Metabolic Studies with Liquid Separation Coupled to Mass Spectrometry

ERIK ALLARD
Dissertation presented at Uppsala University to be publicly examined in C4:301, BMC, Husargatan 3, Uppsala, Monday, December 14, 2009 at 10:15 for the degree of Doctor of Philosophy. The examination will be conducted in English.

Abstract

Metabolism is the sum of all chemical processes with the purpose to maintain life, as well as enable reproduction, in a living organism. Through the study of metabolism, increased understanding of pharmacological mechanisms and diseases can be achieved. This thesis describes several ways of doing so, including targeted analysis of selected metabolites and investigations of systematic metabolic differences between selected groups through pattern recognition.

A method for exploring metabolic patterns in urine samples after intake of coffee or tea was developed. The methodology was later used with the aim to find biomarkers for prostate cancer and urinary bladder cancer.

Furthermore, a fully automated quantitative method was developed for concentration measurements of the double prodrug xime lagatran and its metabolites in pig liver. The method was then used to study the role of active transporters in pig liver cells.

Moreover, a fundamental study was conducted to investigate how monitoring of small, doubly charged analytes can improve the limit of detection and precision in a quantitative method.

The techniques used for the experiments were liquid separation coupled to electrospray mass spectrometry. Extra efforts were made to make the separation and the ionization as compatible as possible to each other for increased quality of the collected data.

Keywords: liquid chromatography, mass spectrometry, tandem mass spectrometry, method development, capillary electrophoresis, electrospray ionization, time-of-flight, quantitation, metabolomics, metabonomics, pattern recognition, xime lagatran, melagatran, charge state

Erik Allard, Department of Physical and Analytical Chemistry, Analytical Chemistry, Box 577, Uppsala University, SE-75123 Uppsala, Sweden

© Erik Allard 2009

ISSN 1651-6214
urn:nbn:se:uu:diva-110310 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-110310)
Tota oscillandum volutandumque nocte
et potandum quotidie
List of Papers

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


V Allard, E., Danielsson, R., Sjöberg, PJR., Bergquist, J. Exploring liquid chromatography-mass spectrometry fingerprints of urine samples from patients with prostate or urinary bladder cancer. *Manuscript*

Reprints were made with permission from the respective publishers.

**Author’s contribution to the papers:**

**Paper I:** Planned, performed and wrote the paper together with B. Arvidsson.

**Paper II:** Took part in the planning, performed all measurements, took part in the discussion of the results and wrote part of the paper.

**Paper III:** Planned and performed all experiments and wrote the paper.

**Paper IV:** Took part in the planning, performed all experiments, took part in the data evaluation and discussion of the results, and wrote parts of the paper.
Paper V: Planned and performed the experiments, performed parts of the data evaluation, took active part in the discussion of the results, and wrote parts of the paper.
<p>| 1  | Introduction ......................................................................................... | 11 |
| 2  | Metabolic studies................................................................................. | 14 |
| 2.1 | Metabolomics ............................................................................. | 15 |
| 2.2 | Targeted Analysis ....................................................................... | 16 |
| 2.3 | Metabolite profiling ..................................................................... | 17 |
| 2.4 | Metabolic fingerprinting ............................................................. | 17 |
| 2.4.1 | Data handling......................................................................... | 18 |
| 3  | Liquid separation ................................................................................. | 22 |
| 3.1 | Liquid chromatography .............................................................. | 22 |
| 3.1.1 | LC phases ............................................................................... | 23 |
| 3.1.2 | Gradient LC ............................................................................. | 25 |
| 3.1.3 | Elevated temperatures in LC ..................................................... | 27 |
| 3.2 | Capillary electrophoresis ............................................................ | 27 |
| 4  | Mass spectrometry............................................................................... | 31 |
| 4.1 | Ionization .................................................................................... | 31 |
| 4.1.1 | Electrospray ........................................................................... | 32 |
| 4.1.2 | Gas phase ionization techniques ............................................. | 34 |
| 4.2 | Mass analyzers ............................................................................ | 35 |
| 4.2.1 | Quadrupole instruments .......................................................... | 35 |
| 4.2.2 | TOF ....................................................................................... | 37 |
| 4.3 | Hyphenation of liquid separation with ESI ................................ | 39 |
| 4.3.1 | LC-ESI .................................................................................. | 40 |
| 4.3.2 | CE-ESI ................................................................................ | 41 |
| 5  | Quantitation ......................................................................................... | 43 |
| 5.1 | Calibration methods ..................................................................... | 43 |
| 5.1.1 | External standard ........................................................................ | 43 |
| 5.1.2 | Internal standard ...................................................................... | 44 |
| 5.1.3 | Standard addition ...................................................................... | 45 |
| 5.2 | Validation ..................................................................................... | 46 |
| 6  | Concluding remarks ............................................................................. | 49 |
| 6.1 | Future aspects ............................................................................. | 49 |
| 7  | Acknowledgements.............................................................................. | 51 |</p>
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>Sammanfattning på svenska</td>
<td>52</td>
</tr>
<tr>
<td>8.1</td>
<td>Metabolism</td>
<td>52</td>
</tr>
<tr>
<td>8.2</td>
<td>Masspektrometri</td>
<td>53</td>
</tr>
<tr>
<td>8.3</td>
<td>Vätskekromatografi</td>
<td>54</td>
</tr>
<tr>
<td>8.4</td>
<td>Kapillärelektrofores</td>
<td>54</td>
</tr>
<tr>
<td>8.5</td>
<td>Metabola fingeravtryck</td>
<td>55</td>
</tr>
<tr>
<td>8.6</td>
<td>Cancer</td>
<td>55</td>
</tr>
<tr>
<td>8.7</td>
<td>Ordlista</td>
<td>56</td>
</tr>
<tr>
<td>9</td>
<td>References</td>
<td>57</td>
</tr>
</tbody>
</table>
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>APCI</td>
<td>Atmospheric pressure chemical ionization</td>
</tr>
<tr>
<td>APPI</td>
<td>Atmospheric pressure photoionization</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary electrophoresis</td>
</tr>
<tr>
<td>CRM</td>
<td>Charge residue model</td>
</tr>
<tr>
<td>dc</td>
<td>Direct current</td>
</tr>
<tr>
<td>EOF</td>
<td>Electro osmotic flow</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>HILIC</td>
<td>Hydrophilic interaction liquid chromatography</td>
</tr>
<tr>
<td>IEC</td>
<td>Ion exchange chromatography</td>
</tr>
<tr>
<td>IEM</td>
<td>Ion evaporation model</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of quantitation</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass-to-charge ratio</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reaction monitoring</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
</tr>
<tr>
<td>NP</td>
<td>Normal phase</td>
</tr>
<tr>
<td>PC</td>
<td>Principal component</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>Q</td>
<td>Quadrupole mass analyzer</td>
</tr>
<tr>
<td>QqQ</td>
<td>Triple quadrupole mass analyzer</td>
</tr>
<tr>
<td>rf</td>
<td>Radio frequency</td>
</tr>
<tr>
<td>RP</td>
<td>Reverse phase</td>
</tr>
<tr>
<td>S/N</td>
<td>Signal-to-noise ratio</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TOF</td>
<td>Time of flight</td>
</tr>
</tbody>
</table>
1 Introduction

The central theme of this thesis is measurements of metabolites using liquid separation techniques with mass spectrometric detection.

In the first part of the thesis, some relevant aspects of metabolic studies, along with the theoretical background of the instrumentation that has been used will be discussed. In the second part five papers are presented, illustrating different aspects of metabolic studies. Paper I describes a method for concentration measurements of the double prodrug ximelagatran, the two intermediary metabolites N-hydroxymelagatran and ethylmelagatran, and the pharmacologically active metabolic product melagatran, which is a direct thrombin inhibitor. In paper II the hepatic metabolism and disposition of ximelagatran and its metabolites in pig was investigated, making use of the method developed in paper I. Simultaneously a simple in vitro method for quantitative investigations of membrane transporters impact on the disposition of the metabolized drug was explored. During the method development, strong tendencies for the compounds to form doubly charged ions in electrospray ionization were observed. In Paper III the impact of this occurrence on quantitation was investigated, along with suggestions on how to control and utilize the phenomenon in order to improve the quality of analysis. Paper IV is a description of how chemometric tools can be used to identify systematic differences between groups of complex samples. As a model, urine samples collected from individuals after intake of coffee, tea and water was used. In paper V the chemometric tools were developed further and urine samples from patients with prostate cancer or urinary bladder cancer were compared with a control group. The aim of the paper was to suggest a set of biomarkers that potentially can be used for diagnostics of the diseases, and to achieve increased understanding of tumor biology. For a brief overview of the papers, see Table 1.

The first part of the thesis gives a theoretical background to the techniques involved in the research. In chapter 2 different approaches to metabolic studies is given along with a discussion on data handling, which is especially relevant for paper IV and paper V. Chapter 3 considers the liquid separation techniques used in papers I, II, IV and V. In chapter 4 mass spectrometry is discussed along with the challenges of hyphenating mass spectrometry with liquid separation. This section is relevant for all five papers. Chapter 5 deals with quantitation and validation of quantitative meth-
ods, which is of importance for paper I and paper II. Finally, in chapter 6 some concluding remarks are made to put the research in a larger context, along with some speculations about future development.
Table 1. Summary of papers included in this thesis.

<table>
<thead>
<tr>
<th>Paper No</th>
<th>Matrix</th>
<th>Type of study</th>
<th>Sample preparation</th>
<th>Separation</th>
<th>Detection</th>
<th>Results</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Pig liver</td>
<td>Targeted analysis, method development</td>
<td>Centrifugation, dilution, online SPE</td>
<td>Capillary RP-LC (C18)</td>
<td>ESI-MRM-MS</td>
<td>A fully automated quantitative method was developed and validated for ximelagatran and three of its metabolites. Total cycle time was 20 min.</td>
<td>Published in Journal of Chromatography B, 2008 (877) p.291-297</td>
</tr>
<tr>
<td>II</td>
<td>Pig liver</td>
<td>Targeted analysis, pharmacokinetics</td>
<td>Centrifugation, dilution, online SPE</td>
<td>Capillary RP-LC (C18)</td>
<td>ESI-MRM-MS</td>
<td>Investigation of the hepatic ximelagatran metabolism was performed. Suggestion of an in vitro method for impact of membrane transporters was also made.</td>
<td>Accepted by Pharmaceutical Research</td>
</tr>
<tr>
<td>III</td>
<td>Standards</td>
<td>Fundamental research on ESI</td>
<td>Preparation of standards</td>
<td>Direct infusion</td>
<td>ESI-TOF-MS, ESI-3Q-MS, ESI-LIT-MS</td>
<td>Suggestions on how to improve quantitative parameters for ximelagatran by controlling the charge state through selected parameters were made.</td>
<td>Submitted to Rapid Communications in Mass Spectrometry</td>
</tr>
<tr>
<td>IV</td>
<td>Human urine</td>
<td>Metabolic fingerprinting, data handling</td>
<td>Dilution</td>
<td>CE</td>
<td>ESI-TOF-MS</td>
<td>The chemometric tools that were developed successfully determined metabolic fingerprints in urine after intake of coffee and tea. Investigated “hotspots” confirmed previous findings and identified a number of new potential biomarkers.</td>
<td>Published in Analytical Chemistry, 2009 (80) p.8946-8955</td>
</tr>
<tr>
<td>V</td>
<td>Human urine</td>
<td>Metabolic fingerprinting, data handling</td>
<td>Centrifugation, dilution</td>
<td>Microbore RP-LC (C18)</td>
<td>ESI-TOF-MS</td>
<td>The chemometric tools in paper IV were further developed and metabolic fingerprints in urine for prostate and urinay bladder cancer suggested.</td>
<td>Manuscript</td>
</tr>
</tbody>
</table>
Metabolism is the sum of all chemical processes with the purpose to maintain life, as well as enable reproduction, in a living organism. Roughly, metabolism can be described to include (1) catabolism where large molecules with the gain of energy are broken down into smaller compounds, (2) anabolism where small molecules are assembled to larger compounds at the cost of energy, and (3) biotransformation aimed at elimination of endogenous or exogenous compounds through excretion. Biotransformation serve two purposes; first of all it is a way to make the molecules more hydrophilic and thus feasible for excretion, secondly it prepares the molecules for further modifications known as phase II metabolism. The latter generally involves conjugation of glucuronic acids, sulfonates or glutathione to the molecule. The result is an even more hydrophilic compound that can be disposed of by either hepatic (through the liver) or renal (through the kidneys) excretion. Exogenous as well as endogenous compounds are subject to this process and therefore any intermediate in the metabolic pathways may end up in the chain of events that lead to excretion. As a result, a thorough analysis of a urine sample has the potential to reveal information about the metabolic state of an individual. Provided a correct backtracking of the metabolic pathways is performed, the origin of a metabolic anomaly could theoretically be detected from information about metabolites at the end of that pathway\(^1,2\).

It is also important to understand the metabolism at a more detailed level. In pharmaceutical development, for example, it is of high significance to investigate which metabolites are formed from a parent drug and at which rate these are formed. Pharmacokinetics has to be investigated, as well as the distribution of the drug to different parts of the body. Sometimes the intended effect of a drug has a direct influence on a compound in a metabolic pathway, and the effect can be measured directly with analytical methods. For some diseases diagnostics is carried out through chemical analysis of body fluids, and the course of a disease can be monitored in the same manner. Studies of the metabolome include several approaches and can be divided accordingly\(^3\):

I Targeted analysis; the assessment of a limited number of target analytes (see 2.2).

II Metabolite profiling; the investigation of a set of metabolites associated to a specific metabolic system or pathway (see 2.3).
III Metabolic fingerprinting; the use of tools to acquire and explore metabolic fingerprints rather than specific metabolites (see 2.4). Each of these approaches will be further clarified in the text to follow, together with the demands on the analytical method and information about which instrumentation is best suited to be used in the analysis.

2.1 Metabolomics

The term metabolomics has been defined as the systematic study of the unique chemical fingerprints that specific cellular processes leave behind⁴, while the term metabonomics has been defined as the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli of genetic modification⁵. The terminology is confusing to say the least. Metabolomics has to great extent been associated with plant studies⁶ and investigation of metabolic changes in single cell systems. Metabonomics seems to be used when metabolic studies are conducted on animals or humans⁷, and when the whole metabolome is investigated. There are several examples where the two terms are mixed up and it is wise to include both terms when searching for articles online. Since no real consensus so far has been reached in the scientific community regarding the terminology, one can only assume that in the future the two terms will either diverge, with an increased distinction between them, or that they will merge. The concept of metabolomics/-nomics was introduced in 1999⁵, and as can be seen in Figure 1 the interest has increased drastically since then.
In this thesis the term metabolomics is used for one sole reason; there are far more publications using that term, and if the two terms merge it feels safe to assume that this is the one that will be used. When the word metabolomics is used in this thesis it refers to the study of \textit{systematic responses in the endogenous metabolome of a living organism as a result of disease, medication or otherwise induced influences}. \textbf{Paper IV} is a good example of a metabolomic study, where systematic changes to the metabolome were investigated after intake of coffee and tea. In \textbf{paper V} diseases in the urinary tract (prostate cancer and urinary bladder cancer) was studied. A major part of the responses correlated to the diseases can be assumed to originate from direct contact, or close approximation of the tumor cells to the sample matrix. It is therefore a matter of opinion whether this study should be defined as metabolomics or not.

\subsection*{2.2 Targeted Analysis}

There are countless numbers of applications that would fit into this category of chemical analysis, of which \textbf{paper I} and \textbf{paper II} are but two examples. Quantitation of a few selected compounds is made with a priori knowledge about the structure of the analytes. Depending on which matrix the analytes are present in the details of the analytical chain may vary. Typically some sample pretreatment, such as solid phase extraction (SPE) is followed up by a liquid separation technique hyphenated to mass spectrometric (MS) detec-

}\textbf{Figure 1}. Publications with \textquote{\textit{metabolomic*}} and \textquote{\textit{metabonomic*}} listed as key-words according to ISI Web of Knowledge\textsuperscript{8}.
tion. Depending on the type of study, and the nature and function of the analytes, demands on limit of detection (LOD) and accuracy of the analysis may differ. In many cases concentration levels of a few nanomolar has to be quantified with high precision. Sample volumes may range from several mL down to a few μL or even smaller in some cases; analytes may vary in chemical stability or undergo chemical degradation due to interactions with the sample matrix if the samples are not treated with care; contents of the sample matrix may interfere with the analytes during the course of analysis etc. In explorative metabolomics, targeted analysis seldom is the focus since a priori knowledge of which metabolites will be affected in association with a certain biological state rarely are available. After initial studies have been conducted however, a few compounds may be selected as biomarkers, and targeted analysis may be used to monitor these. For further information on quantitation and validation, see chapter 5.

2.3 Metabolite profiling

In some cases it is not enough to measure the levels of a few selected metabolites in order to understand a pathologic state, or the dynamics of a pharmacological substance. Feedback mechanisms may compensate for genetic or otherwise induced changes in the metabolic pathways in such a way that they are not detected if only the product of the metabolic pathway was to be investigated. In other cases a small change in metabolite levels at the top of a metabolic pathway may be amplified further down the metabolic chain through cascade reactions or up-regulatory mechanisms. For both of these examples a more thorough investigation of the metabolic pathway has to be undertaken in order to fully understand the alterations of the system involved. In other situations the relative concentration in a set of metabolites is more interesting than the absolute level of each metabolite, or at least of equal importance. Often the distinction between metabolic profiling and the other two categories, targeted analysis and metabolic fingerprinting, is not that obvious and sometimes they are definitely overlapping. It can be debated if targeted analysis really should be included in the definition of metabolomics given in this thesis, while metabolic profiling however is a perfect example of what should be considered as metabolomics.

2.4 Metabolic fingerprinting

As discussed above, when metabolic pathways are affected by disease, pharmaceutics or other influences, several metabolites can be expected to increase as well as decrease as a result. By comparing detailed data obtained from groups of samples, as was done in paper IV and paper V, a metabolic
fingerprint consisting of systematic differences in levels of the affected metabolites can be obtained. There are several strategies to find the information of value, each with its flaws and merits, but what they all have in common is that the quality of the raw data is crucial for successful extraction of vital information. Since little or no information about the analytes are known a priori, it is hard to define the criterions for the chemical analysis; one group of compounds is often discriminated when parameters are optimized for another group (see 3.1.1). A common strategy to gather qualitative information about the sample content is to use a high precision separation system coupled to a mass spectrometer, and include as many compounds as possible in the analysis. As a consequence a lot of superfluous information is collected, even though important information may still be lost. The data is often presented in two dimensional contour plots (Figure 2) with time on the x-axis, m/z on the y-axis and intensities on the z-axis. When the plot is viewed from above, the z-axis is typically color coded, or as in Figure 2 depicted in grey scale.

![Contour plot with grey scale coded intensities.](image)

**Figure 2.** Contour plot with grey scale coded intensities.

### 2.4.1 Data handling

The quantity of data collected and processed in paper IV and paper V summed up to such amounts that data reduction was a necessity for present day computers to perform the multivariate data analysis. In-house built software tools were created for the work in paper IV, which served as a model to confirm the legitimacy of the techniques involved. The tools were further developed in paper V, why that study will be used to illustrate the discus-
sion on data handling: The data set consisted of 49 samples of which duplicate analysis were performed. Data was collected in both positive and negative mode, resulting in an average 70 million and 115 million data points per run respectively. The data was read into Matlab and organized in a regular mesh with respect to \( m/z \) and retention time. Any data point containing fewer than 3 counts were regarded as noise and discarded. This reduced the amount of data to 40 million data points per run in positive mode and 60 million data points per run in negative mode. Since the data was collected with a time of flight (TOF) instrument, the reported mass values did not appear in an equidistant grid (see 4.2.2), but increased from a resolution of \( \Delta m/z = 0.009 \) for \( m/z \) 60 to \( \Delta m/z = 0.036 \) for \( m/z \) 1000. The stored data, however, was binned in \( m/z \) channels with \( \Delta m/z = 0.1 \), which corresponds to the approximate width of a mass peak. A spline function was then used to define the chromatographic base line, and data points containing non vital information (i.e. noise) could be discarded\textsuperscript{10,11}. The binning and baseline reduction condensed the number of data points in each run to less than one percent of the original amount.

![Image of Chamaeleo Dilepis](image)

**Figure 3.** With the aid of multivariate data analysis, individual differences between the samples can be ignored and systematic trends defined. *Original picture of Chamaeleo Dilepis provided by Joakim Ahlgren.*

The search for systematic differences between groups of contour plots can be resembled to searching for common elements in a series of pictures (see **Figure 3**). A prerequisite for this is that the pixels, i.e. the peaks, are aligned
in m/z as well as in time. Instrumental m/z drift was significantly below the binning level previously described, why no further measures were taken regarding that. Retention times however, varied with as much as 30 s for some peaks. Furthermore, the drift was not completely linear throughout the runs. By defining a master run, and for each run in the data set creating pair wise correlations with the master, peaks with high correlation could be selected (see Figure 4). These were then adjusted to have the same retention times as the master, followed by a linear adjustment of the time intervals between the selected peaks. In paper IV the multi-point alignment had not yet been developed and peaks were therefore aligned in a similar, but less complex manner. During the peak alignment some strong correlations were found more far off in time than expected, and when examined closer it was found that some peaks displayed irregular retention compared to the main part of the sample peaks (see Figure 6). The origin of this phenomenon is discussed in further detail in section 3.1.1.

Figure 4. Left: Retention times of a selected run correlated to those of the master run. Right: A zoomed area of the correlation plot where a peak with strong correlation has been selected.

To further reduce the amount of data before principal component analysis (PCA) was carried out, binning was performed with respect to time. To compensate for the naturally occurring variation of total concentration in the samples, peak intensities were normalized to the same total sum in all samples. Furthermore, a square rooting of the intensities was performed in order to prevent the high intensity peaks from dominating the PCA. A fuzzy PCA\textsuperscript{10, 11} was carried out, and mean values along with confidence intervals were calculated for the scores of each group (see Figure 5). When performing binning, peaks located near the bins edges may slip into different bins in
different runs due to minor drift. A traditional PCA will treat such peaks as different compounds. The concept of fuzzy PCA however, was able to compensate for this as well as for the peaks differing irregularly in retention time. **Paper V** is a work in progress, and currently the “hotspots” contributing to the fingerprints are being defined. The next step would be to perform a structural analysis of these compounds in order to gain increased understanding of tumor biology and ultimately also develop new diagnostic tools for the diseases.

![Image](image_url)

**Figure 5.** Mean scores for PC1 and PC2 according to two-way ANOVA with approximately 95% confidence regions. Left: Prostate cancer (p), bladder cancer (b) and control (c). Right: Male (m) and Female (f).

Throughout the work in both **paper IV** and **paper V** the guiding-star was to perform each operation in such a way that no relevant information were lost. With other strategies, such as data reduction through peak picking, there is always a risk that smaller peaks are overlooked in comparison with large ones. For biological samples collected from several individuals, it is likely that most apparent differences occur from individual genetic variations rather than originating from the factors to be investigated. The principal component analysis performed in **paper IV** showed that the variation of interest did not appear until the third principal component, while in **paper V** significant differences between group means were obtained already for PC1 and PC2 (Figure 5).

Another important concept was to perform as many of the operations as possible interactively so that the result of each step could be visually inspected. Without the semi manual peak alignment, the peaks displaying irregular retention behavior would never have been identified.
3 Liquid separation

The purpose of liquid separation is to transform a mixture of constituents into isolated parts, containing only one compound each. This will facilitate qualitative and/or quantitative measurements of the components. A well performed separation enhances the quality of an analysis method, and is often crucial for selective detection of the investigated compound. Furthermore, sample cleanup may be needed in order to protect sensitive parts of the equipment (for example from clogging) further down the analysis process (see 3.1.1). Also, when electrospray ionization mass spectrometric detection is used, constituents in the sample matrix may interfere with ionization (see 4.1.1) or harm the ion source or mass spectrometer (see 4.3). In paper I and paper II the separation was therefore carried out in two steps, where the first functioned as an online solid phase extraction (SPE). The purpose of the first step was to wash the sample and concentrate the analytes, while the second step was performed to facilitate the detection.

In metabolic fingerprinting, detailed data sets describing complex biological samples are investigated, and the image resolution, i.e. the total information content is therefore of importance. Thus, in paper IV and paper V the separation not only facilitated the detection, but also in itself provided vital information for the data analysis.

One of the challenges associated with liquid separation is the compromise between quality and time consumed since the request for high throughput analysis is often in conflict with the demands for accurate and sensitive methods. Therefore it was important to set up a defined goal in advance for the quality of the method described in paper I, which was later used in paper II. Paper IV and paper V describes explorative methods where the demand on rapid separation is of less importance.

3.1 Liquid chromatography

Chromatography is by the International Union of Pure and Applied Chemistry (IUPAC) defined as “a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (the stationary phase) while the other (the mobile phase) moves in a definite direction”\(^{12}\). In liquid chromatography (LC) the mobile phase consists of a liquid, which commonly is mechanically pumped through a solid
medium. This can consist either of a bed of porous particles, a porous mono-
lithic structure, or in rare cases an open tube. Most common is the use of
silica particles covered with covalently bond molecules of the desired prop-
erty. Due to different distribution of the compounds between the phases,
constituents of a sample introduced to the system will gain distinct velocities
and thus be separated from each other. The only exception of this general
mechanism is size exclusion chromatography. Here the stationary phase is
constructed in such a way that smaller molecules are permitted a longer path
through the media and thus have lower linear velocity than large molecules.

Important contributions to LC theory have been made throughout the
years by scientists such as Giddings, van Deemter, Knox, Guiochon and
Horváth\textsuperscript{13-19}. In the sections to follow some of the aspects that are important
for the work included in this thesis will be discussed. Theoretical arguments
will be explained when found to be needed.

3.1.1 LC phases

LC was employed in paper I, II and V, and in all three cases reverse phase
(RP) was used. Paper I and paper II utilized in-house slurry packed col-
umns with butyl (C4) covered silica particles for sample cleanup and pre
concentration, and octadecyl (C18) covered silica particles for the separa-
tion. In paper V a commercially available C18 column designed to perform
well during extreme gradients and provide good separations for polar anal-
lytes was used. In RP chromatography the retention mechanism is caused by
hydrophobic interactions of the solute with the non polar stationary phase.
Several retention models have been suggested in the literature of which the
partitioning model\textsuperscript{20, 21}, the adsorption model\textsuperscript{22, 23} and the solvophobic the-
ory\textsuperscript{24} are especially worth mentioning.

The elution strength of the mobile phase is controlled by adjusting the
type and amount of organic modifier. Retention can also be influenced by
affecting the protic state of the solute through pH adjustments, or by pairing
the solute to a counter ion and thereby gain increased retention. Since the
analytes and/or sample matrix often have protic properties, the pH may be
affected in the sample plug. In paper I, II and V the samples were therefore
diluted with mobile phase before they were introduced in the separation sys-
"
times broader than that of the larger substance. A possible explanation for both observations could be a secondary retention mechanism besides the hydrophobic interaction with C18. For example an ion exchange interaction with free silanols\textsuperscript{25, 26} would generate concentration dependent retention times due to increased competition for available silanols when total sample concentration is increased. Strong ion pairing with another substance present in the sample is also a possible explanation, provided the pairing substance differs in concentration between the samples. A third explanation could be ion pairing with residue trifluoroacetic acid (TFA) that had previously been used in the LC system. However, the residues would have been gradually washed out with a steadily decreased retention of the compound for each analysis performed, and no such trend could be identified.

**Figure 6.** Overlay of two selected masses (m/z=109 and m/z=539) extracted from two separate samples (A and B). In both runs the compound with m/z=539 have the same retention time, while m/z=109 may differ as much as 30 s.

Although RP is suitable for many organic substances, polar compounds have little or no retention on the stated material. As discussed in the introduction of section 2, compounds found in urine, are expected to be highly hydrophilic, and a retention mechanism complementary to RP needs to be considered for full separation of the sample. Ion exchange chromatography (IEC) could provide a solution since the various packing materials are ionic in na-
ture (commonly cationic or anionic, but mixed columns are also available) and retention is achieved through electrostatic interactions between analyte and stationary phase. Elution strength is controlled by adjusting the ionic strength of the mobile phase, as an increased salt level will displace the analyte from the stationary phase. However, this excludes the neutral compounds as well as either the cations or the anions from effective retention, and much information about the sample is still lost.

Many research groups have therefore turned their attention towards hydrophilic interaction liquid chromatography (HILIC), which actually is a special case of normal phase (NP) chromatography. In practice NP works as the exact opposite of RP; analytes are retained on a polar stationary phase and elution strength increases with increased amount of polarity in the mobile phase. However, the incompatibility of NP with water based matrices makes the technique a non favorable choice for analyzing biological samples. HILIC on the other hand is compatible with water and makes use of several retention mechanisms simultaneously. The general idea is to use a polar surface that is surrounded by a thin water layer and thus create a solvation partitioning system. The polar surface can be constituted in many ways, but of particular interest when trying to achieve retention of a wide spectrum of analytes is of course zwitterionic groups such as the one used in ZIC®-HILIC (Figure 7). This strategy makes it possible to achieve a combination of hydrophilic and IEC interactions. The high content of non polar solvents in the mobile phase could be beneficial for the electrospray ionization process in the detection step. On the other hand, the high salt levels needed to make use of the IEC interactions could prove a challenge for the ionization process (see 4.1.1 and 4.3.1). This material is being considered for the future work on paper V, but has so far been avoided due to the increased complexity of combined retention mechanisms.

![Figure 7. The zwitterionic group attached to the solid phase in ZIC®-HILIC.](image)

3.1.2 Gradient LC

When the analytes display high difference in retention time on the chosen stationary phase, gradient elution may be considered. The eluting strength of the mobile phase is then increased throughout the analysis and can be con-
trolled in such a fashion that an optimal separation is carried out in shortest time possible. In addition the analytes experience what is known as stacking or focusing on the top of the column, with high and narrow peaks as the intended result. The low elution strength in the beginning of the gradient generates a relatively low velocity of the analytes through the column initially, and the zone containing the analytes is compressed on the top of the column. When the zone leaves the column, the analytes in the front instantaneously accelerate to the velocity of the mobile phase and the analyte zone is extended again. With an optimized gradient however, the zone accelerates through the column, more or less keeping its shape throughout the acceleration. The increase in velocity, and consequently zone broadening, when the plug leaves the column is therefore less pronounced. Stacking effects can also be utilized when the elution strength of the sample plug injected is lower than that of the mobile phase, enabling the technique to be used to some extent even in isocratic separations. With the help of stacking an increased signal to noise ratio can generally be achieved, and the technique was utilized in paper I, II and V.

Figure 8. The long tails in the chromatogram from paper I can be explained as an effect of a too rapid increase of organic modifier during the separation and/or secondary retention mechanisms. The elution order is (a) melagatran, (b) OH-melagatran, (c) ethyl-melagatran and (d) ximelagatran.
Figure 8 displays a chromatogram from paper I. The long peak tails have two likely explanations, namely non optimized gradient shape\textsuperscript{33} and secondary interactions with free silanols\textsuperscript{25, 26}. When the elution strength of a gradient increases rapidly, the velocity of solutes on the particle surface increases immediately. The change in mobile phase composition inside the pores will however lag behind, and solutes maintain a stronger interaction with the stationary phase than on the outside. Furthermore, the solutes inside the particle pores have to be transported out to the surface through diffusion before their velocity can increase, and thus lag behind the front of the peak. The tailing can also be explained by secondary interactions with free silanols as discussed in section 3.1.1. During the work with paper III it was observed that ximelagatran indeed has strong affinity for silica capillary walls. However, it is possible that both mechanisms contribute to the tailing. A third possible explanation could be band broadening in the ESI source due to recirculation of sample vapor in the gas phase\textsuperscript{34}. Since the method validation was meeting the in advance set demands, no effort to obtain more appealing chromatographic peaks was made.

3.1.3 Elevated temperatures in LC

In paper V the separations were performed at an elevated temperature with the purpose of decreasing the viscosity of the mobile phase. This causes the pressure drop over the column to decrease, which allows for higher flow rates, thus generating faster analysis. Furthermore, diffusion of the analytes in to, and out of, the particle pores increase, which improves the separation\textsuperscript{35}. In addition the mobile phase exchange in the pores takes place more rapidly, which further improves the retention behavior of the analytes, as well as decreases reequilibration times for the column. Considerations should however be made regarding column stability since elevated temperatures can shorten its lifetime (especially if operated at low pH). Degradation of thermo labile compounds in the sample is also an issue when employing elevated temperatures in LC, but was in paper V neglected due to the benefits of improved separation and decreased back pressure.

3.2 Capillary electrophoresis

Early fundamental studies of what later developed into the separation technique known as capillary electrophoresis (CE) was performed in the beginning of the 20\textsuperscript{th} century by Michaelis\textsuperscript{36}. Important contributions were made in the 30’s by the Swedish chemists Arne Tiselius\textsuperscript{37} who was awarded with the Nobel prize 1948 for his work on electrophoresis and chromatography. Further development was made by Stellan Hjertén\textsuperscript{38}, and in 1981 the concept of electrophoretic separation in a glass capillary was first described\textsuperscript{39}. 


While LC utilizes the distribution of an analyte between a mobile and a stationary phase, capillary electrophoresis (CE) separates the different species based on their electrophoretic mobility in solution in an electric field. Briefly, a CE analysis is carried out as follows: The sample is commonly introduced by hydrodynamic injection into a fused silica capillary (usually 25-75 μm i.d.) that is filled with a background electrolyte. With the aid of a high voltage power supply a potential is applied over the capillary and ions will migrate towards the capillary end of opposite charge. Since the capillary inner walls have a high density of negatively charged silanol groups, cations from the electrolyte will be attracted to the walls. The layer of ions closest to the wall, commonly known as the Stern layer, is considered immobile. However, the cations in the second layer will migrate towards the cathode and drag the surrounding solvent in the same direction, creating an electroosmotic flow (EOF). The sample ions will migrate towards their opposite charge and thus have a different velocity than the EOF. Since the ion mobility is dependent on size and charge, small cations will reach the detector first, followed by large cations, neutral molecules, large anions and finally small anions (Figure 9). Provided that the EOF exceeds the electrophoretic mobility of the anions with highest mobility, all analytes will eventually reach the detector. Several ways to modify the silica wall surface by altering its charge has been developed in order to control the EOF in such a way that it can be minimized, eliminated or reversed.

Figure 9. Schematic figure of a CE setup where migration order can be seen in the enhanced cross-section. Figure made by Andreas Dahlin.
CE displays high peak separation efficiencies due to the flat flow profile generated by the EOF, as well as short run times. However, CE suffers from insufficient repeatability with regards to irreproducible injection volumes as well as fluctuations in migration time. Furthermore the injection volume is normally as small as 50 nM. Therefore CE has so far rarely been the first choice for quantification.

Since the injection usually is performed hydrodynamically, the injection volume will be sensitive to inconsistent back pressure in the system. Variations can be caused by viscosity differences between the samples, or due to temperature dependent viscosity changes of the background electrolyte (which may also affect the EOF). The latter can be minimized by keeping the capillary in a thermostated environment. This is readily done when the detector is integrated in the CE-system, as often is the case for UV detection. However, when coupling CE to mass spectrometry, the capillary will partly be exposed to room temperature which makes complete temperature control impossible. The impact on the injection volume from the non thermostated part, compared to differences of the sample viscosities, can however be debated.

Variation in migration times are often caused by alterations of the capillary wall properties due to interactions with the sample content. When analyzing complex samples as in paper IV, some of the compounds are likely to have high affinity to the silica surface, resulting in visible changes of the EOF from run to run. By regenerating the capillary with sodium hydroxide at regular intervals this effect can be minimized. When mass spectrometry is used the capillary should be removed from the ion source prior to regeneration in order to protect the instrument from contamination. If the ion source is orthogonal to the instrument inlet, turning off the nebulization gas and capillary voltage is sufficient. This inconvenience is not required if UV detection is used. In paper IV the capillary was reconditioned for every twentieth run after being removed from the mass spectrometer. For some compounds migration times still varied up to 20 s in a 6 min run, and manual peak alignment was performed as a part of the data pre treatment. Further details are given in paper IV.

Since sample consumption is low (usually 10-50 nL), CE is a suitable analysis method when sample volumes are limited. Another advantage of CE in comparison to LC is the ability to handle crude samples. An open capillary rarely get clogged, and in most cases the only sample pretreatment needed is dilution in order to lower the ionic strength below that of the background electrolyte. If the sample zone has higher ionic strength than the background electrolyte its field strength will decrease, which will lead to increased band broadening. Lower ionic strength on the other hand, will permit the opportunity of stacking effects, with an up concentration of the analyte as a result. The straightforward sample pre treatment was one of the reasons this technique was chosen for paper IV. It is important to point
out that CE and LC should not be considered as techniques competing with each other, but rather as complementary techniques due to their more or less orthogonal separation principles and complementary strengths.
4 Mass spectrometry

A mass spectrometer is an analytical instrument designed to separate ions according to their mass-to-charge \((m/z)\) ratios and record their intensities. The capacity to separate ions based on their \(m/z\) was first described in the late 19\(^{th}\) and early 20\(^{th}\) century by J.J. Thompson\(^47\). Since then mass spectrometry has been developed into a detection technique that is available in most analytical laboratories. Schematically a MS system consists of an ion source, a mass analyzer and a detector. Several technical solutions are available for all three components; each with its own merits for certain types of analysis. In this section of the thesis, theoretical as well as practical aspects of ion sources and mass analyzers will be presented, with emphasis on the aspects that are of importance for \textit{papers I} through \textit{V}.

4.1 Ionization

In order to separate and detect molecules in a mass spectrometer, they need to be present as ions in gas phase. There are several techniques to accomplish this, and a brief explanation will be given for those of highest interest of the work presented in this thesis. In some cases the choice of ionization technique is crucial to the quality of the analysis, but in some cases the choice is of less importance. When transferring an analyte from a liquid phase and ionizing it in the process, atmospheric pressure ionization (API) techniques have a number of advantages over other methods. First, they are “soft” techniques, which mean that labile species in general remain intact after ionization. Second, they can handle relatively high flow rates, which make them suitable to hyphenate directly with LC. Electrospray ionization\(^48\) (ESI) has been used in all projects discussed in this thesis, but nevertheless a brief introduction to atmospheric pressure chemical ionization\(^49, 50\) (APCI) and atmospheric pressure photo ionization\(^51, 52\) (APPI) is presented in this section, since they are considered for the future work on \textit{paper V}. All API techniques should be regarded as complementary to each other and the choice of technique depends on the nature of the analytes and the separation conditions prior to detection, as will be discussed in more detail further on.
4.1.1 Electrospray

In ESI an electric field is applied between the liquid outlet and the inlet of the mass spectrometer, generated by applying an electric potential (usually 2-5 kV) on either the liquid outlet or the mass spectrometer inlet, letting the other act as a counter electrode\textsuperscript{53, 54}. At the outlet electrode electrochemical reactions occur, forming excess charges in the solution as a result\textsuperscript{53-55}. In positive ion mode the outlet acts as an anode, and cations will be attracted towards the mass spectrometer inlet, deforming the liquid surface at the outlet into a cone shape commonly referred to as Taylor cone\textsuperscript{56}. At the point where the force of the Coulombic repulsion exceeds the surface tension, known as the Rayleigh limit, droplets with an excess charge will break free from the Taylor cone\textsuperscript{57}. The droplet formation is sometimes pneumatically assisted with the use of nebulizing gas\textsuperscript{58}. According to the partitioning model\textsuperscript{59}, two separate phases exist in an electrospray droplet. Because of mutual charge repulsion, excess charges will reside in the surface layer, while the net charge of the interior is zero since the ions of opposite charge residing there will balance each other out. Ions of mutual charge may change places with each other, enabling a transfer of ions between the layers. Furthermore, in any of the layers charges may be transferred from an ion to an uncharged molecule.

As solvent evaporates from the droplet, its size will decrease and the charge density increase until the Rayleigh limit is approached and the droplet is subject to coulombic fission, generating smaller charged droplets. Usually the environment is heated to facilitate the evaporation of solvent. According to the charge residue model (CRM), the newly formed droplets undergo the same process over and over again until droplets containing only one ion remains\textsuperscript{60, 61}. When the last of the solvent has evaporated the ion has been transferred to gas phase. The ion evaporation model (IEM) initially describes the same process, but instead of evaporation of the last solvent remains, Coulombic forces pushes the ions free from the surface\textsuperscript{62, 63} (Figure 10). Probably both mechanisms are valid, and depending on the analyte as well as the constitution of the sprayed solution, either of them is dominating. CRM is believed to dominate for large molecules, while IEM is a more accurate description of gas phase ion generation from smaller molecules\textsuperscript{64-66}. 
Figure 10. The two dominating explanations of transfer of ions to gas phase in ESI is the Ion Evaporation Model (IEM) and the Charge Residue Model (CRM). Figure made by Andreas Dahlin.

Several mechanisms have been suggested to explain the difference in ionization rate when comparing different analytes. The distribution of the analyte to the droplet surface clearly affects its ionization rate since the excessive charges must reside there. Since a molecule with a non-polar region is more likely to exist in the surface layer due to the non-polar surrounding gas phase, it is more likely to be ionized. Along the same line of thoughts, a droplet consisting of a mix of water and organic solvent is believed to have a higher concentration of organic modifier at the surface, which will enhance the effect. Furthermore, a large molecule should statistically be more probable to be found close to an excessive charge than a small one. Different species could also interact with each other, transferring charges between them, especially since some molecules have a higher charge affinity than others due to their internal structure.

Competition for the excess charges exists between the constituents of the droplet and can cause what is known as ion suppression. This can be observed as decreased ionization of molecules with relatively low proton affinity when (1) excess charges are scarce, (2) elevated levels of matrix constituents with high proton affinity are present, or (3) salt levels in the solvent are high. High salt levels may also give rise to adduct formation or ion clusters, generating complex mass spectra that are complicated to interpret. All these phenomena are examples of what is commonly referred to as matrix effects. In order to minimize the risk of matrix effects, separation was performed in two steps in paper I and paper II as described in section 3.1.1. The impact of matrix effects on the precision of a quantitative method can be minimized.
by the use of an isotopically labeled internal standard\textsuperscript{70} (see 5.1.2), which was utilized in both papers. The concept of excess charge competition is discussed in paper III where the mechanisms behind the formation of singly and doubly charged ions are discussed.

Besides the peak band broadening that may arise when hyphenating liquid separation with ESI (see section 4.3), band broadening can also occur in the gas phase due to recirculation of sample vapor\textsuperscript{34}.

4.1.2 Gas phase ionization techniques

When conducting a study where low abundant small molecules are present and the matrix effects described above threatens to hide vital information, ESI may not be the most beneficial choice of ionizing technique. Instead of ionizing the analytes in solution and then transfer the ions to gas phase, the option of gas phase transfer before ionization can be considered. In APCI\textsuperscript{54, 71, 72} the liquid from the separation is nebulized with the assistance of a high pressure gas flow similar to the setup in ESI. The aerosol formed is then passed through a heated compartment where the solvent is vaporized, before the resulting gas is carried along a corona discharge needle, where electrons are emitted and ionization is induced (Figure 11). Through a chain reaction, primary ions formed induce the formation of secondary reactant gas ions, originating from the solvent used in the separation. The analytes are then ionized by proton transfer or adduct formation with the reactants as they collide with each other. Since the process occurs at atmospheric pressure, the gas density is sufficiently high for the collisions, i.e. charge transfers, to occur with high efficiency.

![Figure 11. The principle of atmospheric pressure chemical ionization (APCI). Figure made by Andreas Dahlin.](image)

APPI is one of the most recently introduced ionization techniques and is set up in a similar fashion as APCI, with a UV lamp replacing the corona dis-
charge needle\textsuperscript{51,52}. Photons that are emitted from the lamp and have a higher energy than the ionization potential of the analytes are absorbed by the molecules and ions are thus formed. Depending on which type of UV lamp is used, ionization of the solvents can be avoided without decreased analyte ionization, since these in general have lower ionization potential than the solvents (Table 2). It should be noted that even though the solvents do not ionize, they still absorb and thus consume photons. The charge transfer mechanisms from a relatively high abundant reactant similar as in APCI can be utilized by adding a dopant such as toluene or acetone to the solvent.

Table 2. A compilation of common UV lamps, dopants and solvents used in APPI. The table illustrates which energy the photons emitted from respective lamp type carries, and the ionization energy of respective dopant and solvent.

<table>
<thead>
<tr>
<th>Energy (eV)</th>
<th>Lamps</th>
<th>Dopants</th>
<th>Solvents</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.4</td>
<td>Xe</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.83</td>
<td></td>
<td>Toluene</td>
<td></td>
</tr>
<tr>
<td>9.7</td>
<td></td>
<td>Acetone</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Kr</td>
<td></td>
<td>Methanol</td>
</tr>
<tr>
<td>10.84</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.2</td>
<td>Ar</td>
<td></td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>12.2</td>
<td></td>
<td></td>
<td>Water</td>
</tr>
<tr>
<td>12.62</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Neither APCI nor APPI should be regarded as competitive techniques to ESI, but as complementary options. Since they are more tolerant to salts and matrix effects than ESI, they should be considered when working with CE or complex samples where the analytes are not sufficiently separated. In metabolomics these techniques are therefore a powerful alternative and are considered for the continued work on \textit{paper V} in case the results that are achieved using ESI are not satisfying.

4.2 Mass analyzers
4.2.1 Quadrupole instruments

A quadrupole consists of four conducting parallel rods acting in pairs. By applying alternating positive and negative electric potential on the rods, ions will accelerate either away from or towards the centre axis of the rods. An oscillating trajectory of ions passing through the quadrupole is created by adjusting the direct current (dc) potential and radio frequency (rf) of the applied electric field. For ions within a certain $m/z$ window the trajectory will be stable, while all other ions will collide with the rods and thus be filtered away. By adjusting the dc and rf potentials, a quadrupole can either be set to filtering out a few selected $m/z$ or to stepwise scan a wider mass window in
cycles of a defined period of time. Since quadrupole instruments do not continuously register the ions that enter them, sensitivity will suffer for each additional $m/z$ observed, i.e. the duty cycle is low in scan mode. As a consequence, depending on the mass range covered, data acquisition in scan mode is rather slow. Therefore quadrupole instruments are not the best suited instrument type for that kind of task. The time spent in the quadrupole increases with $m/z$, which means increased mass resolution. Resolving power, which is the ability to yield distinct signals for two ions with a small mass difference, is nevertheless considered to be poor for quadrupoles, why identification of multiply charged species can be difficult.

The triple quadrupole (QqQ) instrument is equipped with three quadrupoles in sequence, which permits several different approaches for detection. In product ion scan mode a precursor ion, also referred to as parent ion is selected in the first quadrupole and fragmented by collision with inert gas in the second quadrupole. This type of fragmentation is called collision induced dissociation (CID) and can in theory to some extent take place anywhere in the instrument, but preferably occurs in the collision cell where the impacts can be properly controlled\textsuperscript{73}. The third quadrupole is operated in scan mode which determines the $m/z$ of the fragments. By employing this technique, information about the chemical structure of unknown species can be acquired along with information about the stability of the precursor ion. Triple quadrupole instruments are therefore excellent tools to investigate the nature of “hotspots” that has been identified in a metabolic pattern recognition study. Some of the planned experiments for paper V include experiments of this kind. In paper III the techniques was used to study the fragmentation patterns of a singly charged species compared to that of the doubly charged precursor ion originating from the same molecule. A comparison of the stability of the two ions was also made.

In multiple reactions monitoring (MRM) mode a parent ion is selected in the first quadrupole, fragmented by collision with inert gas molecules in the second, followed by selection of a fragment to monitor in the third quadrupole. This results in a highly selective detection where even two compounds of the same mass can be separated from each other, provided they have a different fragment pattern. As a result of the high selectivity, background noise is reduced immensely with beneficial outcome for the signal to noise ratio. Since the dwell time is also important for S/N the acquisition can be divided into several parts, which allows fewer $m/z$ to be monitored simultaneously. The quantitative method that was developed in paper I and later employed in paper II was therefore managed in MRM mode, with the acquisition split into two parts.
4.2.2 TOF

The basic principle of a TOF instrument is to accelerate ions to a certain kinetic energy, measure the time of flight between two given points in a field free space, and then calculate the m/z ratio. The concept of the TOF analyzer was first described in 1946\textsuperscript{74}, and the first design of a TOF mass spectrometer was published in 1955\textsuperscript{75}.

The first TOF instruments were linear and made use of a pulsing ion source, creating packages of ions that were accelerated through the instrument towards the detector. Modern instruments are generally constructed in a slightly different manner with an orthogonal flight tube\textsuperscript{76}, which allows them to utilize ion sources working in a continuous mode. After the ions have entered the TOF instrument they are transported in an unbroken beam to the flight tube where the mass separation takes place (Figure 13). Ion packages are accelerated along the flight tube by an electric field generated by what is commonly called a pusher. Repeated pulses give all ions with the same number of charges an equal amount of kinetic energy according to

\[ E_k = \frac{mv^2}{2} \]

where \( E_k \) is kinetic energy, \( m \) is mass and \( v \) is velocity. Species with low m/z will therefore move faster in the flight tube than species with high m/z and thus reach the detector in shorter time. The flight time dependence of m/z is plotted in Figure 12 and can be described as

\[ t = d \sqrt{\frac{m}{2E_k}} \]

where \( t \) is time and \( d \) is distance. Multiply charged ions are affected stronger by the electric field and will travel with the same speed as the singly charged species of corresponding m/z. When the ion impact with the detector is registered, m/z can be calculated from the flight time. Mass resolution increases with increased mass\textsuperscript{54}, but since the instrument registers the impacts with a fixed time interval the data points will be closer for low masses (Figure 12).
Since the time difference of the impacts of ions with different m/z is dependent on the distance they travel, a longer flight path will provide enhanced mass resolution in the instrument. Therefore an electrostatic reflector\textsuperscript{54, 77}, also called a reflectron, can be placed at the end of the flight tube and by working as an ion mirror effectively increase the flight path towards the detector, which in that case is placed at the inlet of the flight tube\textsuperscript{54}. Furthermore, the reflectron will compensate for the higher speed of ions that from the start are positioned further back in the pusher and thus are accelerated more than ions with the same m/z in the front (Figure 13).
TOF instruments “continuously” register ions over the complete mass range, as opposed to quadrupole instruments in scan mode where one \( m/z \) is measured after the other, and consequently offer high resolution mass scans without loss in sensitivity or time resolution. Thus a TOF instrument is the ideal choice for investigations where acquisitions of wide mass range in combination with high time resolved detection of efficient peaks are demanded. In pattern recognition studies like paper IV and paper V, TOF instruments are therefore often utilized.

When investigating multiply charged species the isotopic peaks will have a distance of less than one mass unit to each other in the mass spectrum\(^{54}\). Instruments with poor mass resolution might then lack the ability to distinguish between the peaks. The high mass resolution in TOF was therefore utilized for some of the experiments in paper III.

### 4.3 Hyphenation of liquid separation with ESI

Several challenges have to be taken into account when using ESI together with different separation techniques. The optimum conditions with regards to pH, solvent additives, flow rate etc. for any given separation technique, rarely matches the optimum conditions for any given API technique\(^{53, 78-80}\). Furthermore, strong ion pairing with the analyte that result in neutralization\(^{62}\) and non volatile buffers causing instrument or ion source contamination are preferably avoided\(^{79, 80}\). However, there are a number of ways to overcome most of these challenges and the techniques described in the two following
paragraphs have either been used or considered, when conducting the experiments on which this thesis is based.

4.3.1 LC-ESI

Analytical LC flow rates can range from a few nL/min up to a couple mL/min. Depending on the construction of the ESI interface, too low or too high flow rates may cause difficulties to maintain a reliable and efficient ESI. Furthermore, an interface with large internal volume will cause extensive band broadening if flow rates are low. Incomplete evaporation of the ESI droplets is an issue for high flow rates. Provided the spray is formed in a heated environment this can however generally be dealt with. However, the most direct way to avoid the problem is to develop the separation method using a column that is adapted for the same flow rates as the interface, or if flow rates are too high simply split the flow post column. The ESI interface used in paper I and paper II was well suited for flow rates in the μL/min range, which was one of the reasons a capillary column appropriate for 10 μL/min was utilized. Furthermore, the spray current displays a square root dependence on flow rate. The number of excess charges per unit volume will therefore decrease with increased flow. The consequence of this for forming multiply charged ions was investigated in paper III.

Some mass spectrometry manufacturers provide ESI interfaces where the positioning of the sprayer can be adjusted, which will affect the sampling rate of ions. In paper I and paper II an interface with such a feature was used and care was taken to find the most beneficial positioning in order to gain as high S/N as possible. The sprayer should be adjusted with respect to flow rate as well as the flow of the nebulization gas, but the m/z of the analyte is important as well. As a general rule, the sprayer should be placed closer to the orifice for large analytes, such as proteins, compared to small compounds.

If the separation step utilizes a mobile phase with poor characteristics for the ionization, post column modifications can be considered. By adding a flow of a solution with suitable composition through a tee connection, parameters such as pH and/or organic modifier content can be adjusted, with increased ionization efficiency as a consequence. Signal suppression due to ion pairing with the analyte can also be minimized. In case of gradient elution, the electrospray conditions change over time and optimization of the instrumental parameters might therefore be challenging. However if the analytes of interest elute during similar conditions, optimization can often be as straightforward as for isocratic elution. In paper I and paper II, the compounds eluted during fairly comparable conditions. Furthermore, isotopically labeled internal standards (see 5.1.2) were used for all four analytes, which compensated for slight variation in retention time. No post column adjustments were therefore made. In paper V however, the gradient spanned from
100 % H₂O initially, to 100 % ACN at the end of the run. All eluting compounds in the investigated m/z range were of interest and data was collected in both positive and negative mode. A post column modification that contributed to 20 % of the total flow was therefore used in order to stabilize the organic modifier content as well as adjusting the pH for the negative mode run. The added flow consisted of 90 % 2-propanol and a buffer that, in positive mode matched the column flow, and in negative mode roughly neutralized the pH.

4.3.2 CE-ESI

The first hyphenation of CE and MS was made by Olivares et al in 1987. In CE, the separation is performed in a fused silica capillary, which in itself does not conduct electricity. In order to define the electric field strength over the capillary and gain contact with the liquid inside the capillary, most commonly a sheath liquid is used. A solution typically consisting of a compromise between the separation medium and what would be optimal for the ESI is bridging the gap between the capillary outlet and the surrounding stainless steel interface (Figure 14) as done in paper IV. The added liquid can give rise to moderately decreased signal intensities due to dilution, but additives to the medium also permits an increase in signal intensity by for example adduct formation or adjustment of the pH. Furthermore, the sheath liquid will decrease the ionic strength in the droplets formed by diluting the relatively high level of buffer salts present in CE, which otherwise may have a negative effect on the spray quality (see section 4.1.1). The combined benefits and disadvantages of dilution can therefore be debated.

Since the separation media in CE usually is water based, the volatility of the effluent may be too low to form efficient electrospray droplets. By adding an organic modifier such as methanol or 2-propanol to the sheath liquid, the surface tension can be lowered and additional beneficial effects on ionization efficiency gained (see section 4.1.1). This possibility was utilized in paper IV.
During the work with paper IV it was observed that extensive band broadening took place due to diffusion into the sheath liquid if the nebulization gas flow was too low. Too high nebulization gas flow on the other hand, created problems with the conductive bridge between the capillary tip and surrounding steel tube. Careful optimization of the gas flow is therefore advised in order to generate as efficient ESI as possible. Furthermore, a high gas flow may lower the pressure at the capillary end (venturi effect), which will have a suction effect on the separation liquid inside the capillary\textsuperscript{94}. This will lead to increased band broadening, partly due to decreased control of the sample plug size but also as a result of the induced parabolic flow profile during the separation\textsuperscript{94}. The effect can be minimized by tapering of the capillary end, which was done in paper IV.

Instead of using a sheath liquid, a conductive agent can be applied to the surface of the outer capillary wall to create a sheathless interface. Commonly metal particles\textsuperscript{95} or graphite particles\textsuperscript{96} are used. This technique was considered for paper IV, but since the resulting increase in capillary outer diameter was incompatible with the interface provided by the instrument manufacturer the idea was abandoned.

Regardless of which technical solution is chosen, the high salt and/or protolytic content used in CE may threaten to contaminate or in other ways be harmful to the MS instrumentation. Even though flow rates are low it might therefore be wise to consider an orthogonal interface when coupling CE to MS.
5 Quantitation

In order to study metabolic processes, quantitation of the analytes is a necessity. The measurements can either be absolute as in paper I and paper II, or relative. The latter is of relevance in for instance metabolic profiling and metabolic fingerprinting, where the ratio between certain metabolites sometimes are of greater interest than their absolute concentration (as discussed in sections 2.3 and 2.4). Furthermore, for pattern recognition there is no demand to know which chemical compound is the cause of a measured response.

The performance of a quantitative method can be described by performing a method validation. A set of parameters are used to define how close the measurements are to the true value, how much they spread when repeated measurements are made and in which concentration interval the method can be used.

5.1 Calibration methods

In order to correlate the acquired response of a measurement with the analyte concentration, a calibration curve has to be created. There are three dominating techniques for doing this; external standard, internal standard and standard addition.

5.1.1 External standard

When working with an external standard, the analyte responses ($R_{\text{analyte}}$) of standard samples with known concentrations of the analyte is used to create the calibration curve. A mathematical relationship between concentration and response can then be achieved, and the unknown concentration of a sample with a measured response thus be calculated (Figure 15).
5.1.2 Internal standard

In order to compensate for deviations in response due to instrumental variations such as matrix effects or variations in injection volume an internal standard (IS) can be used\textsuperscript{97}. The IS can also compensate for differences that might occur in the sample handling, such as variations in dilution, evaporation, adsorption etc. The IS, which is added in a fixed amount to all samples, acts as a reference and instead of creating a calibration curve based on \( R_{\text{analyte}} \), the response ratio between analyte and IS is used (\( R_{\text{analyte}}/ R_{\text{IS}} \)) (Figure 16). In order to be subject to the same fate as the analyte, the IS should have as similar physical and chemical properties as possible. When using mass spectrometric detection, isotopically labeled analytes (commonly using \(^2\text{H}, ^{13}\text{C}, ^{15}\text{N}, ^{18}\text{O}\)) are therefore preferred. Chemical transformation, chemical interactions and matrix effects are then very similar, even if some minor discrepancies can occur in retention times\textsuperscript{98}. However, there are reports that IS can contribute to both enhancement- and suppression effects in ESI\textsuperscript{99, 100}. When working with isotopically labeled IS it is important that the mass differs sufficiently from the analyte so that it does not interfere with the naturally occurring isotopic variations. As a rule of thumb the difference should never be below 4 Da for relatively small molecules. However, depending on molecule size and which atoms are present, a larger difference should be taken into consideration. In paper I and paper II isotopically labeled IS was used for all four analytes and was added in the dilution step.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure15.png}
\caption{The principle of external standard calibration.}
\end{figure}
prior to the analysis. Since enzymatic degradation of the analytes was studied in paper II, the IS used could not be added earlier in the experiment. However, it would have been theoretically possible to add another compound which would not have been affected by the enzymes in order to compensate deviations in that part of the experiment\textsuperscript{101}. The possibility was discussed during the planning of the experiments, but it was decided that the extra work did not match the potential benefit. Besides extended method development of the analysis method, the labor of finding an IS with similar physicochemical properties as the analyte without being subject to enzymatic degradation in the rather complex medium (homogenized pig liver) would have been intricate to say the least.

![Figure 16. The principle of internal standard.](image)

5.1.3 Standard addition

If the analyte of interest is of endogenous origin it is not viable to prepare standards in the biological matrix. A blank matrix sample, completely free from the analyte, will simply be impossible to find. It is of course possible to create the calibration curve from pure standard solutions. However, unpredictable consequences of matrix effects in the ESI as well as different interactions in the separation step will then be a possible outcome when analyzing the real samples. This problem can however be circumvented by using the method of standard addition instead. The sample is divided into several sub samples which all but one are spiked with an increasing amount of ana-
lyte. The responses are plotted and a mathematical correlation defined similar to what has been described for the previous methods. When extrapolating by setting the response ($R_{\text{analyte}}$) to zero, the unknown concentration ($U$) of the sample can be calculated (Figure 17). The extrapolation renders the method less precise than previously described methods. By addition of a suitable IS the accuracy of the results can however be increased due to minimized impact of variation in the sample preparation or instrumental output. Furthermore, standard addition can be impractical when the amount of sample is scarce. The method will be considered for the continued work described in paper V.

![Figure 17. The principle of calibration with standard addition.](image)

5.2 Validation

The purpose of a validation is to ensure that the quality of a quantitative method is according to in advance set criterions. Furthermore, the validation is a description of how well a method performs.

Regulatory instances such as U.S. Department of Health and Human Services; Food and Drug Administration\(^{102}\) (FDA) in the United States and The European Medicines Agency\(^{103}\) (EMEA) in the European Union provide guidelines for the biomedical and drug manufacturing industry as well as biomedical laboratories\(^{104},^{105}\). The guidelines are continuously updated in order to adapt to new analytical procedures and demands due to new is-
Small differences in the definition and interpretation of validation parameters exist between countries, why biomedical applications often have to be approved several times if they are to be used internationally. The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) is a project with the purpose to improve the harmonisation regarding guidelines worldwide in order to reduce the need for duplicate testing.

The method described in paper I followed the guidelines provided by FDA where it was possible. As the method was used to generate the data for paper II it was important to define the quality of the quantitation in order to later on make the correct interpretation of the measurements. An explanation of common parameters used for validation along with a short description of how to test them according to the FDA and/or EMEA guidelines can be compiled as follows:

**Accuracy (trueness)** is a measure of the deviation from the known true value. At least five identical samples with known concentrations are investigated (FDA).

**Linearity** is the ability to make a measurement that is directly proportional to the analyte concentration. A minimum of five concentrations are plotted and visually inspected. For a linear relationship, appropriate statistical methods such as calculation of a regression line by the method of least squares, is used. In addition an analysis of the deviation of the actual data points is recommended.

**Sensitivity** is the response change due to a change in analyte concentration.

**Selectivity** is the ability to quantify the analyte in the presence of other compounds expected to be present, including metabolites, impurities, degradation products and matrix constituents. Blank samples of the relevant matrix are tested for interfering signals.

**Precision** is the closeness of agreement (degree of scatter) between a series of measurements of the same sample or several samples taken from the same homogenous sample source. Precision can be divided into *repeatability*, *ruggedness* and *reproducibility*.

**Repeatability** is a measure of how results spread for repeated analysis of identical samples under the same conditions. At least three samples (EMEA) or five samples (FDA) are measured in the low, medium and high concentration regions of the linear range.

**Ruggedness (intermediate precision)** is a measure of how results spread for repeated analysis of identical samples with variations of the conditions in the same laboratory. Tests are carried out in the same way as for *repeatability* with small changes in the performance, such as different operators or instruments.
Reproducibility is the precision between different laboratories. Tests are carried out in the same way as for ruggedness, but on different sites.

Robustness (EMEA) is defined as how well the method withstands deliberate variations of the conditions. The method is provoked by changes in parameters like pH, temperature and composition of the solutions, whereupon the measurements are compared to previously obtained results.

Limit of detection (LOD) is the lowest concentration possible to detect (S/N > 3) within the limits of precision and accuracy. Decreasing concentrations are measured until the boundary is approached.

Limit of quantitation (LOQ) is the lowest concentration possible to quantify (S/N > 10) within the limits of precision and accuracy. The method for determination is performed as for LOD.

Depending on the type of application the quantitative method is intended for, the demands on each parameter vary. Validation is not only performed during method development, but should be performed on a regular basis in order to ensure that the quality of the method doesn’t change with time.
6 Concluding remarks

A chain is never stronger than its weakest link, and that is true for the analytical chain as well. It is the task of the analytical chemist to find that link and reinforce it! When conducting the experiments on which this thesis is based, great effort was made to leave as few weak spots as possible. The hyphenation of different techniques often requires compromises to be made in order for the various parts to perform well together. Optimal conditions for LC and CE are often non favorable for the ESI process, development of high quality quantitation requires rigorous method development and often involves time demanding separation, information rich data sets are a necessity for successful pattern recognition but result in challenges when it comes to data handling, etc. Careful considerations were therefore always taken regarding the compromises when planning and conducting the experiments.

In this thesis it has been demonstrated that the hyphenation of liquid separation techniques with mass spectrometry, along with proper treatment of the collected data, can provide valuable information regarding metabolism. Metabolomics can contribute to increased understanding of the chemical processes in the human body, and be used to develop new diagnostic tools. A method for exploring metabolic patterns in urine samples was developed on a model and later used with the aim to find biomarkers for prostate cancer and urinary bladder cancer.

Furthermore, targeted analysis of a few selected compounds can be used to study cellular mechanisms at a detailed level. A fully automated quantitative method was developed for measurements double prodrug and its metabolites. The method was later used to study active transporters in pig liver cells.

Moreover, even though the laboratory techniques described in this thesis are considered standard techniques since many years, much is still to learn about fundamental aspects. A study was conducted to further elucidate the mechanism of ESI-MS and to describe how to improve LOD and precision for quantitative methods.

6.1 Future aspects

Metabolomics is a relatively new and quickly growing “omic” science. The metabolome is a highly dynamic system and displays a wide genetic variety.
Nevertheless it is just a matter of time before the human metabolome is fully mapped and understood. This will to a large extent be done through the other “omic” sciences; genomics, transcriptomics and proteomics, but studies like the ones presented in this thesis will play a highly significant roll as well. With enhanced computing power, improved software, better analytical tools and increased numbers of well equipped laboratories worldwide, the day that we have full understanding of the metabolome might not even be far away. Present day cell phones already have enough capacity to handle the file size needed to contain highly detailed analytical data, as well as to upload that information to any computer in the world. With proper in-field analytical equipment it will therefore be possible to perform advanced diagnostics at home, at work or outdoors. The computers are nearly there and the knowledge about the metabolome is just a matter of man-hours. It seems the development of portable analytical equipment will be the bottle neck…
Acknowledgements

Family, friends and colleagues have helped me make this thesis a reality. To all of you who have contributed in one way or another:

Tack!

Erik Allard
Uppsala 9/11 2009
Den här avhandlingen beskriver olika sätt att mäta förekomsten och halten av nedbrytningsprodukter som bildas i kroppen. Avhandlingen är baserad på fem delarbeten. Gemensamt för samtliga arbeten är (utöver att de alla behandlar sätt att mäta nedbrytningsprodukter) vissa av de laborativa mätinstrument som använts för att utföra mätningarna, nämligen masspektrometrar. Denna instrumentgrupp kan mäta halten och massan av kända ämnen som finns närvarande i ett prov, samt undersöka den kemiska strukturen hos okända ämnen. Arbetena presenteras i tur och ordning i varsitt avsnitt av avhandlingen och den teoretiska bakgrunden till de tekniker som använts behandlas i avhandlingens kappa. För att underlätta för läsaren återfinns sist i den här sammanfattningen en ordlista för de begrepp som är skrivna i kursiv stil första gången de dyker upp i texten.

8.1 Metabolism

Samlingstermen för alla de kemiska reaktioner som sker i djur och växter är metabolism, eller vad vi i dagligt tal refererar till som ämnesomsättning. I metabolismen knyts nedbrytningen av ämnen – katabolism – samman med uppbyggnaden av strukturer i kroppen – anabolism (jfr anabola steroider) – i ett nätverk. De nedbrytningsprodukter som är resultatet av katabolismen används helt enkelt som byggstenar i anabolismen. Stärkelse i bröd bryts till exempel först ner till sockerarter som sedan kan ge kroppen energi eller lagras som fett. Metabolism pågår ständig i hela kroppen, men vissa organ deltar mer aktivt än andra. Levern spelar en avgörande roll som kroppens ”reningsverk”, där främmande substanser bryts ned och omvandlas. Vad som möjliggör detta är att leverns celler innehåller mycket enzym. En grup av proteiner som underlättar och påskyndar kemiska reaktioner i kroppen. I cellernas väggar finns även transportproteiner med två möjliga funktioner; de pumpar antingen ämnen in i cellerna, där dessa kan brytas ned, eller ut ur cellerna. Förhållandet mellan transportproteiner och ämnen som förs in i kroppen på olika sätt blir därför mycket viktigt för vår metabolism. Ämnen som pumpas in snabbt och effektivt i cellerna bryts ned och görs tillgängliga för ämnesomsättningen med hög hastighet, emedan ämnen som pumpas ut med hög hastighet förblir oförändrade i kroppen under längre tid. När vi tillsför läkemedel till kroppen är det därför av stor betydelse att vi har kun-

Under utvecklingen av nya läkemedel är det även viktigt att veta vilka ämnen läkemedlet bryts ner till och hur fort detta sker. Om nedbrytningsprodukterna är skadliga, eller om läkemedlet bryts ner så fort att man måste ta nya doser med korta intervall, är läkemedlet helt enkelt inte speciellt användbart. Studier av dessa två aspekter ligger till grund för beslut om hur ofta och i hur stora doser ett läkemedel ska tas. I **arbete II** tillämpas därför mätmetoden som beskrivs i det första arbetet. Genom att tillsätta läkemedlet till provrör innehållande celler från grislever bestämdes dess nedbrytningshastighet och transportproteinernas roll i läkemedelsmetabolismen.

**8.2 Masspektrometri**

8.3 Vätskekromatografi


8.4 Kapillärelektrofores

I arbete IV användes en alternativ separationsmetod som kallas kapillär-elektrofores. Här separeras inte provkomponenterna med avseende på deras vatten- och fettlöslighet, utan med avseende på deras storlek och elektriska laddning. Ena änden av en tunn glaskapillär (0,05 mm i innerdiameter) kopplas till masspektrometern och fylls med vätska som innehåller en saltlösning, samt de ämnen som ska separeras. Salter som löses upp i en vätska formar joner, och lösningen är därför bra på att leda elektrisk ström. Glaskapillärens väggar har naturligt en negativt laddad yta som kommer att dra till sig positivt laddade joner från lösningen. När elektrisk ström leds genom vätskan börjar de positiva jonerna längs väggarna att vandra mot minuspolen och med hjälp av friktionskraft dra med sig den omgivande vätskan. På så vis skapas ett vätskeflöde mot masspektrometern som är placerad vid kapillärens minuspol. De ämnen i provet som skall analyseras och som är närvarande som positiva joner kommer inte bara att följa med vätskeflödet, utan dessutom vandra med något högre hastighet mot minuspolen. Stora positiva joner bromsas dock upp relativt kraftigt av vätskan runtomkring, medan små joner enklare tar sig fram i det omgivande lösningsmedlet. På motsvarande sätt
vandrar negativa joner i den andra riktningen, mot kapillärrens pluspol, men så länge som vätskeflödets hastighet överstiger deras vandringshastighet kommer de ändå röra sig i riktning mot masspektrometern. Oladdade (neutrala) molekyler följer med vätskeflödet och rör sig med samma hastighet som detta, oavsett vilken storlek de har. Resultatet blir att de ämnen som är små och positivt laddade när masspektrometern först, följt av större positiva joner, alla neutrala molekyler, stora negativa joner och sist små negativa joner.

8.5 Metabola fingeravtryck


8.6 Cancer

Baserat på den metod som utvecklades i arbete IV pågår nu försök att hitta motsvarande fingeravtryck för prostatacancer och cancer i urinblåsan, även denna gång i urin. Båda sjukdomarna kräver i dagsläget någon form av ingrepp vid diagnostisering. I fallet prostatacancer tas ett vävnadsprov och för diagnos av bläscancer inspekteras urinblåsans vägg visuellt med hjälp av en kamera som förs in i patientens kropp via urinröret. Båda metoderna är obe-
hagliga för patienten, kräver kvalificerad personal och är kostsamma. Målsättningen med arbete V är därför att undersöka möjligheten att i framtiden kunna ställa diagnoser på de två sjukdomarna med hjälp av ett vanligt urinprov.

8.7 Ordlista

Metabolism – Ämnesomsättning. Alla de kemiska reaktioner som sker i kroppen. Delas in i anabolism och katabolism.

Anabolism – Uppbyggande kemisk reaktion i kroppen.

Katabolism – Kemisk nedbrytning i kroppen.

Enzym – Struktur som underlättar och snabbar upp metabolism.

Transportprotein – Protein som transporterar ämnen in och/eller ut ur kroppens celler.

Kappa – Sammanfogande text som integrerar olika delar i en avhandling baserad på flera delarbeten.

Masspektrometri – En teknik som mäter ett ämnes vikt och halt.

Detektionssteg – Det steg i analysen där provinnehållet registreras av en masspektrometer.

Separationssteg – Det steg i analysen som föregår detektionssteget och som för att underlätta detta delar upp provinnehållet.

Jonisering – Den process där ett ämne förses med en eller flera elektriska laddningar.

Vätskekromatografi – Separationsmetod som utnyttjar ämnens vatten-/fettlöslighet.

Kapillärelektrofores – Separationsmetod som utnyttjar i vilken grad ett ämne är elektriskt laddat i lösning, samt ämnets storlek.
9 References


89. Klampfl, C.W., Recent advances in the application of capillary electrophoresis with mass spectrometric detection. Electrophoresis, 2006. 27(1): p. 3-34.


99. Liang, H.R., et al., Ionization enhancement in atmospheric pressure chemical ionization and suppression in electrospray ionization between target drugs and stable-isotope-labeled internal standards in quantitative liquid chromat-


Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology 690

Editor: The Dean of the Faculty of Science and Technology

A doctoral dissertation from the Faculty of Science and Technology, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology”.)

Distribution: publications.uu.se
urn:nbn:se:uu:diva-110310