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Microbial Enzymes to Produce Flavonoid Metabolites, Their Biological Activity, and Use in Metabolic Studies

název disertace

Komise pro obhajoby doktorských disertací v oboru Mikrobiologie, virologie a mykologie

Jméno uchazeče: Kateřina Valentová

Pracoviště uchazeče: Mikrobiologický ústav AV ČR, v. v. i.

Místo a datum ……..

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List of Abbreviations

ADME – Absorption, Distribution, Metabolism and Excretion
ABTS – 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)
CAT – catalase
CYP – cytochrome P450
DAD – diode array detector
DPPH – 2,2-diphenyl-1-picrylhydrazyl
FRAP – ferric reducing antioxidant power
FCR – Folin–Ciocalteu reagent
GPx – glutathione peroxidase
HPLC – high-performance liquid chromatography
HR-MS – high-resolution mass spectrometry
LC-MS – liquid chromatography–mass spectrometry
LC-MS/MS – tandem liquid chromatography–mass spectrometry
LPS – lipopolysaccharide
MS – mass spectrometry
NQO1 – NAD(P)H quinone dehydrogenase 1
Nrf2 – nuclear factor erythroid 2–related factor 2
OATP – organic anion transporting polypeptide
Ptgs2 – prostaglandin-endoperoxide synthase 2
SOD – superoxide dismutase
SPE – solid-phase extraction
SULT – sulfotransferase
UGT – UDP-glucuronosyltransferase
UHPLC-HRMS – ultra-high-performance liquid chromatography–high-resolution mass spectrometry
XO – xanthine oxidase

Summary

This dissertation addresses the application of microbial enzymes for the preparation of flavonoid and other (poly)phenol metabolites, the evaluation of their biological activity, and their utilization in metabolic studies. Flavonoids and structurally related polyphenols represent a major class of plant secondary metabolites with well-documented antioxidant, anti-inflammatory, cardioprotective, and chemopreventive properties. However, following dietary intake, these compounds undergo extensive biotransformation in humans, including deglycosylation, C-ring cleavage, and Phase I and Phase II conjugation reactions such as sulfation, glucuronidation, and methylation. Consequently, circulating and tissue-exposed forms differ substantially from their native plant-derived structures. The limited commercial availability of authentic conjugated metabolites has significantly restricted mechanistic and pharmacokinetic investigations.

The first part of this thesis focuses on bacterial and microbial enzymes as selective and sustainable biocatalysts for the synthesis of flavonoid metabolites. Emphasis is placed on microbial glycosidases and transglycosylation strategies, quercetin 2,3-dioxygenases involved in flavonol C-ring cleavage, and bacterial aryl sulfotransferases enabling regioselective sulfation. Enzymatic glucuronidation using microbial systems, together with chemoenzymatic and chemical approaches, is also discussed. The work highlights the advantages of enzymatic methods in terms of regioselectivity, mild reaction conditions, environmental compatibility, and suitability for the preparation of authentic human metabolites and structurally defined standards.

The second part evaluates the biological activity of sulfated, glucuronidated, glycosylated, and microbially transformed metabolites. The data demonstrate that conjugation does not necessarily abolish biological activity; instead, structural modification may alter target selectivity, cellular uptake, metabolic stability, and interaction with enzymes, transporters, and signaling pathways. Specific attention is devoted to antioxidant and anti-inflammatory mechanisms, modulation of enzyme systems, and cardiovascular effects.

The third part integrates in vitro and in vivo metabolic studies, including microbial transformation models, cellular systems, animal experiments, and human intervention studies. The role of gut microbiota in shaping the polyphenol metabolome and contributing to inter-individual variability is emphasized. Advanced analytical methodologies, particularly LC–MS-based metabolomics, are presented as indispensable tools for metabolite identification and quantification.

Collectively, this work demonstrates that microbial enzyme technology, combined with biological evaluation and metabolic studies, provides a comprehensive platform for elucidating the fate and function of dietary polyphenols in humans and for generating well-defined metabolites required for mechanistic research.

Table of Content

Introduction and Theoretical Background

Dietary (poly)phenols represent a structurally diverse group of plant secondary metabolites with well-documented biological effects[1-2]. Among them, flavonoids constitute a major subclass[3] (Figure 1) with reported antioxidant, anti-inflammatory, cardioprotective, chemopreventive, and metabolic effects.

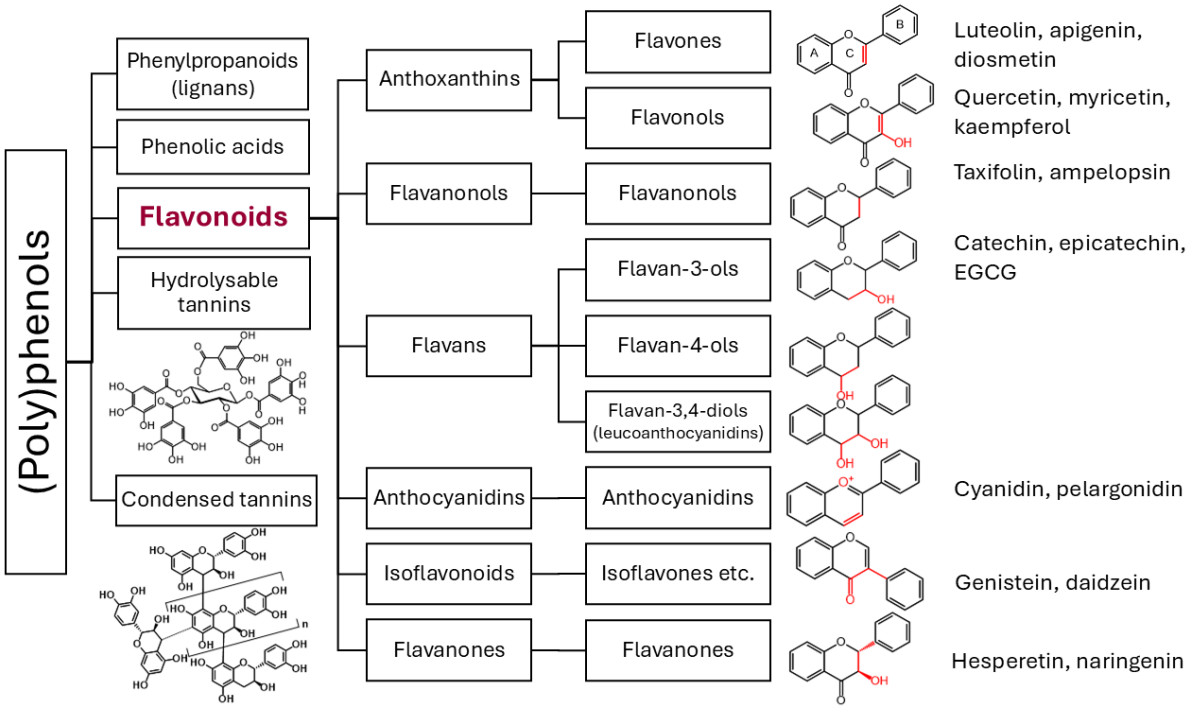

Figure 1: (Poly)phenols and their Main Subgroups

However, after oral intake, these compounds undergo extensive biotransformation in the human body[4]. Following consumption, polyphenols undergo enzymatic deglycosylation, microbial C-ring cleavage, Phase I oxidation and Phase II conjugation (sulfation, glucuronidation, methylation – mainly in intestinal and liver cells, Figure 2). Their bioavailability is generally low, and the circulating forms in plasma are predominantly phase II conjugates, particularly sulfates, glucuronides, and methylated derivatives[5-6]. Consequently, increasing evidence clearly demonstrates that the biologically relevant forms in humans are not the native dietary compounds, but rather their metabolites formed after ingestion[6]. Nevertheless, mechanistic and biological studies have historically focused on the unconjugated compounds, largely due to the limited availability of authentic metabolite standards. Chemical synthesis of conjugated metabolites is often multi-step, poorly regioselective, and yields low quantities of products.

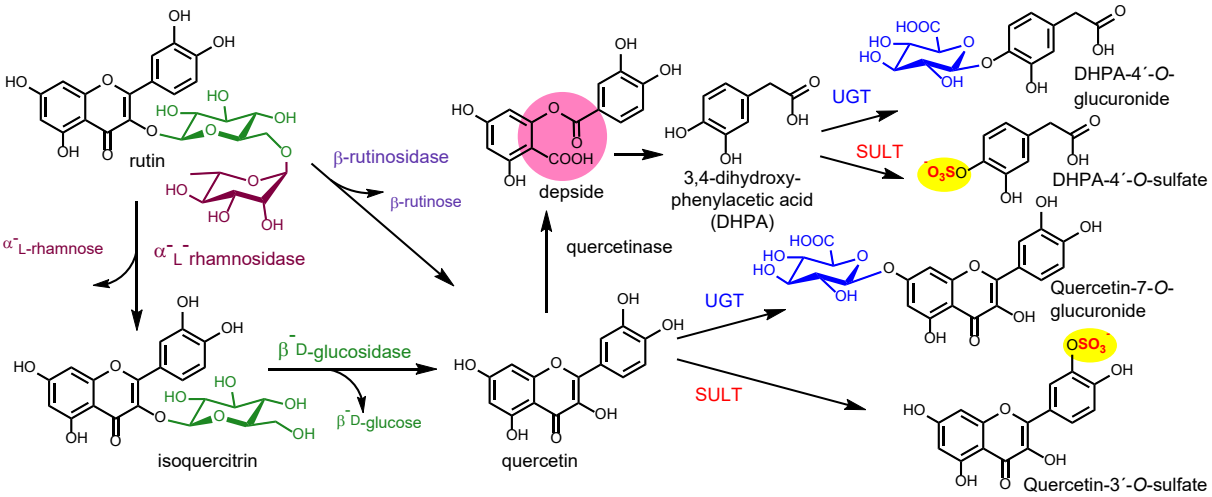

Figure 2: Selected Pathways of Flavonoid Metabolism in Humans. While deglycosylation and C-ring fission is catalyzed by microbial enzymes (glycosidases and quercetinase, respectively) in the gut, conjugation is mediated by UDP-glycuronosyltransferases (UGT) and sulfotransferases (SULT) mainly in intestinal and liver cells.

In this context, bacterial enzymes, particularly glycosidases, dioxygenases, aryl sulfotransferases, and other tailored biocatalysts, have emerged as versatile, sustainable, and highly selective tools for producing authentic human metabolites and structurally novel derivatives of flavonoids and polyphenols.

The central hypothesis of this work was:

Microbial enzymes can serve as highly selective, sustainable, and versatile tools for the preparation of authentic flavonoid metabolites required for biological and metabolic studies.

Aims of the Dissertation

The main objectives of this dissertation were:

1. To develop and optimize enzymatic and chemoenzymatic methods for the preparation of selectively (de)glycosylated, sulfated and other flavonoid metabolites.
2. To evaluate their biological activity and compare it with that of the parent compounds.
3. To employ the prepared metabolites as authentic standards in metabolic studies.

Methodological Approach

The work employed fungal glycosidases, fungal quercetinase, bacterial aryl sulfotransferases and fungal glucuronidases expressed in heterologous hosts (*E. coli*, *Pichia pastoris)*. Reaction conditions were systematically optimized with respect to selection of suitable sulfate donors, pH and temperature parameters, substrate concentration and reaction time, preparative-scale synthesis and purification procedures.

The prepared metabolites were isolated and purified using chromatographic techniques. Structural identification and confirmation were performed by HPLC and UHPLC, LC–MS/MS and high-resolution mass spectrometry (HRMS), nuclear magnetic resonance (NMR) spectroscopy.

Biological evaluation included antioxidant and anti-inflammatory assays, enzyme modulation studies, and selected *in vitro* models relevant to intestinal metabolism and bioavailability. The synthesized metabolites were further applied in metabolic profiling studies in both animals and humans to enable accurate identification of conjugated species in biological matrices.

1. Microbial Enzymes to Produce Flavonoid Metabolites

1.1 Microbial Glycosidases for Selective Hydrolysis and Transglycosylation Strategies

Microbial glycosidases were employed for selective hydrolysis and transglycosylation of flavonoids and phenolic acids. Enzymes such as β-D-glucosidases[7], α-L-rhamnosidases[8], and β-rutinosidases[9]. enabled:

- controlled deglycosylation of natural flavonoid glycosides (Figure 3)
- selective modification of glycosylation patterns
- preparation of novel glycosylated derivatives (Figure 4)

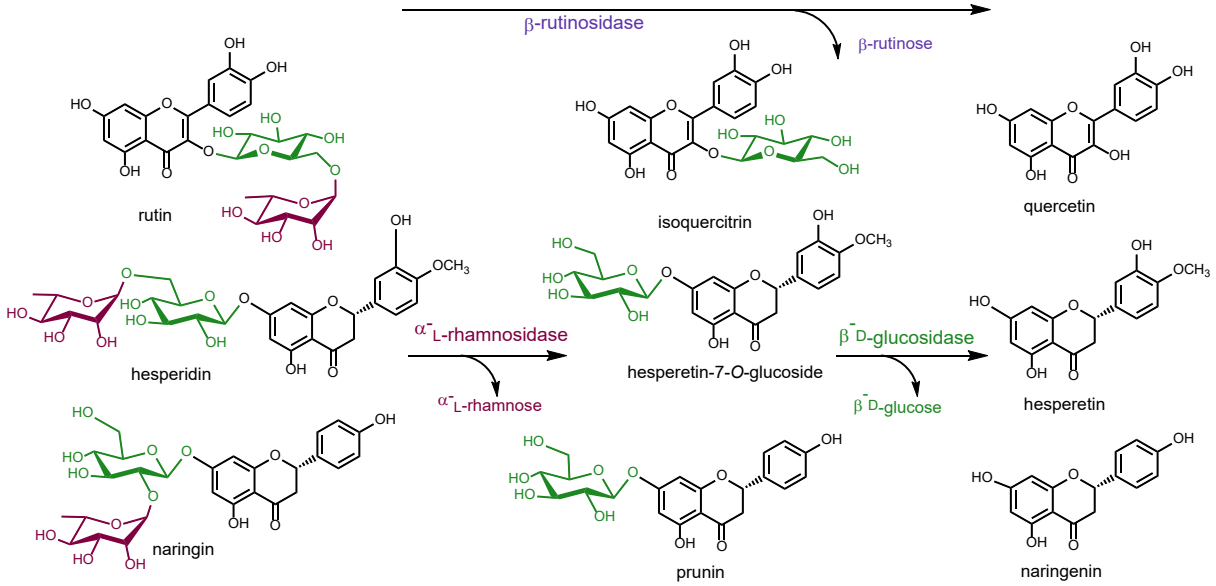

Figure 3: Deglycosylation of Rutin, Hesperidin, and Naringin by Glycosidases (adapted from ref.[10])

A key achievement was the first description of rutinosidase-catalyzed formation of not only glycosyl esters, but also phenolic glycosides from hydroxycinnamic acids[11] (Figure 4). These reactions demonstrated that microbial glycosidases can operate beyond simple hydrolysis and can be redirected toward synthetic applications. One of the β-rutinosidases studied in our laboratory accepts not only rutinosides, such as rutin, but also glucosides such as isoquercitrin. And it catalyzes not only its hydrolysis but is also able to transfer glucose or even glycosylated glucose to mostly simple alcohols[12-13].

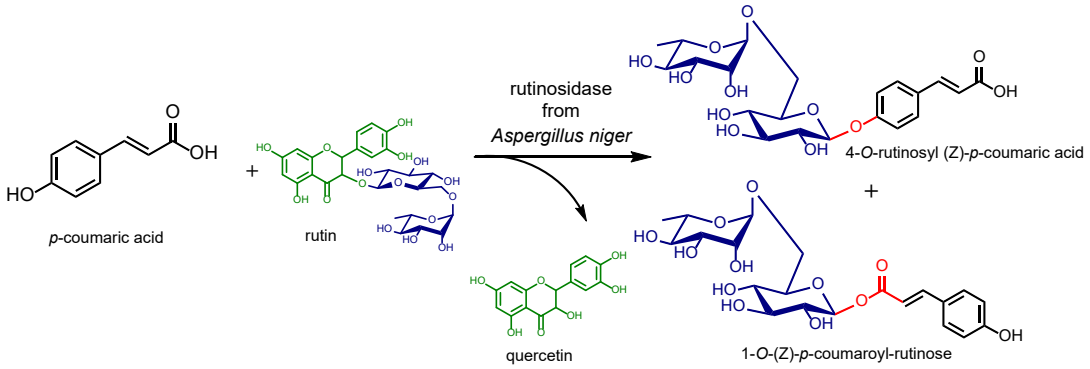

Figure 4: Rutinosylation of *p*-Coumaric Acid with Rutinosidase from *Aspergillus niger* (from ref.[11])

The work provided new tools for modifying physicochemical properties such as solubility and stability, with implications for food chemistry and metabolite research.

1.2 Engineering of Quercetin 2,3-Dioxygenase

A fungal quercetin 2,3-dioxygenase from *Penicillium chrysogenum* (PcQueD) was biochemically characterized and subsequently engineered using an evolution-inspired rational design strategy. Using quercetin labeled with ^{13}C at position C-3, we demonstrated that the carbon monoxide released during *Pc*QueD-mediated conversion originates solely from this carbon atom (Figure 5).

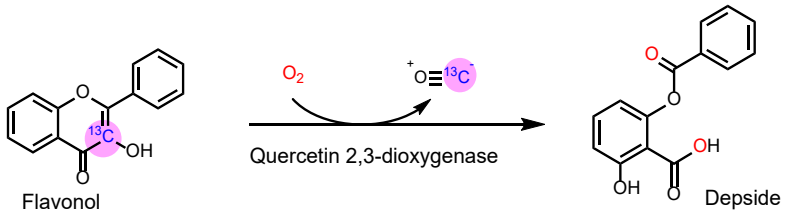

Figure 5: Reaction Catalyzed by a Quercetin 2,3-Dioxygenase.

The enzyme displayed broad pH tolerance and catalytic properties. Importantly, PcQueD also shows low but detectable activity toward a range of synthetic flavonols (namely those with bulky substituents and small substrates lacking some OH groups).

Targeted remodeling of the substrate-binding cavity resulted in:

- up to 1000-fold increased activity toward selected artificial flavonols
- altered substrate specificity
- improved conversion of bulky or structurally modified derivatives

This study demonstrated that a limited number of carefully selected mutations can profoundly reshape catalytic performance while maintaining structural integrity[14]. The engineered enzyme variants represent powerful tools for generating defined flavonol degradation products, which are otherwise difficult to synthesize chemically.

1.3 Bacterial Aryl Sulfotransferases

Bacterial aryl sulfotransferases (ASTs) represent a central part of this work. Bacterial ASTs are valuable tools for regioselective sulfation of polyphenols, including flavonoids, flavonolignans, and phenolic acids. One of the most studied bacterial ASTs is the enzyme from *Desulfitobacterium hafniense*[15]. This enzyme, produced heterologously in *E. coli* shows broad substrate tolerance and allows for the sulfation of hydroxylated aromatic substrates using synthetic donors such as *p*-nitrophenyl sulfate (*p*NPS), phenyl sulfate (PheS), or 4-methylumbelliferyl sulfate (MUS)[16]. These reactions proceeded under mild aqueous conditions and demonstrated high regioselectivity. This AST preferentially sulfate at the C-3'-OH position of flavonols, at the C-4'-OH position of flavanonols and at C-20-OH of flavonolignans (Figure 6), depending mostly on the reaction conditions[17-18]. More than 40 sulfated metabolites of flavonoids, flavonolignans, and phenolic acids were prepared using this enzyme, including sulfated simple phenols, phenolic acids, flavonoids and their glycosides, and flavonolignans[16-24] (Figure 7).

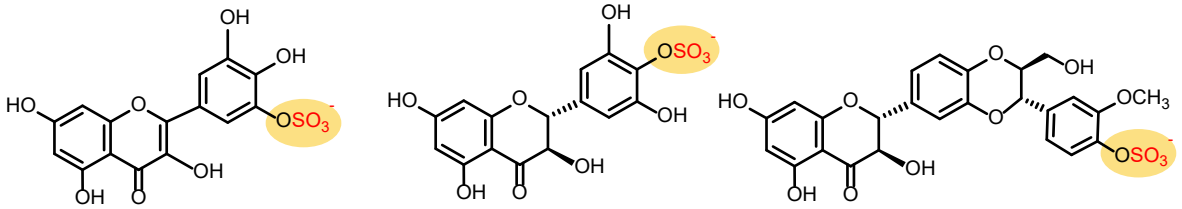

Figure 6: Regioselectivity of AST from *Desulfitobacterium hafniense* in the Sulfation of Flavonols, Flavanonols, and Flavonolignans

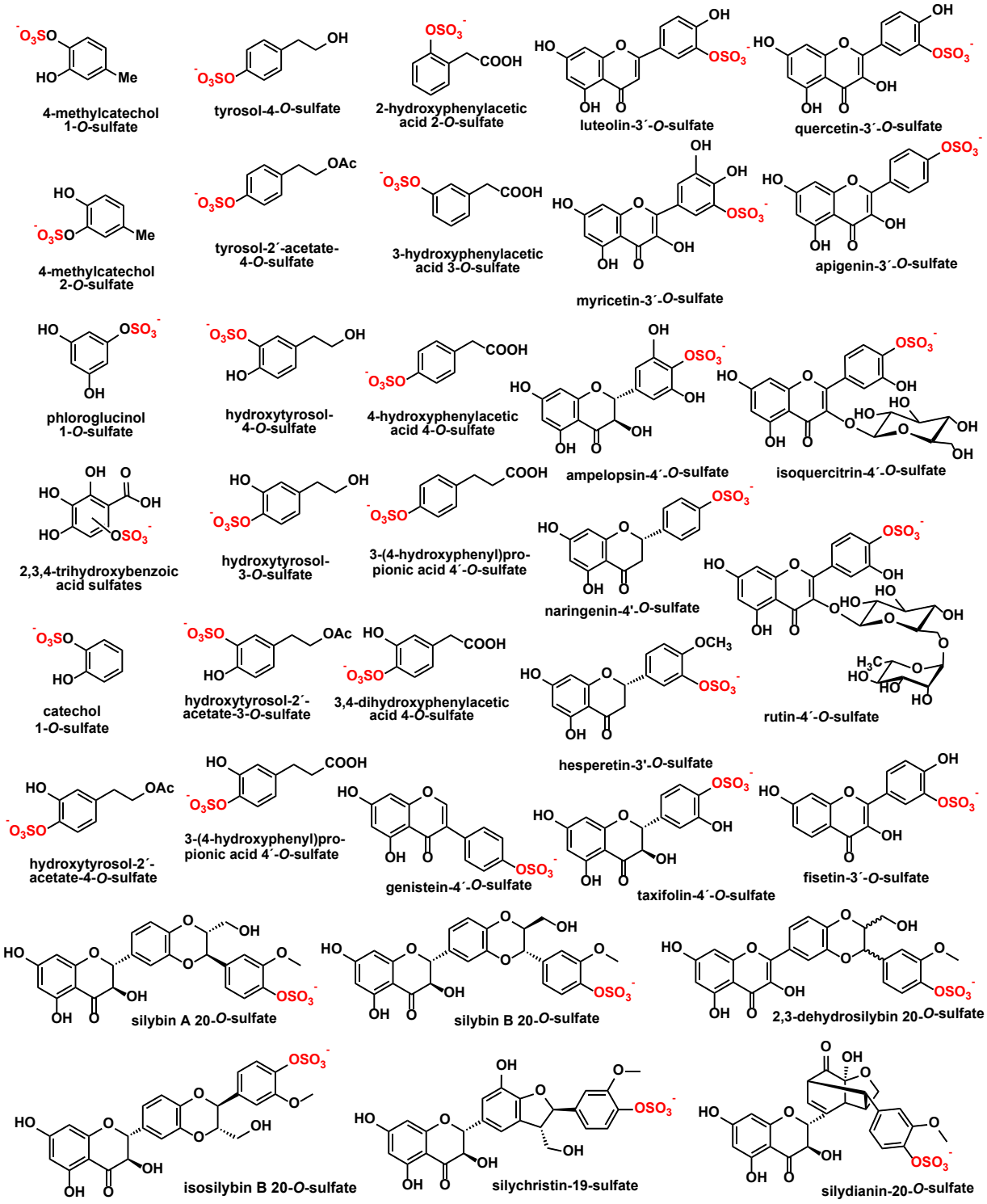

Figure 7: Overview of Sulfated (poly)phenols Prepared using AST from *Desulfitobacterium hafniense*[16-24]

Protein engineering approaches further expanded substrate scope and improved catalytic efficiency. We now possess a library of seven enzymes from two clusters, of which four are more efficient catalysts than the previously described enzymes from *D. hafniense* and *E. coli*[25]. Furthermore, site-directed mutagenesis is now employed to alter the donor or acceptor binding pocket deleting a loop located above the active site (Figure 8). The mutant enzymes were expressed, produced, purified,

and characterized. Kinetic analysis further revealed that the introduced mutations affected individual enzymes differently. Moreover, the effect of the mutations was substrate dependent. The work is still in progress.

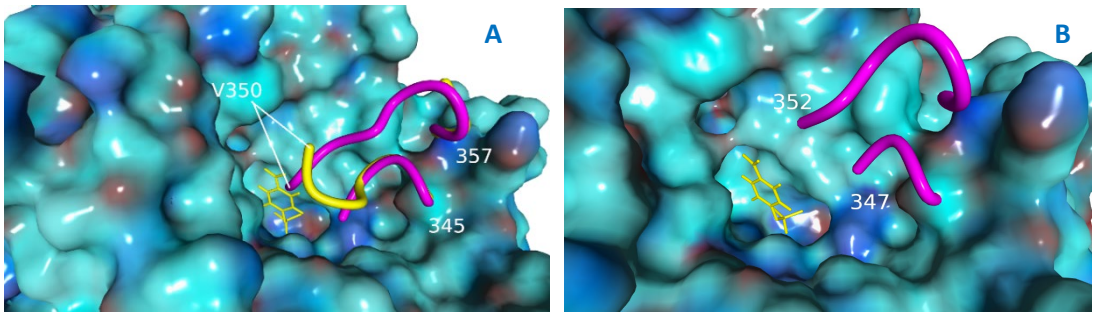

Figure 8: Overlay of the Structure of the Active Site of *Ec*AST: (A) showing the loop blocking the active site; (B) with the loop deleted.

Bacterial aryl sulfotransferases provide a practical and eco-friendly approach to synthesize sulfated polyphenols. ASTs bridge a critical gap between *in vivo* metabolic studies and *in vitro* bioactivity testing, supporting the development of more accurate pharmacokinetic models. The enzymatically produced sulfates served as authentic standards for LC–MS metabolomic studies and enabled direct comparison with human plasma metabolites.

1.4 Glucuronidation Strategies

Glucuronidation represents one of the most important Phase II metabolic reactions of phenolic compounds in mammals, plants, and microorganisms. The conjugation of glucuronic acid to hydroxylated xenobiotics and endogenous metabolites significantly increases their polarity, thereby facilitating transport, storage, and excretion[26]. Despite their relevance, phenolic glucuronides are rarely commercially available, and their preparation remains challenging due to issues of regioselectivity, stereochemical control, and scalability. In the laboratory, two principal strategies are employed for their synthesis: (i) enzymatic glucuronidation, using either mammalian or microbial enzymes, and (ii) chemical synthesis, typically based on protected glucuronic acid donors. Whole-cell biocatalysis employing species of *Streptomyces*, namely *S. tubercidicus* DSM 40273, *S. nigrescens* DSM 40276, and *S. coeruleorubidus* DSM 40145 = NRRL B-2569 is now investigated as a sustainable alternative to microsomal systems[27-29]. In addition to transferases, β-glucuronidases represent another class of enzymes relevant to glucuronide chemistry.

Under appropriate conditions, glucuronidases might be capable of catalyzing transglycosylation reactions, transferring glucuronic acid residues from activated donors to suitable acceptor molecules – here the field is practically unexplored. Recombinant glucuronidases from microbial sources such as *Penicillium brasilianum* or *Aspergillus tamarii*, but also from plants or even human enzymes, can be heterologously expressed, for example, in *Pichia pastoris* and purified. This strategy represents a promising alternative route for glucuronide synthesis that bypasses the need for UDP-activated donors. The work is in progress.

1.5 Chemoenzymatic Approaches to Polyphenol Conjugation

Enzymatic sulfation proved to be less effective for some phenolic acids, such as 2-hydroxyphenylacetic acid (2-HPA), 3-hydroxyphenylacetic acid (3-HPA), and 4-hydroxyphenylacetic acid (4-HPA), which were finally sulfated using methods of chemical synthesis[22] (Figure 9).

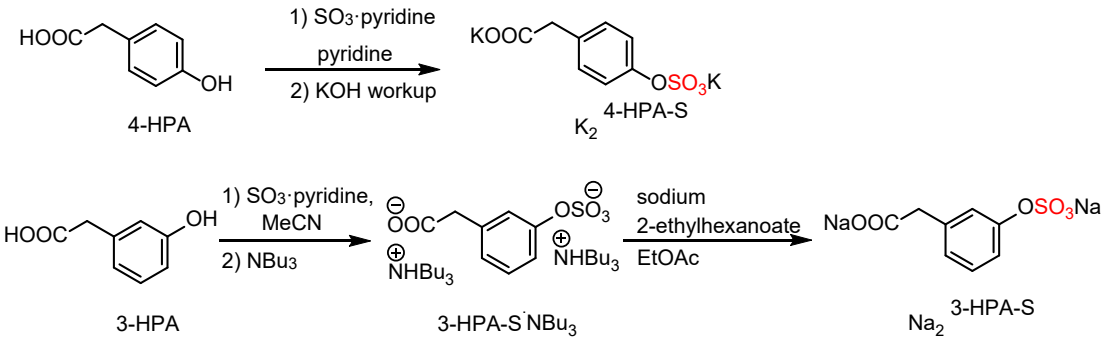

Figure 9: Chemical Sulfation of 3-Hydroxyphenylacetic Acid (3-HPA) using SO_3·pyridine

Chemical synthesis remains an indispensable method for the preparation of phenolic glucuronides when enzymatic approaches fail to provide sufficient yields or selectivity. Most chemical strategies rely on the reaction of 2,3,4-triaceto-1-bromo-α-D-glucuronic acid methyl ester (perAc-GlcA-Br) with phenols under basic conditions, e.g., the Koenigs-Knorr glycosidation[30-32] or the use of protected glucuronic acid derivatives, such as acetylated methyl esters or trichloroacetimidate donors (Schmidt imidate). Chemical synthesis is particularly valuable for producing glucuronides of phenolic acids and simpler phenols. In our hands, we used it for the glucuronidation of phenolic acids[33] (Figure 10).

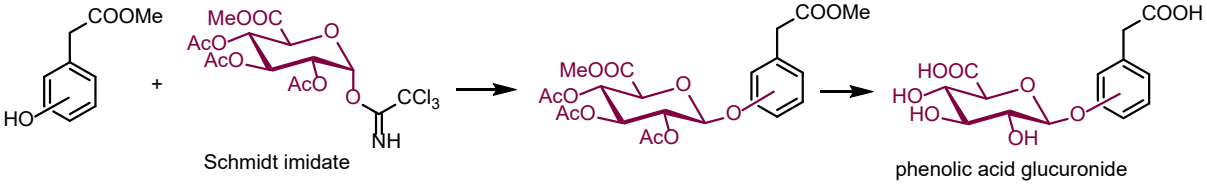

Figure 10: Glucuronidation of Phenolic acids with the Schmidt imidate[33]

1.6. Conclusion 1 – Enzymes to Produce Metabolites

This chapter demonstrates the successful development of regioselective enzymatic methods for the preparation of flavonoid metabolites. The novelty lies mainly in the systematic optimization of bacterial aryl sulfotransferases for preparative-scale synthesis and in the broad substrate scope achieved. The author's original contribution consists of methodological development, experimental optimization, structural elucidation of novel conjugates, and establishment of a reproducible enzymatic toolbox for metabolite synthesis.

2. Biological Activity of Flavonoid Metabolites

Biological effects of flavonoids and other (poly)phenols include antioxidant and anti-inflammatory actions, modulation of signaling pathways, enzyme activity, and gene expression. While early research focused on parent aglycones, increasing evidence shows that their Phase II metabolites—mainly sulfates and glucuronides, as well as microbiota-derived products—exhibit distinct and sometimes enhanced biological properties. These conjugates differ from parent compounds in solubility, stability, tissue distribution, and interaction with molecular targets such as transcription factors (e.g., Nrf2[19,34]), enzymes (e.g., NAD(P)H/quinone oxidoreductase 1 (NQO1)[19], xanthine oxidase (XO)[24,35], cytochromes P450[36-38]), and membrane transporters (e.g., organic anion-transporting polypeptides (OATPs[37-39], Figure 11).

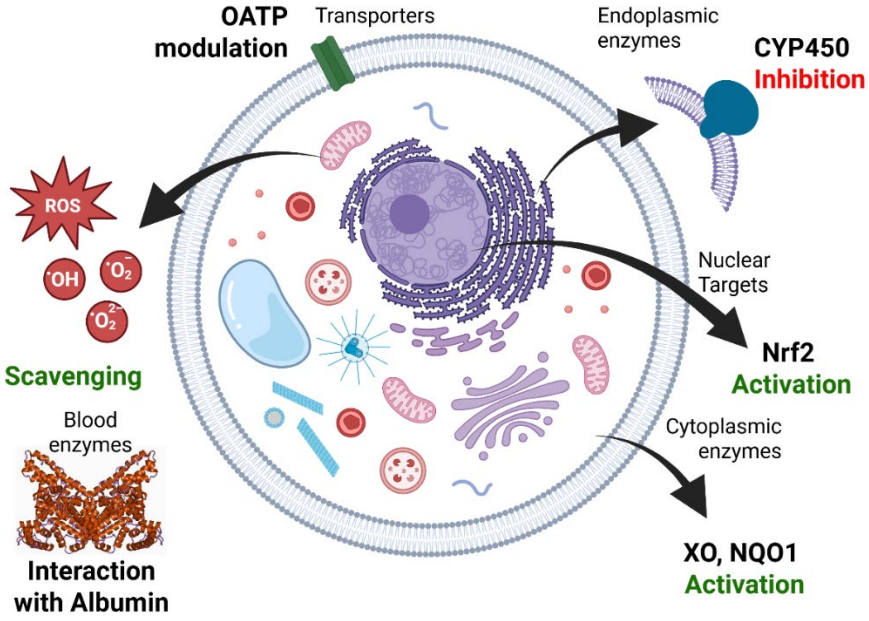

Figure 11: Cellular and Molecular Targets of Flavonoids and Their Metabolites. OATP: Organic Anion Transporter, CYP450: cytochrome P450, ROS: Reactive Oxygen Species, Nrf2: Nuclear factor erythroid 2-related factor 2, NQO1: NAD(P)H/Quinone Oxidoreductase 1.

This chapter summarizes our studies comparing flavonoid metabolites with their parent molecules, focusing on oxidative stress modulation, vascular and immune effects, and enzyme/transporter interactions, with emphasis on structure–activity relationships.

2.1 Antioxidant and Anti-inflammatory Properties

Antioxidant activity is among the most studied properties of flavonoids and their metabolites. Although *in vitro* antioxidant assays do not fully reflect *in vivo* efficacy[40-41], they remain valuable for understanding structure-dependent redox properties. Polyphenols scavenge reactive oxygen and nitrogen species, chelate metals, and modulate antioxidant enzymes. Structural modification via sulfation may retain, reduce, or occasionally enhance these effects[10,42].

Our studies showed that enzymatically sulfated (poly)phenols often partially retain antioxidant activity in cell-free assays (DPPH, ABTS, FRAP, FCR) and in anti-lipoperoxidant models[18-21,43]. Activity was strongly structure-dependent. For example, positional isomers of quercetin sulfates differed markedly: quercetin-4′-*O*-sulfate was more effective in lipid peroxidation inhibition, likely due to stronger interaction with membrane bilayers, as supported by molecular dynamics simulations[18].

Beyond direct radical scavenging, flavonoid metabolites modulate endogenous antioxidant defense. Sulfated flavonolignans showed modest NQO1 induction in Hepa1c1c7 cells, comparable to parent compounds. In macrophages, sulfation generally reduced Nrf2 activation compared to aglycones[19,43].

2.2 Modulation of Enzymes and Transporters

Flavonoids and their metabolites interact with metabolic enzymes and membrane transporters, influencing both biological effects and potential drug–nutrient interactions. Conjugation alters physicochemical properties and often modifies enzyme inhibition profiles.

Cytochrome P450 enzymes (CYP2C9, CYP2C19, CYP3A4) are prominent targets. Sulfated flavonoids generally inhibited CYP isoforms less strongly than parent compounds, although exceptions were observed. These interactions may affect drug metabolism depending on context and concentration.

Other enzyme targets include xanthine oxidase (XO), UGTs, and SULTs. While aglycones such as naringenin and luteolin inhibited XO effectively, sulfation typically reduced this inhibition[24,36-39,44] (Table 1). Such modulation may still be relevant for conditions linked to oxidative stress and purine metabolism.

Transport processes are also influenced by conjugation. Several sulfate metabolites modulated OATP1B1 and OATP2B1 more strongly than their parent compounds[37-39] (Table 1), suggesting potential effects on hepatic uptake and intestinal absorption. Thus, conjugation does not abolish biological interaction but reshapes pharmacokinetic behavior.

Table 1: Effect of Sulfation on the Interactions of Polyphenols with Albumin, Cytochromes P450 (CYPs), Organic Anion-Transporting Polypeptides (OATPs) and Xanthine Oxidase (XO)

Compound	**Interaction compared to parent compound with**							**Ref.**
	albumin	CYP				OATP		XO	
		2C9	2C19	2D6	3A4	1B1	2B1		
2,3-Dehydrosilychristin-19-*O*-sulfate	=	-	-	/	++	n.d.	n.d.		36
Ampelopsin-4'-*O*-sulfate	/	/	0	n.d.	-	++	++		38
Isosilybin-A-20-*O*-sulfate	-	0	/	/	--	n.d.	n.d.		36
Luteolin-3',7-di-*O*-glucuronide	=	0	+	n.d.	=	--	=	--	24,39
Luteolin-3'-*O*-glucuronide	+	0	-	n.d.	=	-	=	-	24,39
Luteolin-3'-*O*-sulfate	++	=	=	n.d.	=	-	=	=	24,39
Luteolin-7-*O*-glucuronide	=	0	-	n.d.	=	--	=	-	24,39
Myricetin-3'-*O*-sulfate	++	/	/	n.d.	=	++	++		38
Naringenin-4'-*O*-glucuronide	=	0	0	n.d.	--	=	++	--	24,39
Naringenin-4'-*O*-sulfate	=	-	--	n.d.	-	++	++	=	24,39
Quercetin-3'-*O*-glucuronide	-	-	n.d.	n.d.	n.d.	n.d.	n.d.		44
Quercetin-3'-*O*-sulfate	+	=	n.d.	n.d.	n.d.	n.d.	n.d.		44
Silybin A-20-*O*-sulfate	=	0	++	/	--	n.d.	n.d.		36
Silybin B 20-O-sulfate	=	--	-	/	--	n.d.	n.d.		36
Silychristin-19-*O*-sulfate	-	0	-	/	--	n.d.	n.d.		36

++ increased, + slightly increased, = retained, - slightly decreased, -- decreased, **0** abolished, **/** no effect, n.d. not determined

2.3 Effect on the Cardiovascular System

Silymarin flavonolignans and their sulfates were evaluated for vasorelaxant and anti-aggregation effects. Most compounds induced partial vasorelaxation in isolated rat aorta, though none achieved

complete relaxation within the tested range. Silychristin-19-O-sulfate exhibited stronger vasorelaxant activity than its parent compound[45]. Anti-platelet effects were generally weak and comparable between parent compounds and metabolites. Overall, sulfation modestly modified vascular activity without fundamentally altering the biological profile[45].

2.4 Mutagenicity and Immunomodulatory Activity

Conjugation significantly influences genotoxic and immunomodulatory effects. Glycosylation of quercetin reduced mutagenicity in the Ames test: isoquercitrin showed negligible activity and rutin was non-mutagenic, while quercetin itself displayed mutagenic potential. All compounds retained antimutagenic properties against oxidative DNA damage, although efficacy decreased in the order quercetin > rutin > isoquercitrin. Thus, glycosylation reduces pro-oxidative risk while preserving protective capacity[46].

In vivo, flavonols exhibited distinct immunomodulatory profiles without toxicity. Quercetin enhanced lymphocyte proliferation and IgM production, rutin stimulated NK cell activity and T-cell responses, and isoquercitrin showed milder effects[46].

Sulfation further modifies immune signaling. In LPS-stimulated macrophages, several flavonoids and selected sulfates reduced Ptgs2 and Nos2 expression. Although sulfated metabolites were usually weaker modulators, some (e.g., myricetin-3'-*O*-sulfate) showed comparable or stronger activity than aglycones[43]. Overall, conjugation reshapes rather than eliminates immunomodulatory potential.

2.5 Summary and Therapeutic Outlook

Flavonoid metabolites exert multi-level biological effects and should not be considered merely inactive detoxification products. Structural modifications significantly influence solubility, transport, receptor binding, and enzyme interaction. Sulfated and glycosylated derivatives often retain antioxidant and anti-inflammatory properties and modulate key targets such as Nrf2, CYP enzymes, XO, and OATPs. In some cases, metabolites act as selective modulators distinct from their parent compounds.

Therapeutically, these metabolites may contribute to prevention or management of cardiovascular, metabolic, inflammatory, and neurodegenerative disorders. Their dual role in redox regulation and enzyme modulation supports their relevance as nutraceuticals or adjunct therapeutic agents.

2.6 Conclusion 2 – Bioactivities of Metabolites

Conjugated flavonoid metabolites retain or selectively modify biological activity and cannot be regarded as inert detoxification products. Comparative evaluation of parent compounds and authentic sulfated derivatives enables precise structure–activity analysis. The author contributed substantially to experimental design, biological evaluation, and interpretation of structure-dependent effects.

3. *In Vitro* and *In Vivo* Metabolic Studies

Metabolism of flavonoids and other (poly)phenols is a multistep process involving host enzymes and gut microbiota[47]. After ingestion, compounds undergo limited Phase I reactions (oxidation, reduction, hydrolysis) and extensive Phase II conjugation (sulfation, glucuronidation, methylation), which determine circulating forms and biological fate.

In vitro models (hepatocytes, microsomes, intestinal cells, microbial fermentations) allow mechanistic characterization of pathways, whereas *in vivo* human and animal studies provide insight into absorption, distribution, metabolism, and excretion (ADME). Integrating both approaches is essential for linking structure with bioavailability and biological effects. This chapter summarizes metabolic studies with emphasis on interindividual variability, and host–microbiota interplay.

3.1 Microbial Transformation of Flavonoids and Polyphenols

Gut microbiota plays a key role in polyphenol metabolism. Due to limited absorption in the small intestine, many compounds reach the colon, where microbial enzymes initiate deglycosylation followed by ring cleavage, dehydroxylation, decarboxylation, and reduction. Genera such as *Eubacterium*, *Clostridium*, *Bacteroides*, and *Eggerthella* participate in these processes[48]. The extent and pattern of metabolism depend on substrate structure and individual microbiota composition.

Using anaerobic fecal batch fermentations, we showed that silymarin flavonolignans undergo dose-dependent microbial degradation. At lower concentrations, demethylation, reduction, and decarbonylation were observed, occasionally yielding cysteine conjugates[49]. Age-related differences were demonstrated: fecal samples from elderly donors produced distinct catabolite profiles compared to young individuals, indicating substantial interindividual variability[50] (Figure 12).

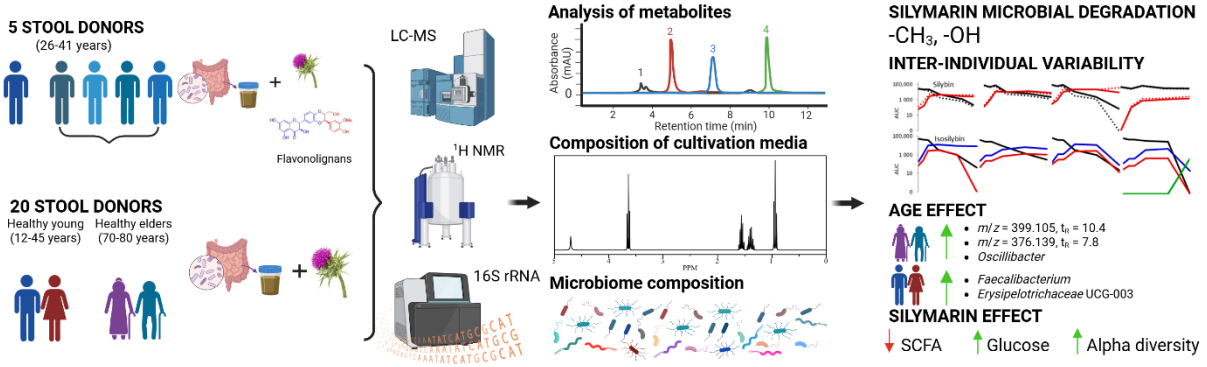

Figure 12: Mutual Interactions of Silymarin Flavonolignans with Human Gut Microbiota.

Similar findings were obtained for ferulic acid. *In vitro* fermentations revealed rapid formation of reduced and dehydroxylated metabolites (e.g., dihydroferulic derivatives)[51]. These microbial products are subsequently conjugated by host Phase II enzymes after absorption. Thus, systemic exposure reflects combined microbial and host metabolism, underscoring the need to integrate both pathways when evaluating bioavailability.

3.2 Host Phase I and Phase II Metabolism

After absorption, flavonoids and microbial catabolites undergo metabolism mainly in the intestine and liver. Phase I oxidation by cytochrome P450 (CYP) enzymes plays a minor but detectable role, while Phase II conjugation predominates. Relevant *in vitro* models include mainly hepatocytes, hepatic or intestinal microsomes, and cytosols or CYP-containing bactosomes (Figure 13).

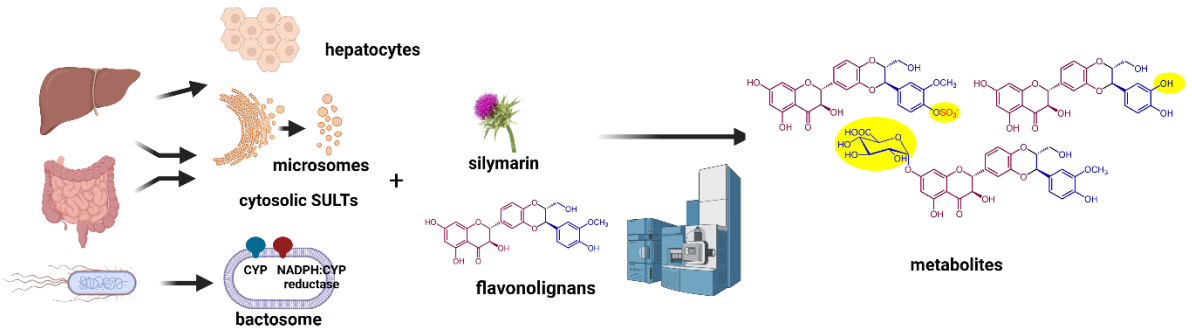

Figure 13: *In vitro* Models for Metabolic Studies

Human cytosolic sulfotransferases (SULTs) catalyze formation of mono-sulfated metabolites. We identified several isoforms (SULT1A1, 1A3, 1B1, 1C4, 1E1) responsible for sulfation of flavonoids and silymarin constituents. SULT1E1 and SULT1C4 showed high catalytic efficiency, whereas SULT2A1 exhibited negligible activity. Regioselectivity depended on hydroxyl group accessibility, with frequent formation of 4'-*O*-sulfates. Major intestinal and hepatic isoforms consistently produced authentic human metabolites, indicating predictable sulfation patterns *in vivo*[43,52].

UDP-glucuronosyltransferases (UGTs) represent another major pathway. Isoforms such as UGT1A1, 1A3, 1A8, 1A9, and 2B7 catalyzed formation of mono- and di-glucuronides of flavonolignans and flavonoids, typically at accessible hydroxyl groups (e.g., C-3 or C-7) [53-54].

CYP isoforms (including CYP2C9, CYP2C19, CYP3A4) mediated *O*-demethylation, hydroxylation, and other oxidative reactions of flavonolignans *in vitro*[55]. Although less extensive than conjugation, oxidative metabolism contributes to metabolic diversity and potential drug–flavonoid interactions.

3.3 Analytical Approaches to Metabolite Identification

Reliable metabolite identification requires advanced analytical techniques due to structural diversity and low concentrations. High-performance liquid chromatography (HPLC) coupled with MS, UV, or fluorescence detection represents the primary platform.

We optimized HPLC-DAD and LC-MS methods for silymarin analysis, enabling detection of known components and identification of previously unrecognized constituents[56]. A pentafluorophenyl-based gradient method was developed for enzymatic sulfation mixtures, compatible with MS detection and suitable for routine analysis[23].

HPLC-MS/MS allows discrimination of positional isomers (e.g., sulfates and glucuronides) via fragmentation patterns[49,57-60]. Availability of authentic standards is crucial for accurate identification and quantification; chemoenzymatically synthesized metabolites proved indispensable in confirming conjugates detected in biological matrices. Our standards were included in interlaboratory metabolomics initiatives and retention-time prediction platforms (e.g., PredRet), supporting cross-system comparability[60].

Sample preparation (SPE, protein precipitation, enzymatic deconjugation) critically influences recovery and specificity. In microbiota-focused studies, untargeted HR-MS and NMR approaches[50-51] facilitate discovery of novel catabolites, though they require robust data processing.

Integration of targeted and untargeted strategies, supported by authentic standards, provides comprehensive metabolic profiling.

3.4 Animal and Human Intervention Studies

Intervention studies are essential for confirming physiological relevance of metabolites identified in vitro. They account for interindividual variability, host–microbiota interactions, and real-life

pharmacokinetics. Early pilot studies with cranberry juice, silymarin, and combination supplements[61-63] demonstrated rapid urinary excretion of sulfated and glucuronidated metabolites and improvements in antioxidant markers. In a study with *Lonicera caerulea* berries, daily intake increased urinary phenolic acid derivatives and modulated oxidative stress markers[64]. A 90-day silymarin study identified multiple flavonolignan metabolites in urine and feces, emphasizing predominance of Phase II conjugates and notable interindividual differences[58] (Figure 14).

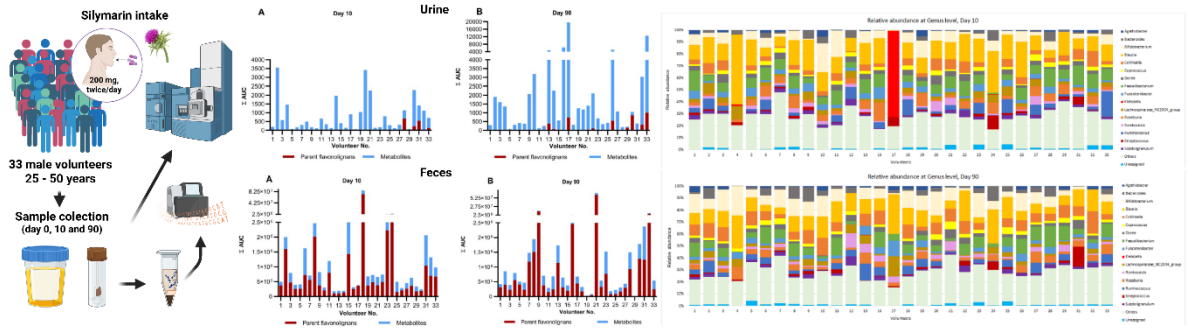

Figure 14: Metabolic profiling of silymarin constituents in urine and feces of healthy volunteers[58]

In a recent rat study with hawthorn extract, two hydroxyphenylacetic acid glucuronides were identified in plasma using UHPLC-HRMS, confirming in vivo formation of laboratory-prepared standards[33] (Figure 15).

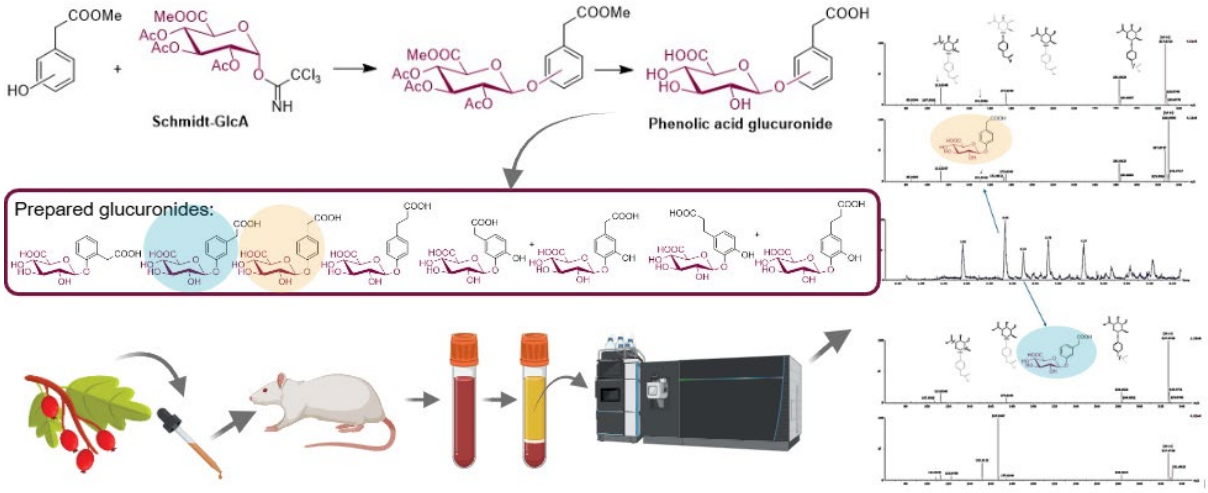

Figure 15: Glucuronidated Hydroxyphenylacetic Acids Identified in Rat Plasma After Administration of a Hawthorn Extract[33]

Overall, human and animal studies validate metabolic pathways observed in vitro and provide crucial pharmacokinetic and functional data.

3.5 Conclusion 3 – Metabolic Studies

This chapter underscores the importance of authentic metabolite standards for accurate metabolic profiling. The originality lies in integrating enzymatic synthesis with *in vitro* and *in vivo* metabolism studies, enabling unequivocal identification of conjugated species. The author contributed to preparation of analytical standards, experimental design, metabolic analyses, and interpretation of metabolomic data.

Conclusion

This dissertation demonstrates that microbial enzymes constitute powerful and versatile tools for the selective modification of flavonoids and other (poly)phenols. Through the application of microbial glycosidases, quercetin 2,3-dioxygenases, aryl sulfotransferases, and microbial whole-cell glucuronidation systems, it is possible to prepare authentic human metabolites and structurally defined derivatives that are otherwise difficult to access by purely chemical means.

The results confirm that enzymatic approaches provide high regioselectivity, operate under mild and environmentally compatible conditions, and are adaptable to reaction engineering and scale-up. These characteristics make microbial biocatalysis particularly suitable for generating reference standards required for metabolic, pharmacokinetic, and bioactivity studies.

Biological evaluation of conjugated metabolites reveals that structural modification profoundly influences their physicochemical properties, cellular uptake, metabolic stability, and molecular targets. Conjugation does not merely represent a detoxification pathway but may modulate or preserve specific biological activities. The work further highlights the crucial role of gut microbiota in shaping the metabolic fate of dietary polyphenols and contributing to inter-individual variability.

By integrating enzyme technology, synthetic chemistry, biological testing, and advanced analytical methodologies, this dissertation provides a comprehensive framework for studying polyphenol metabolism. The findings contribute to a deeper understanding of the relationship between structure, metabolism, and biological function and support the rational development of bioactive metabolites for nutritional, pharmacological, and analytical applications.

Overall, this work provides:

- systematic exploitation of microbial enzymes for preparation of authentic human flavonoid metabolites
- integration of enzyme engineering with metabolite synthesis
- comprehensive evaluation of biological activity of conjugated derivatives
- methodological platform connecting biocatalysis, metabolomics, and pharmacology

The interdisciplinary approach bridges microbiology, enzymology, organic chemistry, analytical chemistry, and biomedical research.

Author’s Contribution

The applicant's own scientific contribution consisted mainly in the conceptual and experimental development of chemoenzymatic approaches to the preparation of conjugated flavonoid metabolites, especially sulfated and glucuronidated derivatives, and in clarifying their biological effects and metabolic relationships. The applicant played a key role in designing a research concept focused on the use of bacterial arylsulfotransferases and other enzymes for the regioselective synthesis of flavonoid conjugates.

She personally carried out a substantial part of the experimental work, including the optimization of enzymatic reactions, the preparative preparation of metabolites, and their isolation. She actively participated in the design and implementation of biological experiments and in the interpretation of the data obtained, particularly in relation to structural-biological contexts. In more recent work, the focus of experimental work lies with students and postdoctoral fellows whom the applicant trains or has trained (K. Káňová, B. Petránková, K. Brodsky, V. Kolaříková/Janouchová).

In the publications included in the dissertation (40 works in total), she often appears as the first author (7 times) or corresponding author (18 times). She was responsible for the experimental design, evaluation of results, and preparation of manuscripts. A significant part of the work was done in collaboration with other domestic (13 times) or foreign (15 times) institutions. Here, the focus of the applicant's work is mainly on participating in the design of the experiment and providing unique substances for testing. Overall, her contribution represents a comprehensive set of original results linking enzymatic synthesis, analytical characterization, and biological evaluation of natural polyphenol metabolites.

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List of Publications Included in the Dissertation

Chapter 1: Bacterial Enzymes to Produce Flavonoid Metabolites:

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Chapter 2: Biological Activity of Flavonoid Metabolites:

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Chapter 3: *In Vitro* and *In Vivo* Metabolic Studies:

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