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# Comparison of two arylsulfatases for targeted mass spectrometric analysis of microbiota-derived metabolites

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ABSTRACT

Sulfation of metabolites is the second highest phase II modification in humans, which plays a critical role in the xenobiotics clearance process and gut microbiota-host co-metabolism. Besides the main function to remove xenobiotics from the body, sulfated metabolites have also been linked to inflammation, bacterial pathogenesis and metabolic disorders. A better understanding of how these metabolites impact the human body has turned into an important research area. Analytical methods for selective identification of this metabolite class are scarce. We have recently developed an assay utilizing the arylsulfatase from *Helix pomatia* due to a high substrate promiscuity combined with state-of-the-art metabolomics bioinformatic analysis for the selective identification of *O*-sulfated metabolites in human samples. This enzyme requires a multistep purification process as highest purity is needed for the developed mass spectrometric assay. In this study, we have utilized a new and recombinant overexpressed arylsulfatase (ASPC) for the selective identification of organic sulfate esters in human urine samples. We have compared the substrate conversion in urine samples and substrate specificity of this enzyme with purified arylsulfatase from *Helix pomatia*. Our analysis of urine samples revealed that both enzymes can be utilized for the selective analysis and discovery of sulfated metabolites with high promiscuity as demonstrated by equal hydrolysis of 108 substrates including sulfated conjugates of 27 metabolites of microbial origin. Importantly, we also identified 21 substrates in human urine samples that are exclusively hydrolyzed by ASPC and application of this enzyme increases the discovery of unknown sulfated metabolites with a higher scaffold diversity.

## 1. Introduction

Trillions of microbes reside in the human gut. Humans are considered to be a complex superorganism based on the magnitude of the symbiotic gut microbiome [1–5]. Over the past decade, it has been revealed that these microbial communities equal the number of human cells in the body but with an increased biochemical repertoire that is mostly orthogonal to the human metabolism. Elucidation of the impact of the gut microbiome on human health and disease development is an emerging research field that is considered as important as the human genome sequencing [6,7]. Humans ingest or are exposed on a daily basis to xenobiotics such as diet, environmental pollutants and pharmaceuticals, which are an important part of the human exposome [8–11]. The gut microbiome contributes strongly to the metabolic transformation of molecules and processing in the gastrointestinal tract. Metabolic conversions result in compound structures that alter bioavailability, bioactivity and effects on the human body [12]. Microbiota-derived molecules can be absorbed by the human gut and are further metabolized by the human detoxification machinery. This two-step process increases the hydrophilicity of these metabolites for excretion from the human body [13,14]. Phase I conversion includes oxidation, reduction or hydroxylation of metabolites to incorporate a polar functional group that afterwards undergoes methylation, sulfation or glucuronidation as part of the phase II metabolism process to increase the hydrophilicity of compounds. Especially, the phase II modification of *O*-sulfated metabolites is a compound class that has been considered as a signature for the co-metabolism of microbes and their human host. Investigation of sulfated metabolites has been explored to identify uncharacterized metabolic links between

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the gut microbiome and the human host [15,16]. Some of these compounds have been linked to various diseases, *e.g.* phenyl sulfate and ethylphenyl sulfate are two prominent examples of this co-metabolism and have been associated with diabetic kidney disease and autism [17,18]. Phenol and ethylphenol are products of tyrosine metabolism through the gut microbiome that are further converted into their corresponding sulfated analogues through hepatic sulfation by the human host. Various additional sulfated metabolites derived from diet and drug metabolism have been reported in human samples [19–22]. For example, vanillic acid 4-*O*-sulfate has been associated with chocolate and tea consumption [23]. Detailed investigation of this metabolite class is crucial to understand the molecular impact of microbiome-host co-metabolism on human health. Many studies of xenobiotic metabolism have even demonstrated a larger contribution of microbial cells than human cells to the conversion and clearance of metabolites [12,24].

For the selective analysis and discovery of these microbiota-derived metabolites to determine their toxic or beneficial properties, a myriad of analytical challenges must be solved [25–27]. Analytical methods for the selective analysis of sulfated metabolites are scarce. Mainly mass spectrometric methods have been utilized for investigation of sulfated metabolites but these are limited for single compound classes [28–31]. We have recently developed an arylsulfatase-based mass spectrometric metabolomics method for selective analysis of sulfate esters and discovery of unknown metabolites [15,32]. An enzyme with broad substrate specificity is crucial for identification of *O*-sulfates through selective hydrolysis. We have previously utilized the commercially available and promiscuous arylsulfatase from *Helix pomatia* (Hp-AS) [32]. However, this commercially available enzyme is a crude extract that also contains glucuronidases, peptidases and oxidases, which would lead to undesired metabolite conversation and more challenging data analysis as described previously [15,16,32]. This crude extracted enzyme requires four steps of untagged purification that is not feasible for large scale analysis. While the promiscuity of this enzyme has been validated, we have also demonstrated that Hp-As is limited in the conversion of some substrate classes. In this study, we have now evaluated the versatility of another commercially available purified arylsulfatase (ASPC$^{TM}$ for simplification referred to ASPC in this manuscript) for application in our assay and the discovery of microbiota-derived compounds.

## 2. Material and methods

### 2.1. General information

All reagents and solvents were purchased from Sigma-Aldrich or Fischer Scientific and were used without further purification. HPLC grade solvents were used for HPLC purification and mass spectrometry grade for UHPLC-ESI-MS analysis. All biochemical reactions were performed with HPLC or LC–MS grade solvents. Solutions were concentrated *in vacuo* on a Speedvac Concentrator Plus System (Eppendorf, Hamburg, Germany). High-resolution mass spectra were acquired on a SYNAPT G2-S High Definition Mass Spectrometry (HDMS) using an electrospray ionization (ESI) source with an ACQUITY UPLC I-class system and equipped with a Waters ACQUITY UPLC® HSS T3 column (2.1 μm, 100 × 1.8 mm). The arylsulfatase ASPC$^{TM}$ was provided by Kura Biotech Inc.

### 2.2. Human samples

Healthy donor urine 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 Stockholm ethical committee *via* Karolinska University Hospital (Ethical approval number: Dnr 2017/290−31). Urine samples were collected using routine clinical collection protocols and all patient codes have been removed in this publication. All samples were stored at -80 °C.

### 2.3. UPLC-MS/MS analysis

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 A) 0.1 % formic acid in MilliQ water and B) 0.1 % formic acid in LC–MS-grade methanol. The column temperature was 40 °C with the following gradient: 0−2 min, 0% B; 2−15 min, 0–100 % B; 15−16 min, 100 % B; 16−17 min, 100−0% B; 17−21 min, 0% B, with a flow rate of 0.2 mL/min.

The samples were introduced into the Q-ToF 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 MSE 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.

### 2.4. Enzymatic assay

A mixture of 12 sulfated compounds was prepared (500 μM each in 50 mM ammonium acetate). In every assay 1 U of sulfatase was used with a starting concentration of 50 μM for each compound. Aliquots were collected at different time points, the enzyme was precipitated using methanol to quench the enzymatic reaction. The supernatant was collected after centrifugation (5 min, 13,600 rpm) and dried under vacuum in a Speedvac. Samples were reconstituted in 5% acetonitrile in water prior to UPLC-MS analysis.

### 2.5. Urine sample preparation

40 μL each from 10 different urine samples were pooled to constitute 400 μL sample. Ice cold methanol (1.6 mL) was added to urine sample for protein precipitation. The sample was vigorously shaken for 30 s and then cooled at 4 °C for 30 min. Upon protein precipitation and centrifugation at 13,780 *g* for 5 min, equal amounts of the supernatant containing the extracted urine metabolite mixture was transferred into two separate tubes and dried *in vacuo* at ambient temperature. The residue of one tube was dissolved in 400 μL of 50 mM ammonium acetate buffer for the Hp-AS enzymatic assay and control sample. The residue of the other tube was dissolved in 200 μL of instant buffer for the ASPC enzymatic assay and control sample. Aliquots of the purified arylsulfatase Hp-AS and arylsulfatase ASPC utilized in the enzymatic assay (11 U for urine), while aliquots of corresponding arylsulfatase solutions were denatured by heating at 100 °C for 30 min and used as negative control. The ASPC control and enzymatic assays were shaken (300 rpm) for 22 h at 40 °C and Hp-AS control and enzymatic assays were shaken (300 rpm) for 22 h at 25 °C. All samples were 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.

### 2.6. Data analysis

Data analysis was performed using the XCMS metabolomics software package under R (version 1.1.414), using a script designed to identify features with a *m/z* difference of 79.9568 Da [33]. 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 cutoff in favor of the control group, a p-value cut-off of 0.0001 and an intensity level higher than a 20,000 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.

## 3. Results and discussion

In our previous studies, we have purified an arylsulfatase from *H. pomatia* with high substrate promiscuity and demonstrated its application for the detection of a large number of sulfated metabolites. However, this commercially available enzyme is only available as a crude extract and also contains glucuronidases, peptidases and oxidases. The developed purification method is time consuming, a multistep procedure and requires suitable equipment. We have now evaluated the commercially available and recombinant overexpressed arylsulfatase ASPC, which is an ultra-pure aqueous preparation and thus free from secondary enzymes. ASPC can be directly utilized without any further purification steps. This enzyme has not yet been characterized for broad substrate specificity. We have conducted a comprehensive analysis of human urine samples to compare the potential of ASPC with purified Hp-AS to evaluate substrate promiscuity and versatility for the discovery of unknown sulfated metabolites.

### 3.1. Comparison of substrate specificity

In an initial analysis, we compared the substrate conversion and substrate specificity between ASPC and Hp-AS using our developed mass spectrometric assay [32]. We selected 12 sulfated metabolites to cover several compound classes including metabolites involved in microbiota-host co-metabolism. Briefly, each organic sulfate was incubated with ASPC or Hp-AS and quenched samples were collected at different time points. These were analyzed by UPLC-MS and the specific mass spectrometric signal of each sulfate substrate was integrated. This hydrolysis assay revealed that ASPC and Hp-AS convert most tested substrates with similar efficiency (Fig. 1). The four substrates 4-methylumbelliferyl sulfate (**1**), methylurolithin sulfate (**2**), estrone-3-sulfate (**3**), and *p*-cresyl sulfate (**4**) are completely hydrolyzed by both enzymes (Fig. 1A). Interestingly, we also observed differences in the hydrolysis of substrates. For example, the metabolite *N*-acetylserotonin sulfate (**5**) is completely hydrolyzed by ASPC, while only <20 % was hydrolyzed by Hp-AS (Fig. 1B). In contrast to this observation, dihydroisoandrosterone-3-sulfate (**6**) is partially hydrolyzed (38 %) by HP but not at all by ASPC (Fig. 1C). Other compounds including L-tyrosine sulfate (**7**), 2-aminophenol sulfate (**8**) and 4-aminophenol sulfate (**9**) were not converted by any enzyme (Table 1).

### 3.2. Sulfatase assay in a pooled urine sample

Based on these results, we sought to compare hydrolysis and metabolite identification of both enzymes in human urine samples [15]. We optimized the hydrolysis conditions and determined 40 °C as an optimum temperature for ASPC (data not shown). In order to increase the metabolite diversity, we pooled 10 urine samples from different individuals (40 μl each). Ice-cold methanol was added to precipitate proteins and enzymes. After centrifugation, the supernatant was divided into four equal fractions of 100 μL. Assay I and assay III were treated with ASPC arylsulfatase and purified arylsulfatase from *H. pomatia*, respectively. Assay II and assay IV were treated with denatured ASPC and Hp-AS to serve as the control sample to compensate for any background signals during the mass spectrometric analysis (Fig. 2).

We adjusted the concentrations of both enzymes to 85 U/mL to avoid unnecessary dilution factor effects. We treated each urine sample fraction with equal amounts of each enzyme (11 U) for 22 h. Upon quenching the assays with methanol, metabolites were extracted, the sample reconstituted and analyzed *via* UPLC-MS. Each sample was injected six times for UPLC-MS/MS analysis in negative mode with a randomized sample list to reduce technical errors. The UPLC-MS data for all four assays was processed using the XCMS software package in R to obtain a list of features of potential sulfated metabolites [33,34]. The unbiased principal component analysis demonstrates a clear separation of enzyme-treated and control sample based on overall features (Fig. S1). Next, we specifically selected features that were upregulated in the control sample compared with the corresponding enzyme treated sample using the following criteria: 1) fold change >1.5; ii) intensity >20,000; and iii) 10 ppm mass accuracy. Finally, we prepared a list of features with a difference of *m/z* = 79.9568 (the loss of a sulfate moiety) that are potential sulfated metabolites. We performed UPLC-MS/MS fragmentation for all these metabolites and confirmed the presence of 163 sulfate esters. We matched the fragmentation pattern for each metabolite with MS/MS databases including METLIN, SIRIUS and HMDB or used our in-house library [35–37]. Furthermore, we unambiguously validated the structure of 36 metabolites using commercially available or synthesized standards (Table S1) [38].

### 3.3. Classification of metabolites into three groups based on the conversion by ASPC and Hp-AS

We have divided all identified sulfated metabolites from both enzymatic assays into three major classes: i) metabolites that are equally hydrolyzed by both the enzymes; ii) metabolites that are hydrolyzed exclusively by ASPC; and iii) metabolites that are hydrolyzed exclusively by Hp-AS (Fig. 3A). We also identified 21 metabolites that did not fit into any of these three groups with clear preference for any enzyme, which are unassigned (Table S2; confidence levels for metabolite structure validation are provided). As mass spectrometry is not quantitative, metabolites with a hydrolysis of more than 90 % were defined as completely hydrolyzed (termed as good substrates), metabolites that are hydrolyzed between 70 %–90 % were defined as intermediate substrates and metabolites that are hydrolyzed below 70 % were defined as poor substrates. Moreover, metabolites with less than 20 % conversion rate were considered as unhydrolyzed substrates. An overview of the sulfated metabolite distribution in each class is depicted in Fig. 3B.

#### 3.3.1. Equally hydrolyzed metabolites

We identified more than 100 sulfated metabolites that were equally hydrolyzed by Hp-AS as well as ASPC. Compounds were of diverse metabolite scaffolds including indoles, cinnamic acids,

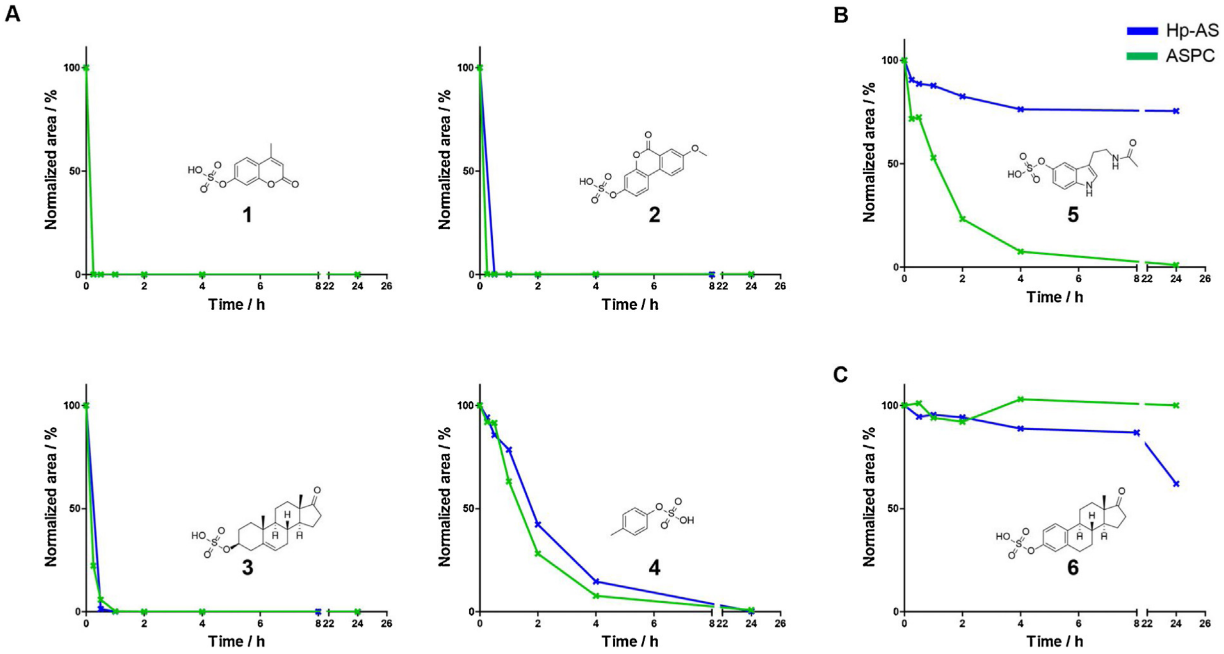
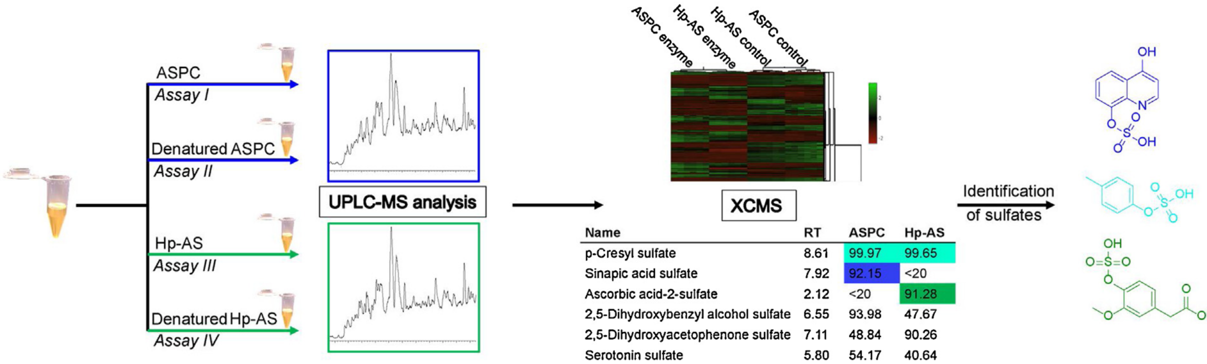


**Fig. 1.** Comparison of substrate hydrolysis for the two arylsulfatases Hp-AS and ASPC for sulfated substrates in *in vitro* enzymatic assays; (A) metabolites equally hydrolysed; (B) representative metabolite exclusively hydrolyzed by ASPC; (C) representative metabolite that is exclusively hydrolyzed by Hp-AS.

**Table 1**
Enzymatic assay hydrolysis percentages after 24 h of sulfated substrates for both enzymes Hp-AS and ASPC.

| Name | m/z | RT/min | ASPC% | Hp-AS% |
|---|---|---|---|---|
| 4-Methylumbelliferyl sulfate | 255.000 | 8.74 | 100 | 100 |
| Methylurolithin sulfate | 321.007 | 11.47 | 100 | 100 |
| Estrone-3-sulfate | 350.12 | 12.24 | 100 | 100 |
| *p*-Cresyl sulfate | 187.0071 | 8.59 | 100 | 100 |
| *N*-Acetyltyramine-*O*-sulfate | 258.0425 | 7.08 | 77.3 | 100 |
| *p*-Coumaric acid sulfate | 242.9969 | 7.8 | 70.2 | 100 |
| D-Mannose-6-sulfate | 260.02 | 1.49 | 42 | <20 |
| 4-Aminophenol sulfate | 188.0023 | 1.95 | 5 | 23.3 |
| 2-Aminophenol sulfate | 188.0023 | 4.24 | <20 | 0 |
| L-Tyrosine sulfate | 260.0226 | 4.85 | <20 | <20 |
| Dihydroisoandrosterone-3-sulfate | 367.1575 | 13.09 | 0 | 38 |
| *N*-Acetylserotonin sulfate | 297.0531 | 7.11 | 100 | 0 |



| Name | RT | ASPC | Hp-AS |
|---|---|---|---|
| p-Cresyl sulfate | 8.61 | 99.97 | 99.65 |
| Sinapic acid sulfate | 7.92 | 92.15 | <20 |
| Ascorbic acid-2-sulfate | 2.12 | <20 | 91.28 |
| 2,5-Dihydroxybenzyl alcohol sulfate | 6.55 | 93.98 | 47.67 |
| 2,5-Dihydroxyacetophenone sulfate | 7.11 | 48.84 | 90.26 |
| Serotonin sulfate | 5.80 | 54.17 | 40.64 |

**Fig. 2.** Schematic representation of the workflow for comparison of substrate promiscuity of the two arylsulfatases Hp-AS and ASPC in a pooled human urine sample using mass spectrometry-based metabolomics analysis.

phenylacetic acids, and phenols. In total, 108 molecules were equally converted by both sulfatases, including 66 good substrates, 34 intermediate substrates, and eight poor substrates (Table S3; confidence levels for metabolite structure validation are provided). Among the identified metabolites hydrolyzed equally by both sulfatases are common metabolites from dietary sources, microbiome-host co-metabolism as well as previously undetected metabolites. This demonstrates the high similarity of ASPC and Hp-AS and is the first large scale validation for the promiscuity of ASPC in human samples. Examples for dietary metabolites are the sulfated analogues of *p*-coumaric acid (**10**), homovanillic acid (**11**), 3,4-dihydrocinnamic acid (**12**), caffeic acid (**13**), ferulic acid (**14**), 3-hydroxyphenylpropionic acid (**15**), 2-methoxyphenol (**16**), 4-vinylphenol (**17**), 4-hydroxybenzoic acid (**18**), and 3,4-

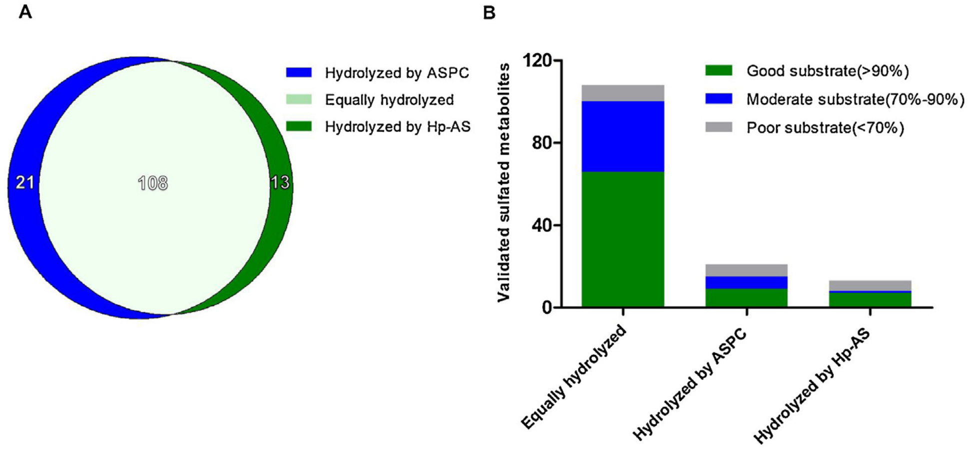


**Fig. 3.** Classification of 142 metabolites based on sulfatase hydrolysis. (A) Metabolites that are either exclusively hydrolyzed by ASPC (blue), equally hydrolyzed by both the enzymes (light green), or exclusively hydrolyzed by Hp-AS (green); (B) Metabolites that are hydrolyzed either with more than 90 % were defined as completely good substrates (green), between 70 %-90 % were defined as intermediate substrates (blue) and below 70 % were defined as poor substrates (grey). 21 Metabolites of the validated 163 sulfated metabolites could not be unambiguously assigned to any of these three groups (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

dihydroxyphenylacetic acid (**19**). We also identified the sulfated metabolites of hippuric acid (**20**), 3-hydroxyhippuric acid (**21**), dihydroxyconiferyl alcohol (**22**), methyl dioxindole-3-acetate (**23**), 4-hydroxyphenylpyruvic acid (**24**) and cytosine (**25**), which are not part of the HMDB metabolite collection. We have recently reported their presence in human samples for the first time in a dietary intervention study [39].

Importantly, this group of equally hydrolyzed metabolites also contains sulfated conjugates of 27 microbiota-derived metabolites and demonstrates the applicability of ASPC for the detection of metabolites from the co-metabolism of host and microbiota (Fig. S3, Table S3). Many of these metabolites have been associated with the development of human diseases or have beneficial properties. The most prominent microbiota-derived metabolites indoxyl sulfate (**26**), *p*-cresyl sulfate (**4**) and 4-ethylphenyl sulfate (**27**) are produced by hepatic sulfation of the microbiota-derived metabolites indole, *p*-cresol and 4-ethylphenol. Indole is produced from tryptophan *via* the tryptophanase enzyme found in *E. coli* and *Bacteroides thetaiotaomicron* [40], while *Clostridium difficile* and other bacteria that belong to *Coriobacteriaceae and Clostridium clusters XI and XIV*, facilitate the conversion of tyrosine into *p*-cresol [41,42]. All three sulfated metabolites have also been considered as uremic toxins due to their link with chronic kidney disease [43]. Indoxyl sulfate (**26**) has also been associated with glomerulosclerosis and endothelial malfunction [44,45]. 4-Ethylphenyl sulfate (**27**) was related to anxiety like behaviour in mice and linked to Parkinson's disease in humans [18]. Gut microbes also contribute to the biosynthesis of several neurotransmitters that human can also produce endogenously. In our study, we have detected serotonin sulfate (**28**) and its unconjugated analogue serotonin is a neurotransmitter produced by *Candida, Streptococcus, Escherichia, and Enterococcus* [46]. It has also been linked to diseases such as IBD, cardiovascular diseases and osteoporosis [47]. Furthermore, a correlation was identified for tyramine sulfate (**29**) and ferulic acid sulfate (**14**) with the improvement of depression as well as higher blood pressure in patients [48,49]. A link between the sulfated conjugate of the microbiota-derived metabolite dopamine, dopamine 4-sulfate (**30**) and Parkinson's disease has been reported [50]. The two metabolites phenyl sulfate (**31**) and 3-hydroxyphenylpropionic acid sulfate (**15**) have been linked to colorectal cancer [51,52], while 5′-(3′,4′-dihydroxyphenyl)-gamma-valerolactone sulfate (**32**) was identified to have a protective effect against urinary tract infections [53].

Most of these microbiota-derived sulfated metabolites are present in bacterial pathways that metabolize aromatic amino acids (AAAs) (Fig. 4). The AAA degradation pathway includes biological or neurological active molecules that are essential to maintain crucial biological functions in humans [54]. Especially, the production of several neurologically active molecules has been associated with the gut-brain axis. For example, microbial degradation of tyrosine produces the neurotransmitters tyramine and even contributes to the concentration of dopamine in humans [55]. Gut bacterial strains of *Enterococcus* and *Enterobacter* are involved in the decarboxylation of tyrosine to form tyramine [56]. Tyramine is further acetylated by an arylalkylamine *N*-acetyltransferase to produce the metabolite *N*-acetyltyramine [57,58]. Another metabolite of bacterial origin, homovanillic acid, is a metabolite of catecholamines and also derived from *Bifidobacterium* [59]. Homovanillic acid and its sulfated conjugates (**11**) have been detected in urine samples of neuroblastoma patients and are used for diagnosis of this disease [60,61]. The production of *p*-coumaric acid results in elevated levels of 4-hydroyphenylpropionic acid that has been associated with autism and schizophrenia in rats. Phenylacetic acid and 4-hydroxyphenylacetic acid are deaminated products of phenylalanine and tyrosine, respectively [62]. Bacteria capable to metabolize these AAA substrates include *B. thetaiotaomicron*, *Bacteroides ovatus*, *Eulonchus halli*, and *Clostridium bartletti* [62].

### 3.3.2. Sulfated metabolites hydrolyzed by ASPC

Application of ASPC enabled the detection of new sulfates of diverse scaffolds, which play an important role in nutritional and human disease development studies in addition to yet unknown metabolites in HMDB. We identified 21 sulfated metabolites only hydrolyzed by ASPC including nine good, six intermediate and six poor substrates (Fig. 5A and Table 2; confidence levels for metabolite structure validation are provided). Additionally, the three metabolites dihydroisoferulic acid-4-sulfate (**33**), coniferyl aldehyde sulfate (**34**) and 2,5-dihydroxybenzyl alcohol sulfate (**35**) were completely desulfated by ASPC but were also partially (less than 50 %) converted by Hp-AS (Table S2). As a representative example for this category, extracted ion chromatogram (EIC) peaks for hesperetin sulfate (**36**) from the mass spectrometric analysis of urine samples demonstrate selective hydrolysis by ASPC only (Fig. 5B). Compound **36** was completely hydrolyzed upon treatment with ASPC, while no conversion was observed in the case of Hp-AS. As the MS/MS fragmentation spectrum was not present

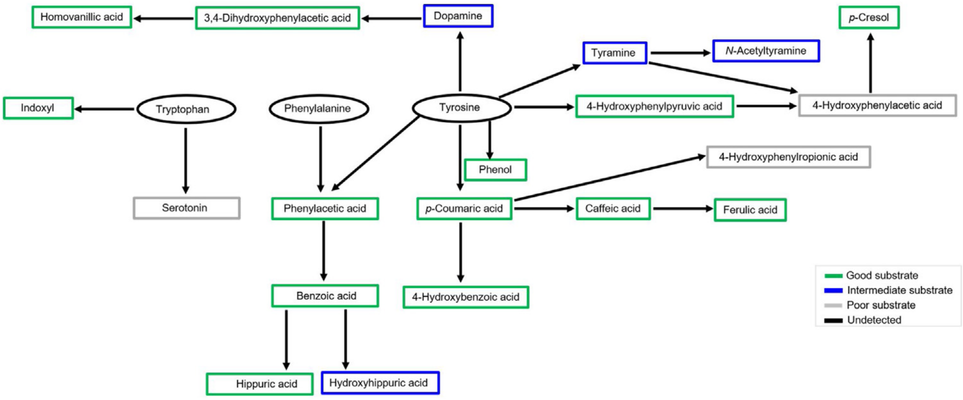


**Fig. 4.** Many gut microbiota-derived sulfated metabolites detected are part of the specific microbial aromatic amino acid degradation pathway. The pathway of the corresponding unsulfated metabolite are shown for simplification. Metabolites are highlighted as good (green), intermediate (blue), poor (grey), and undetected (black) substrates for ASPC and Hp-AS (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

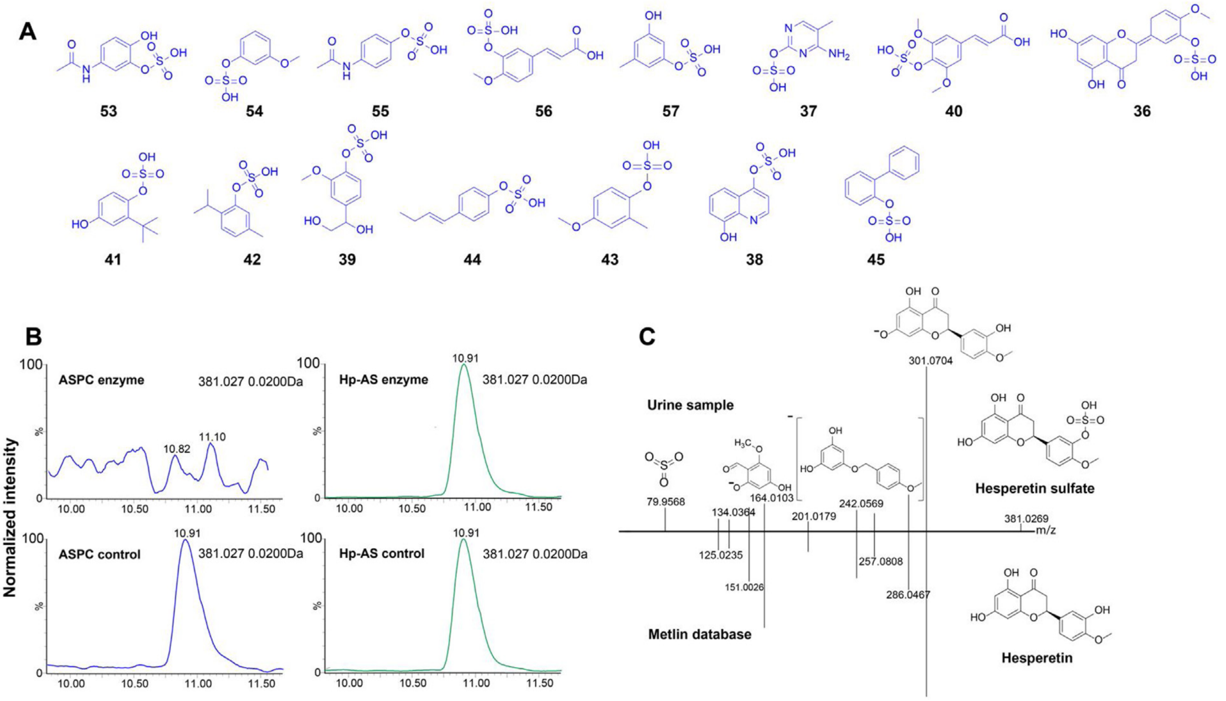


**Fig. 5.** (A) Sulfated metabolites hydrolyzed by ASPC. One potential structure is depicted for metabolites with more than one phenolic or aliphatic alcohol; (B) Mass spectrometric peak areas represent the hydrolysis comparison of hesperetin sulfate between both enzymes;(C) Structure validation of hesperetin sulfate by comparison of the fragmentation spectra with the corresponding unsulfated metabolite in the database METLIN (20 V).

in any database, we compared it with the unsulfated hesperetin fragmentation spectrum in METLIN [35]. Both spectra resulted in the same fragmentation pattern with an additional sulfur trioxide signal for the natural sulfated metabolite (Fig. 5C).

Our analysis demonstrates that ASPC has a high substrate preference for the three heterocyclic compounds 5-methylcytosine sulfate (**37**), quinoline-4,8-diol sulfate (**38**), and *N*-acetylserotonin sulfate (**5**, Tables 2 and S2; confidence levels for metabolite structure validation are provided). Furthermore, the three metabolites dihydroisoferulic acid-4-sulfate (**33**), 3-methoxy-4-hydroxyphenylglycol sulfate (**39**) and hesperetin sulfate (**36**), in this class have not been identified in previous arylsulfatase-based studies due to the inability of Hp-AS to hydrolyze these compounds. Compound **39** has been linked to pediatric septic acute kidney injury [63]. Hesperetin sulfate (**36**) is a known product of citrus fruit and may be a molecular reason for the cardioprotective effects of citrus fruit consumption [64]. Sinapic acid sulfate (**40**) is another interesting metabolite, which has been associated with the consumption of tea and rye. The deconjugated compound sinapic acid is an antioxidant that is commonly found in fruits, vegetables, and cereal grains [65]. Furthermore, we also identified two structurally similar compounds, 2-tert-butylhydroquinone sulfate (**41**) and thymol sulfate (**42**), that belong to the phenylpropane compound class. The role of **42** in human xenobiotic metabolism has been associated with colorectal cancer [51]. Thymol is an important dietary component found in different plant species such as *Thymbra capitate*, *Centipeda minima* and *Carum copticum* [66]. Thymol is known for its antibacterial, anti-inflammatory, antioxidant, and analgesic properties [66].

Moreover, sulfated analogues of coniferyl aldehyde (**34**) and 4-hydroxy-3-methylanisol (**43**) have been identified for the first time in humans, as they have only been registered in HMDB as their corresponding phenolic compounds. Additionally, sulfateconjugates of 5-methylcytosine (**37**), 2-tert-butylhydroquinone (**41**),

**Table 2**
Sulfated metabolites hydrolyzed by ASPC. (Level 1: Validation with authentic synthetic or commercial standards; Level 2a: Metabolite structure validation based on unambiguous matching of MS$^2$ spectra with experimental spectra from literature or library sources; Level 2b: Identification of the molecular formula and MS$^2$ fragmentation pattern comparison using computational tools; Level 3: MS$^2$-validation of sulfate ester moiety in the metabolite; n.s. = no structure.).

| Name | *m/z* | RT/min | ASPC% | Level of confidence |
|---|---|---|---|---|
| 4-Hydroxyacetaminophen sulfate (**53**) | 246.0066 | 6.99 | 98.9 | 2b |
| 3-Methoxyphenol sulfate (**54**) | 203.0009 | 8.44 | 96.6 | 1 |
| Paracetamol sulfate (isomer 2) (**55**) | 230.0119 | 7.39 | 96.4 | 2b |
| Isoferulic acid sulfate (**56**) | 273.0063 | 8.52 | 95.0 | 2b |
| 3-Hydroxy-5-methylphenyl sulfate (**57**) | 203.0008 | 8.61 | 93.5 | 2b |
| n.s. | 273.0425 | 10.61 | 93.3 | 3 |
| 5-Methylcytosine sulfate (**37**) | 204.0035 | 8.41 | 92.8 | 2b |
| n.s. | 247.0263 | 8.87 | 92.6 | 3 |
| Sinapic acid sulfate (**40**) | 303.0167 | 7.92 | 92.2 | 1 |
| Hesperetin sulfate (**36**) | 381.0270 | 10.91 | 88.9 | 2a |
| 2-tert-Butylhydroquinone sulfate (**41**) | 245.0474 | 9.55 | 88.6 | 2b |
| Thymol sulfate (**42**) | 229.0531 | 12.30 | 88.5 | 2a |
| 3-Methoxy-4-hydroxyphenylglycol sulfate (**39**) | 263.0215 | 5.63 | 84.3 | 2b |
| *p*-Butenylphenol sulfate (**44**) | 227.0367 | 11.86 | 83.5 | 2b |
| 4-Hydroxy-3-methylanisol (**43**) | 217.0156 | 7.96 | 82.5 | 2b |
| n.s. | 285.0426 | 10.98 | 69.9 | 3 |
| n.s. | 342.9771 | 4.92 | 68.6 | 3 |
| n.s. | 203.0020 | 6.07 | 64.3 | 3 |
| Quinoline-4.8-diol sulfate (**38**) | 239.9954 | 8.09 | 63.0 | 2a |
| 2-Biphenylol sulfate (**45**) | 249.0271 | 11.62 | 37.9 | 2a |
| n.s. | 249.0058 | 5.66 | 36.4 | 3 |

*p*-butenylphenol (**44**), and 2-biphenylol (**45**) were identified in our recent study but have not been reported in HMDB yet. 2-Tert-butyl hydroquinone is used as a food antioxidant and considered as a potential biomarker of fats and oils consumption. Several studies have demonstrated the anti-carcinogenic effect of this compound [67]. Coniferyl aldehyde is involved in the phenylpropanoid biosynthesis pathways in plants and considered as a potential marker for the consumption of pear, walnut and citrus [68,69]. Comparison of the hydrolysis differences of coniferyl aldehyde sulfate (**34**) between two enzymes demonstrates complete conversion with ASPC only. MS/MS fragmentation spectra validate the molecular structure (Fig. S2).

### 3.3.3. *Sulfated metabolites hydrolyzed by Hp-AS*

Application of Hp-AS in this pooled urine sample also facilitated the detection of additional sulfated metabolites of bile acids, vitamin C, chromane structures, and polyphenols, which are beneficial to the human according to health and nutritional studies. Metabolites only hydrolyzed by Hp-AS consist of 13 sulfate esters including six good, one intermediate and six poor substrates (Fig. 6A and Table 3; confidence levels for metabolite structure validation are provided). These metabolites are also of diverse scaffolds without a clear structure specificity that could be an indication why these compounds are only hydrolyzed by Hp-AS. Phloroglucinol is a metabolite produced by the bacterium *Pseudomonas fluorescens* [70] and its sulfated analogue (**46**) that we have detected here is not part of HMDB.

Surprisingly, we identified five non-aromatic sulfates. Ascorbic acid-2-sulfate (**47**) and 6-hydroxycyclohexa-1,3-dien-1-yl sulfate (**48**) have shown to be hydrolyzed by Hp-As in our previous studies, which confirms the reproducibility of our results. We have also detected 5,6-dihydroxycyclohexa-1,3-dien-1-yl sulfate (**49**), which is an analogue of **48** and has to the best our knowledge not been detected before. Dihydroisoandrosterone-3-sulfate (**6**) was completely hydrolyzed in urine samples and mirrors our *in vitro* enzymatic assay observation using **6** as a pure substrate (Fig. 1C). This is the first detection of **6** in human samples. No signal was obtained for this compound in urine samples treated with Hp-AS, which validates complete hydrolysis of these molecules by Hp-AS, while no conversion was observed for treatment with ASPC (Fig. 6B). The complete conversion of this metabolite in the pooled urine sample compared to the partial conversion for the pure compound can be explained by higher units of enzyme in urine sample (11 U) as compared to the enzymatic assay (1 U). Moreover, we compared the MS/MS fragmentation pattern of **6** identified in the urine sample with a commercial standard. Both spectra resulted in the same fragmentation pattern, which validates the presence of **6** in urine samples at the highest confidence level (Fig. 6C).

Another interesting molecule is the bile acid conjugate *N*-[(3a,5b,7b)-7-hydroxy-24-oxo-3-(sulfooxy)cholan-24-yl]-glycine (**50**), which is not an aromatic phenolic compound. This secondary alcohol was also converted by Hp-AS by about 47 %. Due to the applied bioinformatic analysis we can detect it using our assay despite the incomplete conversion [15]. Additionally, the two metabolites 4-((2R,3R)-3,4,5,7-tetrahydroxychroman-2-yl) phenyl sulfate (**51**) and 2,4-dihydroxy acetophenone sulfate (**52**) were fully converted by HP-AS as well but also partially (less than 50 %) converted by ASPC (Table S2). These three compounds have not been detected in our previous studies using this arylsulfatase in non-pooled urine samples. **52** is a polyphenolic metabolite [71], whereas **51** belongs to the compound class of chromanes, which is a structural feature present in pharmaceutical compounds and also a core structure of tocopherols [72,73].

## 4. Conclusions

In summary, we have compared the substrate conversion of the arylsulfatase ASPC to the previously utilized arylsulfatase Hp-AS in enzymatic assays and human urine samples. Both enzymes equally hydrolyzed more than 100 sulfated molecules from different compound classes. We have demonstrated that application of ASPC is similar to Hp-AS as more than 130 sulfated metabolites were detected including metabolites containing diverse scaffolds. We have not identified any pattern in substrate specificity for each arylsulfatase and future studies are required to determine specific substrate preferences. Furthermore, this study also led to the identification of more than 27 microbiota-derived sulfated metabolites in a pooled urine sample, which further demonstrates the tremendous potential of this method for identifying and analyzing unknown metabolic links of gut microbiome and host related to human health and disease development. ASPC can also be utilized for large-scale analysis and discovery of sulfated metabolites and does not require purification prior to application. Our comprehensive analysis of this arylsulfatase validates the high promiscuity of

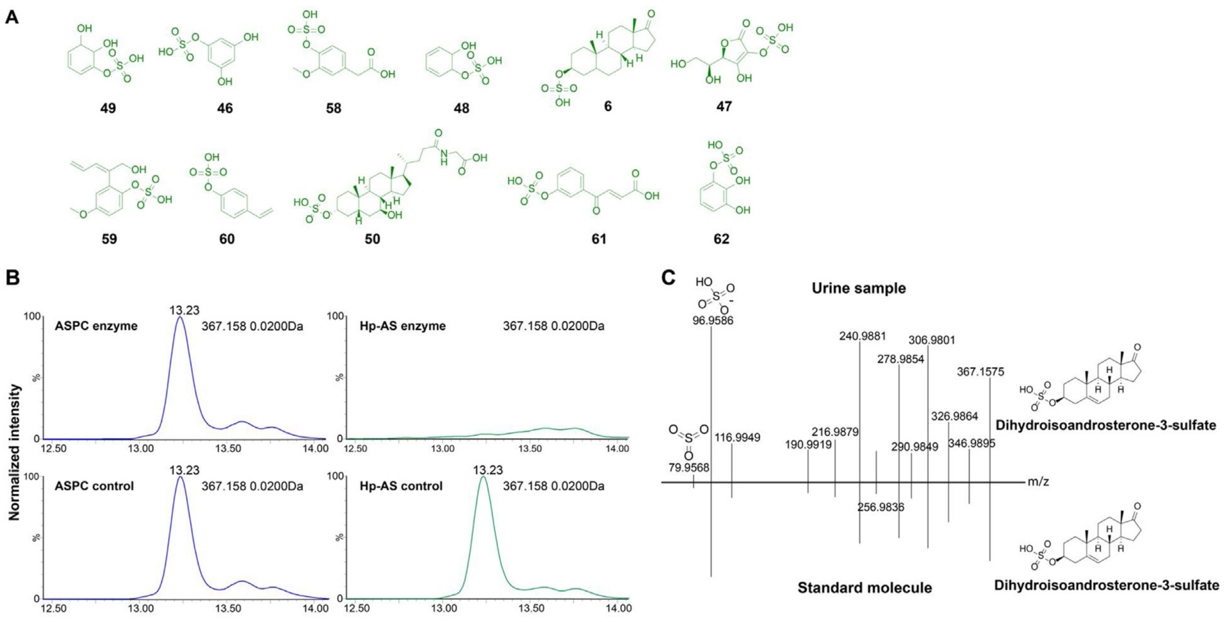


**Fig. 6.** (A) Sulfated metabolites hydrolyzed by Hp-AS. One potential structure is depicted for metabolites with more than one phenolic or aliphatic alcohol; (B) Mass spectrometric peak areas represent the hydrolysis comparison of dihydroisoandrosterone-3-sulfate between both enzymes; (C) Structure validation of the natural compound dihydroisoandrosterone-3-sulfate by comparison with the fragmentation spectra of a commercial standard.

**Table 3**
Sulfated metabolites hydrolyzed by Hp-AS. (Level 1: Validation with authentic synthetic or commercial standards; Level 2a: Metabolite structure validation based on unambiguous matching of MS$^2$ spectra with experimental spectra from literature or library sources; Level 2b: Identification of the molecular formula and MS$^2$ fragmentation pattern comparison using computational tools; Level 3: MS$^2$-validation of sulfate ester moiety in the metabolite; n.s. = no structure).

| Name | *m/z* | RT/min | Hp-AS% | Level of confidence |
|---|---|---|---|---|
| 5,6-Dihydroxycyclohexa-1,3-dien-1-yl sulfate (**49**) | 206.9958 | 6.23 | 99.7 | 2b |
| Phloroglucinol sulfate (**46**) | 204.9800 | 5.27 | 98.0 | 2b |
| Homovanillic acid sulfate (isomer) (**58**) | 261.0062 | 7.14 | 93.2 | 2a |
| 6-Hydroxycyclohexa-1,3-dien-yl sulfate (**48**) | 191.0011 | 5.69 | 92.0 | 2b |
| Dihydroisoandrosterone-3-sulfate (**6**) | 367.1575 | 13.23 | 91.5 | 1 |
| Ascorbic acid-2-sulfate (**47**) | 254.9797 | 2.12 | 91.3 | 2b |
| 2-(1-Hydroxypenta-2,4-dien-2-yl)-4-methoxyphenol sulfate (**59**) | 285.0435 | 7.90 | 75.3 | 2b |
| n.s. | 267.9570 | 4.57 | 56.8 | 3 |
| Vinylphenol sulfate (isomer) (**60**) | 199.0053 | 4.95 | 56.0 | 2a |
| n.s. | 199.0053 | 4.79 | 47.0 | 3 |
| *N*-[(3a,5b,7b)-7-Hydroxy-24-oxo-3-(sulfooxy)cholan-24-yl]-glycine (**50**) | 528.2625 | 14.67 | 46.8 | 2b |
| 4-(3-Hydroxyphenyl)-4-oxobut-2-enoic acid sulfate (**61**) | 270.9895 | 7.27 | 39.0 | 2b |
| 3-Hydroxy-2-methyl-4-pyrone sulfate (**62**) | 204.9803 | 5.56 | 34.6 | 1 |

this enzyme similar to Hp-AS and represents an additional promising tool for the investigation of sulfated metabolites in general as well as co-metabolism of host and gut microbiome in any human sample type.

## CRediT authorship contribution statement

**Abhishek Jain:** Conceptualization, Data curation, Formal analysis, Methodology, Writing - original draft, Visualization. **Mario S.P. Correia:** Formal analysis, Methodology, Writing - review & editing. **Hannes Meistermann:** Formal analysis, Writing - review & editing. **Miroslav Vujasinovic:** Writing - review & editing. **J.-Matthias Löhr:** Writing - review & editing. **Daniel Globisch:** Conceptualization, Writing - original draft, Writing - review & editing, Visualization, Supervision.

## Declaration of Competing Interest

The authors report no declarations of interest.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2020.113818.

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