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Mag. Veronika Fitz

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1 Introduction

1.1 Introduction to metabolomics and exposomics

Metabolites are substrates and products of the most fundamental processes in a living cell: energy generation, energy storage and synthesis of macromolecules. Metabolomics is a research field that aims at comprehensively capturing and interpreting these endogenous metabolic transformation products. Nucleic resonance spectroscopy (NMR) and mass spectrometry (MS) are the analytical techniques most frequently employed. The analytes of metabolomics comprise the small molecules of a biological sample and they cover a wide polarity range, including water soluble metabolites (amino acids, nucleobases, sugars etc.) and lipids.[1] Because of their special chemical properties, lipids demand dedicated analytical workflows that divert from those of water-soluble metabolites. The research field lipidomics evolved to address these analytical needs more specifically, leaving the water-soluble metabolites for metabolomics as it is defined in this thesis.

The samples of a metabolomics analysis are usually taken from cell culture, tissue, body fluids or excretions. The complex sample matrix brings about analytical challenges compared to an analysis in pure water: complex sample preparation, critical stability of analytes, low analyte concentrations, narrow linear range and many possibilities to interfere with the analysis.[2] These characteristics are shared by a pharmacokinetic study of a drug, for example. However, a metabolomics analysis poses additional challenges: It usually tries to capture a plethora of analytes simultaneously. The targeted analytes are biologically intended to be quickly transformed in metabolic pathways coordinated by enzymes that are very likely to be present in the sample. Metabolites are frequently degraded by ubiquitous surrounding conditions (e.g. oxidized by air oxygen). Their abundance levels in the sample stretch over a wide concentration range. And, in contrast to more established “omics” disciplines like genomics and proteomics, the analytes of metabolomics are usually small molecules of very different physicochemical properties. The optimization of a metabolomics method is always a tradeoff between metabolite coverage, certainty of identification, robustness, reproducibility and duration of the analysis (in-depth vs. high throughput) and should orient itself along the aim of the study.

There are two general concepts of metabolomics analyses: the targeted and the untargeted approach. Both aim at maximizing the parameters listed above (coverage, time-efficiency, robustness, etc.), but with different emphases. An untargeted metabolomics approach is chosen to explore differences between two groups of individuals in an unbiased manner to get an idea about how and, subsequently, why they are different. The two compared groups could be one with good response to a treatment and one resistant to the treatment, to name an example. In untargeted analysis, every detected signal is of possible importance and the volume of data to be evaluated is accordingly very high. This prevents manual data evaluation. Instead, peak picking algorithms are applied to discrimi-

nate “real” signals from noise. Databases are mined to annotate the measured accurate masses and fragment spectra obtained from the so-picked peaks with compound names. Finally, the annotated compounds are digitally mapped on a metabolic network to point out metabolic pathways that might be involved in or affected by the underlying biological process. The depth of the analysis is probably more valuable for creating a hypothesis than its speed and a suitable analytical method therefore covers as many compounds as possible. Samples are often evaluated via relative quantification of the detected features, whereas it is rarely necessary to report absolute levels of metabolites. In fact, it is not even necessary – and also not possible – to unambiguously identify each measured “feature”. A feature, as defined here, is a reported but not systematically identified signal that is not yet proven to really stem from an analyte. This nomenclature was put in place to avoid inappropriate confidence in the meaning of the annotation. However, it is not a standardized term.[3] An untargeted metabolomics experiment often evaluates the generated data by using multivariate statistics. Principal component analysis, for example, helps to spot and focus on the most obviously altered independent features comparing the two (or more) groups, which, in the best case, again results is a distinct and meaningful grouping of the analyzed individuals, indicating a real and graspable difference between them. To maximize information output, it is recommendable to unambiguously identify these important features subsequently by targeted comparison with authentic standards. Having the limitations of technical accuracy and precision in mind, the most altered features can probably be monitored most precisely. They are, however, not necessarily the most decisive ones for the underlying biological process. They are merely the measurable result of the biological process and are selected by the capability of the analytical method used to analyze them. Conducting and evaluating an untargeted metabolomics experiment is truly a highly complex procedure with many key points to consider and a need for a suitable analytical method.

This thesis focuses on the other type of metabolomics approaches: the targeted experiment. Its scope is narrowed to a subset of analytes in a hypothesis driven manner and its aim is to test a hypothesis created from an untargeted experiment or other omics data, for example. The advantage of a targeted approach is that the analytical setup can be optimized for capturing the analytes of interest. As such confirmative studies typically involve a greater batch size, time-efficiency is of pronounced importance for a suitable analytical method. Increased robustness, reproducibility and accuracy as well as better characterization of the method render quantitative results more reliable.

One application that falls into the field of a targeted metabolomics approach is the quantification of a metabolomic biomarker. A biomarker is a reliably quantifiable measurand that is linked to and reflects the state of a biological process. Marchand et al. (2018) describe a *metabolomic* biomarker as a “meta-biomarker” comprising a set of metabolites whose levels are monitored together, thereby creating a “disease signature”. While classical single-compound biomarkers produce false results

when faced with random fluctuations of the target compound, a “meta-biomarker” should be more robust.[4] To develop a metabolomic biomarker, metabolites would be selected in an untargeted approach and their conclusiveness confirmed by thorough targeted evaluation. The really interesting thing about the metabolome, in contrast to genome, transcriptome and proteome, is that it depicts the phenotype of an organism. Metabolites are the ultimate result over all summarized genome damages and countering reparations, transcription activation and suppression, enzyme activation and inhibition, or disruption of negative or positive feedback loops. They reflect – at least in theory – what effectively happened in the organism. A metabolomic biomarker could be employed to detect and determine the extent of a disease, to monitor desired or adverse drug effects or stratify patients for the best treatment.[5] However, before a new biomarker for human use enters daily medicinal and clinical routine in the European Union, it has to prove itself in clinical performance studies as required by regulation (EU) 2017/746 on in vitro diagnostic medical devices.[6] Proper technical validation of the analytical method used to quantify the biomarker is an indispensable prerequisite for such a performance study and for an official medicines authority’s approval, accordingly.

The term “exposomics” denotes the assessment of all environmental factors an organism is subjected to from pre-natal development onward. Its subsequent goal is to elucidate the impact of these exposures on the organism. The motivation behind this is the justified assumption that many chronic diseases do not result from genetic susceptibility alone, but from its interplay with exposure to environmental stressors.[7] An exposomics experiment is often of epidemiological character and usually investigates the effects of a long-term exposure to low concentrations of chemical substances, but also immaterial environmental factors such as radiation. To gain a thorough impression of the biological system and its perturbations, exposomics research tries to connect quantitative information coming from different sources: impacts from the organism’s surrounding (the external exposome) and substance concentrations in blood, excretions, tissues and so on (the internal exposome), genetic effects (covered by the research field of genomics), the corresponding reaction of gene transcription and translation into proteins (transcriptomics and proteomics) and finally the investigation of metabolic reactions to the perturbation (metabolomics).[8] It is not trivial to integrate those different system levels conclusively. However, a biological system with little knowledge beforehand can most probably not be explained via one “omics” approach alone and to address an explorative biological questions it is necessary to combine them with other “omics” disciplines. A prerequisite for any conclusive data integration and interpretation is good data quality generated by validated analytical methods.

1.2 Definition and purpose of validation

> "The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. […] The analytical procedure refers to the way of performing the analysis. It should describe in detail the steps necessary to perform each analytical test. This may include but is not limited to: the sample, the reference standard and the reagents preparations, use of the apparatus, generation of the calibration curve, use of the formulae for the calculation, etc." (ICH Q2(R1))[9]

In other words, the aim of validating an analytical procedure is to make sure that it is capable of producing reliable data before the samples of interest are measured. The analytical procedure comprises sampling, sample preparation, measurement, data evaluation, statistical procedures and reporting of the results. This laborious task may be more approachable if it is first subdivided in smaller validation objectives (e.g. testing sampling and sample stability, capability and suitability of the analytical technique, raw data processing, statistical evaluation, etc.) and finally validated in an overarching validation exercise. This study focuses on the validation of the analytical method only, which by the definition used here comprises sample preparation, measurement and raw data evaluation.

In an experiment that tries to find differences in the metabolite levels of two compared groups, the analyst desires a certain level of confidence that a measured difference between the two groups is due to an actual biological difference rather than a random bias of the analytical procedure. This confidence is provided by quality assurance measures of which validation for a clearly defined application is, next to the implementation of quality control samples, for example, an integral part. Without quality assurance, the extent of method inherent variability is not defined and the analyst would have to assume that the observed difference might, in the worst case, completely stem from a broad scatter of results (i.e. poor precision) or bad recovery of the true value (i.e. bad accuracy) of the used method. Beyond its confirmatory function, validation helps to characterize the capabilities and limitations of the analytical method and if it actually suits the analytical question. This process is often referred to as system suitability or fitness-for-purpose testing and has more of an explorative character. Since this institute is located in the European Union, the European Medicines Agency's (EMA) concept of method validation is of utmost interest for validating a bioanalytical procedure. According to the *Guideline on Bioanalytical Method Validation* issued by the agency in 2012[10], validation of a bioanalytical method is a process that creates evidence to serve as a basis for confidence. Confidence that the entire analytical workflow (sampling, sample storage until analysis, sample analysis, data evaluation) is suitable for the intended purpose and free of inherent flaws, that is. Limitations of the method have to be revealed, described and taken in to consideration. The goal is to preclude the

generation of misleading results. The necessary evidence is created by meeting or failing specifications that were defined by the analyst beforehand. Official validation guidelines provide suggestions on which requirements to set and which target values are deemed appropriate. It is important to understand that the EMA's guideline was developed to guide stakeholders through a highly regulated operating environment. In this environment, an analytical method is usually readily developed, characterized and documented to a very high extent before it is considered for validation. It is also usually embedded in a regulated environment: Instrumentation is calibrated and qualified; personnel, data treatment and data storage systems are qualified as well, otherwise validation is regarded as inconclusive. Validation means proving that the assembly of these qualified elements really works out the way that we expect it to. The definition of requirements is an integral part of any qualification or validation exercise, thus sufficient effort and consideration should be dedicated to this task. It becomes evident that a method validation guideline from the medicinal sector is very much in place to confirm the method's capabilities rather than explore them. Assuring the reliability of an analytical procedure is a subject of heavy legal regulation in clinical research because inadequate data quality can compromise the conclusions drawn from it and ultimately jeopardize a human's well-being. However, the critical consideration of data quality is also necessary for other research topics and an ethical obligation for the analyst towards the customer or scientific community. To maintain both statistical significance and scientific seriousness of the results, it is necessary to have a good knowledge of the analytical workflow and to be aware of the crucial steps involved, to control these steps and evaluate their impact on the results. An appropriate degree of method validation can be a suitable instrument for gaining the required knowledge of the method, to identify and help to improve its weaknesses and eventually demonstrate its capability to produce reliable results.

1.3 Aim of the thesis

The aim of the thesis is to try validating a newly developed dual-chromatography LC-MS method developed in our working group for simultaneous metabolomics and exposomics analysis in a way that approximates the validation needs for a clinical study. It is recognized that this endeavor is very challenging as it tries to link basic university research with a highly regulated application environment. It seems self-explaining that guidelines adapted to medicines authorities' strict provisions are not suitable for guiding a validation exercise in basic university research. However, it is necessary to translate innovative procedures into the required regulatory framework before they can exert their benefits in the "outside world". With this work, I would like to shine a light on the missing link between the two worlds and describe the issues this "clash of cultures" involves. Maybe this will help to address them more specifically in future projects.

2 Validation Plan

2.1 Validation plan template

For drafting a validation strategy suited to this LC-MS metabolomics-exposomics method, the guidelines for bioanalytical method validation published by the European Medicines Agency (EMA)[10] and the United States Food & Drug Administration (U.S. FDA)[11] were taken into consideration. Special guidance for the validation of a bioanalytical method was desired because it poses several specific problems compared to an analysis in aqueous environment. With blood, for example, matrix and sample preparation are complex, stability of analytes is critical, analyte concentrations are low (usually in a magnitude of 10^{-9} g to 10^{-6} g), the linear range is narrow (one to two as compared to five powers of ten in purely aqueous solvent) and many factors can interfere with the analysis.[2] EMA and U.S. FDA are governmental agencies that are, amongst other tasks, responsible for scientifically evaluating and monitoring the safety of medicines related proceedings in the European Union and in the United States of America, respectively. The guidelines on bioanalytical method validation issued by these institutions serve as an orientation for the goal of the validation, the figures of merit including cut-off values that would be important to satisfy their requirements, and the tools to achieve the necessary evidence. Guidelines are nonbinding recommendations. That means that a measurement facility operating in EMA's or U.S. FDA's area of responsibility is legally allowed to divert from the suggested strategy for validation and adapt it to its needs. If eventually the official approval of a health authority is to be obtained, though, it is advisable to closely follow the suggestions of the guidelines and justify deviations very conclusively. After all, these guidelines reflect the authorities' current opinion of best practice behavior. Official authority approval of an analytical method is, for example, necessary when it is part of a clinical trial. In general, an interventional experiment on a human being is classified as clinical trial and has to be authorized by a competent authority. Clinical trials are legally requested for a new drug application, to expand the indications (i.e. targeted illnesses) of an authorized drug or to introduce a biomarker for patient stratification. They can also be conducted to establish new or systematically evaluate existing therapeutic treatment. In basic research and nonclinical studies, on the other hand, analytical methods are not required to comply with the guidelines, as such stringent validation is neither necessary nor feasible for exploratory investigation. Still, the suggestions for validation can guide efforts to achieve data reliability and aid the development of an analytical method.

The U.S. FDA's guideline seems to understand the needs of a less regulated analytical institution better than the EMA's guideline. It includes several very specific tables that can be used as templates in the development of an own validation plan. Furthermore, the U.S. FDA's guideline is of newer date and seems a bit more reflective of novel analytical techniques. Since the general concepts for meth-

od validation published by the two authorities are largely harmonized and despite our analytical institute being situated in the European Union, this work will closely follow the suggestions of U.S. FDA's guideline *Bioanalytical Method Validation - Guidance for Industry* (2018)[11]. A convenient summary of the U.S. FDA's opinions on the validation of a quantitative chromatographic assay is given in appendix VII, table 1 of the guideline.

2.2 Adaptation of template

Although the U.S. FDA's guideline explicitly suggests its applicability not only for the quantification of drugs and their metabolites, but also for the quantification of biomarkers (i.e. endogenous compounds), the document focuses on the quantification of single or few analytes and not on the simultaneous detection and quantification of a whole set of chemically diverse compounds, as it is the case with metabolomics. At this point, it is helpful to investigate literature specifically dealing with quality assurance problems for metabolomics analyses. There is a vivid scientific community working on improvements in the metabolomics field and some have tried to move towards harmonization to raise transparency and quality of the published results.[12–14] There are also quality initiatives that provide a lot of content for general issues in analytical chemistry. Eurachem, for example, is a European network of organizations that have the "objective of establishing a system for the international traceability of chemical measurements and the promotion of good quality practices." (https://www.eurachem.org/) Among other content, the network provides guidance documents for chemical analysis and the quality issues associated with it. Although the target figures of the Eurachem guideline *The Fitness for Purpose of Analytical Methods – A Laboratory Guide To Method Validation and Related Topics*[15] are a bit less strict than the provisions of the EMA's or U.S. FDA's guidelines, the content and questions addressed in the named documents are more or less the same.
The noble intention for this thesis would be to design a validation plan specifically adapted to metabolomics that could meet the requirements for validation defined by international competent medicines authorities. As this is a very challenging endeavor with unclear feasibility, I would, in the following, like to explain some problems that a suitable validation plan should address and outline some ideas on how to approach them. Special attention is given to internal standardization with isotopically enriched biomass extract to enhance reproducibility and accuracy of absolute quantification and circumvent matrix effect issues.

2.2.1 Matrix effect

One central idea of the U.S. FDA's guideline is to validate the quantification of target analytes in the authentic matrix of the sample. That is because, due to the complexity of a biological sample matrix, there is a considerable risk that it will influence the measurement result compared to the results

obtained from measuring a matrix-free sample or standard prepared in pure solvent. To investigate and account for possible interferences coming from the sample matrix, the guideline suggests that the calibration curve should be prepared by spiking blank matrix with different amounts of standard compound. Blank matrix is defined as a matrix that is representative of the samples but does not contain the analytes of interest. Matrix, in this context, means the blend of components that accompany the analyte in the sample but are not the intentional target of the analysis.[16] Obtaining blank matrix poses a problem for metabolomics analysis: The analytes of interest are ubiquitously present in every biological matrix due to their central role for the primary metabolism of living organisms. To increase trust in the analytical results, we ought to prove the resilience of the analytical method towards matrix variability, though. Indeed, there are several elements in our analytical method that can be expected to be influenced (i.e. produce altered signal intensities) when confronted with varying matrices. Mass spectrometry *per se* is not an absolute quantitative technique and absolute quantification in a mass spectrometry assay relies on fitting the signal intensity of a compound obtained from the sample to the corresponding signal intensities obtained from compound-specific calibration standards, e.g. in the form of an external calibration curve. The idea of preparing the calibration curve in authentic blank matrix is to introduce the same matrix-induced signal intensity alteration from the sample to the reference standards and thus render the comparison of the signals valid. Since this matrix-match of the calibration curve is not a viable route for metabolomics, two other strategies are conceivable. Both come with specific limitations. We could either use calibration standards prepared in pure solvent with the risk of overlooking matrix effects; or we could spike an actual representative sample, e.g. a pooled plasma sample, with known amounts of analyte and subtract the signal of the non-spiked sample. A response curve could then be constructed out of the signal deltas (so-called standard addition method). This may, however, involve the problem that we measure a very low delta at a high concentration level, so we can never be sure if the linearity of the curve can really be extrapolated to low concentrations. In addition, spiking high standard concentrations brings the risk of overstocking the calibrator with compounds that are already present in the matrix in a high concentration and leaving the linear dynamic range of these compounds with the need to dilute the samples and prepare a calibration curve based on the diluted matrix.[17]

The considerate application of a compound specific isotopically labelled internal standard could mark the middle ground between these approaches – if it were demonstrated to sufficiently balance matrix effects and hence supported comparability of the sample signal to a solvent-based calibration curve. This would be an advantage when samples with different matrices, e.g. extracts from different tissues, were to be analyzed in one sequence. Different sample types could then justifiably refer to only one calibration curve. This strategy is intriguing, but it needs to be supported by adequate evidence. An ideal validation protocol would deliver sufficient data on the matrix effects of a specific

sample type to enable an informed decision whether it would be allowable to use solvent-based calibration standards or the standard addition method would have to be pursued. A thinkable validation strategy could be to prepare quality control samples (QCs) spiked with different amounts of standard mixture and evaluate concentration recovery under the use of a solvent-based calibration curve: After extraction, one pooled sample per sample type is spiked with different concentrations of standard analyte and the signal deltas to the corresponding non-spiked pooled sample are fitted on the calibration curve. For this comparison, all calibrators and QCs should contain internal standard and the QCs should cover the whole calibration range. For detailed information, see chapter 2.2.5 on page 13.

2.2.2 Internal standardization

The U.S. FDA's guideline requires the use of internal standards. In the analytical community, the use of suitable isotopically labelled internal standards for mass spectrometry is considered to widely balance measurement variability coming from varying sample extraction efficiencies, matrix effects, or loss of instrument sensitivity in the course of a measurement sequence. The best effects are achieved by using compound-specific internal standards, e.g. fully ^{13}C-labelled metabolites, as it is widely agreed that the differences in chemical behavior (solubility in the extraction solvent, chromatographic retention, ionization efficiency, stability in the ion source, detectability by the mass spectrometer and signal susceptibility to matrix effects) of ^{13}C-labelled analogues compared to their non-labelled isotopologues are negligible (see examples [18,19]). For most hydrophilic small molecules, a mass spectrometer can distinguish the labelled internal standards from the target analytes by their differing accurate mass. Selectivity in this respect is thus provided. The limiting factor for compound-specific isotopically labelled internal standards for metabolites, however, is their commercial availability. A practical solution for this is to use fully ^{13}C-labelled biomass extracts as internal standard, e.g. from the yeast *Pichia pastoris* grown on fully ^{13}C-labelled glucose as the only carbon source.[20] The same aliquot of internal standard is added to each biological sample before sample extraction and to each standard (see chapter 3.1.2 on page 18).

Despite the mostly poor characterization of the cell extracts used as internal standard, this procedure should in principle conform to the U.S. FDA's guideline, since it does not demand extensive characterization of the internal standard (i.e. certificate of analysis including purity and stability data). The advantage of using an isotopically enriched biomass extract is that it comprises a great diversity of metabolites without being too costly. It is this complexity, though, that introduces new pitfalls that need to be addressed by a suitable method validation plan. While the principal overlap in compound species between yeast and human metabolome is probably constant, the concentrations in the internal standard can markedly deviate from the concentrations present in the biological sample.

When applying yeast extract as internal standard it would therefore be necessary to define suitable maximal and minimal signal intensity ratios between target analyte and internal standard compound to ensure the reliability of the calculated ratios. The amount of internal standard that is able to sufficiently match the concentrations of the labeled compounds with those of the target compounds present in the samples and calibration standards needs to be evaluated. This endeavor is surely not trivial considering that the metabolites in biological samples spread across a huge concentration range – Nandania et al. (2018), for example, estimated the median concentration of adenine in human adult serum to be as low as 0.007 µmol L^{-1}, whereas the median concentration of alanine was estimated to be several hundred µmol L^{-1}.[21] Stemming from different organisms, the concentrations might also widely differ between internal standard and samples. A systematic one-fits-all solution for all compounds is hardly imaginable. After all, we are trying to match two, potentially highly variable, biological extracts. Moreover, by adding the matrix of the yeast extract, we add to the complexity of the sample, which in turn bears the risk of adverse effects on sensitivity, linearity and measurement reliability. In fact, Büscher et al. (2019) explicitly described matrix effects introduced by a ^{13}C-labeled yeast extract by comparing the slopes of calibration curves prepared in pure solvent and in yeast matrix (for the sake of completeness, I should mention that the extract was obtained with a different extraction procedure than our internal standard). It becomes clear that, although compound-specific internal standardization is considered a gold standard in quantitative mass spectrometry, it is crucial to evaluate the benefit and validate the use of this complex internal standard for each specific experimental design. The U.S. FDA's guideline on bioanalytical method validation in deed requests proof that the applied internal standard does not interfere with the detection of the target analytes. In our case, this means that we have to strictly preclude any non-labelled contamination because it would erroneously contribute to the target analyte's signal. This can be assessed by investigating the zero-calibrators for ^{12}C-signals. We also have to make sure that the internal standard exerts beneficial as opposed to detrimental impact on the measurement reliability of each targeted analyte. This can be assessed by comparing measurement accuracy and precision of quality control samples (QC) with and without the addition of internal standard. The QCs should be distributed across the whole calibration range to clarify if problems occur in high (internal standard signal might get suppressed up to an extent where it is not valid for quantification anymore) or low concentration ranges (internal standard signal might be too intense and target compound signal too weak to give a reliable ratio). This will allow defining the linearity range for each compound and will also help to elucidate the optimal span of signal ratios for future quantification exercises. For details on the preparation of such QC samples, see chapter 2.2.5 on page 13.

2.2.3 Calibration curves and working range

A calibration curve is prepared for each compound by dilution of an authentic reference standard. For easier handling, the standards can be combined to equimolar mixtures (in the following referred to as “primary mixes”). To assure unambiguous peak identification, isomeric compounds should not be contained in the same primary mix. Likewise, an unstable compound should not be put into the same primary mix as a compound that is also its degradation product. This way, problems with in-source fragmentation of the target analytes can be revealed and taken into consideration for specificity evaluation. The primary mixes are measured once in positive and once in negative polarity mode in a concentration of 1.00 µmol L^{-1}. If there are no major problems with in-source fragmentation and chromatographic overlap of isobars, the primary mixes can be combined to form one master mix. The master mix will be used to create the calibration curves. The calibrators, i.e. dilutions of master mix, are prepared in pure solvent and each is spiked with internal standard. According to the guideline, the quantitation range and concentration of the calibrators should be chosen based on the expected analyte concentrations. In metabolomics, the target compounds in the biological sample spread across a wide concentration range. To approach this issue without too much effort, I suggest measuring dilutions of calibration standard that cover a wide concentration range in relatively narrow increments. The curves could then be “cut” during data treatment according to the linearity assessment of each target compound. For example, twelve calibration standards will cover a concentration range from 0.01 µmol L^{-1} to 10 µmol L^{-1} regardless of the compound concentrations in the sample. The calibrator concentrations are 0.01 µmol L^{-1}, 0.03 µmol L^{-1}, 0.05 µmol L^{-1}, 0.10 µmol L^{-1}, 0.30 µmol L^{-1}, 0.50 µmol L^{-1}, 0.75 µmol L^{-1}, 1.00 µmol L^{-1}, 3.00 µmol L^{-1}, 5.00 µmol L^{-1}, 7.50 µmol L^{-1} and 10.00 µmol L^{-1}. To check for irregularities concerning the creation of the calibration curves, each calibration standard is prepared in duplicate. Additionally, zero-calibrators (with internal standard but without reference standard) and a blank (pure solvent) are prepared. Each calibrator is injected twice per MS polarity, which is four injections in positive and four in negative mode for each calibrator concentration. The blank is injected as often as necessary. The calibration curves are constructed by plotting the area ratios of the ^{12}C- and ^{13}C-compound (i.e. signal from reference and internal standard) against the nominal concentrations of the calibrators. For compounds without internal standard, it is, in theory, possible to plot the non-standardized areas. This is a less desirable case, though, because it results in reduced accuracy, and since the U.S. FDA’s guideline requests the use of an internal standard for quantification, values obtained this way should be regarded as purely informational. For linearity assessment, the curves are studied calibrator by calibrator for each compound. In accordance with the U.S. FDA guideline, non-zero calibrators should be ± 15 % of the theoretical concentrations, except at LLOQ where the calibrator is allowed to deviate ± 20%. If a calibrator differs from the nominal concentration more than ± 15%, it should be discarded. However, for each

compound, a minimum of six non-zero calibrator levels should meet the above criteria. The narrow increments of the calibrator concentrations should assure that an adequate number of calibrator levels remains after linearity assessment.

2.2.4 Quality control to assess technical accuracy and precision

Aside from biological variability, measurement variability can also originate from instability of the analytical system (mass drift, retention time shift). This can be observed by implementing quality control samples (QCs) distributed across the whole measurement sequence. Statistical comparison of QC results reveals trends like mass drift of the mass spectrometer, retention time shifts due to the chromatographic system, loss in ionization efficiency due to gradual sample precipitation in the ion source, and so forth. It renders information about intra- and inter-experiment reproducibility and, if QC samples with known concentrations are applied, also about the accuracy of the analysis. The stability of the analytical system can be improved by optimizing sample preparation and analytical method parameters. The remaining instability should be precisely characterized so that the effects on data reliability can be accounted for during data interpretation or problems possibly circumvented by thoughtful design of the sample and standards acquisition queue.

The U.S. FDA's guideline on bioanalytical method validation asks for accuracy and precision to be demonstrated by use of QCs at LLOQ, low, mid and high concentration. Technical accuracy and precision checks seem informative based on the assumption that the sample matrix-specific alterations of these figures will mostly be compensated by the internal standard. I suggest five replicates of all twelve calibration standards (0.01 µmol L^{-1} to 10.00 µmol L^{-1}) in three runs to calculate technical accuracy and precision of the analytical method. Accuracy is additionally calculated for standard-spiked pooled sample QCs in the course of matrix effect assessment (see chapter 2.2.5 on page 13).

2.2.5 Quality control to assess matrix effects

To describe matrix effects coming from the sample matrix, but also from the internal standard, quality control samples could be prepared as follows (scheme inspired by [21]): a pooled biological sample is prepared and divided into eight portions. Four aliquots are spiked with IS before extraction, four are not, and all sample portions are extracted. These are the eight QC stock samples. Two replicates are spiked with IS after the extraction, leaving two stock samples without IS. Six of the eight QC stock samples are divided into six portions and each portion is spiked with different concentrations of standard (0.05 µmol L^{-1}, 0.30 µmol L^{-1}, 0.75 µmol L^{-1}, 3.00 µmol L^{-1}, 5.00 µmol L^{-1}, 7.50 µmol L^{-1}). Each of the QCs as well as the QC stock with IS is injected to the mass spectrometer twice. A chart of the different QCs is given in **Figure 1**. QCs without IS (1a, 1b) are compared to the corresponding solvent-based calibration standards to quantify the matrix effect for each compound. QCs with IS added before extraction (3a, 3b) should be compared to the QCs without IS (1a, 1b) to assess the benefit of the internal standardization at different concentration levels. QCs with IS added after extraction (2a, 2b) and before extraction (3a, 3b) may be compared to assess extraction efficiency. Matrix-specific accuracy and precision are calculated from QCs with IS added before extraction (3a/3b corrected for the signal intensities from 4a/4b and fitted on solvent-based calibration curve) at the different concentration levels.

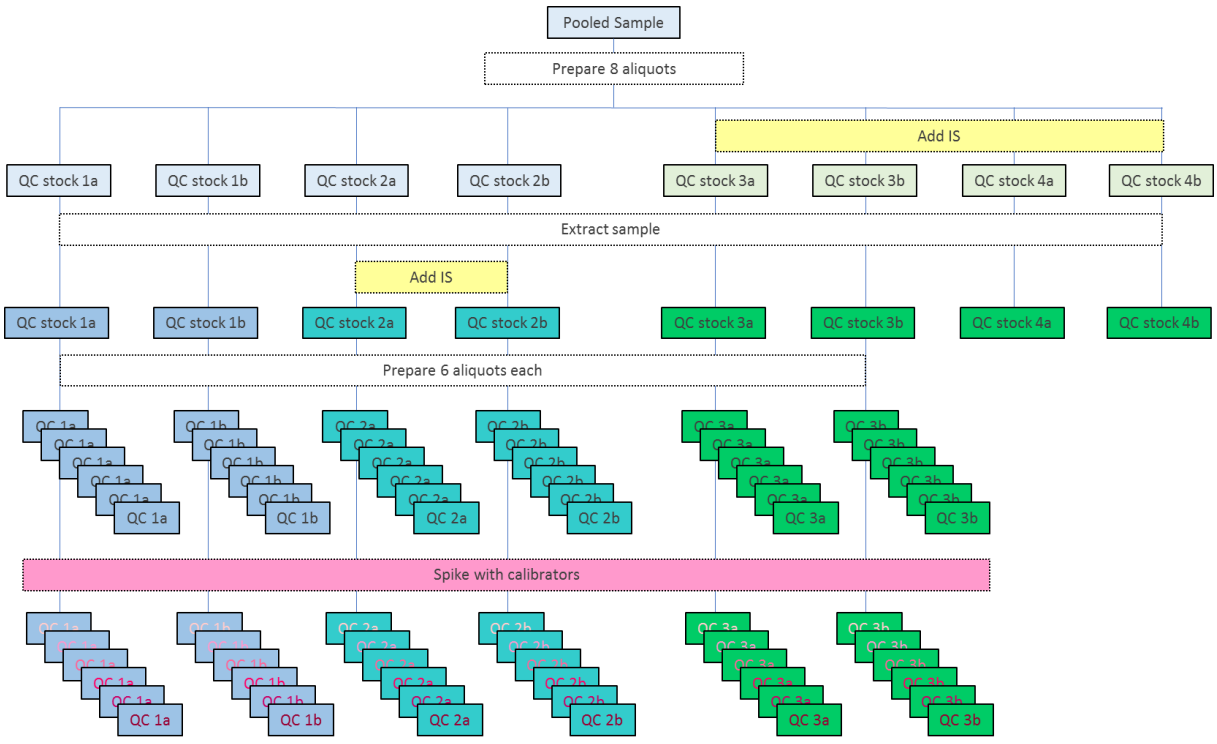

Figure 1 Systematic preparation of quality control samples to maximize information output. QC: quality control, IS: internal standard.

3 Materials for Validation

3.1 Analytical method

3.1.1 Rationale for method design

The presented HPLC-HRMS method with 2D-LC was developed for the simultaneous analysis of hydrophilic metabolites and small-molecule xenobiotics. While HILIC-separation is popular for the analysis of polar metabolites, small-molecule xenobiotics like pharmaceuticals are frequently a bit more lipophilic to keep a balance between water solubility and the ability to penetrate biological membranes. They are therefore regularly analyzed via reversed-phase chromatography. The basic idea for this method was to broaden the polarity range of the chromatographic separation and yield good retention for an extended set of analytes without sacrificing time-efficiency of the analysis. The combination of liquid chromatography and high-resolution mass spectrometry, hyphenated by an electrospray ion source, is especially fruitful because the two techniques complement each other regarding the chemical properties they exploit for compound discrimination. This, in turn, ameliorates the selectivity of the analysis. The combination is highly automatized, enabling a sample throughput suitable for processing biological replicates. The features described render this method appealing for integrating metabolomics into exposomics research.

High-resolution profile mass scans

Compared to other techniques for compound analysis, mass spectrometry provides a high degree of selectivity and a relatively wide dynamic range. Thanks to its selectivity, mass spectrometry is able to distinguish between analytes even in complex analyte mixtures and biological sample matrix. It is relatively fast and inexpensive, and workflows can be automatized to a high degree.[22] The Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ mass spectrometer is capable of producing high-resolution data with the possibility to introduce fragmentation, if required. In contrast to a triple quadrupole mass spectrometer, which more or less restricts the investigator to a targeted workflow, it extensively records sample information across the entire specified mass range. This property is an advantage for explorative metabolomics experiments especially, as any bit of information that describes the sample is potentially valuable for hypothesis generation. In cases where the investigator is interested in a defined subset of compounds, i.e. in a targeted experiment, recording excess information is not a disadvantage either, since it is always possible to restrict data evaluation to those compounds and leave the rest of the data. After all, the rest of the data possibly gets more interesting in a later state of the research project where the scientific question has been adapted to

results obtained in the meantime. In this case, the required information may be extracted from the data set in retrospect without the need to perform a new experiment.

Enabling absolute quantification

The general principle for quantification in LC-MS is that the signal intensity of a compound – separated by LC and additionally discriminated by MS – is plotted against time, thereby creating a peak shape with a quantifiable area under the curve. This area correlates with a certain analyte concentration. To establish the connection between an (absolute) concentration and its corresponding peak area, it is necessary to measure a dilution series of known concentrations of the analyte. A function is derived that describes the connection between signal intensities and concentrations. This concept is called external calibration. It relies on the stability and comparability of signal intensities. In an LC-MS experiment there are, however, several factors aside from its concentration that impact an analyte's signal intensity, resulting in marked intensity fluctuation between repeated injections of the same sample. Direct comparison of the peak areas is even less allowable when the signals are recorded at different days or from different matrices. To counterbalance signal intensity biases, a ^{13}C-labeled yeast extract is employed as internal standard for samples as well as reference standards. The strategy rests on the assumption that the original amount of ^{13}C-compound is the same in each spiked sample and standard and the fully ^{13}C-labeled compound is prone to ion suppression, day-by-day fluctuation or time-dependent loss of instrument sensitivity, chemical degradation, etc. to the same extent as its non-labeled analogue. **Figure 2** exemplarily shows the chromatographic peak areas recorded for eight replicate standard injections of tryptophan. The analyte areas (red bar) vary between the injections although they are derived from the very same standard solution. The signal of the co-eluting internal standard (IS, purple) is suppressed or enhanced in the same way as the analyte signal, so that the ratio between analyte and internal standard is more or less the same across all replicate injections. This, in theory, allows quantitatively comparing signal intensities of intra- and inter-experiment replicate injections, samples and calibration standards, and possibly even different sample matrices.

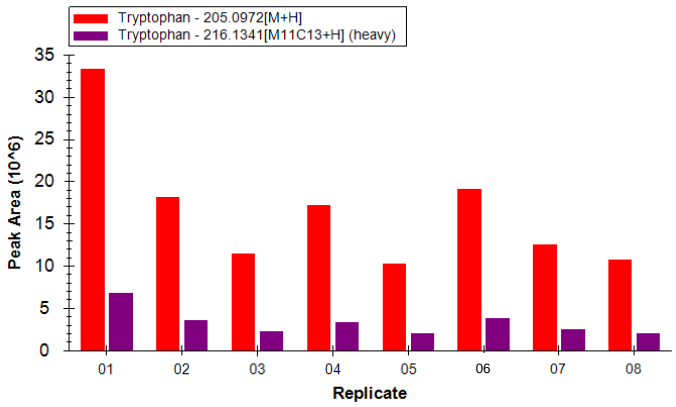

Figure 2 Intra-experiment area variability between replicate injections of standard. Red bar: ^{12}C-tryptophan (analyte), purple bar: ^{13}C-tryptophan (IS). Eight replicate QC injections (1 µmol L^{-1} standard mixture) distributed across ~35 hours of one experiment, recorded in positive mode. Figure exported from Skyline.

Positive and negative electrospray ionization

Electrospray Ionization (ESI) is a soft atmospheric pressure ionization technique that generates intact mass signals with little fragmentation. This provides a high signal intensity for the molecular ion and enables accurate mass determination in combination with high resolution MS. In an ESI source, small molecules like our target analytes (< 900 g mol^{-1}) are usually loaded with one charge. The charging polarity, positive or negative, is defined in the method script by choosing the according ionization mode. The preferred charge that yields the best possible signal intensity depends on the chemical properties of the analyte. An analyte that is not ionized in the respective polarity is not "visible" to the mass spectrometer and cannot be analyzed. Serving the high chemical diversity of analytes in a metabolomics-exposomics experiment, both ionization polarities are recorded in order to yield the best possible signal for each analyte. The predominant argument for an ESI source, however, is that it is one of few interfaces suitable for hyphenating mass spectrometry with liquid chromatography.

Addressing spray quality and ionization efficiency

During a chromatographic run, analyte enriched eluent is constantly eluting from the column and continuously vaporized in the ion source in order to be introduced to the mass spectrometer. Uniformity of the spray is important, as it would otherwise compromise stability and reliability of the detected signal. With the presented method, spray stability is supported by two high-pressure binary pumps employed for gradient formation and flow delivery.

The quality of the spray is reflected by the so-called TIC variation. TIC is short for total ion current and its variation should be below 10% when the ion source is faced with a continuous eluent flow. This parameter is routinely checked before a measurement series is started. However, spray quality is never perfect and likely to worsen with analysis time, as sample matrix components tend to accumulate at the inlet. To counterbalance these effects, a species-specific internal standard is employed. It does, of course, not improve absolute signal intensity, but comparability of the signals by building a ratio of compound and IS area.

For a compound that is in principle ionizable, it is usually still only part of the theoretically available analyte molecules that is really ionized. This is reflected by the concept of ionization efficiency. In addition to the chemical properties of the analyte, ionization efficiency is also influenced by eluent composition and co-eluting compounds. Eluent composition can support or hamper ionization. A rule of thumb says that an eluent containing a high content of organic solvent ameliorates ionization as compared to a purely aqueous eluent (see for example [23]). Due to their complementing gradient compositions, combining HILIC and RP flows prior to ionization provides the spray with a certain amount of organic solvent during the whole run duration of 20 minutes.

Reducing complexity of the analytical sample

Measures to reduce sample complexity before mass-spectral analysis, i.e. sample extraction and chromatography, pose an integral part of the presented analytical procedure; likewise do measures to counterbalance intensity fluctuations during mass-spectral analysis.

For this analytical method, we chose a non-selective sample extraction procedure (protein precipitation by adding methanol) to avoid analyte loss. Removing high molecular sample components is important to preclude unnecessary matrix effects and pollution of the analytical platform. The procedure yields an extract with quite a wide analyte scope appropriate for non-targeted analysis, if required.[24] This non-selective extraction procedure, however, implies that the analytical sample is not only dense with analytes, but also with unwanted accompanying compounds of low molecular weight. The equal suitability for non-targeted and targeted metabolomics is considered an advantage of the presented workflow. However, the complexity of the analytical sample renders chromatographic separation and internal standardization all the more important.

Two obstacles for the accuracy of an absolute analyte quantification are linked to sample extraction: First, considering the chemical diversity of the analytes and their varying solubility in the extraction solvent, sample extraction is probably incomplete. This means that there is a lower amount of a specific analyte in the analytical sample than there was in the biological sample. Addition of a species-specific internal standard before sample extraction is considered to counterbalance this analyte loss. A limitation of this approach might be the fact that the internal standard is spiked to the sample in a dissolved form, whereas the actual analyte might be bound to the sample matrix more tightly and therefore not extracted as easily.

Second, the sensitivity of several elements of the presented analytical method is potentially reduced by accompanying matrix. Although the predominant portion of it is removed by dedicated sample preparation, the prepared analytical sample will still contain some matrix elements specific to its biological origin. Being selected by the same extraction process, the physicochemical properties of the remaining matrix components are similar to those of the target analytes and are likely to interfere with their detection. Since metabolomics focuses the entirety of small molecules in a biological sample, their exhaustive extraction is actually desired instead of their elimination from the analytical sample, posing the additional problem of detection interferences between analytes.

Ionization efficiency in the electrospray ion source depends, among other things, on the composition of the analyte mixture present in the ion source in the moment of ionization. Ionization is a complex process and subject to various influencing factors, but a general trend is that the chance for the desired analyte molecules to be ionized decreases with an increasing amount of non-target molecules competing for ionization. Accompanying matrix potentially lowers the analyte's signal intensity and reduces the sensitivity of the analytical process towards a target compound. In this regard, it is advis-

able to reduce sample complexity as far as possible by sample extraction and chromatographic separation prior to ionization. Under the assumption that a fully ^{13}C-labeled compound elutes at the exact same moment and is equally affected by ion suppression as its unlabeled "natural" counterpart, the presented analytical workflow should be able to outweigh the described intensity fluctuation by implementation of the species-specific internal standard.

A similar competition between analyte and matrix compounds appears in the curved linear trap (c-trap) of the orbitrap-based mass spectrometer Q Exactive™ HF (see schematic overview in **Figure 4**, page 21). The main purpose of the c-trap, a sort of ion trap, is to accumulate the continuously eluting ions, reduce their kinetic energy, and direct ion packages of defined volume to the orbitrap mass analyzer.[25] The magnitude of the ion package is customized by setting the advanced gain control (AGC) target value. The AGC target value correlates to the number of ions in the package. It does, however, not reflect the actual ion count. The AGC target has to be considered during method optimization because a compound's signal intensity is linked to the amount of ions that the signal is based on. If the target analyte is accompanied by many other ions, the AGC-target value will be reached quite fast. The fraction of desired analyte ions in the package will be rather low, though, rendering the analyte's signal intensity lower than it could be. The extent to which the AGC target value can be increased in order to yield a greater absolute amount of analyte ions is linked to the size of the ion trap and is therefore limited. While a high AGC target value might be expected to correlate with good sensitivity, the c-trap is, in reality, overpopulated with ions at a certain point, resulting in a rapid decrease in mass accuracy and resolution. This is called space-charge-effect. It roots in repulsive charges that cause excess ions to be randomly ejected from the ion trap.[26] The application of our internal standard to improve measurement accuracy has to be reflected critically in this case: Being a minimally purified fermentation extract, we potentially introduce a highly complex matrix to our already complex analytical sample. This could adversely affect measurement sensitivity towards the target analytes due to increased competition in c-trap and ion source. With this in mind, the reduction of sample complexity by sample extraction and chromatography prior to detection appears all the more important.

3.1.2 Technical description

Plasma sample extraction

The extraction of the plasma sample is performed according to the study of Simón-Manso et al. (2013)[27] with the adaptations from Schwaiger et al. (2018)[28]: After storage at -80°C, the sample is allowed to thaw completely at room temperature. An aliquot of 50 µL is mixed with 50 µL of t internal standard (IS) solution (see below) or 50 µL water. 400 µL methanol are added to reach a final volume of 500 µL (80 % methanol, *V*/*V*), yielding a 1:10 dilution of plasma sample and IS, respective-

ly. The mixtures are vortexed thoroughly and then kept on ice for 30 minutes. After vortexing again, the samples are kept at -20°C overnight. The samples are thawed and centrifuged (14 000 * g, 4°C, 15 min) and two 200 µL aliquots of the supernatant are dried in a vacuum centrifuge. The aliquots are reconstituted in 200 µL 50%/50% (*V*/*V*) acetonitrile/water, vigorously vortexed for 3 min to ensure complete dissolving of the sample and centrifuged (14 000 * g, 4°C, 10 min) prior to being transferred to an HPLC vial.

Internal standard for samples, calibration and QC

A fully ^{13}C-labeled cell extract from *Pichia pastoris* (purchased at ISOtopic solutions e.U., Vienna, Austria) is used as an internal standard for absolute quantification of the metabolites. The yeast species can be cultivated in minimal growth media containing only ^{13}C-enriched glucose as a carbon source. It thereby generates a broad spectrum of metabolites with > 99 % isotopic enrichment.[20] The dried cell extract is stored at -80°C. For the experiment, an extract aliquot derived from two billion cells is thawed at room temperature and reconstituted in 2 mL water. The final content is 10 % (*V*/*V*) of this solution in samples, calibrators and QCs.

Setup for HILIC-RP liquid chromatography

A Vanquish™ Duo UHPLC system (Thermo Scientific™) equipped with an autosampler with two injection units, two binary pumps and a column compartment is used for parallel hydrophilic interaction liquid chromatography (HILIC) and reversed phase liquid chromatography (RP-LC). A post-column T-piece is installed prior to introduction to the electrospray ionization (ESI) source to combine the flows of the two separations before entering the mass spectrometer. **Figure 3** gives a schematic overview over the analytical setup. HILIC separation is carried out on an Acclaim™ Trinity P2 LC column (2.1 x 100 mm, 3 µm, Thermo Scientific™) under acidic conditions with 25 mol L^{-1} ammonium

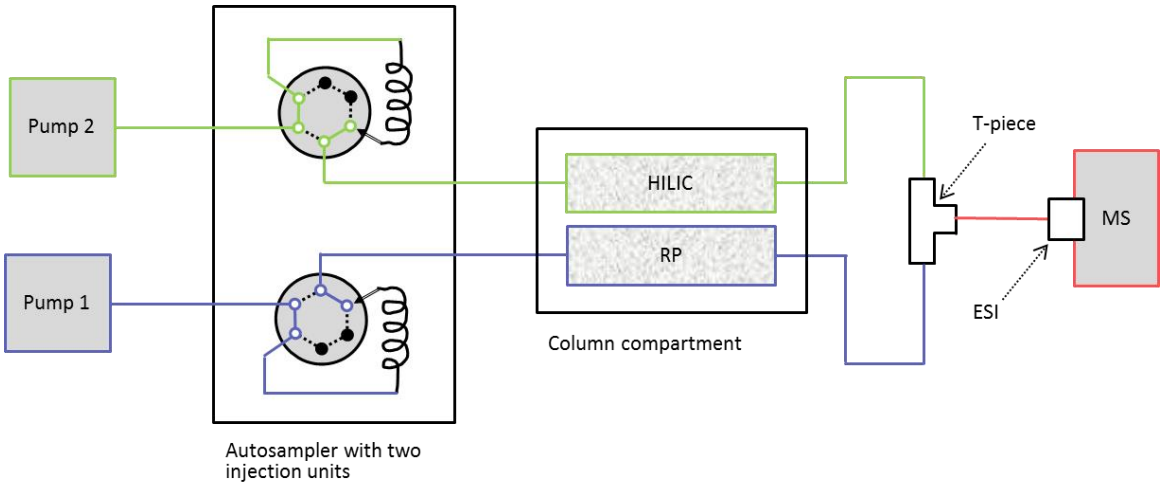

Figure 3 LC-MS setup for parallel HILIC and RP-LC separation. The two six-port valves of the autosampler are set to injection configuration. The eluent flows enter the mass spectrometer simultaneously.

acetate (pH 4.5) as eluent A and 100 % acetonitrile as eluent B. RP-LC separation is carried out on a Hypersil GOLD™ C18 Selectivity LC column (2.1 x 100 mm, 1.9 µm, Thermo Scientific™) using water with 0.1 % formic acid as eluent A and acetonitrile with 0.1 % formic acid as eluent B. The gradients applied to each separation are given in Table 1. Injection volume is set to 5 µL and the flow rate to 250 µL min^{-1} for both separations. The column compartment temperature is 40°C and the samples are kept in the autosampler at 6°C. The two injection units draw sample from the same vial and prepare it for injection. At the start of the analytical run, one injection unit injects its sample portion on the HILIC- and the other on the RP-column, both at minute zero. Orthogonal separation of the two identical sample portions is carried out in parallel, resulting in a total run time of 20 min.

Table 1 Gradients for HILIC and RP-LC separation.

Time [min]	HILIC Eluent B [%]	RP-LC Eluent B [%]
0.0	90	5
2.0	90	5
13.0	10	95
15.0	10	95
15.1	90	5
20.0	90	5

High-resolution mass spectrometry (HRMS)

High-resolution mass spectrometry is performed on a Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ mass spectrometer (Thermo Scientific™, see next page, **Figure 4**) equipped with an electrospray ion source (ESI). The ESI source parameters are the following: sheath gas 50, auxiliary gas 14, spray voltage 2.8 kV in negative and 3.5 kV in the positive mode, capillary temperature 270°C, S-Lens RF level 45 and auxiliary gas heater 380°C. Full mass scan data are acquired in profile mode in a scan range of 60–900 m/z. Positive and negative mode data are acquired separately with a resolution of 120 000 at m/z 200. The automatic gain control target is set to 10^6 and the maximum injection time is 200 ms.

3.2 Provided data set

3.2.1 Materials

Standards for calibration and QC

HPLC-grade standards for the generation of compound-specific external calibration curves of metabolites and xenobiotics were purchased from Sigma Aldrich (Vienna, Austria) or Carbosynth (Berkshire, UK). All standards were combined to a mixture in advance comprising 163 compounds in equimolar concentrations. The mixture was aliquoted and the aliquots dried and stored at -80°C until the day of the experiment. One aliquot was thawed at room temperature, dissolved in water and diluted to obtain suitable calibrator concentrations. Each calibrator was spiked with 50 µL internal standard and acetonitrile was added. The final calibrators contained 50 % (*V*/*V*) acetonitrile and had the following concentrations: 0.01 µmol L^{-1}, 0.05 µmol L^{-1}, 0.10 µmol L^{-1}, 0.50 µmol L^{-1}, 1.00 µmol L^{-1}, 5.00 µmol L^{-1} and 10.00 µmol L^{-1}. A zero-calibrator containing no standard mix and only internal standard in 50 % (*V*/*V*) acetonitrile was also prepared. Another 1.00 µmol L^{-1} standard was prepared as QC sample and spiked with IS in the same way as the calibrators. For details on the internal standard, see page 18, chapter 3.1.2.

Eluents

Acetonitrile, methanol and water were of LC-MS grade and ordered at Fisher Scientific (Vienna, Austria) or Sigma Aldrich (Vienna, Austria). Ammonium acetate was ordered as eluent additive for LC-MS at Sigma Aldrich. Formic acid was also of LC-MS grade and ordered via VWR International (Vienna, Austria).

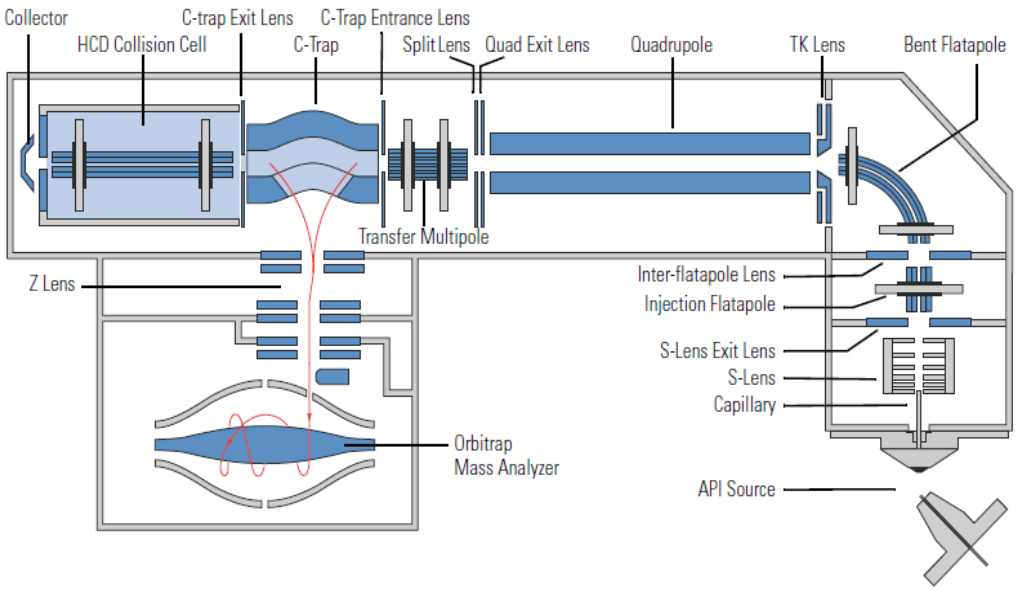

Figure 4 Schematic view of Q Exactive HF mass spectrometer (Thermo Scientific). Source: *Exactive Series – Operating Manual (Revision A)*, Thermo Scientific™ (April 2017). API: atmospheric pressure ionization (here: ESI), c-trap: curved linear trap (ion trap), HCD: higher-energy collisional dissociation.

Standard reference material 1950

Standard reference material (SRM) 1950 - Metabolites in Frozen Human Plasma was purchased from the National Institute of Standards & Technology (NIST, Gaithersburg, USA). This reference material is prepared from pooled plasma samples taken from 100 individuals at the age of 40 to 50 years after an overnight fast and reflects the US American population's "normal" plasma, i.e. without the impact of extreme diets, sports or overt diseases.[29] It contains lithium heparin as anticoagulant. For each batch of reference standard, the National Institute of Standards and Technology provides certified values for cholesterol, total glycerides, selected fatty acids, amino acids, vitamins and carotenoids, as well as clinical markers, hormones and electrolytes. A NIST-certified value reflects the highest possible confidence in the accuracy of the measurement result; the values are assigned by combining the means of data sets obtained from different analytical methods. Its associated uncertainty margin takes all known or suspected sources of bias into account. NIST-certified values can be used to establish metrological traceability. Reference and information values are provided for further compounds: they do not meet the very strict confidence criteria for a NIST-certified value because there is a lack in statistical agreement among multiple analytical methods or a full evaluation of measurement uncertainty could not be performed, for example. Nevertheless, these values are elaborated under trustworthy conditions and are still valuable for comparison. In our validation study, SRM 1950 was used as an exemplary human plasma sample to perform an accuracy check of the analytical system with highly recognized external reference. The sample was prepared according to the extraction protocol described in chapter 3.1.2 (page 18).

3.2.2 Data acquistition

A test data set was acquired with the sample preparation and instrument settings as described earlier. A 1.00 µmol L^{-1} standard with IS was used as QC. The calibrators contained IS. For comparison and evaluation of the IS, one 5.00 µmol L^{-1} and one 10.00 µmol L^{-1} standard were prepared without IS. An SRM 1950 extract was used as sample and investigated with and without IS. Additionally, an extract of ^{12}C-*Pichia pastoris* (i.e. without isotopic enrichment) was measured. Types, concentration levels and replicate numbers of samples and standards were not chosen along the validation requirements outlined above, however, but rather from an explorative chemical-analytical point of view. **Figure 5** (page 23) gives an overview of the measured samples and standards, their concentrations and replicates.

SRM 1950 with IS was injected five times in a row and full-MS data were acquired. The calibration curve, including the zero-calibrator, was measured once. A total of ten blank and six QC injections were distributed evenly across the positive mode sequence. The described sample lineup was measured again in negative mode, thereby producing separate positive and negative mode data files. An-

	1	2	3	4	5	6	7	8	9
A	Blank	QC with IS 1.00 µM							Standard Mix 5.00 µM
B	Standard Mix 10.00 µM	Standard Mix with IS 0.01 µM	Standard Mix with IS 0.05 µM	Standard Mix with IS 0.10 µM	Standard Mix with IS 0.50 µM	Standard Mix with IS 1.00 µM	Standard Mix with IS 5.00 µM	Standard Mix with IS 10.00 µM	Zero Calibrator
C	*P. pastoris* (12C) Extract	*P. pastoris* (12C) Extract with IS	SRM1950 Extract	SRM1950 Extract with IS	SRM1950 Extract *				
D									
E									
F									
G									
H									
I									

Figure 5 "Autosampler rack" for the acquisition of the test data set. Green: reference standards (in solvent), orange: SRM 1950, red: 12C-*P. pastoris* extract, dark shade: with IS, light shade: without IS, blank: pure solvent. Each labeled square represents one sample vial. The 12C-*P. pastoris* extract data were not evaluated for this work, but they contributed to the total duration of the sequence.

other two positive mode QC measurements were interspersed in the negative mode sequence. Five replicate injections of the 0.5 µmol L^{-1} calibration standard in positive and negative mode, respectively, served for LOD and LLOQ estimation. After each polarity switch, blank or QC injections were measured to avoid or investigate sensitivity problems caused by the switch. The entire test data set for this validation exercise was generated in one run sequence with a total duration of approximately 57 hours.

For additional characterization of the 2D-LC setup, SRM 1950 and the 5.00 µmol L^{-1} standard, both without IS, were also measured in this sequence under exclusion of either the HILIC or the RP column, in 1D-LC setup, so to speak. Since we are expecting two peaks for each compound in the 2D setup (one showing the retention time of HILIC and one of RP separation), the 1D data are useful to assign the peaks correctly to either column. In the same run sequence, the 2D setup was also tested in data-dependent MS2 mode with adapted instrument parameters. However, for this validation study, data evaluation will be limited to the full MS scan mode of the 2D-LC setup.

3.3 Raw data evaluation

To test the new LC-MS method regarding its suitability for combined metabolomics and exposomics analysis, the test plasma sample SRM 1950 was searched for 148 endogenous metabolites and 15 xenobiotics. The target metabolites were selected because of their presumed importance in cancer metabolism. The xenobiotics were chosen after a rough untargeted pre-evaluation of the data set and are meant to be a placeholder for pharmaceuticals or toxicants with similar chemical properties. A list of the compounds investigated in targeted data evaluation, including molecular formula and assigned compound class as well as the adducts and exact masses used for ion chromatogram extraction of positive and negative mode data is given in Table S1 of the supplement. The list comprises all standards contained in the standard mixture. They were not necessarily recovered with the presented method.

Identification, retention time analysis and absolute quantification were performed in a targeted data evaluation approach in Skyline (64-bit, version 4.2.0.19072, MacCoss Lab Software). This open source software was originally developed for protein analysis at the University of Washington. Since then, the software has faced several updates and dedicated features for small molecule analysis have been implemented continuously. Assuming that $[M+H]^+$ and $[M-H]^-$ are the most prominent compound adducts, only those two adducts were investigated in the positive and negative mode data, respectively.

Prior to raw data treatment in Skyline, the profile mode data were centroided with the open source software msConvert (ProteoWizard, see **Figure 6**, page 25). The software is able to transform several proprietary file formats into an open format, e.g. mzML. The purpose of centroiding data is to reduce its dimensionality. In profile mode data, a peak has three dimensions: time, intensity and m/z distribution. The centroid of a peak divides the peak's area under the mass range distribution curve in half, thereby reducing the peak's m/z range to a discrete value. The centroid's intensity still reflects the intensity (i.e. area under the curve) of the entire previous mass-range peak.

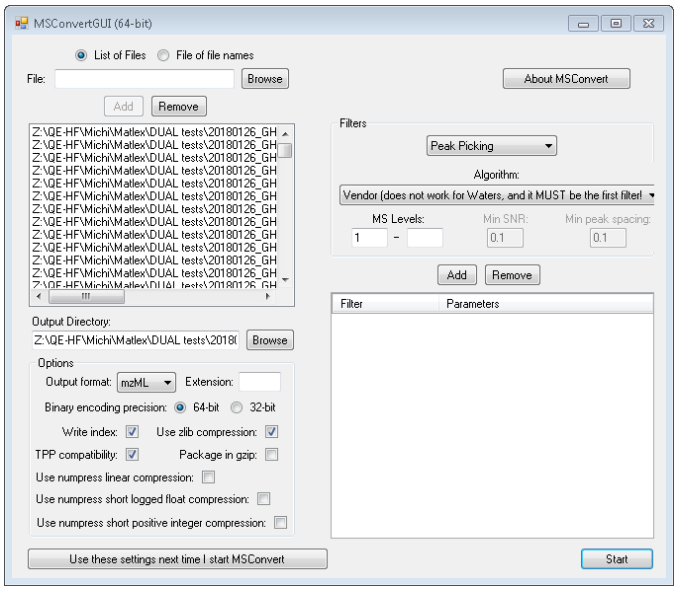

Figure 6 Graphic user interface of msConvert. The open-source software was used for data-centroiding.

Confronted with centroided data, the data evaluation software Skyline includes a signal to the extracted ion chromatogram only if the centroid lies within the specified mass extraction window. The advantage is that m/z signals that have been grouped to peaks are either collectively included or excluded from the extracted ion chromatogram. If the data are not centroided, it might happen that Skyline does not integrate the whole mass-range of a peak although the entire mass range might really be derived from one analyte mass. **Figure 7** shows the mass spectrum at of an extracted ion chromatogram peak at minute 11.63. The data file is not centroided. The blue window indicates the mass deviation allowed for ion chromatogram extraction, 5 ppm in this case. The software counts all signals that deviate at maximum 5 ppm from the m/z specified for ion chromatogram extraction as one peak. The peak shown in the extracted ion chromatogram, i.e. the "normal" intensity-over-time view, only reflects the integrated area under the curve within the blue window. This means that only a part of all m/z that were previously assigned to this analyte mass by the peak-picking algorithm contribute to the intensity in the extracted ion chromatogram. To include the whole sum of m/z derived from one mass it would be possible to broaden the mass extraction window, e.g. to 10 ppm.

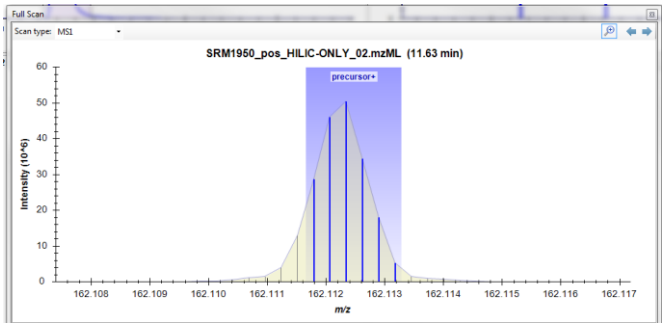

Figure 7 Mass spectrum of a non-centroided peak. The blue window indicates the mass window allowed for ion chromatogram extraction. Only signals from within the blue window contribute to the extracted ion chromatogram. Sample: SRM 1950, column: HILIC, extraction window: 5 ppm. Figure exported from Skyline.

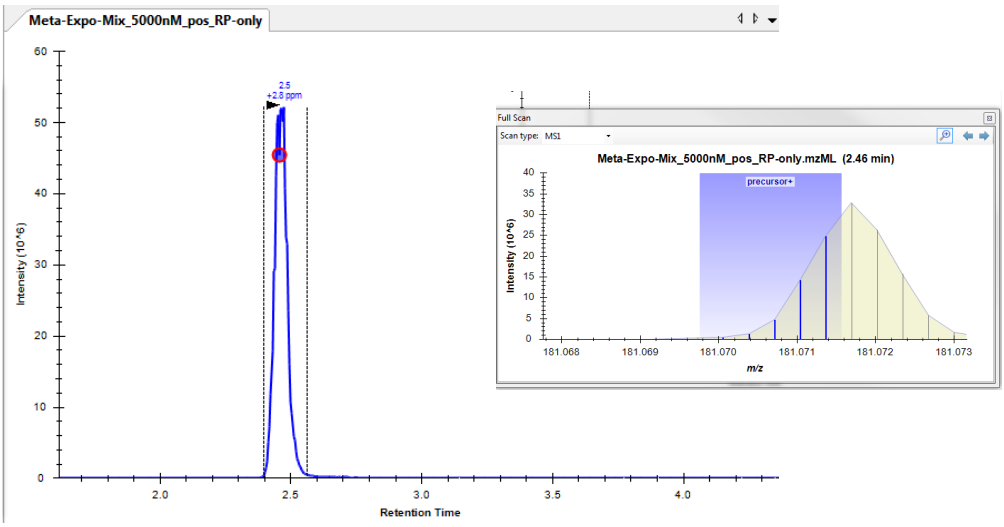

Figure 8 Extracted ion chromatogram (left) and mass spectrum (right) from a non-centroided data file. The chomatographic peak (left window) is erroneously extracted for inositol ([M+H]$^+$ adduct m/z 181.0707). The mass spectrum at minute 2.46 (right window) reveals that the chromatographic peak is derived from a signal overlap of a nearby mass. Sample: 5 µmol L^{-1} standard mix, column: RP, extraction window: 5 ppm. Figure exported from Skyline.

However, this would raise the risk of accidentally including other m/z signals that happen to be close to the m/z of the analyte under observation. **Figure 8** further illustrates the importance of centroiding data previous to ion chromatogram extraction. This RP peak was found in an extracted ion chromatogram from profile mode data of a 5 µmol L^{-1} standard mix injection at minute 2.5, although the target analyte, inositol (monoisotopic mass 180.0633 g mol^{-1}), would not be expected to show any retention on reversed phase. Looking at the mass spectrum at minute 2.46 (indicated by the small red circle in **Figure 8**, left window), it is easy to see that the centroid of the generated mass-range peak lies, in reality, outside of the allowed mass extraction window. The mass-range peak is only overlapping with the set window by chance and the peak depicted in the extracted ion chromatogram is most likely not the analyte of interest. In this case, the mass range peak is probably derived from theobromine (monoisotopic mass 180.0647 g mol^{-1}), which has a very similar m/z and expectedly good retention on RP. In a centroided data file, this mass-range peak would have been correctly excluded.

Once centroided, data treatment was continued in Skyline. Positive and negative mode data were evaluated separately. It would, in theory, be possible to investigate positive and negative mode data simultaneously in one Skyline file by loading the according adduct m/z for ion chromatogram extraction at the same time. However, simultaneous quantitative data treatment currently founders on Skyline's disability to calculate two calibration curves (one for positive and one for negative mode data) for the same analyte. Hence, the following strategy was pursued for both polarities separately: A list with compound names, sum formulas and adducts was loaded (see Supplement, Table S1). In the used Skyline version, this list was termed "transition list" and the settings "transition settings", although only full MS data were investigated. The according m/z for ion chromatogram extraction were calculated by Skyline automatically. For targeted analysis of centroided full HRMS data, the

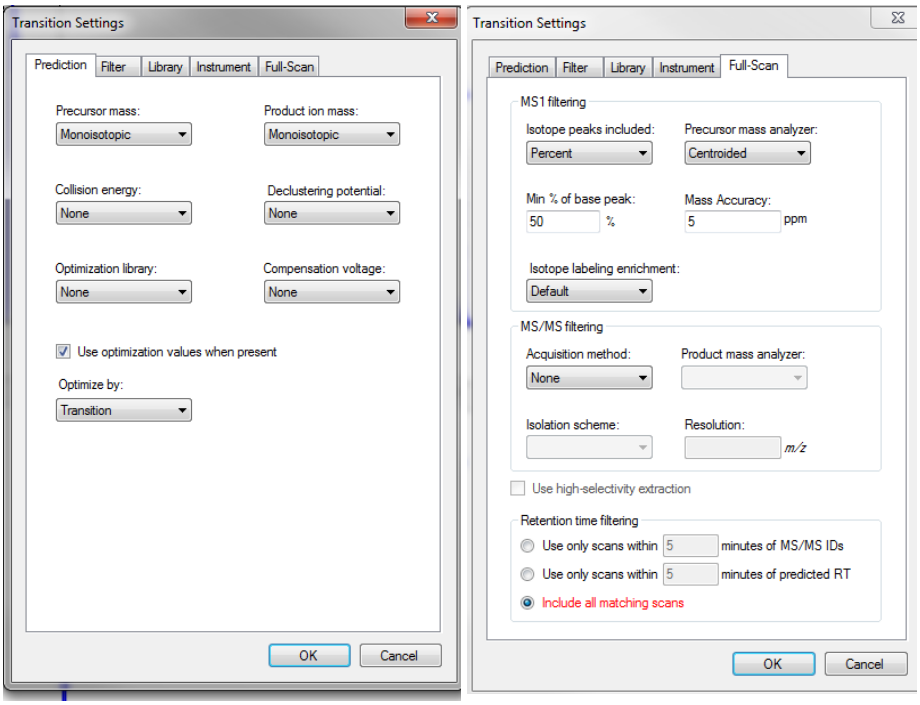

Figure 9 Settings for ion chromatogram extraction in Skyline. m/z for ion chromatogram extraction were based on monoisotopic masses calculated from the adducts of the loaded sum formulas ("Precursor mass: monoisotopic"). Centroids ("Precursor mass analyzer: Centroided") were extracted with a mass deviation window of 5 ppm ("Mass accuracy: 5 ppm"). Only the most abundant isotopologue was investigated, less abundant isotopologues were excluded by setting intensity threshold to 50% oft he base peak ("Min % of base peak: 50%"). Only full MS data were analyzed. Figure exported from Skyline.

options in the "Prediction" tab and the "Full-Scan" tab were set as shown in **Figure 9**. The other tabs were left with default settings.

Targeted compound identification was conducted by accurate mass extraction and retention time comparison with authentic standard compounds. Chromatographic resolution and retention time analysis are valuable for producing orthogonal identity information and essential for the discrimination of isomers. The authentic reference standards employed for retention time comparison were combined to a multi-component mixture comprising 148 endogenous metabolites and 15 xenobiotics, most of which were drug substances or their metabolites. Indeed, this mixture contained several isomers (3'-AMP and 5'-AMP, betaine and valine, glutamic acid and N-acetylserine, leucine and isoleucine, ribose-5-phosphate and ribulose-5-phosphate, sarcosine and alanine, ATP and dGTP, citric acid and isocitric acid, homoserine and threonine; fructose, galactose, glucose, mannose and inositol; fructose-6-phosphate, glucose-1-phosphate and glucose-6-phosphate; fructose-1,6-bisphosphate and glucose-1,6-bisphosphate). The accurate masses of isomers are identical and their MS1 mass traces are extracted in the same ion chromatogram. Isomer identities could therefore not be derived from the multi-component standard mixture alone. Retention time assignment could eventually be achieved by comparison with archive data where isomer-free standard mixtures had been measured under similar chromatographic conditions (i.e. same column, eluents and gradient, but not in dual

setup). Chromatographic separation in the dual setup was, of course, a prerequisite for this identification strategy. See chapter 4.2.1 (page 36) for details on isomer separation.

After the compound identities had been thoroughly assigned by retention time comparison with authentic standards, it was necessary to ensure that the peak of choice was integrated in each data file independent of Skyline's peak choosing algorithm. This is important because the algorithm is easily mislead in samples with pronounced baseline noise and insufficient signal intensities. The software tends to prefer an intense noise signal to a weak or missing compound signal and this would lead to incorrect automatic integrations. Isomers pose a similar challenge to the peak choosing abilities of Skyline. The strategy was therefore to manually set the integration boundaries in one representative data file and export these integration boundaries (start and end time of the chromatographic integration window) to a .csv file. Using an Excel macro programmed in-house by my colleague Yasin El Abiead, a file was created that assigned the integration boundaries for each compound to each data file. After loading the rest of the data files to Skyline, this peak boundary list was re-imported. The result was that the integration boundaries were set identically in all files.

In the next step, absolute compound quantification was pursued. Due to the eluent flow configuration of the presented dual setup, each compound adduct produced, in general, two peaks in the extracted ion chromatogram: one eluting from the HILIC column, and one eluting from the RP column. Considering the opposite chemistries of these two columns, we expected that each compound would show good retention on one or the other column. The "better" peak was chosen for integration and quantification. The decisive factor in this peak quality assessment was the extent of retention and chromatographic separation. By this procedure it was mostly the HILIC peaks picked for endogenous metabolites and mostly the RP peaks picked for the exogenous substances.

Calibration curves were calculated by Skyline after defining the calibrator files. Calibration curve quality was assessed manually: Calibration levels that showed peaks of insufficient intensity (less than 15 consecutive data points above baseline) were discarded as well as calibration levels that exceeded the lower or upper end of the linear range. Adequacy of the peak intensities and linearity were estimated by visual inspection and verified via recovery of the nominal calibrator concentrations and R^2 of the calibration curve.

4 Validation

4.1 Comparison validation plan vs. provided data set

Several institutions well recognized in the medicinal and pharmaceutical regulatory sector – the ICH (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use), EMA (European Medicines Agency) and U.S. FDA (United Stated Food and Drug Administration) among them – formulate a definition for analytical method validation. These definitions are largely harmonized and include the following key points: To call an analytical method validated, the user has to prove that the method (1) *continuously* fulfils (2) *all specifications* and requirements deemed necessary for a specific analysis. These requirements are derived from the (3*) specific application purpose* of the method and the specifications are defined accordingly. Since an absolute, unequivocal proof is, of course, not possible, the guidelines request a (4) *high level of confidence* to be provided by conducting (5) *systematic experiments*. By all definitions, validation roots in the thorough definition of the application purpose and the requirements to be fulfilled by an analytical method in order to obtain conclusive and adequately reliable results. The requirements are translated into specifications and numerical acceptance criteria and put down in a validation plan together with the experimental assessment strategy. The actual process of validation comprises conducting the assessment experiments, comparing the results with the specifications and evaluating whether or not the method meets the requirements. If it does, the method is ready for use. If it does not, a risk assessment is put in place: Can the method arguably be used with restrictions or does is have to be revised?

The goal of the proposed project was to validate a novel analytical method for metabolomics and exposomics according to U.S. FDA's bioanalytical method validation guideline. This endeavor failed. One of the main reasons for this is that the application purpose was not defined specifically in advance. Defining the necessary requirements for this very general application case was overly difficult and so was setting up an appropriate experimental strategy. The validation plan could hence not be completed as intended. In addition, the provisions of U.S. FDA and EMA for method validation gradually turned out to not be ideally suited for the present state of development of the analytical method. Likewise, the provided data set was obtained to characterize the technical features of the method and not to serve as a data basis for validation. Calibration did not satisfy the guideline's provisions since ten calibrators spread across four powers of ten of analyte concentration could, for many compounds, not guarantee that at least six calibrators in the linear range met the acceptance criteria. Neither did quality control and QC concentration levels (LLOQ, low, mid and high QC for each compound). One data set obtained in a single experimental run would not fulfill the criteria in any case because the provisions request to prove a stable performance over a longer timespan (days, weeks). Matrix effect was not sufficiently assessable since calibrators and QCs were prepared in pure solvent.

Inspired by the study of regulatory validation provisions, the available data were netvertheless examined to test the presented method regarding its suggested application for metabolomics and exposomics research.

4.2 Results of data evaluation

4.2.1 Qualitative results

103 of 163 standard compounds were detected by targeted evaluation of the QC data (1 µmol L^{-1} standard mixture, eight replicate injections) obtained in positive ionization mode. For 54 of these, an internal standard signal was present. **Figure 10** compares the amount of recovered metabolites sorted by compound class.

Chromatographic separation and column choice

In general, endogenous metabolites showed better retention on the HILIC column. For differentiated analysis, the analytes were grouped into six compound classes – amino acid derivatives, purine derivatives, pyrimidine derivatives, carbohydrates and derivatives, "other metabolites", which comprised endogenous metabolites such as small organic acids and "exposome", which contained non-endogenous compounds such as drug substances. Investigated compounds and their respective compound classes are listed in Table S1 of the Supplement.

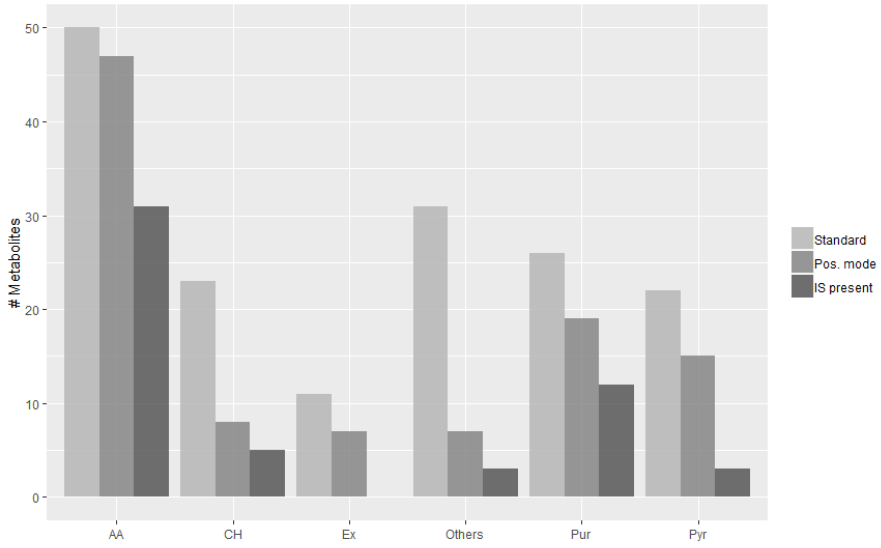

Figure 10 Number of compounds per compound class. "Standard": contained in the standard mixture, "Pos. mode": found in positive ionization mode, "IS present": found in pos. mode and compound-specific internal standard present. AA = amino acids and derivatives, CH = carbohydrates, Ex = exposomics/xenobiotics, Pur = purine derivatives, Pyr = pyrimidine derivatives.

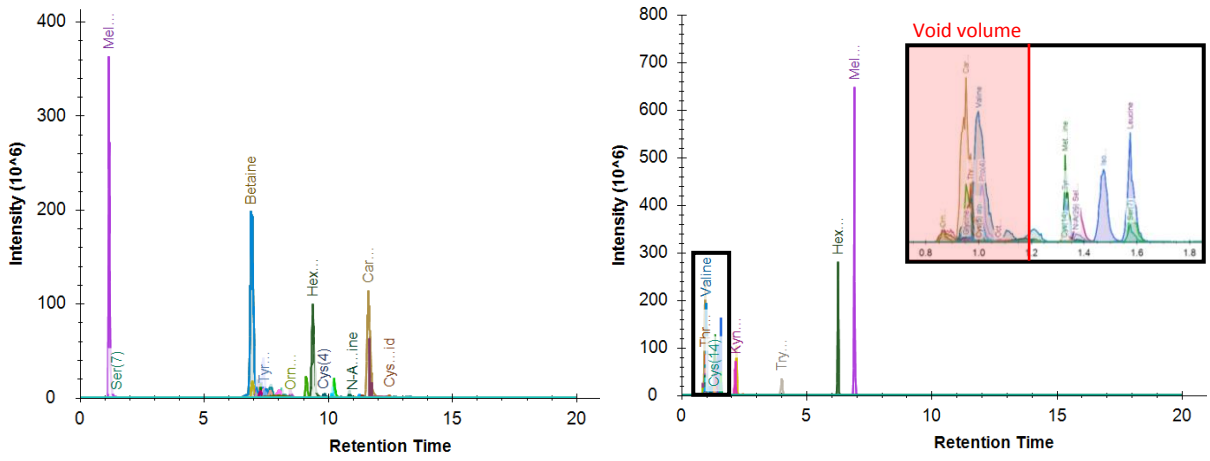

Figure 11 Extracted ion chromatograms of amino acid derivatives. Left: HILIC only, right: RP only; 5 µmol L^{-1} standard, positive ionization mode. For the majority of amino acid derivatives, separation was better on the HILIC column. Under RP elution conditions, many amino acid derivatives elute in the void volume (zoom, red window). Graphs exported from Skyline.

Figure 11 shows a comparison of extracted ion chromatograms of the amino acid derivatives obtained by 1D HILIC and RP analysis of a 5 µmol L^{-1} standard mixture in positive ionization mode over a run time of 20 min. The HILIC chromatogram is depicted on the left, the RP chromatogram on the right hand side. With the HILIC column, the available separation space was exploited more efficiently than with the RP column. Only melatonin eluted in the void volume. In contrast, melatonin, tryptophan, kynurenine and phenylalanine (here hidden behind the kynurenine peak) as well as hexanoylcarnitine showed satisfying retention on the RP column. Selenomethionine, methionine, cysteine, serine, leucine and isoleucine showed acceptable retention under RP conditions. The latter two were even baseline separated, in contrast to the separation on HILIC. Many of the more hydrophilic amino acids, however, eluted in the estimated void volume of the column (< 1.2 min).

Figure 12 and **Figure 13** show the same for analysis for the purine and pyrimidine derivatives (5 µmol L^{-1} standard, positive mode; HILIC on the left, RP on the right). The separation of the purine derivatives profited from the better retention on the HILIC column compared to the RP column (**Figure 12**). The pyrimidine derivatives showed better retention on the HILIC column and similar ionization efficiencies in both polarity modes. The HILIC separation was by no means ideal, though: thymine, thymidine, uracil, uridine, 2'-deoxyuridine, 5-methyluracil and N4-acetylcytidine showed very broad and sometimes split peak shapes (**Figure 13**, left zoom). Additionally, the respective HILIC and RP peaks of thymine and thymidine overlapped in the dual setup. The peak shape of the pyrimidine-based nucleoside-monophosphates, on the other hand, was quite satisfying (**Figure 13**, right zoom). Nucleoside-triphosphates were neither visible in positive, nor in negative mode.

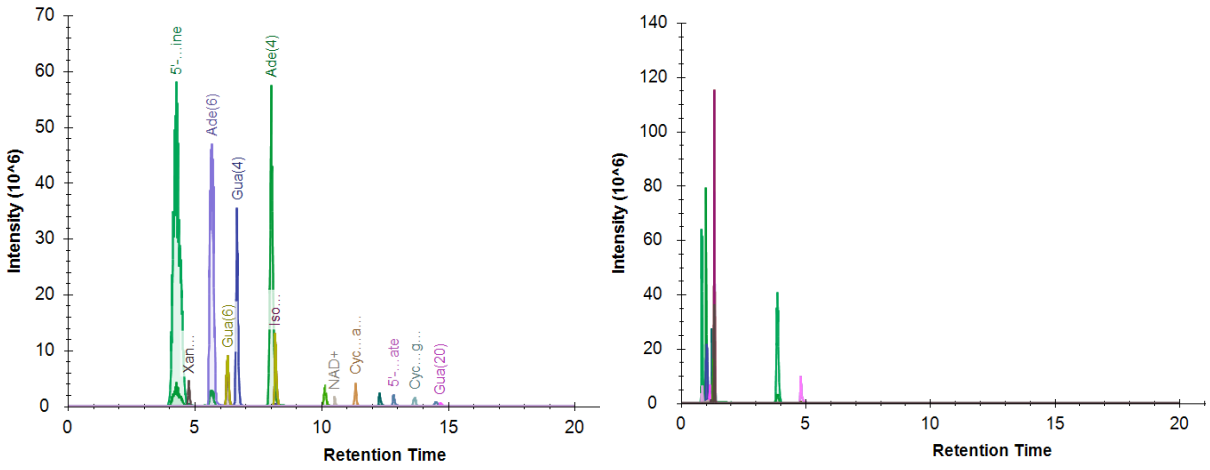

Figure 12 Extracted ion chromatograms of purine derivatives. Left: 1D HILIC, right: 1D RP. 5 µmol L^{-1} standard, positive ionization mode. Graphs exported from Skyline.

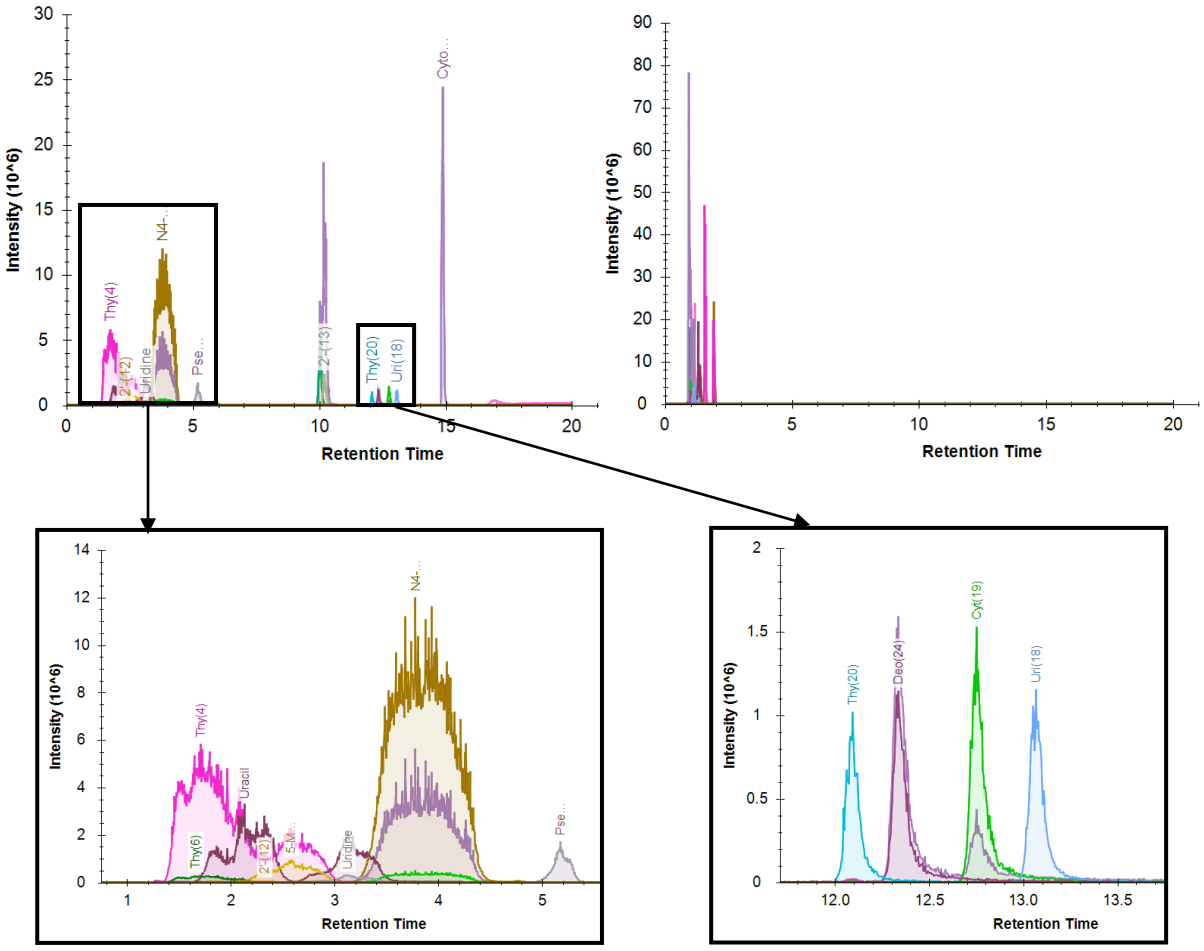

Figure 13 Extracted ion chromatograms of pyrimidine derivatives. Left: 1D HILIC, right: 1D RP, left zoom: nucleosides and nucleobases, right zoom: nucleotside-monophosphates. 5 µmol L^{-1} standard, positive ionization mode. Graphs exported from Skyline.

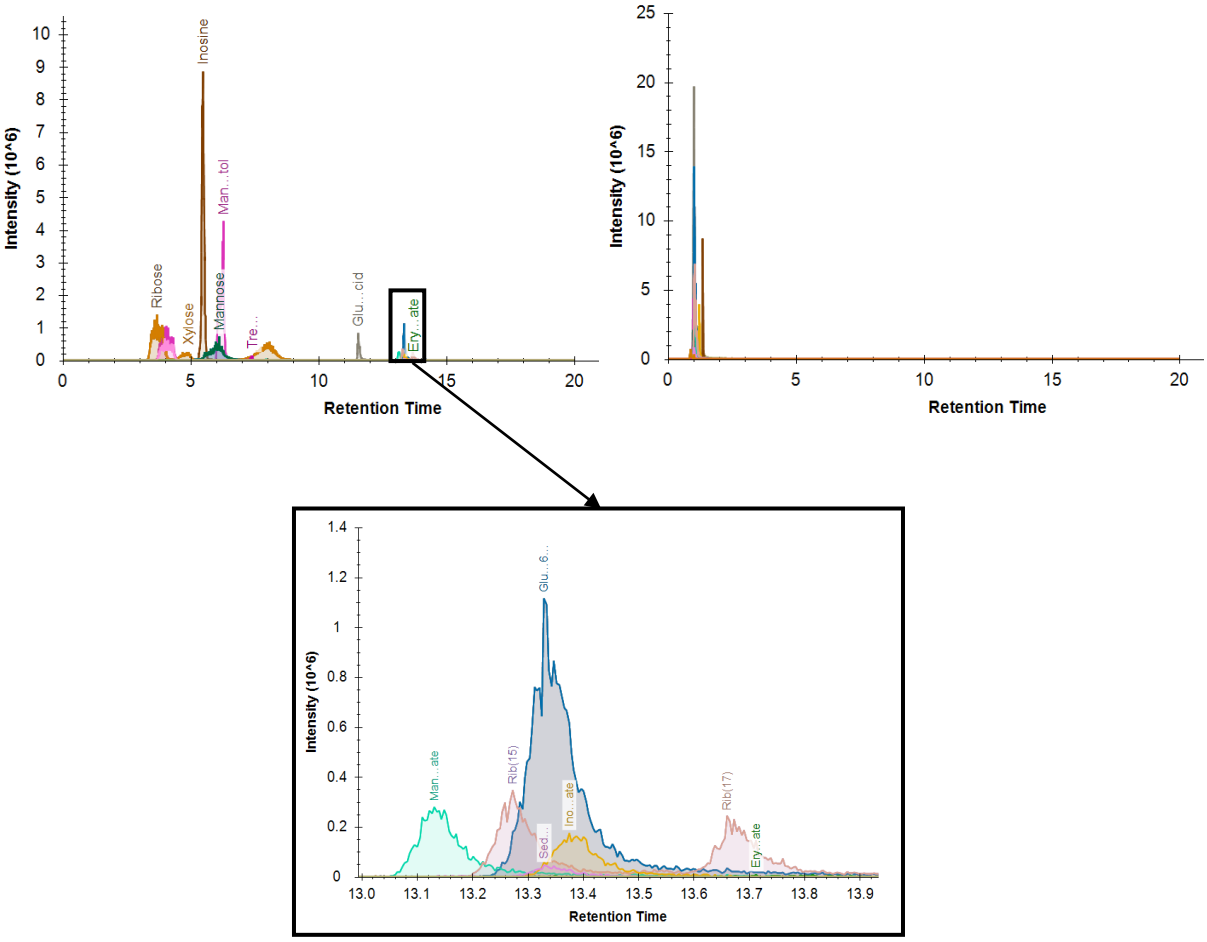

Figure 14 Extracted ion chromatograms of carbohydrate derivatives. Left: 1D HILIC, right: 1D RP, zoom: sugar-monophosphates. 5 µmol L^{-1} standard, negative ionization mode. All hexose isomers co-elute on HILIC (left, only mannose is labeled).

Figure 14 depicts the extracted ion chromatograms for the carbohydrate derivatives contained in the standard mixture and measured in negative ionization mode (c = 5 µmol L^{-1}, HILIC on the left, RP on the right). Monosaccharides were better ionized in negative ionization mode and showed practically no retention on the RP column (all eluted < 1.5 minutes). Retention on the HILIC column was quite satisfying, although the hexose isomers fructose, galactose, glucose, inositol and mannose could not be separated. Sugar-monophosphates were ionized equally well in positive mode and negative mode. They were also practically not retained on the RP column. On the HILIC column they showed pronounced retention, but while ribose- and ribulose-5-phosphate were baseline separated, fructose-6-phosphate, glucose-1-phosphate and glucose-6-phosphate co-eluted (**Figure 14**, zoom; only glucose-6-phosphate is labeled).

The small organic acids, which accounted for the majority of the "other metabolites" group, were better ionized in negative mode. **Figure 15** shows the extracted ion chromatograms acquired in negative mode (c = 5 µmol L^{-1}). For ease of visualization, the figure shows the extracted ion chromatograms obtained in dual setup. All compounds from the RP column eluted before minute 5.7, whereas the usable signals (i.e. other than noise) from the HILIC column appeared after minute 5.7. The biotin peaks – one from RP, one from HILIC – indicate the border. Chromatographic conditions of the HILIC separation were not ideal for many of the small organic acids. The signals were either weak with a lot of background noise, split, or not visible at all. However, mevalonic acid, lactic acid, dihydroxyisovaleric acid, 3-methyl-2-oxovaleric acid, alpha-ketoisovaleric acid and pyruvic acid were found to have satisfying retention and peak shape on the HILIC column (**Figure 15**, right zoom). Interestingly, for the other small organic acids, the RP separation seemed to be a bit more favorable in terms of peak shape. Chromatographic separation was practically not given, though, and many of the compounds eluted in the void volume (**Figure 15**, left zoom). In general, identification of the small organic acids was ambiguous and reliable quantification hardly possible.

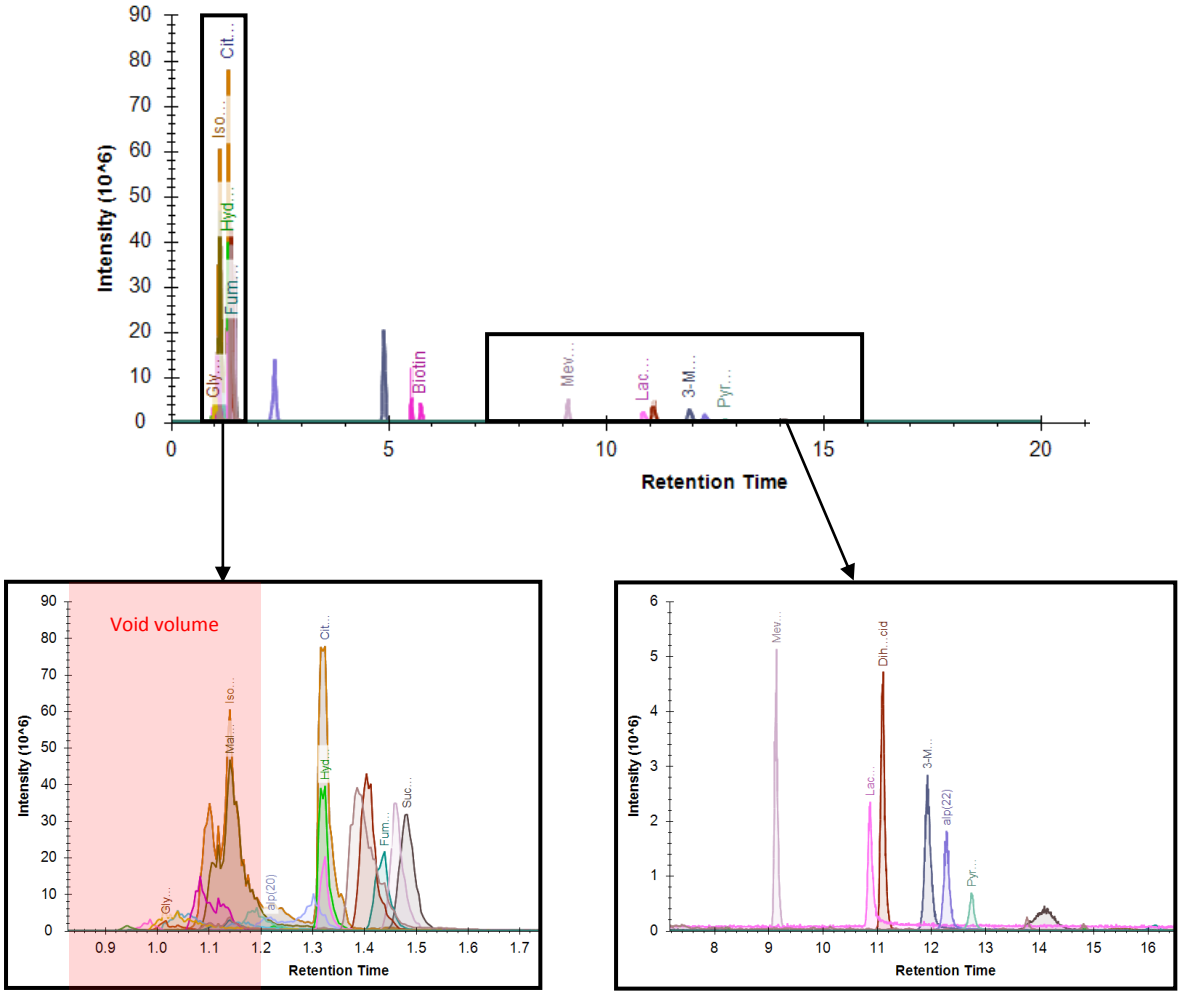

Figure 15 Extracted ion chromatograms of "other" metabolites (small organic acids etc.). Graph obtained from 2D setup, zoom left: peaks that eluted from RP, zoom right: peaks that eluted from HILIC. 5 µmol L^{-1} standard, negative ionization mode. Graphs exported from Skyline.

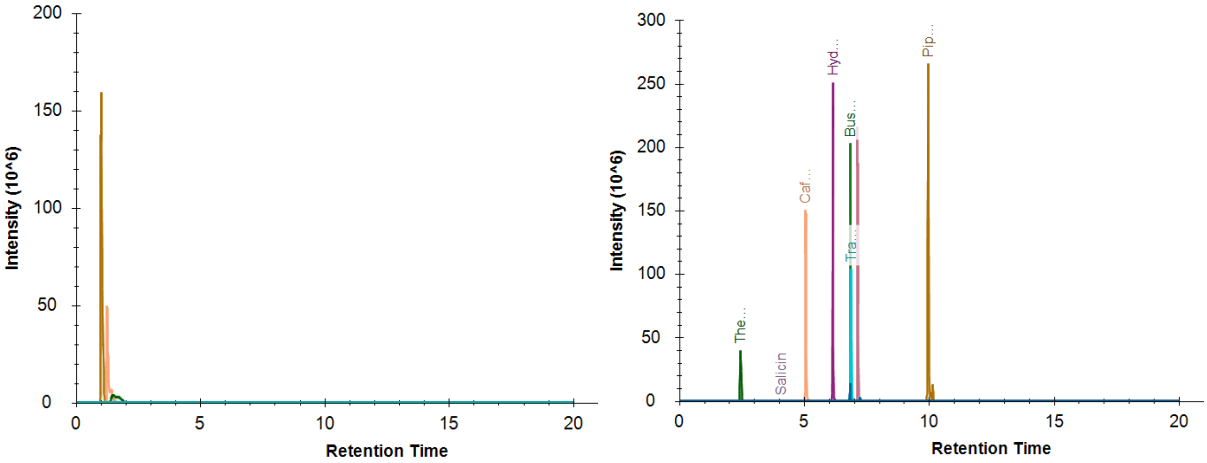

Figure 16 Extracted ion chromatograms of xenobiotics. Left: 1D HILIC, right: 1D RP. 5 µmol L^{-1} standard, positive ionization mode. Graphs exported from Skyline.

Figure 16 depicts the analysis of the "exposomic" compounds contained in the standard mixture (c = 5 µmol L^{-1}, positive mode; HILIC on the left, RP on the right). While the pharmaceuticals displayed insufficient or no retention at all on the HILIC column, they were perfectly retained and separated under RP elution conditions. The targeted compounds buspirone, caffeine, hydroxybupropione, piperine, quetiapine, theobromine and trazodone were visible in positive ionization mode. Acetylsalicylic acid, salicylic acid and salicine were visible in negative mode with equally good retention and peak shape (negative not depicted; see Supplement Table S3). Like many small molecule pharmaceuticals, all the compounds had a phenolic moiety.

To sum up, a better metabolite separation was achieved under HILIC conditions. Accordingly, the HILIC peak was preferably integrated for metabolite quantification. Better separation on the HILIC column was, however, regularly associated with broader and less intense peaks with a jagged outline when compared to the RP separation. Broad and jagged peak shape might reduce the reliability of the area count and introduce a pronounced inter-replicate area variability especially for compounds with already low signal intensity. This is why, for some metabolites, the RP peak was chosen in spite of the better chromatographic separation on the HILIC column. All investigated pharmaceuticals showed better retention, peak shape and separation under RP chromatography conditions and the RP peak was therefore chosen for xenobiotic quantification.

Isomer separation

Table 2 states the isomeric metabolites contained in the standard mixture and whether or not they were separated on the HILIC column. The order of elution is indicated if possible. For most compounds, separation on the RP column was worse compared to HILIC or compounds had no retention at all.

Table 2 Separation of isomeric metabolites.

Isomers	Separation status and elution order on HILIC column
3‘-AMP/5‘-AMP	Baseline separated (5'-AMP – 3'-AMP)
Betaine/valine	Baseline separated (betaine – valine)
Glutamic acid/N-acetylserine	Almost separated (glutamic acid – N-acetylserine)
Leucine/isoleucine	Almost separated (leucine – isoleucine) (RP: baseline separated, isoleucine – leucine)
Ribose-5-phosphate/ribulose-5-phosphate	Baseline separated (ribose-5-phosphate – ribulose-5-phosphate)
Sarcosine/alanine	Baseline separated (sarcosine – alanine)
ATP/dGTP	Peaks barely visible
Citric acid/isocitric acid	Peaks not visible (RP separation suboptimal)
Homoserine/threonine	Coelute (threonine – homoserine)
Hexoses (fructose/galactose/glucose/inositol/mannose)	Coelute
Hexose-phosphates (fructose-6-phosphate/ glucose-1-phosphate/glucose-6-phosphate)	Coelute

HILIC retention time analysis

A common concern regarding the HILIC technique is that retention times are not stable, especially in comparison with an RP column. The provided data set allowed to check for intra-experiment retention time drifts by analysis of eight QC injections (c = 1 µmol L^{-1}) evenly distributed across a measuring time of approximately 35 hours. Compounds were separated in dual setup and measured in positive ionization mode. The HILIC peaks of 73 compounds were integrated in each of the eight QC injection replicates and the relative standard deviation (RSD [%]) of the retention time was calculated for each compound. A high RSD indicates retention time instability, e.g. retention time drift during the measurement sequence. **Figure 17** shows a boxplot of the retention time RSDs for the 73 compounds that showed retention on HILIC and were found in positive mode. Only four compounds exhibited a retention time RSD (N=8) greater 1.5 %: uracil (4.5 %), N4-acetylcytidine (3.8 %), uridine (3.6 %) and thymine (3.1 %). If the elevated retention time RSD were caused by retention time drift, we would expect that the retention time move to one direction progressively with analysis time. The delta of the retention times should steadily increase or decrease compared to the first injection. To assess for this trend, the retention time of each QC injection was compared to the first QC injection (time 00:00) and the difference was calculated (**Figure 18**). The plot shows a random deviation of retention

times as opposed to a systematic trend. This suggests that the elevated retention time RSD is, for these substances, not due to a retention time drift.

A plausible explanation for the observed retention time instability can be obtained by looking at the peak shapes of the compounds in question (page 32, **Figure 13**, left zoom): the peaks are very broad with a jagged outline. Working with default parameters, Skyline seems to assign the retention time to the intensity maximum of a peak rather than, for example, to the mean of its area or its peak basis. Faced with jagged and flat peaks it tends to produce fictitious retention time variation. This theory is supported by the low retention time RSD of compounds the peaks of which showed a steeper outline like cytidine monophosphate (0.05 %), deoxycytidine monophosphate (0.10 %), thymidine monophosphate (0.07 %) and uridine monophosphate (0.08 %) (page 32, **Figure 13**, right zoom). It is also interesting to note that the four compounds with markedly high retention time RSD elute at low retention times, maybe even partly in the void volume. Bad peak shape and high retention time RSDs can both be regarded as a symptom of insufficient retention on the HILIC column under the elution conditions applied.

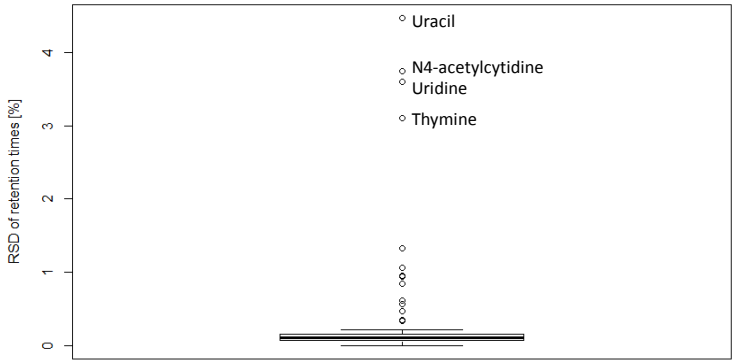

Figure 17 Retention time RSDs of 73 compounds found on HILIC. RSDs calculated from eight QC injections (= 1 µmol L^{-1} standard mixture) distributed over ~35 hours. Positive ionization mode.

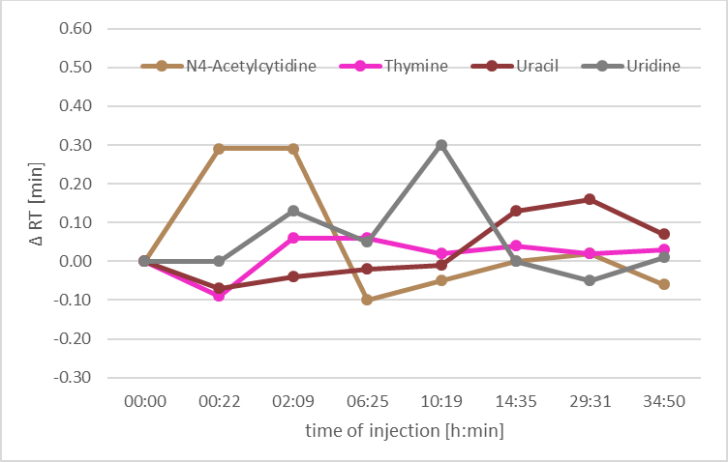

Figure 18 Inter-replicate-injection retention time comparison for retention time RSD outliers. Eight replicate injections of 1 µmol L^{-1} standard distributed across ~35 hours measurement time. Retention times of the later injections are normalized to the retention time of the first (time 00:00) injection. The absence of a clear trend indicates that the high RSD is not due to retention time drift.

Retention time RSDs of the exposomics substances, representing the reversed phase elution, were all below 1.5 %. Summarizing, it can be stated that there was no considerable retention time drift in the dual setup within almost 35 hours of measuring time. The available data did not, however, allow an inter-experiment or long-time evaluation of retention time stability.

Instrument sensitivity and sequence dependent intensity variation

The dual-chromatography data were analyzed for sensitivity loss throughout the measurement sequence. Compound areas obtained in positive ionization mode from eight QC injections were plotted exemplarily against injection time. In **Figure 19**, the signal area of each QC injection is normalized to the mean compound area and plotted against measurement time (103 compounds, found either on HILIC or RP in positive mode). Two things are noteworthy: There is quite a high random variability of signal intensity between the injections. We can, however, still see a trend towards lower intensity as measurement time proceeds. For most of the compounds, the area RSDs of the replicates injected within 35 hours ranged between 10 % and 30 % (see boxplot **Figure 20**). Some compounds, indicated as outliers of the boxplot, had a higher RSD. Closer investigation revealed that the chromatographic signal of guanosine diphosphate was an assembly of spikes rather than a peak, inevitably resulting in a poorly reproducible area count. 4-Hydroxyproline evoked the impression that a second peak of lower intensity was co-eluting. Leucine was not completely separated from isoleucine. Phenylalanine, sarcosine, tryptophan, methionine and inosine, on the other hand, displayed quite desirable peak shape and the reason for their outstanding area variability could not be uncovered. **Figure 21** exemplifies the peak shapes of those compounds with the highest area RSD. The chromatographic analyte peak is represented by the red line, the internal standard signal by the purple line. The black lines parallel to the y-axis mark the integration boundaries.

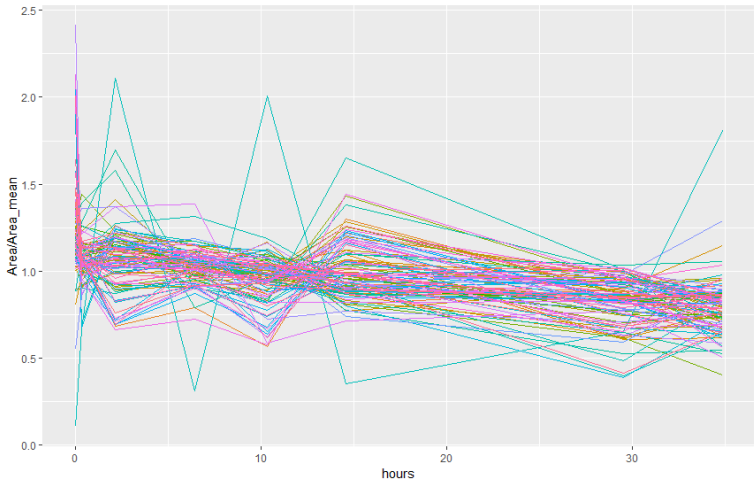

Figure 19 Normalized areas of 103 compounds. Eight QC injections (1 µmol L^{-1} standard) over ~35 hours. Areas are normalized to the respective mean area of the compound. Positive ionization mode.

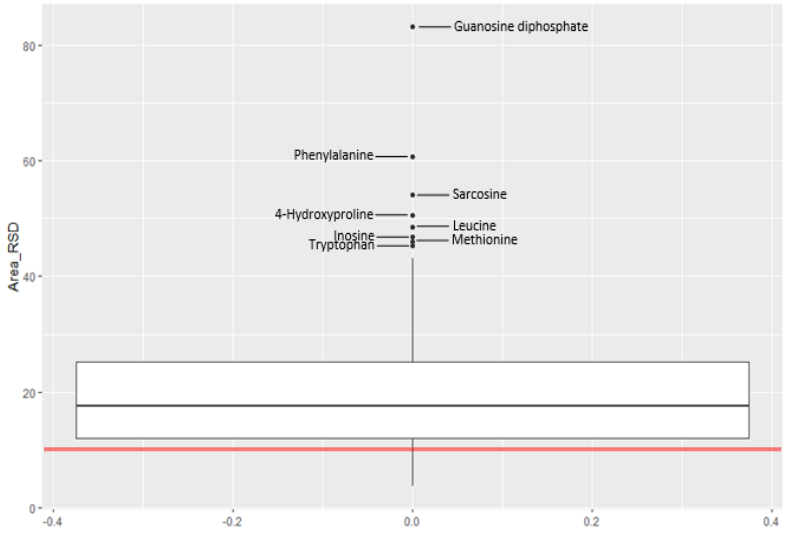

Figure 20 Area RSDs of 103 compounds. Eight QC injections over ~35 hours, positive ionization mode. The red line indicates 10 % RSD.

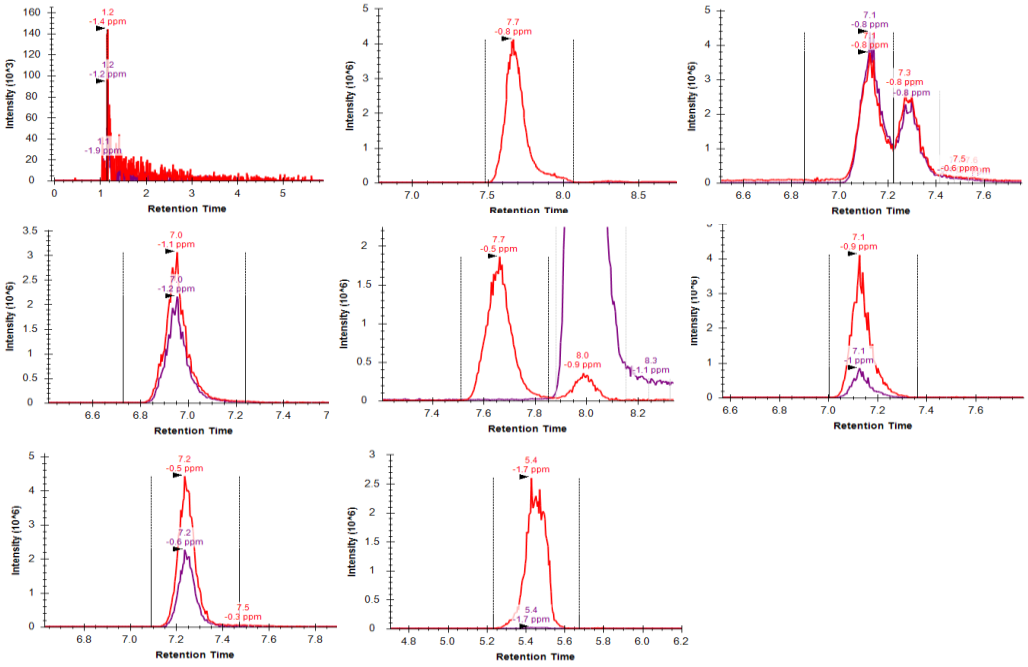

Figure 21 Peak shapes of compounds that displayed extraordinary area RSD. Row one: guanosine diphosphate, 4-hydroxyproline, leucine; row two: phenylalanine, sarcosine, tryptophan; row three: methionine and inosine (from left to right). Red line: compound signal, purple line: IS signal, black lines parallel to y-axis: integration boundaries. Ion chromatograms extracted from a QC file (1 µmol L^{-1} standard), positive ionization mode

For 56 compounds an internal standard signal was present in positive mode. **Figure 22** gives their area-ratios (compound area divided by internal standard area, "ratio to heavy"), normalized to the mean area-ratio of the compound and plotted against measurement time. To ensure full comparability of the visual impressions, **Figure 23** plots the non-normalized areas of the (reduced) set of compounds shown in **Figure 22**. For many compounds, the implementation of the internal standard could level out sequence dependent as well as random variation of signal intensities. Most of the compounds where an internal standard was present profited from building the "ratio to heavy" in terms of signal intensity RSD (N=8 QC injections). However, for cysteine and 3'-adenosine-monophosphate, applying the internal standard led to an increase in RSD (**Figure 24**).

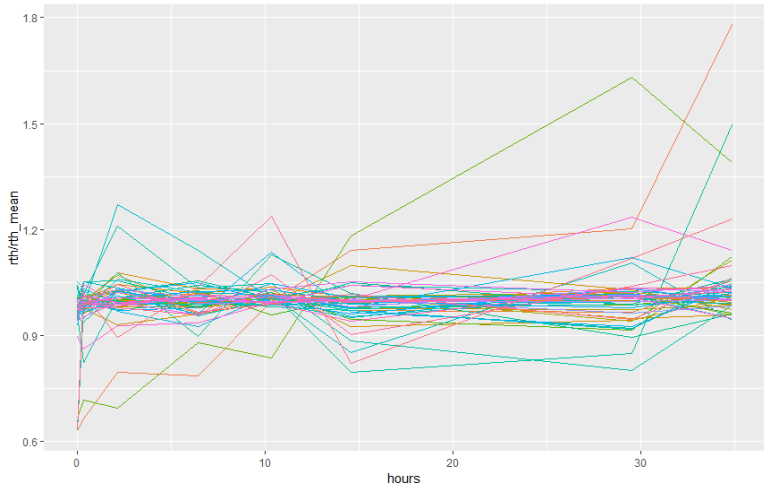

Figure 22 Normalized "ratios to heavy" of 56 compounds. Eight QC injections (1 µmol L^{-1} standard) over ~35 hours. Each replicate area is normalized to the internal standard and the respective mean reatio-to-heavy of the compound. Positive ionization mode.

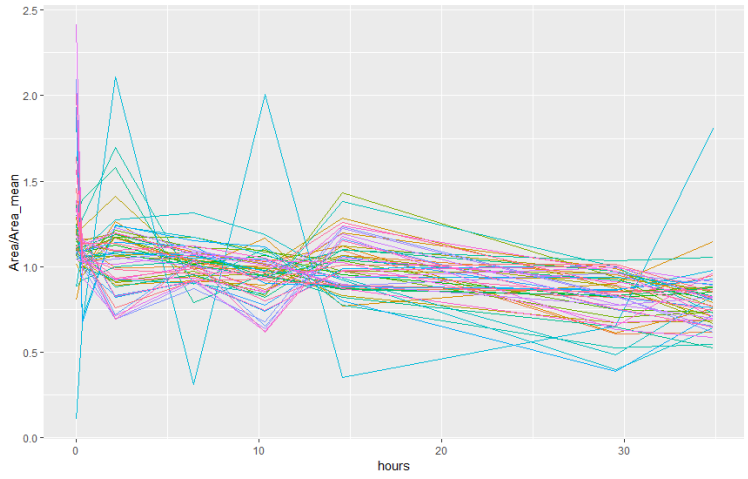

Figure 23 Normalized areas of 56 compounds. Eight QC injections (1 µmol L^{-1} standard) over ~35 hours. Only compounds with IS are depicted. Areas are normalized to the respective mean area of the compound. Positive ionization mode.

The boxplots in **Figure 25** visualize the beneficial impact of internal standardization on intra-experiment intensity variability per compound class. Only those compounds for which an internal standard was present are depicted and only positive mode data were analyzed. The boxplot on the left hand side shows RSDs of the non-normalized areas, the one on the right the RSDs after normalization to the respective internal standard. For ease of comparison, the 10 % marks are indicate by a red line. Some compounds appeared to have a ratio-to-heavy RSD greater than 50 %. In all of these cases, the internal standard turned out to be an assembly of random signals and spikes that were erroneously kept during the manual integration process. These compounds were excluded from the boxplots.

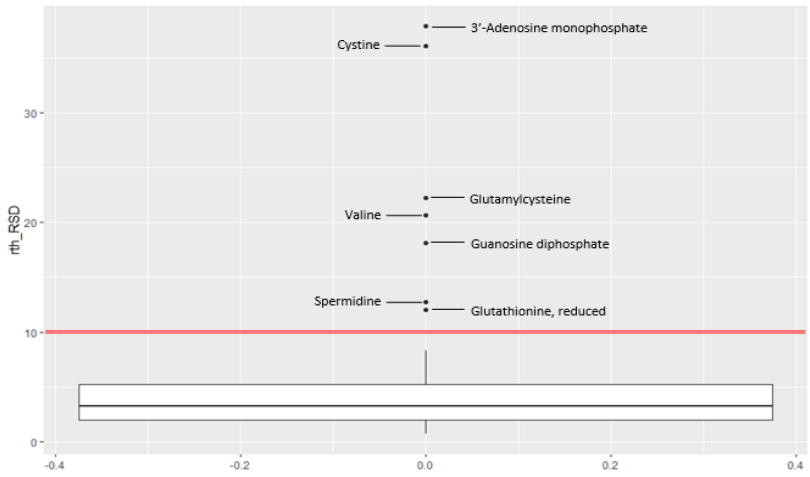

Figure 24 "Ratio-to-heavy" RSDs of 56 compounds for 8 QC injections over ~35 hours. Eight QC injections over ~35 hours, positive ionization mode. The red line indicates 10 % RSD.

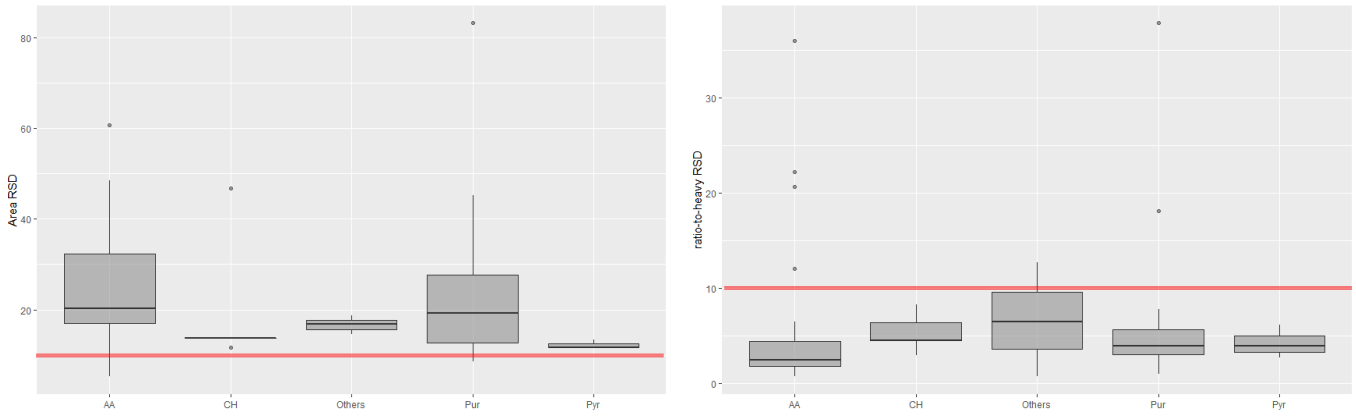

Figure 25 Impact of internal standardization on the signal intensity variability. Left: RSDs of non-standardized areas per compound class, right: RSDs of ratio-to-heavy per compound class. RSDs were calculated from eight replicate QC injections over ~35 hours. Only those 56 compounds for which an IS was present were included in the calculations. The red line indicates the 10 % mark.

Non-targeted data evaluation

Although targeted data evaluation was the goal of the validation, it is also interesting to illuminate the method's potential for untargeted data evaluation. Since its capability for metabolite detection was abundantly tested during targeted data evaluation, the following report will focus xenobiotics only. The investigated full MS files were recorded using the method described in chapter 3.1.2 (page 18). MS^2 data were recorded using an adapted method (runtime 20 minutes, positive and negative scans separately) that alternates full MS and fragmentation scans. The parameters for full MS scans were: scan range 60 – 900 m/z, resolution 60 000, AGC target 10^6, max. injection time 100 ms. The parameters for data dependent (top 10) MS^2 fragmentation scans were: scan range 60 – 900 m/z, resolution 30 000, AGC target $10*5^5$, max. injection time 60 ms, isolation window 1 m/z, normalized collision energy 30 eV, including the following fragmentation settings: min. AGC target $1.00*10^3$, intensity threshold $1.7*10^4$, apex trigger 3 – 8 s, charge exclusion 2 – 8 and > 8, dynamic exclusion 8 s, "if idle pick others".

The data were analyzed using the proprietary software Compound Discoverer™ (version 3.0, Thermo Scientific™). Mass tolerances for compound grouping, composition prediction and mass list search were set to 5 ppm. Mass tolerance for assigning annotations was set to 3 ppm. The database search (node "Search ChemSpider") comprised The Human Metabolome Database (HMDB, www.hmdb.ca)[30], Kyoto Encyclopedia of Genes and Genomes (KEGG, www.genome.jp/kegg), NIH Clinical Collection and PubMed (www.ncbi.nlm.nih.gov/pubmed). An overview of the employed workflow is given in **Figure 26**.

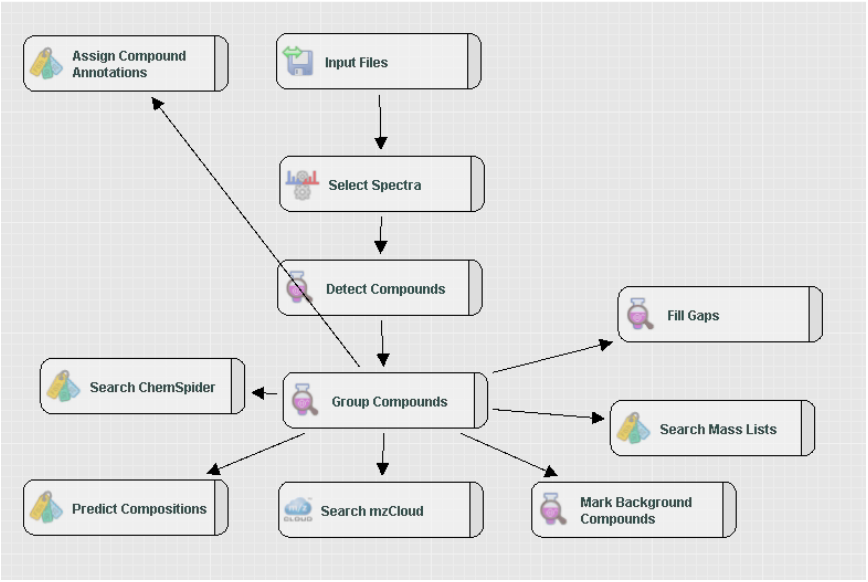

Figure 26 Workflow employed for non-targeted data evaluation in Compound Discoverer.

Table 3 shows drug substances and drug metabolites that were identified via non-targeted evaluation of five replicate injections of SRM 1950 measured via 2D setup in positive ionization mode. The reported compounds fulfil the requirements for level 2 identification according to the rating suggested by Schymanski et al. (2014)[31]: accurate mass and MS^2 spectrum match with library (mzCloud). The following quality parameters were ensured by filtering the data: no background compound, retention time greater 1.2 min (estimated void volume of RP column), inter-replicate CV of areas below 30 %. Caffeine, hydroxybupropione, piperine and theobromine were compared with authentic standards for retention time match and quantification during targeted data evaluation, resulting in level 1 identification for these compounds. Trazodone and quetiapine were additionally found via targeted data evaluation, but not via non-targeted data evaluation applying the quality filters described previously. Salicylic acid was found in negative mode data via targeted data evaluation. Supplementary information on description and typical blood concentrations was taken from HMDB and https://www.pharmazeutische-zeitung.de/ausgabe-292018/arzneimitteltherapie-nach-mass/. Typical blood concentrations are reported to get a rough idea about the concentrations to be expected in a human blood sample.

Table 3 Drug substances and drug metabolites found in SRM 1950 via non-targeted evaluation. Based on five replicate injections of SRM 1950, positive ionization mode. Data evaluated in Compound Discoverer 3.0. LOD and LLOQ are calculated for the presented 2D-chromatography setup as described in chapter 4.2.2. * Targeted RT comparison with authentic standard.

Compound	Description	Typical blood concentrations	Verified *
Allopurinol	Uricostatic	---	No
Benzoylecgonine	Cocaine metabolite	---	No
Caffeine	Psychostimulant	~ 26 - 129 µmol L^{-1}	Yes LOD: 0.06 µmol L^{-1} LOQ: 0.22 µmol L^{-1}
Ecgonine	Cocaine metabolite	---	No
Gabapentine	Anticonvulsant	~ 0.31 µmol L^{-1}	No
Hydroxybupropione	Metabolite of bupropione (antidepressant)	---	Yes LOD: 0.04 µmol L^{-1} LOQ: 0.15 µmol L^{-1}
3-Hydroxycotinine	Nicotine metabolite	~ 0.20 - 0.50 µmol L^{-1}	No
Nicotine	Psychostimulant, neurotoxin	~ 0.14 – 1.35 µmol L^{-1}	No
Paracetamol	Analgesic, antipyretic	~ 66 - 199 µmol L^{-1}	No
Paraxanthine	Caffeine metabolite	---	No
Piperine	e.g. from pepper	---	Yes LOD: 0.01 µmol L^{-1} LOQ: 0.03 µmol L^{-1}
Theobromine	e.g. from cocoa	~ 0.9 - 1.3 µmol L^{-1}	Yes LOD: 0.01 µmol L^{-1} LOQ: 0.05 µmol L^{-1}

4.2.2 Quantitative results

The results of targeted data evaluation are summarized in Table S2, Table S3 and Table S4 the Supplement. Table S2 gives a summary of the quantitative results (retention times and retention time standard deviations, linear range, QC recovery, estimated LOD and estimated LLOQ) for all compounds detected in the standard mixture in positive mode. Table S3 gives the same for negative mode data. All calibration curves were calculated via linear regression and weighted 1/x, giving more importance to the lower concentrated calibrators of the calibration curves. Linear range was evaluated visually and calibrators that differed more than 15 – 20 % from their nominal concentration were discarded.

Zero calibrator and blank evaluation revealed that many amino acid derivatives, especially carnitine, glutamine and glutamic acid, showed carryover in the blank injections after injection of a high concentrated standard. For cytidine monophosphate, cytosine, deoxycytidine monophosphate, guanosine monophosphate, reduced glutathionine, thymidine monophosphate, uridine monophosphate, adenosine monophosphate, deoxyadenosine monophosphate, glutamylcystein in positive mode and lactic acid, pyruvic acid and lysine in negative mode, there was a contamination peak visible in the zero calibrator. The blank injections, which were distributed evenly across the sequence, lacked a typical carryover pattern, i.e. a pronounced signal increase after injection of a high concentrated standard. The signal intensities were more or less the same in each blank. The source of the observed blank and zero calibrator contaminations – carryover during sample preparation or instrumental analysis, incomplete ^{13}C-labeling of the internal standard, contamination of HPLC vials and other consumables – would need to be investigated in further experiments. For quantification, all zero calibrators were included into the calibration curve, equalling blank correction.

Detection limits (LOD) and lower quantification limits (LLOQ) were estimated following Eurachem *The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics* (2014)[15]. The guide recommends calcultating LOD and LLOQ based on replicate injections of a blank sample or a low concentrated sample. However, neither is feasible in a metabolomics experiment: The target analytes are ubiquitously present in any biological sample; they are numerous and spread across a wide concentration range. For a situation like this, the guide suggests ten replicate measurements of reagent blanks spiked with a low concentration of analyte. The present data set provided five replicate measurements of a 0.5 µmol L^{-1} standard. The lower number of replicates undermines the statistical power of this calculation. In addition, “low concentration” is a relative magnitude that refers to the linear range of the analysis and is therefore linked to its sensitivity towards the respective target analyte. For compounds that are well above the LOD and LLOQ due to their high abundance in the biological sample (e.g. amino acids), it will not make so much difference

if LOD and LLOQ are really calculated at an adequately low concentration. A concentration of 0.5 µmol L^{-1} probably yields a useful overview of the method's sensitivity towards these analytes. However, for lower abundant compounds (e.g. nucleic bases), it might make a difference, since 0.5 µmol L^{-1} marks or even exceeds the upper end of the expectable concentration range. For these compounds, a 0.5 µmol L^{-1} standard hardly passes as a "low concentrated standard" and the calculated limits of detection and quantification are probably less adequate. This is even truer considering that 0.5 µmol L^{-1} refers to the concentration in the analytical sample, which is diluted 1:10 compared to the biological sample. In view of the limited data available, the reported LODs and LLOQs should be understood as rough estimates only. In Table S2 and Table S3, LOD was calculated as 3*s0 and LLOQ as 10*s0, where s0 is the concentration standard deviation of five replicate injections of a 0.5 µmol L^{-1} standard.

Table S4 of the Supplement gives the results of targeted evaluation of the SRM 1950 data. Specific issues are reported in the column "comments" and certified/reference values for SRM 1950 are stated for comparison. Depending on the chemical preferences of the analyte, either positive or negative mode data were chosen for quantification. Likewise, either the HILIC or the RP peak was chosen, whichever showed better chromatographic retention. The quantification strategy implied the use of a compound specific, fully ^{13}C-labelled internal standard. However, manual data assessment revealed that internal standardization with ^{13}C-labeled yeast extract is not a straightforward process. The adequacy of its use was therefore evaluated compound by compound. Reasons to refrain from internal standardization were: (1) The ^{13}C-labelled equivalent of the targeted compound is not present in the yeast extract. (2) The internal standard peaks seem too weak to be used for reliable ratio calculation. (3) The internal standard peaks are suppressed with increasing standard concentration up to a point where the internal standard peak intensity is not suitable for quantification anymore. (4) The internal standard peak intensity is unproportionally high compared to the analyte peak intensity. The decision whether a compound could be quantified with internal standardization or not was, for the sake of simplicity as well as reproducibility, based on the average recovery of the theoretical concentrations of the calibration standards as well as the 0.5 µmol L^{-1} and the 1.0 µmol L^{-1} QC standards (the closer to 100 %, the better). The decision was made widely regardless of the actual concentration in the sample. The concentration values given in Table S4 are based on five replicate injections of the same analytical sample spiked with internal standard and refer to the concentration in the biological sample, whereas LOD and LLOQ (calculation see above) refer to the concentration in the analytical sample.

Equation 1 Analytical sample concentration of second most abundant isotopologue. *c* (sec.isotopologue): Concentration of second most abundant isotopologue in analytical sample, *A* (sec.isotopologue): Peak area of second most abundant isotopologue, *d* (monoisotopic) and *k* (monoisotopic): Calibration curve intercept and slope, where the calibration curve is calculated by monoisotopic mass extraction.

$$c\,(\text{sec.isotopologue}) = \frac{A\,(\text{sec.isotopologue}) - d\,(\text{monoisotopic})}{k\,(\text{monoisotopic})}$$

Equation 2 Analytical sample concentration calculated via second most abundant isotopologue. *c*: Compound concentration in the analytical sample, *f* (sec.isotopologue): Abundance of second most abundant isopopologue relative to monoisotopic mass (for tryptophan e.g. 0.1218).

$$c = \frac{c\,(\text{sec.isotopologue})}{f\,(\text{sec.isotopologue})}$$

The concentration of some compounds exceeded the highest calibrator concentration (= upper limit of quantification, ULOQ). These compounds were quantified by use of the second most abundant isotopologue, if possible. As before, calibration curves were calculated from the ion chromatograms extracted by means of $[M+H]^+$ or $[M-H]^-$ adduct m/z, where M is the exact monoisotopic mass of the respective molecule. A second set of ion chromatograms was extracted by means of $[M'+H]^+$ or $[M'-H]^-$ adduct m/z, where M' is the exact mass of the second most abundant isotopologue. The area of the second most abundant isotopologue adduct was fitted on the calibration curve and the concentration in the analytical sample was calculated (**Equation 1**). The resulting value was divided by the average natural abundance of this isotopologue (**Equation 2**). The natural isotopologue distribution was calculated via the open-source online tool enviPat Web 2.4 (see **Figure 27**).[32]

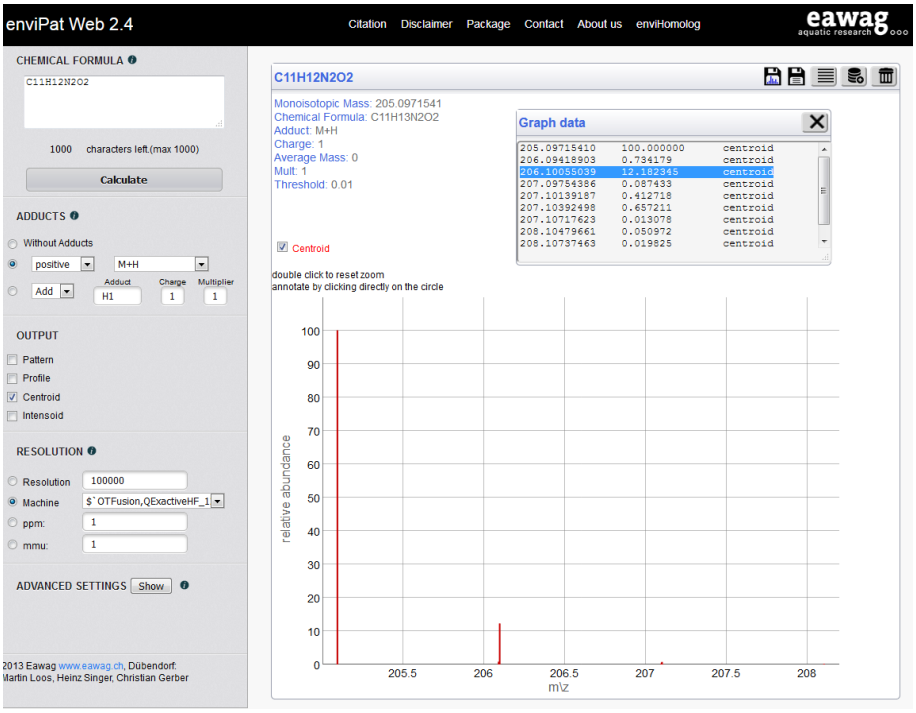

Figure 27 Graphic user interface of the enviPat web-application. The picture exemplarily shows a query for tryptophan (adduct $[M+H]^+$, instrument QExactive HF, resolution of 120 000 at m/z 200). For compounds with a concentration > ULOQ in the analytical sample, this software was used to calculate the abundances of the second most abundant isotopologues given in the window "Graph data". The abundance of the second most abundant isotopologue is marked in blue.

For an accuracy check with external reference, the concentration values obtained for SRM 1950 were compared to those stated in NIST's official certificate of analysis.[29] This was, however, only possible for very few compounds. **Figure 28** shows their recovery. The concentrations for the depicted compounds were calculated from the corresponding HILIC peaks because they showed better retention on HILIC compared to RP. To provide some level of confirmation for the non-certified compounds, their concentrations were compared to archive data obtained from the same sample but by use of a different dual LC-MS setup, if possible. **Figure 29** (page 48) shows the calculated concentrations obtained via 2D setup with Acclaim Trinity P2, pH=4.5 relative to those obtained in a dual setup with SeQuant ZIC pHILIC, pH=9.2 published by Schwaiger et al. (2018)[28]. The depicted compounds showed better retention on HILIC. A logarithmic concentration scale was chosen in order to fit all compounds in one chart. **Figure 30** describes the same information in a different form since the logarithmic scale in **Figure 29** might evoke a misleading impression of the actual value differences: Recovery of pHILIC value [%] = $c_{mean,trinity}$ / $c_{mean,pHILIC}$ * 100. Threonine and homoserine are not reported here because they co-elute under the conditions of the presented metabolomics-exposomics-setup. Leucine and isoleucine were summarized for comparison because they co-elute in the reference setup.

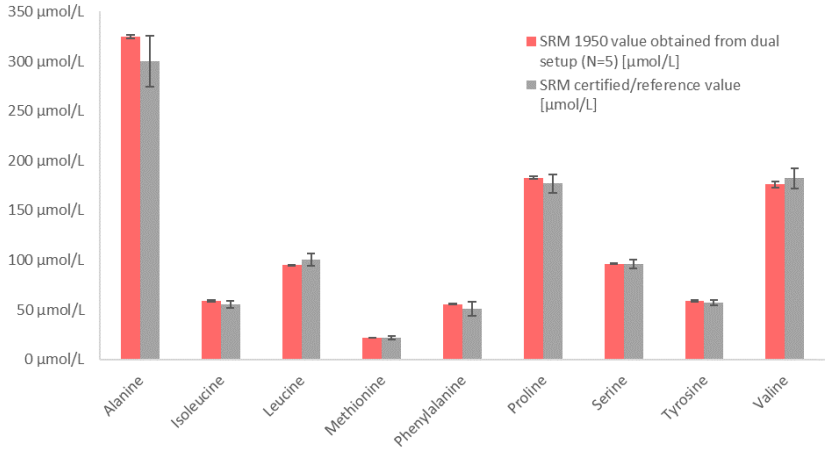

Figure 28 Comparison of SRM 1950 metabolite concentrations with NIST-certified/reference values. Pink bar: values obtained via presented 2D setup in either positive or negative ionization mode (absolute quantification by use of internal standardization and external calibration). Grey bar: NIST-certified/reference value. Whiskers represent standard deviation (N=5) or certified uncertainty, respectively.

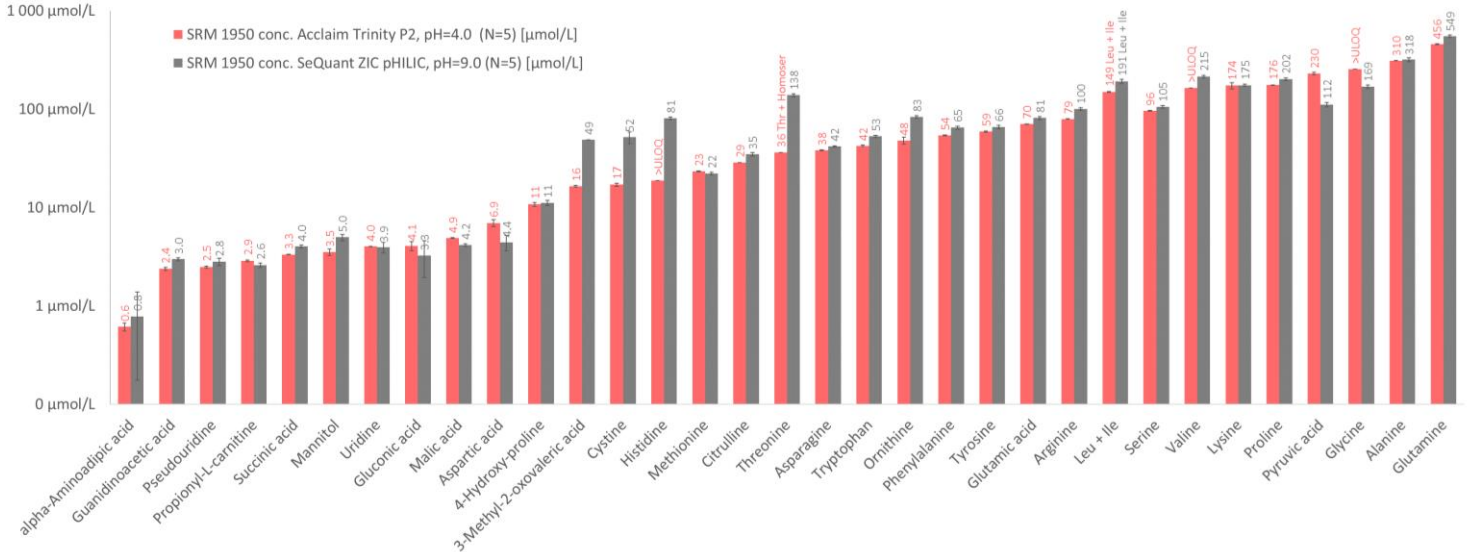

Figure 29 Comparison of SRM 1950 metabolite concentrations with archive data. Pink bar: values obtained via presented dual setup (Acclaim Trinity P2, pH=4.5), grey bar: values obtained by Schwaiger et al. (2018) (SeQuant ZIC pHILIC, pH=9.2). Sample: SRM 1950 (N=5 replicate injections), polarity: positive and negative. Absolute quantification in Skyline by use of internal standardization and external calibration. Threonine and homoserine co-elute in presented 2D setup and are not depicted.

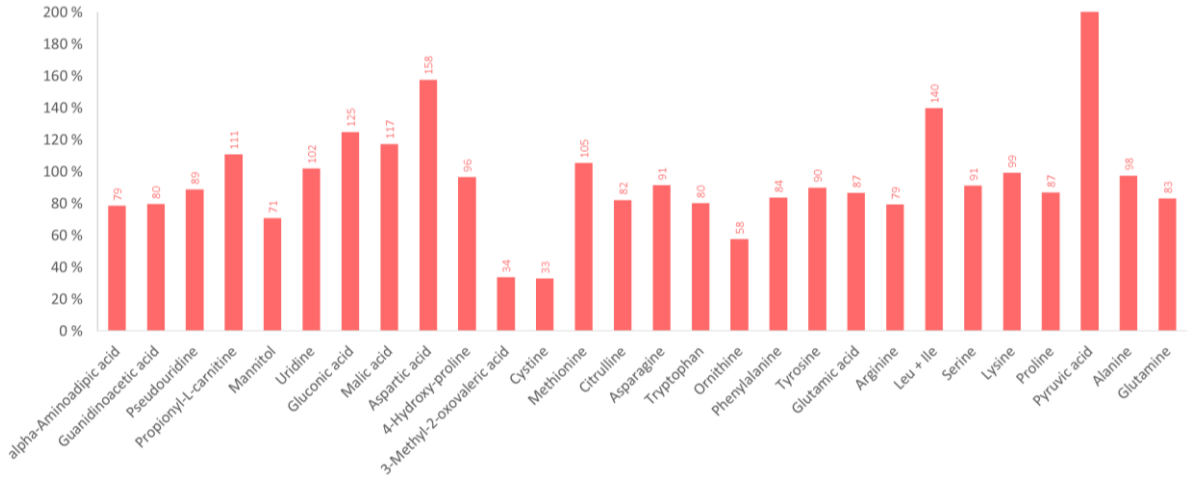

Figure 30 Recovery of SRM 1950 concentration reported by Schwaiger et al. (2018). Sample: SRM 1950 (N=5), chromatography: Acclaim Trinity P2, pH=4.5, polarity: positive and negative. Absolute quantification in Skyline by use of internal standardization and external calibration. Threonine and homoserine co-elute in the presented 2D setup and are not depicted. Leucine and isoleucine were summarized for comparison because they co-elute with the reference method.

5 Conclusion

Although the presented novel analytical method could not be validated according to U.S. FDA's guideline Bioanalytical Method Validation, this work offers important insights into regulatory method validation. A basic characterization of the analytical method was achieved and several issues specific for validating a metabolomics method were revealed.

5.1 Characterization of the analytical method

The provided data set was exploited to gain deeper insight into the capabilities and weak spots of the presented dual-chormatography HPLC-HRMS method for combined metabolomics and exposomics research. Overall, the investigated hydrophilic endogenous metabolites showed good retention and separation under acidic HILIC chromatography conditions, while a great fraction of them eluted in or near the void volume under RP conditions. Amino acids and purine derivatives showed satisfying retention and separation as well as pyrimidine-based nucleoside monophosphates. Pyrimidine-based nucleosides and nucleobases were assessable as well, but the HILIC chromatography conditions were less favorable compared to their nucleoside monophosphates: The peaks eluted near the void volume and their shapes were broad and jagged, resulting in a higher area and retention time variability. Although their peak shapes were on average quite broad, carbohydrate derivatives were sufficiently retained on the HILIC column. However, hexose and hexose monophosphate isomers could not be separated. Some organic acids could be analyzed by the presented analytical method quite well. For a great fraction of them, however, the method was not ideal and resulted in unreliable identification and quantification. The pharmaceuticals investigated during targeted data evaluation showed good retention and separation under reversed phase conditions, as expected. In line with that, untargeted data evaluation revealed some more small-molecule xenobiotics in SRM 1950. All the compounds except gabapentine had an aromatic moiety, a quite common structural element for small-molecule pharmaceuticals. Their expectable blood concentrations fall within the range of detectable magnitudes of the analytical method (i.e. nano- to mycromolar). Summarizing, these findings can be rated as a proof of principle that the presented dual setup is attractive for simultaneous metabolomics and exposomics investigation.

The implementation of the internal standard could level out sequence dependent as well as random variation of signal intensities for many compounds. On the other hand, we encountered some compounds for which building the ratio-to-heavy increased inter-replicate variability. Considering that the internal standard is a biological extract of which neither exact composition nor stability are explicitly known, it is crucial to judge the application of the internal standard for each compound and

each concentration in each experiment individually. After all, the signal intensities of ^{12}C- and ^{13}C-compound should probably stay within a certain range of proportion in order to build a reliable and meaningful ratio. The strategy chosen for this data evaluation was to visually evaluate each peak and accept or reject it. This is, however, poorly reproducible and hardly feasible for the multitude of target analytes in a metabolomics experiment and it would be desirable to evade this manual process in future experiments. Further investigation is duly needed on this topic.

Some of the obtained concentration values were in good agreement with the NIST-certified values, but comparison was only possible for a handful of compounds. The comparison provides verification for those few compound concentrations and, to some extent, that the data evaluation process was in principle correct. The verification can by no means be extrapolated to the rest of the compounds, though. To provide reference for some additional compounds, the concentration values were compared to SRM 1950 results obtained via another 2D-chromatography HPLC-HRMS setup[28]. While some concentration values were in acceptable agreement, most of them differed markedly between the two analytical setups. Against this background, the importance of critical method validation and value verification in absolute quantitative metabolomics becomes all the more obvious. It is, however, important to stress that the values compared are not only produced by different analytical methods, but probably also underwent different data evaluation processes. Raw data evaluation, as performed in our working group, is still a very manual and individual process and a strict comparison of calculated concentrations would probably be more conclusive with a standardized evaluation procedure. The presented comparison therefore serves as a rough orientation in terms of magnitudes rather than judge the validity of the analytical technique.

Susceptibility to matrix effects was not sufficiently assessable in the given experiment since calibrators and QCs were prepared in pure solvent.

5.2 Issues specific for metabolomics

There is one feature of a metabolomics investigation that poses a special obstacle to a fully U.S. FDA conforming validation. Most specifications in the guideline, for example the ones concerning calibration curve, quality controls, selectivity and specificity, rest on the assumption that any alteration caused by matrix effect can be levelled out by using standard-spiked blank matrix as a basis for calibrators and QCs. This is, however, not easy to accomplish in a metabolomics experiment since a biological sample matrix free of primary metabolites is hardly imaginable. A validation neglecting this issue would probably fail to achieve official medicines authorities' approval. I think it is necessary to explore and develop a special strategy for addressing matrix effects in metabolomics during valida-

tion and subsequent sample analysis. Species-specific internal standardization, extensively characterized, appears as a suitable approach.

6 Outlook

The issues that currently separate this metabolomics method from meeting official medicines authorities' provisions can be overcome, but further investigation is needed. One promising tool is the species-specific internal standard that was used in the present experiment. It would be interesting to quantify and compare potential positive and negative effects of its application regarding measurement sensitivity (signal intensity, peak area, lower limit of detection), linearity and precision (linearity range, lower limit of quantification), as well as accuracy of the result (comparison with certified reference standard). A good characterization of the internal standard might allow to stratify sample types, analytes and concentration ranges in order to maximize the benefit of its application. Additionally, an automatized way to make the qualitative decision of normalizing or not normalizing to the internal standard needs to be developed for quantitative data evaluation, as well as numeric criteria that allow to unerringly distinguish between "good" signals (i.e. peaks usable for quantification) and "bad" ones (spikes, noise).

For future validation endeavors that aim at translating university research into regulated environment, it seems reasonable to temporarily refrain from clinical application and take a preclinical research application as an intermediate goal. For preclinical research in toxicology assessment of new chemicals or pharmaceuticals, European law requests that results are obtained following the provisions of Good Laboratory Practice (GLP) issued by the OECD. GLP guidelines are not so much aimed at routine analysis but are more project-oriented and might therefore be closer to a university research situation. Approaching the requirements for preclinical investigation is still a very challenging task, but at this point probably a bit more doable than aiming at clinical application.

7 Abstract

The central motif of this work is to face the challenge of validating an absolute quantification procedure based on a newly developed LC-MS method for simultaneous metabolomics and exposomics analysis in a way that approximates the validation needs for a clinical study. The presented method is a novel HPLC-HRMS approach employing simultaneous chromatography under complementing elution conditions: Hydrophilic interaction liquid chromatography (HILIC) provides good separation conditions for the more hydrophilic metabolites, whereas reversed phase (RP) chromatography poses a better environment for analyzing compounds of higher lipophilicity, a property quite common for small molecule pharmaceuticals. To get a sense of a medicines authority's regulatory expectations of analytical method validation, the U.S. FDA's guideline *Bioanalytical Method Validation – Guidance for Industry* is sought for advice. The guideline conveniently suggests strategies how to evaluate the capabilities of a chromatographic method. It serves as a starting point for approaching the specific issues of an HPLC-HRMS metabolomics method.

An already acquired data set is exploited to gain deeper insight into the capabilities and weak spots of the novel dual-chromatography HPLC-HRMS method and in deed suggests its versatility for combined metabolomics and exposomics research. With the exception of a few targeted metabolites with overlapping HILIC and RP peaks, the 2D chromatography concept saves time without considerable drawbacks for the quality of the analysis. This work especially maps out the benefits of internal standardization with a fully ^{13}C-labelled metabolite extract from *Pichia pastoris* while, on the other hand, also discussing its possible pitfalls. Analyzing the data arises questions that will have to be addressed by further research.

Linking basic university research with a highly regulated application environment is an ambitious endeavor, which, in the course of this work, turned out to be hardly feasible for the current state of method development. Nonetheless, the thesis collates general issues specific for a full validation of a metabolomics method according to medicines authorities' provisions. Among them are the complexity of matrix effect assessment and the problem that an approach via blank matrix, as suggested by the guideline, is not viable for metabolomics. Internal standardization with a fully ^{13}C-labeled yeast cell extract is presented as a possible solution. Dedicated evaluation is needed but lies beyond the scope of this thesis.

8 Zusammenfassung

Im Zentrum dieser Arbeit steht eine neuartige instrumentelle HPLC-HRMS Methode, welche für die simultane Analyse von (hydrophilem) Metabolom und (internem) Exposom entwickelt wurde. Die gleichzeitige Erfassung endogener Metabolite und weniger polarer Xenobiotika wird durch Erweiterung des Polaritätsspektrums mittels paralleler HILIC- und RP-Chromatographie ermöglicht, ohne dadurch die Laufzeit der Analyse zu erhöhen. Der Auftrag der Arbeit besteht nun darin, die Methode so weit als möglich nach behördlichen Empfehlungen zu validieren, wie sie im Rahmen klinischer Studien verlangt werden. Die Leitlinie *Bioanalytical Method Validation – Guidance for Industry* der U.S.-amerikaischen Lebensmittelüberwachungs- und Arzneimittelbehörde *U.S. Food and Drug Administration* (U.S. FDA) dient dabei als Orientierungshilfe zur Einschätzung der behördlichen Erwartungen.

Durch Auswertung bereits vorhandenen Datenmaterials gelingt eine grundlegende Charakterisierung der Methode, welche die Eignung für die kombinierte *metabolomics-exposomics*-Forschung untermauert. Das besonderes Augenmerk der Untersuchung gilt dabei den Vorteilen, aber auch möglichen Komplikationen durch den ^{13}C-markierten *Pichia pastoris*-Zellextrakt, der hier als interner Multikomponentenstandard zur Anwedung kommt.

Eine vollständige Validierung nach U.S. FDA kann letzten Endes nicht erzielt werden. Die Gründe dafür – validierungsstrategische wie *metabolomics*-spezifische – werden in der Arbeit ausführlich diskutiert. Möglicherweise können künftige Validierungsvorhaben davon profitieren.

9 Abbreviations

1D, 2D	one-dimensional, two-dimensional, here: referring to (parallel) chromatography
AGC target	Advanced gain control target
CC	Calibration curve
c-trap	Curved linear trap
EMA	European Medicines Agency
ESI	Electrospray ionization
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High-performance liquid chromatography
HRMS	High-resolution mass specrtometry
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IS	Internal standard, here: metabolite extract of fully 13C-labeled yeast
IT	(Maximum) injection time
GLP	Good Laboratory Practice (OECD)
LC	Liquid chromatography
LOD	Limit of detection
LLOQ	Lower limit of quantification
$[M+H]^+$	Ionized adduct formed by protonation of analyte molecule
$[M-H]^-$	Ion formed by proton loss of analyte molecule
MS	Mass spectrometry
MS1	Full MS scans, no intentional fragmentation of molecular ion
MS2	MS scans with deliberate fragmentation of molecular ion
NIST	National Institute of Standards an Technology (USA)
OECD	Organisation for Economic Co-operation and Development
QC	Quality control, here: standard with c = 1 µmol/L
RP	Reversed Phase
RT	(Chromatographic) retention time
SRM 1950	Standard Reference Material 1950 - Metabolites in Frozen Human Plasma (NIST)
TIC	Total Ion current
U.S. FDA	United States Food and Drug Administration
ULOQ	Upper limit of quantification, here: highest acceptable calibrator concentration of calibration curve

10 Bibliography

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11 Supplement

Table S1 163 compounds investigated during targeted data evaluation. The given m/z were used for ion chromatogram extraction from positive and negative mode data, respectively.

Compound name	Compound class	Neutral formula	Pos. adduct	Pos. m/z	Neg. adduct	Neg. m/z
1-Methylhydantoin	Others	C4H6N2O2	[M+H]	115.0502	[M-H]	113.0357
1-Methylnicotinamide	Others	C7H8N2O	[M+]	136.0631	[M-H]	135.0564
2-Carbamoylaminobutanedioic acid	Others	C5H8N2O5	[M+H]	177.0506	[M-H]	175.0360
2'-Deoxycytidine	Pyrimidine deriv.	C9H13N3O4	[M+H]	228.0979	[M-H]	226.0833
2'-Deoxyuridine	Pyrimidine deriv.	C9H12N2O5	[M+H]	229.0819	[M-H]	227.0673
3'-Adenosine monophosphate	Purine deriv.	C10H14N5O7P	[M+H]	348.0704	[M-H]	346.0558
3-Methyl-2-oxovaleric acid	Others	C6H10O3	[M+H]	131.0703	[M-H]	129.0557
3-Methylcytidine	Pyrimidine deriv.	C10H15N3O5	[M+H]	258.1084	[M-H]	256.0939
3-Phosphoglycerate	Others	C3H7O7P	[M+H]	187.0002	[M-H]	184.9857
4-Hydroxy-proline	Aminoacid deriv.	C5H9NO3	[M+H]	132.0655	[M-H]	130.0510
5'-Adenosine monophosphate	Purine deriv.	C10H14N5O7P	[M+H]	348.0704	[M-H]	346.0558
5'-Deoxy-5'-methylthioadenosine	Purine deriv.	C11H15N5O3S	[M+H]	298.0968	[M-H]	296.0823
5-Methyluridine	Pyrimidine deriv.	C10H14N2O6	[M+H]	259.0925	[M-H]	257.0779
6-Phosphogluconate	Others	C6H13O10P	[M+H]	277.0319	[M-H]	275.0174
Acetylsalicylic acid	Exposome	C9H8O4	[M+H]	181.0495	[M-H]	179.0350
Adenine	Purine deriv.	C5H5N5	[M+H]	136.0618	[M-H]	134.0472
Adenosine	Purine deriv.	C10H13N5O4	[M+H]	268.1040	[M-H]	266.0895
Adenosine diphosphate	Purine deriv.	C10H15N5O10P2	[M+H]	428.0367	[M-H]	426.0221
Adenosine triphosphate	Purine deriv.	C10H16N5O13P3	[M+H]	508.0030	[M-H]	505.9885
Alanine	Aminoacid deriv.	C3H7NO2	[M+H]	90.0550	[M-H]	88.0404
alpha-Aminoadipic acid	Aminoacid deriv.	C6H11NO4	[M+H]	162.0761	[M-H]	160.0615
alpha-Ketoglutaric acid	Others	C5H6O5	[M+H]	147.0288	[M-H]	145.0142
alpha-Ketoisovaleric acid	Others	C5H8O3	[M+H]	117.0546	[M-H]	115.0401
alpha-Tocopherol	Others	C29H50O2	[M+H]	431.3884	[M-H]	429.3738

Compound name	Compound class	Neutral formula	Pos. adduct	Pos. m/z	Neg. adduct	Neg. m/z
Arginine	Aminoacid deriv.	C6H14N4O2	[M+H]	175.1190	[M-H]	173.1044
Argininosuccinic acid	Aminoacid deriv.	C10H18N4O6	[M+H]	291.1299	[M-H]	289.1154
Asparagine	Aminoacid deriv.	C4H8N2O3	[M+H]	133.0608	[M-H]	131.0462
Aspartic acid	Aminoacid deriv.	C4H7NO4	[M+H]	134.0448	[M-H]	132.0302
Betaine	Aminoacid deriv.	C5H11NO2	[M+H]	118.0863	[M-H]	116.0717
Biotin	Others	C10H16N2O3S	[M+H]	245.0954	[M-H]	243.0809
Bisphenol A	Exposome	C15 H16 O2	[M+H]	229.1223	[M-H]	227.1078
Buspirone	Exposome	C21H31N5O2	[M+H]	386.2551	[M-H]	384.2405
Caffeine	Exposome	C8H10N4O2	[M+H]	195.0877	[M-H]	193.0731
Carnitine	Aminoacid deriv.	C7H15NO3	[M+H]	162.1125	[M-H]	160.0979
Choline	Others	C5H14NO	[M+H]	105.1148	[M-H]	103.1003
cis-Aconitic acid	Others	C6H6O6	[M+H]	175.0237	[M-H]	173.0092
Citric acid	Others	C6H8O7	[M+H]	193.0343	[M-H]	191.0197
Citrulline	Aminoacid deriv.	C6H13N3O3	[M+H]	176.1030	[M-H]	174.0884
Cyclic adenosine monophosphate	Purine deriv.	C10H12N5O6P	[M+H]	330.0598	[M-H]	328.0452
Cyclic guanosine monophosphate	Purine deriv.	C10H12N5O7P	[M+H]	346.0547	[M-H]	344.0402
Cystathionine	Aminoacid deriv.	C7H14N2O4S	[M+H]	223.0747	[M-H]	221.0602
Cysteic acid	Aminoacid deriv.	C3H7NO5S	[M+H]	170.0118	[M-H]	167.9972
Cysteine	Aminoacid deriv.	C3H7NO2S	[M+H]	122.0270	[M-H]	120.0125
Cysteinylglycine	Aminoacid deriv.	C5H10N2O3S	[M+H]	179.0485	[M-H]	177.0339
Cystine	Aminoacid deriv.	C6H12N2O4S2	[M+H]	241.0311	[M-H]	239.0166
Cytidine	Pyrimidine deriv.	C9H13N3O5	[M+H]	244.0928	[M-H]	242.0782
Cytidine monophosphate	Pyrimidine deriv.	C9H14N3O8P	[M+H]	324.0591	[M-H]	322.0446
Cytidine triphosphate	Pyrimidine deriv.	C9H16N3O14P3	[M+H]	483.9918	[M-H]	481.9772
Cytosine	Pyrimidine deriv.	C4H5N3O	[M+H]	112.0505	[M-H]	110.0360
Deoxyadenosine monophosphate	Purine deriv.	C10H14N5O6P	[M+H]	332.0754	[M-H]	330.0609
Deoxyadenosine triphosphate	Purine deriv.	C10H16N5O12P3	[M+H]	492.0081	[M-H]	489.9936
Deoxycytidine monophosphate	Pyrimidine deriv.	C9H14N3O7P	[M+H]	308.0642	[M-H]	306.0497

Compound name	Compound class	Neutral formula	Pos. adduct	Pos. m/z	Neg. adduct	Neg. m/z
Deoxycytidine triphosphate	Pyrimidine deriv.	C9H16N3O13P3	[M+H]	467.9969	[M-H]	465.9823
Deoxyguanosine triphosphate	Purine deriv.	C10H16N5O13P3	[M+H]	508.0030	[M-H]	505.9885
Dihydrocholesterol	Others	C27H48O	[M+H]	389.3778	[M-H]	387.3632
Dihydroxyacetone phosphate	Carbohydrate deriv.	C3H7O6P	[M+H]	171.0053	[M-H]	168.9907
Dihydroxyisovaleric acid	Others	C5H10O4	[M+H]	135.0652	[M-H]	133.0506
Erythrol	Carbohydrate deriv.	C4H10O4	[M+H]	123.0652	[M-H]	121.0506
Erythrose-4-phosphate	Carbohydrate deriv.	C4H9O7P	[M+H]	201.0159	[M-H]	199.0013
Flavinadenin dinucleotide	Purine deriv.	C27H33P2N9O15	[M+H]	786.1644	[M-H]	784.1499
Fructose	Carbohydrate deriv.	C6H12O6	[M+H]	181.0707	[M-H]	179.0561
Fructose-1,6-bisphosphate	Carbohydrate deriv.	C6H14O12P2	[M+H]	341.0033	[M-H]	338.9888
Fructose-6-phosphate	Carbohydrate deriv.	C6H13O9P	[M+H]	261.0370	[M-H]	259.0224
Fumaric acid	Others	C4H4O4	[M+H]	117.0182	[M-H]	115.0037
Galactose	Carbohydrate deriv.	C6H12O6	[M+H]	181.0707	[M-H]	179.0561
Gluconic acid	Carbohydrate deriv.	C6H12O7	[M+H]	197.0656	[M-H]	195.0510
Glucose	Carbohydrate deriv.	C6H12O6	[M+H]	181.0707	[M-H]	179.0561
Glucose-1-phosphate	Carbohydrate deriv.	C6H13O9P	[M+H]	261.0370	[M-H]	259.0224
Glucose-6-phosphate	Carbohydrate deriv.	C6H13O9P	[M+H]	261.0370	[M-H]	259.0224
Glutamic acid	Aminoacid deriv.	C5H9NO4	[M+H]	148.0604	[M-H]	146.0459
Glutamine	Aminoacid deriv.	C5H10N2O3	[M+H]	147.0764	[M-H]	145.0619
Glutamylcysteine	Aminoacid deriv.	C8H14N2O5S	[M+H]	251.0696	[M-H]	249.0551
Glutathione, oxidized	Aminoacid deriv.	C20H32N6O12S2	[M+H]	613.1592	[M-H]	611.1447
Glutathione, reduced	Aminoacid deriv.	C10H17N3O6S	[M+H]	308.0911	[M-H]	306.0765
Glycine	Aminoacid deriv.	C2H5NO2	[M+H]	76.0393	[M-H]	74.0248
Glyoxylic acid	Others	C2H2O3	[M+H]	75.0077	[M-H]	72.9931
Guanidinoacetic acid	Aminoacid deriv.	C3H7N3O2	[M+H]	118.0611	[M-H]	116.0466
Guanine	Purine deriv.	C5H5N5O	[M+H]	152.0567	[M-H]	150.0421
Guanosine	Purine deriv.	C10H13N5O5	[M+H]	284.0989	[M-H]	282.0844
Guanosine diphosphate	Purine deriv.	C10H15N5O11P2	[M+H]	444.0316	[M-H]	442.0171

Compound name	Compound class	Neutral formula	Pos. adduct	Pos. m/z	Neg. adduct	Neg. m/z
Guanosine monophosphate	Purine deriv.	C10H14N5O8P	[M+H]	364.0653	[M-H]	362.0507
Guanosine triphosphate	Purine deriv.	C10H16N5O14P3	[M+H]	523.9979	[M-H]	521.9834
Hexanoylcarnitine	Aminoacid deriv.	C13H25NO4	[M+H]	260.1856	[M-H]	258.1711
Histidine	Aminoacid deriv.	C6H9N3O2	[M+H]	156.0768	[M-H]	154.0622
Homocysteine	Aminoacid deriv.	C4H9NO2S	[M+H]	136.0427	[M-H]	134.0281
Homoserine	Aminoacid deriv.	C4H9NO3	[M+H]	120.0655	[M-H]	118.0510
Hydroxybupropion	Exposome	C13H18ClNO2	[M+H]	256.1099	[M-H]	254.0953
Hydroxyglutaric acid	Others	C5H8O5	[M+H]	149.0444	[M-H]	147.0299
Inosine	Carbohydrate deriv.	C10H12N4O5	[M+H]	269.0880	[M-H]	267.0735
Inosine monophosphate	Carbohydrate deriv.	C10H13N4O8P	[M+H]	349.0544	[M-H]	347.0398
Inositol	Carbohydrate deriv.	C6H12O6	[M+H]	181.0707	[M-H]	179.0561
Isocitric acid	Others	C6H8O7	[M+H]	193.0343	[M-H]	191.0197
Isoguanosine	Purine deriv.	C10H13N5O5	[M+H]	284.0989	[M-H]	282.0844
Isoleucine	Aminoacid deriv.	C6H13NO2	[M+H]	132.1019	[M-H]	130.0874
Kynurenine	Aminoacid deriv.	C10H12N2O3	[M+H]	209.0921	[M-H]	207.0775
Lactic acid	Others	C3H6O3	[M+H]	91.0390	[M-H]	89.0244
Leucine	Aminoacid deriv.	C6H13NO2	[M+H]	132.1019	[M-H]	130.0874
Lysine	Aminoacid deriv.	C6H14N2O2	[M+H]	147.1128	[M-H]	145.0983
Malic acid	Others	C4H6O5	[M+H]	135.0288	[M-H]	133.0142
Mannitol	Carbohydrate deriv.	C6H14O6	[M+H]	183.0863	[M-H]	181.0718
Mannitol-1-phosphate	Carbohydrate deriv.	C6H15O9P	[M+H]	263.0526	[M-H]	261.0381
Mannose	Carbohydrate deriv.	C6H12O6	[M+H]	181.0707	[M-H]	179.0561
Melatonine	Aminoacid deriv.	C13H16N2O2	[M+H]	233.1285	[M-H]	231.1139
Methionine	Aminoacid deriv.	C5H11NO2S	[M+H]	150.0583	[M-H]	148.0438
Methionine sulfone	Aminoacid deriv.	C5H11NO4S	[M+H]	182.0482	[M-H]	180.0336
Mevalonic acid	Others	C6H12O4	[M+H]	149.0808	[M-H]	147.0663
N4-Acetylcytidine	Pyrimidine deriv.	C11H15N3O6	[M+H]	286.1034	[M-H]	284.0888
N-Acetylaspartic acid	Aminoacid deriv.	C6H9NO5	[M+H]	176.0553	[M-H]	174.0408

Compound name	Compound class	Neutral formula	Pos. adduct	Pos. m/z	Neg. adduct	Neg. m/z
N-Acetylaspartylglutamic acid	Aminoacid deriv.	C11H16N2O8	[M+H]	305.0979	[M-H]	303.0834
N-Acetylserine	Aminoacid deriv.	C5H9NO4	[M+H]	148.0604	[M-H]	146.0459
NAD+	Purine deriv.	C21H27N7O14P2	[M+H]	664.1164	[M-H]	662.1018
NADH	Purine deriv.	C21H29N7O14P2	[M+H]	666.1320	[M-H]	664.1175
NADP+	Purine deriv.	C21H28N7O17P3	[M+H]	744.0827	[M-H]	742.0682
NADPH	Purine deriv.	C21H30N7O17P3	[M+H]	746.0984	[M-H]	744.0838
Nicotinamide	Others	C6H6N2O	[M+H]	123.0553	[M-H]	121.0407
Octopamine	Aminoacid deriv.	C8H11NO2	[M+H]	154.0863	[M-H]	152.0717
Ornithine	Aminoacid deriv.	C5H12N2O2	[M+H]	133.0972	[M-H]	131.0826
Oxaloacetic acid	Others	C4H4O5	[M+H]	133.0131	[M-H]	130.9986
Palmitic acid	Others	C16H32O2	[M+H]	257.2475	[M-H]	255.2330
Phenylalanine	Aminoacid deriv.	C9H11NO2	[M+H]	166.0863	[M-H]	164.0717
Phosphocreatine	Aminoacid deriv.	C4H10N3O5P	[M+H]	212.0431	[M-H]	210.0285
Piperine	Exposome	C17H19NO3	[M+H]	286.1438	[M-H]	284.1292
Proline	Aminoacid deriv.	C5H9NO2	[M+H]	116.0706	[M-H]	114.0561
Propionylcarnitine	Aminoacid deriv.	C10H19NO4	[M+H]	218.1387	[M-H]	216.1241
Pseudouridine	Pyrimidine deriv.	C9H12N2O6	[M+H]	245.0768	[M-H]	243.0623
Pyruvic acid	Others	C3H4O3	[M+H]	89.0233	[M-H]	87.0088
Quetiapine	Exposome	C21H25N3O2S	[M+H]	384.1740	[M-H]	382.1595
Ribose	Carbohydrate deriv.	C5H10O5	[M+H]	151.0601	[M-H]	149.0455
Ribose-5-phosphate	Carbohydrate deriv.	C5H11O8P	[M+H]	231.0264	[M-H]	229.0119
Ribulose-5-phosphate	Carbohydrate deriv.	C5H11O8P	[M+H]	231.0264	[M-H]	229.0119
S-Adenosylhomocysteine	Purine deriv.	C14H20N6O5S	[M+H]	385.1289	[M-H]	383.1143
S-Adenosylmethionine	Purine deriv.	C15H22N6O5S	[M+H]	399.1445	[M-H]	397.1300
Salicin	Exposome	C13H18O7	[M+H]	287.1125	[M-H]	285.0980
Salicylic acid	Exposome	C7H6O3	[M+H]	139.0390	[M-H]	137.0244
Sarcosine	Aminoacid deriv.	C3H7NO2	[M+H]	90.0550	[M-H]	88.0404
Sedoheptulose-7-phosphate	Carbohydrate deriv.	C7H15O10P	[M+H]	291.0476	[M-H]	289.0330

Compound name	Compound class	Neutral formula	Pos. adduct	Pos. m/z	Neg. adduct	Neg. m/z
Selenomethionine	Aminoacid deriv.	C5H11NO2Se	[M+H]	198.0028	[M-H]	195.9882
Serine	Aminoacid deriv.	C3H7NO3	[M+H]	106.0499	[M-H]	104.0353
Serotonine	Aminoacid deriv.	C10H12N2O	[M+H]	177.1022	[M-H]	175.0877
Spermidine	Others	C7H19N3	[M+H]	146.1652	[M-H]	144.1506
Spermine	Others	C10H26N4	[M+H]	203.2230	[M-H]	201.2085
Squalen	Others	C30H50	[M+H]	411.3985	[M-H]	409.3840
Succinic acid	Others	C4H6O4	[M+H]	119.0339	[M-H]	117.0193
Theobromine	Exposome	C7H8N4O2	[M+H]	181.0720	[M-H]	179.0575
Thiamine	Pyrimidine deriv.	C12H17N4OS	[M+H]	266.1196	[M-H]	264.1050
Threonine	Aminoacid deriv.	C4H9NO3	[M+H]	120.0655	[M-H]	118.0510
Thymidine	Pyrimidine deriv.	C10H14N2O5	[M+H]	243.0975	[M-H]	241.0830
Thymidine monophosphate	Pyrimidine deriv.	C10H15N2O8P	[M+H]	323.0639	[M-H]	321.0493
Thymidine triphosphate	Pyrimidine deriv.	C10H17N2O14P3	[M+H]	482.9965	[M-H]	480.9820
Thymine	Pyrimidine deriv.	C5H6N2O2	[M+H]	127.0502	[M-H]	125.0357
Trazodone	Exposome	C19H22ClN5O	[M+H]	372.1586	[M-H]	370.1440
Trehalose	Carbohydrate deriv.	C12H22O11	[M+H]	343.1235	[M-H]	341.1089
Tryptophan	Aminoacid deriv.	C11H12N2O2	[M+H]	205.0972	[M-H]	203.0826
Tyrosine	Aminoacid deriv.	C9H11NO3	[M+H]	182.0812	[M-H]	180.0666
Uracil	Pyrimidine deriv.	C4H4N2O2	[M+H]	113.0346	[M-H]	111.0200
Urea	Others	CH4N2O	[M+H]	61.0396	[M-H]	59.0251
Uridine	Pyrimidine deriv.	C9H12N2O6	[M+H]	245.0768	[M-H]	243.0623
Uridine diphosphate	Pyrimidine deriv.	C9H14N2O12P2	[M+H]	405.0095	[M-H]	402.9949
Uridine monophosphate	Pyrimidine deriv.	C9H13N2O9P	[M+H]	325.0431	[M-H]	323.0286
Uridine triphosphate	Pyrimidine deriv.	C9H15N2O15P3	[M+H]	484.9758	[M-H]	482.9613
Valine	Aminoacid deriv.	C5H11NO2	[M+H]	118.0863	[M-H]	116.0717
Xanthine	Purine deriv.	C5H4N4O2	[M+H]	153.0407	[M-H]	151.0261
Xylose	Carbohydrate deriv.	C5H10O5	[M+H]	151.0601	[M-H]	149.0455

Table S2 Figures of merit for the presented dual-chromatography HPLC-HRMS method - positive ionization mode. The linear range of the calibration curve was evaluated visually. Additionally, calibrators that diverged > 15-20 % of the nominal concentration were discarded. Mean RT incl. SD and mean QC recovery incl. SD are calculated from five replicate injections of a 1 µmol/L standard. LOD and LLOQ are estimated from five replicate injections of a 0.5 µmol/L standard according to Eurachem (2014, see main text). Xenobiotics are marked in grey.

Compound	mean RT [min]	RT SD [min]	Neutral formula	pos m/z	pos linear range lower end [µmol/L]	pos linear range upper end [µmol/L]	pos internal standard	R^2 pos calibration curve	LOD pos [µmol/L]	LLOQ pos [µmol/L]	mean QC recovery pos [%]	QC recovery SD pos [%]	Comment
2-Carbamoylaminobutanedioic acid	1.1	0.02	C5H8N2O5	177.0506	0.05	1.00	---	0.9979	0.04	0.14	99	8	---
2'-Deoxycytidine	10.2	0.04	C9H13N3O4	228.0979	0.01	0.50	---	0.9927	0.04	0.13	85	10	---
3'-Adenosine monophosphate	14.5	0.02	C10H14N5O7P	348.0704	0.05	5.00	---	1.0000	0.03	0.11	106	12	---
3-Phosphoglycerate	1.0	0.01	C3H7O7P	187.0002	0.10	1.00	---	0.9983	0.08	0.26	95	12	Only 3 calibrators!
4-Hydroxy-proline	7.8	0.04	C5H9NO3	132.0655	0.05	10.00	---	0.9996	0.09	0.29	133	63	---
5'-Adenosine monophosphate	12.8	0.00	C10H14N5O7P	348.0704	0.01	0.50	U13C 5'-Adenosine monophosphate	0.9990	0.04	0.15	100	3	---
5'-Deoxy-5'-methylthioadenosine	4.2	0.03	C11H15N5O3S	298.0968	0.01	0.50	U13C 5'-Deoxy-5'-methylthioadenosine	0.9998	0.00	0.01	98	2	---
5-Methyluridine	2.5	0.10	C10H14N2O6	259.0925	0.05	10.00	---	0.9998	0.04	0.13	99	18	---
Adenine	8.0	0.01	C5H5N5	136.0618	0.01	5.00	U13C Adenine	0.9993	0.01	0.04	94	3	---
Adenosine	5.7	0.03	C10H13N5O4	268.1040	0.01	5.00	U13C Adenosine	0.9996	0.01	0.02	96	1	---
Adenosine diphosphate	1.1	0.04	C10H15N5O10P2	428.0367	0.10	5.00	U13C Adenosine diphosphate	0.9988	0.05	0.15	103	4	---
Alanine	8.0	0.00	C3H7NO2	90.0550	0.10	5.00	U13C Alanine	0.9993	0.11	0.35	95	5	---
alpha-Aminoadipic acid	10.1	0.00	C6H11NO4	162.0761	0.01	5.00	U13C alpha-Aminoadipic acid	0.9998	0.01	0.03	99	1	---
Arginine	0.9	0.00	C6H14N4O2	175.1190	0.05	10.00	U13C Arginine	0.9973	0.02	0.08	89	2	---
Argininosuccinic acid	11.2	0.01	C10H18N4O6	291.1299	0.05	10.00	U13C Argininosuccinic acid	0.9995	0.01	0.03	101	3	---
Asparagine	8.3	0.01	C4H8N2O3	133.0608	0.05	10.00	U13C Asparagine	0.9971	0.02	0.06	89	1	---
Aspartic acid	11.4	0.00	C4H7NO4	134.0448	0.05	5.00	U13C Aspartic acid	0.9997	0.03	0.10	100	2	---
Betaine	6.9	0.02	C5H11NO2	118.0863	0.50	10.00	U13C Betaine	0.9994	0.01	0.02	109	42	---
Buspirone	6.9	0.01	C21H31N5O2	386.2551	0.01	1.00	---	0.9937	0.09	0.28	93	4	Compound ID ambigous.
Caffeine	5.1	0.01	C8H10N4O2	195.0877	0.01	10.00	---	1.0000	0.06	0.22	122	19	---
Carnitine	11.6	0.01	C7H15NO3	162.1125	0.01	5.00	U13C Carnitine	0.9997	0.04	0.15	98	5	---

Compound	mean RT [min]	RT SD [min]	Neutral formula	pos m/z	pos linear range lower end [µmol/L]	pos linear range upper end [µmol/L]	pos internal standard	R^2 pos calibration curve	LOD pos [µmol/L]	LLOQ pos [µmol/L]	mean QC recovery pos [%]	QC recovery SD pos [%]	Comment
Citrulline	8.5	0.01	C6H13N3O3	176.1030	0.05	10.00	U13C Citrulline	0.9986	0.01	0.03	95	1	---
Cyclic adenosine monophosphate	11.3	0.01	C10H12N5O6P	330.0598	0.05	5.00	---	0.9934	0.01	0.04	119	18	---
Cyclic guanosine monophosphate	13.7	0.01	C10H12N5O7P	346.0547	0.01	5.00	---	0.9998	0.05	0.16	105	13	---
Cystathionine	9.8	0.01	C7H14N2O4S	223.0747	0.01	5.00	U13C Cystathionine	0.9992	0.02	0.07	98	2	---
Cysteic acid	12.4	0.01	C3H7NO5S	170.0118	0.05	10.00	---	1.0000	0.05	0.15	102	15	---
Cystine	9.8	0.01	C6H12N2O4S2	241.0311	0.05	10.00	---	0.9974	0.03	0.11	104	17	---
Cytidine	10.0	0.01	C9H13N3O5	244.0928	0.01	0.50	U13C Cytidine	0.9982	0.05	0.16	102	13	---
Cytidine monophosphate	12.7	0.01	C9H14N3O8P	324.0591	0.01	1.00	U13C Cytidine monophosphate	0.9999	0.04	0.14	109	7	---
Cytosine	14.9	0.01	C4H5N3O	112.0505	0.01	5.00	U13C Cytosine	0.9998	0.01	0.04	106	3	---
Deoxyadenosine monophosphate	12.3	0.01	C10H14N5O6P	332.0754	0.05	5.00	---	1.0000	0.04	0.14	98	19	---
Deoxycytidine monophosphate	12.3	0.01	C9H14N3O7P	308.0642	0.01	5.00	---	0.9997	0.05	0.16	103	12	---
Glutamic acid	10.8	0.00	C5H9NO4	148.0604	0.05	10.00	U13C Glutamic acid	0.9971	0.02	0.07	88	2	---
Glutamine	8.1	0.01	C5H10N2O3	147.0764	0.05	10.00	U13C Glutamine	0.9974	0.02	0.05	90	2	---
Glutamylcysteine	11.3	0.01	C8H14N2O5S	251.0696	0.01	5.00	---	1.0000	0.04	0.12	117	43	---
Glutathione, oxidized	1.3	0.00	C20H32N6O12S2	613.1592	0.10	10.00	---	0.9988	0.10	0.35	118	8	---
Glutathione, reduced	11.0	0.01	C10H17N3O6S	308.0911	0.01	1.00	U13C Glutathione, reduced	0.9989	0.07	0.23	133	17	---
Glycine	8.3	0.01	C2H5NO2	76.0393	0.10	10.00	U13C Glycine	0.9946	0.11	0.36	90	2	---
Guanidinoacetic acid	9.1	0.00	C3H7N3O2	118.0611	0.01	0.50	---	0.9990	0.04	0.15	110	33	---
Guanine	6.7	0.01	C5H5N5O	152.0567	0.05	5.00	U13C Guanine	1.0000	0.03	0.12	105	4	---
Guanosine	6.3	0.01	C10H13N5O5	284.0989	0.01	1.00	U13C Guanosine	0.9972	0.05	0.18	108	8	---
Guanosine monophosphate	14.7	0.01	C10H14N5O8P	364.0653	0.01	5.00	---	0.9997	0.04	0.12	104	9	---
Hexanoyl-L-carnitine	9.4	0.01	C13H25NO4	260.1856	0.01	1.00	---	0.9997	0.02	0.07	103	7	---
Hexose monophosphates	13.3	0.01	C6H13O9P	261.0370	0.10	10.00	---	0.9999	0.09	0.29	107	13	Hexose-P disambig. not possible.
Histidine	0.9	0.01	C6H9N3O2	156.0768	0.01	1.00	---	0.9996	0.02	0.05	97	7	---

Compound	mean RT [min]	RT SD [min]	Neutral formula	pos m/z	pos linear range lower end [µmol/L]	pos linear range upper end [µmol/L]	pos internal standard	R^2 pos calibration curve	LOD pos [µmol/L]	LLOQ pos [µmol/L]	mean QC recovery pos [%]	QC recovery SD pos [%]	Comment
Homocysteine	1.1	0.01	C4H9NO2S	136.0427	0.05	1.00	---	0.9956	0.02	0.08	109	24	---
Homoserine + threonine	8.0	0.01	C4H9NO3	120.0655	0.01	10.00	U13C Homoser+Thr	0.9998	0.03	0.11	106	4	Homoser and Thr coelute.
Hydroxybupropion	6.2	0.01	C13H18ClNO2	256.1099	0.01	0.50	---	0.9999	0.04	0.15	89	8	---
Inosine	5.5	0.02	C10H12N4O5	269.0880	0.01	1.00	U13C Inosine	0.9976	0.09	0.29	100	6	---
Inosine monophosphate	13.4	0.01	C10H13N4O8P	349.0544	0.05	1.00	U13C Inosine monophosphate	0.9989	0.05	0.18	105	3	---
Isoguanosine	8.2	0.08	C10H13N5O5	284.0989	0.01	1.00	---	0.9999	0.07	0.25	116	28	---
Isoleucine	7.3	0.01	C6H13NO2	132.1019	0.50	10.00	U13C Isoleucine	0.9962	0.03	0.11	98	2	---
Kynurenine	7.1	0.01	C10H12N2O3	209.0921	0.01	10.00	---	0.9999	0.04	0.14	115	46	---
Leucine	7.1	0.01	C6H13NO2	132.1019	0.50	10.00	U13C Leucine	0.9949	0.03	0.10	98	2	---
Lysine	0.9	0.00	C6H14N2O2	147.1128	0.05	1.00	U13C Lysine	0.9961	0.04	0.14	111	3	---
Mannitol-1-phosphate	13.1	0.01	C6H15O9P	263.0526	0.05	10.00	---	1.0000	0.04	0.13	107	14	---
Melatonine	1.1	0.01	C13H16N2O2	233.1285	0.01	0.10	---	0.9980	0.03	0.10	64	6	Only 3 calibrators!
Methionine	7.2	0.00	C5H11NO2S	150.0583	0.05	5.00	U13C Methionine	0.9997	0.01	0.05	96	1	---
Methionine sulfone	7.4	0.01	C5H11NO4S	182.0482	0.05	1.00	---	0.9998	0.02	0.06	107	21	---
N4-Acetylcytidine	4.1	0.14	C11H15N3O6	286.1034	0.01	1.00	---	0.9975	0.05	0.17	100	23	---
NAD+	10.5	0.01	C21H27N7O14P2	664.1164	0.05	5.00	---	0.9996	0.03	0.11	109	16	---
NADP+	1.1	0.03	C21H28N7O17P3	744.0827	0.50	5.00	---	0.9993	0.08	0.25	112	19	Only 3 calibrators!
Nicotinamide	2.0	0.01	C6H6N2O	123.0553	0.01	5.00	U13C Nicotinamide	1.0000	0.00	0.02	99	1	---
Ornithine	0.9	0.00	C5H12N2O2	133.0972	0.05	5.00	U13C Ornithine	0.9992	0.03	0.11	94	2	---
Phenylalanine	6.9	0.01	C9H11NO2	166.0863	0.01	1.00	U13C Phenylalanine	0.9999	0.03	0.11	101	2	---
Phosphocreatine	1.0	0.01	C4H10N3O5P	212.0431	0.50	5.00	---	0.9995	0.07	0.24	108	15	Only 3 calibrators!
Piperine	10.0	0.01	C17H19NO3	286.1438	0.01	0.50	---	0.9998	0.01	0.03	84	8	---
Proline	7.4	0.01	C5H9NO2	116.0706	0.05	5.00	U13C Proline	0.9987	0.01	0.03	96	1	---
Propionylcarnitine	10.2	0.01	C10H19NO4	218.1387	0.01	5.00	---	1.0000	0.01	0.03	103	6	---

Compound	mean RT [min]	RT SD [min]	Neutral formula	pos m/z	pos linear range lower end [µmol/L]	pos linear range upper end [µmol/L]	pos internal standard	R^2 pos calibration curve	LOD pos [µmol/L]	LLOQ pos [µmol/L]	mean QC recovery pos [%]	QC recovery SD pos [%]	Comment
Pseudouridine	5.2	0.05	C9H12N2O6	245.0768	0.05	1.00	---	0.9989	0.10	0.35	129	28	Only 3 calibrators!
Quetiapine	7.2	0.01	C21H25N3O2S	384.1740	0.01	0.50	---	0.9999	0.08	0.26	96	8	---
Ribose-5-phosphate	13.2	0.01	C5H11O8P	231.0264	1.00	10.00	---	1.0000	---	---	111	19	Only 3 calibrators!
Ribulose-5-phosphate	13.6	0.01	C5H11O8P	231.0264	0.50	10.00	---	0.9999	0.08	0.25	107	19	---
S-Adenosylhomocysteine	10.1	0.01	C14H20N6O5S	385.1289	0.01	1.00	U13C S-Adenosylhomocysteine	0.9968	0.05	0.18	105	4	---
S-Adenosylmethionine	0.8	0.01	C15H22N6O5S	399.1445	0.05	1.00	---	0.9884	0.04	0.14	89	10	---
Sarcosine	7.7	0.06	C3H7NO2	90.0550	0.05	10.00	---	1.0000	0.08	0.28	151	76	---
Selenomethionine	7.3	0.01	C5H11NO2Se	198.0028	0.05	10.00	---	0.9995	0.06	0.21	118	47	---
Serine	8.3	0.00	C3H7NO3	106.0499	0.05	10.00	U13C Serine	0.9980	0.03	0.11	91	2	---
Serotonine	1.6	0.00	C10H12N2O	177.1022	0.01	1.00	---	0.9997	0.02	0.07	106	10	---
Spermidine	0.8	0.00	C7H19N3	146.1652	0.05	1.00	U13C Spermidine	0.9988	0.08	0.25	103	12	---
Spermine	0.9	0.01	C10H26N4	203.2230	0.50	5.00	---	0.9998	0.17	0.58	104	5	Only 3 calibrators!
Theobromine	2.4	0.03	C7H8N4O2	181.0720	0.01	1.00	---	0.9997	0.01	0.05	105	13	---
Threonine + homoserine	8.0	0.01	C4H9NO3	120.0655	0.01	10.00	U13C Homoser+Thr	0.9998	0.03	0.10	106	4	Homoser and Thr coelute.
Thymidine	1.6	0.01	C10H14N2O5	243.0975	0.50	10.00	---	1.0000	0.06	0.18	105	15	HILIC and RP peak overlap!
Thymidine monophosphate	12.1	0.01	C10H15N2O8P	323.0639	0.01	1.00	---	0.9994	0.03	0.10	103	13	---
Thymine	1.5	0.04	C5H6N2O2	127.0502	0.05	1.00	---	0.9998	0.01	0.04	98	10	HILIC and RP peak overlap!
Trazodone	6.9	0.01	C19H22ClN5O	372.1586	0.01	1.00	---	0.9995	0.07	0.25	96	12	---
Tryptophan	7.1	0.00	C11H12N2O2	205.0972	0.05	10.00	U13C Tryptophan	0.9992	0.02	0.07	95	2	---
Tyrosine	7.4	0.01	C9H11NO3	182.0812	0.01	1.00	U13C Tyrosine	0.9999	0.02	0.05	103	1	---
Uracil	1.9	0.08	C4H4N2O2	113.0346	0.10	10.00	---	0.9997	0.04	0.14	104	13	---
Urea	2.7	0.03	CH4N2O	61.0396	0.50	10.00	U13C Urea	0.9998	0.10	0.32	110	9	---
Uridine	3.1	0.10	C9H12N2O6	245.0768	0.50	10.00	---	0.9992	0.03	0.10	96	20	---

Compound	mean RT [min]	RT SD [min]	Neutral formula	pos m/z	pos linear range lower end [µmol/L]	pos linear range upper end [µmol/L]	pos internal standard	R^2 pos calibration curve	LOD pos [µmol/L]	LLOQ pos [µmol/L]	mean QC recovery pos [%]	QC recovery SD pos [%]	Comment
Uridine monophosphate	13.0	0.01	C9H13N2O9P	325.0431	0.05	5.00	U13C Uridine monophosphate	0.9985	0.02	0.07	99	4	---
Valine	7.5	0.01	C5H11NO2	118.0863	1.00	10.00	U13C Valine	0.9916	0.24	0.78	70	28	Only 3 calibrators!
Xanthine	4.7	0.01	C5H4N4O2	153.0407	0.01	5.00	---	1.0000	0.03	0.08	111	23	---

Table S3 Figures of merit for the presented dual-chromatography HPLC-HRMS method - negative ionization mode. The linear range of the calibration curve was evaluated visually. Additionally, calibrators that diverged > 15-20 % of the nominal concentration were discarded. Mean RT incl. SD and mean QC recovery incl. SD are calculated from five replicate injections of a 1 µmol/L standard. LOD and LLOQ are estimated from five replicate injections of a 0.5 µmol/L standard according to Eurachem (2014, see main text). Xenobiotics are marked in grey.

Compound	mean RT [min]	RT SD [min]	Neutral formula	neg m/z	neg linear range lower end [µmol/L]	neg linear range upper end [µmol/L]	neg internal standard	R^2 neg calibration curve	LOD neg [µmol/L]	LLOQ neg [µmol/L]	mean QC recovery neg [%]	QC recovery SD neg [%]	Comment
2'-Deoxycytidine	10.2	0.02	C9H13N3O4	226.0833	0.50	10.00	---	0.9999	0.05	0.16	102	4	---
2'-Deoxyuridine	2.0	0.05	C9H12N2O5	227.0673	0.05	10.00	---	0.9989	0.11	0.35	104	4	---
3'-Adenosine monophosphate	14.5	0.01	C10H14N5O7P	346.0558	0.50	10.00	---	0.9998	0.07	0.23	103	3	---
3-Methyl-2-oxovaleric acid	11.9	0.01	C6H10O3	129.0557	0.05	10.00	U13C 3-Methyl-2-oxovaleric acid	0.9990	0.07	0.23	96	3	---
3-Phosphoglycerate	1.1	0.01	C3H7O7P	184.9857	0.05	5.00	U13C 3-Phosphoglycerate	0.9975	0.06	0.19	73	21	Compound ID ambigous.
4-Hydroxy-proline	7.7	0.03	C5H9NO3	130.0510	0.05	5.00	---	0.9984	0.14	0.46	104	15	---
5'-Adenosine monophosphate	12.8	0.01	C10H14N5O7P	346.0558	0.10	5.00	U13C 5'-Adenosine monophosphate	0.9999	0.05	0.18	106	7	---
5'-Deoxy-5'-methylthioadenosine	4.2	0.03	C11H15N5O3S	296.0823	0.10	5.00	---	0.9999	0.13	0.44	110	9	---
5-Methyluridine	2.5	0.14	C10H14N2O6	257.0779	0.05	5.00	---	0.9999	0.09	0.31	102	6	---
Acetylsalicylic acid	6.8	0.00	C9H8O4	179.0350	0.50	10.00	---	0.9994	0.84	2.81	161	67	---
Adenine	8.0	0.01	C5H5N5	134.0472	0.01	5.00	U13C Adenine	0.9998	0.05	0.18	104	5	---
Adenosine	5.6	0.02	C10H13N5O4	266.0895	0.50	10.00	---	0.9989	0.25	0.85	109	20	---
Adenosine diphosphate	1.1	0.06	C10H15N5O10P2	426.0221	0.50	5.00	U13C Adenosine diphosphate	0.9968	0.14	0.47	105	9	Only 3 calibrators!
Alanine	---	---	C3H7NO2	88.0404	1.00	10.00	U13C Alanine	0.9974	---	---	---	---	Only 3 calibrators!
alpha-Aminoadipic acid	10.1	0.00	C6H11NO4	160.0615	0.05	10.00	---	0.9997	0.04	0.13	94	5	---
alpha-Ketoglutaric acid	1.2	0.00	C5H6O5	145.0142	0.05	10.00	---	0.9997	0.04	0.12	95	5	Coelution with unidentified compound.
alpha-Ketoisovaleric acid	12.3	0.01	C5H8O3	115.0401	0.05	10.00	---	0.9997	0.12	0.40	100	4	---
Argininosuccinic acid	11.2	0.00	C10H18N4O6	289.1154	0.50	10.00	U13C Argininosuccinic acid	0.9985	0.04	0.15	98	4	---
Asparagine	8.2	0.00	C4H8N2O3	131.0462	0.10	5.00	U13C Asparagine	0.9987	0.06	0.18	94	2	---

Compound	mean RT [min]	RT SD [min]	Neutral formula	neg m/z	neg linear range lower end [µmol/L]	neg linear range upper end [µmol/L]	neg internal standard	R^2 neg calibration curve	LOD neg [µmol/L]	LLOQ neg [µmol/L]	mean QC recovery neg [%]	QC recovery SD neg [%]	Comment
Aspartic acid	11.4	0.00	C4H7NO4	132.0302	0.05	5.00	U13C Aspartic acid	0.9987	0.03	0.08	98	5	---
Citric acid	1.3	0.00	C6H8O7	191.0197	0.01	10.00	U13C Citric acid	0.9995	0.01	0.05	99	1	Peak ID ambigous.
Citrulline	8.5	0.01	C6H13N3O3	174.0884	0.10	10.00	U13C Citrulline	0.9969	0.04	0.14	91	2	---
Cyclic adenosine monophosphate	11.3	0.01	C10H12N5O6P	328.0452	0.50	10.00	---	0.9996	0.05	0.16	115	11	---
Cyclic guanosine monophosphate	13.7	0.01	C10H12N5O7P	344.0402	0.10	5.00	---	0.9996	0.07	0.22	101	6	---
Cystathionine	9.8	0.01	C7H14N2O4S	221.0602	0.10	5.00	U13C Cystathionine	0.9999	0.05	0.18	100	1	---
Cysteic acid	12.4	0.00	C3H7NO5S	167.9972	0.05	1.00	---	0.9998	0.06	0.21	104	6	---
Cystine	9.8	0.02	C6H12N2O4S2	239.0166	0.50	10.00	---	0.9998	0.07	0.25	104	4	---
Cytidine	10.0	0.01	C9H13N3O5	242.0782	0.10	10.00	---	0.9994	0.06	0.19	96	6	---
Cytidine monophosphate	12.7	0.01	C9H14N3O8P	322.0446	0.05	1.00	---	0.9953	0.06	0.22	110	4	---
Deoxyadenosine monophosphate	12.3	0.01	C10H14N5O6P	330.0609	0.05	5.00	---	0.9998	0.07	0.25	98	5	---
Deoxycytidine monophosphate	12.3	0.01	C9H14N3O7P	306.0497	0.10	10.00	---	0.9999	0.06	0.19	97	3	---
Dihydroxyacetone phosphate	14.1	0.01	C3H7O6P	168.9907	0.50	10.00	---	0.9974	0.19	0.64	108	16	---
Dihydroxyisovaleric acid	11.1	0.01	C5H10O4	133.0506	0.05	5.00	U13C Dihydroxyisovaleric acid	0.9999	0.03	0.10	99	2	---
Flavinadenin dinucleotide	4.8	0.01	C27H33P2N9O15	784.1499	0.05	5.00	---	0.9988	0.05	0.16	95	4	Peak ID ambigous.
Fumaric acid	1.4	0.01	C4H4O4	115.0037	0.01	5.00	U13C Fumaric acid	0.9999	0.01	0.05	99	1	---
Gluconic acid	11.5	0.01	C6H12O7	195.0510	0.10	5.00	U13C Gluconic acid	0.9992	0.03	0.11	111	11	---
Glutamic acid	10.8	0.00	C5H9NO4	146.0459	0.10	5.00	U13C Glutamic acid	0.9993	0.06	0.19	96	2	---
Glutamine	8.1	0.01	C5H10N2O3	145.0619	0.05	10.00	U13C Glutamine	0.9976	0.03	0.09	92	2	---
Glutamylcysteine	11.3	0.01	C8H14N2O5S	249.0551	0.50	10.00	---	0.9976	0.11	0.38	115	12	---
Glutathione, reduced	11.0	0.01	C10H17N3O6S	306.0765	0.50	10.00	---	1.0000	0.20	0.66	149	68	---
Guanidinoacetic acid	9.1	0.00	C3H7N3O2	116.0466	0.50	10.00	---	0.9998	0.07	0.24	93	6	---
Guanine	6.7	0.02	C5H5N5O	150.0421	0.05	10.00	---	0.9994	0.16	0.53	110	18	---
Guanosine	6.3	0.00	C10H13N5O5	282.0844	0.05	5.00	---	1.0000	0.12	0.40	114	12	---

Compound	mean RT [min]	RT SD [min]	Neutral formula	neg m/z	neg linear range lower end [µmol/L]	neg linear range upper end [µmol/L]	neg internal standard	R^2 neg calibration curve	LOD neg [µmol/L]	LLOQ neg [µmol/L]	mean QC recovery neg [%]	QC recovery SD neg [%]	Comment
Guanosine monophosphate	14.7	0.01	C10H14N5O8P	362.0507	0.10	5.00	---	0.9959	0.07	0.25	95	5	---
Hexanoylcarnitine	9.4	0.02	C13H25NO4	258.1711	5.00	10.00	---	0.9612	0.21	0.71	18	8	---
Hexose monophosphates	13.3	0.00	C6H13O9P	259.0224	0.05	10.00	---	0.9997	0.08	0.28	94	4	Hexose-P disambig. not possible.
Hexoses	6.1	0.07	C6H12O6	179.0561	0.50	10.00	U13C Hexose	0.9964	0.27	0.91	100	14	Hexose dis-ambiguation not possible.
Histidine	0.9	0.01	C6H9N3O2	154.0622	0.05	5.00	U13C Histidine	0.9995	0.03	0.09	97	1	---
Homoserine + threonine	8.0	0.00	C4H9NO3	118.0510	0.05	10.00	U13C Homoser+Thr	0.9985	0.02	0.07	91	3	Homoser and Thr coelute.
Hydroxyglutaric acid	1.3	0.00	C5H8O5	147.0299	0.01	10.00	U13C Hydroxyglutaric acid	1.0000	0.01	0.05	99	1	---
Inosine	5.5	0.02	C10H12N4O5	267.0735	0.05	1.00	---	0.9999	0.56	1.87	132	40	---
Inosine monophosphate	13.4	0.01	C10H13N4O8P	347.0398	0.10	5.00	---	0.9991	0.07	0.23	98	2	---
Isocitric acid	1.1	0.00	C6H8O7	191.0197	0.05	1.00	---	0.9846	0.20	0.67	101	7	Peak ID ambigous.
Isoguanosine	8.2	0.01	C10H13N5O5	282.0844	0.05	5.00	---	0.9993	0.15	0.50	101	8	---
Isoleucine	7.3	0.01	C6H13NO2	130.0874	0.10	5.00	U13C Isoleucine	0.9997	0.06	0.20	91	5	---
Kynurenine	7.1	0.01	C10H12N2O3	207.0775	0.10	5.00	---	0.9993	0.10	0.34	103	18	---
Lactic acid	10.9	0.01	C3H6O3	89.0244	---	---	---	---	---	---	---	---	Calibration failed.
Leucine	7.1	0.00	C6H13NO2	130.0874	0.10	5.00	U13C Leucine	0.9994	0.12	0.41	95	4	---
Lysine	0.9	0.00	C6H14N2O2	145.0983	0.10	1.00	U13C Lysine	0.9806	0.05	0.17	69	2	Only 3 calibrators!
Malic acid	1.1	0.01	C4H6O5	133.0142	0.01	5.00	U13C Malic acid	0.9999	0.02	0.06	99	1	Peak ID ambigous.
Mannitol	6.1	0.00	C6H14O6	181.0718	0.05	5.00	U13C Mannitol	0.9996	0.07	0.24	107	7	Coelution with unidentified compound.
Mannitol-1-phosphate	13.1	0.01	C6H15O9P	261.0381	0.10	5.00	---	0.9992	0.05	0.16	95	5	---
Melatonine	1.2	0.01	C13H16N2O2	231.1139	0.50	10.00	---	0.9992	---	---	106	7	---
Methionine	7.3	0.01	C5H11NO2S	148.0438	0.05	5.00	U13C Methionine	0.9989	0.04	0.12	92	2	---

Compound	mean RT [min]	RT SD [min]	Neutral formula	neg m/z	neg linear range lower end [µmol/L]	neg linear range upper end [µmol/L]	neg internal standard	R^2 neg calibration curve	LOD neg [µmol/L]	LLOQ neg [µmol/L]	mean QC recovery neg [%]	QC recovery SD neg [%]	Comment
Methionine sulfone	7.4	0.01	C5H11NO4S	180.0336	0.05	5.00	---	0.9997	0.66	2.21	133	58	---
Mevalonic acid	9.1	0.00	C6H12O4	147.0663	0.05	5.00	U13C Mevalonic acid	0.9999	0.02	0.06	99	2	---
N4-Acetylcytidine	4.0	0.29	C11H15N3O6	284.0888	0.05	5.00	---	0.9989	0.03	0.09	95	4	---
N-Acetylaspartic acid	1.2	0.01	C6H9NO5	174.0408	0.01	1.00	---	0.9970	0.08	0.28	109	5	---
N-Acetylaspartylglutamic acid	2.6	0.13	C11H16N2O8	303.0834	0.05	10.00	---	0.9994	0.07	0.24	96	4	---
N-Acetylserine	11.0	0.00	C5H9NO4	146.0459	0.05	5.00	---	0.9996	0.23	0.75	108	16	---
NAD+	10.5	0.00	C21H27N7O14P2	662.1018	0.50	10.00	---	0.9991	0.09	0.32	107	3	---
NADH	1.3	0.00	C21H29N7O14P2	664.1175	0.50	10.00	U13C NADH	0.9997	0.07	0.23	109	4	---
Ornithine	0.9	0.01	C5H12N2O2	131.0826	0.05	10.00	U13C Ornithine	0.9995	0.04	0.12	99	2	---
Phenylalanine	6.9	0.01	C9H11NO2	164.0717	0.05	10.00	U13C Phenylalanine	0.9967	0.02	0.08	88	2	---
Proline	7.4	0.00	C5H9NO2	114.0561	1.00	10.00	U13C Proline	0.9965	---	---	128	16	Only 3 calibrators!
Pseudouridine	5.2	0.02	C9H12N2O6	243.0623	0.01	5.00	U13C Pseudouridine	0.9991	0.06	0.18	90	8	---
Pyruvic acid	12.7	0.01	C3H4O3	87.0088	0.05	10.00	U13C Pyruvic acid	0.9946	0.20	0.65	83	8	---
Ribose	3.6	0.08	C5H10O5	149.0455	0.50	5.00	---	0.9999	0.08	0.27	101	5	Only 3 calibrators!
Ribose-5-phosphate	13.2	0.00	C5H11O8P	229.0119	0.50	10.00	---	0.9998	0.05	0.16	96	6	---
Ribulose-5-phosphate	13.6	0.05	C5H11O8P	229.0119	0.50	10.00	---	0.9992	0.05	0.18	100	3	---
S-Adenosylhomocysteine	10.1	0.01	C14H20N6O5S	383.1143	0.10	10.00	---	0.9992	0.07	0.23	96	5	---
Salicin	4.0	0.03	C13H18O7	285.0980	0.10	10.00	---	0.9998	0.09	0.31	99	5	---
Salicylic acid	7.2	0.00	C7H6O3	137.0244	0.01	10.00	---	0.9999	0.30	0.99	129	29	---
Sedoheptulose-7-phosphate	13.3	0.01	C7H15O10P	289.0330	1.00	10.00	---	0.9987	0.13	0.42	111	8	Only 3 calibrators!
Selenomethionine	7.2	0.01	C5H11NO2Se	195.9882	0.50	5.00	---	0.9997	0.21	0.70	111	24	Only 3 calibrators!
Serine	8.3	0.01	C3H7NO3	104.0353	0.05	10.00	U13C Serine	0.9979	0.05	0.15	90	5	---
Succinic acid	1.5	0.00	C4H6O4	117.0193	0.01	5.00	U13C Succinic acid	1.0000	0.01	0.04	100	1	---
Threonine + homoserine	8.0	0.00	C4H9NO3	118.0510	0.05	10.00	U13C Homoser+Thr	0.9985	0.02	0.07	91	3	Homoser and Thr coelute.

Compound	mean RT [min]	RT SD [min]	Neutral formula	neg m/z	neg linear range lower end [µmol/L]	neg linear range upper end [µmol/L]	neg internal standard	R^2 neg calibration curve	LOD neg [µmol/L]	LLOQ neg [µmol/L]	mean QC recovery neg [%]	QC recovery SD neg [%]	Comment
Thymidine	1.9	0.03	C10H14N2O5	241.0830	0.01	5.00	---	0.9997	0.06	0.20	106	5	HILIC and RP peak overlap!
Thymidine monophosphate	12.1	0.01	C10H15N2O8P	321.0493	0.05	5.00	---	0.9990	0.07	0.25	95	5	---
Thymine	1.5	0.02	C5H6N2O2	125.0357	0.01	5.00	---	0.9996	0.03	0.10	107	6	HILIC and RP peak overlap!
Trehalose	7.4	0.02	C12H22O11	341.1089	1.00	10.00	---	0.9892	0.08	0.28	113	19	Only 3 calibrators!
Tryptophan	7.1	0.00	C11H12N2O2	203.0826	0.05	5.00	U13C Tryptophan	0.9992	0.04	0.14	92	1	---
Tyrosine	7.4	0.01	C9H11NO3	180.0666	0.05	10.00	U13C Tyrosine	0.9951	0.03	0.10	87	2	---
Uracil	1.8	0.03	C4H4N2O2	111.0200	0.05	5.00	---	0.9995	0.08	0.26	107	4	---
Uridine	3.1	0.02	C9H12N2O6	243.0623	0.05	10.00	---	1.0000	0.09	0.30	107	9	---
Uridine diphosphate	1.2	0.01	C9H14N2O12P2	402.9949	0.50	10.00	U13C Uridine diphosphate	1.0000	0.17	0.55	124	19	---
Uridine monophosphate	13.0	0.01	C9H13N2O9P	323.0286	0.10	10.00	---	0.9993	0.04	0.13	96	5	---
Valine	7.5	0.01	C5H11NO2	116.0717	0.50	10.00	U13C Valine	0.9951	0.13	0.44	97	8	---
Xanthine	4.7	0.01	C5H4N4O2	151.0261	0.01	1.00	---	0.9999	0.55	1.85	115	40	---
Xylose	4.9	0.06	C5H10O5	149.0455	0.50	10.00	---	0.9995	0.26	0.85	98	4	---

Table S4 Detected and quantified compounds of SRM 1950. Values are based on five replicate injections of the same analytical sample spiked with internal standard (^{13}C-yeast extract). Samples are diluted 1:10 during sample preparation. The table states the calculated concentrations for the biological sample, whereas LOD and LLOQ refer to the analytical sample concentration. Compounds the concentration of which exceeded ULOQ were quantified using the second most abundant isotopologue, if possible. CC: calibration curve, LOD: limit of detection, LLOQ: lower limit of quantification, ULOQ: upper limit of quantification (= highest concentrated calibrator of calibration curve). Xenobiotics are marked in grey.

Compound	RT [min]	Neutral formula	Polarity f. quant.	Adduct f. quant.	Isotopologue for quant.	m/z of selected adduct	LOD for selected polarity [µmol/L]	LLOQ for selected polarity [µmol/L]	Internal standard for quantification	R^2 of CC in selected polarity	Mean conc. [µmol/L]	Conc. RSD [%]	NIST cert./ref. values [µmol/L]	Comment
2-Carbamoylamino-butanedioic acid	1.10	C5H8N2O5	pos	[M+H]	M+0	177.0506	0.04	0.14	---	0.9979	<LLOQ	10.6	---	---
3-Methyl-2-oxovaleric acid	11.94	C6H10O3	neg	[M-H]	M+0	129.0557	0.08	0.27	---	0.999	16.4	1.7	---	---
4-Hydroxy-proline	7.67	C5H9NO3	neg	[M-H]	M+0	130.0510	0.14	0.46	---	0.9984	10.8	4.6	---	---
5'-Adenosine monophosphate	12.84	C10H14N5O7P	pos	[M+H]	M+0	348.0704	0.04	0.15	U13C 5'-Adenosine monophosphate	0.999	<LLOQ	44.5	---	---
5-Methyluridine	2.52	C10H14N2O6	neg	[M-H]	M+0	257.0779	0.09	0.31	---	0.9999	<LLOQ	1.9	---	Peak shape barely sufficient.
Adenosine	5.65	C10H13N5O4	pos	[M+H]	M+0	268.1040	0.01	0.02	U13C Adenosine	0.9996	<LLOQ	4.7	---	---
Alanine	8.00	C3H7NO2	pos	[M+H]	M+0	90.0550	0.11	0.35	U13C Alanine	0.9993	310	0.5	300 ± 26	---
alpha-Aminoadipic acid	10.14	C6H11NO4	pos	[M+H]	M+0	162.0761	0.01	0.03	U13C alpha-Aminoadipic acid	0.9998	0.61	9.3	---	---
Arginine	0.86	C6H14N4O2	pos	[M+H]	M+0	175.1190	0.02	0.08	U13C Arginine	0.9973	79.4	0.8	81.4 ± 2.3	---
Argininosuccinic acid	11.24	C10H18N4O6	pos	[M+H]	M+0	291.1299	0.01	0.03	U13C Argininosuccinic acid	0.9995	<LLOQ	27.1	---	---
Asparagine	8.25	C4H8N2O3	pos	[M+H]	M+0	133.0608	0.02	0.06	U13C Asparagine	0.9971	38.1	0.9	---	---
Aspartic acid	11.36	C4H7NO4	pos	[M+H]	M+0	134.0448	0.03	0.10	U13C Aspartic acid	0.9997	6.94	8.3	---	---
Caffeine	5.07	C8H10N4O2	pos	[M+H]	M+0	195.0877	0.06	0.22	---	1	4.50	5.7	---	---
Citrulline	8.47	C6H13N3O3	pos	[M+H]	M+0	176.1030	0.01	0.03	U13C Citrulline	0.9986	28.6	0.2	---	---
Cystathionine	9.83	C7H14N2O4S	pos	[M+H]	M+0	223.0747	0.02	0.07	U13C Cystathionine	0.9992	<LLOQ	15.1	---	---
Cystine	9.81	C6H12N2O4S2	pos	[M+H]	M+0	241.0311	0.03	0.11	---	0.9974	17.1	3.8	7.8 ± 0.4	---
Cytidine	10.02	C9H13N3O5	pos	[M+H]	M+0	244.0928	0.05	0.16	U13C Cytidine	0.9982	<LLOQ	5.6	---	---
Cytosine	14.87	C4H5N3O	pos	[M+H]	M+0	112.0505	0.01	0.04	U13C Cytosine	0.9998	<LLOQ	32.7	---	---
Deoxyadenosine monophosphate	12.28	C10H14N5O6P	neg	[M-H]	M+0	330.0609	0.07	0.25	---	0.9998	<LLOQ	14.9	---	---
Fumaric acid	1.44	C4H4O4	neg	[M-H]	M+0	115.0037	0.01	0.05	U13C Fumaric acid	0.9999	0.57	2.4	---	---

Compound	RT [min]	Neutral formula	Polarity f. quant.	Adduct f. quant.	Isotopo-logue for quant.	m/z of selected adduct	LOD for selected polarity [µmol/L]	LLOQ for selected polarity [µmol/L]	Internal standard for quantification	R^2 of CC in selected polarity	Mean conc. [µmol/L]	Conc. RSD [%]	NIST cert./ref. values [µmol/L]	Comment
Gluconic acid	11.53	C6H12O7	neg	[M-H]	M+0	195.0510	0.03	0.11	U13C Gluconic acid	0.9992	4.07	11.0	---	---
Glutamic acid	10.84	C5H9NO4	pos	[M+H]	M+0	148.0604	0.02	0.07	U13C Glutamic acid	0.9971	70.4	0.7	---	massive carryover ($160*10^3$) in blank after SRM
Glutamine	8.12	C5H10N2O3	pos	[M+H]	M+1	148.0791	0.02	0.05	U13C Glutamine	0.9974	456	1.2	---	massive carryover ($90*10^3$) in blank after SRM
Glutamylcysteine	11.35	C8H14N2O5S	pos	[M+H]	M+0	148.0791	0.04	0.12	---	1	<LLOQ	---	---	carryover ($10*10^3$) in blank after SRM
Glutathione, reduced	11.04	C10H17N3O6S	pos	[M+H]	M+0	148.0791	0.07	0.23	U13C Glutathione, reduced	0.9989	<LLOQ	---	---	carryover ($10*10^3$) in blank after SRM
Glycine	8.27	C2H5NO2	pos	[M+H]	M+0	148.0791	0.11	0.36	U13C Glycine	0.9946	>ULOQ	---	245 ± 16	> ULOQ, but no isotop. present; carryover ($10*10^3$) in blank after SRM
Guanidinoacetic acid	9.10	C3H7N3O2	pos	[M+H]	M+0	148.0791	0.04	0.15	---	0.999	2.37	3.8	---	---
Guanosine	6.28	C10H13N5O5	pos	[M+H]	M+0	284.0989	0.05	0.18	U13C Guanosine	0.9972	<LLOQ	---	---	---
Guanosine monophosphate	14.72	C10H14N5O8P	pos	[M+H]	M+0	364.0653	0.04	0.12	---	0.9997	<LLOQ	---	---	---
Hexanoylcarnitine	9.36	C13H25NO4	pos	[M+H]	M+0	148.0791	0.02	0.07	---	0.9997	<LLOQ	---	---	---
Hexoses	6.06	C6H12O6	neg	[M-H]	M+0	179.0561	0.27	0.91	U13C Hexose	0.9964	>ULOQ	---	4560 ± 56	hexose disambig. not possible. >>ULOQ, but (M+1) quant. even worse.
Histidine	0.87	C6H9N3O2	pos	[M+H]	M+0	148.0791	0.02	0.05	---	0.9996	>ULOQ	---	72.6 ± 3.6	> ULOQ, but no isotopol. present
Homocysteine	1.07	C4H9NO2S	pos	[M+H]	M+0	136.0427	0.02	0.08	---	0.9956	2.62	6.8	8.5 ± 0.2	---
Hydroxybupropion	6.15	C13H18ClNO2	pos	[M+H]	M+0	256.1099	0.04	0.15	---	0.9999	<LLOQ	---	---	---
Hydroxyglutaric acid	1.32	C5H8O5	neg	[M-H]	M+0	147.0299	0.01	0.05	U13C Hydroxyglutaric acid	1	0.49	10.4	---	---
Isoleucine	7.29	C6H13NO2	pos	[M+H]	M+0	132.1019	0.03	0.11	U13C Isoleucine	0.9962	60.4	1.4	55.5 ± 3.4	---
Kynurenine	7.13	C10H12N2O3	pos	[M+H]	M+0	209.0921	0.04	0.14	---	0.9999	<LLOQ	---	---	---
Lactic acid	10.87	C3H6O3	neg	[M-H]	M+0	89.0244	0.21	0.71	U13C Lactic acid	0.8647	2509	3.2	---	---

Compound	RT [min]	Neutral formula	Polarity f. quant.	Adduct f. quant.	Isotopo-logue for quant.	m/z of selected adduct	LOD for selected polarity [µmol/L]	LLOQ for selected polarity [µmol/L]	Internal standard for quantification	R^2 of CC in selected polarity	Mean conc. [µmol/L]	Conc. RSD [%]	NIST cert./ref. values [µmol/L]	Comment
Leucine	7.13	C6H13NO2	pos	[M+H]	M+0	132.1019	0.03	0.10	U13C Leucine	0.9949	89.0	0.8	100.4 ± 6.3	---
Lysine	0.86	C6H14N2O2	pos	[M+H]	M+0	147.1128	0.04	0.14	U13C Lysine	0.9961	174	7.2	140 ± 14	---
Malic acid	1.15	C4H6O5	neg	[M-H]	M+0	133.0142	0.02	0.06	U13C Malic acid	0.9999	4.88	1.1	---	Unident. compound coelutes.
Mannitol	6.12	C6H14O6	neg	[M-H]	M+0	181.0718	0.07	0.24	U13C Mannitol	0.9996	3.52	7.4	---	---
Methionine	7.25	C5H11NO2S	pos	[M+H]	M+0	150.0583	0.01	0.05	U13C Methionine	0.9997	23.4	1.0	22.3 ± 1.8	---
Methionine sulfone	7.37	C5H11NO4S	neg	[M-H]	M+0	180.0336	0.66	2.21	---	0.9997	<LLOQ	---	---	---
N4-Acetylcytidine	4.10	C11H15N3O6	pos	[M+H]	M+0	286.1034	0.05	0.17	---	0.9975	<LLOQ	---	---	---
N-Acetylaspartic acid	1.20	C6H9NO5	neg	[M-H]	M+0	174.0408	0.08	0.28	---	0.997	<LLOQ	---	---	Peak shape barely sufficient.
Nicotinamide	1.99	C6H6N2O	pos	[M+H]	M+0	123.0553	0.00	0.02	U13C Nicotinamide	1	<LLOQ	---	---	---
Ornithine	0.86	C5H12N2O2	pos	[M+H]	M+1	134.0999	0.03	0.11	U13C Ornithine	0.9992	47.9	8.3	---	Peak ID correct?
Phenylalanine	6.94	C9H11NO2	pos	[M+H]	M+1	167.0894	0.03	0.11	U13C Phenylalanine	0.9999	54.2	0.8	51 ± 7	---
Piperine	9.96	C17H19NO3	pos	[M+H]	M+0	286.1438	0.01	0.03	---	0.9998	<LLOQ	---	---	---
Proline	7.37	C5H9NO2	pos	[M+H]	M+1	117.0737	0.01	0.03	U13C Proline	0.9987	176	0.5	177 ± 9	---
Propionylcarnitine	10.22	C10H19NO4	pos	[M+H]	M+0	218.1387	0.01	0.03	---	1	2.87	1.6	---	---
Pseudouridine	5.25	C9H12N2O6	neg	[M-H]	M+0	243.0623	0.06	0.18	U13C Pseudouridine	0.9995	2.48	2.6	---	---
Pyruvic acid	12.75	C3H4O3	neg	[M-H]	M+0	87.0088	0.20	0.65	U13C Pyruvic acid	0.9991	230	3.3	---	---
Quetiapine	7.16	C21H25N3O2S	pos	[M+H]	M+0	384.1740	0.08	0.26	---	0.9999	<LLOQ	---	---	---
Ribose	3.56	C5H10O5	neg	[M-H]	M+0	149.0455	0.08	0.27	---	0.9999	<LLOQ	---	---	Peak shape barely sufficient.
S-Adenosyl-homocysteine	10.13	C14H20N6O5S	pos	[M+H]	M+0	385.1289	0.05	0.18	U13C S-Adenosyl-L-homocysteine	0.9968	<LLOQ	---	---	SRM-conc.only carryover from standard?
Salicylic acid	7.23	C7H6O3	neg	[M-H]	M+0	137.0244	0.30	0.99	---	0.9999	<LLOQ	---	---	---
Sarcosine	7.70	C3H7NO2	pos	[M+H]	M+0	90.0550	0.08	0.28	---	1	<LLOQ	---	---	---
Serine	8.26	C3H7NO3	pos	[M+H]	M+0	106.0499	0.03	0.11	U13C Serine	0.998	96.1	0.9	95.9 ± 4.3	---
Succinic acid	1.49	C4H6O4	neg	[M-H]	M+0	117.0193	0.01	0.04	U13C Succinic acid	1	3.33	0.3	---	---
Theobromine	2.44	C7H8N4O2	pos	[M+H]	M+0	181.0720	0.01	0.05	---	0.9997	3.93	3.0	---	---

Compound	RT [min]	Neutral formula	Polarity f. quant.	Adduct f. quant.	Isotopologue for quant.	m/z of selected adduct	LOD for selected polarity [µmol/L]	LLOQ for selected polarity [µmol/L]	Internal standard for quantification	R^2 of CC in selected polarity	Mean conc. [µmol/L]	Conc. RSD [%]	NIST cert./ref. values [µmol/L]	Comment
Threonine + homoserine	7.98	C4H9NO3	pos	[M+H]	M+0	120.0655	0.03	0.10	U13C Threonine + homoserine	0.9998	36.2	0.4	119.5 ± 6.1	Homoser and Thre coelute, proper quant. not possible.
Trazodone	6.90	C19H22ClN5O	pos	[M+H]	M+0	372.1586	0.07	0.25	---	0.9995	<LLOQ	---	---	---
Tryptophan	7.13	C11H12N2O2	pos	[M+H]	M+1	206.1002	0.02	0.07	U13C Tryptophan	0.9992	42.3	1.5	---	---
Tyrosine	7.43	C9H11NO3	pos	[M+H]	M+1	183.0844	0.02	0.05	U13C Tyrosine	0.9999	59.2	1.5	57.3 ± 3	---
Uracil	1.83	C4H4N2O2	neg	[M-H]	M+0	111.0200	0.08	0.26	---	0.9995	<LLOQ	---	---	Very weak, peak shape barely sufficient.
Urea	2.68	CH4N2O	pos	[M+H]	M+0	61.0396	0.10	0.32	U13C Urea	0.9998	>ULOQ	---	3900 ± 80	> ULOQ, but no isotopologue present.
Uridine	3.08	C9H12N2O6	neg	[M-H]	M+0	243.0623	0.09	0.30	---	1	4.01	0.8	---	---
Uridine monophosphate	13.04	C9H13N2O9P	pos	[M+H]	M+0	325.0431	0.02	0.07	U13C Uridine monophosphate	0.9985	<LLOQ	---	---	---
Valine	7.51	C5H11NO2	neg	[M-H]	M+0	116.0717	0.13	0.44	U13C Valine	0.9951	>ULOQ	---	182.2 ± 10.4	> ULOQ, but (M+1) quant. results even less accurate.
Xanthine	4.74	C5H4N4O2	pos	[M+H]	M+0	153.0407	0.03	0.08	---	1	<LLOQ	---	---	---