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Analytical method development in liquid chromatography- mass spectrometry based metabolomics

IDA ERNGREN

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Abstract

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Metabolomics is the analytical field which aims at analyzing all small molecules, metabolites, in a biological system simultaneously. Currently no analytical methods are able to capture the entire metabolome, therefore, the analytical methods are often developed to be as general as possible. However, as research within the metabolomics field is generally driven by biological questions method development is often overlooked. Moreover, method development in metabolomics is very challenging, as evaluation of the methods are difficult since they are not developed for any particular metabolites. Method development is very important though, data quality and accuracy of relative quantitations is paramount if metabolomics is to be used to answer the biological questions at hand.

The articles included in the thesis focus around both analytical method development and applications of metabolomics. In the first paper, head and neck cancer cell lines with different sensitivity to ionizing radiation was investigated using LC-MS based metabolomics. A theory on how the radiation resistant (UM-SCC-74B) cell line could alter its metabolism to handle redox status, DNA repair and DNA methylation was formulated. In the second article the sampling of sponge samples (Geodia barretti) was investigated with regard to its effects on detected metabolite profiles and data quality. It was found that freezing the samples directly was the best alternative which allowed for analysis of most metabolite classes. Storing the samples in solvent lead to a substantial extraction of metabolites to the solvent. For metabolomics, the solvents were more useful than the actual sponge samples that had been stored in solvent. In article three the problems caused by high concentrations of inorganic ions in biological samples in HILIC-ESI-MS analyses was described. The inorganic ions can affect relative quantitation and lead to erroneous results and overly complicated datasets inflated by the extra signals caused by cluster formation. To mitigate the problems caused by the inorganic ions a sample preparation method was developed in article four. The method used cation exchange SPE to trap alkali metal ions which, resulted in less ion-suppression, higher signal intensities of relevant metabolites as well as reduced adduct and cluster formation.

In conclusion, this thesis have described projects where metabolomics have been applied to answer biological questions as well as analytical method development in LC-MS based metabolomics. Limitations with current methods was described and possible solutions to improve the methods has been presented.

Keywords: Analytical pharmaceutical chemistry, metabolomics, liquid chromatography, mass spectrometry, hydrophilic interaction liquid chromatography

Ida Erngren, Department of Medicinal Chemistry, Analytical Pharmaceutical Chemistry, Box 574, Uppsala University, SE-75123 Uppsala, Sweden.

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List of Papers

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

I Lindell-Jonsson, E.*, **Erngren, I.***, Engskog, M., Haglöf, J., Arvidsson, T., Hedeland, M., Pettersson, C., Laurell, G., Nestor, M. (2019), Exploring radiation response in two head and neck squamous carcinoma cell lines through metabolic profiling, *Frontiers in Oncology*, 9: 1-16. * Equal contribution

II **Erngren, I.**, Smit, E., Pettersson, C., Cardenas, P., Hedeland, M. (2020) The effects of sampling and storage conditions on the metabolite profile of marine sponge *Geodia barretti*. *In manuscript*

III **Erngren, I.**, Haglöf, J., Engskog, M., Nestor, M., Hedeland, M., Arvidsson, T., Pettersson, C. (2019), Adduct formation in electrospray ionisation-mass spectrometry with hydrophilic interaction liquid chromatography is strongly affected by the inorganic ion concentration of the samples, *Journal of chromatography A*, 1600: 174-185

IV **Erngren, I.**, Nestor, M., Pettersson, C., Hedeland, M. (2020) Improved sensitivity in hydrophilic interaction liquid chromatography-electrospray-mass spectrometry after removal of sodium and potassium ions from biological samples, *Submitted manuscript*

Additional papers not included in this thesis:

Niklison-Chirou, M. V., **Erngren, I.**, Engskog, M., Haglöf, J., Picard, D., Remke, M., Redmond McPolin, P. H., Selby, M., Williamson, D., Clifford, S. C., Michod, D., Hadjiandreou, M., Arvidsson, T., Pettersson, C., Melino, G., Marino, S. (2017) TAp73 is a marker of glutamine addiction in medulloblastoma, *Genes and development*, 31: 1738-1753

Elmsjö, A., Haglöf, J., Engskog, M. K. R., **Erngren, I.**, Nestor, M., Arvidsson, T., Pettersson, C., (2018), Selectivity evaluation using the co-feature ratio in LC/MS metabolomics: Comparison of HILIC stationary phase performance for the analysis of plasma, urine and cell extracts, *Journal of Chromatography A*, 1568: 49-56

Contents

Abbreviations

EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionization
HILIC	Hydrophilic interaction liquid chromatography
LC	Liquid chromatography
LLE	Liquid-liquid extraction
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MVDA	Multivariate data analysis
OPLS-DA	Orthogonal projection to latent structures – discriminant analysis
PBS	Phosphate buffered saline
PCA	Principal component analysis
RP	Reversed phase
SPE	Solid phase extraction
UHPLC	Ultra high performance liquid chromatography

Introduction

Metabolomics is the analytical field which aims at analyzing all small molecules, metabolites, in a biological system simultaneously [1]. The comprehensive analysis of all small molecules in a biological system, the metabolome, is thought to reflect the sample phenotype better than the genome, transcriptome or proteome as the metabolites are the downstream products in a biological system [2]. However, the metabolome also interacts with, and regulate processes on protein, RNA and DNA levels, meaning that the metabolome could be considered both a regulator of the phenotype as well as biomarker of the phenotype [3]. Metabolomics can be conducted as untargeted analyses without any prior selection of metabolites or as targeted analyses of a selection of metabolites, however, in this thesis metabolomics will refer to untargeted methods.

Often metabolomics is referred to as a method for biomarker discovery, though, it has also been widely used to study biochemical processes [4,5], disease development [6–9], mode of action of drugs [10–13], toxicity [14,15] and bioengineering [16,17]. Despite the large number of studies in biomarker discovery, very few of the discovered biomarkers are validated and no biomarkers discovered using metabolomics are used clinically today [18–20]. However, there are many examples of small molecular biomarkers that are used clinically for diagnosis, screening of inborn errors of metabolism and to assess risk of future health issues [20]. Identifying small molecular biomarkers are complicated as the discovered biomarkers are often common metabolites, involved in numerous metabolic processes, which make it difficult to link them to certain disease states. Moreover, many metabolite concentrations change with age or other normal physiological changes, making the biomarker discovery even more complicated. This means that often multiple biomarkers or patterns of biomarkers are needed to differentiate between disease states, however, that is an important strength of metabolomics where large number of metabolites can be screened simultaneously.

The vast range of metabolites making up the metabolome put huge demands on the analytical methods used within metabolomics. The metabolites range in physicochemical properties such as hydrophilicity/lipophilicity, acidity/basicity and molecular structure as well as the concentration range in which they are found in biological systems. Liquid chromatography - mass spectrometry (LC-MS) has become the most widely used technique for metabolomics due to its sensitivity, selectivity and the possibilities to use different

chromatographic techniques as well as ionization modes to widen the metabolite coverage [21]. Using LC-MS it is common to combine reversed phase (RP) and hydrophilic interaction liquid chromatography (HILIC) in both positive and negative ionization mode to widen the metabolome coverage. However, despite the combination of chromatographic modes and different ionization polarities, the entire metabolome of any biological system cannot be covered.

Method development in metabolomics is very challenging as the outcome goals is not always obvious. Should method development in metabolomics strive towards greater coverage of the metabolome, or towards better data quality, or possibly both? Moreover, how should the methods be evaluated and validated as they are not developed for any particular metabolites. To date no validation guidelines for analytical methods used in metabolomics exist, even though validation and standardization of the analytical methods used in metabolomics have been called for [22,23]. Furthermore, all choices regarding sample collection, preparation, extraction, storage, analysis, data processing and data analysis will affect the final outcome, which, is true for targeted and untargeted analyses alike. However, in untargeted analyses those changes are difficult to evaluate as the exact composition of the sample is often unknown, making the method development process difficult to overview. Often methods for metabolomics are evaluated by counting the number of detected features (*m/z* retention time pairs), or the number of reliable features (based on CV %), assuming that more features equals more detected metabolites and better metabolome coverage. However, more features do not necessarily mean more detected metabolites but rather more adduct formation, cluster formation or higher degree of in-source fragmentation and is therefore a blunt tool for evaluation of method suitability. Some efforts have been made to develop tools for evaluating untargeted analytical methods, although no have been widely recognized [24,25].

As metabolomics studies are often conducted with the biological questions and applications in focus rather than analytical chemistry or method development, the method development has often been overlooked. However, if metabolomics is to ever reach its promised potential, method development will be an important factor for many years to come.

Aims

The overall aim of this doctoral project was to apply liquid chromatography – mass spectrometry (LC-MS) based metabolomics in interdisciplinary projects as well as to characterize and develop the sampling, sample handling and analytical methods for metabolomics.
More specifically the aim in each paper was to

- Investigate the differences in metabolic response to ionizing radiation in head and neck cancer cell lines with different radiation sensitivity/resistance using LC-MS based metabolomics. Furthermore, the overall aim was to elucidate the metabolic mechanisms behind radiation resistance and to suggest possible new ways of sensitizing head and neck cancer cells to irradiation (**I**).
- Investigate how sampling and sample storage techniques of sponge samples affect the detected metabolite profiles and the data quality for metabolomics studies as well as to provide recommendations for future metabolomics studies of sponge samples (**II**).
- Describe the effects of high sample concentrations of inorganic ions in HILIC-ESI-MS and its implications for untargeted metabolomics analyses (**III**)
- Present a sample preparation method to mitigate the problems caused by inorganic ions in biological samples in HILIC-ESI-MS analyses (**VI**).

The metabolomics work flow

The metabolomics work flow can be summarized by four major steps 1) sample generation and collection, 2) sample preparation, 3) analysis and finally 4) data analysis and biological interpretation (*Figure 1*). The following discussion will focus on those four steps of the metabolomics work flow as well as discuss the methods and results of the four papers included in this thesis in relation with the respective steps they cover. Under the section about sample generation and collection, paper **II** is discussed in more detail as the aim of the project focused around the sample collection of marine sponges, however, cell culture and collection of plasma are discussed as well (paper **I**, **III** and **IV**). Paper **IV** is discussed in more detail under the section about sample preparation while paper **III** is discussed mainly under the section about LC-MS analysis. Paper **I** is discussed more in depth under the section data analysis and biological interpretation as the aim of the project was focused around the biological interpretation of the results. However, as all projects in some way involve all steps from sample preparation to data analysis they will be discussed briefly under all sections. This discussion about the metabolomics workflow will focus around LC-MS being the main technique for the analysis of samples and the selection of collection techniques, sample preparation methods as well as data analysis methods will be discussed for their suitability in combination with LC-MS.

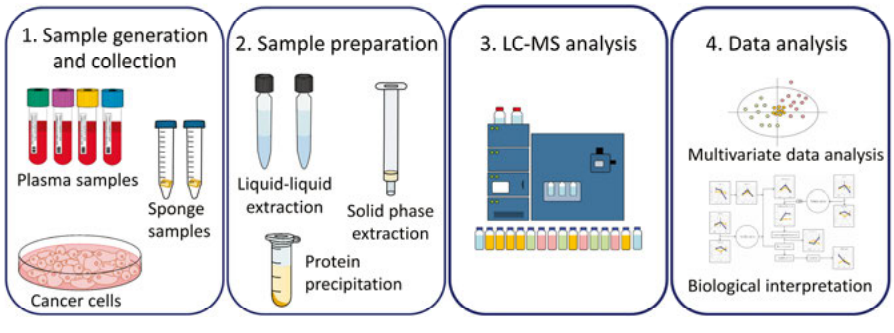

Figure 1. Schematic figure describing the metabolomics workflow and the main steps to a metabolomics analysis.

Sample generation and collection

The sample generation and collection is the first step of any metabolomics study, except for the study design. Choosing a suitable collection method depending on the investigated sample material can have large effects not only on the variation and data quality but also on what metabolites are detected and the concentrations of metabolites [26,27]. It is also important to quickly stop the metabolism after sample collection to reduce variation between samples as well as metabolite degradation or transformation. For the most commonly used sample materials in metabolomics, such as blood, urine and cells different protocols have been evaluated and discussed, however, no guidelines or consensus have been reached regarding the most suitable methods for sample handling [28–36]. Blood samples are most often separated as soon as possible upon collection to either serum or plasma. The differences and possible advantages with using either serum or plasma as well as the differences between the anti-coagulants used for plasma have been studied extensively [28,29,35]. Higher metabolite concentrations have been reported for serum, while issues with higher variation between samples as well as more background ions have been reported as the drawbacks [33,35–37]. Plasma samples are generally considered to be more reproducible, however, the anticoagulants added to the samples have been reported to cause ion-suppression and the most commonly used anticoagulants all add to the inorganic ion content of the samples (**IV**), heparin is added as a lithium salt, EDTA is often added as its respective potassium salt and citrate is added as its sodium salt [35,36]. The addition of inorganic ions can affect the ionization as well as the adduct formation (**III, IV**) [35,38].

Primary cells as well as cell lines are often used in metabolomics to study metabolic responses to stimuli such as toxins [39], pharmaceuticals [13,40,41], gene knock out or enzyme inactivation [42,43] or as in paper **I**, the metabolic response to ionizing radiation. For cell metabolomics, choosing the appropriate cell model for the question at hand is very important, as well as choosing the appropriate growth medium and culture conditions. An important factor to consider is the number of cells, and the differences in number of cells between the samples, which should be harmonized as far as possible for a fair comparison of relative abundances of metabolites. A problem that can arise if cells in one or several sample groups are treated with e.g. cytotoxic substances or ionizing radiation is that the survival rate between sample groups can affect the cell density between sample groups and thus the metabolite concentrations of the final samples [44].

In cell metabolomics, both the extracellular metabolome, i.e. the growth medium or rather time dependent changes in the growth medium, as well as the intracellular metabolome can be analyzed [44]. Analyzing the extracellular metabolome can give information about the excretion of metabolites from the

cells as well as uptake from the growth medium, which, reflect upon the metabolic state of the cells and the continuous transport in and out of the cells [44,45]. The intracellular metabolome is the more common choice for metabolomics and often mean that the cells are separated from the growth medium as well as rinsed to remove as much growth medium as possible before cell lysis and metabolite extraction. Phosphate buffered saline (PBS) is the most commonly used solution for rinsing the cells to avoid cell rupture and metabolite leaching. However, the PBS contains high concentrations of non-volatile salts e.g. sodium phosphate and NaCl, which adds to the inorganic ion content of the samples and can have large effects on the LC-MS analysis further on. Intracellularly the concentration of K^+ is much higher than the concentration of Na^+, however, in the cell samples which were analyzed in the studies described in paper **I**, **III** and **IV**, the concentration of Na^+ was much higher than the concentration of K^+, indicating that the large amounts of Na^+ were added to the samples during cell harvesting. The effects of high concentrations of Na^+ ions in cell samples analyzed with HILIC-ESI-MS was described in paper **III**, which is discussed in more detail under the LC-MS section. There are alternatives to rinsing the cells with saline, e.g. water or more MS-friendly buffers as ammonium acetate [34,46]. After separating the cells from growth medium, the cell metabolism is quickly quenched, often using liquid nitrogen or addition of organic solvents and the cells are lysed by freeze-thaw cycles or ultra-sonication [44].

Sponges can be found in almost all marine and fresh-water habitats, they are primitive, filter-feeding animals thought to be among the first multicellular life-forms to inhabit the earth [47]. Sponges have gained a lot of attention due to the diversified and pharmacologically interesting metabolites they produce as well as their importance for many eco-systems [48–53]. For sponges no guidelines or recommendations for sample collection for metabolomics studies exist. Currently the samples are collected in several different ways, either the samples are frozen directly upon collection, frozen after collection, however not always stated how long after collection and a third common sampling technique is to store the samples in ethanol directly upon collection [54–59]. Storage of the samples in ethanol is the most common way historically, often used by biologists to fixate the samples and remove as much water as possible for future histology examinations and DNA extractions. When storing the samples in ethanol it is common to exchange the solvent repeatedly a few hours after collection to reduce the water content of the samples as much as possible, the exchanged solvents are discarded. During metabolite extraction for metabolomics the ethanol is often filtered away where after the sponge is freeze-dried and then extracted. We hypothesized that a substantial part of the metabolites would be extracted to the ethanol and that exchanging the solvent repeatedly and discarding it would lead to substantial depletion of metabolites from the sponge samples (**II**).

The effects of sampling and storage of sponge samples for metabolomics was therefore investigated in paper **II**. Three specimens of the marine deep-sea sponge *Geodia barretti* (Geodiidae family, Tetractinellida order) were collected at the Swedish west-coast in Krugglöbranten, Koster sea outside the marine station at Tjärnö (*Figure 2*). Upon collection of the sponges, pieces approximately 1×0.5×0.5 cm where sampled from the interior of the sponges and directly stored in either ethanol or methanol, or flash frozen using liquid nitrogen alternatively frozen later at the marine station upon arrival after the collection cruise. From each of the three sponge specimens, three pieces were taken for each treatment. The ethanol and methanol was exchanged once after collection but the solvents were saved and analyzed to investigate what had been extracted to the solvent from the fresh sponges.

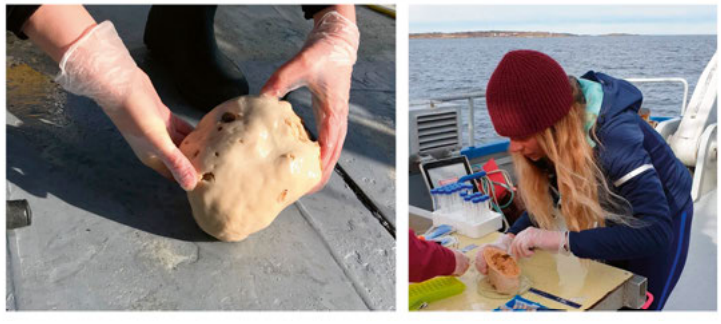

Figure 2. To the left; photograph of sponge Geodia barretti just fresh out of the water being prepared for measuring and photographing before sampling (photo by Ida Erngren). To the right; sampling from the interior of the sponge using a scalpel and tweezers (photo by Karin Steffen).

There was a substantial extraction of metabolites from the sponge samples to the fixation solvents, ethanol or methanol, and there was a substantial decrease in the number of metabolites that could be found in the second extracts as well as in the sponge samples that had been stored in solvent (**II**). The frozen samples together with the first extracts of the sponge samples were the most metabolite rich samples. These results highlight the importance for biologists that collect specimens in ethanol to not discard the ethanol extracts as they contain much valuable information on the sponge metabolome. Furthermore, the results also show that ethanol preserved specimens still hold a value and can be used for chemistry studies, especially if lipids or peptides are of interest (**II**). Our results should increase the value of museum collections, not only for the unique and diverse specimens they hold but also for the ethanol preserving these same specimens. However, it should be stressed that we did not look into long term stability of metabolites in neither ethanol preserved samples or frozen samples, which needs to be evaluated before samples are compared.

Even though the specimens collected in ethanol could be used for metabolite profiling, they should not be used for relative quantifications. The relative abundances in different extracts or samples may not necessarily reflect the initial concentrations of the samples depending on the number of extracts made from the sponges, the ratios between solvent and sponge (volume to weight) etc. (**II**).

The variation between samples was the lowest in the flash frozen samples, followed by the frozen samples (frozen at marine station). However, the difference in variation between the flash frozen and frozen samples were surprisingly small. The samples that were frozen at the marine station were stored in ambient temperature on the boat deck in quite different time periods, ranging from 45 min to almost 2 hours in contrast to the flash frozen samples that were frozen directly after collection and then kept frozen until extraction (**II**).

Sample preparation

An ideal sample preparation for metabolomics should be as general as possible and extract all metabolites from a biological sample while removing interferences that can hamper the analysis. In reality there is always a compromise between extracting as much of the metabolome as possible and removing enough interferences to avoid any detrimental effects on the down-stream LC-MS analysis. Removing proteins from the samples prior to the LC-MS analysis can reduce the risk for LC column degradation and retention time drift, while other components as inorganic ions can cause issues with the electrospray ionization and matrix effects. Moreover, the large differences in physicochemical properties e.g., polarity, protolytic properties, charge states and size of the metabolites present in most biological samples are another obstacle for extraction of all metabolites present in any biological sample [60]. For that reason, minimal pretreatment of the samples is often used for untargeted metabolomics, e.g. protein precipitation for plasma or dilute-and-shoot for urine. In some cases liquid-liquid extraction (LLE) is used to fractionate the metabolites based on polarity, where lipophilic analytes are extracted to the organic solvent and the hydrophilic metabolites remain in the aqueous phase. The use of LLE to separate the metabolites based on polarity allow for the different fractions of metabolites to be analyzed separately with specialized chromatographic methods for the respective fractions, i.e. RP for the lipophilic fraction and HILIC for the hydrophilic fraction [27,44,61]. Separating the lipids and the hydrophilic metabolites can reduce the matrix effects from phospholipids during the HILIC analysis of hydrophilic metabolites and similarly the matrix effects during analysis of lipids can be reduced as hydrophilic interferents are removed. However, an issue with the approach for hydrophilic metabolites is that the inorganic ions remain in the aqueous phase and are often enriched in the final sample (**I**, **III**, **IV**).

Depending on the chromatographic separation used in the LC-MS analysis the inorganic ions cause different issues, in reversed phase the inorganic ions are not retained and have limited effects on retained analytes. However, in HILIC the inorganic ions are retained and co-elute with analytes, this cause ion-suppression, excessive adduct formation and cluster formation (**III**, **IV**). The effects of high inorganic ion concentration on ionization, adduct formation and cluster formation in HILIC-ESI-MS was described in paper **III** and will be discussed in more detail under the section LC-MS-analysis.

In bioanalysis desalting of the samples is very common to alleviate the issues caused by inorganic ions in LC-MS using either LLE or RP-SPE, however, as discussed that is not possible for the hydrophilic metabolites. In paper **IV** we therefore developed an SPE method (*Figure 3*) to remove inorganic ions from biological samples while keeping most the small hydrophilic metabolites. The SPE method was based on a strong cation exchange solid phase, where the inorganic ions could be trapped while most of the hydrophilic metabolites were either not retained or could be eluted and later combined with the non-retained metabolites.

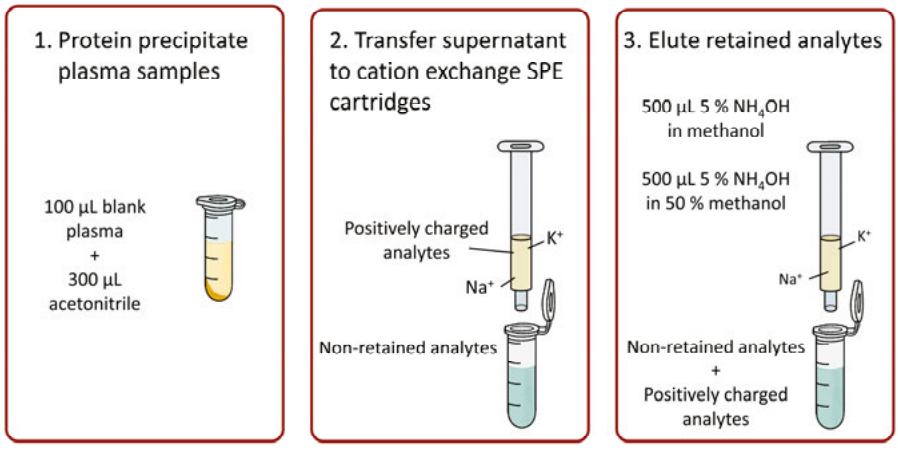

*Figure 3. Schematic summary of the developed SPE method in paper **IV**.*

SPE is often used to achieve high selectivity for a smaller number of analytes, however, in this case the SPE method was used more as a filtration step to remove the inorganic ions while still achieving a wide coverage of the hydrophilic metabolome. The method was evaluated using pooled drug-free blank plasma with Na-EDTA as anticoagulant, where the plasma was first protein precipitated using acetonitrile. Protein precipitation is the most common sample pretreatment of plasma samples for untargeted metabolomics, therefore, plasma samples treated with SPE was compared to samples treated with only protein precipitation [60].

To evaluate sample preparation methods used in metabolomics or other untargeted screening applications is very challenging as the methods should be as general as possible and are not developed for a limited set of analytes. For the evaluation of the developed SPE method we chose to first evaluate differences in signal intensity of a set of selected metabolites between the only protein precipitated samples and the SPE treated sample (**IV**). The metabolites were of different substance classes, polarity, molecular sizes and represented some of the most common and important metabolite classes present in a plasma sample. The signal intensity increased for the metabolites that co-eluted or eluted in close proximity to the alkali metal ion clusters using the SPE method as compared to the only protein precipitated samples. Moreover, the peak area of the Na^+ formate cluster decreased by 92.8 % and the K^+ formate cluster was not detectable in the SPE treated samples (**IV**). To investigate if the increase in signal intensity was due to decreased ion-suppression or decreased adduct formation, post-column infusion experiments were performed. The post-column infusion to study matrix effects was first described by Bonfiglio et al. [62] and is especially useful to study the matrix effects for untargeted methods as the matrix effects and ionization can be assessed over the entire chromatogram and not only at the retention time of a set of analytes (*Figure 4*). Using post-column infusion it is also possible to study the adduct formation over the entire chromatogram and how the adduct formation is affected by other components of the sample, i.e. inorganic ions. During the post-column infusion, isotopically labeled analogs of six metabolites; hypoxanthine, inosine, guanine, tryptophan, valine and glutamine was infused, the isotopically labeled standards were chosen as they are not naturally present in the plasma samples, however, they can be expected to ionize as their non-labeled analogs (**IV**).

In *Figure 4* a post-column infusion chromatogram of $[M+H]^+$ of guanine is presented, there the reduced ion-suppression from Na^+ and K^+ in the SPE treated samples can be observed as disappearing negative peaks. In the samples treated with only protein precipitation the ion-suppression was almost 100% during the elution of the Na^+ cluster (**IV**). Studying the post-column infusion chromatograms of the sodium adducts of the infused analytes indicated a reduced adduct formation in the SPE treated samples, however, not as large difference as might be expected. This indicates that the main reason for the increase in signal intensity was mainly due to reduced ion-suppression and but to some extent to reduced adduct formation as well.

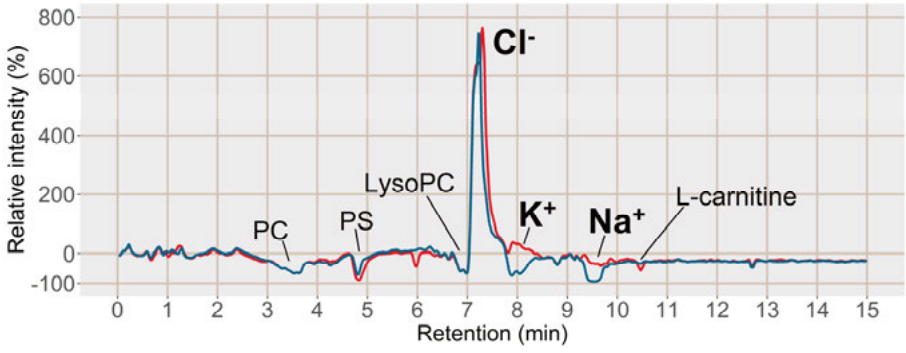

Figure 4. Post-column infusion chromatogram of guanine (2*^{13}C, ^{15}N) with the relative intensity in % as compared to the injection of a solvent blank on the y-axis 100*(Intensity$_{MCX\ or\ PPT}$/Intensity$_{blank}$)-100. Blue line =injection of plasma treated with protein precipitation, red line=injection of plasma treated with SPE. Each line is based on the average of 3 samples of each type, PPT, SPE MCX or a solvent blank. Major suppressions/enhancements are annotated in the chromatogram, PC= phosphatidylcholines, PS=phosphatidylserines, LysoPC= Lyso-phosphatidylcholines, K^+=potassium formate cluster K^+(HCOOK), *m/z* 122.926, Na^+=sodium formate cluster $Na^+(HCOONa)_3$), *m/z* 226.954, Cl^-=potassium chloride cluster, K^+(KCl), *m/z* 112.8963 (**IV**).

To study global differences in the collected data between the two sample treatments, multivariate data analysis was used. The multivariate data analysis was used to find any metabolites that were lost in the SPE treatment that were not discovered in the first selection of studied metabolites. The multivariate data analysis did not reveal any major metabolite losses other than those that were discovered in the previous experiments, however, the multivariate data analysis highlighted how the reduced alkali ion concentration also lead to reduced adduct and cluster formation. These results also highlight why it can be so problematic to use the number of features as a quality measure when evaluating analytical methods for metabolomics. More features is not necessarily a measure of more detected metabolites or better metabolome coverage, but rather more adduct formation or cluster formation leading to inflation of the number of features (**IV**).

Finally, we studied the linearity of response of six isotopically labelled standards that were spiked in eight different concentrations. In untargeted metabolomics linear response of all metabolites is often assumed for the relative quantifications between sample groups or samples, however, it is rarely studied or validated. For this evaluation we wanted to investigate if the linearity of response was changed as the ion-suppression from the alkali metal ions was reduced using the SPE method. The linearity of response was improved for some of the metabolites, most notably for $[M+H]^+$ of valine, that suffers from poor sensitivity and high degree of ion-suppression from Na^+,

where the reduction of ion-suppression lead to an increased sensitivity and thereby also a better linearity (**IV**).

In conclusion, using the SPE method to trap the Na^+ and K^+ ions from precipitated plasma we were able to reduce the concentrations of Na^+ and K^+ substantially. The reduction of alkali metal ion concentration in the plasma samples resulted in less ion-suppression, higher signal intensity of metabolites co-eluting with the ion-clusters as well as less adduct and cluster formation of the metabolites. Data analysis and metabolite annotation is one of the most time consuming and challenging steps in any metabolomics study and the reduced cluster and adduct formation, achieved by removing the inorganic ions should help to reduce the dataset complexity by removing many redundant MS signals. Moreover, the increase in signal intensities of many metabolites co-eluting with the inorganic ions, made it possible to detect more metabolites as their $[M+H]^+$ adducts, which are important in samples where the inorganic ion concentration can differ between samples and the Na^+ or K^+ adducts can suffer from poor reproducibility.

LC-MS analysis

Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) has become the most widely used technique for metabolomics due to its sensitivity and metabolite coverage [21]. Often data from orthogonal chromatographic techniques, e.g. reversed phase (RP) and hydrophilic interaction liquid chromatography (HILIC) is combined for a broader coverage of the metabolome. Reversed phase is the most widely employed chromatographic mode in metabolomics, however, it is most suited for non-polar to moderately polar analytes [63]. The retention of polar, ionic species e.g. amino acids and organic acids, is poor and they tend to elute with the void volume. For those types of metabolites, HILIC is much better suited. In all projects included in this thesis, HILIC has been used, especially as analysis of polar metabolites has been the main focus, RP was only used in paper **II**. Often when both HILIC and RP are employed, the samples are separated during extraction in polar and non-polar fractions, or aqueous and organic fractions that can be analyzed in HILIC and RP respectively.

In untargeted metabolomics high-resolution full-scan mass spectrometry is most commonly used due to its mass accuracy and ability to collect data from the entire *m/z* range in each scan with high sensitivity. The mass accuracy typically range between 0-10 ppm depending on the instrument. The most commonly used MS instruments in metabolomics are time of flight (TOF) instruments due to their short scan times and high mass accuracy [23]. The short scan times are especially important in UHPLC-MS applications where the chromatographic peaks are narrow, in some cases just a few seconds. The data acquisition has to be fast for sufficient number of data points across the peaks,

especially if the data are to be used for quantitative purposes, relative or absolute. Higher resolutions instruments, e.g., Orbitraps are not as commonly used, despite their superior mass accuracy, mainly due to slower scan rates as well as issues with saturation effects and limited dynamic range for complex samples [23,64,65]. The high resolution and mass accuracy is especially valuable for metabolite annotation and identification as it reduces the list of potential candidates substantially, however, unambiguous identification is generally not possible based solely on accurate mass [66]. Most often several different types of information e.g., monoisotopic mass, retention time, fragmentation from MS/MS or similar experiments are needed for identification, the metabolite identification process will be discussed in more detail further down in the discussion under data analysis.

Quality control

In metabolomics it is common practice to create a quality control (QC) sample, the QC sample is most commonly created by pooling small aliquots of all study samples [26,67,68]. The sample can be used to monitor the analytical performance throughout the analysis but also to identify analytically reproducible features during the subsequent data analysis. Monitoring the analytical stability throughout the analysis can be done by multivariate data analysis and principal component analysis to ensure that the QC samples cluster tightly together or the QC chromatograms can be overlaid and visually inspected to make sure they overlap. However, to be able to set acceptance criteria and evaluate the analytical performance, the evaluation has to be performed on peak level using the raw data.

Furthermore, the QC sample is often used to pre-condition the analytical system by repeated injections to ensure stable conditions prior to starting the analysis of study samples [26,69,70]. The pre-conditioning of the analytical system is thought to be due to build-up of sample components in the chromatography system as well as in the MS instrument that after repeated injections reach a steady state where retention times and detector response stabilize [69–71]. Typically 5-15 injections are necessary to reach stable conditions, the number of injections that are necessary are dependent on the sample type.

In all metabolomics projects included in this thesis QC samples, test samples (standard mix samples) and blank samples (solvent and extraction/processing blanks) have been used to ensure that the analytical system is ready and that the collected data is good-enough to proceed with data analysis (**I**,**II** and **IV**). A schematic figure of the general analysis procedure and the monitored parameters are presented in *Figure* 5. Solvent blanks are injected to begin with to assess the background in the system and to identify potential contaminant ions, secondly extraction blanks are analyzed to evaluate potential background ions from the extraction. Prior to starting the analysis of QC

samples, a test mix is analyzed, the test mix is used to control the mass accuracy and the calibration of the instrument, furthermore the retention, peak shape and signal intensity of the analytes can be controlled and compared to previous analyses. As the same test mix is used, column performance and detection sensitivity can be monitored over time. The test mix used for HILIC applications contained hypoxanthine, cytidine, phenylalanine, tryptophan and glutamine, meaning that the same analytes can be monitored in the QC samples as well as they are present in most sample types.

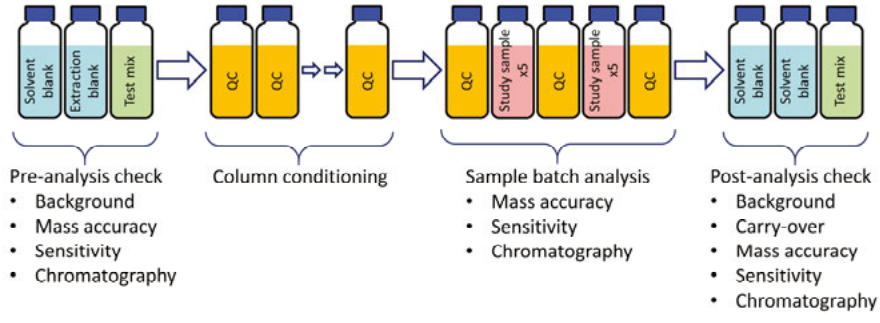

Figure 5. Schematic figure of the general analysis order and the included quality control steps as well as the controlled parameters in each step.

During column conditioning, the same analytes as in the test mix (if possible) are monitored, the signal intensity and retention times are monitored to ensure that it stabilizes prior to starting the actual sample analysis. The QC samples injected interspaced between the study samples can be evaluated after analysis to control that the signal intensities and retention times were stable as well as to check that the mass accuracy did not deteriorate during the run. Finally solvent blanks analyzed after the run are used to check for issues with carry over as well as to check that the background has not changed over the run.

Adduct formation in ESI-MS applications

Electrospray ionization (ESI) is one of the most widely used ionization techniques in MS, especially due to its compatibility with LC, broad range of applicability and has since its introduction revolutionized the field of bioanalysis [72]. Most commonly in ESI, protonated ($[M+H]^+$) or deprotonated ions ($[M-H]^-$) are formed meaning it is possible to easily calculate the molecular weights of analytes which can be used in metabolite annotation. However, ESI is prone to matrix effects as well as adduct and cluster formation, which could be considered the major drawbacks with the technique [62,73,74]. Adducts are defined as ionic species that is formed by the adduction of an ion, Na^+ or K^+, to a molecule, M e.g., $[M+Na]^+$ [75]. The protonated ions, $[M+H]^+$ are per definition also adducts, however, for simplicity in this discussion the protonated

ions will not be referred to as adducts. Similarly a cluster ion can be defined as an ion that is formed by two or more molecules, M, (of one or more chemical species) joined by noncovalent forces and one or more ionic species, Na^+ or K^+ e.g., $[nM + mHCOONa + Na]^+$ [75].

Adduct formation has often been said to lower the sensitivity as the analyte signal is split between several MS signals, that is not necessarily true though and there are several examples where adduct formation have been used to increase sensitivity [76–81]. However, adduct formation can generate other issues as it leads to more complicated MS spectra and unpredictable ionization processes with variations in ionization efficiencies between adducts (*Figure 6*) [38,73,82]. Adducts are formed in both positive and negative ionization mode but are more common in positive ionization mode. They are commonly formed with alkali metal ions but also with mobile phase additives and components [74,83]. The alkali metal ions can originate from the samples, solvents, mobile phase additives and glassware.

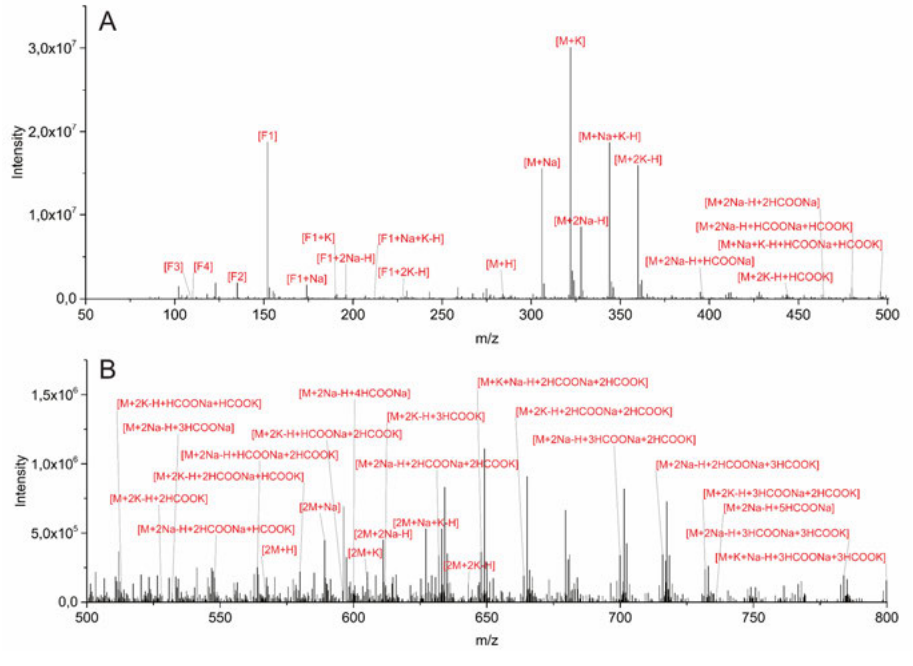

Figure 6. Mass spectrum of guanosine injected as a solution containing guanosine, sodium formate (20 mM) and potassium formate (20 mM). The spectrum is divided in A, m/z range 50–500 and B m/z range 500–800, note the different scales on the y-axis. Fragmentation of guanosine was labelled as FX in order of intensity, with F1 being the fragment with the highest intensity (paper **III**).

Inorganic ions are present in all biological samples in varying concentrations depending on the sample types, they can also be added during sample collection and preparation as was discussed under the respective sections. In HILIC these ions are retained and using ESI-MS they can be detected as cluster ions with mobile phase components or other inorganic ions. The retention of inorganic ions in HILIC has previously been used to analyze counter ions in pharmaceutical formulations [84,85]. However, in biological samples the retention of mainly Na^+ and K^+, but many other inorganic ions as well, lead to co-elution with analytes and the retention of Na^+ and K^+ in HILIC or more precisely the co-elution with metabolites and the effects of the co-elution was the focus in paper **III**.

During data analysis in paper **I** we noticed that the adduct formation was much more extensive for metabolites co-eluting with Na^+ or K^+. The extensive adduct formation in that retention time range could result from the fact that metabolites eluting closely together are metabolites with similar structures and physicochemical properties, and thus are equally prone to forming adducts. However, it could also be a result of the co-elution with the inorganic ions. To investigate how the elution of Na^+ and K^+ affected adduct formation, a selection of metabolites were infused post-column when solutions with sodium and potassium formate was injected (*Figure 7*).

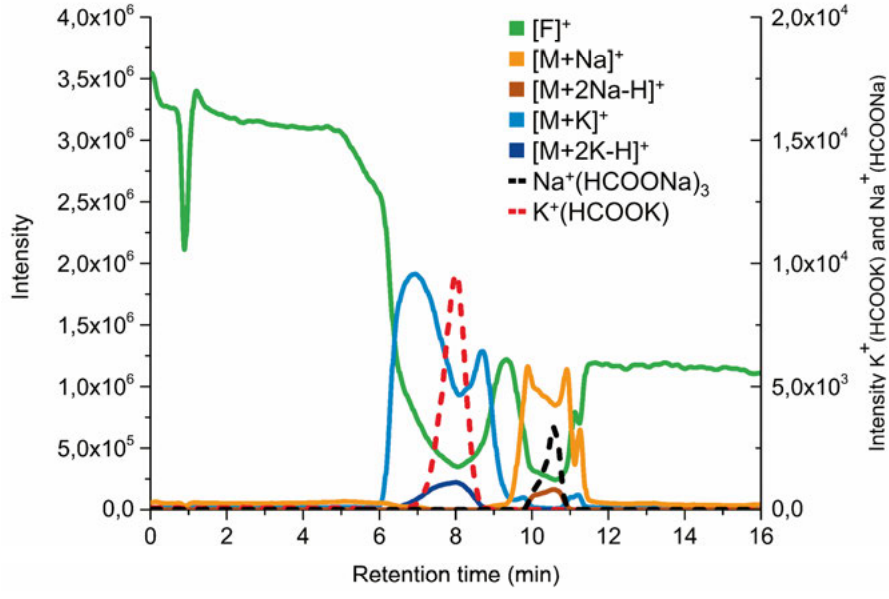

Figure 7. Post-column infusion chromatogram of guanosine when 10 mM sodium formate and 10 mM potassium formate was injected. The investigated adducts of guanosine were $[M+K]^+$, $[M+2K-H]^+$, $[M+Na]^+$, $[M+2Na-H]^+$ as well as the fragment [F, 152.0876]$^+$. The chromatographic peaks of $K^+(HCOOK)$, *m/z* 122.9252 and $Na^+(HCOONa)_3$, *m/z* 226.8525, intensity on the right y-axis (paper **III**).

The selection of metabolites consisted of metabolites from different substance classes, with different physicochemical properties, different retention times and with very different adduct formation in the chromatographic system. The post-column infusion then allowed for the investigation on how metabolites ionized over the entire chromatogram and not only at their normal retention time, which meant that it was possible to study how metabolites that normally do not elute closely to Na^+ or K^+ ionized when they ionized together with the inorganic ions. It should be emphasized though, that the infusion of analytes post-column, where they are mixed in the mobile phase flow just before the ion-source, do not necessarily replicate the ionization if the analytes are injected, separated chromatographically and reach the ion source as narrow chromatographic bands. Therefore, the ionization also has to be investigated in the normal settings where samples are injected. In the post-column infusion experiments all metabolite adduct formation profiles followed the elution of Na^+ and K^+ very closely (*Figure* 7). However, there were differences between the metabolites as to what adducts were favored, the most commonly formed adducts were $[M+X]^+$, $[M+2X-H]^+$, $[2M+X]^+$ or $[2M+2X-H]^+$, where the X denotes any of the alkali metal ions. Moreover, a substantial cluster formation of analytes was detected as well, both clusters of multiple analyte molecules with alkali metal ion or analytes clustered with different combinations of sodium and potassium formate, typically with the formula $[M+X+nHCOOX]^+$ or $[M+2X-H+nHCOOX]^+$. As can be seen in the spectrum presented in *Figure 6* the cluster formation can add extreme complexity to the spectrum. The spectrum in *Figure 6* was collected from a standard solution containing a limited number of analytes as well as sodium and potassium formate, which highlight how complex metabolomics data can be considering the added complexity that a biological sample would provide as well.

Data analysis and biological interpretation

Multivariate data analysis

During the LC-MS analysis, huge amounts of data is collected, often tens to hundreds of samples are analyzed using full scan MS resulting in thousands of MS peaks in each sample. For data visualization as well as to find structures, trends and to pinpoint the differences between sample groups among those thousands MS signals, chemometrics and multivariate data analysis (MVDA) is commonly used. Multivariate data analysis refers to statistical methods and models that simultaneously analyze multiple variables for each individual observation [86–88]. However, prior to MVDA the three-dimensional MS data has to be converted to a two-dimensional matrix. The created two-dimensional matrix contains the samples as observations in rows and features, *m/z* – retention time pairs as variables. To correct for instrumentation

drifts or batch effects, normalization techniques can be applied, as in paper **I** where the QC samples injected throughout the analysis were used to normalize for the inter-day variation and batch effects during LC-MS analysis. To reduce the noise in the data, centering and scaling of the data is often performed [89,90]. In metabolomics data the most common scaling methods are auto scaling where the variable values are divided by the variable standard deviation or pareto scaling where the variable value is divided by the square root of the standard deviation [89–91]. Pareto scaling decrease large fold-changes more than smaller fold-changes, resulting in less influence of the large fold-changes on the final models, as well as handling noisy data better than auto scaling. Pareto scaling was applied in paper **I**, **II** and **IV** [89,91].

Principal component analysis (PCA) is a unsupervised projection method that is especially valuable when studying sample clustering, grouping, trends and to find outliers as an initial step in the MVDA [90,92]. PCA is based upon a number of principal components, the principal components are linear combinations of the variables that describe the total variance in the dataset [87]. The first principal component (PC) describe the largest portion of the total variance, whereas the second PC describe the second highest portion of the variance that is not correlated to the first PC in the data set and so on [87]. In paper **I**, **II** and **IV**, PCA was used to find sample clustering and groups, to look for trends in the data based on injection order or sample batch as well as to look for outliers (*Figure 8*). In *Figure 8* a PCA score plot from paper **II** is presented, the aqueous extracts of sponge samples, analyzed in negative ionization mode, each sample is represented by a data-point and the data points are colored based on sample groups. Each sample group is made up by three samples from three sponge specimens, the labels denote the specimen number. Hence, from the PCA scores plot it is possible to study how the sample groups cluster and relate to each other, it is also possible to get an indication of the biological variation (between specimens) as well as the variation between technical replicates (between replicates from the same specimen). The QC samples that was injected in between the study samples (red dots) are clustered very tightly together indicating stable conditions throughout the analysis and that the variation between injections are much lower than the variation within and between the sample groups.

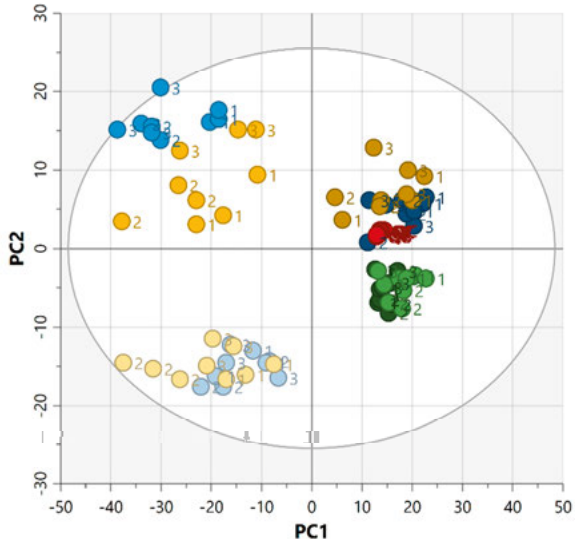

Figure 8. PCA score plot of the aqueous extracts analyzed in negative ionization mode in HILIC-ESI-QTOF-MS in paper **II**. Each sample is represented by a data-point and the data points are colored based on sample groups. Each sample group is made up by three samples from three sponge specimens, the labels denote the specimen number.

In paper **I**, PCA was used, together with other statistical methods, to ensure that batch effects or trends due to injection order was not evident in the data after normalization since the samples were prepared and analyzed in different batches. PCA is as discussed valuable for visualization of the data and to give an overview of the dataset, however, using PCA for feature selection to pinpoint the differences between two or more samples groups can be difficult.

To find the discriminating features between sample groups, supervised projection models as partial least squares (PLS) or orthogonal projection to latent structures - discriminant analysis (OPLS-DA) are more commonly used. In supervised models, information on sample group belonging is used to classify the samples. In OPLS-DA, qualitative data as the sample class belonging is correlated to the variables containing quantitative data (features and intensity) and the variation in the dataset can be divided in one part that is linearly related to the sample class discrimination and a second part that is not [86,90]. This means that OPLS-DA is able to distinguish between within-group variation and between-group variation, making it a very powerful tool for discrimination. Moreover, OPLS-DA can be used with tools as the S-plot and shared and unique structure (SUS) plot for feature selection and further on metabolite identification to find the important differences between sample groups (**I**, **II** and **IV**).

Metabolite identification

Performing MVDA to investigate the differences between sample groups result in lists of features important for the discrimination between the groups. However, to be able to interpret the data in a biological context, the lists of features has to be transformed to biologically relevant information i.e., metabolites. Metabolite identification is often one of the most challenging and time consuming steps of a metabolomics study due to the complexity of the LC-MS data and the vast number of metabolites present in any biological system.

Depending on how well characterized the investigated biological system is the metabolite identification can be more or less challenging. Human and mammalian cells are generally well characterized and the culture of cells in laboratories are performed in controlled environments, meaning that the metabolome in large is made up by metabolites used in cell metabolism and homeostasis as well as what is added in form of culture media (**I**). Other human and mammalian sample types, e.g., plasma or urine, are also generally well characterized, however, in addition to all endogenous metabolites a large amount of exogenous metabolites from food, drugs and so on have to be considered as well (**IV**). For the human metabolome, several databases are available for metabolite searching, e.g. human metabolome database (HMDB) [93–95], Metlin [96], lipidmaps, among others. For more unusual sample types, as the sponges investigated in paper **II**, very little is known about the metabolome and the metabolites they produce, which, make the metabolite identification very difficult as no comprehensive databases exist.

The resulting lists of features from the MVDA contain information on *m/z* as well retention times, the *m/z* values can be used to search in data bases for matching molecular weights. However, experimental *m/z* values, even with low mass errors below 5 ppm is generally not enough for unambiguous annotation of metabolites due to the large number of possible metabolites and isobaric compounds. Therefore additional information and experiments are often necessary. Additional information can be matching of fragments to available reference spectra if available, matching of retention time with in-house databases if such exist as well as using isotopic patterns and abundance to calculate theoretical elemental compositions. Several different guidelines and systems for reporting the level of identification rigor have been proposed, they are generally very similar and are based upon 4–5 levels of confidence in identification [22,97,98]. The system most commonly used in metabolomics, have four levels of identification and for metabolite identification, the highest level of confidence, comparison with an authentic standard has to be performed and several different parameters in the same analytical system has to be used, e.g., retention time, accurate mass, fragmentation or MS/MS [22]. For the lower confidence levels, 2–3, matching experimental data with available spectra from databases is enough. However, depending on if there is just one probable

structure or several different structures remaining after matching, e.g., difficulties to tell the exact position of a methyl group rendering several different possibilities, the levels are divided between probable structure and tentative candidates. Finally the lowest level(s) of identification would be just an exact mass of interest or an elemental composition.

The possibilities for identification using standards is limited by the availability of standards and that the metabolites are known previously. In many cases though, identification at level two or even three can be enough to draw conclusions regarding the metabolic alterations and affected pathways. However, in other cases the exact position of a methyl group or the stereo chemistry could be decisive for a metabolite's biological effects and identification with level 1 is necessary.

Biological interpretation

Following metabolite identification, the list of identified metabolites has to be put into context for the research question at hand, e.g, the difference in metabolic response to ionizing radiation between cell lines of different radiation sensitivities (**I**). To connect the changed metabolites to metabolic pathways and processes, pathway enrichment analysis is often performed [99]. The pathway enrichment analysis process the list of altered metabolites and calculate whether any particular metabolic pathways are more represented than others, based on statistics and possibilities [99]. In paper **I** the freely-available source MetaboAnalyst was used for pathway enrichment analysis [100]. However, following the analysis, the results still have to be put into context, related to the current biological system and the possible implications of changes in the highlighted pathways.

If possible, the results can be combined and correlated with the results from genomics, transcriptomics or proteomics analyses. In paper **I** two cell lines, UM-SCC-74A and UM-SCC-74B, were analyzed, the cell lines originated from the same patient but displayed different radiation sensitivities [101]. However, no changes in the genome could be detected to explain the developed resistance in the second cell line, UM-SCC-74B. Therefore it was hypothesized that the developed resistance was due to alterations in metabolism rather than genetic alterations. Furthermore, to test whether the results are valid, and if an enzyme activity actually is upregulated, additional assays and experiments can be performed. In paper **I** we found alterations in the nicotinamide and nicotinic acid metabolism pathway, following irradiation in the radiation resistant cell line UM-SCC-74B, however, the pathway was not altered in the radiation sensitive cell line UM-SCC-74A. In the UM-SCC-74B cell line the NAD^+ levels were almost completely depleted 24 h after irradiation which could indicate an increase in ADP-ribosylation by poly(ADP-ribose)ploymerases (PARPs) to initiate DNA repair. The differences in PARP1

activity between the two cell lines was confirmed using an ELISA assay measuring a cleaved and inactivated fragment of PARP1. In UM-SCC-74B, the levels of cleaved PARP1 was unchanged after irradiation, whereas it was increased in UM-SCC-74A after irradiation (**I**). The differences in PARP1 activity between the two cell lines also provide a possible target for sensitizing cells to radiation treatment to improve treatment outcomes and previously inhibition of PARP have been demonstrated to increase radiation sensitivity [102–109].

Concluding remarks

In conclusion this thesis consists of four different articles or manuscripts related to metabolomics. The first two are application oriented and have a clear interdisciplinary focus (**I**, **II**) while the other two are more oriented towards analytical chemistry and method development (**III**, **IV**). When starting this doctoral project the aim was to apply metabolomics analyses in relevant projects and to characterize and develop the analytical methods used, in large those aims have been achieved.

In paper **I**, LC-MS based metabolomics was used to study radiation resistance in head and neck cancer cells with aim to investigate the differences in metabolic response between two cell lines with different radiation sensitivities. Moreover, the aim was also to try to elucidate the mechanisms behind radiation resistance and how some cancer cells are able to survive ionizing radiation. Prior to starting the project the hypothesis was that as no changes in the genome that could explain the developed resistance have been detected, the developed radiation resistance might be due to an altered metabolism. In the study we were able to detect the differences in metabolic response after radiation treatment between the two cell lines and could formulate a hypothesis on how the more radiation resistant cell line alters its metabolism to be able to control redox status and DNA repair mechanisms as well as to change DNA methylation. Understanding the metabolism behind radiation resistance is important as it can provide tools for predicting radiation treatment outcomes as well as possibilities to sensitize tumors to radiation and thereby improve treatment outcome.

In paper **II** the aim was to investigate how sampling and sample storage techniques of sponge samples affect the detected metabolite profiles and the data quality for metabolomics studies. Metabolomics have become more and more widely used within the research of sponges. However, the collection and sampling are performed in many different ways and there are currently no recommendations on how to collect sponge samples for metabolomics. We therefore collected *Geodia barretti* sponges to investigate how the detected metabolite profiles and the data quality was affected by the sampling technique. We found that freezing the samples as fast as possible after collection should be the preferred method for quantitative as well as qualitative investigations. We also found that if the specimens are stored in solvent, much of the metabolome is extracted to the solvent thus the solvents should not be discarded which is generally the practice. Moreover, many museums and other institutions hold large and unique collections of sponge specimens, the results

suggest that both the solvents they are stored in and the sponges could be used for qualitative investigations. However, the long-term stability of metabolites in ethanol preserved specimens needs to be investigated as well.

During data analysis of the first project (**I**), the extensive adduct and cluster formation was identified as an issue that hampered the data analysis and metabolite identification. The reasons behind the extensive adduct formation was therefore investigated which led to paper **III** where the problems with high concentrations of inorganic ions in the samples were described. The issues in HILIC-ESI-MS analyses described in paper **III**, are especially important to acknowledge and control in metabolomics analyses as it is not possible to compensate for the variations using internal standards for all metabolites as would be possible in many other fields. Furthermore, some disease states and medical treatments may affect the alkali metal ion concentrations of biological fluids, causing effects on the quantitative signals of metabolites leading to erroneous results and conclusions. This requires careful consideration in method development of biological samples in HILIC-ESI-MS analyses and all steps from sampling to analysis should be considered and controlled.

To mitigate the problems with the inorganic ions in biological samples, a sample preparation was developed in paper **IV**, the sample preparation was based on SPE. However, the SPE cartridges were used more as filtration units to trap the alkali metal ions while retained metabolites could be eluted. This allowed for a significant reduction of sodium and potassium concentrations in plasma samples, with reduced ion-suppression, reduced adduct and cluster formation of metabolites as well as increased signal intensities of metabolites as a result. The developed sample preparation could significantly improve the data quality in metabolomics analyses using HILIC-ESI-MS.

Populärvetenskaplig sammanfattning

Metabolomik är en gren inom den analytiska kemin där man har som mål att kunna analysera så många substanser som möjligt med så få experiment som möjligt. Substanserna som analyseras är små molekyler i biologiska system som ofta kallas metaboliter, därav benämningen metabolomik. I en metabolomikstudie är målet ofta att jämföra en eller flera grupper med varandra för att kunna identifiera skillnaderna, exempelvis kanske man vill veta hur en grupp sjuka patienter skiljer sig från friska individer. Man kan också undersöka processer i celler eller vävnader, i artikel **I** undersökte vi hur cancerceller med olika strålningskänslighet reagerade på just strålning för att få bättre förståelse kring varför vissa cancertyper utvecklar strålningsresistens. Alltså vet man inte på förhand exakt vad man letar efter utan experimenten designas för att söka så brett som möjligt för att kunna identifiera skillnaderna på metabolitnivå. Detta brukar kallas att studien är hypotesgenererande, resultaten som genereras när studien är slutförd kan alltså vara en hypotes gällande skillnaderna på metabolitnivå mellan de sjuka patienterna och de friska individerna. Detta står i kontrast till mer traditionell forskning då man allt som oftast startar med en hypotes och därefter designar experiment för att kunna testa om hypotesen stämmer.

Metaboliter inom metabolomiken syftar på små molekyler i ett biologiskt system. Målet för analyserna är därför inte stora makromolekyler som proteiner eller DNA som utgör våra gener, utan mindre byggstenar och signalsubstanser som aminosyror, sockerarter, vitaminer, organiska syror och så vidare. Det biologiska systemet kan vara celler odlade i labbet, blodprover från människor eller försökdjur, urinprover, vävnadsprover eller växtprover m.m. I de flesta biologiska system är antalet metaboliter otroligt stort, man uppskattar att hos oss människor finns ungefär 8500 kroppsegna metaboliter och upp till 40 000 exogena metaboliter från mat, läkemedel m.m.

I dagsläget går det inte att analysera alla dessa metaboliter med bara ett eller ett fåtal experiment, i verkligheten sker alltid ett urval beroende på hur man utformar sina experiment, och vilka metoder man använder. Det betyder också att när man designar sina experiment och väljer vilka metoder man ska använda sig av så ställs man inför ett väldigt viktigt avvägande, ska man försöka att analysera så många metaboliter som möjligt eller ska man utveckla metoderna så att de är mer precisa för ett mindre antal metaboliter. Något som försvårar detta är att eftersom man utvecklar metoderna för att vara så generella som möjligt och inte för ett mindre urval av metaboliter, så är det väldigt

svårt att utvärdera hur bra de analytiska metoderna fungerar. Att försöka utvärdera och förbättra just de analytiska metoderna som används inom metabolomik var ett av huvudsyftena med arbetet för den för avhandlingen. Under arbetet med avhandlingen har de flesta stegen i en metabolomikstudie berörts, så som provinsamling, förvaring, provupparbetning, analys och utvärdering av data.

I artikel **II** utvärderades hur provtagningen och förvaringen efter provtagningen av svampdjur påverkar analysresultaten för en metabolomikstudie. Svampdjuren samlades in i Kosterhavet utanför Tjärnö marina laboratorium, och frystes sedan in direkt på båten eller lite senare, alternativt så lades proverna i etanol eller metanol. Inom forskningsfältet av marina djur eller växter är det väldigt vanligt att förvara prover i etanol och vi ville därför undersöka hur förvaringen i etanol påverkar provkvalitén och analysresultaten samt jämföra med frysning som är vanligare i andra forskningsfält. Vi fann att förvaringen i etanol extraherar mycket av metaboliterna till etanolen och att förvaringen urlakade själva svampdjursproverna, det betyder att etanolen bör sparas och inte kastas om man förvarar sina prover i etanol. I övrigt så fann vi att frysning direkt var bättre för variationen mellan prover. Resultaten kan ha stor inverkan på hur man ser på museisamlingar, där det finns många unika samlingar med stort värde ofta förvarade i etanol och där etanolen i sig kan innehålla mycket information om svampdjurens kemi.

I ett annat projekt (artikel **III**) studerades hur oorganiska joner som natrium- och kaliumjoner kan påverka analysen av metaboliter. Natrium- och kaliumjoner finns i alla biologiska prover och följer ofta med i provupparbetningen när man analyserar polära metaboliter, de kan sedan under provanalysen störa analysresultatet och ge upphov till många extra detektorsignaler samt släcka detektorsignalen för viktiga och intressanta metaboliter. Detta kan ge felaktiga analysresultat och kan också leda till att dataanalysen och utvärderingen av resultaten försvåras.

För att komma runt problemet med de oorganiska jonerna i biologiska prover så utvecklades en provupparbetningsmetod för att kunna isolera och ta bort de oorganiska jonerna från blodplasmaprover utan att ta bort några av de polära metaboliterna i proverna (artikel **IV**). Detta gjordes genom att använda ett negativt laddat absorptionsmaterial, där alla positivt laddade joner fastnade, natrium och kalium joner så väl som positivt laddade metaboliter, men de positivt laddade metaboliterna kunde sedan frigöras från absorptionsmaterialet med hjälp av olika tvättsteg. På så sätt kunde de oorganiska jonerna separeras från de organiska metaboliterna och det ledde till ökad känslighet för många av de viktigaste metaboliterna som aminosyror.

Sammanfattningsvis innehåller den här avhandlingen fyra delarbeten som berör olika delar av arbetsgången inom metabolomik. Två av arbetena har utförts i samarbete med andra forskningsgrupper och har ett tydligt tvärvetenskapligt fokus (**I** och **II**). De andra projekten, fokuserar kring metodutveckling och grundforskning (**III** och **IV**). I artikel **I** så studerades hur metabolismen

hos cancerceller med olika strålningskänslighet reagerade på joniserande strålning, målet var att kunna bilda en hypotes om vad som skiljer strålningsresistenta cancerceller från strålningskänsliga cancerceller. En teori kring hur strålningsresistenta cancerceller kan ställa om sin metabolism för att överleva joniserande strålning kunde presenteras. I arbetet med artikel **I** identifierades ett antal förbättringsmöjligheter i de analytiska metoderna som användes, framförallt problemen med de oorganiska jonerna som sedan studerades i artikel **III** och sedan presenterades ett förslag för att lösa de beskrivna problemen i artikel **IV**. Slutligen så undersöktes hur provinsamlingen av svampdjur kan påverka analysresultaten och vilka metaboliter man kan detektera beroende på hur man behandlar proverna (artikel **II**).

Acknowledgement

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