Mass Spectrometry of Biologically Active Small Molecules
Focusing on polyphenols, alkaloids and amino acids

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Doctoral Thesis
Department of Analytical Chemistry
Stockholm University
2010
Academic dissertation for the Doctor of Philosophy degree in Analytical Chemistry to be publicly defended on Friday, January 29th at 13:00 in Magnélisalen, Kemiska övningslaboratoriet, Svante Arrhenius väg 16, Stockholm, Sweden. The defence will be held in English.

**Opponent:**
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Abstract

The main foci of this dissertation are on advanced liquid chromatography (LC) separation and mass spectrometry (MS) techniques for the analysis of small bioactive molecules. In addition to discussing general aspects of such techniques the results from analyses of polyphenols, alkaloids and amino acids published in five appended studies are presented and discussed. Various MS methods were applied in the studies, and several prior work-up/separation techniques (see below), but LC was used in most cases, since it can often provide valuable corroborative and/or complementary (e.g. quantitative) information to the data generated by emerging MS techniques such as laser desorption/ionization (LDI) MS. High availability, relatively convenient handling and well understood principles make LC the method of choice for separating analytes in many kinds of scientific investigations. LC systems can also be conveniently and robustly coupled to various kinds of detectors. Most importantly (in the context of these studies) they are frequently coupled nowadays to MS instruments to separate and analyze diverse analytes. In LC-MS analytes are separated in two stages: firstly they are separated and pre-concentrated in narrow bands using LC and then separated according to their mass-to-charge ($m/z$) ratios in an MS instrument. Furthermore, valuable information about their molecular structure can be acquired through MS detection (far more than can be acquired using most other on-line LC detection techniques). Some MS instruments can even provide highly accurate molecular weight measurements and sufficient mass resolution to identify unknown compounds in complex mixtures based purely on MS data, thus making prior separation unnecessary. However, prior separation is essential for analyzing substances in most complex matrices, and in many other applications it greatly improves the analysis – especially if ultra-high performance LC (UHPLC) is used. The advantages of using UHPLC rather than HPLC for the analysis of polyphenols (PPs) in tea and wine were evaluated in one the study this thesis is based upon. The UHPLC methods typically offered higher separation efficiency, together with substantial reductions in run time and solvent consumption compared to HPLC. The phenolic composition of red
wine was also examined, using novel LDI techniques, following solid phase extraction (SPE) for clean up/pre concentration. In this case, LDI could be used directly after sample work up (in conjunction with a time-of-flight mass spectrometer, TOF MS, offering relatively high mass accuracy and resolution) allowing LC separation to be entirely omitted. The results obtained with this elegant and straightforward approach show that it is not always necessary to use an external matrix or special surface to facilitate the LDI process since for instance, if an analyte contains roughly seven or eight conjugated \( \pi \) electrons, it is very likely to be readily desorbed/ionised. A class of small aromatic molecules (medicinally important alkaloids) also proved to be amenable to straightforward analysis, by thin layer chromatography (TLC) work-up followed by LDI-MS. This is the first report of the use of TLC-LDI without the addition of an external matrix. Finally, a method for monitoring neurotoxins (\( \beta \)-N-methyl-amino-L-alanine and 2,3-diaminobutyric acid) in complex biological matrices was developed and applied. Here, efficient LC separation coupled with tandem MS detection was essential, due to the complexity of the matrices, the low concentrations and mutual isobaric interferences of analytes. Therefore, neither LC nor MS could be used alone, and both techniques had to be carefully optimized and coupled to obtain satisfactory results. Overall, the studies show that careful attention to the physicochemical properties of analytes can provide insights that can greatly facilitate the development of appropriate methods to analyze them, e.g. by LDI. Such developments may make as important contributions as technological advances, as demonstrated by the analyses based on UHPLC and LC-MS/MS methods. Collectively, the studies described in this dissertation have enriched our repertoire for analysing small bioactive molecules.
List of papers

This doctoral thesis is largely based on the following papers, which are referred to in the text by the corresponding Roman numerals. For convenience, the studies described in them are sometimes referred to as Studies I-V.

I  Analysis of phenolic compounds by high performance liquid chromatography and ultra performance liquid chromatography
Zdeněk Spáčil, Lucie Novaková, Petr Solich
Talanta 78 (2008) 189-199

The author was responsible for all the experimental work, data evaluation and for writing the paper.

II  Matrix-less laser desorption/ionisation of polyphenols in red wine
Zdeněk Spáčil, Mohammadreza Shariatgorji, Nahid Amini, Petr Solich, Leopold L. Ilag
Rapid Communications in Mass Spectrometry 23 (2009) 1834-1840

The author was responsible for all the experimental work, data evaluation and for writing the paper.

III  Matrix free TLC-LDI mass spectrometry for facile separation and identification of medicinal alkaloids
Mohammadreza Shariatgorji, Zdeněk Spáčil, Gianluca Maddalo, Leopold L. Ilag
Rapid Communications in Mass Spectrometry 23 (2009) 3655-3660

The author was responsible for some of the experimental work and data evaluation, and partly for writing the paper.
IV Analytical protocol for identification of BMAA and DAB in biological samples
Zdeněk Spáčil, Johan Eriksson, Sara Jonasson, Ulla Rasmussen, Leopold L. Ilag, Birgitta Bergman
Analyst, 135 (2010) 127-132

The author was responsible for a major part of the experimental work, data evaluation and for writing the paper.

V Transfer of a cyanobacterial neurotoxin within a temperate aquatic ecosystem suggests pathways for human exposure
Sara Jonasson, Johan Eriksson, Lotta Berntzon, Zdeněk Spáčil, Leopold L. Ilag, Lars-Olof Ronnevi, Ulla Rasmussen, Birgitta Bergman
(manuscript)

The author was responsible for a major part of the LC-MS/MS experimental work and some of the data evaluation.
Papers not included in this doctoral thesis:

Simultaneous liquid chromatographic determination of metals and organic compounds in pharmaceutical and food-supplement formulations using evaporative light scattering detection
Zdeněk Spáčil, Jana Folbrová, Nikolaos Megoulas, Petr Solich, Michael A. Koupparis
*Analytica Chimica Acta* 583 (2007) 239-245

Fast assay of glucosamine in pharmaceuticals and nutraceuticals by capillary zone electrophoresis with contactless conductivity detection
Pavel Jáč, Petr Los, Zdeněk Spáčil, Marie Pospíšilová, Miroslav Polášek;
*Electrophoresis* 29 (2008) 3511-3518

Ultra-high performance liquid chromatography analysis of phenolic compounds
Lucie Novaková, Zdeněk Spáčil, Marcela Seifrtová, Petr Solich

Comparison of positive and negative ion detection of tea catechins using tandem mass spectrometry and ultra high pressure liquid chromatography
Zdeněk Spáčil, Lucie Novaková, Petr Solich (manuscript)
Abbreviations

$\alpha$ Selectivity
ACQ 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate
ALS-PDC Amyotrophic lateral sclerosis/parkinsonism dementia complex
APCI Atmospheric pressure chemical ionization
APPI Atmospheric pressure photoionization
BEH Ethylene bridged hybrid
BMAA $\beta$-N-methyl-amino-L-alanine
C$_8$, C$_{18}$ Octyl, octadecyl silica
CI Chemical ionization
CID Collision-induced dissociation
Da Dalton
DAB 2,3-diaminobutyric acid
DC Direct-current
DE Delayed extraction
$d_p$ Particle diameter
EM Electron multiplier
ESI Electrospray ionization
FD Fluorescent detection
FWHM Full width at half maximum
ICR Ion cyclotron resonance mass spectrometer
$HETP$ The height equivalent to the theoretical plate
HPLC High performance liquid chromatography
HTLC High temperature liquid chromatography
IT Ion trap
$k'$ Capacity factor
$l$ Column length
LC Liquid chromatography
LDI Laser desorption/ionization
MCP Microchannel plate
MP Mobile phase
MS Mass spectrometry
MS/MS Mass spectrometry/mass spectrometry
MALDI Matrix-assisted laser desorption/ionization
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>m/z</td>
<td>Mass of an ion in unified atomic mass units divided by its charge number</td>
</tr>
<tr>
<td>N</td>
<td>Number of theoretical plates</td>
</tr>
<tr>
<td>p_c</td>
<td>Peak capacity</td>
</tr>
<tr>
<td>ppm</td>
<td>Part per million</td>
</tr>
<tr>
<td>PPs</td>
<td>Polyphenols</td>
</tr>
<tr>
<td>Q</td>
<td>Quadrupole</td>
</tr>
<tr>
<td>QIT</td>
<td>Quadrupole ion trap</td>
</tr>
<tr>
<td>QPAs</td>
<td>Quaternary protoberberine alkaloids</td>
</tr>
<tr>
<td>RF</td>
<td>Radio frequency</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RP</td>
<td>Reversed phase</td>
</tr>
<tr>
<td>SP</td>
<td>Stationary phase</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>SRM</td>
<td>Selected reaction monitoring</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin-layer chromatography</td>
</tr>
<tr>
<td>TQ</td>
<td>Tandem quadrupole</td>
</tr>
<tr>
<td>TOF</td>
<td>Time-of-flight</td>
</tr>
<tr>
<td>t_R</td>
<td>Retention time</td>
</tr>
<tr>
<td>u</td>
<td>Linear velocity</td>
</tr>
<tr>
<td>UHPLC</td>
<td>Ultra high pressure liquid chromatography</td>
</tr>
<tr>
<td>w_b</td>
<td>Baseline width</td>
</tr>
</tbody>
</table>
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Aim

The general aim of the experimental work presented in this doctoral thesis was to develop and evaluate in detail analytical techniques for the identification and determination of important biologically active molecules present in natural matrices. For these purposes appropriate sample workup techniques had to be selected and optimised in order to address the considerable complexity of natural samples and obtain accurate, precise and reproducible results. More specifically, methods were developed for analyzing several groups of health-promoting polyphenolic compounds, medicinal alkaloids and neurotoxic amino acids based on advanced liquid chromatography (LC) or thin-layer chromatography (TLC) and mass spectrometry (MS) techniques. The requirements for the novel methods were that they should provide high throughput without compromising either the selectivity or sensitivity of their detection. Hence, careful optimisation of the separation and detection methods, based on detailed understanding of instrument parameters and their interactions were required. Other important criteria were convenience, cost effectiveness and environmental friendliness. The results (presented in five appended publications) highlight the potential utility of the novel approaches for analyzing various substances of interest to the pharmaceutical, nutraceutical and food processing industries.
Introduction

Since natural products are widely consumed commodities, accurate information about their contents is of great importance. Natural products often contain small organic molecules that provide proven or purported health benefits and in some cases, potentially toxic substances. Hence, knowledge of their contents of such molecules provides valuable information about the quality and geographical origins of the raw material and final products. It can also provide valuable information on the suitability of the production technology, storage conditions, and/or the products’ potential toxicity. Thus, such knowledge is important for both consumers and regulatory authorities, highlighting the importance of developing robust, sensitive and rapid analytical methods capable of screening as many compounds as possible.

Further, there is considerable interest in functional foods, which can be ascribed in part to increasing awareness of the health-promoting effects of polyphenols (PPs). For instance, a number of clinical studies report their important role in prevention of serious pathologies such as cancer, cardiovascular diseases and diabetes[1-5] mainly via their ability to scavenge free radicals[6]. There is a particularly wide spectrum of natural PPs in plants, a diverse class of secondary metabolites, which can be divided into various sub-classes, including flavonoids, phenolic acids, coumarins, anthocyanins and stilbenes[7]. The total dietary intake of PPs from everyday commodities such as fruits, vegetables, herbs, juices, teas, coffee, beers and wines amounts to about 1 g/d, but is strongly dependent to the individual’s dietary habits. For instance, fruits like apples, grapes, pears and cherries contain up to 200-300 mg PPs per 100 mg fresh weight, while a typical glass of red wine or cup of tea or coffee contains about 100 mg of PPs. Also chocolate, cereals and dry legumes are also relatively rich in PPs. Thus, daily intake varies substantially and can be enhanced using food supplements[5].

Alkaloids, which are present in many plant families, form another broad group of natural products with substantial medicinal importance. For instance, quaternary protoberberine alkaloids (QPAs)
are traditionally used to restore normal intestinal health, and also exhibit potent antimicrobial, antifungal, antiprotozoal, antiviral, anticancer, antiarrhythmic, immunosuppressive and anti-inflammatory activities[8-16]. Hence, methods for improving their analysis were also developed in the studies this thesis is based upon.

Toxin monitoring is also essential, in addition to the analysis of natural health-promoting compounds. Therefore, a method to analyze two neurotoxins – the amino acids β-N-methyl-amino-L-alanine (BMAA) and 2,3-diaminobutyric acid (DAB)– produced by free living taxa of cyanobacteria[17] were developed. BMAA is a structurally simple molecule with neurotoxic effects that have been intensively studied in the context of the neurodegenerative disease amyotrophic lateral sclerosis/parkinsonism dementia complex (ALS-PDC)[18]. The compound has been reported in brain tissue samples from patients, who died from ALS-PDC[19-21]. However the presence of BMAA in cyanobacteria and brain tissues are controversial[22]. Both positive and negative findings in biological samples have polarized the scientific community studying BMAA. Since determination of BMAA is purely an analytical problem and currently used analytical protocols have been questioned based on published negative data, a novel analytical protocol for unambiguous determination of BMAA in complex biological matrixes is highly desirable. The sample pretreatment, clean-up procedure and detection method applied have to meet stringent requirements in terms of sensitivity, selectivity and separation efficiency. All doubts concerning BMAA detection have to be clarified and properly designed studies of long term toxicity on animal models have to be performed to evaluate its neurotoxicity and confirm (or refute) speculations about BMAA bioaccumulation in the food chain.

The abovementioned small molecules co-occur with both, structurally related compounds and compounds with widely differing chemical and physical properties, which may interfere with their analysis. Further, some of the ballast compounds are usually present at levels several orders of magnitude higher than those of some analytes. Mass spectrometry (MS) has powerful analytic advantages in such cases, since it is extremely sensitive, providing attomole sensitivities, but also major disadvantages since it is also relatively sensitive to contaminants, in particular surfactants and charged non-volatile
species. Analogously to the suppression of fruity aroma notes in red wines by the presence of ethanol, an analyte signal may be suppressed by undesirable interaction with other compounds. Therefore a basic rule for analytical chemists applying MS is to use minimal amounts of additives/reagents to avoid compromising the analysis. Paraphrasing a Swedish proverb we can simply say, “Add less, see more”. In a good analytical laboratory this can be relatively easy to accomplish with the use of standards, but the composition of natural matrices is mostly beyond the analyst’s control. This obstacle posed by nature presents substantial problems for the analyst to resolve, but also provides the driving force for the development of analytical chemistry as an essential multidisciplinary science. As in all scientific research, a rational systematic approach (preferably combined with flashes of inspiration), is essential for solving such analytical problems. This requires sound knowledge of in/organic chemistry and physics for thorough understanding of analytical instrumentation, accurate judgment of analyte behavior and an active approach to address possible technical and chemical analytical pitfalls.
Polyphenols (PPs)

In recent years considerable attention has focused on the health-promoting effects of PPs, due to their ability to scavenge excess reactive oxygen species (ROS, e.g. superoxide, hydroxyl and peroxyl radicals) and reactive nitrogen species (RNS, e.g. nitric oxide and peroxynitrous acid)[23]. The oxidative stress[24] caused by ROS/RNS damages cellular lipids, proteins and DNA, thus inhibiting their normal functions[6], and abundant evidence indicates that increased oxidative damage is associated with the development of most age-related degenerative diseases, such as cancer[1-3] and cardiovascular diseases[25]. Therefore, the potentially protective effects of PPs against such conditions have been intensively studied, and several recent reviews have summarized their major activities against cancer[1-3], inflammation[3,4] and coronary diseases[5]. In Studies I and II underlying this thesis methods for analyzing PPs of several classes (flavonoids, phenolic acids and coumarins) were explored.

![Structural types of the most common flavonoid aglycones](image)

**Figure 1.** Structural types of the most common flavonoid aglycones

a) flavan-3-ol; b) flavonol; c) flavone; d) flavanone; e) isoflavone; f) anthocyanin.

Flavonoids comprise the largest group of PPs and are structurally related to a chroman-type skeleton with a phenyl substituent at the C₂
or C₃ position. The basic skeleton is composed of three rings differentially hydroxylated at positions 3, 5, 7, 3’, 4’ or 5’, and the hydroxylic groups are frequently modified (methylated, acetylated, prenylated or sulphated). In nature PPs are often present as O- or C-glycosides[26]. They are classified into six families (flavanols, flavonols, flavones, flavanones, isoflavones and anthocyanins, Figure 1) according to their substitution pattern and the degree of oxidation of the central pyran ring[7]. Flavonoids are plant pigments, largely responsible for the colour of flowers, fruits and sometimes leaves. More than 6500 flavonoid products of secondary plant metabolism have been characterized in various plant species to date, but their total number may exceed 8000[27]. The phenolic acids are secondary plant metabolites derived from either hydroxybenzoic or cinnamic acid. Examples (frequently found in tea and herbal infusions) include gallic, caffeic, vanillic, ferulic and chlorogenic acids and their esters[28], Figure 2. Coumarins are also often found in phenolic fractions of plant extracts, and more than 1000 have been described in the plant family Fabaceae (which includes sweet clover and tonka bean). However, these compounds (2H-1-benzopyran-2-ones) generally have lower antioxidant activity than flavonoids[29,30], Figure 2.

<table>
<thead>
<tr>
<th>derivatives of benzoic acid</th>
<th>R1</th>
<th>R2</th>
</tr>
</thead>
<tbody>
<tr>
<td>gallic acid</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>protocatechuic acid</td>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td>vanillic acid</td>
<td>H</td>
<td>OH₃</td>
</tr>
<tr>
<td>syringic acid</td>
<td>OCH₃</td>
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<table>
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<th>derivatives of cinnamic acid</th>
<th>R1</th>
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<tbody>
<tr>
<td>caffeic acid</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>chlorogenic acid</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>ferulic acid</td>
<td>OH</td>
<td>OCH₃</td>
</tr>
<tr>
<td>4-coumaric acid</td>
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<tr>
<td>cinnamic acid</td>
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<table>
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<th>R1</th>
<th>R2</th>
<th>R3</th>
</tr>
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<tr>
<td>aesculin</td>
<td>OH</td>
<td>glucose</td>
<td>H</td>
</tr>
<tr>
<td>scopoletin</td>
<td>OH</td>
<td>OCH₃</td>
<td>H</td>
</tr>
<tr>
<td>4-hydroxycoumarin</td>
<td>H</td>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td>6-methylcoumarin</td>
<td>H</td>
<td>CH₃</td>
<td>H</td>
</tr>
</tbody>
</table>

Figure 2. Typical examples of phenolic acids and coumarins.
**Quaternary protoberberine alkaloids (QPAs)**

UIPAC defines alkaloids as basic nitrogen compounds (mainly heterocyclic) occurring mostly in the plant kingdom (although some are of animal origin). The alkaloids form an extensive group of secondary metabolites, with diverse structures, limited distribution in organisms and important biological activities. Their function in plants is uncertain, but they probably participate in protective mechanisms against herbivores and parasites. On the other hand, some insects are attracted by alkaloid-producing plants. The isoquinoline alkaloids are the broadest group, including protoberberines and many other important groups. Approximately 25% of all characterized alkaloids with a protoberberine skeleton isolated from natural sources to date are quaternary protoberberine alkaloids (QPAs)[31]. Berberine and palmatine are two important examples of QPAs, found in the Berberis and Coptis plant genera, **Figure 3**. Diverse biological effects, e.g. antioxidant, anticancer[8], anti-inflammatory[9], antiarrhythmic[10], antimicrobial[11], antiprotozoal[12], antifungal[13], antiviral[14] and immunosuppressive activity[15] is attributed to these compounds, hence methods for analysing them were investigated in Study III. Although berberine and palmatine[16] are structurally related, and thus some of their biological effects are similar, differences in their antibacterial[32] and antioxidant activities[33] have been reported. Since both alkaloids contain a quaternary nitrogen in a polycyclic planar structure, it was hypothesised that these structural properties would also make them amenable to sensitive MS detection. This hypothesis was experimentally confirmed and a convenient method, based on TLC followed by LDI-MS, was developed for separating and identifying QPAs.

![berberine](image1.png) ![palmatine](image2.png)

**Figure 3. Structures of studied quaternary protoberberine alkaloids.**
β-N-methyl-amino-L-alanine (BMAA)

The non-protein amino acid β-N-methyl-amino-L-alanine (BMAA) was originally isolated in 1967 from seeds of the cycad tree Cycas micronesica Hill (Cycadaceae) as a product of endophytic cyanobacteria[34]. Since then, production of BMAA by other free-living taxa of cyanobacteria has been reported [17]. BMAA has been found to potentiate injury to motor neurons at concentrations as low as 10 µM[35] and selectively kill motor neurons at a concentration as low as 30 µM by AMPA/kainate receptor activation[36]. Neurotoxic effects of BMAA have been studied in the context of the neurodegenerative disease ALS-PDC among Chamorro people of Guam (Western Pacific) exposed to BMAA [18]. Recently, further interest in BMAA has been aroused by evidence that it is associated with the protein fraction[37] and recent experiments have demonstrated its incorporation into proteins[38]. This important finding has prompted speculations about its bioaccumulation in the food chain, which could have profound ecological and medical implications since cyanobacterial blooms commonly contaminate water bodies and near-shore marine ecosystems. Based on this intriguing hypothesis, BMAA might be a causative agent of increasing morbidity of neurodegenerative diseases such as amyotrophic lateral sclerosis, Parkinson’s disease and ALS-PDC. Accordingly, BMAA has been reported in brain tissues from patients who died of ALS-PDC[19-21]. However, the findings of BMAA in cyanobacteria and brain tissue samples have been challenged[39-42], thus requiring verification[22]. In addition, the possibility of confusing BMAA and 2,3-diaminobutyric acid (DAB) has been mentioned[40]. A novel protocol including complete sample clean-up and HPLC-MS/MS analysis for the separation and semi-quantification of BMAA in complex samples was developed and presented in Studies IV and V. In addition to the clear medical relevance of these studies, the developed methodology could be used in environmental risk assessment and has implications for sustainable measures to preserve the integrity of ecosystems.

Natural products

PPs are abundant micronutrients in the human diet, with an average consumption of around 1g/day[43]. The phenolic content of raw food
sources varies considerably, not only between different types but also between cultivars of the same type and are further dependent on growing conditions, time of harvest, geographical location, weather/microclimate and soil[44]. In addition, the content of PPs in the natural products strongly influences their quality and is markedly affected by processing technology and storage conditions. The summary of these factors is given in Figure 3. Since food production has become a tightly regulated, multi-billion dollar business, sensitive and high-throughput analyses are highly desirable for profiling, process optimization and quality control in the increasingly complex nutraceutical and agriculture industries of the 21st century. Among food products, tea and wine are known to be rich sources of polyphenols.

Tea contains antioxidant phenolic compounds, especially polyphenols, and after water it is the most frequently consumed beverage worldwide. It is made by steeping processed leaves, buds, or twigs of *Camellia sinensis* (L.) Kuntze (Theaceae) in hot water for a few minutes. Tea infusions are rich natural sources of alkaloids (caffeine, theobromine, theophylline, theacrine, adenine and xanthine), phenolic compounds (phenolic acids, flavanols and flavonols), amino acids (L-theamine), metals and vitamins (ascorbic acid)[45].

![Figure 3](image.png)

**Figure 3.** Factors affecting the composition of tea and wine.
The polyphenolic fraction of tea contains flavanols (catechins), flavonols (myricetin, quercetin and kaempferol) and condensation products (proanthocyanidins). (+)-Catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECg) and (-)-epigallocatechin gallate (EGCg) are the most abundant phenols in tea leaves, constituting up to 30% of their dry mass[46]. The contents of particular catechins in tea infusions are dependent on the processing applied to the tea leaves. EGCg is the major component of unfermented green tea[47], accounting for ca. 10-50% of the total green tea catechins. Its antioxidant activity is 100 times greater than that of vitamin C and 25 times greater than that of vitamin E[48]. After fermentation the major catechin is GC, while the content of EGCg is decreased approximately 10-fold[49]. Besides catechins small amounts of flavonols 3-O-glycosides also occurs in tea leaves. Both red and wine also have substantial PP contents. However, the contents are higher in red wine since (unlike white wines) they are produced by fermenting grapes with the skins present. To date, more than 200 polyphenols classified as phenolic acids, flavonoids, stilbenes or anthocyanins have been identified in red wine, and grape seed extract is one of the best-known sources of flavones[5]. These compounds have substantial health effects, for example constituents including the natural phytoalexin resveratrol, quercetin, delphinidin and (+) catechin have been intensively studied and shown to have pharmacological properties partially explaining the beneficial effects of moderate red wine consumption against coronary diseases[50]. They also have organoleptic properties, for instance monomeric PPs, such as (+) catechin, contribute to bitter taste, while polymeric tannins are highly astringent, and anthocyanins are responsible for the colour of red grapes and red wine[51]. The PPs and tannins comprise a significant proportion of the nonvolatile matrix of wine and affect the aroma volatility and perception. For instance gallic acid significantly decreases the volatility of 2-methylpyrazine (which has a nutty odour), through specific $\pi-\pi$ stacking stabilized by hydrogen bonds between the galloyl ring of phenolic compounds and the aromatic ring of the odorant[52].

**Sample pre-treatment**

Simple, convenient one-step sample pre-treatment methods are presented in Studies I-III. Target analytes were present in the samples
at appreciable levels (mg.g\(^{-1}\) or g.L\(^{-1}\)), and were either separated from the complex sample matrix chromatographically (Studies I and III) or selectively ionized/desorbed (Study II), according to their structural properties. In the analysis of PPs reported in Study I, the only pre-treatment of the analyzed tea and wine samples (prior to direct injection into an LC system) was filtration through a membrane with 0.22 µm pores, allowing rapid workflow. Similarly, one-step sample pretreatments were applied in the analyses described in Studies II and III. Prior to LDI analysis polyamide resin-based SPE was used to extract phenolic acids from wine samples in Study II and silica gel TLC for isolating of berberine and palmatine in Study III. Totally different analytical strategies had to be applied during the work described in Studies IV and V to meet demanding requirements. The target analytes were expected to be present at low concentrations in the samples (<1.0 µg.g\(^{-1}\) dry weight) and the sample matrices was very complex. Therefore, multiple-step sample pretreatment procedures were developed, involving cell disruption, protein extraction, hydrolysis, solid phase extraction and analytes derivatization prior to HPLC-MS/MS analysis. The developed protocols for isolating and pre-concentrating analytes followed by HPLC-MS/MS analysis showed great potential, with general applicability to widely varying types of biological samples, ranging from prokaryotic cyanobacteria to eukaryotic fish.

**Cell disruption and protein hydrolysis**

The biological materials analyzed in Studies IV and V were dissolved in methanol/water (80/20, v/v) and the resulting suspensions were subjected to three freeze/thaw cycles in liquid nitrogen to disrupt the prokaryotic and eukaryotic cells. Then the samples were sonicated in an ice-water bath, to prevent protein degradation. The protein concentration was subsequently measured in triplicate samples using an RC/DC kit (BioRad, Sweden) in order to standardize the biological material to protein equivalents. After that each dried sample was redissolved in 6 M hydrochloric acid and hydrolyzed in a conventional way (24 h, 110°C). The hydrolysate was then filtered and solvent was evaporated under a gentle airflow at 55°C. Finally, the pellet was reconstituted in 20 mM hydrochloric acid and subjected to SPE.
**Solid phase extraction (SPE)**

In the solid phase extraction (SPE) the complex sample is passed through solid particles serving as adsorbent material. The solid adsorbent is insolated in either a disk cartridge or a column. Thus, the target analyte(s) can be extracted and preconcentrated very quickly and conveniently. Discovery DPA-6S SPE cartridges filled with polyamide resin-based material from Supelco (Bellefonte, PA, USA) were used for the efficient extraction of phenolic acids from the wine samples in **Study II**. The interaction between PPs and polyamide resin is based on reversed-phase and hydrogen-bonding mechanisms. In addition to compounds with hydroxyl groups and phenols, aromatic carboxylic acids are also efficiently extracted through the formation of hydrogen bonds with the polyamide sorbent, thus the material is very suitable for extracting phenolic acids. The SPE procedure was applied as follows: an SPE cartridge was conditioned with methanol (3 mL) and water (3 mL), then a wine sample was applied (2 mL), the cartridge was subsequently washed with 0.1% TFA and the analytes were eluted with methanol:0.1% TFA solution (80:20, v/v).

In **Studies IV** and **V** Isolute HCX mixed mode sorbent (Sorbent AB, Sweden) was used to extract basic analytes. This sorbent has strong cation exchange functional groups, allowing many interferences to be removed by applying a suitable washing and elution protocol. Therefore, levels of co-extracted materials were low and the risk of ion suppression effects during subsequent LC-MS/MS analysis was minimized. The SPE elution protocol was selected following consideration of both the nature of the analytes and MS compatibility. The optimized version was as follows: the SPE cartridge was flushed with methanol (1 mL), water (1 mL) and conditioned with 0.1% FA (1 mL), then the sample was loaded dissolved in 0.1% FA, the SPE cartridge was washed with 0.1% FA then finally, two elution fractions were collected for further analysis, the first eluted with 1 mL FA in 25% methanol and the second with 2% ammonium hydroxide in 100% methanol. Three SPE columns were tested in trials using this protocol, packed with: i) Strata-X-C, polymeric strong cation exchange sorbent (Phenomenex, Torrance, CA, USA), ii) Oasis MCX strong cation exchange and reversed phase sorbent and iii) Isolute HCX strong cation exchange and reversed phase sorbent (Sorbent AB, Sweden). Given the chemical character of the analytes, reversed phase retention is insignificant for them. However cleaner LC-MS/MS spectra were
acquired using mixed mode rather than pure strong cation exchange columns, Figure 4. Of the Oasis MCX and Isolute HCX mixed mode columns, the latter exhibited higher potential for analyte pre-concentration with lesser breakthrough and thus was selected for further analyses.

Figure 4. LC-MS/MS (SRM) spectra acquired using SPE sample pre-treatment: a) Strata-X-C; b) Oasis MCX; c) Isolute HCX-3.

**ACQ derivatization**

The reagent 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (ACQ) was used for pre-column derivatization of target analytes in Studies IV and V. During the reaction both primary and secondary amines are converted into stable fluorescent derivatives and the excess reagent is hydrolyzed to yield 6-aminoquinoline, N-hydroxysuccinimide and carbon dioxide. The reaction has a well understood mechanism and provides quantitative yields achieved in borate buffer (pH 9.0) with approx. 4:1 molar excess ACQ[53]. The ACQ reagent is commercially available from Waters Corp. and is generally used for HPLC-FD analysis of amino acids[54]. Since derivatization significantly increases the hydrophobicity of analytes, ACQ derivatives exhibit favourable chromatographic properties (narrow peaks) using RP-LC, which is essential for separating derivatized BMAA from isomeric DAB. In addition, the effects of the cyanobacterial matrix on the derivatization have been reported in detail[55], and according to my experience these ACQ derivates are effectively ionized in the ESI source, resulting in sensitive MS detection. This can be explained through the presence of hydrophilic and lipophilic groups in the molecule of the derivate, causing preferential deposition on the ESI droplet interphase compared to
underivatized molecule resulting in highly efficient ionization process. Derivatized molecules of BMAA and DAB are shown in Figure 5.

![Figure 5. Structures of BMAA and DAB derivatized with ACQ and their specific fragments.](image)

**Thin layer chromatography (TLC)**

Thin layer chromatography (TLC) is the simplest type of planar chromatography. The stationary phase (SP), usually silica gel, aluminium oxide or cellulose is coated on a glass, metal or plastic plate. The sample is applied to the plate, which is then inserted vertically (or nearly vertically) into a developing chamber containing a shallow pool of mobile phase (MP), so the bottom edge of the plate (below the point where the sample was applied) is immersed in the MP. MP is drawn up the TLC plate via capillary action and separation is achieved through adsorption, partitioning and ion-exchange interactions of the solutes between the SP and MP. Due to its convenience and rapidity TLC is frequently used in many scientific applications e.g. to identify plant material in botanical taxonomy[56], to isolate active compounds from extracts[57,58] and to monitor reactions in organic chemistry. In Study III silica gel TLC plates with aluminium support were used with an MP consisting of butanol:glacial acid:water (14:3:4 v/v) to isolate QPAs from plant
extracts. The combination of TLC with MALDI requires extraction of analytes from the TLC plate, their transport to the surface followed by matrix deposition and crystallization[59]. However, these extraction steps can be avoided when LDI is used. Therefore, TLC has promising potential for sample pre-treatment/separation prior to LDI analysis. The analyte is isolated from the sample matrix and preconcentrated into a chromatographic band, but in contrast to SPE no time-consuming evaporation of elution solvent is needed. In addition the analyte is already adsorbed to a porous silica SP after the TLC, and since the TLC plate support is conductive it can be used for direct LDI analysis after placing it on a modified stainless steel MALDI target. The sample bands can be either excised or the whole plate can be placed on the MALDI target and simply secured with tape. Another potential advantage is that it theoretically provides a more even distribution of the analyte(s) on a porous silica surface than in LDI analyses using stainless steel MALDI targets, but this has to be evaluated experimentally.

**Column chromatography**[60]

The term “chromatography” was coined at the beginning of the 20th century by Russian botanist Mikhail Tswett[61], who used a column packed with calcium carbonate to separate plant pigments. However, his technique was ahead of its time and ignored for several decades before being reintroduced in 1941 by Martin and Synge[62-65], who were awarded the Nobel Prize in chemistry for their invention of partition chromatography in 1952. Column chromatography separation is accomplished by continuously passing an MP over an SP packed in a column. When the sample is injected, it moves with the MP and the sample’s components partition between the MP and SP. Components whose distribution ratio favours SP require a longer time to pass through the column. Thus, solutes with similar distribution ratios can be separated using a suitable MP, SP and sufficient time. Column chromatography exploits the interactions between the analyte molecules and both the MP and SP, which depend on their physicochemical character. Thus, separation is determined (to varying degrees) by liquid-solid adsorption, liquid-liquid partitioning, ion exchange and size exclusion. The stronger the interaction with the SP, the longer the time required for the analyte to travel through the analytical column. Nowadays, SP media generally consist of porous
particles providing a large surface area. Silica beads typically ranging between 2.5-5 μm in diameter are used in traditional HPLC, but silica-organic hybrid materials with particle sizes below 2 μm are increasingly used following the development of UHPLC. In silica-organic hybrid materials the inorganic structure of silica gel is reinforced by organic polymeric material, resulting in higher chemical and physical stability. In addition to columns packed with particulate sorbents there are special types of columns such as capillary and monolithic columns. In capillary columns the SP is arranged in a thin layer around the column’s inner wall, analogously to planar chromatographic systems. Therefore, their inner diameter is narrower than in particulate columns, but they are much longer in order to provide sufficient surface area. This type of column is commonly used in gas chromatography and, at smaller scale in LC applications. The SP medium used in a monolithic column is prepared by polymerization of organic or inorganic monomers, resulting in a single piece of porous cross-linked polymer or silica. In such material the MP flows through macropores with lower back pressure than through particulate sorbents and mesopores provide sufficient surface area for efficient separation. However, the benefit of monolithic columns for LC-MS applications is limited due to the relatively high flow-rates used.

**High performance liquid chromatography (HPLC)**

The acronym HPLC originally denoted high-pressure liquid chromatography, indicating the switch from reliance on atmospheric pressure and gravity flow to the use of pressurized pumping systems. The early 1970s saw a tremendous leap in technology and the term high-performance liquid chromatography was established by Prof. Horváth, while the acronym HPLC remained the same[66]. Conventional HPLC instruments can operate at back-pressures up to 6 000 psi (41 370 kPa or 401 bars). The definition of several terms is necessary for proper understanding of chromatographic principles. The retention time ($t_R$) of an analyte is the time between the introduction of the solute and the time it appears at highest concentration (peak maximum) in the eluate passing through the detector, and baseline width ($w_b$) is determined by the intersection with the baseline of tangential lines drawn though the inflection point on either side of the peak. The chromatographic resolution, which is a
quantitative measure of the degree of separation between two chromatographic peaks A and B, is defined as:

\[ R = \frac{t_{R,B} - t_{R,A}}{0.5(w_{b,B} + w_{b,A})} = \frac{2\Delta t_R}{w_{b,B} + w_{b,A}} \]  

(Eq. 1)

The resolution of 1.5 corresponds to almost baseline separation of two equally large peaks. This equation clearly shows that the resolution may be improved by either increasing \( \Delta t_R \) or decreasing \( w_b \). While \( \Delta t_R \) can be increased by enhancing the interaction of the solutes with the column or changing the column’s selectivity, \( w_b \) depends on kinetic variables associated with the solute’s movement within and between MP and SP, which are governed by several factors and their overall effects on peak broadening are referred to as column efficiency. The capacity factor \((k')\) measures how strongly a solute is retained by the SP and the ratio of capacity factors for two solutes, showing the column’s relative selectivity for them, is expressed by a coefficient \((\alpha)\). The column efficiency can also be defined in terms of the number of theoretical plates \((N)\), a series of small zones or plates where partitioning between MP and SP occurs. The number of theoretical plates is calculated from the column length \((l)\) and the height equivalent of the theoretical plate \((HETP)\) according to:

\[ N = \frac{l}{HETP} \]  

(Eq. 2)

The number of theoretical plates of a column is not fixed and depends on both the properties of the column and the solute. Another important consideration is peak capacity \((p_c)\), the estimated number of solutes that can be baseline-resolved by a given column. The peak capacity (number of peaks resolved per unit time in gradient separations)[67,68] can be calculated by solving:

\[ p_c = 1 + \sqrt{N} \ln \frac{V_{max}}{V_{min}} \]  

(Eq. 3)

where \( V_{min} \) and \( V_{max} \) are the smallest and largest volumes of mobile phase in which a solute can be eluted and detected. This estimate may help to exclude from consideration columns that do not have sufficient theoretical plates to separate a complex mixture. In practice, the peak capacity is usually less than the theoretically estimated value, due to deviations from ideality in the retention and partitioning of the analytes. The ideal chromatographic peak is of Gaussian profile, but
sometimes peak shapes are distorted due either to column overload (fronting) or uneven retention of the analyte by the SP (tailing). From defined terms and their relationships the resolution between two chromatographic peaks A and B can be expressed in another way:

\[
R = \frac{1}{4} \sqrt{N_B} \left( \frac{\alpha - 1}{\alpha} \right) \left( \frac{k'_B}{1 + k'_B} \right)
\]

(Eq. 4)

which includes terms corresponding to column efficiency, column selectivity and capacity factor. The parameters of these three factors can be varied to obtain the desired resolution and analysis time for a pair of solutes. One way to improve resolution is to adjust \(k'_B\), which is particularly effective when the original capacity factor is small (in cases where the solute is weakly retained by the SP). In LC the mobile phase’s solvent strength is easily modified either by concentrating the organic constituent or the temperature [69], and hence changing \(k'_B\). An increase in \(k'_B\) is observed when the MP has a low solvent strength and thus solutes spend proportionally more time in the SP. Increasing the volume of SP (e.g. using a longer column) will also lead to an increase in \(k'_B\). Note that any improvement in resolution obtained by increasing \(k'_B\) generally comes at the expense of a longer analysis time. In some cases \(k'_B\) is insufficient to resolve early eluting solutes, but increasing \(k'_B\) leads to an unacceptably long retention time for later eluting solutes. This “general elution problem” can be solved by incremental adjustments of \(k'_B\) over time. In LC this can be done by gradient elution [70], in which the mobile phase’s solvent strength is gradually increased. Gradient elution also has favourable effects on peak width, because a band migrates through the column in MP of continually increasing strength, therefore the band tail always moves faster than the band front. The resulting narrowing of the band is referred to as gradient compression [71]. The second factor, \(\alpha\), has to be adjusted especially in cases when it is nearly equal to 1. The chromatographic conditions have to be altered, so they are more selective for one of the solutes. This can be done by altering either the SP or MP, although in LC changing the composition of the MP (e.g. pH) is more frequent. In situations where the values of \(k'_B\) and \(\alpha\) are given, the number of theoretical plates needed to achieve a desired resolution can be calculated from Eq. 4. The column efficiency expressed in \(N\) can be doubled by simply doubling the column length, but this also doubles the analysis time. A more favourable approach is
to reduce $HETP$ without affecting the analysis time. The experimental factors contributing to the broadening of a solute’s chromatographic band have to be examined in order to determine how $HETP$ can be decreased. These factors are: multiple paths, longitudinal diffusion, mass transfer in the SP and mass transfer in the MP. The multiple paths factor expresses the effect of solute molecules passing through the chromatographic column along paths of various lengths. The principal contribution to this variation is nonhomogeneous packing of the SP (e.g. variations in particle size and packing). Thus, the contribution of multiple paths to $HETP$, $H_p$ is given by:

$$H_p = 2\lambda d_p$$  \hspace{1cm} (Eq. 5)

where $d_p$ is the average diameter of the particulate packing material and $\lambda$ is Eddy dispersion accounting for the consistency of the packing. Typical values of $\lambda$ for well-packed columns are 0.8–1.0[72]. A narrow distribution in particle size and more even packing results in a smaller value of $\lambda$, while $H_p$ is eliminated completely in capillary columns. The longitudinal diffusion factor is the result of the solvent’s diffusion from the most concentrated centre of a chromatographic band to the band’s edges. Thus, the contribution of longitudinal diffusion to $HETP$, $H_d$ is:

$$H_d = \frac{2\gamma D_m}{u}$$  \hspace{1cm} (Eq. 6)

where $D_m$ is the solute’s diffusion coefficient in the MP, $u$ is the linear velocity of the MP and $\gamma$ is a constant related to the column packing. From this definition it is clear that the effect of $H_d$ on $HETP$ is minimized by a high MP velocity. The mass transfer defines the time required for a solute molecule to diffuse through the SP and MP. The separation process takes place though the solvent’s interactions with the SP and MP, but first the solvent has to diffuse to the interface between the two phases to reach a true equilibrium. Whenever the solute’s movement is insufficient to reach a true equilibrium band broadening occurs. The contributions of mass transfer $H_{SP}$ and $H_{MP}$ are given by:

$$H_{SP} = \frac{qk'd_f^2}{(1 + k'')^2 D_{SP}}u$$  \hspace{1cm} (Eq. 7)

$$H_{MP} = \frac{fn(d_p^2, d_c^2)}{D_{MP}}u$$  \hspace{1cm} (Eq. 8)
where $d_f$ is the thickness of the SP, $d_c$ is the column’s diameter, $D_{SP}$ is the solute’s diffusion coefficient in the SP and $q$ is a constant related to the column packing material\[73\]. $HETP$ is least affected by mass transfer when MP linear velocities are slow, particles of packing materials have small diameters and films of SP are thin. The net $HETP$ is a sum of the abovementioned contributions:

$$HEPT = H_p + H_d + H_{SP} + H_{MP}$$ \hfill (Eq. 9)

**Ultra-high pressure liquid chromatography (UHPLC)**

As noted 40 years ago by Knox\[74\], chromatographic performance can be enhanced in terms of efficiency and rapidity by using an SP with small particles (sub-2-µm) at ultra-high pressures (>400 bar). However, the practical development of UHPLC separations began in 2004 when Waters Corp. introduced the first commercial UHPLC instrument\[75\], packed with a sub-2-µm porous ethylene bridged hybrid (BEH) material that Waters had also developed\[76\]. Since “UPLC” is a trademark registered by Waters, and hence should not be used in scientific texts, the term UHPLC will be used here instead. UHPLC is a revolutionary technique, capable of operating at backpressures up to 15 000 psi (~103 MPa or 1034 bars) and thus established new standards for LC separations. Its main advantage compared to conventional HPLC is higher separation efficiency achieved by the use of sub-2-µm particle sorbents\[77\]. The principles underlying HPLC and UHPLC are governed by the van Deemter equation, an empirical formula describing the relationship between the linear velocity $u$ (flow rate) and $HETP$:

$$HETP = A + \frac{B}{u} + Cu$$ \hfill (Eq. 10)

The van Deemter equation is basically a modified form of Eq. 9, where $A$ stands for $H_p$, $B/u$ for $H_d$ and $Cu$ for $H_{SP}$ and $H_{MP}$. Typical values are roughly 1 for $A$, 2 for $B$ and $C$ values for well-packed columns vary from 0.1-0.2 \[78,79\]. The formula highlights the role of $u$ and suggests that the number of theoretical plates can be increased without increasing the column length by decreasing one or more of the terms in the equation. The terms can be simply altered by adjusting $u$, but assuming that parameter $B$ is inversely proportional to $u$ and parameter $C$ is directly proportional to $u$, column efficiency will
always be limited at low linear velocity by longitudinal diffusion and at high linear velocity by the two mass transfer terms. Thus, the linear velocity is optimal ($u_{opt}$) when the combined, counteracting effects of these parameters are minimised[80]. On the other hand, both parameters $A$ and $C$ are functions of the particle size and thus efficiency can be improved decreasing the particle size[81]. The use of sub-2-µm particles allows the resolution per unit time to be improved because column efficiency and $u_{opt}$ are both inversely proportional to $d_p$. However, small particles induce even greater back pressures. According to Darcy’s law the latter is inversely proportional to the square of $d_p$ at $u_{opt}$:

$$\Delta P = \phi \cdot \eta \cdot l \cdot u \cdot \frac{1}{d_p^2}$$  \hspace{1cm} (Eq. 11)

where $\eta$ is the viscosity of the MP and $\phi$ the flow resistance[82,83]. The advantage of smaller particles will be increased speed and peak capacity. The other way to decrease analysis time involves increasing the temperature of the MP above ambient temperature. This significantly affects all the parameters of a separation except the mass of the packing material in the column. Consequently changes in the column efficiency, selectivity and $u_{opt}$ values are observed. The dependence of $HETP$ on the column temperature can be explained by its effects on the molecular diffusivities and the rate constants of the mass transfer kinetics[84]. Thus, the overall result depends on whether the effects of axial dispersion or those of the mass transfer resistances are the main contributors to band spreading. As a consequence the $u_{opt}$ value increases with increasing temperature, while the minimum value of $HETP$ is affected insignificantly. Although controversial results concerning the effects on $HETP$ have been reported[85], high temperature LC (HTLC) is no longer recognised as a technique that significantly improves column efficiency [86,87]. Nevertheless, HTLC leads to faster separations for a given efficiency and thus, together with UHPLC, fits into the concept of efficient and rapid LC separations[86]. The use of sub-2-µm particles and narrow column diameters emphasize the need for ultra-high purity solvents. The filtration of MP through a membrane filter with 0.22 µm pores before use is also recommended to prevent column clogging. A very effective way to prolong column life-time is to use pre-column or pre-column filters.
**Reversed phase liquid chromatography**

Reversed phase (RP) LC is a type of LC separation, utilizing a nonpolar SP and a polar MP, that can be used to separate a wide spectrum of analytes. The most common RP-LC SP media are $n$-octyl ($C_8$) or $n$-octadecyl ($C_{18}$) hydrocarbon chains bonded to a silica surface, for which the separation mechanism is based on hydrophobic interactions between the analytes and non-polar functional groups of the SP. The bonded SP is attached via reaction with an organochlorosilane, thus some of the silanol groups are derivatized[88]. However a great number of silanol groups remain unreacted and available for interactions with analytes. This causes distorted peak shapes and excessive tailing, particularly for basic compounds. The majority of modern RP-LC SP media are formed by a reaction with trimethylchlorsilane to reduce the number of free silanol groups. This procedure, so-called “endcapping”[89], also improves column stability[90]. The activity of silanol groups can also be modified by adjusting the MP composition and pH. Therefore, organic amines such as triethylamine, trioctylamine and cetyltrimethylammonium salt are used as MP additives, due to their high affinity to silanols, thus eliminating their effects. The number of ionized silanols is decreased by using an MP with low pH, providing another way to mitigate their undesirable effects. Indeed, acidic aqueous MP is frequently used in LC-MS separations to improve ionization of analytes in the ion source. Since RP-LC separations are carried out with an aqueous MP the analytes must have sufficiently high solubility in polar solvents. RP-LC was the method of choice in **Studies I, IV and V.**

**Mass spectrometry**[91]

The importance of mass spectrometry (MS) has greatly increased in recent decades in large part due to the development of techniques for the analysis of biological macromolecules such as nucleic acids[92,93], proteins[94-96], carbohydrates[97,98] or lipids[99-101]. Considerable resources and great effort invested in the development of ionization techniques for MS instruments culminated in the award of a Nobel Prize in 2002 to John B. Fenn and Koichi Tanaka for the development of soft desorption ionisation methods for mass spectrometric analyses of biological macromolecules. This success
enabled routine use of MS, in conjunction with separation techniques such as electrophoresis and liquid chromatography, resulting in the development of so-called “hyphenated” techniques e.g. LC-MS. A mass spectrometer has three major parts: the ion source, the mass analyzer and the detector. Many ion sources, the analyzer and the detector operate at low pressure (typically $10^{-3}$-$10^{-10}$ Pa) and thus require efficient pumping (provided by a rotary vacuum pump combined with a turbo-molecular pump). Modern MS systems are controlled by a computer, which also records and processes acquired MS data.

**Ion sources**

The role of the ion source is to facilitate the process whereby analyte atoms, molecules, adducts or clusters are ionized and transferred into gas phase. The gas phase ions thus generated are introduced into the mass analyzer. Some ion sources operate in atmospheric pressure – including Atmospheric pressure chemical ionization (APCI), Atmospheric pressure photoionization (APPI) and Electrospray ionization (ESI) sources – and others in vacuum, e.g. Chemical ionization (CI), Electron ionization (EI), Laser Desorption/Ionization (LDI) and Matrix-Assisted Laser Desorption/Ionization (MALDI) sources. Another way of classifying ion sources is by the degree of analyte fragmentation during the ionization and gas phase transfer. Soft ion sources (APCI, APPI, CI, ESI, LDI and MALDI) mainly produce intact molecular ions and thus some of them can be used for the analysis of large fragile molecules or even noncovalently bound complexes. Other ion sources (e.g. EI) produce intense fragment ions along with the molecular ion. ESI and LDI ion sources will be discussed below in more detail, because they were used in Studies II-V.

**Electrospray ionization (ESI)**

This mode of ionization was first described by Dole in 1968[102] and coupled to MS by Fenn in 1984[103] and shortly thereafter applied also to the analysis of large biomolecules[104]. The invention of ESI sources represented a breakthrough in the facility of LC/MS coupling, hence they soon supplanted other contemporary interfaces and ionization techniques such as thermospray[105] and particle beam...
interfaces[106]. Hyphenated techniques based on ESI-MS connections became routine and reliable with enormous potential in a number of sciences, e.g. chemistry, biochemistry, medicine and pharmacy[107,108].

In ESI-MS a sample is dissolved in a polar, volatile solvent and transported though a metal capillary with a high positive or negative potential (3-5 kV). Charged droplets are nebulized and shrunk through evaporation supported by a flow of nitrogen gas. The increase in density of surface charge leads to Coulombic explosions, resulting in smaller droplets and repetition of this process leads to the release of quasi-molecular ions. ESI is useful for compounds with medium to very high hydrophilicity and molecular weights ranging from c.a. 100 to 100 000 Da, though ionization of large (glyco)proteins and noncovalent biocomplexes up to 2 MDa has been reported[109].

**Laser desorption/ionization (LDI)**

The first experiments on desorption/ionization by a laser beam were performed in 1963[110], but the real boom in this ionization technique came in the late 1980s, when soft laser desorption (SLD) was introduced by Tanaka[111] and concurrently MALDI was described by German scientists Karas and Hillenkamp[112,113]. Since MALDI was developed shortly before SLD and is still widely used the true impact of Tanaka’s discovery has been debated[114]. However Tanaka was the first to report use of LDI to ionize proteins. Nowadays, LDI is usually mediated by a 4 ns pulsed nitrogen laser beam (337 nm), and is also used for the analysis of small molecules. This is especially favourable when analytes can function as matrices, readily absorbing the irradiation of a laser beam. In such cases it is not necessary to add any matrix at all. Applications of LDI in this mode are presented in Studies II and III, Figure 6.
Figure 6. Electron delocalization trend is showing the increase in LDI detection sensitivity with the increasing number of conjugated $\pi$ electrons.

There are no differences in the hardware used for LDI and MALDI sources, both kinds of ionization operate under high vacuum ($<10^{-3}$ Pa) and are usually combined with a Time of Flight (TOF) mass analyzer. In principle MALDI is a special case of LDI, demanding sample preparation (notably a UV-absorbing matrix). In LDI the sample is placed on a conductive target plate and resonantly excited by a laser pulse, typically of nanosecond duration. The absorbed laser energy causes an explosive ablation, ejecting both neutral and charged species into the gas phase. LDI and MALDI, in particular, is the softest ionization technique, enabling analysis of intact biomolecules up to about 1 MDa.

**LDI sample deposition**

In **Study II** the “dried droplet” sample deposition protocol for LDI analyses was optimized to provide more even distribution on the LDI target plate after air-drying under ambient conditions. Standards were spotted both in pure water and water with varying proportions of...
methanol. Use of pure methanol was inconvenient, due to its low surface tension, which causes excessive spread of the droplet. On the other hand, standards spotted in pure water required longer times for air-drying, but formed well-defined spots with eccentric distribution and less spread of analytes. This was particularly favourable when standards at very low concentrations were analyzed since in such cases somewhat stronger signals were obtained compared to those from dried droplets spotted in water/methanol solvent. Conversely, a high proportion of organic modifier (50-90%) allowed short air-drying times and more even spread of analytes, judging by more consistent signals generated from within the spot. The more consistent sample spread can be explained by the “Marangoni effect” describing mass transfer in a liquid layer due to differences in surface tension. The principle is that a liquid with a high surface tension pulls more strongly on the surrounding liquid than one with a low surface tension, thus the presence of a gradient in surface tension will naturally cause the liquid to flow away from regions of low surface tension[115]. The gradient in surface tension is created though the unequal evaporation of water and methanol. Since these spreading effects of solvents with organic modifier resulted in more even signals for wine samples than pure water, finally a solution of methanol/0.1 mol.L⁻¹ TFA (80:20 v/v) was used for spotting standards and samples, Figure 7.

Figure 7. Wine samples spotted on LDI target plate using “dried droplet” method.

Since these spreading effects of solvents with organic modifier resulted in more even signals for wine samples than pure water, finally a solution of methanol/0.1 mol.L⁻¹ TFA (80:20 v/v) was used for spotting standards and samples. The underlying mechanisms of LDI
processes are not yet fully understood, so the role of TFA in signal generation should also be considered, since the stronger signals acquired from the “dried droplets” of aqueous solution might have been due to more favourable interactions between co-crystallized TFA and the analytes, due to TFA acting as a proton donor. The results confirmed that TFA is essential to generate signals. Problems associated with the homogeneity of the “dried droplets” in Study II were resolved by accumulating and statistically processing spectra recorded from points across the whole sample spots. Typically, signals from 20 laser shots were collected randomly from 10 different locations within a radius of 700 microns from each spot centre and accumulated into one 200-laser shot spectrum. A more reliable way of obtaining homogeneous spots is probably to use ink jet spraying techniques[116]. However, another approach to obtain more homogenous sample spots was suggested in Study III, in which TLC sample pretreatment was used and the analytes were then analyzed directly on a TLC plate by LDI. In this study LDI was not used for sample quantification, but in theory the distribution of analytes on a porous silica TLC plate should be more even than on the surface of the steel target plate.

**LDI adducts**

The formation of various singly charged potassium and sodium adducts, possibly caused by substitution of hydroxylic hydrogens, was observed. An effort was made to promote the formation of just one adduct by adding potassium or sodium chloride to the standard solution. However, this neither increased sensitivity nor simplified the mass spectra, indeed it promoted the formation of an even larger number of adducts. The possibility of avoiding the formation of potassium and sodium adducts of different analytes with similar molecular weights, and associated analytical complications, by adding cesium ions was also tested in Study II. Analyte/cesium adducts were observed in the acquired mass spectra, but the sensitivity was significantly lower for the cesium adducts than for potassium and sodium adducts and protonized analyte molecules.
**Mass analyzers**

Ionized atoms, molecules or clusters can be separated according to their m/z ratios in the mass analyzer. Currently used mass analyzers separate ions by applying electromagnetic forces and can be divided into categories according to their separation principle. Magnetic analyzers use magnetic and electric fields to influence the radius of the accelerated ion trajectory, which depends on the magnetic field strength, m/z and ion velocity. Quadrupole (Q), ion trap (IT) and quadrupole ion trap (QIT) analyzers employ a combination of direct-current (DC) and radio frequency (RF) potentials to keep ions within a certain, narrow m/z range in a stable trajectory. Time-of-flight (TOF) analyzers register the time difference between the signal start (e.g. acceleration of the ion) and the pulse generated when the ion hits the detector. The time of flight in the mass analyzer drift tube is dependent on m/z since the velocities of ions depend on their m/z ratios. In ion cyclotron resonance (ICR) and orbitrap analyzers specific frequencies corresponding to certain m/z ratios are measured. Ions trapped by a strong ICR magnetic field are excited and ions with different m/z ratios are distinguished according to the corresponding ICR frequencies, hence each generated current frequency will correspond to a certain m/z value. Orbitrap analyzers[117,118] trap and store ions in similar fashion to QIT analyzers, but subsequently the frequencies of axial oscillations of ions with different m/z ratios are measured very accurately, resulting in very high m/z separation. The key analytical parameters of mass analyzers (such as their resolution, mass accuracy, m/z range, sensitivity, dynamic range, quantification abilities, scanning speed and compatibility with available ion sources) depend on their mode and underlying principles of operation. Tandem quadrupole (TQ) and TOF mass analyzers will be discussed further in the following sections since understanding their principles and advantages is important for understanding Studies II-V.

**Tandem quadrupole (TQ) mass analyzers**

These widely used mass analyzers consist of three quadrupoles (Q) connected in series. Q1 and Q3 perform as mass filters, since at given values of DC and RF potentials, only ions within a certain narrow m/z window will have stable trajectories through the Q. The other ions will
collide with the rods and thus never reach the detector. Q2 is usually an octopole or a hexapole, which can function as a collision cell, where low energy collision induced dissociation (CID) takes place. This arrangement generally provides high sensitivity (femtomole/attomole), a high dynamic range (typically with linear responses across five orders of magnitude), high scanning speeds (around 10 000 Da/sec) and excellent possibilities for quantification. In contrast, the TQ mass analyzers have low resolution (typically 3000-5000 FWHM) low mass accuracy (±0.2 Da) and a low m/z range (typically 25-2000 m/z).

**TQ scanning modes**

TQ mass spectrometers are typical examples of tandem-in-space instruments capable of operating in four main scanning modes: i) product ion scanning, ii) precursor ion scanning, iii) neutral loss scanning and iv) selected reaction monitoring (SRM). The Q1 and Q3 mass analyzers operating in MS/MS mode, connected through a Q2 collision cell, either scan within a defined m/z range or are fixed at a certain m/z. For example, in product ion scanning mode the Q1 is fixed at one m/z and a full mass spectrum of the fragment ions is produced after passing through the Q3. Conversely, in precursor ion scanning, the Q1 is scans all precursor ions of a fragment selected by the Q3. This scanning mode is useful for finding compounds that produce a common fragment. Neutral loss scanning, in which the Q1 and Q3 both scan at a fixed m/z difference, is used to find compounds that lose a common neutral species. Similarly, SRM scanning is based on selection of ions originating from a fragmentation reaction. In this mode the Q1 is fixed at the m/z of a precursor and the Q3 at the m/z of a product, hence the ion selected by Q1 is only detected if it produces a given fragment, corresponding to the selected reaction. The absence of scanning provides relatively long time periods to focus on the precursor and fragment ions, thus increasing sensitivity. SRM based instruments are often used for demanding applications, especially in bioanalytical assays. This two-step mass filtering process provides high specificity and sensitivity even for highly complex samples and was used for the biological trace analyses described in Studies IV and V. Recently published data show that TQ (SRM) offers 20-fold higher sensitivity for most compounds compared to Q-TOF and QIT and has a linear dynamic range of at least three orders of magnitude, while Q
TOF and QIT have linear dynamic ranges of one and two orders of magnitude, respectively[119]. On the other hand it has to be mentioned that TQ is a scanning device achieving lower sensitivity in full mass scan than the Q-TOF and QIT.

**Time-of-Flight (TOF) mass analyzers**

The TOF principle has been known from 1946[120,121] and is based on the physical law that accelerated ions with the same charge will have the same kinetic energy, but mass-dependent velocities. This is expressed as:

\[
E_k = \frac{1}{2} m \cdot v^2 = z \cdot V \tag{Eq. 12}
\]

where \( m \) is the mass of the ion, \( v \) is the ion velocity after acceleration, \( z \) the charge state and \( V \) the accelerating electric potential. The time of flight \( t \) then depends on the length of the analyzer drift tube \( l \):

\[
t = \frac{l}{v} \tag{Eq. 13}
\]

Linking equations (12) and (13) results in the following relationship from which \( m/z \) can be calculated:

\[
m / z = 2 \cdot V \cdot \frac{t^2}{l^2}, \tag{Eq. 14}
\]

indicating that fast, light ions will fly for shorter times than slower, heavier ions. The most suitable ion sources for a TOF analyzer are pulsed ion sources, such as LDI, but can also be connected to continuous ion sources such as ESI. Conventionally, a high positive or negative potential is applied to the sample plate and the ions in the gas phase are accelerated towards a field-free region of the drift tube. The spread of ion velocities determines the resolution of the TOF analyzer, thus several techniques have been developed to compensate for variations in the velocity of ions with the same \( m/z \) ratio, the most widely being to use an electrostatic ion reflector or delayed extraction (DE). The reflector technique uses a high potential electric field to reflect ions towards the reflector mode detector, instead of reaching the linear mode detector. Faster ions penetrate deeper into the electric field and have a longer path to the reflector mode detector compared to slower ions of the same \( m/z \), thus increasing the resolution by reducing the variation in the time of arrival of ions with the same \( m/z \) at the detector. The mechanism is mass-independent, and thus
compensates for velocity variations across the full mass range, but the sensitivity of detection is lower than in linear detection mode. In DE, which has been widely used in MALDI-TOF instrument, the acceleration voltage is applied after a few hundred nanoseconds delay following the desorption/ionisation process. This causes the slower ions situated close to the sample plate to be exposed to a higher potential than the faster ions further away. Consequently, the slower ions will be more strongly accelerated, gaining more kinetic energy, so ions with the same \( m/z \) will gradually get closer to each other, while travelling though the field-free region and finally detected almost simultaneously. In addition, the detection sensitivity may be somewhat increased by DE, due to a better spread of ions in the gas phase, leading to a lower number of collisions. Since the process is mass-dependent it has to be optimized for the mass region of interest. TOF analyzers characteristically provide very high scanning speeds, high sensitivity (decreasing with increases in mass) and medium dynamic range. The \( m/z \) range of a TOF analyzer is theoretically unlimited and detection of ions up to 1000 000 \( m/z \) has been reported. High resolution in the reflector detection mode (typically 10 000-40 000 FWHM) and high mass accuracy (<5 ppm using internal calibration) are further advantages of this type of mass analyzer.

**Detectors**

The energy of an incoming ion from the mass analyzer is converted into a current signal by a detector (in which the energy from the impact induces the emission of secondary particles such as electrons or protons). The current signal is then registered by electronic devices and transferred to the computer. This process has to be sufficiently efficient, with short recovery times, minimal variations in transit time, linear responses and low noise. The most widely used now are electron multipliers (EM), employing a dynode to amplify the signal. A discrete-dynode EM consists of a string of dynodes connected via resistors and can be constructed in many ways. The microchannel plate (MCP) is another type of EM, consisting of a parallel array of continuous dynodes (channel EMs). The advantage of an MCP is that it provides short transit times, but the drawback is that an electron cascade in one channel drains the neighbouring channels for several microseconds, causing nonlinear responses due to saturation effects.
Scintillation detectors emit secondary photons when struck by a particle and the resulting flash of the light is amplified by a photomultiplier. The detectors are often coupled together in order to increase the gain or dynamic range of the instrument. For example, the Voyager DE-STR MALDI-TOF instrument (Applied Biosystems, Framingham, MA, USA) uses a hybrid high-current detector consisting of a single MCP, a fast scintillator, and a photomultiplier in linear mode, while in reflector mode uses two MCPs stacked together. This ensures superior tolerance for high ion currents in linear mode and higher gains in reflector mode.

**Hyphenated techniques**

Since the advent of routine LC-MS coupling through ESI, hyphenated techniques have been used in various scientific fields. For instance, TQ-MS in combination with online LC separation is often used for quantitative analysis of low-abundance molecules in complex biological mixtures, illustrated in Studies IV and V. The importance of LC-TQ coupling was substantially increased by the introduction of UHPLC, since TQ-MS operating in SRM scan mode can provide very rapid duty cycle (5 ms). Hence, sufficient numbers of data points (minimum 7-12) can usually be acquired over single chromatographic peaks, for reliable quantification even for multicomponent monitoring and high throughput chromatography. Another advantage of UHPLC-MS coupling is the relatively low flow-rate used during UHPLC separation, which provides optimal conditions for atmospheric pressure ionisation sources. Although ESI tolerates a flow-rate of 1 mL.min\(^{-1}\) and even higher rates are technically feasible, high flow rates are likely to reduce signal/noise ratios. Higher sensitivity is generally achieved at low flow rates, usually at a few hundreds of microlitres per minute. Nowadays, conventional HPLC is being increasingly replaced by UHPLC to provide separated compounds for MS analysis, due to its abovementioned advantages and higher separation efficiency. Apart from technical issues that should be considered when coupling LC and MS the composition of the analyzed mixture and (especially) the MP composition should also be considered to ensure that both chromatographic and MS requirements are fulfilled.
Summary of papers

All the studies this thesis is based upon present techniques that are capable of separating analytes according to differences in their physicochemical properties. While LC is based on physicochemical interactions between analytes, the SP and MP, MS separates ionized analyte molecules according to differences in their m/z ratios. Thus, coupling these complementary techniques results in a powerful analytical tool, as shown in Studies IV and V. Study I compares the utility of recently developed UHPLC systems and conventional HPLC for separating phenolic compounds. The results show that the former is superior, in large part because it provides higher separation efficiency, which can be used either simply to improve the separation of analytes per unit time or to decrease analytical runtimes while maintaining the same separation efficiency as in HPLC. LC separation was not used in the study reported in Study II since the LDI-MS technique applied, exploiting the selective ionization/desorption of analytes from the complex samples, required no external matrix and provided sufficient resolution without prior LC. Here, the TOF analyzer provided sufficient separation of ionised molecules according to their mass-dependent time of flight through the drift tube. An analogous principle was exploited in Study III, in which analytes carrying a permanent positive charge were analyzed by LDI after TLC sample pre-treatment. This arrangement provides a slightly faster workflow since the TLC plate is mounted directly on the LDI target and inserted into the LDI source. Studies IV and V deal with trace analyses of extremely complex biological samples. The expected concentrations of the target analytes were less than 100 ng.g\(^{-1}\) of biological material. Therefore, multi-step sample pre-treatment was required and a TQ-MS instrument was selected to address all the demands of this application.

Conclusions and future perspectives

Several groups of important PPs were analyzed in Study I using four gradient elution methods to show the advantages of UHPLC over HPLC. Although the results are immediately applicable in food analyses, the feasibility and advantages of using rapid LC to separate PPs were further evaluated in two following papers not included in this dissertation. The aim was to analyze a wide range of different PPs
in a single chromatographic run, thus achieving simultaneous separation and quantification of 29 PPs in 20 minutes. The last step will be to couple UHPLC separation with MS/MS detection, as already done for catechins. Novel ways to analyse PPs were also explored in Study II, in which an LDI-TOF method was used instead of LC, prompted by a hypothesis that PPs would be amenable to LDI-MS analysis due to their structural similarity with commonly used MALDI matrices. The hypothesis was confirmed experimentally in trials with phenolic acids since their responses to laser desorption/ionisation agreed well with expectations. Additional unpublished experiments on flavonoids (Figure 8) revealed the potential of LDI analysis for other classes of PPs.

**Figure 8.** LDI-MS spectra of quercetin and rutin.

The main drawback of LDI analysis remains the inhomogeneous deposition of analytes, when the dried droplet method is used, thus more effort is required to solve this general, severe deficiency of LDI methods. One way to address it is to acquire multiple spectra from each dried droplet and evaluate the acquired data chemometrically. However, this addresses the symptoms rather than the underlying problems, and initial experiments conducted at the Department of
Analytical Chemistry, Stockholm University, by the author did not yield satisfactory results. Therefore, an approach capable of reducing or completely eliminating inhomogeneity would be more favourable, e.g. flash-freezing. **Study III** presented another practical application of LDI analysis, focused on alkaloids with a permanent positive charge. Here, TLC sample workup/separation was employed and the TLC plate was used directly for desorption/ionization of the analytes. TLC sample workup is easier and faster than SPE and in theory can also be used for PPs. The main focus of the work presented in **Studies IV** and **V** was to provide unambiguous identification of BMAA and DAB. Analyzed AQC derivatives of both compounds exhibited similar RP retention properties and nearly identical MS/MS fragmentation patterns (problems that had not been addressed in previously published LC analyses of BMAA). Therefore my colleagues and I developed an LC-MS/MS method providing chromatographic separation of BMAA and DAB followed by SRM detection of diagnostic transitions. Although the requirements for unequivocal, sensitive and selective identification of both analytes were accomplished the developed method remains semi-quantitative. Future work should be focused on developing techniques to obtain more reliable quantification results, ideally using a deuterated BMAA internal standard to appropriately compensate for matrix effects. Consideration of the physicochemical properties of analytes and their interactions with other entities is essential for any analyst seeking to improve analytical methodology. As shown in the appended papers, and this thesis, the exploitation of insights gained from understanding the structure and chemical properties of analytes, and the operating principles of available equipment (e.g. UHPLC, LDI and LC MS systems), greatly facilitates the development of innovative analytical techniques.
Acknowledgements

First of all I would like to thank my supervisor, Assoc. Prof. Leopold L. Ilag, for encouragement during periods of arduous working and personal circumstances, for giving me opportunities to present my results at scientific meetings abroad and for intriguing philosophical discussions during leisure time. I appreciate your rational and systematic approach, as well as your Christian behaviour.

I would also like to thank my former supervisor, Prof. Petr Solich, for arranging my stay at Stockholm University, for your overall encouragement in my travel ambitions, for your accommodating approach and for the freedom you gave me.

Special thanks are due to Prof. Bo Karlberg and his wife Marianne, who made Stockholm my second home. I will never forget the fishing adventures with Bosse and Marianne’s outstanding cuisine.

I learned that research in analytical laboratory is mainly independent and sometimes even lonely work, which proceeds much more rapidly and effectively with the help of mentors. Thus, many thanks to all my co-authors, including:

Shahram, for original and inspirational ideas, for many scientific and personal dialogues and for kind help with preparation of this dissertation. You gave me an idea of key features of creative analytical research work, and how to approach it.

Johan, for helpful consultations and new ideas during LC-MS/MS method development. You are a very lively person, an inexhaustible raconteur and a lot of fun to work with.

Sara, for dedicated work on the biological aspects of the BMAA project. My work on LC-MS/MS method development would have been much less exciting without seeing the practical benefit of your biological studies.

Gianluca, for introducing me to proteomics, educating me about nightlife in Stockholm and teaching me a few important Italian words. You have been a good friend, an excellent chef and it has been very enjoyable working with you, since you always bring fun to the drab Analytical Chemistry department.

Nana for your advice concerning the tricky LC-MS/MS instrument and practical information about SPE.
There are many other people to thank for contributing to this dissertation and I will never be able to appropriately acknowledge all of them. However, some of my other colleagues, past and present, warrant a special mention, including:

Assoc. Prof. Ulrika Nilsson for your kind approach and for giving me the opportunity to be involved in the tender for the new LC-MS/MS instrument. It was very exciting and informative work and I hope that my opinions were of some help.

Assoc. Prof. Roger Westerholm for your comments on this doctoral thesis.

Gunnar Thorsén for your inexhaustible scientific enthusiasm (spiced with an excellent sense of humour), useful knowledge about LC and plenty of remarkable and inspirational discussions.

Annalisa Ambrosi for your tips regarding the art of handling LC-MS/MS instruments, which saved me enormous amounts of time.

Yasar Thewalin for your practical point of view and willingness to help with paperwork of any kind.

Christoffer Bergvall for your translations from Swedish and never saying no, if I needed help.

Silvia Masala for your skill in making delicious coffee.

Thuy Tran for your contagious smile, always shining in the northern darkness.

Tuula Larsson for your friendly and talkative approach.

Sven Erasmie for swimming with crocodiles at the Kampong.

Anne-Marie Nilsson Hagelroth and Jonas Rutberg for your help with paperwork and IT related problems concerning to this dissertation.

Z celého srdce děkuji svým drahým rodičům, prarodičům a bratru Honzovi za podporu na klikaté cestě životem, za smířlivé přijetí mých světových ambicí a za trpělivé omlouvání z mnoha rodinných aktivit.

Poděkování patří také všem kamarádům, spolubydlícím, známým a kolegům, kteří zůstali o devět rovnoběžek jižněji i těm, které jsem získal ve Stockholmu. Díky Tondo, Pavle, Peťo a Radko, Peťo, Marcelo, Kristýno, Jančo, Katko a Honzo, že jste si našli čas na návštěvu ve Stockholmu.

V neposlední řadě chci poděkovat své osudové lásce Veronice za štěstí, kterým naplňuje můj život. Miluji Tě celým svým srdcem.
References


