	**MRM parent ion**	**MRM daughter ion**	**Calibration curve range (µM)**	**R^2**
Gallic acid	GA	169.1	124.9	0.4-20	0.998
4-Methylgallic acid	4-MeGA	183.1	167.8	0.1-8	0.997
4-methylgallic acid-3-O-sulfate	4-MeGA-sulf	163.1	183.1	0.29-14.7	0.998
Protocatecuic acid-O-sulfate	PA-sulf	233.1	153.1	0.2-16	0.999
Vanillic acid-4-O-sulfate	VA-sulf	147.1	167.1	0.18-18.5	1.000
Caffeic acid-O-sulfate	CA-sulf	259	179	0.1-16	1.000
Ferulic acid-4-O-sulfate	FA-sulf	273	193	0.05-8	1.000
Isoferulic acid-3-O-sulfate	IFA-sulf	273	193	0.05-8	1.000
Dihydrocaffeic acid-O-sulfate	DHCA-sulf	261	181	0.1-8	0.999
Dihydroferulic acid-4-O-sulfate	DHFA-sulf	275	195	0.1-8	0.999
Catechol-O-sulfate	Cat-sulf	189.1	109.1	0.2-20	0.999
4-Methylcatechol-O-sulfate	4-MeCat-sulf	203.1	123.1	0.1-20	0.999
Pyrogallol-O-sulfate	Pyr-sulf	205.1	125.1	0.2-20	0.998
1-Methylpyrogallol-O-sulfate	1-MePyr-sulf	219.1	139.1	0.34-17	1.000
2-Methylpyrogallol-1-O-sulfate	2-MePyr-sulf	219.1	139.1	0.2-20	0.999

Plasma appearance of phenolics

Previously, several metabolites were identified in the urine of volunteers after ingestion of fruit puree, and although only a relative quantification was performed, sulfated-phenolics were more abundant than glucuronidated-phenolics [12]. In this work, plasma of human volunteers was analysed after ingestion of the fruit puree for confirmation of the presence of those sulfated-metabolites by LC-MS/MS (Fig 1). Gallic acid was identified, as well as its conjugated metabolites 4-*O*-methylgallic acid (4-MeGA) and 4-*O*-methylgallic acid-3-*O*-sulfate (4-MeGA-Sulf). Sulfated-metabolites of VA, FA, dihydroferulic (DHFA) and dihydrocaffeic (DHCA) acids were also confirmed in plasma. PA-*O*-sulf was also present, but it was not possible to distinguish between the isomers PA-3-*O*-sulf and PA-4-*O*-sulf, since they co-eluted.

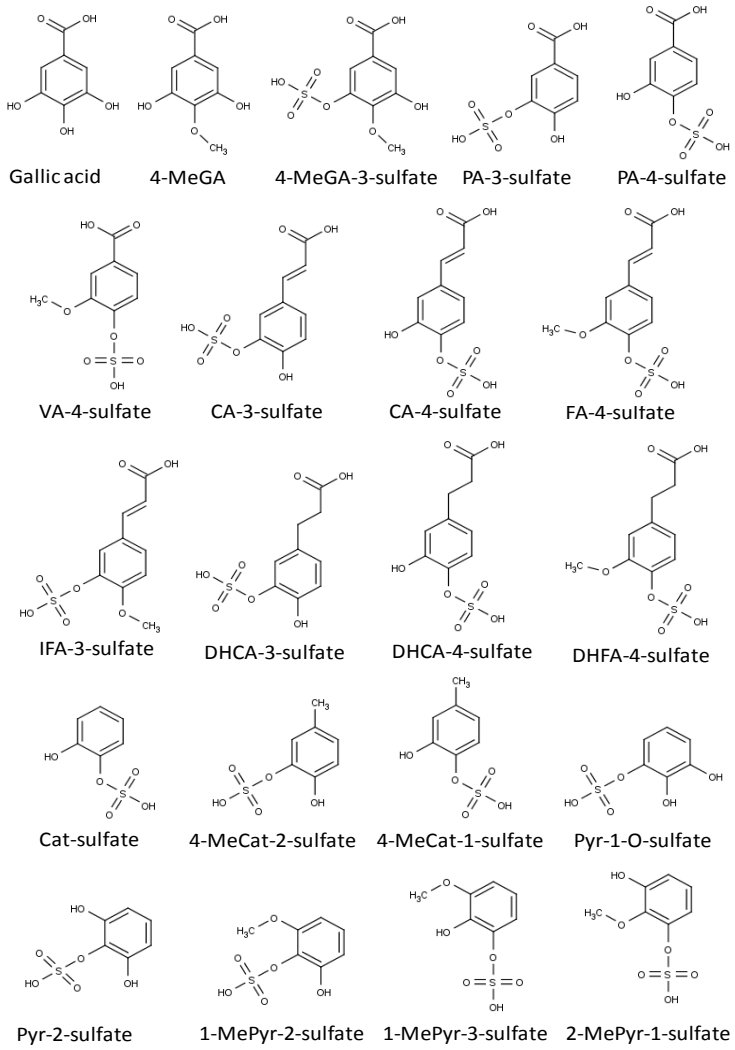


Figure 1. Structure of phenolic metabolites searched for in the plasma from human volunteers: 4-MeGA, 4-methylgallic acid; PA, protocatechuic acid; VA, vanillic acid; CA, Caffeic acid; FA, ferulic acid; IFA, isoferulic acid; DHCA, dihydrocaffeic acid; DHFA, dihydroferulic acid; Cat, catechol; 4-MeCat, 4-methylcatechol; Pyr, Pyrogallol; MePyr, Methylpyrogallol.

Although one peak of sulphated DHCA was found in plasma at low levels, it is possible that this could be two sulfated metabolites, since they co-eluted (Table 2). The presence of sulfated metabolites of catechol (Cat), 4-MeCat, 1-MePyr and 2-*O*-methylpyrogallol (2-MePyr) was also confirmed. Two sulfated-metabolites of pyrogallol (Pyr) were found, corresponding to the synthesized standards.

Table 2 –Concentration in plasma of phenolic conjugated-metabolites and presence in volunteers after ingestion of 500 mL of fruit puree

Metabolite	**Cmax (nM)**[†]	**Tmax (hours)***	**Number of volunteers present**
4-MeGA	300 ± 138	2	7
4-MeGA-sulf	2028 ± 1095	2	9
VA-sulf	1345 ± 1310	4	6
PA-sulf	1055 ± 972	4	6
CA-3-sulf	181 ± 127	1	8
FA-sulf	188 ± 107	1	9
Cat-sulf	12194 ± 5860	6	9
4-MeCat-sulf	636 ± 469	6	7
Pyr-sulf (1)	652 ± 328	6	7
Pyr-sulf (2)	11430 ± 6678	6	8
1-mePyr-sulf	2879 ± 1807	6	9
2-MePyr-sulf	1969 ± 982	6	8

[†]C_{max}, peak plasma concentration calculated from the average concentration in volunteers where it was present.
*T_{max}, time to reach C_{max} of the metabolites for the times of collection. It is no to exclude higher concentration of some metabolites after 6h.

Plasma quantification of phenolic metabolites

The synthesized standards or pure aglycones were used for the quantification of phenolic metabolites (Table 1). For the co-eluting compounds in plasma (sulfates of PA, 1-MePyr and 4-MeCat) quantification was done as corresponding to a single compound. Measurement of plasma metabolites was performed between 0 and 6 h following the ingestion of 500 mL of fruit puree. The pharmacokinetic profile of each metabolite is shown in Fig. 2 and an average maximum concentration of the metabolites when present in six or more volunteers is presented in Table 2. GA, despite being present in all volunteers, was mostly found under the limit of quantification, only being quantifiable in three out of nine volunteers, reaching a maximum concentration of 840±340 nM at 1 h after ingestion of the fruit puree. However, its conjugated metabolites 4-*O*-methylgallic (4-MeGA) and 4-*O*-methylgallic-3-O-sulfate clearly peaked at 2 h. DHFA-*O*-sulf was detected in the plasma of volunteers, however it was under the limit of quantification. DHCA-*O*-sulfate was only quantifiable in four volunteers at 6h where it reached 151±18 nM.

The metabolites Cat-sulf, 4-MeCat-sulf, Pyr-*O*-sulfates and MePyr-*O*-sulfates were found at baseline in several volunteers (Fig. 2). All these metabolites, with exception of 4-MeCat-sulf, had a statistically significant increase over the collection period time in comparison to the baseline. Interestingly, the compounds Cat-sulf and Pyr-sulf even reached concentrations up to 20 μM in some volunteers. However, variability

between plasmatic concentrations between the volunteers was high for all the quantified metabolites.

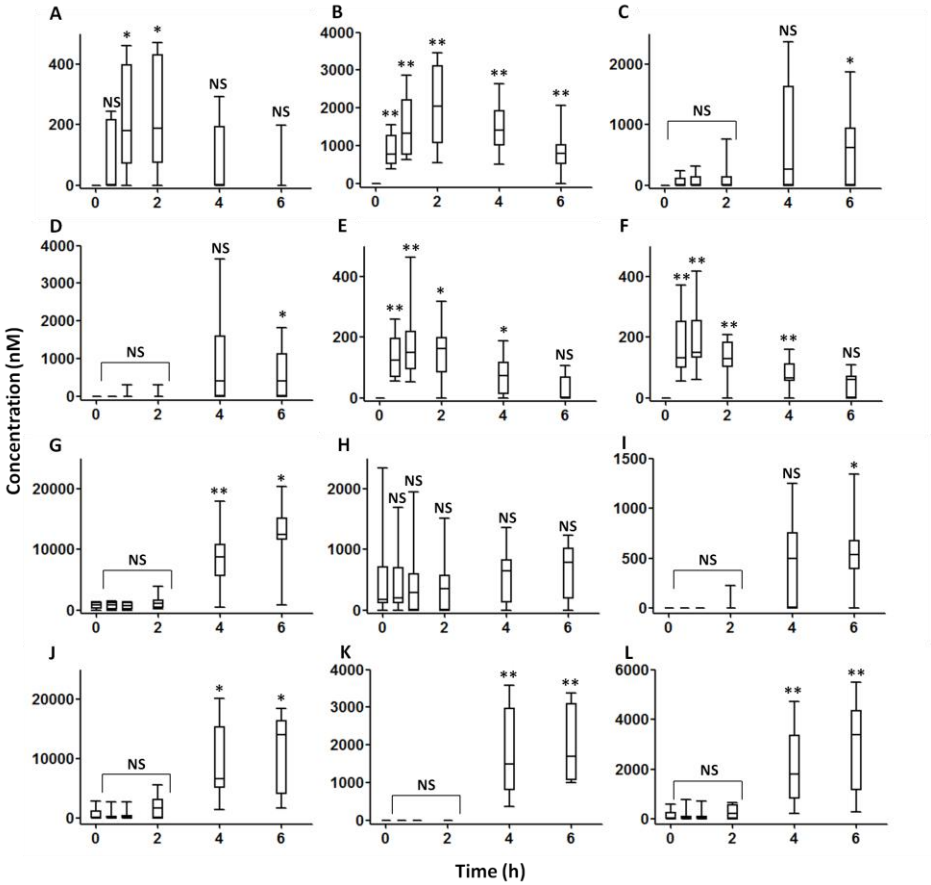


Figure 2. Plasma quantification of phenolic metabolites at baseline, and at 0.5, 1, 2, 4 and 6 h after ingestion of 500 mL of fruit puree. A – 4-MeGA, B – 4MeGA-sulf, C – PA -sulf, D – VA-sulf, E – CA-sulf, F – FA-sulf, G – Cat-sulf, H – 4-MeCat-sulf, I – Pyr-sulf (1), J – Pyr-sulf (2), K – 2-MePyr-sulf, L – 1-MePyr-sulf. Statistical comparison is in relation to baseline. No star, not significant; *, $p < 0.05$; **, $p < 0.01$ relative to time zero.

Comparison between ingestion of fruit puree and (poly)phenol-free meal

Confirmation of the provenance of the analysed metabolites was assessed by the ingestion of a (poly)phenol-free meal (control) as opposed to the ingestion of fruit puree by the same volunteers. Comparative analysis was done in plasma samples collected at baseline, at 2 h and at 4 h and is summarized in Fig 3. At baseline, no differences were observed between ingestion of the fruit puree or the (poly)phenol-free meal. However, for most metabolites, a statistically significant increase was observed after ingestion of the fruit puree at a certain time point. The only exceptions were observed for PA-sulf where an increase was observed both after ingestion of the fruit puree and the control meal and for VA-sulf which at 4 h had a higher increase in plasma after ingestion of the control meal than for the fruit puree.

For Pyr-sulf, although a small increase was observed in plasma of volunteers 2 h after the ingestion of the control meal, at 4 h there was a marked increase in the plasma of volunteers ingesting the fruit puree.

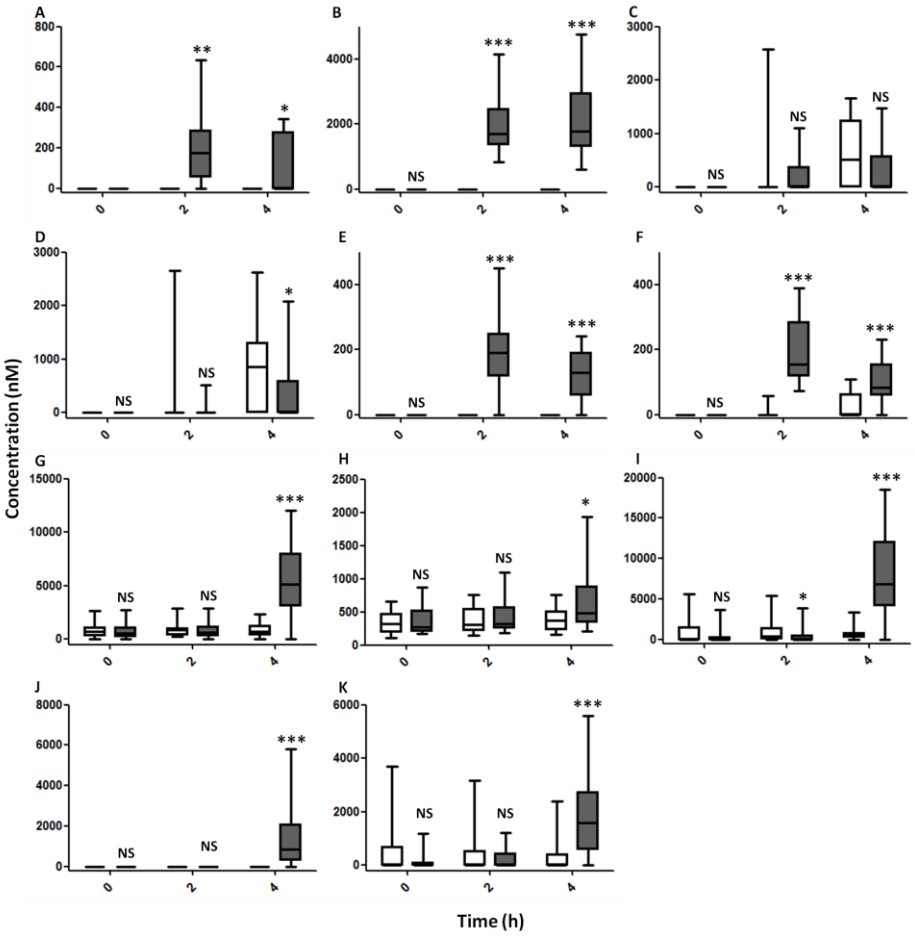


Figure 3. Plasma quantification of phenolic metabolites at baseline, and at 2 and 4h after ingestion of the fruit puree (■) or standard breakfast (□). A – 4-MeGA, B – 4-MeGA-sulft, C – PA-sulf, D – VA-sulf, E – CA-sulf, F – FA-sulf, G – Cat-sulf, H – 4-MeCat-sulf, I – Pyr-sulf (2), J – 2-MePyr-sulf, K – 1-MePyr-sulf. Statistics compare plasma concentrations of each volunteer after ingestion of the fruit puree or standard breakfast. No star, not significant; *, $p < 0.05$; **, $p < 0.01$, $p < 0.005$ relative to time zero.

DISCUSSION

Conjugation reactions, particularly sulfation, glucuronidation and methylation, are known to be involved in the metabolism of phenolics in the human body, generally resulting in stabilisation and increased water-solubility, and therefore modifying their distribution and excretion [16, 17]. Sulfate conjugation is one of the major metabolic pathways for endogenous and exogenous phenolic compounds [18]. Previously, we demonstrated that sulfated phenols were found in urine after ingestion of a fruit puree containing 5 berry fruits [12]. In the present study, some of those compounds were chemically synthesized and used for quantification purposes in plasma collected from volunteers. Confirmation of their provenance was also achieved by comparing the amounts in plasma of volunteers consuming the puree or a (poly)phenol-free standard meal.

GA, a compound present as aglycone in high amounts in the fruit puree, was previously quantified in human plasma as well as its methylated metabolite 4-MeGA after ingestion of GA tablets and black tea[19]. Its kinetics were similar to the present study, reaching a maximum concentration near 1.5 h. Identified for the first time in rats urine by Yasuda and co-workers [20], 4-MeGA-3-sulf was quantified in human plasma for the first time in the present study and was undoubtedly derived from compounds within the fruit puree (Fig. 3). Its kinetic profile was similar to GA and 4-MeGA reaching a maximum concentration at 2 h (Figure 2, Table 1), suggesting that absorption and metabolism of GA was

fast, with the 4-hydroxyl group being regioselectively preferred for methylation and the 3-hydroxyl group for sufation.

Previous studies suggested PA as one of the major metabolites from degradation of cyanidin [21, 22]. Of the compounds studied here, PA-sulf, VA-sulf and FA-sulf were previously identified in plasma as metabolites resulting from the degradation of ^{13}C-labeled cyanidin-3-glucoside in the digestive tract [9]. However, in the present study, only at 6 h was there a significant increase of PA-sulf and VA-sulf compared to the baseline, and an increase of these metabolites was registered also in the control meal. It is unlikely that (poly)phenols are present in the control meal, and so phenolics present after ingestion of the control meal are likely to be derived from endogenous metabolism. A comparable example is dopamine metabolism, where homovanillic acid is a known metabolite, which would probably exist as homovanillic acid-*O*-sulfate [23].

Hydroxycinnamic acid metabolism has been widely studied in relation to coffee consumption and our results are consistent with these data. Sulfation is dominant over glucuronidation for most hydroxycinnamic acids [24], but methylation also appears to have a important role, resulting in FA and isoferulic acid [25]. CA-3-*O*-sulfate and FA-4-*O*-sulfate clearly resulted from ingestion of the puree (Fig. 3) and were quantified in plasma, having similar values and absorption kinetics. These results were in accordance with previous studies proposing rapid absorption kinetics for CA and FA metabolites in plasma [25, 26]. DHCA and DHFA-conjugates were not quantifiable in plasma, being mostly under the limit of quantification. However, these are derived from the action of colonic

bacterial reductases [25], and so would only start to appear at 5 hours and have a T_{max} between 7 and 11 hours [27].

Previously we identified conjugated-phenolic metabolites possibly resulting from catabolism of other (poly)phenols [12]. Their colonic origin is suggested since some unconjugated phenolics have been identified in faeces after ingestion of (poly)phenols [10, 28]. However, our data suggest that absorption in the colon might also occur and these compounds can subsequently be found conjugated in plasma. The origin of these phenolic-conjugates such as catechol, pyrogallol, 2-methylpyrogallol and 1-methylpyrogallol -*O*-sulfated metabolites was a consequence of consuming the fruit puree, and to our knowledge this is the first time that these compounds have been quantified in human plasma and associated with (poly)phenol metabolism. Clearly, this class of metabolites has been neglected in bioavailability studies and, since they reached considerable amounts in the plasma, further studies are needed to access their biological significance. Recently, the importance of colonic catabolism of (poly)phenols and several metabolites has been appreciated. Due to the ability of the colonic microbiota to catalyse reactions such as O- and C-deglycosylation, ester and amide hydrolysis and deglucuronidation, flavonoids, once considered to show poor bioavailability, might result in smaller phenolics with a much higher absorption than the parent compounds [29]. Additionally, new classes of metabolites have been specifically assigned to colonic metabolites of flavonoids, such as valerolactones from catabolism of catechins and urolithins from catabolism of ellagic acid and ellagitannins [28, 30]. These metabolites of

colonic origin might be of great importance for the health effects commonly associated to the ingestion of polyphenols.

Glucuronidation has been described as being a major route for conjugation of phenolics such as acetaminophen [31], quercetin [32], PA and VA [33] and other substances with a 1,2-dihydroxybenzene group [34]. However, sulfation is the primary conjugation route of the hydroxycinnamic acids CA, FA and DHCA [24] and many other sulfated phenolics have been reported, including sulfates of protocatechuic and vanillic acids [35]. Sulfation of phenolics is carried out by cytosolic sulfotransferases (SULTs) which catalyze the transfer of a sulfonate group from 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to a substrate containing a hydroxyl group. The isoforms SULT1A1, SULT1A3/4, SULT1B1, SULT1E1 and SULT2A1 were considered the most relevant in (poly)phenol metabolism in human adults [18]. Besides sulfation, methylation, carried out by catechol O-methyltransferase (COMT), also has great importance in metabolism of phenolic compounds [17]. This activity may be responsible for the conversion of pyrogallol to 1- and 2-methylpyrogallol, GA to 4-MeGA, CA to FA, DHCA to DHFA and PA to VA. Additionally, all of these compounds could be sulfated, and this dual conjugation of compounds will affect re-activation of conjugated compounds via deconjugation reactions [17].

In summary, our results confirm the provenance of several phenolic-conjugates resulting from the ingestion of a (poly)phenol-rich berry fruit puree by humans, since they increase in plasma in comparison to either the baseline or after ingestion of a (poly)phenol-free control meal. Metabolism of fruit (poly)phenols results in methylated, sulfated and

some dual conjugated compounds. We highlight the importance of catabolism in the colon, generating simple phenols that can then be absorbed from the colon and circulate in conjugated form in the blood. Therefore, they contribute indirectly to the bioavailability of food (poly)phenols, and can potentially also be used as markers for (poly)phenol intake.

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Chapter 5

Unraveling the beneficial effects of human bioavailable phenolic-conjugated metabolites on the blood-brain barrier

This chapter is based on the manuscript to be published as:

Unraveling the beneficial effects of human bioavailable phenolic-conjugated metabolites on the blood-brain barrier.

R. C. Pimpão, I. Figueira, I. Palmela, R. B. Ferreira, R. Ventura, K. S. Kim, G. Williamson, D. Brites, M. A. Brito, C. N. Santos. *Unpublished data.*

This chapter contains unpublished data in which the author of this dissertation contributed to the planning of the experimental work, performed the chemical synthesis of compounds and extracted and analyzed the metabolites from the HBMEC experiments and wrote the manuscript (the experiments with the HBMEC were performed by Inês Fugueira).

Contents

ABSTRACT

A great amount of research has focused on the possible protective effects of dietary (poly)phenols against human neurodegenerative disorders. However, the knowledge of the metabolites able to cross the blood-brain barrier and affect the brain is still scarce, as well as their mechanisms of action. With the discovering of novel bioavailable metabolites resulting from the ingestion of (poly)phenols, new opportunities arise for unraveling this process.

In this study, phenolic conjugates found in plasma of humans following the ingestion of (poly)phenols were chemically synthesized and tested on an *in vitro* model of the blood-brain barrier formed by confluent monolayers of brain microvascular endothelial cells (HBMEC). The toxicity of the phenolic-conjugates at physiological concentrations was tested in the HBMEC and their ability to cross the endothelial monolayer was evaluated by HPLC-MS. Additionally, the protective effect of compounds against injury induced by H_2O_2 was evaluated, as well as their effect on the expression and activity of the efflux transporter P-glycoprotein (P-gp).

All the phenolic-conjugates were not toxic and they were able, although only in a very small percentage, to cross the HBMEC. They were also able to protect the cells from H_2O_2 injury. Additionally, the compounds had no effect on P-gp activity and expression. Overall, our results suggest that biovailable phenolic-conjugates may protect the brain function at physiological concentrations at the level of the blood-brain barrier and have the potential to enter the brain.

INTRODUCTION

Epidemiological studies have suggested that a diet rich in fruits and vegetables can reduce the development of neurodegenerative disorders [1]. (Poly)phenols, considered some of the active compounds, were also shown to improve the cognitive performance in aged rats upon dietary supplementation with blueberries and partially reverse some of the degenerative effects of ageing [2]. The potential of (poly)phenols to exert protective actions in neuronal cells was, in other experiments, related with ability of compounds to interact with signaling pathways and alter gene expression [3], although the mechanisms of action at the molecular level remain poorly understood.

The effective uptake of (poly)phenols into the brain is still open to debate. Assays with exsanguinated and perfused animals revealed that (poly)phenols can enter the brain at measurable levels [4-6]. The knowledge of (poly)phenol compartmentalization within the central nervous system, whatsoever, is rather scarce. There is now good evidence from cell culture experiments that (poly)phenols are able to enter at least in some cell types [7, 8] and exert pharmacological effects. To have an activity in the brain, (poly)phenols have to be able to permeate the blood-brain barrier (BBB), an ability that is related with their bioavailability.

However, for most experiments, bioavailability has not been much considered since many of the compounds tested were pure aglycones or plant extracts, used at concentrations much above the physiological range and do not necessarily reflect what occurs *in vivo*.

It is expected that for (poly)phenols to exert protective activities in the brain they necessarily have to be bioavailable and reach the target tissues. However, during digestion only a portion of food polyphenols are absorbed in the small intestine and most of them reach the colon. In the colon (poly)phenols are catabolized by colonic microbiota and degraded into simpler units such as simple phenols, phenolic acids and non-aromatic metabolites, which can still be absorbed [9]. After absorption, metabolites generally go through phase II conjugation, becoming less lipophilic and easier to excrete [10]. However, conjugated-phenolics are able to circulate in the blood and potentially reach the tissues. Moreover, although phase II conjugation usually inactivates compounds, the opposite has been observed, with some compounds being in fact activated [11], and therefore a pharmacological action is not excluded.

Previously, we identified and chemically synthesized bioavailable metabolites of colonic origin resulting from the ingestion of a puree composed of five different berry fruits [12, 13]. Here, we test the effect of these bioavailable (poly)phenol-conjugated compounds in preventing H_2O_2 damage in human brain microvascular endothelial cells (HBMEC) and their ability to permeate across cells in a confluent monolayer, and further accessed if they interfere with expression and activity of the efflux transporter P-glycoprotein (P-gp). For this, we used a cell line of HBMEC as a simplified *in vitro* model of the human BBB [14], considering that brain microvascular endothelial cells are the principal component of such important interface, forming the intimal surface of blood vessels that

selectively limits the passage of most molecules into the brain and regulates the brain extracellular fluid composition [15].

MATERIAL AND METHODS

Reagents

All the used chemicals were purchased from Sigma-Aldrich, unless stated otherwise. Basal medium Roswell Park Memorial Institute (RPMI) 1640 and antibiotic-antimycotic solution were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Non-essential amino acids (NEAA), sodium pyruvate, L-glutamine, fetal bovine serum (FBS) and minimum essential medium (MEM) vitamins were purchased from Biochrom AG (Berlin, Germany). Nuserum IV and rat-tail collagen I were acquired from BD Biosciences (Erembodegem, Belgium). The primary antibodies for the P-gp were from Calbiochem and for caveolin-1were from Cell Signalling. The secondary antibodies Alexa 594 anti-rabbit IgG and Alexa 488 anti-mouse IgG were from Invitrogen. Triton X-100 was obtained from Merck.
ACN (LC-MS grade) was purchased from Fisher Scientific Ltd. (Leicestershire, UK). LC-MS grade water was produced by an Elix/MilliQ purification system (Millipore, Waterford, UK). 4-Methylgallic acid and 2-methylpyrogallol were obtained from Apin chemicals (Oxon, UK) and taxifolin was obtained from Extrasynthese. The sulfated-phenolics were synthesized as previously described in the Chapter IV of this thesis [13]. The synthesized compounds were: 4-methylgallic acid-3-*O*-sulfate (4-MeGA-sulf, 57% yield, 79% purity), vanillic acid-4-*O*-sulfate (VA-sulf, quantitative yield, pure), catechol-*O*-sulfate (Cat-sulf, 66 % yield, pure), 4-

methylcatechol-*O*-sulfates (4-MeCat-sulf, 66% yield, mixture of two compounds indistinguishable, pure), pyrogallol-O-sulfates (Pyr-sulf, 75% yield, mixture of two compounds in equal proportions), 1-methylpyrogallol-O-sulfates (1-MePyr-sulf, mixture of two compounds in equal proportions, 58% yield) and 2-methylpyrogallol-O-sulfate (2-MePyr-sulf, 44% yield, 89% purity). The compounds 4-methylcatechol-*O*-sulfate (4-MeCat-sulf), pyrogallol-*O*-sulfate (Pyr-sulf) and 1-methylpyrogallol-*O*-sulfate (1-MePyr-sulf) are constituted by a mixture of two isomers approximately in equal proportions. Since no distinction in the chromatographic profile can be obtained for isomers of 4-MeCat-sulfates and 1-MePyr-sulfates, we opted for presenting the results relative to the mixture of the isomers. Synthesized compounds were firstly dissolved in DMSO (Fluka), before dilution in the cellular medium.

Cell culture

The cell line used in this study was derived from primary cultures of HBMEC transfected with SV40 large T antigen [16]. HBMEC line was used as a simplified model of the human BBB, as reported previously [14, 17]. This cell line displays good tight and adherens junctions and was shown to present the best barrier properties among four different cell lines as shown by the highest transendothelial electrical resistance (TEER) and the lowest permeability [18]. HBMEC line was cultured in RPMI medium supplemented with 10% FBS, 10% NuSerum IV, 1% NEAA, 1% MEM vitamins, 1mM sodium pyruvate, 2mM L-glutamine, and 1% antibiotic-antimycotic solution. For cell viability and cytoprotection studies, cells

were seeded at a density of 8×10^4 cell/mL in collagen I-coated 96-well plates and treated after 48h in culture. For integrity and transport studies, cells were seeded on collagen I-coated polyester transwell inserts (0.4 μm, Corning Costar Corp., USA) at a density of 8×10^4 cell/insert and treated after 8 days in culture. All experiments were performed at confluence. Endothelial cultures were maintained at 37 °C in a humid atmosphere enriched with 5% CO_2. For the P-gp experiments cells were seeded on collagen I-coated coverslips previously placed in 24-well culture plates.

Cellular viability evaluation

Cell viability assay was performed as previously reported [19] using a 96-well plate. Briefly, 48 h after seeding, HBMEC cells were incubated with tested compounds in relevantly physiological concentration. Toxicity tests involved 24h of incubation of 5 and 10 μM of each compound. Cell viability was assessed using the CellTiter-Blue® Cell Viability Assay (Promega), according to the manufacturer's instructions in a Polarstar Galaxy fluorescent plate reader (BMG Labtechnologies). Non-viable cells rapidly lose their metabolic capacity and thus do not generate fluorescent signal.

Cytoprotective effect

To evaluate the cytoprotective effect of tested compounds, HBMEC were incubated in the presence of H_2O_2 [20]. Briefly, 24 h after seeding cells, growth medium was removed and cells were pre-incubated for 24h with 5 μM of each compound in RPMI medium. After pre-incubation, media was

replaced by new media containing 300 μM H_2O_2, for 24 h. In the end, medium was removed and cell viability was assessed using the CellTiter-Blue® Cell Viability Assay as described above.

Transport of phenolic-conjugates across HBMEC

For the transport studies, HBMEC were cultured on semipermeable filters with two well-defined compartments: the luminal or upper compartment, which can be considered as the "blood-side" where the tested compounds were added, and the abluminal or lower compartment, which is considered the "brain side". At the end of the incubation period, and after transendothelial electric resistance (TEER) measurement, the cultured inserts were used for permeability studies and media from the upper and lower chambers were collected for LC-MS measurements.

Transendothelial electric resistance measurement

TEER was evaluated as reported previously [14]. Briefly, TEER readings were performed using an EndOhm™ chamber coupled to an EVOMX resistance meter (World Precision Instruments, Inc., USA). Readings were collected before the addition of the (poly)phenols-conjugates (time 0) and after 2h of exposure. The percentage of variation from average control readings, after deducting the empty insert values, was calculated.

Cellular permeability evaluation

In order to evaluate the selective paracellular permeability of the HBMEC monolayer after exposure to the polyphenol metabolites, a permeability

assay was conducted with sodium fluorescein (molecular weight: 376 Da). The permeability was determined as described before [14, 21]. The endothelial permeability coefficient *Pe* was calculated as percentage of variation from control as previously described [22].

Permeation assay

Permeation assays were conducted in the incubation medium. The incubation medium consisted of HBSS with calcium and magnesium (Gibco), supplemented with 0.1% FBS. Confluent monolayers of the HBMEC were incubated with 5 or 10 μM of each compound, or no addition (control), for 2h. The monolayer integrity was guaranteed by assessment of TEER and permeability. In the end, the media from the apical and basal compartments were collected and frozen at -80°C until analysis. Individual samples of the HBSS medium containing the compounds at the same concentration used in the experiment were incubated without HBMEC at 37 °C for 2h as a control.

Deproteinization of samples was adapted from a previous publication [23]. To 1 mL of cellular medium was added 139 μL of 50% formic acid, ascorbic acid (final concentration of 1 mM) and taxifolin as internal standard (final concentration of 9 μM). To the mixture, 2.5 mL of ACN was added dropwise to precipitate proteins, and the samples were vortexed before centrifugation at 3200 *g* for 15 min. The supernatant was removed and dried under centrifugal evaporation (Genevac EZ-2 plus; Genevac Ltd, Suffolk, UK). Samples were dissolved in 100 μL of water and analyzed by HPLC-MS.

The percentage of relative passage from the luminal to the abluminal compartments was calculated according to the equation:

% passage= (Amount of compound in the abluminal side) / (Amount in control incubation at 37 °C) x 100

HPLC-MS analysis

HPLC analysis of apical and basal media was conducted on a Shimadzu LC-2010 HT coupled with a LCMS-2020 quadrupole mass spectrometer fitted with an electro spray ionisation source (Shimadzu, Milton Keys, UK). A sample volume of 10 μL was injected and separations were achieved on a Zorbax Eclipse XDB-C18 (4.6x50 mm; 1.8 μm), operated at 30 °C. The mobile phase consisting in 0.5% (v/v) formic acid in water (solvent A) and 0.5% (v/v) formic acid in acetonitrile (solvent B), was used at a flow rate of 0.5 mL/min. The gradient started with 0% B to reach 30% B at 20 min, reaching 90% B at 25 min, maintaining the percentage for four minutes.

Functional assay of P-glycoprotein (P-gp)

Activity of P-gp was determined by measuring cellular accumulation of the P-gp substrate Rhodamine 123 (R123) expressed per mg of protein content [24, 25], and results are shown as fold-change compared to the respective control. In brief, HBMEC treated with bioavailable polyphenol metabolites were washed, and incubated for 1 h at 37 °C with Ringer–HEPES Buffer (pH 7.4) containing 10 mM R123. The solution was quickly removed, HBMEC were washed three times with PBS and solubilized in 0.1 M NaOH. R123 content was determined using a Polarstar Galaxy

fluorescent plate reader (BMG Labtechnologies; excitation at 505 nm, emission at 538 nm). Verapamil (1μM, 30 min pre-incubation) was used as a reference P-gp inhibitor for the positive control. Protein content was evaluated by the Bradford method [26] using Bio-Rad's Protein Assay reagent (Bio-Rad).

Immunofluorescence analysis of P-glycoprotein

For analysis of the P-gp expression in HBMEC, cells were fixed with 4% paraformaldehyde for 20 min. Cells were permeabilized with 0.2% Triton X-100, blocked with 3% BSA and incubated with primary antibodies anti-P-gp (1:50) overnight at 4°C. Incubation with secondary antibody Alexa 488 anti-mouse IgG (1:500) (Invitrogen) lasted for 2 h at room temperature. Nuclei were counterstained with DAPI. Between incubations cells were washed three times with PBS. Coverslips were mounted in Gel Mount (Biomeda, USA) and staining was examined using a Leica DFC 490 camera (Leica, Germany) adapted to an AxioScope.A1 microscope (Zeiss, Germany), ZEN 2012 (blue edition) software by Carl Zeiss Microscopy GmbH, 2011.

Statistical analysis

The results reported in this work are the averages of at least three independent experiments and are represented as the means ± SD. Differences amongst treatments were detected by analysis of variance with the Tukey HSD (honest significant difference) multiple comparison test (a = 0.05) using SigmaStat 3.10 (Systat).

RESULTS

Cytotoxicity of bioavailable compounds to human brain microvascular endothelial cells

The structure of the sulfated compounds synthesized as previously described is shown in Fig. 1. The synthesized compounds were evaluated for cytotoxicity in HBMEC (Fig. 2). Toxicity was evaluated at two concentrations, 5 and 10 μM, which are physiological concentrations found in plasma of humans, as previously determined for these compounds [13]. Quercetin, currently used in assays regarding the effect of (poly)phenols in *in vitro* cellular models, and previously tested for BBB permeability, [27] was also used as a control. Toxicity was measured in terms of cellular viability using CellTiter-Blue® kit, over a period of 24 h. Of the tested compounds only quercetin was toxic, inducing a decrease in cellular viability (Fig. 2). In addition, morphological alteration of brain endothelial cells was observed on the microscope, with the loss of endothelial integrity as a monolayer (data not shown). Further use of quercetin for testing protection against H_2O_2-induced damage and BBB permeability was therefore compromised. On the contrary, the other compounds tested were not toxic at these concentrations and can be safely used for further assays.

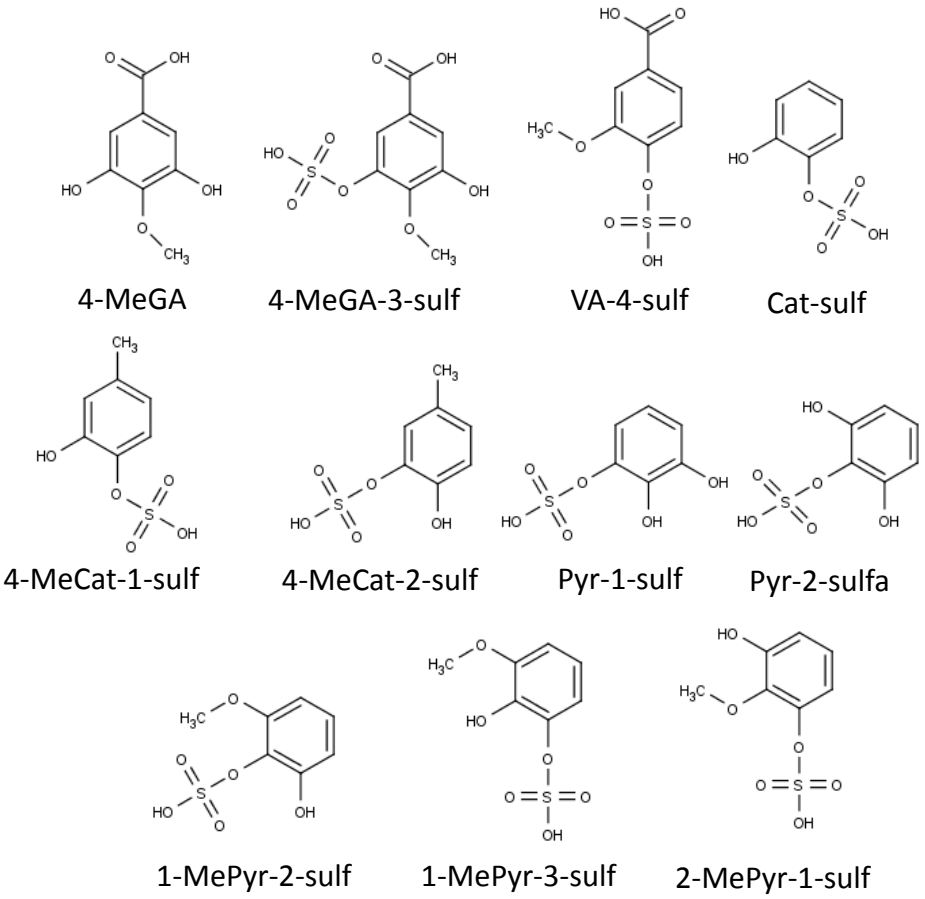

Figure 1. Structure of phenolic metabolites tested for transport across HBMEC: 4-MeGA, 4-methylgallic acid; 4-MeGA-3-sulf, 4-methylgallic acid-3-*O*-sulfate; VA-4-sulf, vanillic acid-4-*O*-sulfate; Cat-sulf, catechol-*O*-sulfate; 4-MeCat-sulf, 4-methylcatechol-*O*-sulfate; Pyr-1-sulf, pyrogallol-1-*O*-sulfate; Pyr-2-sulf, pyrogallol-2-*O*-sulfate; 1-MePyr-2-sulf, 1-methylpyrogallol-2-*O*-sulfate; 1-MePyr-3-sulf, 1-methylpyrogallol-3-*O*-sulfate; 2-MePyr-1-sulf, 2-methylpyrogallol-1-*O*-sulfate.

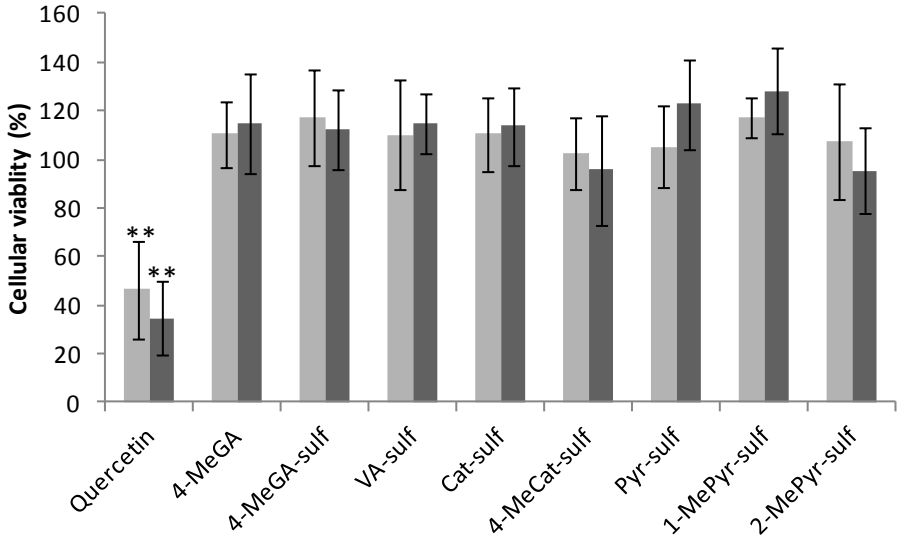

Figure 2. Evaluation of the toxicity of bioavailable polyphenol metabolites in HBMEC. Cell viability of HBMEC was assessed after 24 h of incubation with phenolic-conjugates or no addition (control). Two physiologically relevant concentrations were tested (▪5 and ▪10 μM) and cell viability is expressed as percentage relatively to control (100%). Statistical differences are denoted as ** for $p<0.01$ relatively to control. All values are means ± SD, n=3.

Cytoprotective effect of bioavailable compounds against H_2O_2 insult to human brain microvascular endothelial cells

To test the protection of the (poly)phenol-metabolites against H_2O_2 insult, viability of HBMEC was measured as previously. HBMEC were incubated with the individual compounds at a concentration of 5 μM for 24h prior to H_2O_2 insult (Fig. 3). Incubation of cells with H_2O_2 induced a loss of viability of nearly 70%, which was greatly reduced with a pre-incubation of the (poly)phenol-conjugates. Of these, 4-MeGA, Pyr-sulf, 2-MePyr-sulf and VA-sulf were able to increase the viability of cells to values very close to the control.

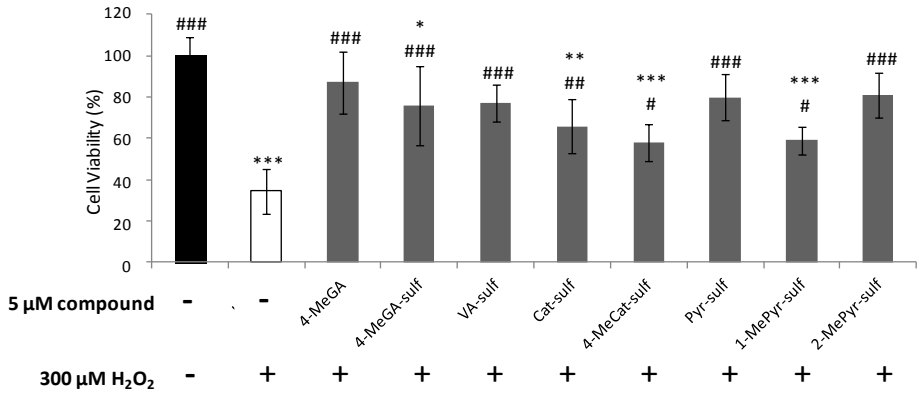

Figure 3**.** Cytoprotective effect of biovailable polyphenol metabolites in injured HBMEC. Cells were preincubated with 5µM of each bioavailable polyphenol metabolite, for 24h and then injured with 300 µM H_2O_2 for 24h. Cell viability is presented as percentage relatively to control. Statistical differences are denoted as *** for p<0.001, ** for p<0.01 and * for p<0.05 relatively to control and as ###p<0.001, ##p<0.01 and #<0.05 relatively to H_2O_2. All values are means ± SD, n=3.

Passage of bioavailable compounds across human brain microvascular endothelial cells

For testing the transport of the conjugated-(poly)phenols across confluent monolayers of HBMEC, the paracellular indicators TEER and permeability were monitored to assure the integrity of the monolayer, and, thus, guarantee that the passage of the metabolites was not due to a disruption of the barrier properties. TEER values for HBMEC treated with (poly)phenol-conjugates did not differ from those of untreated cells used as control, assuring that the tested compounds were not affecting the integrity of the barrier. Additionally, no significant differences were registered for the permeability to sodium fluorescein of HBMEC monolayers treated with the bioavailable compounds in comparison with the control, confirming that the barrier was intact.

The permeation efficiency of each (poly)phenol-conjugate across the barrier at the concentrations of 5 and 10 μM was evaluated 2 h after their addition to the luminal surface. The efficiency of transport was calculated by the relative percentage of compound that passed to the abluminal side in relation to the control without cells, incubated at 37 °C for 2h (Fig. 4). The passage efficiency was higher for the 2-MePyr-sulf and Pyr-sulf, respectively reaching 25% and 17.5% detection in "brain side" for the 10 μM concentration. A large difference in the permeation efficiency was registered between the two concentrations tested in both compounds, as well as for the 4-MeCat-sulf. More consistent values were observed for all the other compounds between the two concentrations. The smallest passage efficiency was observed for 4-MeGal, 4-MeGal-sulf and 1-MePyr-sulf (2%, 3% and 3%, respectively). These results indicate that compounds are able to cross the BBB, though with a small efficiency.

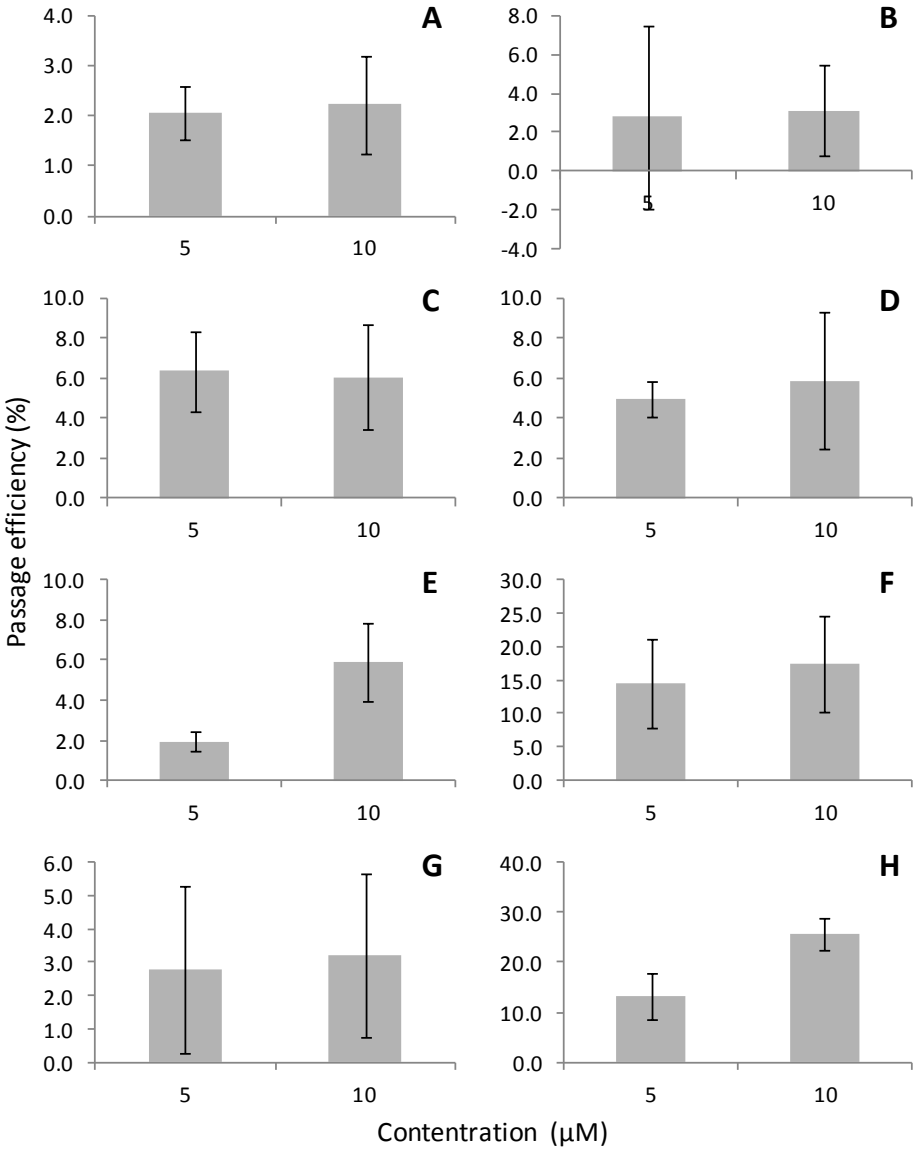

Figure 4. Percentage of passage efficiency of phenolic metabolites across HBMEC after 2h of incubation in a transwell system: (**A**) 4-MeGA, (**B**) 4-MeGA-sulf, (**C**) VA-sulf, (**D**) Cat-sulf, (**E**) 4-MeCat-sulf, (**F**) Pyr-sulf, (**G**) 1-MePyr-sulf, (**H**) 2-MePyr-sulf. Two different concentrations (5 and 10 μM) of each compound were tested and applied at luminal surface of HBMEC and the amount of each compound that reached the abluminal side after 2h was determined by LC-MS. The values are means of at least two independent experiments.

P-gp expression and activity in HBMEC

The presence of P-gp in HBMEC was confirmed by fluorescence microscopy (Supplementary Fig. 1). The results indicate that the cells are expressing the P-gp in normal conditions (Supplementary Fig. 1A) and are expressing as well when incubated with the (poly)phenol-conjugates (Supplementary Fig. 1B-I).

To assess if the presence of the (poly)phenol-conjugates affected the functional activity of P-gp in HBMEC, the uptake of R123 was measured in the presence of the compounds (Fig. 5). No significant differences were found in relation to the control when cells were incubated with the (poly)phenol-conjugates, whereas a much smaller retention of the P-gp substrate was observed for verapamil, a well-known P-gp inhibitor. These results suggest that poly)phenol-conjugates did not alter the P-gp activity.

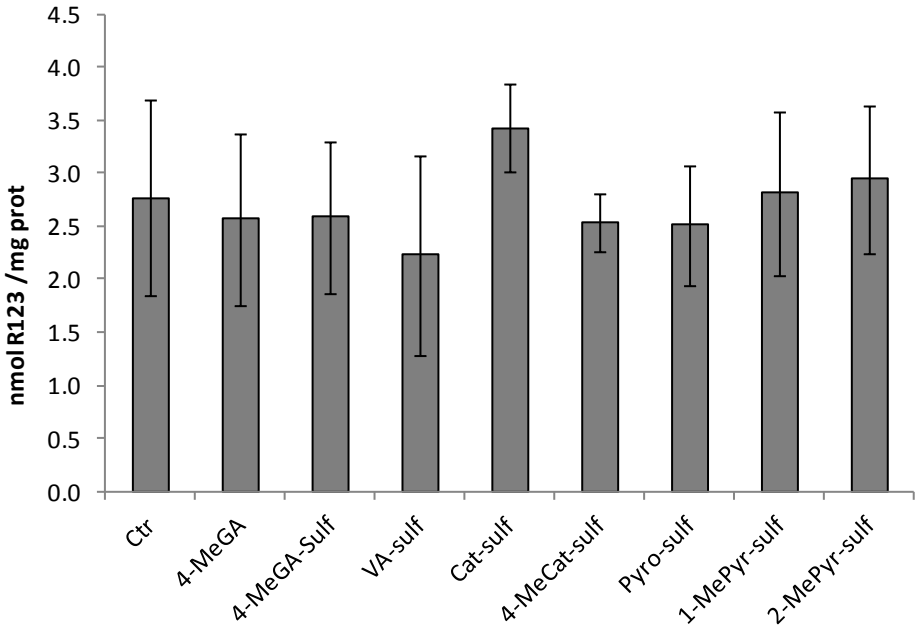

Figure 5. P-glycoprotein (P-gp) activity in HBMEC when incubated with the phenolic-conjugates. The evaluation of P-gp activity was measured by its ability to retain the substrate rhodamine 123 (R123). After 2h of treatment, cells were incubated for 30 min with 10 mM R123. Total protein extracts were collected, R123 accumulation fluorescent emission was determined and results were expressed per mg of protein. Values presented are means ±SD, n=5.

DISCUSSION

Brain endothelial cells are exposed to diverse physical and chemical stimuli, which can affect the integrity of the barrier [28]. Disruption of the BBB has been seen in various states of inflammation, neoplasia, infections (meningitis, encephalitis), trauma and Alzheimer disease [29, 30]. Compounds able to protect the BBB against chemical damage can become very important in prevention of several pathologies. Some (poly)phenols, and particularly flavonoids, have been used in experiments aimed at determining the ability of compounds to permeate the BBB and exert protective actions in the brain or in the BBB itself against chemical insults [31]. However, after ingestion, most flavonoids are found in plasma in conjugated form and in much smaller concentrations that the ingested amount, which renders difficulty in associating these compounds to the expected protective effects [32]. Nevertheless, we recently identified in urine several phenolic-conjugates after ingestion of a (poly)phenols-rich berry fruits puree [12]. These low molecular weight phenolic-conjugates, most of them originated from the colonic break down of more complex (poly)phenols, were also found and quantified in plasma [13] and are therefore promising candidates for the positive health effects associated with the consumption of berries.

Methylated and sulfated small phenolic-metabolites found in urine and plasma of human subjects were chemically synthesized. In the present study, the capacity of such compounds in preventing H_2O_2 damage in HBMEC and their ability to permeate the endothelium, together with P-gp

expression and activity were investigated in a simplified model of the BBB.

Before testing the protective effects of phenolic-conjugates from an oxidative insult, the toxicity of the compounds was evaluated in HBMEC, at two concentrations, 5 and 10 μM. These concentrations are considered to be very close to the physiological concentrations previously detected in plasma for such compounds [13]. The absence of toxicity for these compounds might be related to conjugated groups, methyl and sulfate. With a general function of making compounds less lipophilic and more easily excreted, conjugating reactions usually inactivate the initial compounds. However, in certain instances, "bioactivation" of compounds can occur, thus resulting in unstable metabolic products more toxic than the original [10]. Quercetin, which was toxic for HBMEC in the present study, had been previously reported to be able to permeate across the BBB effectively, despite restraining the proliferation of endothelial cells and inducing apoptosis [33]. Nevertheless, quercetin is not usually found unconjugated in the human body, but conjugated with methyl, sulfate and glucuronide groups [34].

Excluding toxicity in HBMEC from phenolic-conjugates, these were tested for protection of HBMEC against damage caused by H_2O_2 incubation. H_2O_2 has been observed to affect the integrity of the membrane, being able to increase its permeability when administered in lower concentrations (1 mM or less) and to induce a decrease in cell viability when administered at high concentrations (5 mM) [35]. All the phenolic-conjugates demonstrated, to a certain extent, the ability to protect cells against H_2O_2

insult. Previously, hydroxycinnamic acids and anthocyanins from blueberry and cranberry were able to protect HBMEC against H_2O_2 injury. However, the physiological relevance of these results was questioned due to the poor bioavailability of the mentioned compounds [36]. Here, we demonstrate that small phenolics resulting from the colonic degradation of more complex (poly)phenols and bio-conjugation in the human body are potentially able to exert protective effects as well.

For compounds to be able to exert protective effects in the brain they have to be able to cross the BBB and many dietary (poly)phenols have been tested for the ability to permeate this barrier [37]. The ability of phenolics to permeate the BBB was related to the lipophilicity of the compounds, being dependent on the functional and conjugated groups [38, 39]. The more hydroxyl groups and glycosides attached to the aglycones, generally result in less permeable compounds.

Phase II reactions in the human body are expected to make (poly)phenols more hydrophilic with lesser ability to cross the BBB. In fact, it was suggested that phenolic acid-conjugated metabolites from coffee ingestion have a very low ability to cross the BBB and, thus, do not reach the brain [40]. However, our results suggest that although in small concentrations, sulfated-phenolics can permeate the BBB. The same idea was obtained for glucuronidated-conjugates of flavanones, anthocyanins and flavanols [39]. Addition of a methyl group, on the other hand, was observed to increase the permeability in the BBB of compounds [41].

Despite the fact that conjugation of phenolic compounds can reduce their ability to penetrate the BBB, it is possible that they can also alter the brain

function through action at the BBB, and not requiring entering into the brain [42]. In the BBB, the P-gp has the physiological role of protecting the organism against exogenous toxic substances, by exporting these substances out of the tissue at the cost of ATP [43]. They are part of the efflux transport system in the body which eliminates endogenous and exogenous compounds from the body [44]. (Poly)phenols have been described to have inhibitory effects on the P-gp [45] and to affect the activity of the P-gp by interacting directly with it or indirectly by altering the plasma membrane properties of factors contributing to its activity [31]. In HBMEC, we observed that P-gp is expressed and functional, as determined by immunofluorescence analysis and by its ability to efflux R123. However, the phenolic-conjugates did not alter its activity.

In conclusion, this study focused on the protective effects of chemically synthesized bioavailable metabolites in humans from the ingestion of berry fruits. These compounds were able to protect HBMEC from chemical damage induced by hydrogen peroxide. In addition, such compounds were able to cross the HBMEC, although in small quantities, and therefore have the potential to exert protective effects in neural cells. On the other hand, the phenolic-conjugates did not alter the function of the P-gp.

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SUPPLEMENTARY MATERIAL

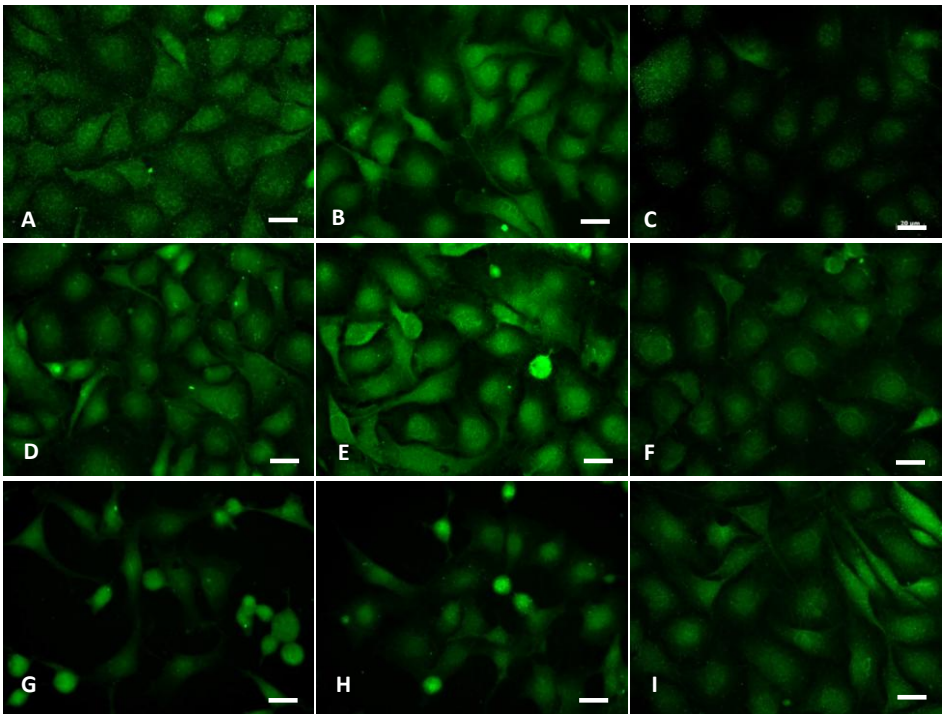

Figure 1. P-glycoprotein expression in HBMEC observed by immunofluorescence analysis: (**A**) control, **(B**) 4-MeGA, (**C**) 4-MeGA-sulf, (**D)** VA-sulf, (**E**) Cat-sulf, (**F**) 4-MeCat-sulf, (**G**) Pyr-sulf, (**H**) 1-MePyr-sulf, (**I**) 2-MePyr-sulf. Scale bar measures 20 μm.

Chapter 6

Discussion and future perspectives

The author of this thesis has written this entire chapter, based on the referred papers and his own results previously described.

Contents

DISCUSSION

(Poly)phenols have been consistently associated with beneficial effects of diets enriched in fruits and vegetables. These diets have been recommended to reduce the risk of developing chronic diseases such as cancer, cardiovascular disease, hypertension, stroke and type 2 diabetes [1]. However, the precise contribution of (poly)phenols is still unknown and there have been several conflicting reports; nevertheless, a great deal of research has been dedicated to the subject. Different approaches have been taken in order to clarify the effects that (poly)phenols might have in the human body, including epidemiological studies, *in vitro* tests and experimental studies on animals, as well as clinical and intervention trials in humans.

The difficulty of proving their effects is in part related to the fact that differences in the dietary content of (poly)phenols lead to subtle and chronic modulations of the metabolism and the health state of individuals and are often obscured by interindividual variability regarding the genetic background, environmental factors and lifestyle [2].

There is also great ambiguity regarding the active compounds in the human body, since recovery from biological samples of the main dietary (poly)phenols has been estimated to be mostly low [3]. Additionally, despite the fact that the absorption and the

concentrations of some (poly)phenols in blood are low, their ingestion is able to affect some biomarkers, suggesting that unknown metabolites are at least partly responsible for the effects [4]. This raises questions concerning the metabolic fate of dietary (poly)phenols and underlines the necessity of identifying the bioavailable metabolites from ingestion of (poly)phenols.

In this study, we aimed to identify some of the most abundant circulating metabolites after ingestion of dietary (poly)phenols, which may be responsible for the associated beneficial health effects. Having this objective in mind, the strategy was to firstly identify and quantify the compounds present in the berry fruits. These fruits were given to the human volunteers in the intervention study and urine samples were used for the screening and identification of the main bioavailable metabolites. Enzymatic and chemical synthesis of standards allowed confirmation of the identification of the metabolites and was used for the quantification in plasma as well as to disclose their possible effects at the level of the blood-brain barrier. Each study of this thesis contributed significant knowledge of the metabolism of dietary (poly)phenols in humans.

(Poly)phenols in berries

Berry fruits are considered one of the main components of healthy diets and constitute some of the richest sources of (poly)phenols, containing a great diversity of compounds [5]. As previously suggested [6], the creation of beverages containing a great diversity of (poly)phenols from multiple sources may be favourable for human health. Therefore, the selection of five berry fruits for the constitution of the fruit puree, to be given to the volunteers, was done to increase the diversity of compounds, strengthening, however, in specific groups of (poly)phenols such as anthocyanins, hydroxycinnamates, and gallic and ellagic acid derivatives (**Chapter II**). Although the content of (poly)phenols in commercial blueberries, blackberries and raspberries has been analysed in many previous publications, they were also analysed in this study since these might vary according to several factors such as the varieties, geographic region, storage conditions, ripeness and climate [5]. Additionally, Portuguese crowberry fruits were analysed in this study for the first time. These fruits and strawberry tree fruits, have a poor commercial exploitation, and can constitute novel sources of (poly)phenols for industry.

Due to the diversity of (poly)phenols in berries, identification and quantification of compounds can be laborious and slow and is limited by the availability of standard compounds. Quantification of

the aglycones after chemical hydrolysis of glycosidic residues has been a methodology previously used particularly for flavonoids or organic acids [7, 8]. In this study (**Chapter II**) we have used a combination of enzymes from *Aspergillus niger* for the hydrolysis of glycosides, and as well of the ester bonds with organic acids, as observed for 5-*O*-caffeoylquinic acid and 3-*O*-caffeoylquinic acid. This additional activity, possibly due to impurities in the commercial enzyme preparation, contributed to making this method an alternative for the commonly used methodology, mostly acidic or alkaline hydrolysis.

Although the identification of aglycones after hydrolysis simplifies the analysis of (poly)phenols in samples, it also has some disadvantages. It can result in an underestimation of some compounds if the hydrolysis is not complete and does not allow identification of all the individual compounds. Additionally, the information of the groups conjugated with the aglycones is lost, and these can be important, as they can influence absorption and metabolism of (poly)phenols in the human organism [9].

Bioavailability of (poly)phenols

Bioavailability is a key issue linking (poly)phenols and health effects [10].

Concerning the bioavailability of (poly)phenols from berries, the focus is usually on flavonoids, particularly anthocyanins, and ellagitannins as well [11]. However, berries comprise a great variety of (poly)phenols including phenolic acids and proanthocyanidins. During digestion, in addition to other events, the hydrolysis of the glycosides and organic acids from the (poly)phenol aglycones takes place [12]. This event is, in part, similar to what occurs *in vitro* during multienzyme hydrolysis with cellulase and hesperidinase (**Chapter II**), and therefore indicative of which metabolites in the berry mixture are most prone to be available for absorption (**Chapter III**).

Additionally, bioavailability studies involving berries, the focus often resides on the (poly)phenols present in the fruits. Only recently, more attention has been given to metabolites of colonic origin, as it was estimated that non-absorbed compounds in the small intestine that are able to reach the colon can go up to 90 to 95% of the ingested dose. In the colon these compounds can be degraded into simpler units by the colonic microbiota and still be absorbed into the blood stream [13]. Following absorption, a high percentage of compounds is found conjugated in plasma and urine samples. Based on this, an *in silico* library of potential bioavailable

metabolites was constructed and was used to search the (poly)phenol metabolites in urine samples of volunteers (**Chapter III**).

For the identification of (poly)phenol-metabolites in biological samples, several methods have been traditionally used. Tandem mass spectrometry following HPLC separation of metabolites has been a very useful tool; however, due to the lack of available standards, identification and quantification has been limited. Often, indirect quantification based on similar compounds or the parent aglycones has been the method of choice, despite some limitations in structural identification of compounds and correct estimation of concentration. Commonly, it is often applied together with the hydrolysis of phenolic conjugates using the enzymes β-glucuronidase and sulfatase. Nevertheless, by this method all information regarding the regioselectivity of conjugation is lost, and quantification might be impaired due to the low efficacy of the mentioned enzymes. In this thesis, enzymatic and chemical synthesis (**Chapters III** and **IV**, respectively) were used for standard production, allowing the identification and quantification of metabolites. SULTS and UGTS were obtained from ox and pig liver, providing the conjugation activity necessary for production of standards which allowed the confirmation of the metabolites in urine samples. Animal models can be of great utility for

comprehending the metabolism of (poly)phenols and the effects of the metabolites. However, differences in conjugating reactions and substrate rate specificity have been observed between mammalian species and therefore, the results obtained with animals cannot always be translated to humans [14].

In this thesis the aim has been to identify the most abundant bioavailable metabolites resulting from the ingestion of the berry puree. The presence of gallic and caffeic acid-derived metabolites in urine was expected, due to their high levels in the fruit puree (**Chapter III)**. Moreover, the other most relevant metabolites identified were estimated to result from colonic catabolism due to their later times of excretion. These simple phenols constituted the most abundant metabolites both in urine (relative quantification, **Chapter III**) as in plasma (absolute quantification, **Chapter IV**). However, it was not possible to confirm that some of the metabolites found in urine originated from the digestion of (poly)phenols from the berries since they were also present in plasma when the control breakfast without (poly)phenols was ingested (**Chapter IV**). This was the case for protocatechuic acid-*O*-sulfate and vanillic acid-*O*-sulfate, which have been previously derived from the ingestion of cyanidin [15]. 4-Methyl-catechol-*O*-sulfate was also not confirmed to be generated from the catabolism of berry (poly)phenols. Additionally, some metabolites found in the

urine of volunteers were not found in plasma or were present in levels under the limit of detection, such as glucuronidated metabolites and dihydrocaffeic and dihydroferulic acids-conjugates (**Chapter IV**). There are several possible explanations for these facts. Some compounds may remain in plasma for a short period of time and be rapidly excreted, having a considerable accumulation in urine over time but not being abundant in plasma. On the other hand, the metabolites originating in the colon generally have a later appearance in plasma, and for some of them later collection times (over 6 hours) would be needed to observe a relevant concentration increase in plasma. In the future, when performing intervention studies to trace the colonic metabolites, the collection of blood and urine samples should be performed at least until 48h. That would allow to find a the time at which the concentration of these metabolites will be higher and follow their concentration until minimum values after the ingestion of (poly)phenols.

Predicting the origin of colonic metabolites

The human colon is inhabited by more than 1000 species of bacteria, where the genera *Bacteroides* spp., *Clostridium* spp. and *Eubacterium* spp predominate. [4, 16]. These microbial communities use diet-derived and host-derived energy sources for growth and are able to perform a great range of biochemical transformations. Several reactions have

been described by the colonic microflora, such as hydrolysis of conjugated groups, backbone rupture and ring fissure, or the loss of functional groups such as methyl, methoxy, hydroxyl, and carboxylic acid, among others [2].

In the present work, although the formation of some phenolic-metabolites can be predicted to occur in the colon, it was not possible to confirm exactly which are the parental compounds. However, there has been an increase of studies on the formation of colonic catabolites from (poly)phenols which can help to predict the formation of such metabolites. Experiments where (poly)phenols are incubated with faecal matter recovered from humans are providing important information on the colonic microbial metabolism. It has been demonstrated that the microbiota can metabolize the non-absorbed phenolics that reach the colon and even recover and metabolize non-extractable phenolics extensively bound to plant polysaccharides, such as ferulic acid [17]. Compounds that are conjugated either with organic acids or with glycosides can be de-conjugated by the microbiota, extending the hydrolysis catalysed by the small intestine epithelium. That is the case for some major compounds in the berry fruit puree, such as anthocyanins and caffeoylquinic acids, which generate, respectively, anthocyanidins and caffeic acid [18, 19]. Anthocyanidins are very unstable and readily break down into different simpler phenolics

depending on the structure of the parental anthocyanidins [20]. Cyanidin results mainly in protocatechuic acid, which we found conjugated in human urine and plasma, and other small phenolics such as phloroglucinaldeyde and several hydroxybenzoic and hydroxyphenylacetic acid-metabolites [15, 21]. Other anthocyanidins can produce a great variety of compounds, some of them found in this study as metabolites, such as gallic, vanillic, caffeic, ferulic, isoferulic dihydrocaffeic and dihydroferulic acids [22]. Caffeic acid, partially resulting from hydrolysis of caffeoylquinic acids, and ferulic acid, initially detected in the puree, but also possibly resulting from the catabolism of other compounds, are described to be metabolized by the microbiota mainly into dihydrocaffeic and dihydroferulic acids, respectively [19]. Other minor catabolic routes can produce several additional metabolites such as (hydroxyphenyl)-propionic acids, protocatechuic acid, and other phenylacetic and benzoic acid-derivatives [19, 23]. Hydroxybenzoic acids such as gallic acid, the main compound detected in the fruit puree, and protocatechuic acid are decarboxylated into pyrogallol and catechol, respectively [19, 20]. Although in other studies these catabolites were described as minor compounds in human samples, in this study, they might be considered the precursors of the most abundant metabolites in urine and plasma of volunteers (**Chapters III and IV**).

Other (poly)phenols initially present in fruits, such as flavonols and flavanols, are degraded by the colonic microbiota into a diversity of phenolic metabolites comprising hydroxyphenylacetic acids, hydroxybenzoic acids, hydroxyphenylpropionic acids and aldehydes, that ultimately can be further metabolised into pyrogallol and catechol as well [20, 24, 25]

The importance of phase II conjugating **reactions**

The result of this work confirms the importance of conjugating reactions in the metabolism of (poly)phenols in humans. All the major metabolites identified in urine were in conjugated form, either with sulfate, glucuronide or methyl groups (**Chapter III**). These reactions can function not only to inactivate the metabolites but as well to increase hydrophilicity and facilitate the elimination of (poly)phenol metabolites as well as other xenobiotics and endogenous compounds. Additionally, conjugation increases the molecular weight of metabolites and facilitates biliary excretion [26].

In the urine samples sulfated compounds were always relatively more abundant than glucuronidated metabolites (**Chapter III**) and in plasma only sulfated metabolites were quantifiable (**Chapter IV**). This suggests that the metabolites here identified are preferrentially sulfated rather than glucuronidated. The same does not occur for other compounds such as quercetin [27], and for approximate 90% of

drugs requiring hepatic clearance out of the body, where glucuronidation is the major conjugation pathway [14]. This may be due to the substrate specificity of the conjugating enzymes.

However, for ingested compounds the hepatic clearance pathway may not be the only predominant route, and extra-hepatic conjugation should not be discounted. The intestine may be an important site of conjugation as well since many conjugating enzymes are expressed in this organ [27]. Moreover, it has been reported that catecholic compounds are poorly glucuronidated [28] and sulfation may be the predominant pathway, as previously determined for hydroxycinnamic acids [29]. In addition to sulfation, methylation is also an important conjugating reaction. Many of the metabolites reported here, in addition to being sulfated or glucuronidated, were also methylated. This dual conjugation has been described as a major conjugation pathway of catecholic drugs [30] and (poly)phenols such as quercetin [31], and epicatechin [32]. In this thesis (**Chapter III),** we consider that methylation might have occurred on gallic, protocatechuic, caffeic, and dihydrocaffeic acids, as well as on pyrogallol.

Regioselectivity appears to be very important in conjugating reactions for compounds with more than one conjugation site. Many of the compounds here reported are conjugated preferentially at a certain position, as for the examples of gallic acid, which was

found methylated in the 4-hydroxyl and sulphated in the 3-hydroxyl groups, and caffeic, dihydrocaffeic and protocatechuic acids which were preferentially methylated in the 3-hydroxyl group. In addition to regioselectivity, substrate specificity has also been described among different isoforms of conjugating enzymes. The SULT1A1 isoform has been reported as the main enzyme responsible for xenobiotic sulfation due to its wide range of substrate acceptability and high abundance in hepatic tissue. However other isoforms are more specific for certain substrates such as SULT1A3, which preferably sulfates catecholamines and SULT1E1 with higher affinity for estrogens [33]. However, no information of the enzyme isoforms which preferably conjugate the (poly)phenol metabolites nor their location was obtained in this study. Comprehension of the substrate specificity and regioselectivity of the conjugatiing enzymes as well as the sites of conjugation is essential for the prediction of metabolism of (poly)phenolic compounds.

Variability between subjects

In the intervention studies performed in this work, a great variability was observed in the metabolism of (poly)phenols between the different human subjects for both in urine (**Chapter III**) and plasma samples (**Chapter IV**). This variability was reflected in

the amount of each metabolite at a certain time point in the biological samples, and also in the metabolites produced, since not all the metabolites were present in each volunteer. A high variability between subjects is common in human studies. It can be explained by several factors such as genetic background, environmental factors and lifestyle. Additionally, there is genetic variability in the conjugatingenzymes between the volunteers, as interindividual variation in enzyme expression has been documented for SULT isoforms [33] and could occur for the other enzymes. Several other factors can also influence the expression and activity of the conjugation enzymes, such as the gender, age and physiopathological conditions, and also single or repetitive exposures to xenobiotics, which can induce or inhibit the enzymes [34]. In addition, variability between subjects can also exist in expression of phase III transporters which eliminate the compounds out of the body. P-gp for example, has broad substrate specificity and is of great importance in limiting the transcellular transport of xenobiotics [35]. The expression of several phase III transporters can also be induced by xenobiotics and therefore varies with individual diets or drug ingestion [36].

Furthermore, these differences can also be caused by variability of the colonic microbiota between subjects. All individuals have a unique signature of intestinal microbiota, and the different bacteria

species can metabolize (poly)phenols in different ways. On the other hand, (poly)phenols also have the ability to modulate the microbiota. Some compounds can inhibit the growth of all or only some bacterial species and therefore have the potential to affect (poly)phenol metabolism. In fact, the diet of each individual can affect its colonic microbiota by modulating its environment and nutrient supply [37].

Based on the results obtained from **Chapters II, III and IV**, and the literature, the general scheme can be proposed for the bioavailability of (poly)phenols from the berries puree (fig 1).

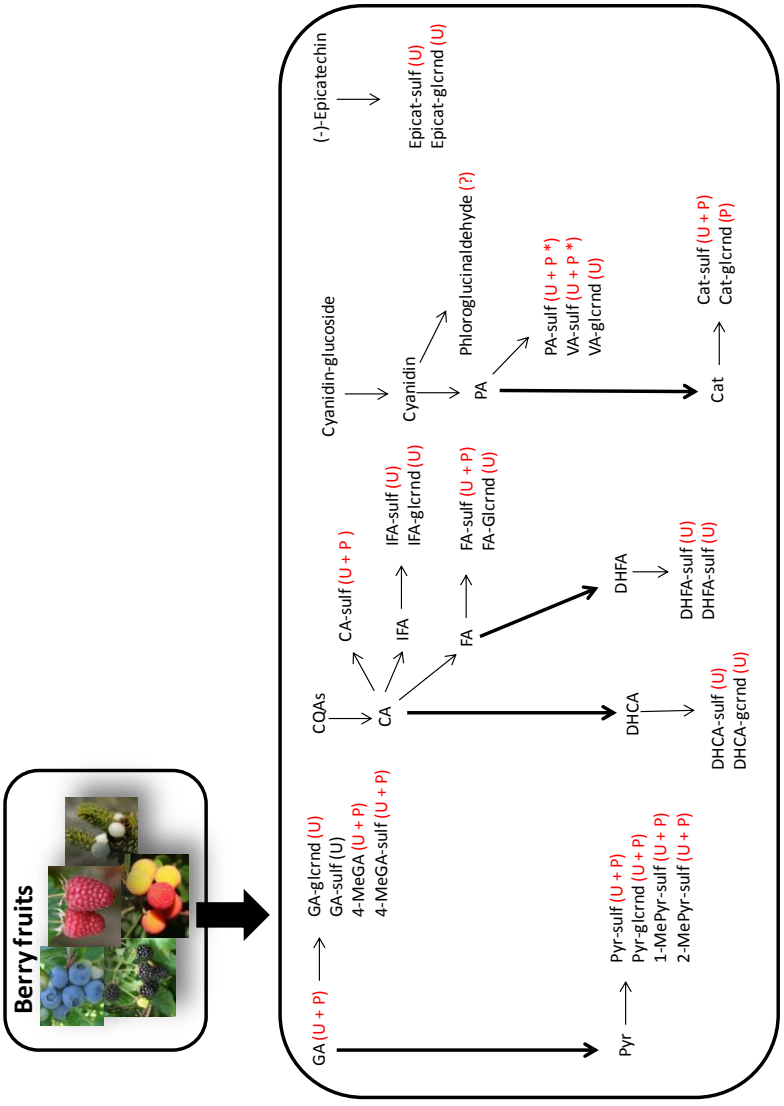

Figure 1. Proposed metabolic pathway of (poly)phenols following the ingestion of berry puree by human volunteers. The identified metabolites urine (U) or plasma (P) are conjugated with methyl, sulfate (sulf) or glucuronide (glcrnd) groups. Bold arrows indicate colonic catabolism. * represents the metabolites which were not confirmed as resulting from the metabolism of (poly)phenols.

Activity of phenolic-conjugates

The effects of (poly)phenols in humans are a function of the nature of the compound, and other contributing factors which include the concentration in the diet, the amount consumed, the transformations in the gastrointestinal tract, absorption, conjugation and excretion [38]. The biological effects attributed to (poly)phenols are due, in large part, to the plethora of bioactive phenolic molecules available in nature, comprising a great variety of chemical structures. This diversity, despite being the basis of the effects of (poly)phenols, makes their study and comprehension difficult. In addition, changes in their structure occurring during food processing, digestion, and also conjugation in the body must also be taken into account.

The biological activities of phenolic-conjugates have not been extensively evaluated due to the difficulty of identification and the lack of available standards.

In this thesis, we intended to identify the phenolic metabolites circulating in the blood and determine their physiological concentration (**Chapter IV**). These metabolites are the most likely to reach the target tissues and exert a biological activity.

Normally, conjugating reactions result in metabolites less biologically active than the parent compounds, thus serving as a detoxification mechanism. However, the opposite might also occur,

and the resulting conjugated metabolites might be more reactive, having higher pharmacological activity or toxicity [14]. Additionally, conjugated compounds being generally more hydrophilic than the parent compounds can easily travel in the blood flow and thus reach the body tissues.

Several studies have mentioned the neuroprotective effect of (poly)phenols either preventing or slowing the progression of neurodegenerative pathologies and the degenerative effect of ageing or contributing to an improvement in cognitive performance [39]. A direct antioxidant effect of (poly)phenols in the brain as commonly been suggested by researchers. However, most *in vitro* studies were performed with plant extracts or (poly)phenol aglycones and the bioavailable conjugated metabolites were usually disregarded. Moreover, the antioxidant effect of (poly)phenols has only been proved *in vitro* and it is thought that the bioavailble (poly)phenol-conjugated metabolites are poor antioxidants due to the loss of antioxidant activity conferred by the conjugation, but also due to the low concentration at which they are present in the blood [40].

Some studies have demonstrated that (poly)phenols can in fact enter the brain, although this ability is dependent on their structure, in particularly their lipophilicity. In this thesis, we demonstrated that the small phenolic-conjugates present in the blood of

volunteers, have a low permeability across the BBB. Of the evaluated metabolites, the transport across the BBB ranged from 2% to 25% of the initial amount, suggesting a limited entrance in the brain (**Chapter V**).

However, when considering the effects of (poly)phenols in the brain, they do not necessarily have to enter the organ to exert an effect. Instead, they can act outside the central nervous system by improving the cerebral blood flow or acting on the communication between peripheral organs and the brain, or even act at the level of the BBB [41]. In fact, in this thesis we demonstrated that conjugated-metabolites of (poly)phenols can have a protective action against an oxidative stress such as hydrogen peroxide at the level of the endothelial cells constituting the BBB (**Chapter V**).

FUTURE PERSPECTIVES

The comprehension of the (poly)phenols effects in the human health passes through the identification of the bioavailable metabolites that circulate in the blood flow and are able to reach the target tissues. In this thesis we were able to identify some of the bioavailable metabolites from the ingestion of berry fruits.

In the future the focus should be on identification of novel metabolites present in human biological samples. Despite the great diversity of (poly)phenols in foods and beverages only a small

portion of metabolites have been thoroughly studied in terms of the digestive process in humans, and even for those there is still a lot to comprehend.

There are several approaches to reach this goal and a combined action is probably the best methodology. Non-targeted approaches can be used to scan and tentatively identify the metabolites in human biological samples. Targeted approaches can be used subsequently for a precise identification of the metabolites and to trace the parental compounds.

Only being in its early days, the study of colonic microbiota and their involvement in (poly)phenols metabolism in humans has a great exploratory potential. Studies suggest a great importance of colonic metabolism in the host wellbeing. Understanding of the evolution of the gut microbiome can provide important clues on the genesis of some metabolic diseases such as obesity and inflammatory bowel disease. Food has the ability to influence the ecology of the microbiota and changes in microbial communities are rapidly observed when there is a variation in the feeding pattern of the host. (Poly)phenols can greatly influence the microbial community and the inverse is verified as well, with the microbiota determining the (poly)phenol catabolites produced in the colon. It is imperative to understand the phenolic metabolites generated in the colon and trace their parental compounds. This can be achieved by

performing studies using isotopic labelled (poly)phenols and tracing and identifying the labelled metabolites generated from their breakdown.

In addition, it will be also important to comprehend which are the microbial species which contribute for the catabolism of (poly)phenols in the colon and how they are affected by the different compounds and changes of diet.

In the future, the comprehension of the impacts of food on the microbiota might lead to the use of a personalized nutrition for a better regulation of the individual microbial community and with benefits for health.

In this thesis, it was also relevant to study the importance of conjugating reactions for the metabolism of (poly)phenols in humans. However there is still a long way for a complete understanding of these processes.

In vitro cellular models can be used to study the absorption and metabolic mechanisms in the gastrointestinal tract. Characterizing the regioselectivity of the conjugating enzymes would provide important insights into enzymatic mechanisms with respect to substrate binding and selectivity. The understanding of regioselectivity of SULTs and UGTs would allow prediction of the site(s) of sulfation and glucuronidation on (poly)phenol-metabolites and identification of the compounds likely to be formed *in vivo*. The

comprehension of the conjugating metabolism of (poly)phenols would also help to predict the impact of food on the metabolism of pharmaceutical drugs.

The discovery of the bioavailable metabolites from the ingestion of (poly)phenols and the chemical synthesis of these compounds enables study of their effects on human health. The effect on the protection at the level of the BBB, demonstrated in this thesis, opens the possibility of using these compounds for reducing the risk of developing neurodegenerative pathologies, and more studies will be needed to verify these effects. The use of *in vitro* disease models would allow testing the activity of these compounds in specific points of the pathological process. If results are favourable, they can be used clinical studies in humans or animals to further test the protective effect of (poly)phenol metabolites.

Finally, there is the possibility of using the identified compounds as biomarkers of the intake of (poly)phenols in humans. The use of biomarkers facilitates the assessment of an accurate estimation of (poly)phenols ingestion, which is essential for a correct association between consumption and the effects of (poly)phenols, determined in epidemiological studies. For that, it is imperative to see if the bioavailable metabolites are specific of (poly)phenols ingestion and sensitive to changes in the consumed amount. These can be achieved by performing intervention studies with specific groups of

(poly)phenols and evaluate the bioavailble metabolites on a large sample of humans, representing of the whole population, and testing differences of dosage.

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