Development of chemical biology tools for metabolomics analysis

Phase II modifications as a link between host and microbiota co-metabolism

MARIO S. P. CORREIA
Abstract

Biomarker discovery is an extremely important field for early disease detection. New selective molecular markers are in high demand, as an earlier disease detection can lead to improved patient care. Furthermore, with the increased knowledge of the importance of the microbiota for human physiology and disease, markers for the interaction between the microbiome and the human host are required. A biomarker can be any biomolecule including metabolites that is altered between a “healthy” state and a disease state. Furthermore, the metabolic interplay between the consortium of trillion of microorganisms and the human is still largely unexplored. The investigation of the co-metabolism represents a tremendous opportunity for the discovery of new metabolites.

Metabolomics is the most recent of the major ‘omics sciences and provides a readout of the phenotype at the time of sample collection. The techniques of choice for metabolomics analysis are mass spectrometry (MS) and NMR spectroscopy. With the most recent developments in both hardware and software, data collected from liquid chromatography-MS can analyze thousands of metabolites at the same time. Despite the technical advances, metabolomics is still at a developmental stage in comparison to genomics or proteomics. Chemical biology tools are required for the selective analysis of functional groups to improve metabolite detection.

We have developed new chemical biological tools for the combination with metabolomics analysis. Using an arylsulfatase from the snail Helix pomatia, we have selectively identified sulfated metabolites in urine and fecal samples. We identified more than 200 sulfates and many these were previously undetected. Several of the identified molecules were products of the co-metabolism of the human host and its microbiome, which have partly been linked to disease development.

We also utilized a purified glucuronidase for identification of glucuronides in urine samples, respectively. We were able to identify close to 200 glucuronides. Additionally, we have developed a new strategy for metabolite validation, based on the enzymatic cleavage of glucuronides.

We have also prepared a comprehensive sulfated metabolite library for efficient structure validation. The understanding of the human and the microbiota co-metabolism was also increased through applying a modified method on a dietary intervention study. This diet was based on (poly)phenolic compounds that are commonly digested by the bacteria and then further metabolized by the human host to their sulfated conjugates. These findings demonstrate the importance of these newly developed tools for metabolomics and the study of these metabolic interactions between the human and the microbiota.

*Keywords:* Metabolomics; Chemical biology; sulfatase; Glucuronides; Phase II modifications; Microbiome; co-metabolism; method development

Mario S. P. Correia, Department of Chemistry - BMC, Box 576, Uppsala University, SE-75123 Uppsala, Sweden.

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To my family
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


* These authors contributed equally

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* These authors contributed equally
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Abbreviations

PDAC  Pancreatic ductal adenocarcinoma
IBD  Inflammatory bowel disease
CKD  Chronic kidney disease
SPE  Solid phase extraction
UPLC-MS  Ultra-performance liquid chromatography mass spectrometry
ESI  Electrospray ionization
qTOF  Quadrupole time-of-flight
LTQ-orbitrap  Linear-quadrupole ion trap-orbitrap
FT-ICR  Fourier transform ion cyclotron resonance
LC-MS  Liquid chromatography mass spectrometry
MS²  Tandem mass spectrometry
HMDB  Human metabolome database
MoNa  MassBank of North America
GNPS  Global Natural Products Social molecular network
NIST  National Institute of Standards and Technology
MWCO  Molecular weight cut-off
SDS-PAGE  Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
MEBOS  4-Methylumbellifery sulfate
NATOG  N-Acetylyramine-\(\beta\)-glucuronide
ArS  Arylsulfatase
RI  Retention time index
GC-MS  Gas-chromatography mass spectrometry
V1  Urine collected before dietary intervention
V2  Urine collected after dietary intervention
EIC  Extracted ion chromatogram
Introduction

One of the most exciting discoveries over the past decade has been the understanding the profound impact of the microbiota on the human body.\textsuperscript{1-3} This consortium of trillions of microorganisms has an endosymbiotic relation with the human host that co-evolved to a beneficial relationship through the exchange of biomolecules.\textsuperscript{4-5} The microbiota is constituted by a variety of microorganisms such as bacteria, archaea, fungi, protists, and viruses. The microbiome exists in the GI tract (the mouth and the gut) and on the skin.\textsuperscript{6-7} The effect of the microbiota in the hosts’ health and disease has been explored and several studies have presented evidence that a microbiome dysbiosis leads to disease development.\textsuperscript{8} There are numerous studies correlating for example the delivery at birth with the onset of diabetes and obesity, among other diseases.\textsuperscript{9-10} Other examples are the correlation between certain types of cancer with an alteration of the microbiome composition. Alterations in the microbiota composition might lead to the increase of undesirable bacteria. This will lead to a dysregulation of the normal microbiome and one of the responses can be the alteration of certain microbial metabolites. Investigation of these metabolic alterations can lead to the discovery of new biomarker molecules.

Biomarker discovery is an initial and crucial step for the development of new diagnostic tools.\textsuperscript{11-12} Through the investigation of small molecules in human samples such as plasma, urine or feces has great potential for the identification of yet unknown biomarkers. Human biological samples can be compared between a disease and a “healthy” state to detect abnormal metabolites quantities. For the investigation of small molecules, the main analytical technique is mass spectrometry coupled with a chromatographic separation system.\textsuperscript{13} Mass spectrometry allows for the analysis of several metabolites in the same analytical experiment, which is an advantage over other techniques as several molecules with different structures, polarity and physical properties can be analyzed from the same sample simultaneously. Further investigation of the biomarker’s biosynthesis has a tremendous potential for the identification of new drug targets and would lead to new opportunities for disease prevention, management, and personalized medicine.

An example of a disease that has been associated with the dysbiosis of the oral and recently the gut microbiome as well is pancreatic ductal adenocarcinoma (PDAC), which is one of the most lethal cancers. Only 30\% of the patients diagnosed with PDAC survive the first year after diagnosis. The mortality increases to over 95 \% considering the 5-year survival rate.\textsuperscript{14} These low
survival rates are due to the absence of symptoms resulting in insufficient diagnostic tools. PDAC can only be detected at an advanced developmental stage through highly invasive biopsies that only allows for late treatment. With the study of the mouth microbiome associated metabolites from bacteria such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* can provide insight to early disease development. Both bacteria have a correlation with the development of PDAC. Understanding microbiota dysbiosis related to pancreatic diseases can lead to new insights of microbiome-metabolism for the development PDAC, which represents a new strategy for biomarker discovery.

**Microbiota and host co-metabolism**

The microbiota present in the human body can perform several biochemical reactions and functions that the host lacks the enzymes for. Considering the number of microorganisms in the human body, there are about the same microbiota derived cells compared to human cells. The microbiome is composed of several millions of different species, which increases the genetic potential encapsulated in the microbiota by a factor of 150 compared with the amount of human genes present. The human microbiome has even been re-

![Human and microbiota co-metabolism](image)

**Figure 1** – Schematic representation of the influence of the human and the microbiota co-metabolism in human physiology and disease.
ferred to as an additional human organ as it influences crucial pathways, including nutrition, detoxification, metabolism, hormonal homeostasis, immune tolerance, and especially inflammation (Figure 1).22-28

An increasing amount of evidence indicates that dysregulation of the microbiota can contribute to a variety of diseases including cancer, diabetes, obesity, cardiovascular diseases, and inflammatory bowel disease (IBD).22-24, 27 The microbiota also aids the human in the detoxification of several metabolites as well as the conversion of several food derived molecules.29 The immune and hormonal system also have been associated with alterations of the natural human flora.30

The human and the microbiome are in a constant metabolic exchange. Further elucidation of the interchange between the microbiota and the host can lead to the identification of new bioactive metabolites. Detailed elucidation of this metabolic interaction is required to identify unknown metabolites and to evaluate their toxic or beneficial properties on the human host.31-33

Phase II metabolism

One of the metabolic alterations profoundly affected by the gut microbiota are phase I and phase II conversions, which are involved in xenobiotic clearance in mammals.34 Enzymes such as cytochrome P450 oxidases introduce polar groups into xenobiotics including microbiota derived metabolites during phase I metabolism.35 After the first conversion, the metabolites are further processed during phase II conjugation. These processes increase the hydrophilicity of the xenobiotics to help the human body excrete the metabolite. It can also lead to bioactivation and the generation of toxic compounds.

In particular, O-sulfated metabolites have been identified as a major feature of the co-metabolism of microbes and their host.36-37 One of the first reports

![Figure 2 – Various indole-containing molecules arise only in the presence of the microbiome and ultimately enter the plasma by means of different routes. Image adapted from 38.](image-url)
on microbiota co-metabolism, performed with gnotobiotic mice, clearly displays the interaction between microbiota and the host for the dietary conversion of tryptophan (Figure 2). Sulfated metabolites have been suggested to be key regulators of bacterial interaction. Sulfatases and sulfotransferases genes are identified in the genomes of several gut bacteria. Two of the most well-known examples are indoxyl sulfate and p-cresyl sulfate, some of the first metabolites associated with the co-metabolism between the human and the microbiota. The unconjugated metabolites are converted from the degradation of aromatic amino acids derived from food by the gut microbes. After absorption in the colon, indole and cresol are further metabolized by the human, prior excretion. Both metabolites have since been described as uremic toxins as they are linked to the development of chronic kidney disease (CKD) as well as cardiovascular diseases, emphasizing the relevance of this metabolite class to human physiology.

One of the main sources for the generation of metabolites produced by the co-metabolism of microbiota and host is food. Especially, a diet that is rich in (poly)phenols such as soybeans, flaxseeds, and raspberries. The main components of soy are the isoflavonoids daidzin and genistin that are converted into bioactive phytoestrogens daidzein and genistein by the gut microbes. Several molecules derived from the metabolites genistein and daidzein, especially phase II modified molecules, have been identified as markers for the consumption of soybeans. Flaxseeds are also rich in polyphenols. The main components in this food are lignans that also belong to the phytoestrogen compound class. The main degradation products of lignans are also produced by the microbiome, namely enterolactone and enterodiol. Anthocyanins are one major component of raspberries. Metabolism by the host’s microbiome produces metabolites such as p-coumaric acid, ferulic acid, and caffeic acid.

Several of the metabolites derived from a (poly)phenol rich diet have been reported as their conjugated phase II analogues. However, a specific study of structurally diverse sulfates in these diets can provide access to unknown beneficial metabolites for the human host.

**Metabolomics analysis**

Global metabolomics analysis aims at studying the small metabolites present in human samples, typically below 1500 Da. Metabolomics analysis can be performed in any human tissue, biological fluid, plants or other organisms. Since the samples are collected at a certain time-point, during a metabolomics experiment, the analysis is performed for the snapshot at that exact time-point. Metabolomics analysis investigates the phenotype of the sample with an overview of all biological processes at the point of sample collection. (Figure 3)
Since metabolomics is the most recent -omics tools, in comparison with genomics, transcriptomics and proteomics, there is still some development required to overcome present limitations. The three main steps for metabolomics analysis are sample collection and preparation, detection of the metabolites and data analysis. There are several advantages/limitations for each separation method, and different analyses provide different information about the samples. Certain extraction methods are more suitable for lipids or for certain drugs than others. For the purpose of this thesis, I will focus on the separation methods that are better suited for the analysis of small and polar metabolites.

Sample collection and preparation
The steps of sample collection and preparation are of critical importance for a successful global metabolomics analysis. Any small alteration of the method during collection or preparation of the samples can lead to major undesired differences in the sensitive mass spectrometric analysis. For biological sample collection, ideally the samples should be collected at the same time of the day, to avoid major circadian alteration of the metabolites. Subjects will have for example different concentrations of metabolites after a meal in comparison to the morning fast. The same can be said for urine sample collection with one major difference. Urine sample collection can be collected in two ways: i) spot urine collection that corresponds to the morning urine; ii) 24-hour urine collection, which measures the overall kidney function, and can be considered an average of all the processes over the day. As for fecal samples, due to the difficulty of collection (usually the individuals collect these themselves and send to the hospital), no specific or optimized protocol exists for the collection, and the methods are still under development. After collection, all biological samples should rapidly be stored at -80 °C, to stop all the biological processes and metabolite degradation.

For a global metabolomics analysis, several aspects should be considered for the sample preparation. The selected method should be unselective to extract most of the metabolite pool. Ideally, it should also be simple and fast, because in more complicated and longer extraction processes, more errors and
metabolite degradation can happen, and it is harder to control all parameters. The most important feature of a sample extraction method is its reproducibility. All samples should be extracted in the same way creating as little variance as possible due to the extraction method.61

Three main sample preparation methods are used in bioanalytical sample preparation: protein precipitation, liquid-liquid extraction, and solid phase extraction (SPE).62-65 Liquid-liquid extraction usually uses two immiscible solvents (aqueous and organic) to separate the metabolites of interest. For example, it is used in the extraction of cell extracts, in which a water:methanol phase and a chloroform phase are used for separation of polar and apolar metabolites that can be collected and analyzed separately.66 Solid phase extraction is the most complex sample preparation method of these three techniques as it requires a matrix for extraction. SPE can more selectively collect metabolites but has higher cost and longer preparation times. SPE is commonly used in extraction of metabolites from diluted environmental samples as it also can be used for concentration of samples with large volumes.67-68 Protein precipitation was the technique of choice for projects in this thesis as it is a fast and reliable technique for metabolomics analysis.69 Protein precipitation is the crucial step to prepare biological samples and avoid biological processes in the collected sample. Typically, in a protein precipitation sample preparation, a 3-4 times volume of a miscible organic solvent (e.g., methanol, acetonitrile, or a mixture) is added to the sample to precipitate the matrix proteins. With protein precipitation, the small polar metabolites stay soluble in the extract and the macromolecules precipitate. Despite the simplicity and quickness of this extraction method, the main drawback is that the extract still contains several of matrix specific molecules that can influence analytical steps.

UPLC-MS

Metabolomics analysis can be performed with different detection techniques. The main detection tools for global metabolomics are mass spectrometry and nuclear magnetic resonance.70 In order to perform metabolite separation prior to the analysis a chromatography step is added, with capillary electrophoresis and gas or liquid chromatography as the most common techniques used.71 Capillary electrophoresis separates the compounds of interest based on an electric field applied to a capillary.72 Analytes are separated based on their ionic strength. In gas chromatography, the solution of interest is vaporized before being introduced into a stationary solid phase.73-74 The compounds are separated in the stationary phase before analysis with different detectors including a MS.

For the scope of this thesis, ultraperformance liquid chromatography-mass spectrometry (UPLC-MS) was the method of choice. UPLC is an optimized ultra-performance liquid chromatography developed by the Waters Corporation and it is ideal for high resolution chromatography.62, 75-76 There are several
types of liquid chromatography columns depending on the composition of the stationary phase. Separation of the compound mixture can either be performed in normal phase analysis, reverse phase or hydrophilic interaction column.\textsuperscript{77} Reverse phase UPLC allows for separation of several compound classes with a solvent gradient that changes from high polarity, usually water, to high content of an organic solvent. For the chromatography analysis, a stationary phase, usually C18 based, is used to help the compounds migrate at a different rate.

Prior to the mass spectrometric analysis, the metabolites need to be charged as mass spectrometry can only separate and detect ionized molecules. A technique that is suitable for LC analysis is electrospray ionization (ESI).\textsuperscript{78} In ESI, the solvent is passed through a steel capillary that is charged at high voltage. After the capillary, the spray forms a Taylor Cone, a fine spray of charged droplets. The droplets decrease in size causing an increase in density of charge. This leads to a coulombic explosion and the analytes are the ionic form present in the gas phase (Figure 4). ESI is used at atmospheric pressure and is the ideal technique for the ionization of metabolites in large volumes of solvents into a gas phase. After the ionization process, the metabolites can be separated and analyzed in the mass spectrometer.\textsuperscript{79}

\textbf{Figure 4 – Schematic representation of the electrospray ionization process.} The analyte solution is pumped through a needle to which a high voltage is applied. A Taylor cone with an excess of positive charge on its surface forms as a result of the electric field gradient between the ESI needle and the counter electrode. Charged droplets are formed from the tip of the Taylor cone, and these droplets evaporate as they move towards the entrance to the mass spectrometer to produce free, charged analyte molecules that can be analyzed for their mass-to-charge ratio. Figure from 78.
Several mass spectrometers are used in metabolomics studies. The most common systems are quadrupole time-of-flight (qTOF), linear-quadrupole ion trap-Orbitrap (LTQ-Orbitrap) or Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometers. The mass spectrometer of choice for metabolomics analysis was a qTOF. A qTOF analyzer combines a quadrupole and a collision cell to the TOF to create higher sensitivity and separation (Figure 5). The TOF separates the molecules by accelerating them at the same velocity and measuring flight time of the analyte. Additionally, the quadrupole and the collision cells are important for later validation experiments of tandem mass spectrometry as UPLC-MS/MS. In a fragmentation experiment, the main ion of interest is selected in the quadrupole, before being fragmented in the collision cell, for the analysis of the fragments of the initial parent ion.

Metabolomics data analysis

For each UPLC-MS analysis, the amount of data generated is extremely large. This data is three-dimensional as it contains information on retention time, mass spectral analysis and peak intensity. To be able to handle these large quantities of data, scientists rely on sophisticated computational tools. There are several software’s that allow for data analysis, such as XCMS, MZMine or Metaboanalyst. In order to convert the analytical data into data that is suitable for interpretation, these softwares perform feature extraction, compound identification and also statistical analysis between the groups of interest. Feature extraction and peak alignment are required to detect a certain mass spectrometric feature to ensure that it is aligned for each sample of the
entire investigated sample set to identify features with the exact same $m/z$ value. After the data preprocessing step, the data gets sorted and can be further investigated.

Metabolite structure identification

One of the main bottlenecks of metabolomics is metabolite structure identification. In LC-MS analysis for a certain metabolite to be validated, several factors need to be considered. Even within the metabolomics community, a consensus has still not been achieved but several researchers have proposed different validation level criteria.92 Schymansky et al. proposed validation criteria with five different levels (Table 1).93 Level 1 corresponds to the highest level of confidence in the validation of the metabolite, in which the compound is either commercially available or synthesized and the retention time, mass and fragmentation all perfectly match. In level 2, the MS$^2$ spectra are compared against available databases for comparison. Metabolites are also considered to be validated at level 2 if the molecule proposed is in accordance with a literature reference. Level 3 is identification of a tentative structure but several possible different regioisomers are possible due to the lack of authentic standards. Only the determination of the unequivocal molecular formula is considered level 4 and only the exact mass of the analyte is known for level 5 metabolites. For our studies, we adapted the validation criteria proposed by Schymansky et al.

Table 1 – Validation level criteria proposed by Schymansky, et al.93

<table>
<thead>
<tr>
<th>Identification level</th>
<th>Criteria</th>
<th>Data</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Level 1</strong></td>
<td>Structure confirmed with reference standard.</td>
<td>MS, MS$^2$, RT and reference standard</td>
</tr>
<tr>
<td><strong>Level 2</strong></td>
<td>2a) Comparison with experimental library</td>
<td>MS, MS$^2$, Library MS$^2$</td>
</tr>
<tr>
<td></td>
<td>2b) Confirmed with spectra and considering experiment</td>
<td>MS, MS$^2$, Experimental data</td>
</tr>
<tr>
<td><strong>Level 3</strong></td>
<td>Possible structure - uncertain position of the substituents</td>
<td>MS, MS$^2$, Experimental data</td>
</tr>
<tr>
<td><strong>Level 4</strong></td>
<td>Unequivocal molecular formula confirmed</td>
<td>MS isotope/adduct</td>
</tr>
<tr>
<td><strong>Level 5</strong></td>
<td>Exact mass only</td>
<td>MS</td>
</tr>
</tbody>
</table>

Metabolite databases for structure identification

Development and defined repository of identified metabolites are essential for successful metabolite identification to draw the correct biological conclusions.
The robust metabolite databases allow for rapid and automated metabolite validation. Several research centers have developed comprehensive metabolite databases such as the Human Metabolome Database (HMDB), Massbank of North America (MoNa), the Global Natural Product Social Molecular Network (GNPS), the National Institute of Standards and Technology (NIST), and METLIN.\textsuperscript{94-100} The number of metabolites reported and characterized in these databases is constantly increasing. Other computational tools are also in rapid development. Tools such as SIRIUS use computer models to predict fragmentation trees spectra.\textsuperscript{101} The calculated fragmentation can be compared with available databases and measured MS/MS spectra. Despite the high quality of these experimental and computational databases that have improved the metabolite identification it is still extremely difficult to distinguish isomeric molecules. Without an authentic standard, the knowledge of the chromatographic system and the specific retention time these databases can only provide a putative metabolite structure.

New tools for metabolomics analysis

Despite the recent developments in metabolomics analysis, the field is still recent when compared with other –omics sciences, such as genomics, transcriptomic or proteomics. In other –omics fields, there are several tools adapted for the analysis of the genome or the proteome, with the use chemical biology tools for the study of methylation patterns in genomics or post-translational analysis in proteomics. Similar tools are still in development for metabolomics analysis.

Recently, two different approaches have been published for the use of chemical biology tools in metabolomics analysis.\textsuperscript{102-103} The new tools allow for the specific analysis of certain metabolite classes of interest and aim to increase mass spectrometric detection of low abundant metabolites. Importantly, these chemical biology tools can be used for targeted large-scale approaches. In a report by Ballet, \textit{et al.} a new chemical biological tool was developed for the study of sulfated metabolites that are associated with microbiota metabolism. In this report, an arylsulfatase was for the first time use for the untargeted analysis of sulfates. In the study by Garg, and coworkers, a chemically synthesized probe was used to capture amines, with increase of the sensitivity of the metabolites of up to 2600 times. A similar probe, aimed for a different reactive group, was reported by Conway, \textit{et al.} and the increase of the mass spectrometric detection for some metabolites reached up to a million times lower concentration detected (Figure 6).\textsuperscript{104}

Metabolomics tools at the interface of chemistry and biology allow for the optimization of the metabolomics workflow and can provide insight on yet undiscovered human metabolites.\textsuperscript{105-106} Furthermore, these tools aim to explore the interaction between the human and the microbiota metabolism,
as several of the metabolite groups described are associated with co-metabolism.\textsuperscript{107}
Aims

In the studies of this thesis, we aimed at developing new chemical biology methods to advance metabolomics applications. These methods were focused on phase II modifications to study human and the microbiota co-metabolism. The detailed aims were:

- Purification and characterization of the arylsulfatase from a commercially available crude mixture from the snail *Helix pomatia*.
- Development of an enzymatic treatment assay with the purified arylsulfatase for urine and fecal samples.
- Identification of sulfates in urine and human samples.
- Development of an enzymatic treatment assay with a purified glucuronidase from *Helix pomatia*.
- Simple preparation method for a library of sulfated metabolites as reference standards.
- Investigation of the global urine sulfatome of a human dietary intervention study.
Methods

*Helix pomatia* arylsulfatase purification

Crude preparation and arylsulfatase purification

The crude arylsulfatase mixture from *Helix pomatia* (1.0 g) was dissolved in buffer A and centrifuged. The supernatant was collected and filtered through Zeba™ Spin Desalting Columns (7K MWCO, 5 mL), which was pre-washed with buffer A. The resulting solution was then filtered using syringe filters (Whatman™ 30mm SPARTAN™ HPLC certified).

Table 2 – Buffers used for the protein purification.

<table>
<thead>
<tr>
<th>Buffer A</th>
<th>Buffer B</th>
<th>Buffer C</th>
<th>Buffer D</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM Tris-HCl buffer, pH 7</td>
<td>50 mM Tris-HCl buffer, pH 7</td>
<td>50 mM sodium phosphate buffer, pH 7</td>
<td>50 mM sodium phosphate buffer, pH 7</td>
</tr>
<tr>
<td>20 mM NaCl</td>
<td>500 mM NaCl</td>
<td>1.5 M ammonium sulfate</td>
<td></td>
</tr>
<tr>
<td>10% Glycerol</td>
<td>10% Glycerol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM DTT</td>
<td>1 mM DTT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The crude mixture was purified in a four step, non-tagged column purification, using an ÅKTA start. The first purification step was a reverse phase, 4-step isocratic elution with buffer B percentage increase (10, 20, 40 and 100 % buffer B concentration steps). For the separation a Hitrap Q HP, 5 × 5 column was used, at a flow rate of 2 mL/min. The fractions corresponding to the 10 % buffer B elution step were collected and pooled. The pooled fraction was then subjected to a buffer exchange into buffer C using a HiPrep 26/10 desalting column, at a flow rate of 5 mL/min. The solution from the buffer exchange was injected into a HiTrap butyl HP, 5 x 5 column and was eluted over a 50 mL salt gradient (from buffer C to buffer D). The relevant fractions in the first eluting peak were pooled and buffer exchanged into a 50 mM solution of ammonium acetate buffer, pH 7.0. The last purification step for the sulfatase was performed using a HiLoad 16/600 Superdex 200 pg with a 50 mM ammonium acetate buffer, pH 7.0. This purification step was performed at the Deindl laboratory with Laura C. Lehmann, at Uppsala University.
At every step of the purification, all fractions were analyzed by SDS-PAGE and the sulfatase and glucuronidase activity was tested using the method described below. The relevant fractions from the last step were pooled and concentrated after SDS-PAGE analysis.

**Glucuronidase purification**

*Helix pomatia* glucuronidase was purified using the same gradient and column as the first step of the arylsulfatase. Instead of the collection of the 10% step fraction, the 20% fraction enriched in glucuronidase was collected, and used as purified fraction.

**Enzymatic assay**

At every step of the purification the eluent was tested for sulfatase and glucuronidase activity to determine which fractions contain the purified enzyme and compared to the crude arylsulfatase mixture. For a quick screening of the enzymatic activity, we used the two substrates 4-methylumbelliferyl sulfate (MEBOS) and *N*-acetyltyramine-*O*-*β*-glucuronide (NATOG) as standards for sulfatase and glucuronidase activity, respectively. For each enzymatic assay, 25 μL of a 1 mM solution of MEBOS in H₂O, 25 μL of a 1 mM solution of NATOG in H₂O, 50 mM ammonium acetate (150 μL, pH 7.0) and 25 μL of each purified fraction were mixed. In parallel, we tested the stability of our standard to hydrolysis in the absence of enzyme, in order to confirm specific enzymatic hydrolysis. Five time-points were collected at 0 min, 30 min, 1 h, 2 h, and 24 h for each assay. At every time-point, 25 μL of the assay mix were collected and the proteins were precipitated with 100 μL of cold methanol for 15 min at 4 °C. After centrifugation at 13,400 rpm for 5 min at room temperature, the supernatant was collected and dried *in vacuo*. The pellet was resuspended in 60 μL of a water/acetonitrile mixture (95/5, v/v). The samples were transferred to HPLC-vials and injected into the UPLC-MS/MS system for mass spectrometric analysis.

**Arylsulfatase activity calculation**

The arylsulfatase activity assay was based on the assay described for *Helix pomatia* arylsulfatase (S9626, Sigma-Aldrich). For each enzymatic assay, 65 μL of 200 mM sodium acetate buffer at pH 5 and 40 μL of a 6.25 mM aqueous solution of 4-nitrocatechol sulfate were mixed. To this mixture were added 5 μL, 7 μL or 10 μL of 50 times diluted purified arylsulfatase. At the same time, an assay was performed without addition of any enzyme as a negative control.
The mixtures were incubated for 30 min at 37 °C. After incubation, 500 µL of 1 M NaOH were added to the reaction and the resulting solutions were transferred into a 96-well plate. Their absorbance was measured at a wavelength of 515 nm.

The amount of units in solution was calculated using the following equation:

\[
\text{Units/mL} = \frac{(A_{\text{Test}} - A_{\text{Blank}}) \times df \times V_T}{\varepsilon_{515} \times V_E \times t}
\]

Details:
- \(A_{\text{Test}}\) – Absorbance measured for the test solutions at 515 nm
- \(A_{\text{Blank}}\) – Absorbance measured for the blank at 515 nm
- \(t\) – Time factor correction (Unit definition for 1 hour)
- \(df\) – Protein dilution factor
- \(V_T\) – Total volume (in mL) of the assay
- \(\varepsilon_{515}\) – Millimolar extinction coefficient of p-nitrocatechol at 515 nm (\(\mu\text{m}^{-1}\text{cm}^{-1}\))
- \(V_E\) – Volume (in mL) of purified arylsulfatase used

Glucuronidase activity calculation

Glucuronidase activity was tested according to the protocol described by Sigma Aldrich (S9626). In order to calculate the activity of glucuronidase in solution, 65 µL of H2O were mixed with 50 µL of a 75 mM potassium phosphate buffer with 1% (w/v) bovine serum albumin, pH 6.8, 25 µL of 3 mM of phenolphthalein-glucuronide and 10 µL of the final purified enzymatic solution. A negative control was also tested, in which no enzyme was added. To stop the reaction, 500 µL of 200 mM glycine buffer, pH 10.4 were added. The resulting solution was transferred to a 96-well plate and the absorbance at 540 nM was measured to monitor the production of phenolphthalein.

At the same time, a phenolphthalein standard curved was prepared, with a quantity range of 1-5 µg. The amount of phenolphthalein was plotted against the absorbance value and test results were based on the measured absorbance.

The amount of units in solution was calculated using the following equation:

\[
\text{Units/mL} = \frac{(\mu\text{g of phenolphthalein released}) \times df}{V_E \times t}
\]

Details:
- \(t\) – Time factor correction (Unit definition for 1 hour)
- \(df\) – Protein dilution factor
- \(V_E\) – Volume (in mL) of purified arylsulfatase used
UPLC-MS/MS

Mass spectrometric analysis was performed on an Acquity UPLC system connected to a Synapt G2 Q-TOF mass spectrometer, both from Waters Corporation (Milford, MA, USA). The system was controlled using the MassLynx software package v 4.1, also from Waters. The separation was performed on an Acquity UPLC® HSS T3 column (1.8 μm, 100×2.1 mm) from Waters Corporation. The mobile phase consisted of E) 0.1% formic acid in MilliQ water and F) 0.1% formic acid in LC-MS-grade methanol. The column temperature was 40 °C and two different gradients were applied.

Table 3 – Buffers used for the protein purification.

<table>
<thead>
<tr>
<th>Gradient 1</th>
<th>Gradient 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time / min</td>
<td>Buffer F / %</td>
</tr>
<tr>
<td>0-2</td>
<td>0</td>
</tr>
<tr>
<td>2-15</td>
<td>0-100</td>
</tr>
<tr>
<td>15-18</td>
<td>100</td>
</tr>
<tr>
<td>18-20</td>
<td>100-0</td>
</tr>
<tr>
<td>20-25</td>
<td>0</td>
</tr>
</tbody>
</table>

The samples were introduced into the qTOF using negative electrospray ionization. The capillary voltage was set to -2.50 kV and the cone voltage was 40 V. The source temperature was 100 °C, the cone gas flow 50 L/min and the desolvation gas flow 600 L/h. The instrument was operated in MS\(^E\) mode, the scan range was \(m/z = 50-1200\), and the scan time was 0.3 s. In low energy mode, the collision energy was 10 V and in high energy mode the collision energy was ramped between 25-45 V. A solution of sodium formate (0.5 mM in 2-propanol:water, 90:10, v/v) was used to calibrate the instrument and a solution of leucine-encephalin (2 ng/μl in acetonitrile: 0.1% formic acid in water, 50:50, v/v) was used for the lock mass correction at an injection rate of 30 s.

Biological sample extraction

Urine and plasma sample preparation

To each urine or plasma sample prepared, four times the volume of ice-cold methanol was added to the sample aliquots for protein precipitation. Each sample was vigorously shaken for 30 s and then cooled at 4 °C for 30 min. The mixture was then centrifuged (5 min, 13,400 rpm, 4 °C) and the supernatant isolated. The solvents were removed under reduced pressure.
Ethical approval

All human samples were obtained in accordance with the World Medical Association Declaration of Helsinki and all patients gave written informed consent. Approval for the study was obtained from the ethical committee at Karolinska Institutet Hospital (Ethical approval number: Dnr 2017/290-31). For the dietary intervention study, all procedures involving human subjects were approved by King’s College London Research Ethics Committee (HR-17/18-5353) and registered at the National Institutes of Health clinicaltrials.gov as NCT03573414.

Fecal sample preparation

Frozen and homogenized fecal samples were cut, lyophilized overnight and suspended in water/DMSO solution (95/5, v/v; 1 mL/60 mg dry fecal sample). Afterwards, the suspension was centrifuged at 4 °C for 5 min (13,780g) and the supernatant was collected. This step was followed by protein precipitation with the addition of ice-cold methanol (4× volume of the fecal extract). Each sample was vigorously shaken for 30 s and cooled at -20 °C for 30 min. The mixture was then centrifuged (5 min, 13,400 rpm, 4 °C) and the supernatant isolated. The solvents were removed under reduced pressure.

Biological sample enzymatic treatment

Upon protein precipitation and centrifugation, the supernatant either containing the extracted fecal or the urine metabolite mixture was dried in vacuo at ambient temperature. The residue was dissolved in 400 μL of 50 mM ammonium acetate buffer (300 μL for feces) and divided into two equal parts for enzymatic and control assays. An aliquot of the purified arylsulfatase was utilized in the enzymatic assay (11 U for urine / 38 U for feces), while the volume of the control assay was adjusted to the same volume with 50 mM ammonium acetate buffer. An aliquot of purified arylsulfatase solution was denatured by heating to 99 °C for 5 min and used as negative control in the enzymatic assay. Both assays were shaken (300 rpm) for 17 h at 21 °C and subjected to protein precipitation by adding cold methanol (4 × the sample volume) for 15 min at 0 °C. After centrifugation (13,780 g for 5 min), the supernatant was collected and dried in vacuo. Afterwards, the remaining pellet was dissolved in 150 μl of water/acetonitrile (95/5, v/v), vigorously shaken for 30 s and then centrifuged (13,780 g for 5 min). Each supernatant was collected and transferred to a HPLC vial for UPLC-MS/MS analysis, alternating injection of control and assay samples to avoid biased results.
Data analysis

Data analysis was performed using the XCMS metabolomics software package in R (version 1.1.414), using a script designed to identify features with an \( m/z \) difference of 79.9568 Da (loss of a sulfate moiety SO\(_3\)). The results were processed using Excel 2016 with applied parameter thresholds to simplify the data set and selectively identify sulfate esters. We applied a 1.5 fold-change in favor of the control group, a \( p \)-value cut-off of 0.0001 and an intensity level higher than a 30,000 ion count. The sulfate esters were confirmed by MS/MS fragmentation experiments. In low energy mode, the collision energy was 10 V and in high energy mode the collision energy was ramped from 30-40 V.

Preparation of sulfate standards

Chemical Synthesis: Method A

The general procedure is as follows: to a solution of the corresponding hydroxyl compound and 3.0 eq NaOH, 4.0 eq NaHCO\(_3\) and 2.5 eq SO\(_3\)•NMe\(_3\) complex were added, as illustrated in Scheme 1. The reaction mixture was stirred at room temperature for 24 h and concentrated using a lyophilizer. The dry crude mixture was purified by preparative HPLC to yield the desired product.

Preparation of Sulfates: Method B

Sulfates were prepared by mixing at least 0.2 mg of a hydroxyl molecule with 1.0 eq of NaOH, 3 eq of NaHCO\(_3\), and 3 eq of SO\(_3\)•NMe\(_3\). The reaction was stirred for 16 h in an inert gas atmosphere.

The solvent of the reaction mixture was removed using a lyophilizer and the remaining solid was re-dissolved in a 5% acetonitrile solution in water and analyzed using UPLC-MS.
Results and discussion

This thesis focused on the development of enzymatic assays using arylsulfatases and glucuronidases to investigate sulfation and glucuronidation phase II modifications in human samples. We began with the purification of an arylsulfatase from a crude commercially available enzyme mixture from the snail *Helix pomatia*. The development of the enzymatic assay coupled with mass spectrometric and bioinformatic analysis for validation of sulfated metabolites in human urine samples is described in Paper I. Paper II describes the characterization of the purified enzyme. The same approach developed for the arylsulfatase was modified for an assay using a β-glucuronidase, purified from the same crude enzyme mixture from *H. pomatia* (Paper III). In this study, we also developed a new metabolite validation method based on the enzymatic conversion. In order to increase the amount of identified sulfated metabolites, we developed a straightforward approach to prepare more than 35 sulfated metabolites as standards for structure validation (Paper IV). In Paper V, we introduced the sulfated metabolome (sulfatome) analysis with a targeted investigation of sulfated metabolites in human urine samples from a dietary intervention.

*Helix pomatia* arylsulfatase crude purification and Characterization

We have developed a four-step untagged enzyme purification to purify the arylsulfatase as demonstrated in Figure 7A. The size of the arylsulfatase was determined to be about 75 kDa (SDS-PAGE). Our successful purification protocol yielded i) a high level of purity and ii) eliminated any glucuronidase activity with only 0.02 % left from the 42 kU initially present in solution (Figure 7B). To monitor and control the purification procedure, both sulfatase and glucuronidase activities of each fraction were determined with hydrolysis of test substrates p-nitrophenol sulfate and phenolphthalein glucuronide.
Since this arylsulfatase has been purified for the first time, we tested its promiscuity in hydrolysis assays with eight different sulfated substrates (Figure 8). These metabolites were either commercially available or chemically synthesized by Dr. Caroline Ballet. The enzyme converted most tested substrates and

Figure 7 – A) SDS-PAGE of the crude *H. pomatia* lysate and the purified arylsulfatase (ArS). B) Glucuronidase activity calculated for the *H. pomatia* crude mixture (Sigma-Aldrich) and the purified arylsulfatase fraction.

Figure 8 – A) Representative hydrolysis experiment of selected sulfated substrates using purified sulfatase; B) Sulfated metabolites tested as substrates in the sulfatase assay. The sulfates tested were indolxyl sulfate (3), *p*-cresyl sulfate (4), ferulic acid 4-sulfate (5), 4-methylumbelliferyl sulfate (6), *N*-acetyltaramine sulfate (7), *L*-tyrosine-*O*-sulfate (8) and estrone-3-sulfate (9). Microbiota metabolites highlighted red. Table 3 – Buffers used for the protein purification.
conversion rates were the highest for phenolic sulfates. As expected by previous studies using the crude mixture, the enzyme was unable to convert sulfated alkylalcohols and had low conversion rates for some compounds including N-acetylserotonine-O-sulfate. This substrate promiscuity observation was crucial for the assay developed, as we were able to optimize the hydrolysis conditions and incubation parameters.

**Purified *Helix pomatia* arylsulfatase characterization**

The arylsulfatase from *Helix pomatia* described in the previous section was purified for the first time and we sought to perform a comprehensive substrate specificity analysis. We determined the enzymatic activity and substrate preference for this enzyme, commonly used in analytical laboratories as the crude mixture, for 20 different sulfated compounds in parallel using a single injection. Furthermore, with this method the declining signal for sulfated metabolites and their increasing signal for their hydrolyzed products can be measured simultaneously. The metabolite structures analyzed in this study are shown in Figure 9a. Representative UPLC-MS chromatograms (EICs) with all analyzed sulfated metabolites demonstrates analysis of these compounds in a single UPLC-MS experiment (Figure 9B).

We optimized the enzyme concentration by testing substrates 4-methylbel-liferylum sulfate and indoxyl sulfate, we decided that the ideal amount to use in our experiments would be 1 U. Using optimized conditions, we performed a time-course experiment for all sulfated substrates. The assay results are depicted in Figure 10 and are separated in two different groups: sulfates that were completely hydrolyzed after 24 h; and the molecules that were either partially or not hydrolyzed under the assay conditions within 24 h. Our analysis clearly determines a substrate preference for phenolic sulfates, especially metabolites with a bi- or tricyclic aromatic scaffold. On the other hand, the arylsulfatase was unable to hydrolyze alkyl sulfates such as mannose-6-sulfate and dihydroandrosterone 3-sulfate. For the completely converted substrates after 24 h, we performed a detailed kinetic analysis (Table 4). $K_m$, $K_{cat}$ and enzyme efficiency values were determined. We identified that the enzymatic efficiency correlated with the velocity of hydrolysis depicted in Figure 10A.
Figure 9 – A) Structures of the tested sulfate ester metabolites. Metabolites tested were indolxyl sulfate (3), p-cresyl sulfate (4), ferulic acid 4-sulfate (5), 4-methyllumbrelliferyl sulfate (6), N-acetyltyramine sulfate (7), L-tyrosine-O-sulfate (8) and estrone-3-sulfate (9), 4-ethylphenyl sulfate (10), resorcinol sulfate (11), p-coumaric acid sulfate (12), vanillic acid sulfate (13), isovanillic acid sulfate (14), 2-methoxy-4-vinylphenyl sulfate (15), mannose-6-sulfate (16), 4-hydroxy-3-methoxyphenylglycol sulfate (17), 3-methylindole-5-O-sulfate (18), 4-nitrophenyl sulfate (19), methyluroolithin sulfate (20), dihydroisoandrosterone 3-sulfate (21) and N-acetylserytonin sulfate (22). The color code distinguishes between natural occurring metabolites including gut microbiota-derived metabolites (red) and potential metabolites based on phase II metabolism (black); B) Normalized extracted ion chromatograms of selected sulfate esters and their separation by UPLC-MS analysis.
Figure 10 – Hydrolysis curves for A) completely hydrolyzed investigated sulfated compounds as well as; B) incompletely converted sulfated metabolites. The declining signal for the time-dependent successful cleavage of the sulfate ester was chosen for data simplification. Assays were performed in triplicate and error bars represent SEM. Numbering corresponds to the previous figure.

In summary, we have performed a comprehensive substrate specificity and kinetic analysis for the purified arylsulfatase extracted from *H. pomatia*. The detailed evaluation of this enzyme provides crucial information for possible applications in drug metabolism, metabolomics analysis and doping control. Furthermore, we developed a new mass spectrometric methodology, which can be applied for kinetic evaluation of other enzymes as well.
Table 4 - Overview of the kinetic parameters determined for the arylsulfatase from Helix pomatia.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>( K_M ) [( \mu M )]</th>
<th>( V_{\text{max}} ) [( \mu M/s )]</th>
<th>( k_{\text{cat}} ) ((s^{-1}) \times 10^5)</th>
<th>Efficiency ((s^{-1} \cdot \mu M^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyluroolithin A sulfate</td>
<td>112</td>
<td>8.21</td>
<td>885</td>
<td>787,000</td>
</tr>
<tr>
<td>4-Methyllum-belliferyl sulfate</td>
<td>175</td>
<td>2.66</td>
<td>286</td>
<td>164,000</td>
</tr>
<tr>
<td>Estrone 3-sulfate</td>
<td>351</td>
<td>3.80</td>
<td>409</td>
<td>117,000</td>
</tr>
<tr>
<td>4-Ethylphenyl sulfate</td>
<td>1,020</td>
<td>0.66</td>
<td>71.5</td>
<td>7,010</td>
</tr>
<tr>
<td>N-Acetyltymamine sulfate</td>
<td>1,486</td>
<td>0.17</td>
<td>18.0</td>
<td>1,210</td>
</tr>
<tr>
<td>Indoxyl sulfate</td>
<td>216</td>
<td>0.02</td>
<td>2.28</td>
<td>1,060</td>
</tr>
<tr>
<td>p-Coumaric acid sulfate</td>
<td>262</td>
<td>0.02</td>
<td>2.35</td>
<td>898</td>
</tr>
<tr>
<td>Ferulic acid 4-sulfate</td>
<td>161</td>
<td>0.01</td>
<td>1.38</td>
<td>862</td>
</tr>
<tr>
<td>Resorcinol sulfate</td>
<td>131</td>
<td>0.01</td>
<td>0.92</td>
<td>696</td>
</tr>
<tr>
<td>p-Cresyl sulfate</td>
<td>1,398</td>
<td>0.08</td>
<td>8.71</td>
<td>623</td>
</tr>
<tr>
<td>Vanillic acid 4-sulfate</td>
<td>341</td>
<td>0.01</td>
<td>1.19</td>
<td>351</td>
</tr>
</tbody>
</table>

Arylsulfatase treatment development and optimization

After the enzyme purification and characterization, we developed the usage of the Helix pomatia arylsulfatase for targeted metabolomics analysis of sulfate metabolites. The assay requires high enzyme purity and the substrate promiscuity determined in the characterization assays. Since the method of choice for the analysis is mass spectrometry-based metabolomics, the composition of the control sample is of crucial importance. UPLC-MS analysis detects both molecules of interest from the sample as well as background and impurities. To enable to compare the enzymatically treated sample with the control sample perfectly, we extracted the same urine sample and divided it into two equal parts. One part of the urine sample was treated with arylsulfatase and the other part with heat denatured arylsulfatase. The denatured enzyme sample is crucial to account for the mass spectrometric background of the enzymatic mixture (Figure 11). The four sulfated metabolites indoxyl sulfate (3), p-cresyl sulfate (4), ferulic acid sulfate (5) and 4-methyllumbiferyl sulfate (6) are representative metabolites derived from co-metabolism that were identified in this study. Prior to the UPLC-MS analysis, each sample was extracted after the enzymatic treatment and reconstituted as described in the methods section.
Each sample was analyzed six times by UPLC-MS. Using the XCMS metabolomics software package in R, we performed retention time correction and peak alignment for each group. In order to selectively identify sulfates, we searched for features that are up-regulated in the control sample (denatured enzyme) and down-regulated or absent in the assay sample. An important parameter of confirmation for the presence of a sulfate ester was the detection of a fragment of 79.9568 Da, corresponding to the loss of SO$_3$. This fragment was one of the key aspects for the selective identification of sulfated metabolites as it can be detected in many cases during LC-MS analysis due to in-source fragmentation. Additional search parameters were applied for comparison of both sample sets. We searched for the hydrolyzed alcohol in the enzyme treated sample that can be detected at a later retention time compared to the corresponding sulfate due to an increased hydrophobicity (Figure 12 / ArS crystal structure from Hanson, *et al.*$^{108}$).
In the beginning of our metabolomics analysis we had 3,620 features (parameters: fold-change > 1.5; p-value < 0.0001 and intensity > 30,000 ion count). By applying the criteria referred above, we decreased the amount to 261 possible sulfate features. In the next step, we performed selective MS² analysis of all 261 possible sulfates and unambiguously validated the presence of a sulfate ester moiety in 206 metabolites. In order to further validate the metabolite structure, we confirmed a total of 36 metabolites based on comparison of MS/MS spectra with HMDB, MZmine and SIRIUS databases. For eight metabolites we determined an additional level of confidence through co-injection experiments of chemically synthesized by Dr. C. Ballet or purchased reference molecules that were used as internal standards. (Table 5).

The use of reference molecules represents the highest level of metabolite validation in structural metabolite determination and metabolomics studies. For this experiment a standard molecule and the natural metabolite detected in urine samples are analyzed in UPLC-MS/MS experiments separately to determine the retention time and high-resolution mass. The next step includes mixing both solutions to compare their chromatographic properties and mass spectra. An example for a structure determination experiment is shown for the microbiota-derived metabolite p-cresol sulfate (Figure 13). The retention times for the natural metabolite, the synthesized molecule and the co-injection perfectly matched illustrated as extracted ion chromatograms (Figure 13A). Furthermore, the fragmentation spectra of the synthesized metabolite and the natural molecule are a perfect match (Figure 13B/C).
One of the most interesting findings of this study was the identification of sulfated metabolites that were derived from the human host microbiota co-metabolism. The sensitivity of this methodology was also supported by the fact that we can detect metabolites that were only partially converted. In addition, several metabolites had been previously related in correlation with disease development, but no method has been reported that can simultaneously analyze these in a single study.

In summary, we describe a new state-of-the-art method that combines chemical biology tools and metabolomics for the selective analysis of sulfated metabolites, a compound class as a direct readout of human host and microbiome co-metabolism. In this study, we identified 206 sulfated metabolites that was three times the number of sulfates described in the most comprehensive metabolite and metabolomics database, the Human Metabolome Database (HMDB).

Figure 13 – Example of sulfate ester validation in urine with p-cresyl sulfate (2). A) UPLC-MS chromatogram comparison of urine sample vs. the synthesized standard molecule; B) MS/MS fragmentation pattern of 2 in urine sample vs. the synthesized standard molecule at a collision energy at 30 V; C) Assignment of MS/MS fragments of 2.
**Helix pomatia** glucuronidase treatment of urine samples

In the manuscript published in *ChemBioChem* entitled “Coupled enzymatic treatment and mass spectrometric analysis for the identification of glucuronidated metabolites in human samples”, we applied our new approach developed in Paper I by replacing sulfatases with glucuronidases. Glucuronides represent the most abundant phase II modification in humans and have also been linked to the co-metabolism with microbes.

The general workflow for this glucuronide detection strategy is shown in Figure 14. Although this method has similarities to the sulfatase workflow, the identification of glucuronides was more complex. Only one fragment from the in-source mass spectrometric analysis was formed in the analysis of sulfated metabolites ($m/z = 79.9568$). In comparison, the neutral loss of 176.0326 is common for glucuronidated metabolites but the glucuronic acid moiety results in four major characteristic mass spectrometric fragments. This adds an additional level of complexity to the analysis as these fragments are in some cases the dominant peaks in the MS² spectra. Overcoming these bioinformatic and analytical limitations, we validated the presence of a glucuronide moiety for 191 metabolites in urine samples and developed a new method for metabolite identification.

![Figure 14 – Workflow for the selective identification of glucuronidated metabolites in human urine samples. The method is based on glucuronidase sample pre-treatment and the use of metabolomics bioinformatic tools. Fragmentation example of indole carboxylic acid-\(O\)-glucurunide (23) and indole carboxylic acid (24). Metabolite identification was conducted by i) mass spectrometric fragmentation (most common analysis); ii) co-injection of authentic standards (highest confidence); and iii) new strategy by using authentic standards of hydrolyzed metabolites for validation through co-injection.](image)

We started with one urine sample that was divided into two equal parts after metabolite extraction (described in Paper I). One sample part was treated with purified glucuronidase (*H. pomatia*), obtained from anion exchange chromatographic purification and the other part was treated with denatured protein to account for
the background. The purification fraction was obtained in the same anion exchange purification step as the arylsulfatase, but present in 20% Buffer B fraction. This fraction was enriched in glucuronidase activity.

After analysis of each sample six times by UPLC-MS, the data was processed with XCMS in the R platform to perform retention time correction, peak picking and statistical analysis. We were able to identify 262 possible glucuronides, out of significantly altered 654 initial features between the two samples. We performed MS/MS fragmentation for all 262 possible features and confirmed the presence of a glucuronide moiety in 191 metabolites. Parameters used for glucuronide validation were identification of the neutral loss of the glucuronide ($m/z = 176.0326$) as well as the three characteristic fragments for the cleaved glucuronide. From the 191 validated glucuronides, we determined the chemical formula for 181 metabolites and identified 40 putative structures based on database fragmentations. Furthermore, we developed an additional level for validation of metabolites due to the setup of our method (Figure 15). The standard method (Strategy A) is based on co-injection experiments and comparison of compound fragmentation comparison of the standard and the natural metabolite that we also described in Paper I (Figure 13). The new level of validation is based on the hydrolyzed molecule upon enzymatic treatment. With the specificity of the enzymatic assay, we can consider that all the new peaks appearing are hydrolysis products from the original glucuronide. Based on this fact we developed strategy B of validation.
An example for the validation of a compound using strategy A is p-cresyl glucuronide (25) (Figure 15). It is evident that both the retention times and the fragmentation spectra perfectly match in this experiment. One example is illustrated for quinoline-2,8-diol (26) in Figure 15. In this case, the co-injection was performed with the enzymatic treated sample, and the retention times between the standard molecule and the hydrolyzed metabolite matched closely. To further increase the confidence for the identified molecule, we also carried out fragmentation experiments, observing identical fragmentation patterns between the hydrolyzed metabolite and the standard is identical. We also identified a total of 9 compounds using strategy B. We can unequivocally distinguish metabolites that are regioisomers, e.g. ferulic acid and isoferulic acid (Figure 16). Our new method also allows for the confirmation of metabolite structures present at low concentrations or with poor ionization properties such as 6,7-dihydroxycoumarin, indole-3-carboxylic acid, indole-3-acetic acid, catechol, and 4-methylcatechol.

In this study we identified 191 glucuronides in a single urine sample including metabolites derived from the human and microbiota co-metabolism. Furthermore, we developed a new strategy for compound identification based on the unique properties of our assay that can be used to distinguish structural

![Figure 16 – Examples of metabolite structure validation using commercial standards (strategy B). In these two examples A) ferulic acid (27) and B) isoferulic acid (28) were identified as hydrolyzed glucuronides. Chromatograms at the top: Hydrolyzed metabolites from the enzyme treated sample; Chromatograms at the bottom: Standard molecules.](image-url)
isomers and allows for metabolite validation in large scale metabolomics studies and will be applied for biomarker discovery.

Library expansion of sulfated metabolite standards

One of the main bottlenecks for metabolite validation, is the availability of authentic synthesized reference standards. Therefore, we developed a straightforward and quick method to produce validation-ready sulfated metabolite standards. Traditional chemical synthesis of metabolites requires several chemical reactions including protecting group chemistry to avoid byproduct formation for most synthetic routes.109-110 This is followed by preparative HPLC purification and NMR characterization to confirm the chemical structure of the synthetic product (Method C).34, 102, 111 Overall the whole process is time consuming, and for the purpose of metabolite structure validation, compounds need to be accessible using simple procedures that can be prepared in any analytical laboratory without an infrastructure for chemical synthesis. In this new developed procedure (Method D), the reactants and solvents were simply removed from the solution. This straightforward method rapidly provides the sulfated molecule standard for validation and can be used for large-scale and parallel preparation of new sulfated compounds (Figure 17).

![Figure 17 – Workflow for the library preparation. Comparison between the obtained product from standard chemical synthesis (Method C) and the two-step preparation described in this manuscript (Method D).](image)

The first step to confirm the suitability of this method, was the comparison of a molecule synthesized by Method C with the same molecule using Method D. In Figure 18, we compare the synthetic products of both methods for 3-methoxyphenol sulfate (2). The two synthetic molecules are identical based on their retention time (Figure 18A) and fragmentation pattern in the UPLC-MS/MS analysis (Figure 18B). Another important confirmation for the feasibility of Method D, was the confirmation that no side reactions were observed, besides the main monohydroxyl group as the sulfation site. Side reactivities
were tested for carboxylic acid moieties, in which all the starting material remained after the reaction (Figure 18C). Primary amines were also tested, with the test sulfation of serotonin. For this compound no bis-sulfated product was identified in the reaction mixture, but we observed two product peaks for monosulfated products in the extracted ion chromatogram. Mass spectrometric fragmentation experiments were performed to distinguish each structure based on their mass spectrometric fingerprint (Figure 18D). Each metabolite was identified by specific MS-fragments for O-sulfated serotonin ($m/z = 79.9582$– the loss of SO$_3$) and the specific fragmentation of N-sulfated serotonin ($m/z = 95.9749$– loss of NSO$_3$).[30] Structurally similar compounds can thus be easily distinguished by analysis of these fragments and the sulfation site easily identified.
Besides the metabolite characterization, another powerful tool provided by this simplified synthesis is the quick validation of regioisomers. These compounds have the same exact chemical formula, differing only in the position of the functional group, and cannot be distinguished solely based on the mass spectrometric fragmentation. Furthermore, chromatographic properties and retention time are difficult to predict for LC-MS-based analysis of similar compounds as a retention index (RI) can currently only be used for GC-MS analysis.112 Depicted in Figure 19 are two different examples of regioisomers that can only be distinguished through their different chromatographic properties. Interestingly, these similar structures can either result in baseline separated or closely eluting and overlapping peaks. Regioisomers 2-hydroxypyridine sulfate and 3-hydroxypyridine sulfate have a retention time difference of over 2.5 min and can clearly be separated (Figure 19A). This is uncharacteristic compared to other structural isomers. In another example, the three metabolites 2-hydroxybenzoic acid sulfate, 3-hydroxybenzoic acid sulfate and 4-hydroxybenzoic acid have similar retention times but each structure can clearly be assigned using synthetic reference standards (Figure 19B). Single UPLC-MS analyses of each prepared standard demonstrate separation of both peaks. Co-injection experiments at equimolar concentrations can be used to distinguish these metabolites in complex biological matrices.

After the validation of Method D, we sulfated 37 additional monohydroxylated metabolites. This allows for the extension of a previously existing synthetic library of 24 previously purchased or synthetic compounds to 62 sulfated reference molecules. In order to characterize these metabolites, most were previously not reported, we performed retention time analysis as well as fragmentation experiments at two different voltages (10 eV and 30 eV) and uploaded this information to the MoNa database.

Figure 19 – Distinction of representative structural regioisomers prepare with Method B. A) Extracted ion chromatogram (EIC) traces of the UPLC-MS analysis of 2-hydroxypyridine sulfate (blue) and 3-hydroxypyridine sulfate (green); B) EIC traces of the UPLC-MS analysis 4-hydroxybenzoic acid sulfate (yellow), 2-hydroxybenzoic acid sulfate (blue), and 3-hydroxybenzoic acid (green).
Sulfatome analysis of a (poly)phenol rich dietary intervention

To expand on the sulfate discovery in human samples performed in paper I, we investigated samples from volunteers of a dietary intervention study based on a diet of flaxseeds, raspberries and soybeans. Samples were obtained from our collaborator Ana Rodriguez-Mateos at King’s College London (UK). For this study we developed a two-step analysis (Figure 20). The first step consisted on expanding on our first sulfate analysis, through the optimization of our enzymatic assay and the discovery of a larger set of sulfated metabolites. The optimization of the previous assay was required, as large amounts of en-

Figure 20 – Overview of the workflow for comprehensive UPLC-MS/MS metabolomics analysis. Urine samples were collected before (V1) and after (V2) dietary intervention. Step 1: Sulfatase-based assay for selective identification of sulfated metabolites. Step 2: Global analysis of the sulfated metabolome (sulfatome).
zyme were used for the denaturation step. In order to circumvent this, we decided to test the samples in batches of four samples, in which the control would be a pool of the same samples. This reduces the control sample to one for four samples and represents an average of these samples. We compared the aforementioned optimization with our preciously developed assay with four separate control samples for each enzymatic assay sample. The results for this analysis were highly similar.

For this study, we initially expanded on our library of sulfated metabolites due to the sample set based on dietary intervention. Applying the same analysis with XCMS and after extensive fragmentation analysis and metabolite validation, we were able to identify a total of 235 sulfated metabolites. From these metabolites, 130 were validated at levels 1 or 2.

Table 6 – Sulfated metabolite validation levels for the sulfatome analysis.

<table>
<thead>
<tr>
<th>Level</th>
<th>Validation criteria</th>
<th>Metabolite no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Authentic standard</td>
<td>27</td>
</tr>
<tr>
<td>2a</td>
<td>MS² spectral match with existing metabolite libraries</td>
<td>59</td>
</tr>
<tr>
<td>2</td>
<td>MS² spectral match assisted with computational tools</td>
<td>44</td>
</tr>
<tr>
<td>3</td>
<td>MS² validation of sulfate ester moiety loss</td>
<td>105</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>35</td>
</tr>
</tbody>
</table>

For the second step of our analysis, we used the identified sulfated metabolites in step 1 to characterize the overall sulfated metabolome (termed sulfatome) alteration in the volunteers and to identify diet dependent markers. Samples were collected for volunteers before they were on the (poly)phenol rich diet (V1) and after three days of diet, the urine was collected again (V2). Comparing the sulfatome of these two samples provides the perfect overview for the sulfated metabolites derived from this diet including several microbiota and host co-metabolites.

Global human sulfatome analysis

We compared the sulfatome changes in 22 volunteers. For dietary studies, the most common analysis performed include certain metabolic signatures at population level. These signatures are affected by several other factors, besides the diet, such as long-term dietary habits, the individual genome, a baseline gut microbiome composition as well as other factors such as physiology, age, sex, and BMI.113-114

To identify diet specific upregulated metabolites, we set a 1.5-fold change alteration between V2 and V1. An inverse factor was also applied for downregulated sulfates. The metabolites that were within these criteria were considered unaltered (fold-change: 0.67<X<1.50). We observed that the diet does
not affect all volunteers in the same way. We ranked subjects based on the percentage of upregulated sulfated metabolites after consumption of the polyphenol-rich breakfast for 3 days (Figure 21A). Despite the large individual variability, it is possible to observe that nine subjects have more than 118 upregulated sulfated metabolites (>50%), while for the other 13 volunteers the

Figure 21 – A) Global human sulfatome analysis. Classification of subjects based on sulfated metabolite level changes of all identified 235 sulfate esters before and after dietary intervention. Individual data is listed according to the number of upregulated sulfated metabolites. B) Individual distribution of 28 metabolites that were significantly upregulated after dietary intervention (V2/V1; p<0.01). (green: upregulated metabolites by at least 1.5-fold; blue: downregulated metabolites by at least 1.5-fold; grey: metabolites that were in-between these factors).
number of upregulated sulfate esters is below 50%. Three outliers were identified that had more than 100 sulfated metabolites downregulated. Extending the scope of this study to a larger sample set, has a high potential to draw conclusions for personalized medicine, dividing subjects in high and low sulfate metabolizers.\textsuperscript{114}

We have also investigated how the 28 most significantly upregulated metabolites ($p < 0.01$) are distributed among the 22 subjects at an individual level (Figure 21B). As expected, none of these metabolites showed a consistent increase in all 22 individuals. For example, 4-ethylphenyl sulfate was more than 1.5-fold upregulated in 20 individuals, while it was more than 1.5-fold downregulated in one individual and remained unchanged in one individual. We only observed a few exceptions for all 28 metabolites.

Discovery of unknown sulfated metabolites
Among the 235 validated sulfated metabolites, we detected 33 sulfated metabolites for the first time that have only been registered in HMDB as their corresponding desulfated compound. Among these compounds 17 polyphenolic compounds, four $N$-heterocyclic compounds, three indole molecules, three new hippuric acids sulfates, and six with diverse scaffolds different from the first four groups that also include aliphatic alcohols (Structures can be found in Paper V). The majority of these newly discovered sulfates are polyphenolic compounds, of which many are derived from main dietary components

(Poly)phenol dietary markers
Several of the products derived from the (poly)phenol metabolism were associated with the nutrition. Soybean derived identified metabolites included five metabolites derived from daidzein metabolism (daidzein sulfate, daidzein-7-glucuronide-4-sulfate, dihydrodaidzein sulfate, tetrahydrodaidzein sulfate, 8,2'-dihydroxyflavone sulfate) and two metabolites derived from genistein metabolism (genistein-7-glucuronide-4-sulfate and 4-ethylphenyl sulfate) (Figure 22A). The results presented are in accordance with previous reports by Hoshida, \textit{et al.}\textsuperscript{45} The two metabolites dihydrodaidzein sulfate and tetrahydrodaidzein sulfate had not been previously reported as metabolites for these diets in humans. They have only been reported in rat urine after high dosage of the pure parent compound daidzein that exceed concentrations in common diets.\textsuperscript{115} Another soy diet product is 4-ethylphenyl sulfate, which was also found to be upregulated in the dietary intervention group (V2).\textsuperscript{116} We also identified significantly altered conjugated metabolites of enterodiol and enterolactone, downstream products of flaxseeds ingestion.

One of the main components in raspberries are anthocyanins. Metabolites such as $p$-coumaric acid, caffeic acid, ferulic acid, and 4-hydroxyphenylpyruvate are common byproducts of anthocyanins, derived from the degradation
by the human microbiota. Three sulfated analogues of these compounds were significantly upregulated (Figure 22B). Dihydroconiferyl alcohol and sinapic acid are downstream products of ferulic acid, and the conjugated form of these two metabolites was also upregulated. Caffeic acid 3-sulfate and ferulic acid 4-O-sulfate have been associated with raspberry rich diets. Sinapic acid sulfate was detected in human urine after tea intake as well as in rat urine after whole rye consumption.

Figure 22 – Investigation of specific diet-induced sulfated metabolites derived from gut microbiome-host co-metabolism. An overview of significantly altered sulfated metabolites after dietary intervention of 22 individuals. Highlighted are the main dietary sources for each metabolite class. A) Depiction of metabolic changes of sulfated analogues of metabolites of phytoestrogens. Scatter plots for sulfated isoflavones present in soy and lignans mainly present in flaxseeds demonstrate semi-quantitative changes of each metabolite. B) Depiction of metabolic changes of anthocyanin-derived sulfated metabolites that are mainly present in raspberries. Values are normalized mass spectrometric peak areas of the EICs for each metabolite (Mean values ± SEM). p-values: * < 0.05, ** < 0.01, *** < 0.001.
In conclusion, we have identified 11 previously unknown sulfated molecules produced and upregulated after consumption of raspberries, flaxseeds and soybeans. These metabolites should be validated in future studies to evaluate their potential as biomarkers for consumption of one of these three diets. This is the first targeted analysis of this co-metabolism compound class and exceeds previous dietary studies that were mainly focused on single compound classes. Furthermore, this comprehensive study also led to the discovery of a plethora of unknown sulfate esters with unknown bioactivity and can be applied for comparative analysis of important sulfated metabolites in any type of human samples to uncover unknown links of human and microbiome co-metabolism.
Conclusion

In this dissertation, we have successfully developed a series of new chemical biology methods for advanced metabolomics analysis and the elucidation of metabolite structures. These methods were targeted for the elucidation of the co-metabolism of the human host with its microbiome.

Essential for the analysis was the purification of an arylsulfatase (ArS) from a crude mixture from *Helix pomatia* for the first time. This enzyme is commonly used in analytical chemistry projects in different research areas. The purification allowed for the selective enzymatic cleavage of sulfated metabolites without background reactions from glucuronidases, oxidases and other enzymes, which was required for our assay development. ArS was present in large quantities for an untargeted analysis of sulfates in urine and fecal samples. Furthermore, the enzyme has a very high substrate promiscuity as demonstrated by the wide array of sulfates converted and identified. The substrates were even further characterized for enzymatic substrate specificity.

The newly developed sulfatase assay was combined with mass spectrometry and bioinformatic metabolomics analysis. The application to human samples allowed for the identification of 206 sulfated metabolites. This was the largest number of sulfates identified in human samples and in a single analysis was three times higher than reported in the most common human metabolite databases. Importantly, many molecules identified were the product of metabolite conversions by the human host of microbiome-derived metabolites.

The method was optimized for targeted analysis of glucuronides in urine samples. This was performed with an enzymatic assay using one glucuronidase purified from *Helix pomatia*. We were able to identify a total of 191 glucuronides in urine samples using optimized and specific data and mass spectrometric fragmentation analysis. In addition, a new method to validate metabolites was presented, based on the hydrolyzed molecule. With the new strategy we identified 11 previously undetected glucuronidated metabolites.

Furthermore, a straightforward method for the preparation of sulfated metabolites was designed to validate the structure of sulfated metabolites. This crucial step in metabolomics analysis is one of the most common bottlenecks for metabolite confirmation. We prepared over 30 new sulfated molecules for structure validation. Our in-house library contains more than 70 sulfates. Several of the prepared sulfates were regioisomers and co-injections with human samples can directly differentiate these metabolites.
For the dietary intervention study, we have extended the scope of the sulfatase assay for investigation of more samples in parallel. Due polyphenol rich diet, we have discovered an even larger number of sulfated metabolites to 236 identified. We have termed this targeted strategy sulfatome analysis to investigate the individual sulfated metabolome. Expanding the scope of this study for a larger sample set has allowed us to divide the subjects into different metabolizer groups and closer to personalized diet. Additionally, we identified 11 previously unknown sulfates associated with the (poly)phenol rich diet. The sulfate metabolites have not been described as markers for this diet before. Notably, these previously undetected metabolites have an unknown bioactivity and can now be investigated to elucidate their impact on human physiology. The importance of the microbiome on this polyphenol diet was evident by several sulfated metabolites identified as products of the co-metabolism with the human.

In conclusion, we have developed several new methods that are important for the elucidation of metabolism of the microbiome and their human host. These methods incorporate enzymatic treatment with mass spectrometric analysis for the identification of new sulfated or glucuronidated. These metabolites are one important gateway between the human host and the gut microbiome with a potential to discover new biomarkers for health and disease.
För att diagnostisera en patient med diabetes på 1600-talet utgick läkaren ifrån sötman på patientens urin. Detta var en av de tidigaste metoder som användes för att upptäcka sjukdomar baserat på biomolekyler. Numera är det mycket lättare då det kan göras med utrustning i hemmet genom ett enkelt nålstick i fingret. Men trots den förbättring som skett av de diagnostiska teknikerna kan inte alla sjukdomar upptäckas snabbt och effektivt. Omfattande forskningsarbete bedrivs ständigt för att framställa fler och bättre diagnostiska verktyg till alla typer av sjukdomar och en del av diagnostiken utvecklas genom upptäckten av små molekyler som vi kan hitta i kroppsvätskan hos människor, till exempel blod och urin.

Dessa små molekyler kallas metaboliter och är exempelvis sockerarter, vitaminer och aminosyror. De spelar en viktig roll på grund av att de kan bidra till en tidig upptäckt av sjukdomar genom deras korrelation med andra mikroorganismer som lever i människans mag-tarmkanal. Dessa så kallade ’hälssamma’ bakterier kallas mikrobiota (tarmflora) och består av biljoner bakterier och andra mikroorganismer och har i samutvecklats i mag-tarmkanalen hos däggdjur. Undersökningar av metabolitinteraktioner mellan människor och deras bakterier kan leda till viktiga upptäckter av nya metabolitmarkörer.

Metaboliter kan upptäckas med masspektrometri, en teknik som möjliggör mätning av metaboliter enligt deras molekylvikt och systematisk analys av metaboliter kallas metabolomik. Detta är ett kraftfullt område som ständigt utvecklas och förbättras.

Sammanfattningsvis så har vi undersökt den allmänt okända sammetabolismen mellan människa och mikrobiota och vi har även påvisat nya metaboliter med potential att bli biomarkörer för sjukdomar, vilket kommer att utforskas i framtida studier.
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