CHARLES UNIVERSITY

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY

Sulfates as phase II metabolites of natural phenolic compounds

DIPLOMA THESIS

Supervisor: Assoc. Prof. Přemysl Mladěnka, PharmD., Ph.D.

Co-supervison: Assoc. Prof. Ing. Kateřina Valentová, Ph.D.

Hradec Králové 2019 Anastasiia Hetman

DECLARATION

Hereby, I declare that this paper is my own work. All the literature and other sources of information I used in this work are mentioned in the list of literature and properly cited throughout the work. This work has not been used to gain equal or different degree.

Anastasiia Hetman

Acknowledgment

My great thanks belong to Assoc. Prof. Ing. Kateřina Valentová, Ph.D. for her consultations, support, and guidance through this research and to Assoc. Prof. Přemysl Mladěnka, PharmD., Ph.D. for his supervision and valuable advice. I would also like to thank prof. Ing. Vladimír Křen, DrSc. for allowing me to work on this thesis at the Laboratory of biotransformation, Institute of Microbiology of the CAS (IM CAS). I am also grateful to Ing. Lucie Petrásková, PhD. for HPLC and MS analyses she provided, and to RNDr. Helena Pelantová, Ph.D. (Laboratory of molecular structure characterization of IM CAS) for NMR analyses. I would also like to thank Pavel Vávra, Ing. Kristýna Káňová, Dr. Christopher Chambers and all the members of the laboratory for their help and advice during this research.

Last but not least I would like to thank Daniel and my family for their support through my studies.

Abstrakt

Univerzita Karlova
Farmaceutická fakulta v Hradci Králové
Katedra farmakologie a toxikologie
Student/ka: Anastasiia Hetman
Školitel: doc. PharmDr. Přemysl Mladěnka, Ph.D.
Školitel specialista: doc. Ing. Kateřina Valentová, Ph.D.
Název diplomové práce: Sulfáty jako metabolity 2. fáze biotransformace přírodních fenolových látek

Přírodní monofenolové látky, které se vyskytují v rostlinách a také vznikají při metabolismu polyfenolů střevní mikrobiotou, jsou absorbovány a procházejí 2. fází biotransformace. To má za následek tvorbu různých konjugovaných metabolitů, jako jsou sulfáty, jejichž biologická aktivita nebyla dosud studována. Tato práce byla zaměřena na přípravu a izolaci čistých sulfatovaných metabolitů fenolových látek, a to 4-methylkatecholu, protokatechové, homoprotokatechové, a 2,3,4-trihydroxybenzoové kyseliny a floroglucinolu za použití arylsulfotransferázy z *Desulfitobacterium hafniense*. Byly připraveny dva sulfatované produkty v dostatečné čistotě, a to 4-methylkatechol-*O*-sulfát (nedělitelná směs 4-methylkatechol-1-*O*-sulfátu a 4-methylkatechol-2-*O*-sulfátu) a floroglucinol-*O*-sulfát. Jejich antioxidační aktivita byla porovnána s aktivitou výchozích látek pomocí následujících šesti *in vitro* testů: zhášení DPPH (1-difenyl-2-pikrylhydrazyl) a ABTS (kyselina 2,2'-azinobis- (3-ethylbenzothiazolin-6-sulfonová)) radikálů, schopnost redukce Folin-Ciocalteauova činidla, železitých iontů a měďnatých iontů a schopnost inhibice lipoperoxidace potkaních jaterních mikrosomů vyvolané *terc*-butylhydroperoxidem. Sulfatace fenolových sloučenin většinou vedla ke ztrátě jejich antioxidační aktivity. Sulfatované produkty byly charakterizovány pomocí HPLC, MS a NMR analýz.

Abstract

Charles University

Faculty of Pharmacy in Hradec Králové

Department of Pharmacology & Toxicology

Student: Anastasiia Hetman

Supervisor: Assoc. Prof. Přemysl Mladěnka, PharmD., Ph.D.

Co-supervisor: Assoc. Prof. Kateřina Valentová, Ph.D.

Title of diploma thesis: Sulfates as phase II metabolites of natural phenolic compounds

Natural monophenolic compounds, which both occur in plants and are formed during gut microbiota metabolism of polyphenols, are absorbed and pass through phase II metabolism. This results in the formation of various conjugated metabolites, such as sulfates, whose biological activity was not studied as yet. This work focused on the preparation and isolation of pure sulfated metabolites of phenolic compounds, namely 4-methylcatechol, protocatechuic, homoprotocatechuic, and 2,3,4-trihydroxybenzoic acid, and phloroglucinol, using the aryl sulphotransferase from *Desulfitobacterium hafniense*. As a result, two sulfated products of sufficient purity were prepared, namely 4-methylcatechol-*O*-sulfate (an inseparable mixture of 4-methylcatechol-2-*O*-sulfate and 4-methylcatechol-1-O-sulfate) and phloroglucinol-*O*-sulfate. Their antioxidantactivity was evaluated and compared with the activity of the parent compounds using the following six *in vitro* tests: DPPH (1-diphenyl-2-picrylhydrazyl) and ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging, ability to reduce Folin-Ciocalteau reagent, ferric and copper ions, and the ability to inhibit the lipoperoxidation of rat liver microsomes damaged by *tert*-butylhydroperoxide. Sulfation of phenolic compounds led mostly to the loss of their antioxidant activity. The sulfated products were characterized by HPLC, MS and NMR analyses.

Table of Contents

1 List of abbreviations

ABTS	2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
AST	aryl sulfotransferases
CUPRAC	cupric reducing antioxidant capacity
DMSO	dimethylsulfoxide
DPPH	1,1-difenyl-2-picrylhydrazyle
FCR	Folin-Ciocalteau reduction
FRAP	ferric reducting antioxidant potential
GAE	gallic acid equivalents
GSH	glutathione
HPCA	homoprotocatechuic acid; 3,4-dihydroxyphenylacetic acid
IC_{50}	the concentration of the tested compound at which 50% of the measured parameter was inhibited
IM CAS	Institute of Microbiology of the CAS
MC	4-methylcatechol
MCS	4-methylcatechol-1-*O*-sulfate and 4-methylcatechol-2-*O*-sulfate (in ratio 64:36)
MRP	multidrug resistance-associated proteins
MS	mass spectrometry
NAPQI	*N*-acetyl-*p*-benzoquinone imine
NMR	nuclear magnetic resonance
Nrf2	nuclear erythroid-related factor 2
PAP	3′-phosphoadenosine-5′-phosphate
PAPS	3′-phosphoadenosine-5′-phosphosulfate
PBS	phosphate buffer solution
PCA	protocatechuic (3,4-dihydroxybenzoic) acid
PCAS	protocatechuic acid-2-O-sulfate and protocatechuic acid-4-*O*-sulfate mixture (in ratio 7:3)
PGS	phloroglucinol-*O*-sulfate
p-NP	*para*-nitrophenole
p-NPS	*para*-nitrophenyl sulfate
SULT	cytosolic sulfotransferase

TBA	thiobarbituric acid
TCA	trichloroacetic acid
TEAC	trolox equivalent antioxidant capacity
THBA	2,3,4-trihydroxybenzoic acid
THBAS	the mixture of two sulfated products of 2,3,4-trihydroxybenzoic acid (monosulfate and another sulfated product, it was not possible to determine exact structures)
TLC	thin layer chromatography

2 Introduction

The role of polyphenols in human health has become a progressively important area of research. Intake of dietary polyphenols is often associated with numerous health benefits, such as prevention of cardiovascular diseases, reducing the cancer risk etc. (Williamson, 2017).

Since the bioavailability of polyphenols is considered to be relatively low, a significant amount of them reaches the distal parts of the intestinal tract, where they are catabolized by gut microbiota into a wide range of phenolic catabolites. Small phenolic compounds are present in various plants and food as well. Some authors propose that small phenolic compounds derived from polyphenol microbial metabolism could be responsible for a considerable part of the health benefits derived from the consumption of food rich in polyphenols (Williamson and Clifford, 2010). During absorption in the digestive tract and then in the liver phenolic compounds undergo mostly phase II metabolism, which results in the formation of sulfated, glucuronidated and methylated conjugates (Pimpao et al. 2014). The biological activity of these conjugated phenolic compounds is barely studied. Therefore, there is a need to prepare pure sulfated phenolic compounds for further studies. Preparation of sulfates is also important since they serve as authentic standards for metabolic studies.

Thus, this work is focused on chemoenzymatic preparation and isolation of pure sulfated metabolites of phenolic compounds, namely 4-methylcatechol, protocatechuic, homoprotocatechuic, and 2,3,4-trihydroxybenzoic acid, and phloroglucinol, using the aryl sulfotransferase from *Desulfitobacterium hafniense*, which appeared in previous studies to be an excellent tool for polyphenol sulfation (Purchartová et al. 2015; van der Horst et al. 2012). The antioxidant activity of the sulfated phenolic metabolites was evaluated and compared with the parent compounds in order to determine the impact of sulfation on the antioxidant activity.

3 Theoretical background

3.1 Polyphenols

Polyphenols are one of the largest group of secondary plant metabolites, which play a significant role in plant physiology, including the effect on their growth, reproduction, defense against predators or pathogens, and signaling function. A lot of polyphenols are present in the human diet, and they are responsible for a wide array of beneficial effects on human health.

3.1.1 Definition

There are several definitions of polyphenols, one of the most recent states: "The term "polyphenol" should be used to define plant secondary metabolites derived exclusively from the shikimate-derived phenylpropanoid and/or the polyketide pathway(s), featuring more than one phenolic ring and being devoid of any nitrogen-based functional group in their most basic structural expression" (Quideau et al. 2011). This definition does not include monophenolic structures such as phenolic acids, which are metabolites of polyphenols or precursors of polyphenolic substances. Despite this definition, there are numerous publications where monophenolic substances are considered as polyphenols (sometimes written (poly)phenols). Thus, 'polyphenol' is not a strict chemical term and it includes flavonoids (e.g. some of them have none or a single hydroxylgroup), phenolic acids, tannins and polymerized compounds (Williamson, 2017).

3.1.2 Classification

Over 8000 polyphenolic substances were discovered to date, 4000 of which are flavonoids. Several attempts have been made at the categorization of polyphenols. Different approaches classify these substances by their source, biological function, or chemical structure. The majority of polyphenols, like flavonols, flavanones, and anthocyanins, occur as glycosides, with one or more sugar moieties linked in majority of cases to the basic core via oxygen atoms (Tsao, 2010).

Generally, polyphenols can be divided into two broad groups. The first one is formed by non-flavonoids such as hydroxybenzoic, cinnamic, and chlorogenic acids, hydrolyzable tannins, coumarins, stilbenoids, lignans, and others. The second group is the

flavonoid group, which includes flavanols or catechins, flavanones, flavones, anthocyanins, isoflavones, and proanthocyanidins. The basic structure of flavonoids consists of a phenylchroman (flavan) structure, which includes two phenyl rings A and B, and an oxygen-containing heterocyclic ring C (**Fig. 1**). This basic structure is modified by methoxy, hydroxy and alkyl groups that are bound to the A- and B- rings. In most cases, as mentioned, flavonoids occur in the form of *O*-glycosides or less frequently as *C*-glycosides. However, some flavonoids exist in the form of oligomeric and polymeric structures, such as the proanthocyanidins (Braune and Blaut, 2016). Sometimes, a third group, consisting of substances isolated from processed food and beverages (coffee, wine) and thus not occurring in intact plants is considered (Williamson and Clifford, 2010).

3.1.3 Polyphenols in the human diet

Many polyphenols are present in the human diet. They are found in plant-based foods like berries, apples, citrus fruits, broccoli, nuts, cereals and many others. These foods contain a complex mixture of polyphenols difficult to characterize (Crozier et al. 2009). Some polyphenols are ubiquitous, such as quercetin, which is found in many fruits and vegetables. Others are specific to a particular food or herb like phloridzin in apples or isoflavones in legumes from the Fabaceae family. *Vaccinium myrtillus* (blueberry) and other berries are known for their high content of flavonoids, particularly anthocyanidins, which are responsible for the pigmentation of the fruit (Veitch, 2007; Routray and Orsat, 2011).

3.1.4 Biological activities

The data from epidemiological studies and meta-analyses suggest that long-term consumption of polyphenol-rich diet have a beneficial effect on human health. It protects against several chronic diseases such as diabetes, neurodegenerative diseases, cardiovascular diseases, and cancer (Arts and Hollman, 2005; Williamson, 2017). However, the exact mechanism of action responsible for this preventive action remains to be discovered. Moreover, the evidence of beneficial effect for specific vegetables, and therefore specific polyphenols is low. Therefore, there is a recommendation to eat as large variety of plant food as possible (Crozier et al. 2009).

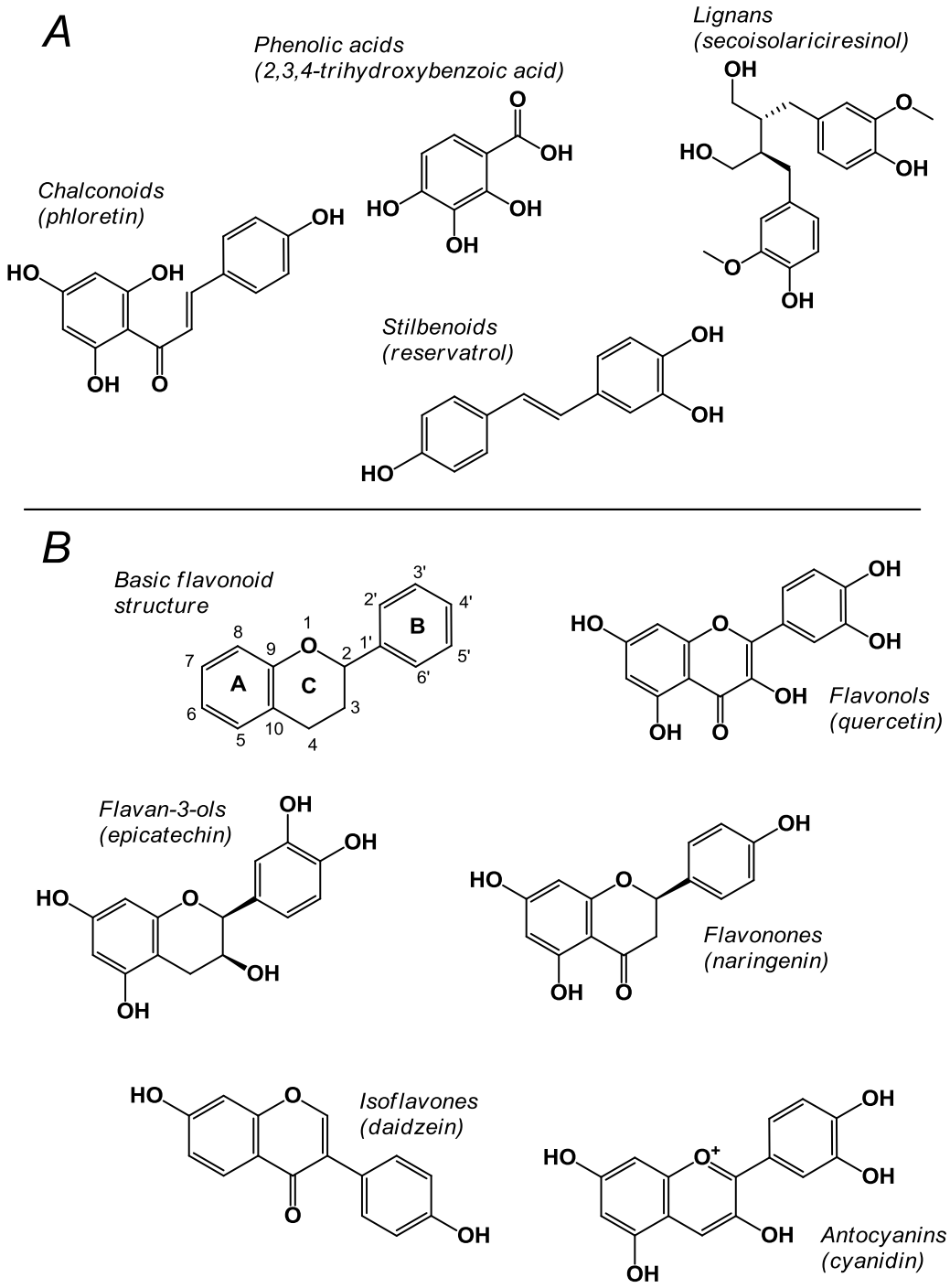


Fig. 1 *Classification of polyphenols: (A) non-flavonoids (stilbenoids, chalconoids, lignans, and phenolic acids), (B) flavonoids (flavanols or catechins, flavanones, flavones, anthocyanins, isoflavones, and proanthocyanidins).*

The antioxidant activities of many flavonoids are stronger than those of vitamins C and E. They act as antioxidants by several mechanisms. The scavenging of reactive oxygen species and metal chelating activity play a role in preventing cell injury caused by free radicals (Procházková et al. 2011). Nevertheless, in the last decade polyphenols got more attention not for their antioxidant activity but first of all for their property to

directly bind to various target proteins. Thus, flavonoids inhibit some radical forming enzymes like lipooxygenase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, decrease platelet aggregation, and leukocyte adhesion. The inhibition of cyclooxygenases was also described. The above-mentioned mechanisms of action may be responsible for protective effects against cardiovascular and neurodegenerative diseases, as well as cancer and diabetes probably in a larger extent than just antioxidant properties of polyphenols (Mladěnka et al. 2010; Quideau et al. 2011).

Polyphenols are the main constituents of many medicinal herbs used in therapy. For instance, isoflavonoids from *Trifolium pratense* and *Glycine max* plants are applied for mitigation of menopausal symptoms. Isolated flavonoids diosmin, rutin or hesperidin have found their usage generally in the treatment of venous diseases. Standardized extracts from *Silybi mariani fructus* consisting of different flavonoids such as silybin, isosilybin, and others are used as a hepatoprotective drug in the treatment of hepatic diseases (Abenavoli et al. 2010).

3.1.5 Pharmacokinetic properties of polyphenols

In general, the bioavailability of most polyphenols in their parent forms is relatively low. However, many factors, such as lipophilicity, molecular weight, food matrix, the presence of different nutrients, etc. can influence it. Low bioavailability is probably caused by their physico-chemical properties, which are the base for limited absorption and strong first pass effect (**Fig. 2**).

It is generally considered that the absorption of polyphenols in the digestive tract starts in the ileum. In the case of the pharmacokinetics of flavonoids, many aglycones are released from their glycosides by intestinal lactase-phlorizin hydrolase (LPH, lactase) located in the brush border membrane of enterocytes lining the villi of the small intestine. Glycosides could also be hydrolyzed by cytosolic β-glucosidase (CBG) located in epithelial cells of the intestine. Thus, more lipophilic aglycones can be quickly absorbed into the blood and the portal vein, which leads to the liver. There and also in the intestinal cells, flavonoids aglycones undergo a „first-pass effect" before entering the systemic circulation. The first-pass metabolism is mediated by phase II metabolism enzymes such as sulfotransferases (SULTs), UDP-glucuronyltransferases (UGTs), and catechol-*O*-methyl transferases (COMT) resulting in the formation of sulfated, glucoronidated and

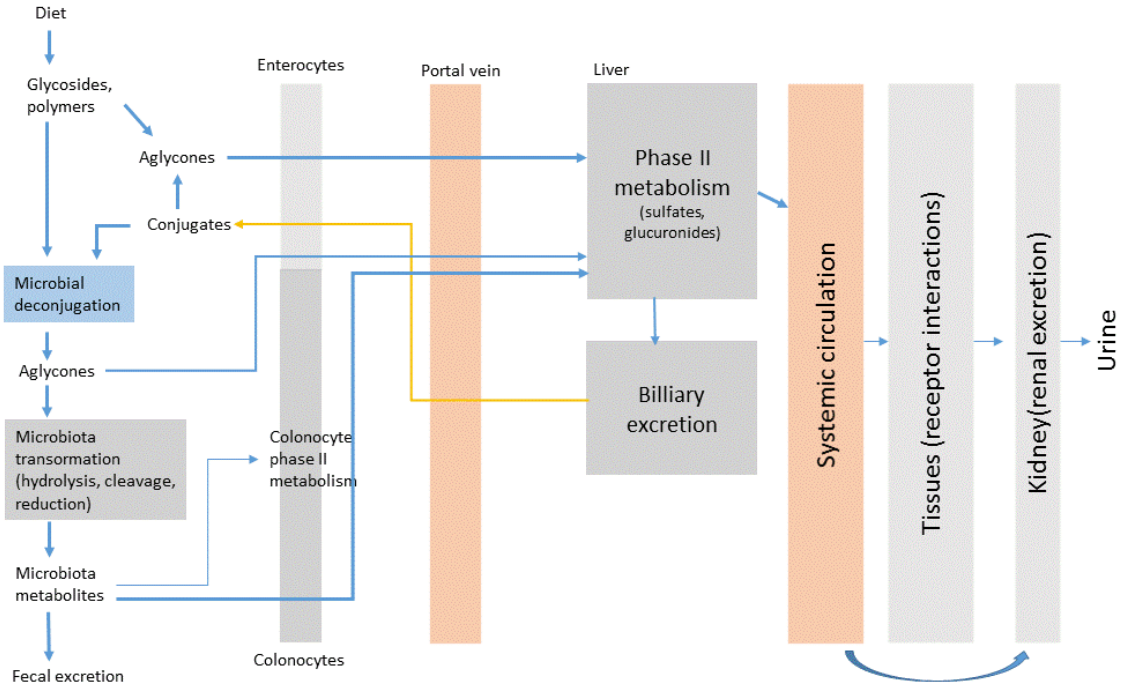


Fig. 2 *Schematic illustration of the metabolic fate of dietary polyphenols. During the absorption, polyphenols are deconjugated (hydrolyzed) by the small intestinal enzymes or by the gut microbiota. Released aglycones, as well as microbial metabolites (small phenolic compounds), could be absorbed and undergo phase I and II metabolism in the liver or the intestinal cells. Some of the absorbed substances could undergo biliary secretion and are subject to enterohepatic circulation. After the entrance into the systemic circulation, they are distributed to the tissues and subsequently excreted in urine (modified from D'Archivio et al. 2010).*

methylated conjugates. Flavonoids are also subject of enterohepatic circulation. Various amounts of conjugated metabolites can be excreted in the bile and re-enter the intestine. Then they are further transformed by bacterial β-glucuronidases back to their aglycones and absorbed again. Thus, the conjugates typically remain in the circulation longer than the parent compounds (Santhakumar et al. 2018; Stevens and Maier, 2016).

Due to the relatively low bioavailability of polyphenols, a significant amount reaches the colon where they are catabolized by microorganisms into small phenolic compounds (see Chapter 3.2). These compounds probably have higher bioavailability than complex polyphenols. Once absorbed they pass through the bloodstream to the liver, and undergo additional structural modifications by conjugation processes. The biological activity of these conjugates is barely studied (Masella et al. 2012).

The bioavailability and distribution of polyphenols are also closely related to the multidrug resistance-associated proteins (MRP), a group of adenosine triphosphate binding cassette (ABC) efflux transporters. While MRP-2 is responsible for transporting intracellular polyphenols back to the intestinal lumen, MRP-1 promotes the passage of flavonoids from the intracellular compartment to the blood (Santhakumar et al. 2018). Various flavonoids and flavonolignans can also inhibit the activity of some of the efflux transporters, such as MRP-1 protein, human P-glycoprotein (known as well as MDR-1, multidrug resistance protein 1), breast cancer resistance protein, and bacterial transporters. The role of these transporters is of particular interest for the research, since they are considered to be some of the main factors contributing to the antineoplastic and bacterial multidrug resistance (Chambers et al. 2019).

3.2 Microbiota and polyphenol metabolism

Generally, a significant amount of polyphenols (90-95% of the total polyphenol intake) reaches the colon (Cardona et al. 2013). Subsequently, they are transformed by gut microbial enzymes into a wide range of low molecular phenolic compounds. The crucial role of gut microbiota for polyphenol metabolism was highlighted by the studies, which proved that germ-free animals did not form phenolic acid metabolites from selected flavonoids (Griffiths et al. 1972). There is a growing number of studies that suggest gut-derived metabolites are at least partially responsible for the biological activity of polyphenols.

3.2.1 Microbiota

An ecosystem of around 10^{13}–10^{14} microorganisms makes the “intestinal microbiota”, which generally consists mostly of anaerobic bacteria. The highest concentration and diversity of microorganisms was found in distal parts of the colon. It has been estimated that approximately 400-500 bacterial species are present in the human microbiome. Despite the great diversity, 98% of all species belong to four bacterial phyla: Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria with different implications on human health. For instance, the ratio of Firmicutes/Bacteroidetes was higher in obese than in normal-weight people. This ratio has decreased during low-caloric diet and weight loss (Duda-Chodak et al. 2015).

The gut microbiota plays a significant role in the homeostasis of the organism. It affects the gut through several mechanisms such as immune signaling, modulation of mucosal barrier function, toxin release, and nutrient metabolism. The dysbiosis, which can be characterized as the disturbance of the microbial ecosystem, is involved in different chronic diseases such as diabetes, allergy, inflammatory bowel disease and others. Intestinal microbiota also plays a significant role in the development of neurodegenerative diseases (Spielman et al. 2018; Serra et al. 2018; Waldecker et al. 2008).

The microbial composition starts to develop at the birth of an individual, and a more stable adult-type of the microbiome evolves after the first 2-4 years of life. A vast diversity among individuals exists in the composition and ratio of different species that form their microbiota. There are multiple factors influencing this diversity, such as diet, genetics, environmental factors, and usage of antibiotics (Duda-Chodak et al. 2015). It has been proposed to divide the population into several "enterotypes" based on three dominant genera *Bacteroides, Prevotella,* and *Ruminococcus*. It leads to individual differences between the microbial metabolization of xenobiotics (polyphenols, drugs, etc.) or nutrients. In some publications, these differences are characterized with the term "gut metabotype". Thus, the ability to produce certain metabolites from polyphenols is closely related to the existence of specific communities of microorganisms (Gross et al. 2010; Espín et al. 2017).

3.2.2 Polyphenol metabolism by microbiota

The great diversity of microorganisms within the gut makes the metabolic capacity of the microbiota to be approximately 100-fold higher than that of the human liver. Unlike the metabolism in the liver, bacterial metabolism of nutrients and xenobiotics does not involve oxygen. The transformation of complex polyphenol structures performed by microbiota results in the formation of more bioavailable low molecular weight products (Duda-Chodak et al. 2015).

It is difficult to define the exact process of microbial degradation of food polyphenols for several reasons. The primary reason for that is the existence of the above-mentioned gut metabotypes, which makes the metabolic pathways of polyphenols individual. Another reason is the variable composition of polyphenols in food (Kemperman et al. 2010). The microbial transformation studies are complicated by the

fact that in some cases, enzymes and microorganisms responsible for these transformations were not characterized, and their specificity remains unknown (Williamson et al. 2010).

Depending on the gut metabotype, some differences were observed in the health effects of polyphenols. For example, individuals with *Bacteroides* enterotype, capsaicin exhibited stronger effects on inflammatory markers than in individuals with *Prevotella* enterotype (Kang et al. 2016).

As microbiota influences polyphenol metabolism, polyphenols can also influence microbiota. Thus, polyphenols have antimicrobial properties against pathogenic strains and change the composition of gut microbiota in favor of beneficial microorganisms such as *Bifidobacterium, Lactobacillus,* but also other beneficial strains such as *Akkermansia muciniphila, Faecalibacterium prausnitzii,* and *Roseburia* spp. The increasing number of *A. muciniphila* and decreasing ratio of Firmicutes/Bacteroidetes (thereby modulation of microbiota to a healthier composition) was observed in mice fed with grape extract rich in condensed tannins (Dueñas et al. 2015; Tomás-Barberán et al. 2016).

Polyphenols and some nutrients, such as dietary fiber and resistant starch are metabolized by gut microbiota to the short-chain fatty acids such as butyrate, succinate, propionate, acetate and lactate. Microbial degradation of polyphenols also results in the formation of phenylacetate and phenylbutyrate, among others. These metabolites, along with short-chain fatty acids, are absorbed and reach tissues and organs, including the brain, where they exhibit important signaling activity and gene expression regulation *via* modulation of histone deacetylases (Waldecker et al. 2008).

In general, microbial transformations of flavonoids can be divided into hydrolysis, cleavage and reduction (Braune and Blaut, 2016; Espín et al. 2017).

3.2.2.1 Hydrolysis

The hydrolysis is the general principle of deconjugation reactions. As mentioned, the majority of flavonoids are present in the diet in the form of *O*- or *C*- glycosides. After deglycosylation, the released aglycone can be absorbed more easily. The species from the Bifidobacteracae family are ones of the most important bacteria with deglycosylation activity. However, species from Lactobacillaceae, Lachnospiraceae and Enterococcaceae are also capable of deglycosylation reactions (Braune and Blaut, 2016).

In some cases, the hydrolysis by bacteria is necessary for absorption. For example, quercetin 4'-*O*-glucoside conjugated with glucose moiety is absorbed in the intestine after deglycosylation by the brush border enzyme LPH. However, flavonoids conjugated with rhamnose moiety (e.g. quercetin-3-*O*-rhamnoglucoside or rutin) cannot be hydrolyzed by LPH. Thus, they reach the colon untouched, and the aglycone can be released there by enzymes of *Bacteroides* species (**Fig. 3**). Isoflavones, ellagitannins, phenylpropanoids, and lignans are other examples of compounds deglycosylated by gut microbiota enzymes. The produced aglycones are not strictly considered as metabolites of the gut microbiome. Nevertheless, the microbial hydrolases play an essential role in increasing the absorption of polyphenols (Williamson et al. 2010).

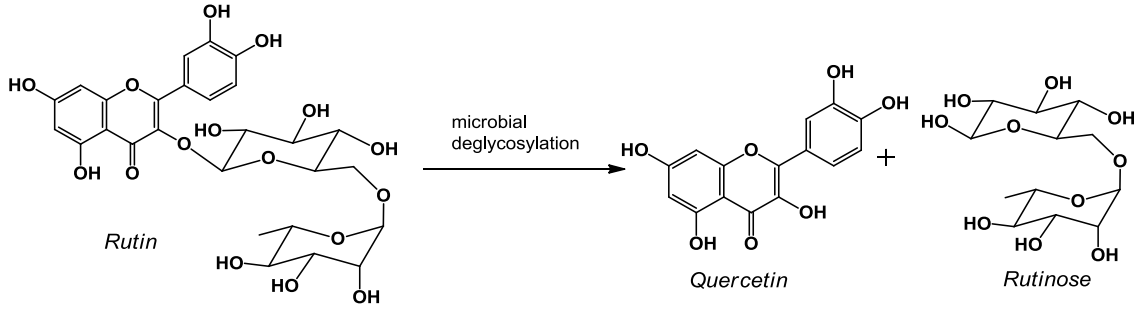


Fig. 3 *The microbial hydrolysis of rutin leading to the formation of quercetin.*

As mentioned in chapter 3.1.5, bacteria also possess β-glucuronidases, which play a significant role in the enterohepatic circulation of polyphenols. They allow polyphenols to be reabsorbed once released to the gut with bile. However, high glucuronidase activity is associated with an increased risk of cancer, since it plays a role in the detoxification and excretion of toxic compounds (Louis et al. 2014).

3.2.2.2 Cleavage

The next relevant step in polyphenol and flavonoid metabolism is the (C-)ring cleavage and demethylation of the side chain. These catabolic reactions run under anaerobic conditions mainly by enzymes of *Clostridium* spp. and some Coriobacteriaceae. *Eubacterium* spp. is also capable of C-ring fission, deglycosylation and other enzymatic activity (Espín et al. 2017; Williamson and Clifford, 2010).

In the case of flavonoids, after the C-ring cleavage, simple phenolic compounds derived from rings A and B are formed. C-ring cleavage can occur at different positions resulting in the formation of different structures. For instance, quercetin is transformed into 3,4-dihydroxybenzoic (protocatechuic), and 3,4-dihydroxyphenylacetic acid that are

derived from the ring B. Other metabolites such as phloroglucinol and 2,4,6-trihydroxy benzoic acid are formed from the ring A (**Fig. 4**, Selma et al. 2009).

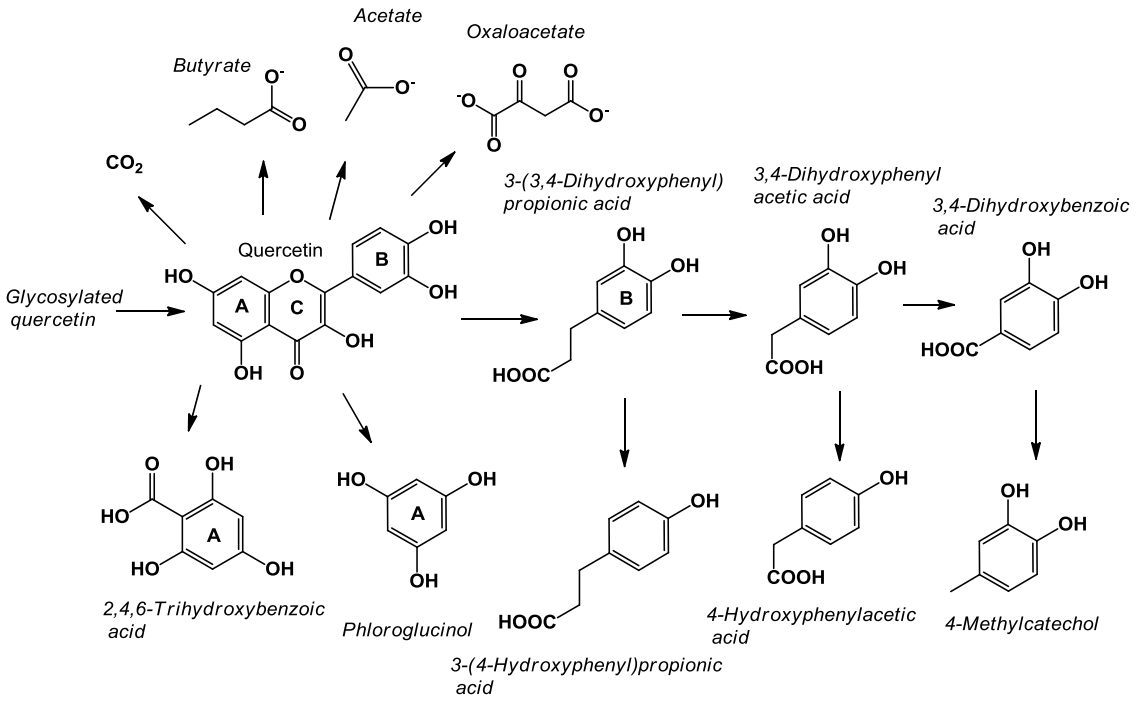


Fig. 4 *Microbial transformations of quercetin resulting in the formation of small phenolic compounds. Some of these catabolites are derived from the ring A (phloroglucinol, 2,4,6-trihydroxybenzoic acid). The others are derived from the ring B (modified from Almeida et al. 2018; Serra et al. 2012).*

Ellagitannins are first hydrolyzed into ellagic acid and then species from the *Gordonibacter* strain from the Coriobacteraceae family catalyze the lactone ring opening and decarboxylation of ellagic acid resulting in the formation of urolithin M5 (**Fig. 5**). The transformation of urolithin M5 to the different types of urolithins such as urolithin A, urolithin B, and isourolithin A depends on the specific strains which catalyze dehydroxylation reactions of urolithin M5. These types of urolithins correspond to the metabotypes A, B and 0 respectively. The B metabotype, which produces urolithin-B as the main metabolite of ellagitannins, is more prevalent in overweight individuals, or in general in patients with metabolic syndrome or colorectal cancer than in healthy individuals. It was also suggested that metabotype B individuals were at higher risk of cardiovascular disease than metabotype A individuals (urolithin A producers) (Espín et al. 2107; Tomás-Barberán et al. 2017).

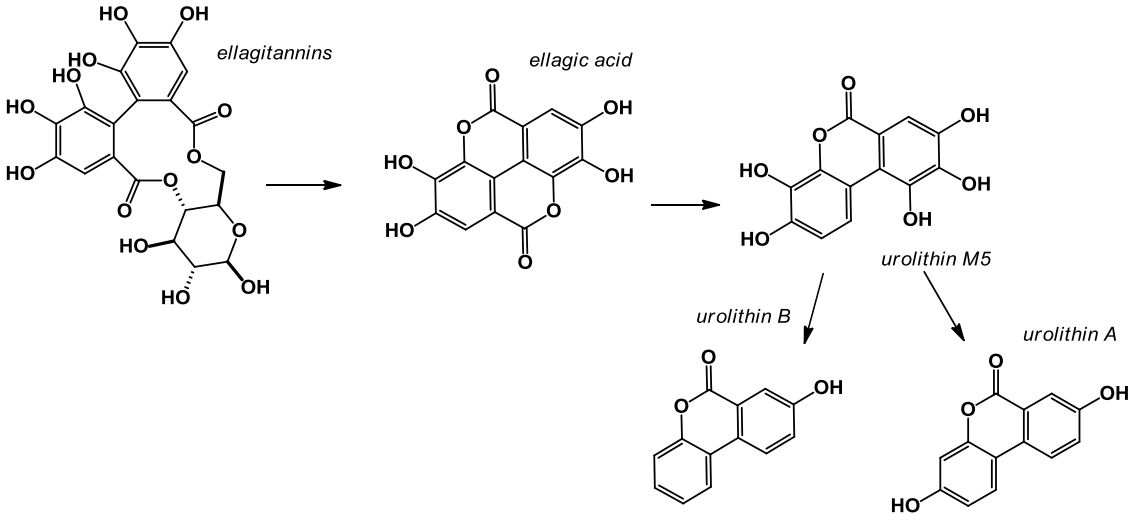


Fig. 5 *Microbial catabolism of ellagitannins results in the formation of urolithins (modified from Espín et al., 2017).*

A methoxy group is often attached to the phenolic rings A- and B- of flavonoids. It was shown that demethylation of flavones, isoflavones, and prenylated flavonones is mediated by *Eubacterium limosum* and *Blautia* spp. (Braune and Blaut, 2016).

3.2.2.3 Reduction

Reduction reactions in polyphenol gut metabolism include hydrogenation of double bonds, carbonyl reduction, and specific dehydroxylations. The hydrogenation of the double bond in caffeic acid results in the formation of 3,4-dihydroxyphenylpropionic acid, which can be subsequently dehydroxylated into monohydroxylated derivatives (**Fig. 4**). Full dehydroxylation of phenolic substances can lead to the production of aromatic compounds (Espín et al. 2017).

Enterodiol, a metabolite of lignans, may undergo dehydrogenation (cyclization) by *Lactonifactor longoviformis* (**Fig. 6**). It leads to the formation of enterolactone. A recent cohort study suggested that high-producers of enterolactone have a lower risk of type 2 diabetes (Sun et al. 2014).

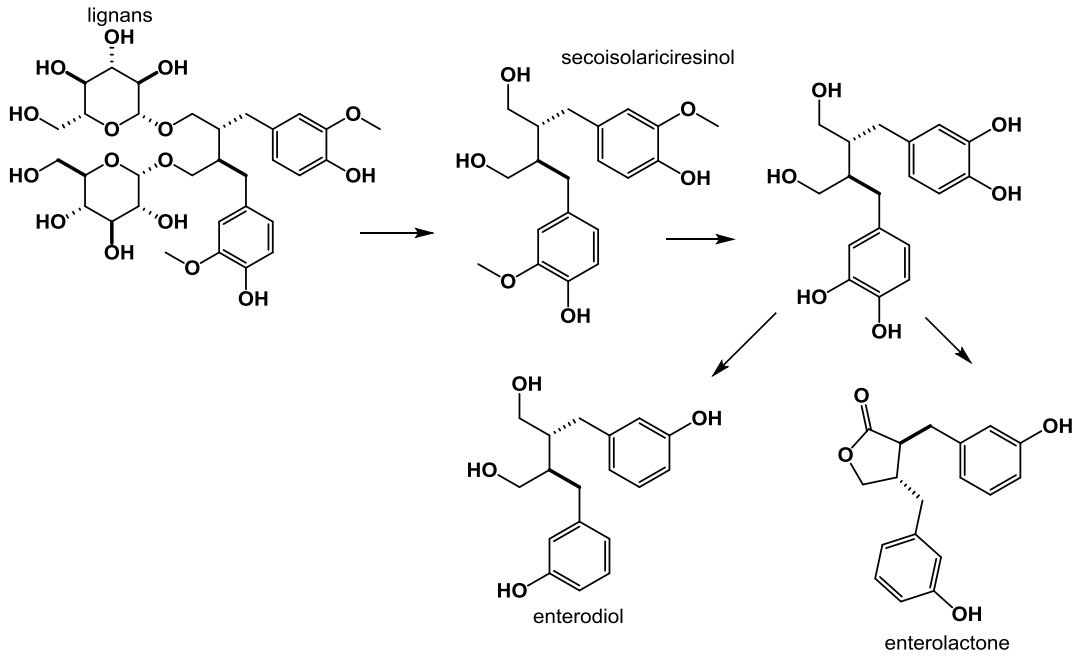


Fig. 6 *Microbial transformation of lignans resulting in the formation of enterodiol and enterolactone (modified from Espín et al. 2017).*

3.3 Selected microbial metabolites of polyphenols and their properties

The majority of simple phenolic compounds related to polyphenols are derived from microbial metabolism. Some of them are present in intact plants as a precursor of polyphenol synthesis. This section is focused on biological activities of selected phenolic compounds.

3.3.1 3,4-Dihydroxybenzoic acid

Also called protocatechuic acid (PCA), 3,4-dihydroxybenzoic acid (**Fig.7**) is known as one of the main microbial metabolites of flavonols and anthocyanins (Santhakumar et al. 2018). It can also be found in many plants. PCA exhibits a variety of biological activities such as antioxidant, neuroprotective, anti-inflammatory and antihyperglycemic effects (Masella et al. 2012). It regulates the renal glucose homeostasis by modulating glucose uptake and production, and strengthen the insulin signaling (Álvarez-Cilleros et al. 2018).

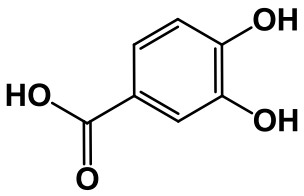


Fig. 7. *The structure of 3,4-dihydroxybenzoic acid*

Protocatechuic acid contains oxidizable catechol moiety, which is important for activation of the nuclear erythroid-related factor 2 (Nrf2). Nrf2 plays a significant role in cytoprotective mechanisms of cells. It is the key regulator for antioxidant response element (ARE) genes, which encode proteins responsible for protection against oxidative stress (Kumar et al. 2014).

3.3.2 3,4-Dihydroxyphenylacetic acid

3,4-Dihydroxyphenylacetic acid is also known as homoprotocatechuic acid (HPCA, **Fig. 8**). Along with 3,4-dihydroxybenzoic acid, HPCA is considered to be the major microbial metabolite of rutin and other polyphenols.

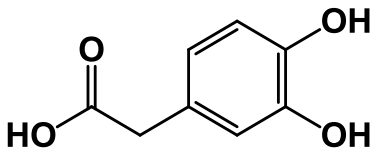


Fig. 8 *The structure of 3,4-dihydroxyphenylacetic acid.*

It has been shown that HPCA has a protective effect against pancreatic beta-cell dysfunction induced by high cholesterol. HPCA also exhibits antiproliferative activity on colon and prostate cancer cells (Carrasco-Pozo et al. 2015; Gao et al. 2006).

The hypothesis of cumulative or additive activities of several circulating metabolites was supported by a recent study. The mixture of 3,4-dihydroxyphenylacetic acid, 2,3-dihydroxybenzoic acid, and 3-hydroxyphenylpropionic acid significantly increased phosphorylation of endothelial nitric oxide synthase and increased nitric oxide production. At the same time, individual metabolites exhibited lower activity (Álvarez-Cillero et al. 2018).

3.3.3 4-Methylcatechol

4-Methylcatechol (MC, **Fig. 9**), also known as 3,4-dihydroxytoluene is one of the catabolites of microbial degradation of quercetin (**Fig. 4**). It was found that 4-methylcatechol reduces the generation of pro-inflammatory cytokines in liposaccharide-

activated macrophages (Su et al. 2014). MC also induces genesis of brain-derived neurotrophic factor, which plays a significant role in the growth and differentiation of new neurons and synapses. Since the lack of brain-derived neurotrophic factor can mediate the neuropathic pain, 4-methylcatechol can mitigate these symptoms (Ishikawa et al. 2014).

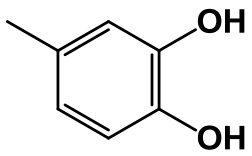


Fig. 9 *The structure of 4-methylcatechol.*

In another study, 4-methylcatechol showed its ability to induce apoptosis in Sertoli cell tumor cells. It also inhibited proliferation of melanoma cells. Moreover, no cytotoxic effects on normal human epidermal melanocytes was observed. Based on its beneficial activities, some authors propose 4-methylcatechol as a potential drug for cancer and neurological diseases (Payton et al. 2011; Li et al. 2015). Our research group recently demonstrated that this compound is causing directly vasodilation and hence decreases arterial blood pressure in spontaneously hypertensive rats (Pourová et al. 2018).

3.3.4 2,3,4-Trihydroxybenzoic acid

2,3,4-Trihydroxybenzoic acid (THBA, **Fig. 10**) is found exclusively in intact plants. For instance, it is contained in *Phaseolus vulgaris,* or common bean, along with ferulic acid, caffeic acid, and other compounds. THBA is also found in some tea plants, lentil cultivars, and *Physalis alkekengi* (Luthria et Pastor-Corrales, 2005; Tao et al. 2015; Xu et Chang, 2010; Lv et al. 2018).

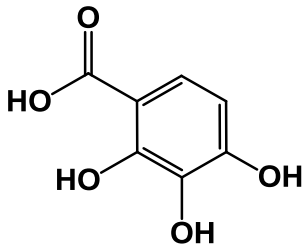


Fig. 10 *The structure of 2,3,4-trihydroxybenzoic acid.*

Some derivatives and analogs of gallic (3,4,5-trihydroxybenzoic) acid, including 2,3,4-trihydroxybenzoic acid, ethyl gallate, and ellagic acid, induced apoptosis in various cancer cell lines such as human stomach cancer and colon adenocarcinoma (Saleem et al.

2002). It was also found that conjugated THBA contained in sweet cherries (*Prunus avium*) exhibited antioxidant and antifungal activity (Wang et al. 2017).

3.3.5 Phloroglucinol

Phloroglucinol (benzene-1,3,5-triol, **Fig. 11**) is a phenolic compound formed during microbial metabolism of flavonoids. It can also be present in the form of phlorotannins in some seaweeds (Shibata et al. 2004). Phloroglucinol exists in the form of two keto-enol tautomers that are in equilibrium (Lohrie and Knoche, 1993).

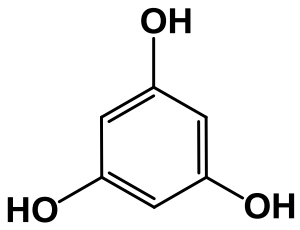


Fig. 11 *The structure of phloroglucinol*

Phloroglucinol is also known as a musculotropic and anti-spasmodic drug used mainly in veterinary medicine (Singh et al. 2009). It also seems to be effective in reducing exacerbation of abdominal pain in patients with inflammatory bowel disease (Chassany et al. 2009). Phloroglucinol has an array of other biological activities such as anti-inflammatory, anti-allergic, antioxidant, and also has promising activity against breast cancer (Crockett et al. 2008; Daikonya et al. 2002; Kim and Kim, 2010; Pádua et al. 2015). Phloroglucinol is commercially widely used in cosmetics, pesticides, dyes and cements (Singh et al. 2010).

3.4 Sulfation and phase II metabolism

The sulfation or sulfurylation is an essential reaction in nature. Its main effect is facilitation of elimination of various substances from the organism. However, in some cases sulfation can lead to new important pharmacodynamic properties which may not be associated with the parent compound.

3.4.1 The role in the human organism

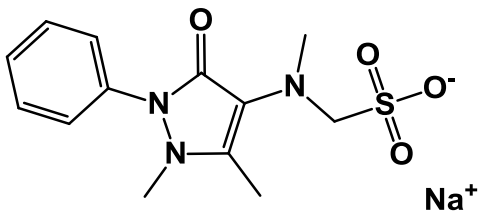
Sulfated molecules play a significant role in many physiological processes such as molecular recognition, hormone regulation, cell signaling, blood coagulation, as well as in some pathological processes (Chapman et al. 2004). For instance, bioavailability of steroid hormones is regulated by sulfation. Sulfated hormones serve as a storage of active

steroids. The active steroids are formed after hydrolysis of the sulfate group by sulfatases (Strott, 2010). Sulfated complex proteins and carbohydrates are important for molecular recognition and signaling pathways processes (Honke and Taniguchi, 2002). Sulfates also play a role in the posttranslational modification of proteins. Tyrosine sulfation is an important modification for regulation of protein-protein interactions involved in leukocyte adhesion, hemostasis, and chemokine signaling (Kehoe and Bertozzi, 2000). Sulfate group is also present in such essential biological molecules as glycosaminoglycans heparin, heparan sulfate, chondroitin sulfate or keratan sulfate (Caterson and Melrose, 2018).

3.4.2 Sulfonates, sulfates, and salts with sulfate ion

Even though sulfonates and sulfates might seem to be similar in structure, they exhibit essential differences. These compounds are formed by different reactions. Sulfonates are products of sulfonation, which results in the formation of the C-S bond. On the other hand, sulfates or organosulfates are products of sulfation, which involves the formation of C-O-S ester bond. For example, the structure of metamizole contains a sulfonate group while sodium lauryl sulfate (SLS) contains a sulfate group (**Fig. 12**, Foster 1997).

A:



B:

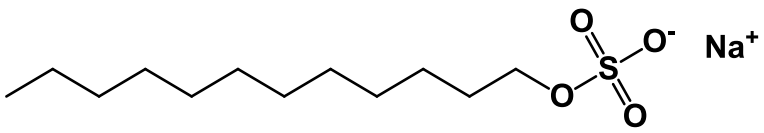


Fig. 12 *Difference between sulfonates and sulfates. A: the structure of metamizole contains a sulfonate group; B: the structure of sodium lauryl sulfate contains a sulfate group.*

The term sulfate is also used for drugs occurring in the form of sulfuric acid salts such as amphetamine sulfate. In this case the sulfate is attached by ion bonds (**Fig. 13**).

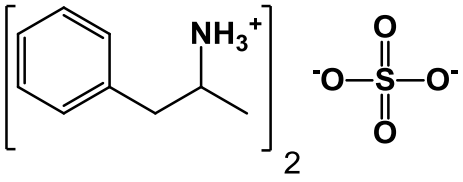


Fig. 13 *The structure of amphetamine sulfate. Sulfate is present in the form of an ion (modified from CorePharma 2016).*

3.4.3 Sulfation and drugs

Addition of a sulfate group to the drug affects its pharmacokinetic properties. In general, the sulfated drug becomes significantly more water-soluble and thus sulfation leads to increased elimination of drugs. Thereby it lowers its toxicity as well as the duration of the pharmacological effect. The addition of the sulfonic acid group to the drug molecule can lead to lowering or abolishing of the drug effect (Hartl and Palat, 1998).

On the other hand, some drugs become pharmacologically active after their sulfation in the organism. For instance, minoxidil is a vasodilator compound used in the treatment of hypertension and alopecia. Both of these therapeutic effects are mediated mostly by its *N*-sulfate conjugate, which is a potent activator of the potassium channel (Buhl et al. 1990).

3.4.4 Biotransformation of xenobiotics

The biotransformation process basically aims to detoxify xenobiotics, mainly by making them more water-soluble, and therefore allowing them to be easily excreted by the kidneys. Nevertheless, the outcome of some biotransformations can be the metabolic activation of the respective compounds. This is the way in which prodrugs work. They become pharmacologically active after they undergo certain types of biotransformation. In some cases, initially nontoxic xenobiotics are transformed into the toxic metabolites. Reduction of the pharmacological effect of drugs or the formation of metabolites with the same activity can also occur. The main biotransformation organ is the liver. However, intestinal cells, skin, lungs and kidneys have also relatively significant enzymatic activity (Murray et al. 2012; De Kanter et al. 2002).

The biotransformation of xenobiotics is divided into the phases I and II. During phase I, the non-synthetic phase, more polar substances are formed. It is accomplished by unmasking or *de novo* formation of functional groups (e.g., OH, -NH, -SH). The oxidation is the primary reaction of phase I metabolism, which is catalyzed by the system of

cytochrome P450 or monooxygenase enzymes. As a result, more polar substances are formed and at the same time, they become "prepared" for phase II metabolism. The changes in CYP enzymes activity is the mechanism of one of the major and clinically relevant types of drug interactions. Some substances can be excreted without phase II (conjugation) if they are sufficiently polar after accomplishing phase I metabolism. Others may undergo directly phase II metabolism without previous reactions of phase I, if they carry an appropriate group (most often hydroxy group) for conjugation process. The activity of biotransformation enzymes may be influenced by several factors such as drug intake, diet, age, genetic factors, environment etc. (Murray et al. 2012; Jančová et al. 2010).

3.4.5 Phase II of biotransformation

Phase II of biotransformation is also called synthetic or conjugational phase. Generally, the metabolites from the Phase I are conjugated with specific molecules at Phase II. Thus, several types of conjugation reactions are distinguished including sulfation, glucuronidation, acetylation, methylation, glutathione and amino acid conjugation. These reactions are accomplished by Phase II enzymes such as sulfotransferases, UDP-glucuronosyltransferases, *N*-acetyltransferases, glutathione *S*-transferases and methyltransferases. As a result, much more polar substances are created, which can be easily excreted with urine or with bile (in the case of glucuronidation). Drug interactions involving enzymes of Phase II metabolism are relatively rare in comparison with cytochromes P450 enzymes. However, the reduced metabolic activity of Phase II enzymes can lead to higher plasmatic levels of some drugs (Jančová et al. 2010; Murray et al. 2012).

Glucuronidation is one of the most common reactions in the metabolism of xenobiotics. Glucuronidation is also typical for many endogenous molecules such as bile acids. Glucuronyl is bound to the substrate *via* oxygen, sulfur or nitrogen. The reaction is catalyzed by the enzymes glucuronyltransferases found in the endoplasmic reticulum and in cytosol (Murray et al. 2012).

Phase II metabolism also includes acetylation. This reaction is a significant metabolic pathway for compounds containing an amino group (sulfonamides, hydrazides, amino acids). A polymorphism of acetyltransferases enzymes exists, which is the cause

of significant differences in the levels of drugs (e.g. isoniazid) that are metabolized by acetyltransferases (Murray et al. 2012).

Conjugation with glutathione (GSH) catalyzed by glutathione-*S*-transferase is an important protective mechanism of the organism against reactive oxygen species. GSH is a tripeptide consisting of glutamic acid, glycine and cysteine. Its biological activity is based on the thiol (SH) of cysteine, which serves as a proton donor. Glutathione is an essential antidote in an overdose of paracetamol since it conjugates and detoxifies *N*-acetyl-*p*-benzoquinone imine (NAPQI), the toxic metabolite of paracetamol (Murray et al. 2012; Moore et al. 1985). It was reported that some flavonoids can modulate the activity and sometimes the expression of glutathione-*S*-transferase. For example, GSH conjugation of NAPQI seemed to be accelerated by flavonoid intake. However, there was also reported the reverse effect in carriers of a particular genotype. Thus, flavonoid intake appears to be mostly beneficial. On the other hand, the intake of high doses of flavonoids (e.g. in dietary supplements) can sometimes significantly increase the toxicity of the drugs which are metabolized through conjugation with GSH (Boušová and Skálová, 2012).

Compounds containing a carboxyl group are often conjugated with endogenous amino acids. Glycine is one of the most common amino acid participating in conjugation reactions. Glycine conjugation is an important reaction responsible for maintaining normal levels of free coenzyme A. Other amino acids such as taurine can be involved in the conjugation of aromatic and heterocyclic carboxylic acids (Badenhorst et al. 2013; Murray et al. 2012).

3.4.6 Sulfotransferases

Sulfation process in organisms is mediated by a variety of sulfotransferases. In eukaryotes, they can be divided into two broad groups: the cytosolic (SULT), and the membrane-associated sulfotransferases. The former ones, also called phenol or aryl sulfotransferases (AST), are responsible for the biotransformation of an array of small endogenous and exogenous molecules such as phenols, hormones, drugs or xenobiotics. The sulfation of larger biomolecules like proteins and carbohydrates is governed by the latter one. In polyphenol metabolism, the isoforms SULT1A1, SULT1A3/4, SULT1B1, SULT1E1 and SULT2A1 are considered to be the most relevant (Ayuso-Fernández et al. 2014; Pimpao et al. 2015).

In the sulfation process, the sulfate group (SO_3^{2-}) is transferred from a donor molecule (usually the 3'-phosphoadenosine-5'-phosphosulfate, PAPS) to O-, N- or S-acceptor group of various substrates (**Fig. 14**). PAPS can be synthesized in all tissues in mammals and appears to be the primary donor of a sulfate group (Jančová et al. 2010). In laboratory conditions, the usage of PAPS brings some problems. First, its by-product (PAP) inhibits the reaction. Second, PAPS is unstable and expensive (Van der Horst et al. 2012; Ayuso-Fernández et al. 2014).

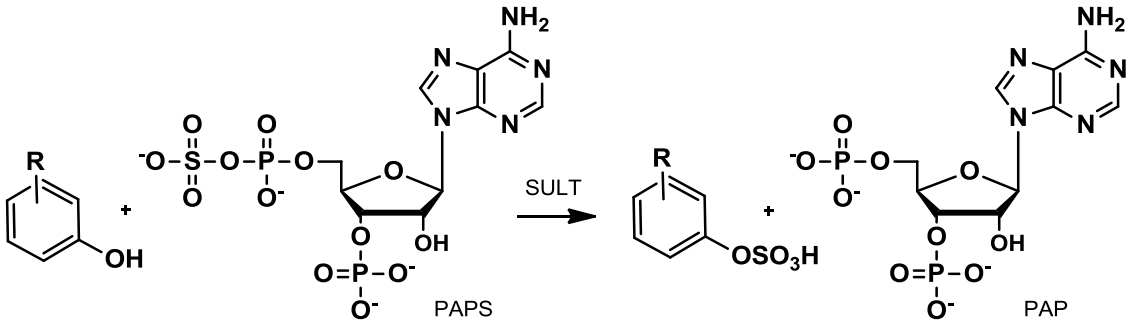


Fig. 14 *Sulfation process mediated by SULT with PAPS as donor molecule and with O-acceptor of sulfate group (modified from Jančová et al. 2010).*

In bacteria, there is a group of PAPS-independent sulfotransferases that use one of the aryl substrates as a cheap and stable sulfate donor (e.g. *para*-nitrophenolsulfate, *p*-NPS) and the other aryl substrate as an acceptor. Bacterial PAPS-independent sulfotransferases are successfully used for the preparative synthesis of sulfates in laboratory conditions. Namely, AST from *Desulfitobacterium hafniense*, *Haliangium ochraceum*, *E. coli* CFT073 or *Citrobacter freundii* are used for this purpose (Van der Horst et al. 2012; Ayuso-Fernández et al. 2014).

Sulfotransferase from *D. hafniense* produces the same sulfated derivatives as the mammalian enzyme arylsulfotransferase IV (AstIV) from rat liver. Thus, products from *D. hafniense* enzyme can be considered as Phase II metabolites (Purchartová et al. 2015).

3.4.7 Sulfated flavonoids

As other xenobiotics, flavonoids undergo phase II biotransformation resulting in the formation of sulfated or/and glucuronidated metabolites. An array of biological activities of sulfated flavonoids have been discovered, including anticoagulant, anti-inflammatory, immunomodulatory, antiviral, and antitumor activities. Sulfation of flavonoids increases the water solubility of flavonoids and promotes interactions with

several biological targets due to the presence of negative charge (Correia-da-Silva et al. 2014).

In addition, approximately 150 naturally occurring sulfated flavonoids, including those with attached sugars, are known to date. Usually, sulfated flavonoids were found in plants growing near aqueous areas rich in mineral salts. That feature is considered to be an ecological adaptation. By bonding with anthocyanin pigments, they seem to play a significant role in co-pigmentation of the plant. Quercetin-3-*O*-sulfate was found to take part in the regulation of the plant growth. However, their role(s) in plant tissues is not completely understood (Teles et al. 2018).

3.4.8 Sulfated simple phenolic compounds derived from microbial transformations

Natural phenolic compounds derived from microbial catabolism undergo biotransformation resulting in the formation of conjugated compounds. An array of sulfated, methylated and glucuronidated phenolic compounds were found in urine and plasma in substantially higher amounts after digestion of polyphenol-rich food. For example, protocatechuic acid-*O*-sulfate, 4-methylcatechol-*O*-sulfate, pyrogallol-*O*-sulfate, caffeic acid-3-*O*-sulfate and other metabolites were detected in plasma in a recent study. The sulfated phenolic metabolites were more abundant in human plasma than glucuronidated phenolic metabolites and metabolites with preserved flavonoid core (Pimpao et al. 2015; Rothwell et al. 2016).

Conjugates can be formed not only in the liver but also in the intestinal cells. Phloroglucinol-aldehyde and protocatechuic acid, derived from anthocyanin microbial metabolism, were shown to be transported inside the Caco-2-cells (a common model of the intestinal barrier) and metabolized there to sulfates and glucuronides (Kay et al. 2009).

Gallic acid conjugates including sulfates were detected in urine with a peak at 2-4 h after ingestion of fruit puree by human volunteers; this can indicate their rapid absorption and elimination. Conjugates were still detected in urine between 8 and 24 h, which means that gallic acid is still being produced, possibly due to degradation of anthocyanins or esters of gallic acid by colonic microbiota (Pimpao et al. 2014). In another study, some pharmacokinetic properties of protocatechuic acid phase II metabolites were characterized. Thus, after consumption of 500 mg of cyanidin-3-glucoside by human volunteers, the elimination half-life of the protocatechuic metabolites

was 30 ± 9 hours with maximal serum concentration of 2.35 ± 0.15 µM (Czank et al. 2013).

Only limited data can be found about biological activities of sulfated phenolic compounds derived from microbial metabolism. However, in a recent study, it was shown that some of the metabolites such as 4-methylcatechol-*O*-sulfate, pyrogallol-*O*-sulfate, and vanillic acid 4-*O*-sulfate are able to pass through the blood-brain-barrier endothelium in significant concentrations. They were also metabolized by brain endothelial cells into an array of novel substances that have never previously studied. However, the mechanism of the transport through the endothelium (simple diffusion or carrier-mediated transport) was not elucidated. Phenolic sulfates exhibited beneficial effects in different neuronal systems. They improved cellular response to oxidative, excitotoxic, and inflammatory injuries (Figueira et al. 2017). Considering the beneficial effect of certain polyphenols or microbial catabolites in humans, the contribution of conjugated metabolites has to be studied in order to determine their possible biological activities.

4 Aim of work

The aims of this work were to

- prepare pure sulfated phenolic derivatives from 4-methylcatechol, protocatechuic acid, homoprotocatechuic acid, 2,3,4-trihydroxybenzoic acid and phloroglucinol using aryl sulfotransferase from *Desulfitobacterium hafniense*;
- study the time course of sulfation for each compound;
- characterize the sulfates using HPLC, MS and NMR;
- test the compounds for their 1,1-diphenyl-2-picrylhydrazyl (DPPH), and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{+\bullet}$) radical scavenging, Folin-Ciocalteau reduction (FCR), ferric reducing antioxidant power (FRAP), cupric reducing antioxidant capacity (CUPRAC) and anti-lipoperoxidant activities in rat liver microsomes.

5 Experimental part

5.1 Chemicals

- 1,1-diphenyl-2-picrylhydrazyl (DPPH, purity at least 95 %, Sigma-Aldrich, Germany)
- 2,3,4-trihydroxybenzoic acid (THBA, purity at least 97%, Acrós organics, UK)
- 3,4-dihydroxybenzoic acid (protocatechuic acid, PCA, purity at least 97%, AlfaAesar, ThermoFisher, Germany)
- 3,4-dihydroxyphenylacetic acid (homoprotocatechuic acid, HPCA, purity at least 98%, Acrós organics, China)
- 4-methylcatechol (4-MC, purity at least 95%, Sigma-Aldrich, India)
- antioxidant assay kit ABTS KF-01-002 (Bioquochem, Spain) https://bioquochem.com/product/abts-antioxidant-capacity-assay-kit/
- antioxidant assay kit FRAP KF-01-003 (Bioquochem, Spain) https://bioquochem.com/product/frap-assay-kit/
- antioxidant assay kit CUPRAC KF-01-005 (Bioquochem, Spain) https://bioquochem.com/product/cuprac-antioxidant-capacity-assay-kit/
- chloroform (purity at least 99,2%, VWR chemicals, France)
- dimethyl sulfoxide (DMSO, 100%, VWR chemicals, France)
- ethyl acetate (purity at least 99,7%, Lachner, Hungary)
- Folin & Ciocalteu's phenol reagent (Merck, Germany)
- formic acid (purity at least 85%, Lachema, Czech Republic)
- gallic acid (purity at least 99 %, Lachema, Czech Republic)
- hydrochloric acid (HCl, 37% solution, purity at least 99%, CertiPUR, Merck, Germany)
- isopropyl-β-D-thiogalactoside (purity at least 99%, Sigma-Aldrich, Czech Republic)
- kanamycin (SERVA Electrophoresis GmbH, Germany)
- methanol (purity at least 99.9%, VWR Chemicals, France)
- phloroglucinol (purity at least 97%, Alfa Aesar, ThermoFisher, Germany)
- *p*-nitrophenylsulfate (*p*-NPS, purity at least 99%, Acrós organics, Germany)
- sodium carbonate (Na_2CO_3, purity at least 99.8%, Lachema, Czech Republic)

- sodium hydroxide (NaOH, purity at least 98.6%, Lachner, Hungary)
- *tert*-butyl hydroperoxide (70% solution, Sigma Aldrich, Czech Republic)
- thiobarbituric acid (TBA, purity at least 99%, Merck, Germany)
- trichloroacetic acid (TCA, purity at least 99%, Lachner, Hungary)
- trifluoracetic acid (purity at least 99%, Sigma Aldrich, Czech Republic)

5.2 Biological material

- Cryopreserved *E. coli* BL21(DE3) cells transformed with the plasmid with the gene of AST from *D. hafniense*.
- The rat liver microsomes (No. M9066) from Sigma-Aldrich (Czech Republic).

5.3 Solutions

- Phosphate buffer saline (PBS):

10× concentrated stock PBS: NaCl (0.137 $mol \cdot l^{-1}$), KCl (2.68 $mmol \cdot l^{-1}$), $NaHPO_4$ (8.96 $mmol \cdot l^{-1}$), KH_2PO_4 (1.47 $mmol \cdot l^{-1}$). The concentrated stock solution was 10× diluted with distilled water before use.

- TBA/TCA:

Thiobarbituric acid (3.75 g) and trichloroacetic acid (180 g) were dissolved in 800 ml of H_2O, then 37% HCl (20.72 ml) was added. The volume was filled up with distilled water to 1000 ml.

- Luria-Bertani (LB) medium:

Tryptone (10 g), yeast extract (5 g), NaCl (5 g) were mixed. Then the volume was filled up with water to 1000 ml and pH was adjusted to 7.0 using NaOH solution.

- Tris-glycine buffer solution:

Tris-base (3.03 g), glycine (1.877 g) were filled up with water to 250 ml, pH was adjusted to 8.9 using HCl solution. The final concentration was 0.1 M.

5.4 Instruments and devices

The following instruments were used for the preparation of sulfates: a thermostatic shaker with cooler KS 4000 ic control (IKA, Germany), centrifuges: Model J-6B (Beckman Coulter, Czech Republic), Centrifuge 5804 R and miniSpin plus (Eppendorf, Germany).

Preparatory HPLC was used for the purification of some samples. It consisted of a Shimadzu LC-8A pump equipped with a Rheodyne manual injection valve (Shimadzu, Japan). FRC-10A, preparative fraction collector was controlled by an operator or by a signal from SPD-20A dual wavelength detector. Asahipack GS-310 20F column (Shodex, USA) was used.

Fraction collection from gel chromatography with Sephadex LH-20 column was carried out with Super Frac (Pharmacia Biotech, USA) automatic fraction collector. The evaporator with a vacuum pump and a device for control of vacuum Rotavapor R-200 (Büchi, Switzerland) was used to evaporate the solvents from the reaction mixtures or purified fractions. For lyophilization of samples, Lyovac GT 2 (Leybold, USA) was used.

HPLC analyses were performed on a Shimadzu Prominence LC analytical system consisting of a Shimadzu LC-20AD binary HPLC pump, a Shimadzu SIL-20ACHT cooling auto sampler, a Shimadzu CTO-10AS column oven, a Shimadzu CBM-20A system controller and a Shimadzu SPD-20MA diode array detector (Shimadzu). The samples were dissolved in acetonitrile/water (5:95, v/v).

Mass spectra in the negative ion mode were measured using LTQ Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, USA) equipped with an electrospray ion source. NMR spectra were recorded on a Bruker Avance III 600 MHz (Bruker, Germany).

All the spectrometric measurements for antioxidant activity tests were carried out with the spectrophotometer Sunrise (Tecan, Switzerland) with programme Magellan™ - Data Analysis Software (Tecan).

5.5 General methods

The following methods used in the thesis were carried out by the author of this thesis (unless stated otherwise):

- Transforming *E. coli* cells with BL21 (de3) Gold plasmid for arylsulfotransferase from *D. hafniense* and their cryopreservation (P. Oubrechtová, IM CAS)
- Preparation of enzymes
- Kinetic study
- Preparation of sulfates

- Purification of sulfates
- Analytical thin layer chromatography
- Analytical High Performance Liquid Chromatography (HPLC, L. Petrásková, IM CAS)
- Mass Spectrometry (MS, L. Petrásková, IM CAS)
- NMR Spectroscopy (H. Pelantová, IM CAS)
- Study of the sulfates stability in saline
- Evaluation of antioxidant and antilipoperoxidation activity of the tested substances

5.5.1 Preparation of enzymes

100 ml of Luria-Bertani medium containing 100 µl of kanamycin (103 mM) was inoculated with the cryopreserved culture of *E. coli* BL21 (DE3) cells, transformed with the plasmid for AST from *Desulfitobacterium hafniense*. Bacterial cultures were grown until the optical density at 600 nm (OD_{600}) reached 0.6, then the expression of the enzymes was induced with 160 µl of 4 mM isopropyl-β-D-thiogalactoside and subsequently cells were incubated at 25 ºC overnight. The mixture was centrifuged at 4668 g for 20 min, and the cells were resuspended in a 100 mM of Tris-glycine buffer (pH 8.9). Then the cells were lysed by sonication (4 cycles, each consisting of 4 min sonication and 4 min cooling) and centrifuged at 17136 g for 10 min. The supernatant containing enzymes was isolated and used for the enzymatic reaction.

5.5.2 Kinetic study

The substrates (MC, HPCA, PCA, THBA, phloroglucinol, 200 mg) were dissolved in 5 ml of acetone, then *p*-NPS (various equivalents in 5 ml of Tris-glycine buffer), 24 ml of Tris-glycine buffer and 2 ml of enzyme solution were added. The mixture was incubated in an inert atmosphere (Ar) at 30 °C for up to 6 hours, and aliquots of about 100 µl were taken from the reaction mixture for HPLC analysis. Monitoring of reaction was performed by TLC analysis (mobile phase: ethyl acetate/chloroform/trifluoroacetic acid, 16:1:0.01). Each aliquot was shortly heated to 95 °C in order to terminate the enzymatic reaction, and stored at -20 °C until HPLC analysis.

5.5.3 Preparation of sulfates

The substrate (MC, HPCA, PCA, THBA, phloroglucinol, 200 mg) was dissolved in 5 ml of acetone, then *p*-NPS (1 or 2 molar equivalents dissolved in 5 ml of Tris-glycine buffer), 24 ml of Tris-glycine and 2 ml of prepared enzyme were added. Then the mixture was incubated in an inert atmosphere (Ar) at 30 °C for 5 hours. The monitoring of reaction was performed by TLC analysis (mobile phase: ethyl acetate/chloroform/trifluoroacetic acid, 16:1:0.01). At the end reaction mixture was shortly heated to 95 °C in order to terminate enzymatic reaction, and stored at -20 °C until purification.

5.5.4 Purification of sulfates

The reaction mixture was partially evaporated in order to remove acetone. Using formic acid, pH was adjusted to 7.5-7.7, and then the reaction mixture was extracted three times with ethyl acetate (3×50 ml). The aqueous phase was evaporated, and the residue was dissolved in 2-5 ml of 80% methanol, centrifuged and loaded on Sephadex LH-20 column with 80% methanol mobile phase. The elution usually took approximately 2 days. Fraction detection was performed by TLC (ethyl acetate/chloroform/trifluoroacetic acid, 16:1:0.01). The fractions were evaporated until the minimum volume was reached, and lyophilized. Fractions with low purity were purified again.

Purification of the reaction mixture with phloroglucinol was made using preparative HPLC, instead of gel chromatography with Sephadex LH-20 column. 100 mg of the reaction mixture was dissolved in 1 ml of 50% methanol, filtered and injected for separation. Separations were performed with 50% methanol at 25 °C. Flow rate: 5 $ml \cdot min^{-1}$. Detection was at 254 and 369 nm.

5.5.5 Characterization of sulfates

5.5.5.1 *HPLC*

2,3,4-Trihydroxybenzoic acid, protocatechuic and homoprotocatechuic acids were analyzed on Chromolith RP 18e, 5 mm column (100 × 3 mm, Merck). Binary gradient elution was used: mobile phase A = 5% acetonitrile, 0.1% formic acid; mobile phase B = 80% acetonitrile, 0.1% formic acid; gradient: 0% B for 0 min, 0–30% B for 0–5 min; 0% B for 5–9 min. The flow rate was 1 ml/min at 25 °C and the injection volume was 1 ml; samples were detected at 260 nm (protocatechuic acid), 260 nm (2,3,4-trihydroxybenzoic acid) and 280 nm (homoprotocatechuic acid).

4-Metylcatechol and phloroglucinol were analyzed on Kinetex Polar C18, 2.6 mm column (50 × 2.1 mm). Binary gradient elution was used: mobile phase A= water, 0.1% formic acid; mobile phase B=80% acetonitrile, 0.1% formic acid; gradient: 0% B for 0 - 3 min, 0–30% B for 0–5.5 min; 0% B for 5.5–9.5 min. The flow rate was 0.4 ml/min at 45 °C and the injection volume was 1 ml; samples were detected at 266 nm (phloroglucinol), 280 nm (methylcatechol).

5.5.5.2 *MS*

The samples were dissolved in MeOH and introduced into the mobile phase flow (MeOH/H_2O 4:1; 100 µl/min) using a 2 µl loop. Spray voltage, capillary voltage, tube lens voltage, and capillary temperature were 4.0 kV, −16 V, −120 V, and 275 °C, respectively.

5.5.5.3 *NMR*

NMR spectra were recorded on a Bruker Avance III 600 MHz spectrometer (600.23 MHz for ^{1}H, 150.94 MHz for ^{13}C) at 30 °C in dimethylsulfoxide (DMSO)-d_6. Residual solvent signal (δ_H 2.500 ppm, δ_C 39.60 ppm) served as an internal standard. NMR experiments: ^{1}H NMR, ^{13}C NMR, gCOSY, gHSQC, and gHMBC were performed using the standard manufacturer's software. ^{1}H NMR and ^{13}C NMR spectra were zero filled to four-fold data points and multiplied by window function before Fourier transformation. A two-parameter double-exponential Lorentz–Gauss function was applied for ^{1}H to improve resolution, and line broadening (1 Hz) was applied to get better ^{13}C signal-to-noise ratio. Chemical shifts are given in δ-scale with digital resolution justifying the reported values to three (δ_H) or two (δ_C) decimal places.

5.5.6 Antioxidant activity measurements

Several tests have been performed to determine the antioxidant and antiradical activity of the tested substances (PCA, MC, phloroglucinol, HPCA, THBA, MCS, PGS). All the stock solutions (100 mM) of the test substances were prepared in DMSO or PB (in the case of PGS) and were further diluted as required for individual tests. All the tests were performed in at least three independent experiments in triplicates.

5.5.6.1 DPPH scavenging

Free radical scavenging activity was determined by measuring the capacity of the compound to inhibit the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. Inhibition of the radical was followed by the decreasing of violet coloration of DPPH. A sample was pipetted into the microplate and further was geometrically diluted in quadruplicates. 15 µl of each diluted sample was mixed with 285 µl of a methanolic solution of DPPH in triplicates. The last replicate was mixed with 285 µl of methanol in order to use it as a blank. After 30 minutes of incubation, the absorbance at 517 nm was measured. The capacity of samples to inhibit DPPH free radicals was expressed as the concentrations that caused a 50% reduction of DPPH absorbance (IC_{50} value). IC_{50} values were obtained from the inhibition curves.

5.5.6.2 $ABTS^{+\cdot}$ scavenging

The capacity of the samples to scavenge the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical ($ABTS^{+\cdot}$) was evaluated using the antioxidant assay kit KF-01-002. All the tests were made according to manufacturer instructions. The reaction was followed by the decoloration of initially green ABTS chromophore. 5 µl of the sample, blank or standard (ascorbic acid at 0-600 µM) were pipetted into the wells of the microplate. Then 200 µl of "ABTS reagent" was added into each well. The reaction mixture was shaken for 4 minutes. Then, the absorbance at 734 nm was measured. Antioxidant activity was expressed as ascorbic acid equivalents (CEAC).

5.5.6.3 *FCR assay*

The reduction capacity of the samples was evaluated using the Folin-Ciocalteau reduction (FCR) assay. This method is based on the reduction of Mo^{6+} to Mo^{5+} in Folin-Ciocalteau reagent that is followed by blue coloration. Folin-Ciocalteau reagent reacts with the samples only under basic conditions, therefore Na_2CO_3 is needed (Huang et al. 2005).

First, gallic acid was diluted (0-4 mM) in order to obtain the calibration curve. 5 µl of the sample (PCA, MC, phloroglucinol, HPCA, THBA with a final concentration of 1 mM) or the standard were mixed with 100 µl of Folin-Ciocalteau reagent solution. PBS was used as a blank. After 5 minutes of incubation, 100 µl of Na_2CO_3 (75 g/l) was added

and the mixture was further incubated for 90 minutes at room temperature. After incubation, the absorbance at 700 nm was measured. The reducing capacity was expressed as gallic acid equivalents (GAE).

5.5.6.4 *FRAP assay*

Ferric reducing antioxidant power (FRAP) was evaluated by the ability of compounds to reduce ferric complex (Fe^{3+}) to ferrous form (Fe^{2+}). The test was performed using the FRAP KF-01-003 antioxidant assay kit and each step was done according to its manual. 10 µl of the sample, blank or standard (Fe^{2+} solution at 0-800 µM) were pipetted into the wells of the microplate. Then 220 µl of the „FRAP working solution“ was added into each well. The reaction mixture was shaken for 4 minutes, and the absorbance at 593 nm was measured. Antioxidant activity was expressed as Fe^{2+} equivalents.

5.5.6.5 *CUPRAC assay*

Cupric reducing antioxidant capacity (CUPRAC) assay is based on the ability of the tested sample to reduce the complex with Cu^{2+} to the complex with Cu^{+}. The formation of Cu^{+} complex provides a yellow color measurable at 450 nm.

The test was performed using the CUPRAC KF-01-005 antioxidant assay kit and each step was performed according to its manual. 40 µl of the sample, blank or standard (trolox solution at 0-2000 µM) were pipetted into the wells of the microplate. Then 40 µl of the “reagent A”, 40 µl of the “reagent B”, 40 µl of the “reagent C” and 40 µl of the ultrapure water were added to each well. After 30 minutes, the absorbance at 450 nm was measured. The results were expressed as trolox equivalent antioxidant capacity (TEAC).

5.5.6.6 *Inhibition of lipid peroxidation*

The method is based on the reaction of thiobarbiturate with malondialdehyde, the end product of lipid peroxidation of microsomes, whose formation was induced with *tert*-butyl hydroperoxide. Malondialdehyde reacts with thiobarbituric acid/trichloroacetic acid (TBA/TCA) resulting in the formation of the colored product. The less coloration the product had, the higher was the ability of the samples to protect microsomes from lipoperoxidation.

Rat microsomes (purchased from Sigma-Aldrich) were suspended in PBS and then centrifuged at 823 g for 5 min. The supernatant was removed. This step was repeated 5 times to eliminate all the sucrose present in the stored microsomes. Then the stock

suspension of microsomes of 1.25 mg/ml concentration was prepared. The reaction mixtures containing 50 µl of a sample, 50 µl of *tert*- butyl hydroperoxide (10 mM in PBS), and 400 µl stock suspension of microsomes (1.25 mg/ml) were prepared in microtubes. A positive control (50 µl of PBS, 50 µl of TBH and 400 µl of microsomes) and a blank (100 µl of PBS and 400 µl of microsomes) were also prepared. The samples were incubated for 1 hour at 37 °C. After incubation, 700 µl of the TBA/TCA mixture was added to each tube and then heated at 90 °C for 15 minutes. Then the tubes were cooled and centrifuged at 12247 g for 10 min. 200 µl of supernatant was pipetted in triplicate on the microplate. Then, absorbance was measured at 535 nm. The activity was calculated as the concentration of the tested compound which caused a 50% reduction in absorbance (IC_{50}).

5.6 Stability in saline

The tested compound was incubated in saline (solution of 0.9% of NaCl in water) at 37 °C for 3 hours. Aliquots of about 100 µl were taken every 30 min and shortly stored at 4 °C until HPLC analysis.

5.7 Statistical analysis

The results of antioxidant tests are expressed as mean ± standard deviation (SD). Student's unpaired t-test was used to determine the statistical significance of the differences between the measured antioxindant activity of parent and sulfated compounds. The results with p value lower than 0.05 were considered to be statistically significant.

6 Results

6.1 Kinetic study

The time course of sulfation of 4-methylcatechol, 3,4-dihydroxyfenylacetic acid, 3,4-dihydroxybenzoic acid, 1,3,4-trihydroxybenzoic acid, and phloroglucinol was investigated in this study. In addition, various amounts of sulfate donor, *p*-NPS, were added in order to determine its influence on sulfate formation. It was expected that the formation of various sulfate derivatives such as disulfates or trisulfates will occur.

6.1.1 Sulfation of 4-methylcatechol

The kinetic study with 4-methylcatechol (MC, **Fig. 15**) was performed with 0.7, 1.5, and 2.28 molar equivalents of *p*-NPS added. The only sulfated product observed during the sulfation of 4-methylcatechol was MCS. MCS appeared as one peak on the chromatogram, and as it was found later, that it is a mixture of two isomers 4-methylcatechol-1-*O*-sulfate and 4-methylcatechol-2-*O*-sulfate in the ratio 64:36. All quantity of MCS was synthesized during the first 20 minutes of the reaction. No MC (the parent compound) was observed afterwards.

6.1.2 Sulfation of phloroglucinol

In the case of sulfation of phloroglucinol (**Fig.16**) the only product which was successfully isolated and characterized was phloroglucinol-*O*-sulfate (PGS). However, the formation of other products was observed. One of these products was the unknown substance (X) that co-eluted with *p*-NPS. In most cases, *p*-NPS and the compound X appeared on chromatograms as a single broad peak (**Fig.17**).

According to NMR analysis of the mixture of *p*-NPS and X, the unknown substance could be phloroglucinol-di-*O*-sulfate. However, at the moment we were not able to confirm its structure and isolate it. The other unknown substances, which appeared during the sulfation were detected only in small amounts, and most of them were formed during the first hour of the reaction. Probably, they arise as a result of the polymerization of components of the reaction mixture.

Fig. 15 *Time-course of 4-methylcatechol (MC) sulfation. The percentages of compounds were calculated from the peak areas. The peak areas of para-nitrophenol (*p-*NP)+para-nitrophenyl sulfate (*p-*NPS)=100%; and MC+4-methylcatechol-*O*-sufate (MCS) =100%. Three various molar equivalents of* p-*NPS were added to reactions: 0.7 equivalents (A); 1.5 equivalents (B); 2.2 equivalents (C). MCS is a mixture of 4-methylcatechol-1-*O*-sufate and 4-methylcatechol-2-*O*-sufate in the ratio 64:36. All experiments were performed once.*

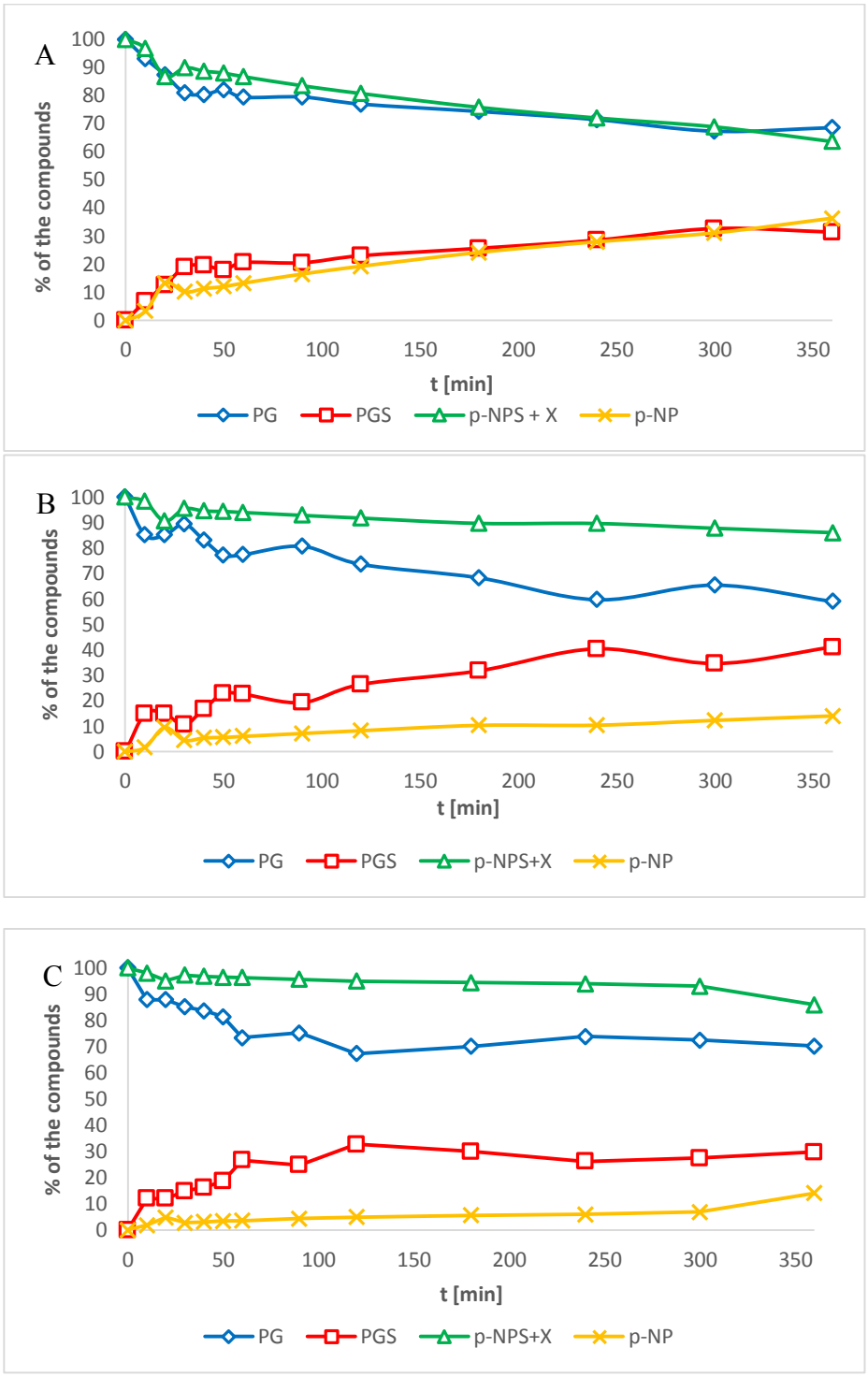


Fig. 16 *Time-course of phloroglucinol (PG) sulfation. The percentages of compounds were calculated from the peak areas. The peak areas of para-nitrophenol (*p*-NP) + para-nitrophenyl sulfate (*p*-NPS)+X=100%; and phloroglucinol + phloroglucinol-*O*-sulfate (PGS)=100%. Three various molar equivalents of* p*-NPS were added to reactions: 1.2 equivalents(A); 2.4 equivalents (B); 3.6 equivalents (C). X is an unknown substance that coeluted with* p*-NPS. All experiments were performed once.*

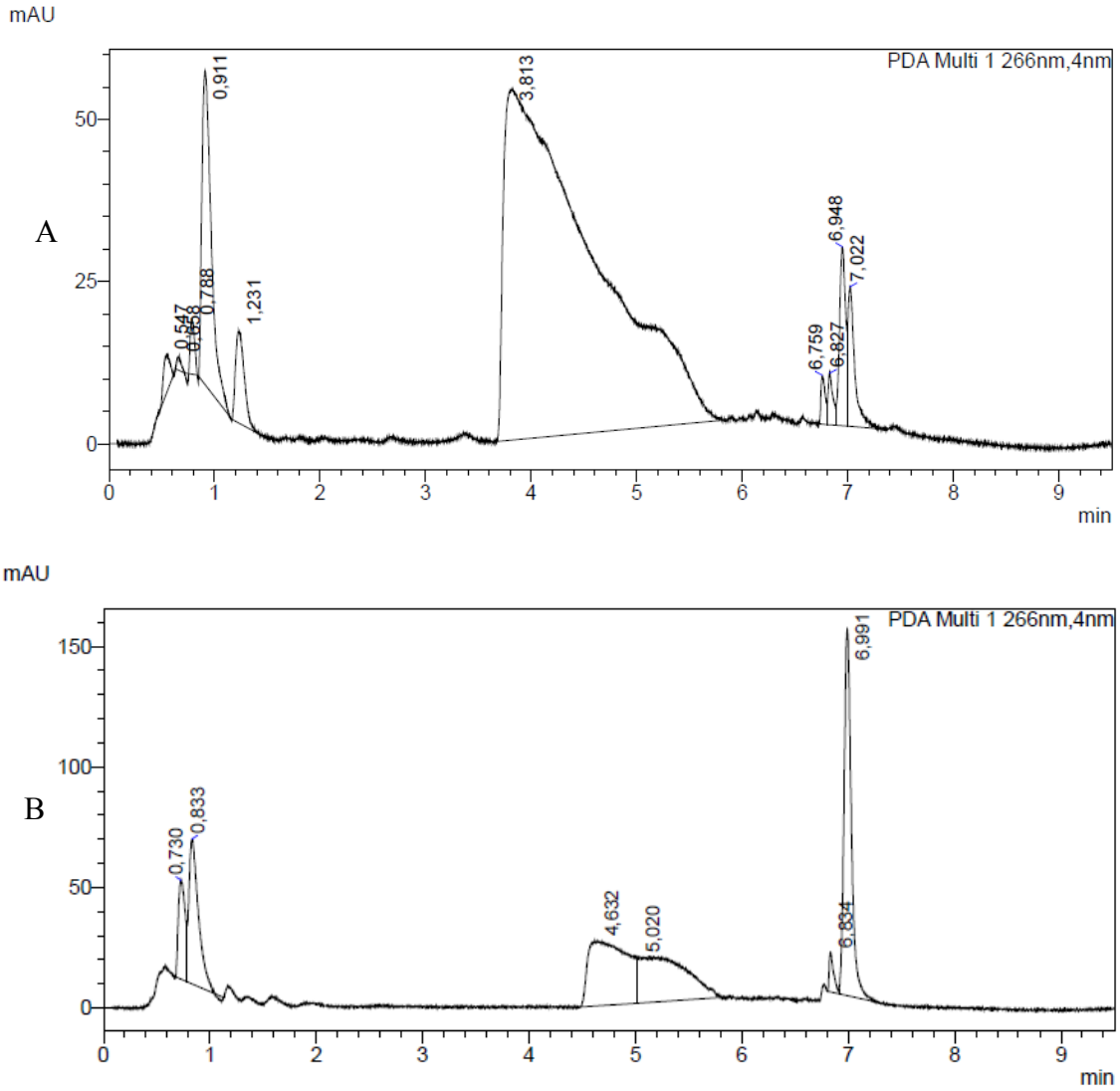


Fig. 17 *HPLC chromatograms from the kinetic study of phloroglucinol sulfation with 1.2 equivalents of* p-*NPS added at (A) 10 min and (B) 6 h after the start of the reaction. Retention times of the peaks in chromatogram B: 0.730-phloroglucinol-*O*-sulfate; 0.833 - phloroglucinol; 4.632 -* p-*NPS; 5.020 - X; 6.834 - an unknown substance; 6.991 -* p-*NP.*

With 2.4 equivalents of *p*-NPS added to the reaction, a slight increase of rate of sulfate formation was observed. In the case of 3.6 equivalents added, the situation with sulfate formation was almost the same as with 1.2 equivalents. Thus, the increase in the amount of *p*-NPS that was added had not significantly influenced the formation of sulfates.

6.1.3 Sulfation of protocatechuic acid

During sulfation of protocatechuic acid (PCA, **Fig. 18**) only one sulfated product was observed, PCAS (the mixture of protocatechuic acid-3-*O*-sulfate and protocatechuic acid-4-*O*-sulfate mixture in the ratio 7:3). The increased amount of *p*-NPS added did not influence the course of sulfation. There was a significant amount of unreacted PCA and *p*-NPS left at the 6th hour of reaction.

6.1.4 Sulfation of 2,3,5-trihydroxybenzoic acid

A kinetic study with 2,3,5-trihydroxybenzoic acid (THBA, **Fig. 19**) showed the formation of THBAS (the mixture of monosulfate and another sulfated product of 2,3,4-trihydroxybenzoic acid. However, we were not able to determine the structures, see chapter 6.3). There were no other sulfated products formed in this reaction mixture. The sulfate was gradually formed during the reaction. The effectiveness of sulfation of THBA was better than of PCA, however, there was still a high amount of unreacted THBA and *p*-NPS at the end of the reaction. Similarly to the kinetic studies with PCA and phloroglucinol, in this case, the increased amount of *p*-NPS added did not significantly affect the course of the sulfation process of THBA.

6.1.5 Sulfation of homoprotocatechuic acid

In the case of homoprotocatechuic acid (HPCA, **Fig. 20**), no formation of sulfated products was observed. However, the amount of *p*-NPS decreased and the amount of *p*-NP increased while HPCA quantity only slightly decreased (down to 82-97 % of its initial quantity) while its relative amount seemed to be constant.

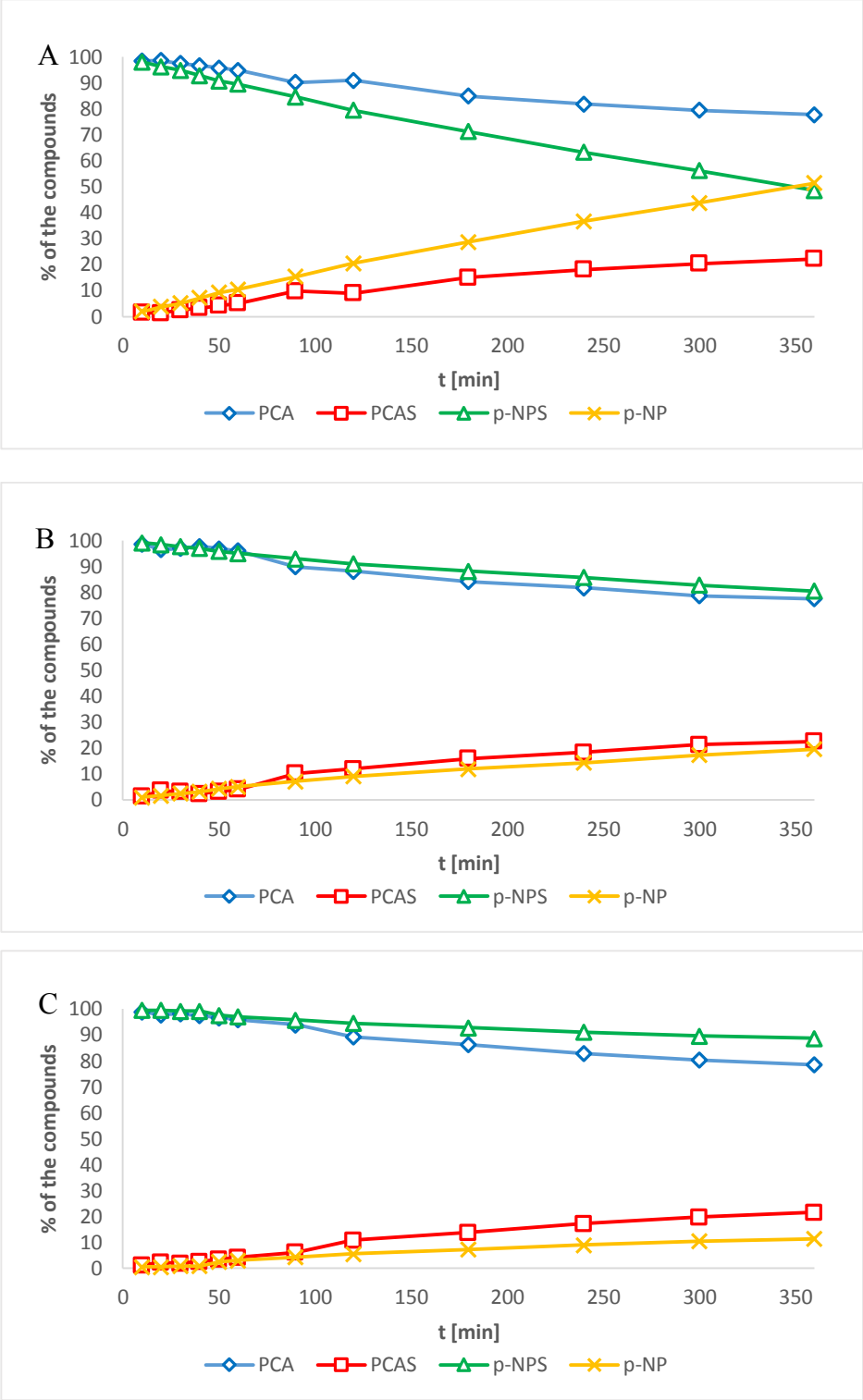


Fig. 18 *Time-course of protocatechuic acid (PCA) sulfation. The percentages of compounds were calculated from the peak areas. The peak areas of para-nitrophenole* (p-*NP)+ para-nitrophenyl sulfate* (p-*NPS)=100%; and PCA + PCAS=100%. Three various molar equivalents of* p-*NPS were added to reactions: 0.7 equivalents(A); 1.5 equivalents (B); 2.2 equivalents (C). PCAS is a mixture of protocatechuic acid-3-*O*-sulfate and protocatechuic acid-4-*O*-sulfate in the ratio 7:3). All experiments were performed once.*

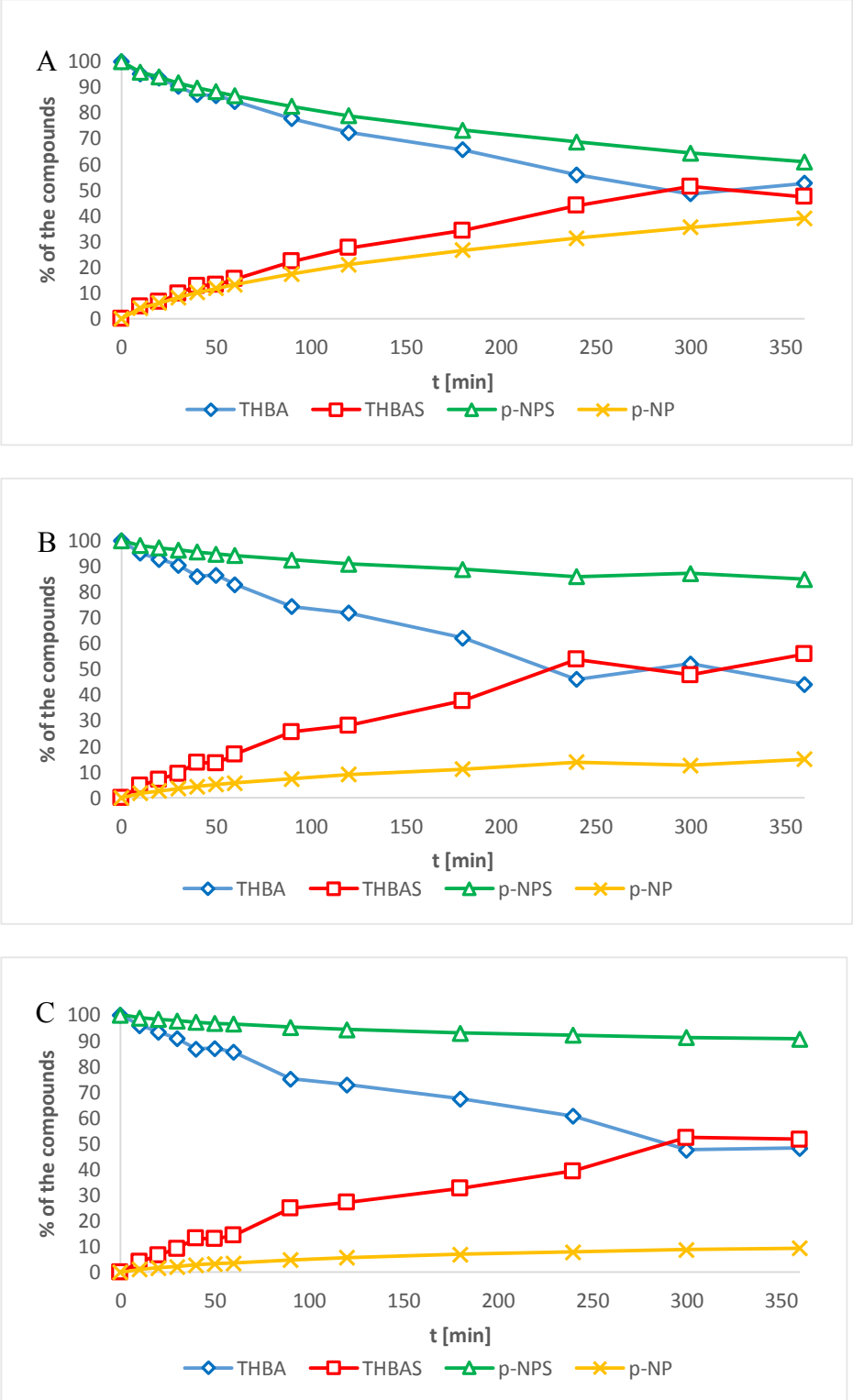


Fig. 19 *Time-course of 2,3,4-trihydroxybezoic acid (THBA) sulfation. The percentages of compounds were calculated from the peak areas. The peak areas of para-nitrophenol (*p-*NP) + para-nitrophenyl sulfate (*p-*NPS)=100%; and THBA+THBAS=100%. Three various molar equivalents of* p-*NPS were added to reactions: 1,2 equivalents(A); 2,4 equivalents (B); 3,6 equivalents (C). THBAS is a mixture of two sulfated products of 2,3,4-trihydroxybenzoic acid (the exact structures were not possible to determine). All experiments were performed once.*

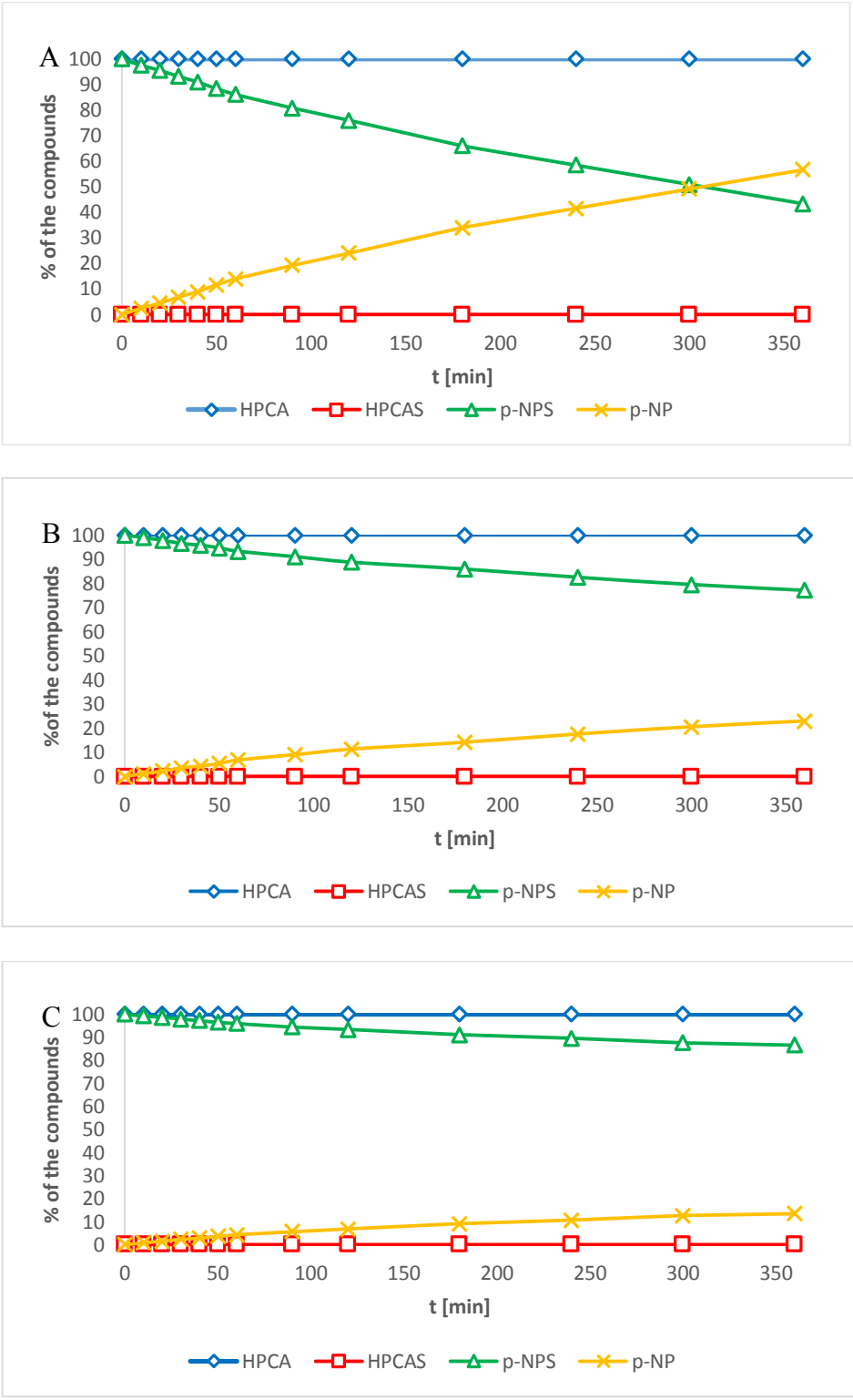


Fig. 20 *Time-course of homoprotocatechuic acid (HPCA) sulfation. The percentages of compounds were calculated from the peak areas. The peak areas of para-nitrophenol (*p-*NP)+para-nitrophenyl sulfate (*p-*NPS)=100%; and HPCA+HPCAS=100%. Three various molar equivalents of* p-*NPS were added to reactions: 0.7 equivalents(A); 1.5 equivalents (B); 2.2 equivalents (C). HPCAS is the sulfate of homoprotocatechuic acid, which was expected to be formed. All experiments were performed once.*

6.2 Purification of sulfated products

The main selected method for reaction mixture purification was gel chromatography. There were attempts to purify reaction mixtures with PCA and MC using preparative HPLC in order to compare the efficiency of the two methods. As a result, the separation of the reaction mixture with preparative HPLC was not more effective than the purification using gel chromatography.

Some complications occurred with the purification of phloroglucinol reaction mixture on Sephadex LH-20 gel chromatography column. As the reaction mixture was passing through the column, a colored contamination gradually started to occur and retained in the column. The contaminant could probably be a product of polymerization of some components of the reaction mixture, and the longtime of purification on the column (35-45 hours) promoted its formation. However, the exact origin of the contamination was not determined. The attempts to eliminate it by repeated flowing of the column with mobile phase (80% methanol) or DMSO had failed. Finally, the contamination was successfully dissolved with ethyl acetate, however, it was accompanied by Sephadex LH-20 gel loss. Therefore, we decided to use preparative HPLC instead of gel chromatography for purification of this reaction mixture. An advantage of preparative HPLC is the rapid passage through the column (about 20 min), and therefore it prevents from possible formation of polymerization products. As in the case of gel chromatography, the fractions of the reaction mixture needed to be purified at least one more time.

Another complication had occurred during the attempts to purify THBA and PCA reaction mixtures. It was found using NMR analysis that the fractions with sulfates contained methanol, formic acid and water. All of these compounds were used during sulfate preparation: methanol was used as mobile phase in gel chromatography, formic acid was used for pH adjustment and water was used in multiple steps, such as dissolving compounds before lyophilization. There were several attempts to purify these fractions and to evaporate the solvents, however, it brought no significant results. Since the fractions with THBAS and PCAS had a low and not precisely determined content of sulfates they were not further used for antioxidant tests.

MCS was obtained as the mixture of two isomers 4-methylcatechol-1-*O*-sulfate and 4-methylcatechol-2-*O*-sulfate (in the ratio 64:36). The above mentioned separation methods failed to separate these two isomers.

6.3 Characterization of sulfated products

The sulfated products were analyzed by HPLC, MS, NMR and characterized by their UV maxima and retention times (**Tab. 1**). Their identity was determined by combination of MS and NMR data.

The highest yields were observed in the case of MC sulfation (ca 58%). In the average 180 mg of MCS were isolated from one reaction. The yields in phloroglucinol sulfation were also relatively high (ca 44%), on average 73 mg of PGS was isolated from one reaction. The yields and the purity of THBAS and PCAS were not possible to determine due to the contamination with the solvents (see chapter 6.2, **Tab. 1**).

Tab. 1 Characterization and the yields of sulfated products

	MCS[a]	**PGS**[b]	**PCAS**[c]	**THBAS**[d]
Purity (%)	93	90	ND[e]	ND
Yields (%)	58	44	ND	ND
UV max.	275/472/329/ 378	272/440/367/ 402	254/380/327/ 476/352	210/256/295/ 375/394
Retention time (min)	1.781	0.730	2.113	2.421
Found m/z $[M - H]^-$	203	205	233	249[f]

[a] *4-methylcatechol-1-*O*-sulfate and 4-methylcatechol-2-*O*-sulfate (in the ratio 64:36)*

[b] *phloroglucinol-*O*-sulfate*

[c] *protocatechuic acid-3-*O*-sulfate and protocatechuic acid-4-*O*-sulfate mixture (in ratio 7:3)*

[d] *the mixture of two sulfated products of 2,3,4-trihydroxybenzoic acid (monosulfate and another sulfated product, exact structures of which could not be determined)*

[e] *not determined*

[f] *the data is related to monosulfate of THBA*

NMR analysis had shown that MCS fractions always contained a mixture of two isomers 4-methylcatechol-1-*O*-sulfate and 4-methylcatechol-2-*O*-sulfate (in the ratio 64:36, **Tab. 2, 3**). PCAS was also formed as a mixture of protocatechuic acid 3-*O*-sulfate and protocatechuic acid 4-*O*-sulfate (in the ratio 7:3, **Tab. 4, 5**). Phloroglucinol-*O*-sulfate was also characterized (**Tab. 6**).

Tab. 2 ^{13}C and ^{1}H NMR data of 4-methylcatechol-1-*O*-sulfate

Atom	**δ_C [ppm]**	***m***	**δ_H [ppm]**	***n* (H)**	***m***	***J* [Hz]**
1	134.11	S	-	0	-	
2	117.70	D	6.630	1	dq	2.1, 0.8
3	148.82	S	-	0	-	
4	138.58	S	-	0	-	
5	122.88	D	6.937	1	d	8.1
6	119.78	D	6.538	1	ddq	8.1,2.1,0.8
1-Me	20.36	Q	2.188	3	m	

δ - chemical shift, m - multiplicity, n - number of hydrogens, J - interaction constant

Tab. 3 ^{13}C and ^{1}H NMR data of 4-methylcatechol-2-*O*-sulfate

Atom	**δ_C [ppm]**	***m***	**δ_H [ppm]**	***n* (H)**	***m***	***J* [Hz]**
1	128.06	S	-	0	-	
2	123.54	D	6.904	1	dq	2.1, 0.8
3	140.46	S	-	0	-	
4	146.76	S	-	0	-	
5	116.88	D	6.693	1	d	8.1
6	125.18	D	6.749	1	ddq	8.1,2.1,0.8
1-Me	19.95	Q	2.175	3	m	

δ - chemical shift, m - multiplicity, n - number of hydrogens, J - interaction constant

Tab. 4 ^{13}C and ^{1}H NMR data of protocatechuic acid 3-*O*-sulfate

Atom	**δ_C [ppm]**	***m***	**δ_H [ppm]**	***n* (H)**	***m***	***J* [Hz]**
1	124.7^{H}	S	-	0	-	
2	123.79	D	7.766	1	d	2.0
3	140.33	S	-	0	-	
4	152.26	S	-	0	-	
5	116.13	D	6.810	1	d	8.4
6	126.06	D	7.516	1	dd	8.4, 2.0
CO	168.16	S	-	0	-	
OH		-	n.d.			

δ - chemical shift, m - multiplicity, n - number of hydrogens, J - interaction constant

Tab. 5 ^{13}C and ^{1}H NMR data of protocatechuic acid 4-*O*-sulfate

Atom	δ_C [ppm]	*m*	δ_H [ppm]	*n* (H)	*m*	*J* [Hz]
1	n.d.	S	-	0	-	
2	117.7[H]	D	7.342	1	d	2.0
3	148.0[H]	S	-	0	-	
4	n.d.	S	-	0	-	
5	121.3H	D	7.209	1	d	8.3
6	120.50	D	7.301	1	dd	8.3, 2.0
CO	n.d.	S	-	0	-	
OH		-	n.d.			

[H] *HSQC/HMBC readout; n.d. - not detected; δ - chemical shift, m - multiplicity, n - number of hydrogens, J - interaction constant*

Tab. 6 ^{13}C and ^{1}H NMR data of phloroglucinol-*O*-sulfate

Atom	δ_C [ppm]	*m*	δ_H [ppm]	*n* (H)	*m*	*J* [Hz]
1, 3	98.87	D	6.102	2	d	2.2
2	154.87	S	-	0	-	
4, 6	158.00	S	-	0	-	
5	97.50	D	5.881	1	t	2.2
1,3-OH	-	-	9.036	2	br s	

δ - chemical shift, m - multiplicity, n - number of hydrogens, J - interaction constant

NMR analysis had also shown that the products of 2,3,4-trihydroxybenzoic acid sulfation were two sulfated products. However, the position and the extent of sulfation of these two sulfated compounds could not be determined. Therefore, in combination with the MS data the formed sulfates of THBA were established to be a mixture of one monosulfate with undetermined site of sulfation and another sulfate with unknown structure.

Log *P* values of all sulfated products and parent compounds calculated using the Molinspiration property engine v2016.1 (Molinspiration Cheminformatics, 2016, **Tab. 7**) clearly showed the increases in compound hydrophilicity after sulfation.

Tab. 7 The calculated Log *P* values of sulfated products and parent compounds

	MC[a]	MCS[b]	THBA[c]	THBAS[d]	PCA[e]	PCAS[f]	PG[g]	PGS[h]
Log *P*	1.42	-1.02	0.90	-1.54	0.88	-1.56	0.43	-1.98

[a] *4-methylcatechol*
[b] *4-methylcatechol-1-*O*-sulfate and 4-methylcatechol-2-*O*-sulfate (in the ratio 64:36)*
[c] *2,3,4-trihydroxybenzoic acid*
[d] *2,3,4-trihydroxybenzoic acid monosulfate*
[e] *protocatechuic acid*
[f] *protocatechuic acid-3-*O*-sulfate and protocatechuic acid-4-*O*-sulfate mixture (in ratio 7:3)*
[g] *phloroglucinol*
[h] *phloroglucinol-*O*-sulfate*

6.4 Stability of sulfates in saline

The stability of 4-methylcatechol-*O*-sulfate (the mixture of 4-methylcatechol-1-*O*-sulfate and 4-methylcatechol-2-*O*-sulfate in a ratio 64:36) and phloroglucinol-*O*-sulfate in the conditions close to the mammal physiology was tested for further studies of these substances. Both substances remained unchanged and no parent compound formation was observed at the end of study (**Fig. 21,22**), thus they were considered to be stable in physiological saline at 37 °C for up to 3 hours.

6.5 Antioxidant activity

The antioxidant and antiradical activity of sulfates with sufficient purity (PGS, MCS) and all the parent compounds was measured with six *in vitro* tests (**Tab. 8**). THBAS and PCAS fractions had not precisely determined content of sulfates, therefore they were not tested (see chapter 6.2).

6.5.1 DPPH scavenging

The results of DPPH test were expressed as the IC_{50} value (concentration of the substance at which 50% of the radical was inhibited, **Tab. 8**). The most active substance was HPCA (47 μM). IC_{50} value of MCS value was higher than the highest concentration tested (i.e., > 10 mM), while IC_{50} of MC, its parent compound, was 69 μM. A similar situation was in the case of PGS and its parent compound phloroglucinol. The IC_{50} value of PGS was higher than the highest concentration tested (i.e., > 10 mM), and phloroglucinol has IC_{50} 1173 μM. Thus, in comparison with sulfated products the parent compound had always a lower activity (higher IC_{50}).

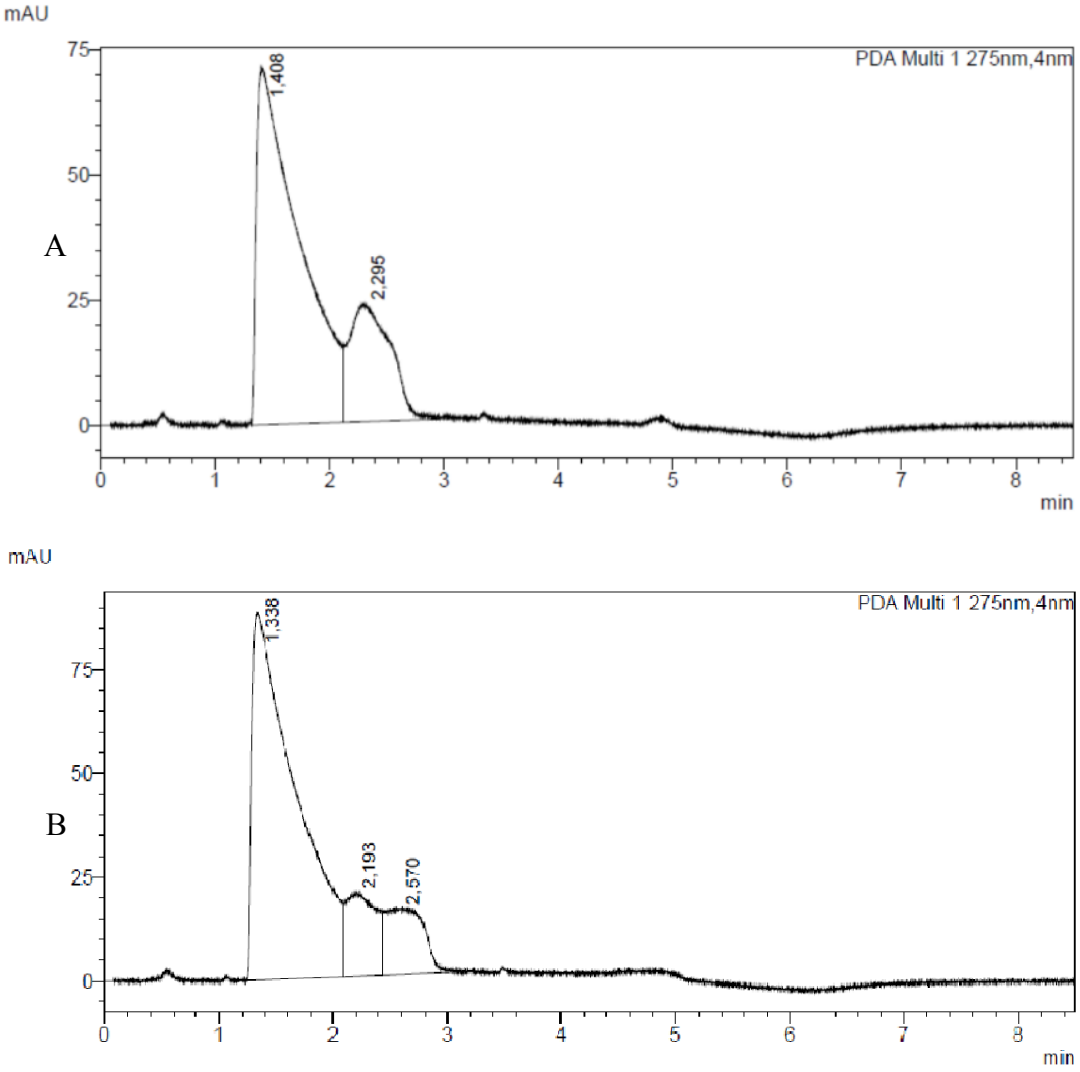


Fig. 21 *The HPLC chromatograms of 4-methylcatechol-O-sulfate (the mixture of isomers) from the study of stability in physiological saline at (A) 30 min and (B) 3 h. The appearance of the new peak at the retention time of 2.570 min in chromatogram B could be attributed to the presence of 4-methylcatechol-O-sulfate with another cation (e.g. Na^+ or K^+). In chromatogram A that peak is not visible due to the presence of a larger peak at the retention time of 2.295 min.*

6.5.2 Folin-Ciocalteau reduction assay

In FCR test, the results were expressed as gallic acid equivalents (GAE, **Tab. 8**). Similarly to other antioxidant tests, the most potent in this test was HPCA (1.8 GAE) followed by THBA (1.10 GAE). MC (0.71 GAE) had slightly higher activity than its sulfate, MCS (0.49 GAE, $p<0.05$). However, unlike in the other tests, the activity of phloroglucinol (0.56 GAE) was slightly lower than its sulfate, PGS (0.70 GAE, $p<0.05$).

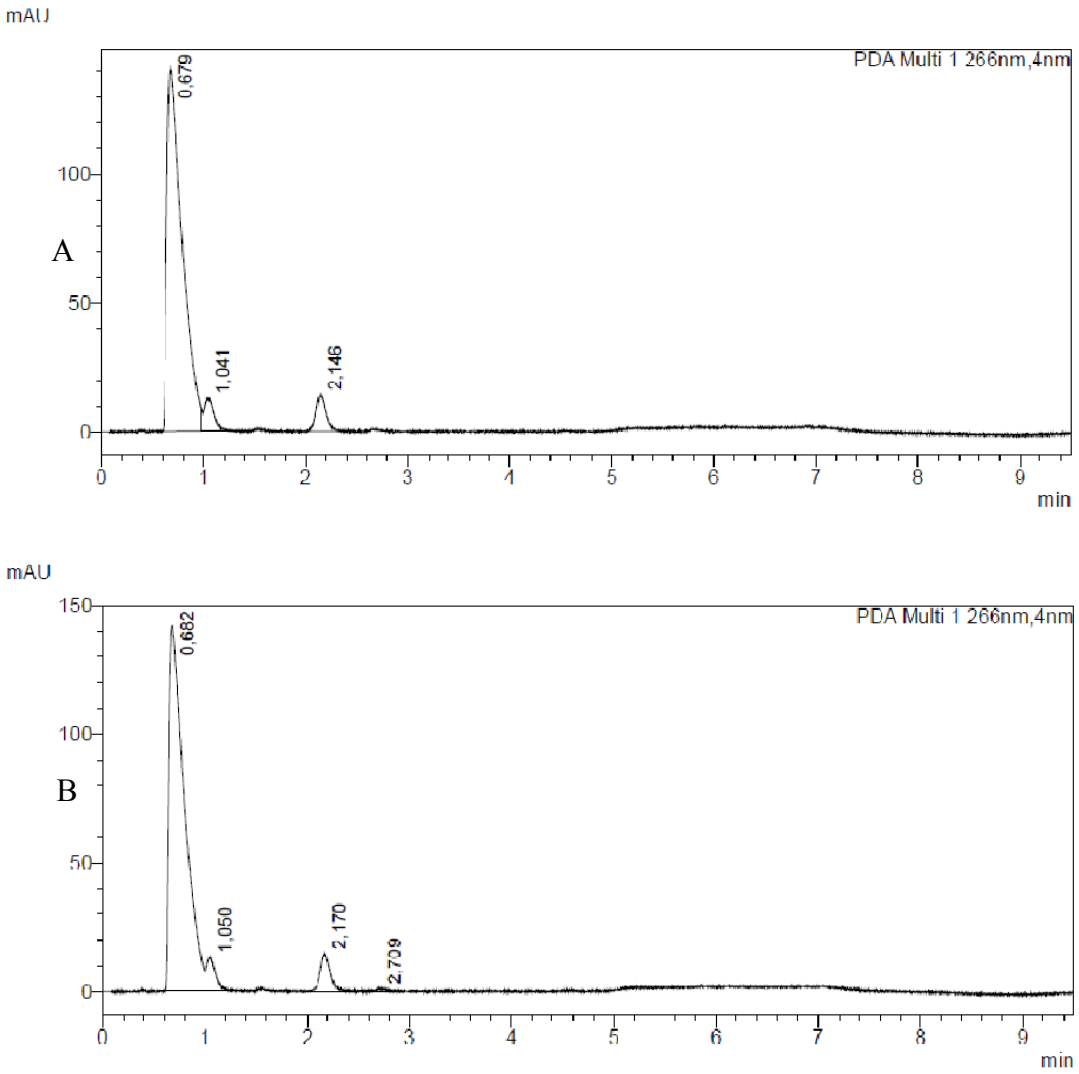


Fig. 22 *The HPLC chromatograms of phloroglucinol-*O*-sulfate from the study of stability in physiological saline at (A) 30 min and (B) 3 h.*

6.5.3 Ferric reducing antioxidant power

In the FRAP test, the results were expressed as Fe^{2+} equivalents (**Tab. 8**). The highest activity was observed in HPCA (2.29 equivalents). MC (1.2 equivalents) was roughly 240 times more potent than MCS (0.005 equivalents, $p<0.05$). Phloroglucinol had very low activity (0.008 equivalents) in comparison with other parent compounds. In the case of PGS, the highest concentration tested (i.e., 50 mM) was not sufficient for a proper FRAP determination (approximately 0.003 Fe^{2+} equivalents). Thus, sulfated products had lower activity than their parent compounds.

Tab. 8 Biological activity of phenolic substances and some of their sulfates

	DPPH (IC_{50}, µM)[a]	ABTS (CEAC)[b]	FCR (GAE)[c]	FRAP (Fe^{2+} equivalents)[d]	CUPRAC (TEAC)[e]	ILP (IC_{50}, µM)[f]
4-methylcatechol (MC)	68 ± 4	0.10 ± 0.00	0.71 ± 0.01	1.2 ± 0.0	2.1 ± 0.1	128 ± 5
4-methylcatechol-*O*-sufate (MCS)[g]	> 10000	0.04 ± 0.00*	0.49 ± 0.02*	0.005 ± 0.000*	0.096 ± 0.003*	9221 ± 152*
homoprotocatechuic acid (HPCA)	47 ± 2	0.23 ± 0.01	1.8 ± 0.1	2.29 ± 0.02	4.2 ± 0.3	1993 ± 41
protocatechuic acid (PCA)	87 ± 2	0.106 ± 0.004	0.98 ± 0.06	1.03 ± 0.04	2.8 ± 0.2	1229 ± 54
2,3,4-trihydroxybenzoic acid (THBA)	75 ± 1	0.210 ± 0.009	1.10 ± 0.02	1.32 ± 0.04	1.6 ± 0.1	1295 ± 40
phloroglucinol	1185 ± 34	0.13 ± 0.00	0.56 ± 0.02	0.008 ± 0.000	0.11 ± 0.01	3192 ± 81
phloroglucinol-*O*-sulfate (PGS)	>10000	0.03 ± 0.00*	0.70 ± 0.01*	<0.00[h]*	0.041 ± 0.004*	20568 ±197*

[a] *1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging*

[b] *2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging (vitamin C equivalents antioxidant capacity)*

[c] *Folin-Ciocalteau reduction (gallic acid equivalents)*

[d] *ferric reducing antioxidant power (equivalents of Fe^{2+})*

[e] *cupric reducing antioxidant capacity (trolox equivalent antioxidant capacity)*

[f] *anti-lipoperoxidant activity in rat liver microsomes*

[g] *mixture of 4-methylcatechol-1-*O*-sulfate and 4-methylcatechol-2-*O*-sulfate in the ratio 64:36*

[h] *the highest concentration tested (i.e. 50 mM) was not sufficient for proper FRAP determination (approximately 0.003 Fe^{2+} equivalents)*

** significant difference of the antioxidant activity of the sulfates vs. the corresponding parent compound ($p<0.05$)*

6.5.4 $ABTS^{+\cdot}$ scavenging

The results of the tests were expressed as equivalents of ascorbic acid (CEAC, (**Tab. 8**). The highest activity was observed in HPCA (0.23 CEAC) followed by THBA (0.21 CEAC). MCS (0.04 CEAC) had about 2 times lower activity ($p<0.05$) than MC (0.10 CEAC). PGS (0.03 CEAC) had a 4 times lower activity ($p<0.05$) than phloroglucinol (0.13 CEAC). Similarly to the DPPH test, sulfated products had lower activity than their parent compounds.

Generally, the reaction was accompanied by decreasing of the intensity of the green color. However, an unexpected change of the color of phloroglucinol (from green to bright yellow) was observed after ABTS reagent was added (**Fig. 23**).

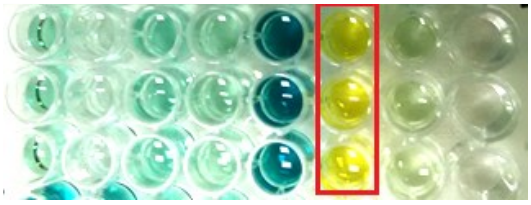


Fig. 23 *The yelllow colored samples with phloroglucinol after ABTS reagent was added.*

6.5.5 Cupric reducing antioxidant capacity

The results of CUPRAC assay were expressed as trolox equivalent antioxidant capacity (TEAC, **Tab. 8**). HPCA was most potent compound (4.2 TEAC), followed by PCA (2.8 TEAC). Sulfated phenolic compounds exhibited a lesser activity than its parent compounds. Thus, MCS (0.096 TEAC) was 20 times less active ($p<0.05$) than its parent compound MC (2.1 TEAC), while PGS (0.041 TEAC) was about 3 times less active ($p<0.05$) than phloroglucinol (0.11 TEAC). Similarly to the results of the FRAP assay, phloroglucinol had very low activity in comparison to other parent compounds.

6.5.6 Inhibition of lipid peroxidation

The results of the test of inhibition of lipoperoxidation were expressed as IC_{50} value (**Tab. 8**). The test results followed a similar trend as DPPH scavenging. The most efficient compound was MC (IC_{50} 127 μM). Its sulfate MCS had roughly 70 times lower activity (IC_{50} 9221 μM, $p<0.05$). PGS had ca 6 times lower activity in comparison to the parent compound, phloroglucinol (IC_{50} 20568 μM and 3191 μM respectively, $p<0.05$). Thus, sulfated phenolic compounds exhibited a lesser activity than their parent compounds.

7 Discussion

The arylsulfotransferase from *Desulfitobacterium hafniense* was shown to be a good tool for sulfation of various substrates. However, no sulfated products were observed in the case of homoprotocatechuic acid (HPCA) sulfation. The reason of ineffective sulfation is possibly related to the structure of HPCA. The carboxylic group could possibly interfere (e.g., due to its shape, or polarity) with the ability of the substrate to bind to the active site of the enzyme known to prefer aromatic and lipophilic substrates (van der Horst et al. 2012).

The reason of contamination of sulfates of protocatechuic (PCAS) and 2,3,4-trihydroxybenzoic acid (THBAS) with methanol, water, and formic acid could be related to the properties of these sulfates that lead to the formation of hydrogen bonds with the solvents. The sulfation of phloroglucinol was complicated by the formation of side products during reaction and purification. However, these complications could be overcome by using preparative HPLC instead of gel chromatography for the the purification (see chapter 6.2). These kinds of complications were not observed in the previous studies of sulfation of flavonoids (Purchartová et al. 2015; Valentová et al. 2018).

No sulfated product was observed in the kinetic study of HPCA sulfation (see section 6.1.5). However, during the reaction the amount of *p*-NPS was decreasing. A possible reason of this outcome could be the decomposition of *p*-NPS, which leads to the formation of *p*-NP. However, in the study of van der Horst et al. (2012) *p*-NPS appeared to be stable in almost similar conditions. Another possible explanation could be that the sulfate of HPCA was produced but quickly decomposed. Alternatively, the formed sulfate could instantly react with some of the reagents of the reaction mixture, which resulted in the formation of various polymeric structures. The last two hypotheses are supported by the fact that the amount of HPCA in the kinetic study was slightly decreasing.

In the case of phloroglucinol, PCA and THBA sulfation studies, the increased amount of added *p*-NPS did not significantly influence the sulfation process. For the sulfation, the optimal ratio of *p*-NPS to a tested compound was 1.2 or less equivalents. Since the amounts of unreacted parent compounds were significant, the effectiveness of sulfation could possibly be increased if the time of the reaction would be prolonged.

However, another study on quercetin sulfation showed that the reaction time of more than 5 hours led to the formation of polymeric insoluble by-products and to the hydrolysis of the sulfated products (Valentová et al. 2017).

The kinetic study of the reaction of 4-methylcatechol (MC) with 0.7 equivalents of *p*-NPS showed that no amount of parent compound was left after 20 min of the reaction (see section 6.1.1). However, with that low *p*-NPS content at least some amount of MC should have remained. It is possible that MC polymerized and subsequently precipitated. Therefore, no MC was observed on the chromatograms of the kinetic studies.

Generally, it was expected that excess of added *p*-NPS will increase the rate of sulfate formation or promote the formation of other derivatives like disulfated or trisulfated phenolic compounds. However, the excessive amount of *p*-NPS did not mostly had an influence on sulfate formation in the present study. One possibility is the potential instability of the formed disulfated or trisulfated compounds, which therefore were not detected. The formation of phloroglucinol-di-*O*-sulfate possibly took place, however the attempts to isolate this compound were unsuccessful (see section 6.1.2). A more likely reason of the absence of disulfates or trisulfates could be that the enzyme had low affinity to the formed hydrophilic monosulfates of phenolic compounds (**Tab. 7**). This is in line with the fact that AST from *D. hafniense* was previously shown to have an affinity to a range of compounds such as estradiol, enkephalin, phenolic compounds and non-phenolic alcohols. Generally, it seems that the enzyme has higher affinity to relatively lipophilic compounds (van der Horst et al. 2012).

NMR analysis had shown that the fractions with MCS (4-methylcatechol sulfates) and PCAS always contained two isomers (see chapter 6.3). It was found that in other studies, MCS was also obtained as a mixture of chromatographically indistinguishable isomers of MCS in the same ratio (64:36) (Figueira et al. 2017, Pimpao at al. 2015).

All the antioxidant and antiradical tests with one exception (PGS in FCR assay) showed lower activity of the sulfated products in comparison to their parent compounds (see chapter 6.5). Generally, the number and position of hydroxyl groups in phenolic compounds are critical for their antioxidant potential. Thus, for example, polyphenols have generally higher activity than simple phenolic compounds. A catechol (-*ortho* dihydroxy) moiety present in MC, PCA, HPCA and a galloyl group (present in THBA) are important for metal ion chelating ability, which is closely connected with the

antioxidant activity (Andjelkovic et al. 2005). The observed weak antioxidant activity of sulfated products is most likely caused by the blocking of this catechol moiety by sulfation. It may also explain a significantly low activity of phloroglucinol (which does not possess catechol- or galloyl- group) in some tests in comparison to other parent compounds (**Tab. 8**).

The relatively high activity of MC (**Tab. 8**, IC_{50} 128 μM) in the test of inhibition of lipoperoxidation could be associated with relatively high lipophilicity of MC (**Tab. 7**), since lipophilic compounds penetrate through lipid membrane of microsomes better and therefore may exhibit higher protection in the microsomal membrane (Yasuda et al. 2013).

No publications with data on antioxidant activities of MCS and PGS were found to compare with results obtained in this work. However, some data regarding the antioxidant activity of parent compounds such as phloroglucinol and PCA are available. In the study of Archana and Vijayalakshmi (2018), DPPH scavenging activity of phloroglucinol was evaluated. Phloroglucinol had IC_{50} of approximately 300 μM (calculated from μg/ml). This value is ca 4 times lower than the value obtained in this work (**Tab. 8**, IC_{50} of 1184 μM). DPPH scavenging activity of PCA was published in the study of Villano et al. (2007). PCA had ca 8 times lower IC_{50} (11 μM) than in this work (87 μM). These dissimilar results could be caused by differences in the experimental setup of DPPH assay. Anyway, the IC_{50} found in this study are clearly supraphysiological or at least in relation to available food kinetic studies (Pimpao et al. 2015).

In the last years, a part of the scientific community has started to doubt the importance of *in vitro* antioxidant tests of polyphenols as a prediction of health benefits in animal or human studies (Gafner 2018). Instead, polyphenols and other biologically active natural compounds had obtained a lot of attention mostly for their action on the receptor level. Therefore, despite the low activity of sulfated phenolic compounds *in vitro*, further *in vivo* or *in situ* studies are needed to discover their potential biological activity.

8 Conclusion

In this work, pure sulfated products of 4-methylcatechol and phloroglucinol were prepared using arylsulfotransferase from *Desulfitobacterium hafniense*. Sulfated product of 4-methylcatechol was obtained as the mixture of two inseparable isomers 4-methylcatechol-1-*O*-sulfate and 4-methylcatechol-2-*O*-sulfate in the ratio 64:36. Phloroglucinol-*O*-sulfate was the only isolated product of phloroglucinol sulfation. Sulfated products of protocatechuic acid and 2,3,4-trihydroxybenzoic acid were also synthesized. Sulfated product of protocatechuic acid was always present as a mixture of two isomers protocatechuic acid-3-*O*-sulfate and protocatechuic acid-4-*O*-sulfate in the ratio 7:3. The products of sulfation of 2,3,4-trihydroxybenzoic acid were obtained as the mixture of a monosulfate with undetermined site of sulfation and another sulfate with unknown structure. The attempts to purify sulfated products of 2,3,4-trihydroxybenzoic acid and protocatechuic acid have not been successful as yet and therefore were not included in antioxidant testing. In the case of homoprotocatechuic acid, no sulfated product was observed. All the sulfated products were analyzed and characterized using HPLC, MS, and NMR.

The studies of time-courses of sulfation (kinetic studies) of all the phenolic compounds were conducted. In the case of 4-methylcatechol sulfation, almost all the amount of sulfates was formed within the first 20 min of reaction. Sulfation of other substances was mostly constant and sulfates were formed gradually during all 6 hours of the reaction. In addition, various amounts of sulfate donor (*p*-NPS) were added at kinetic study in order to determine its influence on sulfate formation. As a result, the excessive amount of added *p*-NPS generally had not increased sulfate formation and had no influence on the formation of other sulfate derivatives.

The antioxidant activity of sulfates with sufficient purity (PGS, MCS) and of all the parent compounds was measured with six *in vitro* tests, such as DPPH (1-diphenyl-2-picrylhydrazyl) and ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging, ability to reduce Folin-Ciocalteau reagent, ferric ions and copper ions, and inhibition of lipoperoxidation in rat liver microsomes damaged by *tert*-butylhydroperoxide. As a result, the sulfation had diminished the antioxidant activity of parent compounds. However, the Folin-Ciocalteau reduction assay had shown a slightly higher activity of phloroglucinol-*O*-sulfate comparing to its parent compound.

Pure sulfated phenolic compounds prepared in this work could be used in further studies and contribute to the better understanding of the biological activity of phase II metabolites of phenolic compounds.

9 References

Abenavoli L, Capasso R, Milic N, Capasso F. Milk thistle in liver diseases: past, present, future. Phytother Res 2010;24(10):1423-32.

Almeida AF et al. Bioavailability of quercetin in humans with a focus on interindividual variation. Compr Rev Food Sci Food Saf 2018;17(3):714-31.

Alvarez-Cilleros D, Martin MA, Ramos S. (-)-Epicatechin and the colonic 2,3-dihydroxybenzoic acid metabolite regulate glucose uptake, glucose production, and improve insulin signaling in renal NRK-52E cells. Mol Nutr Food Res 2018;62(4):1-9.

Alvarez-Cilleros D, Ramos S, Goya L, Martin MA. Colonic metabolites from flavanols stimulate nitric oxide production in human endothelial cells and protect against oxidative stress-induced toxicity and endothelial dysfunction. Food Chem Toxicol 2018;115:88-97.

Andjelkovic M et al. Iron-chelation properties of phenolic acids bearing catechol and galloyl groups. Food Chem 2006;98(1):23-31.

Archana I, Vijayalakshmi K. Antioxidant potential of phloroglucinol; an *in vitro* approach. Int J Pharm Sci Res 2018;9(7):2947-51.

Arts ICW, Hollman PCH. Polyphenols and disease risk in epidemiologic studies. Am J Clin Nutr 2005;81(1):317-25.

Ayuso-Fernandez I, Galmes MA, Bastida A, Garcia-Junceda E. Aryl sulfotransferase from Haliangium ochraceum: A versatile tool for the sulfation of small molecules. Chemcatchem 2014;6(4):1059-65.

Badenhorst CPS, van der Sluis R, Erasmus E, van Dijk AA. Glycine conjugation: importance in metabolism, the role of glycine N-acyltransferase, and factors that influence interindividual variation. Expert Opin Drug Metab Toxicol 2013;9(9):1139-53.

Boušová I, Skálová L. Inhibition and induction of glutathione S-transferases by flavonoids: possible pharmacological and toxicological consequences. Drug Metab Rev 2012;44(4):267-86.

Braune A, Blaut M. Bacterial species involved in the conversion of dietary flavonoids in the human gut. Gut Microbes 2016;7(3):216-34.

Buhl AE, Waldon DJ, Baker CA, Johnson GA. Minoxidil sulfate is the active metabolite that stimulates hair-follicles. J Invest Dermatol 1990;95(5):553-7.

Cardona F, Andres-Lacueva C, Tulipani S, Tinahones FJ, Queipo-Ortuno MI. Benefits of polyphenols on gut microbiota and implications in human health. J Nutr Biochem 2013;24(8):1415-22.

Carrasco-Pozo C, Gotteland M, Castillo RL, Chen C. 3,4-dihydroxyphenylacetic acid, a microbiota-derived metabolite of quercetin, protects against pancreatic beta-cells dysfunction induced by high cholesterol. Exp Cell Res 2015;334(2):270-82.

Caterson B, Melrose J. Keratan sulfate, a complex glycosaminoglycan with unique functional capability. Glycobiology 2018;28(4):182-206.

Chambers CS et al. Defying multidrug resistance! Modulation of related transporters by flavonoids and flavonolignans. J Agric Food Chem 2019 (in press).

Chapman E, Best MD, Hanson SR, Wong CH. Sulfotransferases: Structure, mechanism, biological activity, inhibition, and synthetic utility. Angew Chem Int Ed 2004;43(27):3526-48.

Chassany O et al. Acute exacerbation of pain in irritable bowel syndrome: efficacy of phloroglucinol/trimethylphloroglucinol - a randomized, double-blind, placebo-controlled study. Aliment Pharmacol Ther 2007;25(9):1115-23.

Correia-da-Silva M, Sousa E, Pinto MMM. Emerging sulfated flavonoids and other polyphenols as drugs: nature as an inspiration. Med Res Rev 2014;34(2):223-79.

Crockett SL, Wenzig EM, Kunert O, Bauer R. Anti-inflammatory phloroglucinol derivatives from Hypericum empetrifolium. Phytochem Lett 2008;1(1):37-43.

Crozier A, Jaganath IB, Clifford MN. Dietary phenolics: chemistry, bioavailability and effects on health. Nat Prod Rep 2009;26(8):1001-43.

Czank C et al. Human metabolism and elimination of the anthocyanin, cyanidin-3-glucoside: a C-13-tracer study. Am J Clin Nutr 2013;97(5):995-1003.

Daikonya A, Katsuki S, Wu JB, Kitanaka S. Anti-allergic agents from natural sources (4): Anti-allergic activity of new phloroglucinol derivatives from Mallotus philippensis (Euphorbiaceae). Chem Pharm Bull 2002;50(12):1566-9.

D'Archivio M, Filesi C, Vari R, Scazzocchio B, Masella R. Bioavailability of the polyphenols: status and controversies. Int J Mol Sci 2010;11(4):1321-42.

De Kanter R et al. Drug-metabolizing activity of human and rat liver, lung, kidney and intestine slices. Xenobiotica 2002;32(5):349-62.

Duda-Chodak A, Tarko T, Satora P, Sroka P. Interaction of dietary compounds, especially polyphenols, with the intestinal microbiota: a review. Eur J Nutr 2015;54(3):325-41.

Dueñas M et al. A survey of modulation of gut microbiota by dietary polyphenols. Biomed Res Int 2015;850902:1-15.

Espín JC, Gonzalez-Sarrias A, Tomás-Barberán FA. The gut microbiota: A key factor in the therapeutic effects of (poly) phenols. Biochem Pharmacol 2017;139:82-93.

Figueira I et al. Polyphenols journey through blood-brain barrier towards neuronal protection. Sci Rep 2017;11456:1-16.

Gao K et al. Of the major phenolic acids formed during human microbial fermentation of tea, citrus, and soy flavonoid supplements, only 3,4-dihydroxyphenylacetic acid has antiproliferative activity. J Nutr 2006;136(1):52-7.

Griffiths LA, Barrow A. Metabolism of flavonoid compounds in germfree rats. Biochem J 1972;130(4):1161-2.

Gross G et al. *In vitro* bioconversion of polyphenols from black tea and red wine/grape juice by human intestinal microbiota displays strong interindividual variability. J Agric Food Chem 2010;58(18):10236-46.

Hartl J, Palát K. Farmaceutická chemie I. In: Strukturální faktory ovlivňující účinek léčiv. Prague: Karolinum, 1998:40

Honke K, Taniguchi N. Sulfotransferases and sulfated oligosaccharides. Med Res Rev 2002;22(6):637-54.

Huang DJ, Ou BX, Prior RL. The chemistry behind antioxidant capacity assays. J Agric Food Chem 2005;53(6):1841-56.

Ishikawa K et al. 4-Methylcatechol prevents derangements of brain-derived neurotrophic factor and TrkB-related signaling in anterior cingulate cortex in chronic pain with depression-like behavior. Neuroreport 2014;25(4):226-32.

Jančová P, Anzenbacher P, Anzenbacherová E. Phase II drug metabolizing enzymes. Biomed Pap 2010;154(2):103-16.

Kang C et al. Healthy subjects differentially respond to dietary capsaicin correlating with specific gut enterotypes. J Clin Endocrinol Metab 2016;101(12):4681-9.

Kay CD, Kroon PA, Cassidy A. The bioactivity of dietary anthocyanins is likely to be mediated by their degradation products. Mol Nutr Food Res 2009;53:92-101.

Kehoe JW, Bertozzi CR. Tyrosine sulfation: a modulator of extracellular protein-protein interactions. Chem Biol 2000;7(3):57-61.

Kemperman RA, Bolca S, Roger LC, Vaughan EE. Novel approaches for analysing gut microbes and dietary polyphenols: challenges and opportunities. Microbiol-Sgm 2010;156:3224-31.

Kim MM, Kim SK. Effect of phloroglucinol on oxidative stress and inflammation. Food Chem Toxicol 2010;48(10):2925-33.

Kumar H, Kim IS, More SV, Kim BW, Choi DK. Natural product-derived pharmacological modulators of Nrf2/ARE pathway for chronic diseases. Nat Prod Rep 2014;31(1):109-39.

Li CJ. et al. 4-Methylcatechol-induced cell damage in TM4 Sertoli cells. Cell Biol Int 2015;39(6):770-4.

Lohrie M, Knoche W. Dissociation and keto-enol-tautomerism of phloroglucinol and its anions in aqueous solution. J Am Chem Soc 1993;115(3):919-24.

Louis P, Hold GL, Flint HJ. The gut microbiota, bacterial metabolites and colorectal cancer. Nat Rev Microbiol 2014;12(10):661-72.

Luthria DL, Pastor-Corrales MA. Phenolic acids content of fifteen dry edible bean (Phaseolus vulgaris L.) varieties. J Food Compos Anal 2006;19(2-3):205-11.

Lv H. et al. Chemical constituents from the calyxes of Physalis alkekengi L. var. franchetii (Mast.) Makino. Biochem Syst Ecol 2018;78:63-5.

Masella R, Santangelo C, D'Archivio M, LiVolti G, Giovannini C, Galvano F. Protocatechuic acid and human disease prevention: biological activities and molecular mechanisms. Curr Med Chem 2012;19(18):2901-17.

Mladěnka P, Zatloukalová L, Filipský T, Hrdina R. Cardiovascular effects of flavonoids are not caused only by direct antioxidant activity. Free Radic Biol Med 2010;49(6):963-75.

Moore M, Thor H, Moore G, Nelson S, Moldeus P, Orrenius S. The toxicity of acetaminophen and *N*-acetyl-para-benzoquinone imine in isolated hepatocytes is associated with thiol depletion and increases cytosolic Ca^{2+}. J Biol Chem 1985;260(24):3035-40.

Murray RK, Bender DA, Botham KM, Kennelly PJ, Rodwell VW, Weil PA. Harperova ilustrovaná biochemie. Prague: Galén, 2012

Pádua D, Rocha E, Gargiulo D, Ramos AA. Bioactive compounds from brown seaweeds: Phloroglucinol, fucoxanthin and fucoidan as promising therapeutic agents against breast cancer. Phytochem Lett 2015;14:91-8.

Payton F, Bose R, Alworth WL, Kumar AP, Ghosh R. 4-Methylcatechol-induced oxidative stress induces intrinsic apoptotic pathway in metastatic melanoma cells. Biochem Pharmacol 2011;81(10):1211-8.

Pimpao RC, Ventura MR, Ferreira RB, Williamson G, Santos CN. Phenolic sulfates as new and highly abundant metabolites in human plasma after ingestion of a mixed berry fruit puree. Br J Nutr 2015;113(3):454-63.

Pimpao RC. et al. Urinary metabolite profiling identifies novel colonic metabolites and conjugates of phenolics in healthy volunteers. Mol Nutr Food Res 2014;58(7):1414-25.

Pourová J et al. Two flavonoid metabolites, 3,4-dihydroxyphenylacetic acid and 4-methylcatechol, relax arteries ex vivo and decrease blood pressure in vivo. Vasc Pharmacol 2018;111:36-43.

Procházková D, Boušová I, Wilhelmová N. Antioxidant and prooxidant properties of flavonoids. Fitoterapia 2011;82(4):513-23.

Purchartová K. et al. Prokaryotic and eukaryotic aryl sulfotransferases: sulfation of quercetin and its derivatives. Chem Cat Chem 2015;7(19):3152-62.

Quideau S, Deffieux D, Douat-Casassus C, Pouysegu L. Plant Polyphenols: Chemical properties, biological activities, and synthesis. Angew Chem Int Ed Engl 2011;50(3):586-621.

Ripps H, Shen W. Review: Taurine: A "very essential" amino acid. Mol Vis 2012;18(274-77):2673-86.

Rothwell JA et al. Systematic analysis of the polyphenol metabolome using the Phenol-Explorer database. Mol Nutr Food Res 2016;60(1):203-11.

Routray W, Orsat V. Blueberries and their anthocyanins: factors affecting biosynthesis and properties. Compr Rev Food Sci Food Saf 2011;10(6):303-20.

Saleem A, Husheem M, Harkonen P, Pihlaja K. Inhibition of cancer cell growth by crude extract and the phenolics of Terminalia chebula Retz. fruit. J Ethnopharmacol 2002;81(3):327-36

Santhakumar AB, Battino M, Alvarez-Suarez JM. Dietary polyphenols: Structures, bioavailability and protective effects against atherosclerosis. Food Chem Toxicol 2018;113:49-65.

Selma MV, Espin JC, Tomás-Barberán FA. Interaction between phenolics and gut microbiota: role in human health. J Agric Food Chem 2009;57(15):6485-501.

Serra A, Macia A, Romero MP, Reguant J, Ortega N, Motilva MJ. Metabolic pathways of the colonic metabolism of flavonoids (flavonols, flavones and flavanones) and phenolic acids. Food Chem 2012;130(2):383-93.

Serra D, Almeida LM, Dinis TCP. Dietary polyphenols: A novel strategy to modulate microbiota-gut-brain axis. Trends Food Sci Technol 2018;78:224-33.

Shibata T, Kawaguchi S, Hama Y, Inagaki M, Yamaguchi K, Nakamura T. Local and chemical distribution of phlorotannins in brown algae. J Appl Phycol 2004;16(4):291-6.

Singh IP, Sidana J, Bansal P, Foley WJ. Phloroglucinol compounds of therapeutic interest: global patent and technology status. Expert Opin Ther Pat 2009;19(6):847-66.

Singh IP, Sidana J, Bharate SB, Foley WJ. Phloroglucinol compounds of natural origin: Synthetic aspects. Nat Prod Rep 2010;27(3):393-416.

Spielman LJ, Gibson DL, Klegeris A. Unhealthy gut, unhealthy brain: The role of the intestinal microbiota in neurodegenerative diseases. Neurochem Int 2018;120:149-63.

Stevens JF, Maier CS. The chemistry of gut microbial metabolism of polyphenols. Phytochem Rev 2016;15(3):425-44.

Strott CA. Sulfonation and molecular action. Endocr Rev 2002;23(5):703-32.

Su KY, Yu CY, Chen YP, Hua KF, Chen YLS. 3,4-Dihydroxytoluene, a metabolite of rutin, inhibits inflammatory responses in lipopolysaccharide-activated macrophages by reducing the activation of NF-kappa B signaling. BMC Complement Altern Med 2014;14:9.

Sun Q. et al. Gut microbiota metabolites of dietary lignans and risk of type 2 diabetes: a prospective investigation in two cohorts of U. S. women. Diabetes Care 2014;37(5):1287-95.

Tao MK. et al. Methylenebisnicotiflorin: a rare methylene-bridged bisflavonoid glycoside from ripe Pu-er tea. Nat Prod Res 2016;30(7):776-82.

Teles YCF, Souza MSR, de Souza MDV. Sulphated flavonoids: biosynthesis, structures, and biological activities. Molecules 2018;23(2):11.

Tomás-Barberán FA, Selma MV, Espin JC. Interactions of gut microbiota with dietary polyphenols and consequences to human health. Curr Opin Clin Nutr Metab Care 2016;19(6):471-6.

Tomás-Barberán FA. et al. Urolithins, the rescue of "old" metabolites to understand a "new" concept: Metabotypes as a nexus among phenolic metabolism, microbiota dysbiosis, and host health status. Mol Nutr Food Res 2017;61(1):35.

Tsao R. Chemistry and biochemistry of dietary polyphenols. Nutrients 2010;2(12):1231-46.

Valentová K et al. Chemoenzymatic preparation and biophysical properties of sulfated quercetin metabolites. Int J Mol Sci. 2017;18(11):17.

Valentová K et al. Sulfated Metabolites of Flavonolignans and 2,3-Dehydroflavonolignans: Preparation and Properties. Int J Mol Sci 2018;19(8).

van der Horst MA, van Lieshout JFT, Bury A, Hartog AF, Wever R. Sulfation of various alcoholic groups by an arylsulfate sulfotransferase from Desulfitobacterium hafniense and synthesis of estradiol sulfate. Adv Synth Catal 2012;354(18):3501-8.

Veitch NC. Isoflavonoids of the Leguminosae. Nat Prod Rep 2007;24(2):417-64.

Villano D, Fernandez-Pachon MS, Moya ML, Troncoso AM, Garcia-Parrilla MC. Radical scavenging ability of polyphenolic compounds towards DPPH free radical. Talanta 2007;71(1):230-5.

Waldecker M, Kautenburger T, Daumann H, Busch C, Schrenk D. Inhibition of histone-deacetylase activity by short-chain fatty acids and some polyphenol metabolites formed in the colon. J Nutr Biochem 2008;19(9):587-93

Wang M, Jiang N, Wang Y, Jiang DM, Feng XY. Characterization of phenolic compounds from early and late ripening sweet cherries and their antioxidant and antifungal activities. J Agric Food Chem 2017;65(26):5413-20.

Williamson G, Clifford MN. Colonic metabolites of berry polyphenols: the missing link to biological activity? Br J Nutr 2010;104: S48-S66.

Williamson G. The role of polyphenols in modern nutrition. Nutr Bull 2017;42(3):226-35.

Xu BJ, Chang SKC. Phenolic substance characterization and chemical and cell-based antioxidant activities of 11 lentils grown in the northern United States. J Agric Food Chem 2010;58(3):1509-17.

Yasuda D, Takahashi K, Ohe T, Nakamura S, Mashino T. Antioxidant activities of 5-hydroxyoxindole and its 3-hydroxy-3-phenacyl derivatives: The suppression of lipid peroxidation and intracellular oxidative stress. Bioorg Med Chem 2013;21(24):7709-14.

9.1 Citations of websites

CorePharma, LLC. Medication Guide for mixed salts of an amphetamine product. In: U.S. National Library of Medicine website. Rev. September, 2016. Available at URL: https://dailymed.nlm.nih.gov/dailymed/fda/fdaDrugXsl.cfm?setid=4cc84b5d-9b56-4b80-a201-6e4075727d44. Accessed 02.03.2019.

Foster N. Sulfonation and Sulfation Processes. In: Chemithon Enterprises. 1997. Available at URL: http://www.chemithon.com/Resources/pdfs/Technical_papers/Sulfo%20and%20Sulfa%201.pdf. Accessed 29.06.19.

Gafner S., Scientific journals increasingly skeptical of antioxidant research. HerbalGram [serial online]: Volume 15, Issue 1, 01.2018. In: *American botanical council.* Available at URL: http://cms.herbalgram.org/heg/volume15/01January/JournalsSkepticalofAssays.html. Accessed 14.05.2019.

Molinspiration Cheminformatics. In: Molinspiration property engine v2016.1. Available at URL: http://www.molinspiration.com. Accessed 3.9.2018.