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Development of Techniques and Methods for the Quantitative Analysis of Endogenous Substances by Microcolumn Liquid Chromatography Coupled to Mass Spectrometry

BY

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Abstract

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Liquid chromatography-mass spectrometry (LC-MS) is a powerful technique for the analysis of endogenous compounds. The introduction of electrospray ionization (ESI) as an interface between LC and MS has contributed strongly to a trend towards miniaturization of LC, due to the possibility to perform ESI at low flow rates. In this thesis, several aspects regarding the design of miniaturized LC systems and electrospray emitters were investigated. In addition miniaturized LC-ESI-MS have been used for the qualitative and quantitative analysis of endogenous polar compounds, peptides and protein digests.

The performance of miniaturized LC-MS was compared using different electrospray emitter configurations. The results indicated that the efficiency of the LC system is rather independent of the configuration of the emitter.

The lifetime of gold-coated fused silica electrospray emitters based on vapor deposited adhesion layers of titanium were investigated. The long lifetime of the emitter facilitates the use in LC-MS experiments, exemplified LC-MS by analysis of neuropeptides.

The ESI voltage is shown to interfere with liquid chromatographic separations performed in packed porous graphitic carbon capillary column. This interference is ascribed to the presence of an electric field over the conductive column in absence of a ground point between the column and the ESI emitter.

The solid supported enhanced microdialysis for analysis of neuropeptides were compared with conventional microdialysis. The difference between the two methodologies were evaluated by LC-MS analysis of the microdialysates. The solid supported method gave in general higher relative recoveries.

Finally, a method of standard addition was developed to determine total level of tryptophan and two of its metabolites in human plasma by capillary LC-ESI tandem mass spectrometry. The method was applied in a clinical study of multiple sclerosis patients treated with cytokines (IFN Beta 1a, 1b). The results show that the intervention effects the tryptophan metabolism.

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PAPERS INCLUDED IN THE THESIS

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

- I **Comparison between different sheathless electrospray emitter configurations regarding the performance of nanoscale liquid chromatography time-of-flight mass spectrometry analysis**
Ardeshir Amirkhani, Magnus Wetterhall, Stefan Nilsson, Rolf Danielsson and Jonas Bergquist
submitted to *Journal of Chromatography A*
- II **Gold-coated fused-silica sheathless electrospray emitters based on vapor-deposited titanium adhesion layers**
Stefan Nilsson, Oliver Klett, Malin Svedberg, Ardeshir Amirkhani, Leif Nyholm
Rapid Communication Mass Spectrometry, 17(14), 1535-40, 2003
- III **Interference of the Electrospray Voltage on Chromatographic Separations using Porous Graphitic Carbon Columns**
Anna Törnkvist, Stefan Nilsson, Ardeshir Amirkhani, Lena M. Nyholm and Leif Nyholm
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- IV **A Feasibility Study on Solid Supported Enhanced Microdialysis of Neuropeptides**
Andreas Pettersson, Ardeshir Amirkhani, Björn Arvidsson, Karin Markides and Jonas Bergquist
Accepted for publication in *Analytical Chemistry*

V Quantitation of tryptophan, kynurenine and kynurenic acid in human plasma by capillary liquid chromatography - electrospray ionization tandem mass spectrometry

Ardeshir Amirkhani, Eva Heldin, Karin E. Markides and Jonas Bergquist

Journal of Chromatography B, 780(2), 381-7, 2002

VI Beta-interferon effects the tryptophan metabolism in the plasma of multiple sclerosis patients

Ardeshir Amirkhani, Cecilia Rajda, Björn Arvidsson, Krisztina Bencsik, Krisztina Boda, Erika Seres, Karin E. Markides, László Vécsei, Jonas Bergquist

Submitted to Neurology

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I was responsible for planning in Paper I. The experimental work, analysis of data and writing was made in collaboration with coauthors. I was responsible for experimental work and planning of the LC-MS experiments as well as writing of Paper II. I was involved in planning and discussion in Paper III. I was responsible for automation of the LC-MS system and planning of the LC-MS experiments in Paper IV. The experimental work, analysis of data and writing the LC-MS experiments was made in collaboration with coauthors. I was responsible for the planning of the LC-MS experiments, the experimental work and writing Paper V. I was responsible for automation of the LC-MS system in Paper VI (B. Arvidsson carried out the experimental work for LC-MS analysis). The analysis of data and writing was made in collaboration with coauthors.

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Abbreviations

APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
APPI	atmospheric pressure photo ionization
CID	collision induced dissociation
CRM	charge residue model
ESI	electrospray ionization
i.d.	internal diameter
IDO	indolamine-2,3-dioxygenase
IEM	ion evaporation model
IS	internal standard
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantification
m/z	mass-charge-ratio
MRM	multiple reaction monitoring
MS	mass spectrometry
ODS	octadecylsilane
PGC	porous graphitic carbon
S/N	signal-to-noise ratio
SIM	selected ion monitoring
TOF	time-of-flight
UV	ultraviolet

1. Introduction

Analytical chemists have to analyze a variety of complex samples and answer a range of questions about the quality and quantity of different compounds of interest (analytes) in the sample. A complex sample contains a range of components that put a restriction on detection of analytes as well as interpretation of the data. Therefore sample preparation and separation methods are often crucial steps in an analysis method. Suitable sample preparation, separation and detection techniques are chosen by considering the physical/chemical properties of analytes and sample matrix as well as analytical demands, available instruments and cost.

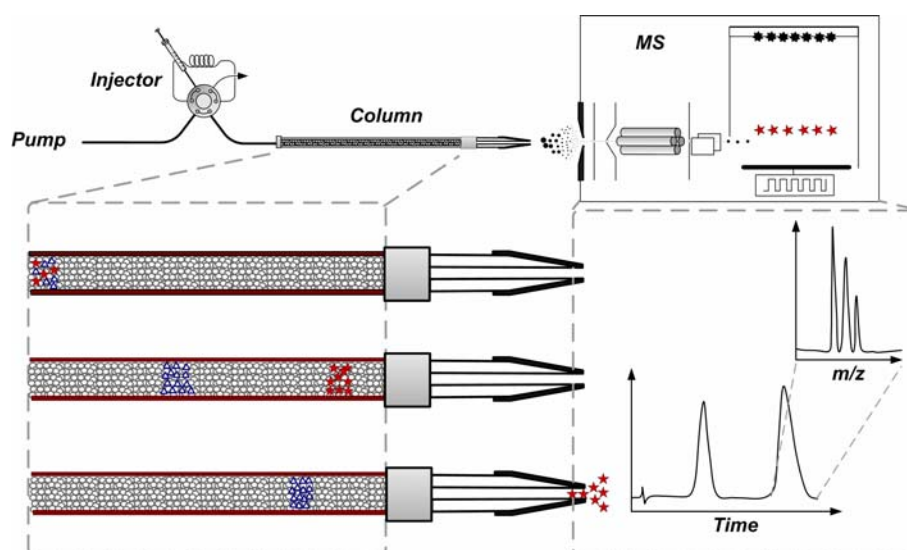


Figure 1: Schematic figure showing an LC-ESI-MS, liquid chromatography electrospray mass spectrometry (a time-of-flight mass analyzer). Separation in LC relies on the sample compounds affinity for a stationary phase, while MS relies on mass-to-charge (m/z) ratio of the ions.

Liquid chromatography-mass spectrometry (LC-MS) is one of the most important analytical tools used for study of thermolabile, nonvolatile and less volatile compounds and this method has been used for analyzing a wide variety of biological samples. Mass spectrometry is regarded not only as a detector but also as an orthogonal separation technique to LC by utilizing

different physical properties of an analyte. Separation in LC relies on the sample compounds affinity for a stationary phase, while MS relies on mass-to-charge (m/z) ratio of the ions derived from the sample (see Fig. 1). An ionization technique is necessary for transfer of analytes in the liquid phase into a mass analyzer, which operates under vacuum. A wide variety of ionization methods for LC-MS have been developed over the years. Among these, atmospheric pressure ionization (API) methods such as electrospray are dominant.

Miniaturized LC has established itself as a complementary separation technique to conventional liquid chromatography. This is mainly owing to lower sample volume requirements, lower flow rates and enhanced detection performance with the use of concentration sensitive detectors [1,2]. Miniaturization of LC started in the late 1960s by Horvath et al. who reported the use of 1.0 mm internal diameter (i.d.) columns [3,4]. The improvement in LC systems by addressing extra-column band broadening and new packing procedures as described by Ishii et al. [5,6] and Scott et al. [7,8] in the mid 1970s, have contributed strongly to the breakthrough of miniaturization of LC systems. Introduction of electrospray as ionization technique has been another driving force for miniaturization due to the possibility or even advantages of performing ESI at low flow rates.

This thesis deals with the improvements and designs of miniaturized packed column LC-ESI systems. In addition, miniaturized LC-ESI systems have been used for qualitative and quantitative analysis of endogenous compounds.

2. The miniaturization of LC

2.1 Nomenclature

The lack of standardization of the terminology describing miniaturized LC is a problem that leads to some confusion when terms like micro LC and capillary LC are used. Unfortunately the use of these terms seems to follow a sporadic trend rather than a standard definition. The most common classification of the LC techniques is listed in Table I [9]. Microcolumn LC is used in this thesis as a general term to describe small internal diameter (i.d.) packed LC-columns and this terminology has also been used frequently at different leading conferences [1].

Table I. Nomenclature for classification of packed column LC techniques [9]

Column i.d.	Typical flow rate in reversed phase	Classification
3.2-4.6 mm	0.5-2.0 mL	conventional LC
1.5-3.2 mm	100-500 $\mu\text{L}/\text{min}$	microbore LC
0.5-1.5 mm	10-100 $\mu\text{L}/\text{min}$	micro LC
150-500 μm	1-10 $\mu\text{L}/\text{min}$	capillary LC
10-150 μm	10-1000 nL/min	nano LC or nanoscale LC

2.2 Fundamental aspects in microcolumn LC

2.2.1 Flow rate and pressure drop

The flow rate through a column can be expressed either as a volumetric flow rate (F) or as a linear flow velocity (u).

The relation between volumetric flow and linear flow can be expressed as

$$F = \frac{u\pi d_c^2 \varepsilon_{tot}}{4} \quad (1)$$

where d_c is the column internal diameter and ε_{tot} is the total column porosity (having a value of 0.6-0.8 for columns packed with porous particles).

The mobile phase flow through the column gives rise to a pressure drop across the packed bed. This pressure drop (ΔP) can be expressed as

$$\Delta P = \frac{\phi \eta L_c u}{d_p^2} \quad (2)$$

where ϕ is the flow resistance parameter, η is the viscosity of the mobile phase, L_c is the column length and d_p is the diameter of the packing material [10]. The relationship between the volumetric flow rate and the pressure drop can thus be expressed as

$$\Delta P = \frac{4\phi \eta L_c F}{d_p^2 \pi d_c^2 \varepsilon_{tot}} \quad (3)$$

by combining Equations 1 and 2.

Equation 3 shows that a constant volumetric flow velocity through a column also can be achieved by applying constant pressure on a column. Equation 2 shows that the pressure drop across a column is independent of column diameter for a given linear flow velocity as long as the flow resistance parameter and the total column porosity is not changed. The flow resistance parameter and the total column porosity also seem to be independent of the column i.d. (265-44 μm) [11,12]. Kennedy et al. observed a slight downward trend in the flow resistance parameter (ϕ) as the column diameter was decreased (50-20 μm) [13]. The observed trend, a decrease in the flow resistance through a packed column, depends on the manner of which particles organize themselves in the center and close to the column wall respectively, which is known as “wall-effects”. Decreasing the column i.d. probably provides more homogeneously but also loosely packed columns.

Providing a low volumetric flow at a constant flow rate is a difficult task and only a few LC systems is claiming to be able to do this. Some conventional LC systems can be operated in a constant pressure mode, which can be used for performing isocratic separation on a microcolumn (an example is the separations performed in Paper III). Pre-column flow splitting of the initial flow rate is another way to accomplish a constant pressure on

the column. Pre-column flow splitting is especially useful when separation is performed in a gradient mode. It should be noticed that although the pump is operating in a constant flow mode, the flow through the microcolumn is provided at a constant pressure. The constant pressure in this case is caused by the passage of the mobile phase through a restrictor. Using a split makes the pressure applied on the microcolumn to be flexible according to changes in the mobile phase viscosity due to almost simultaneous passage of mobile phase through the restrictor and the microcolumn. It is important to notice that the flow rate provided by constant pressure should be calibrated frequently due to risk of the partial plugging of the column. Introduction of a restrictor (a capillary with sufficiently small i.d.) between the split and the microcolumn allows implementation of a pressure sensor at this point for monitoring the pressure drop over the microcolumn. The LC-system used in Papers II, IV, V and VI follows this design. The recorded pressure drop for each chromatographic run was compared with previous runs to detect any change in the obtained pressure drop profile. Any change indicates changes in flow rate through the microcolumn, which was adjusted either by recalibration of the LC system or by removing the clogging from the connecting tubing. In Paper I, a fused-silica transfer capillary (1 m length, 19 μm i.d.) was used both as means for transporting the mobile phase and an on-line flow meter. The pressure drop over the capillary was measured by connecting the outlet of the capillary to a pressure sensor. The pressure drop (difference between pressure measured at the inlet of the transfer capillary subtracted from pressure measured by the pressure sensor at the outlet of the transfer capillary) over the capillary (ΔP) is dependent on the flow rate through the capillary. The volumetric flow rate (F) through the capillary can thus be expressed as:

$$F = \frac{\pi \Delta P r_t^2}{8 \eta L_t} \quad (4)$$

where r_t , and L_t presenting the internal radius and the length of the capillary (tube) respectively [10]. Equation 4 can be used for calculation of the flow rate through the capillary and subsequently the microcolumn. The effect of the surrounding temperature and composition of the mobile phase on viscosity must also be considered in the calculation. The viscosity of most usual mobile phases (when neglecting buffer effects) can be estimated by the tabled reference values for organic modifier-water mixtures at different temperature [14].

Recently, LC pumps have become commercially available that provide constant flow rate by the use of an active split. An active split is capable of adjusting the pressure applied on the pressure drop through the restrictor to

compensate for the changes in the flow resistance parameter (as a result of a partial plugging of the microcolumn).

2.2.2 Band broadening

The apparent number of theoretical plates (N_a) expresses the efficiency of a chromatographic system, which can be described in volumetric terms as

$$N_a = \frac{V_R^2}{\sigma_{total}^2} \quad (5)$$

where V_R is the retention volume of a compound and σ_{total}^2 is the total volumetric variance of the corresponding eluted peak.

The total volumetric variance expresses the over all loss of efficiency due to the band broadening throughout a chromatographic system. This overall loss of efficiency corresponds to the loss of efficiency in each part of the system. As the individual losses are considered to be independent quantities, the total volumetric variance can be written as

$$\sigma_{total}^2 = \sigma_{column}^2 + \sum \sigma_{extracolumn}^2 \quad (6)$$

where σ_{column}^2 is the column variance due to band broadening within the column and $\sigma_{extracolumn}^2$ terms are various volumetric inputs from the rest of the chromatographic system.

The difference between N_a and N should be noted. The number of theoretical plates (N) describes the efficiency of a column, which often is determined by minimizing extracolumn band broadening. If extracolumn band broadening effects are minimized then the obtained σ_{total}^2 is assumed to be equal to the column band broadening (σ_{column}^2). At this circumstances the efficiency of the column can be described as

$$N = \frac{V_R^2}{\sigma_{column}^2} \quad (7)$$

2.2.2.1 Column band broadening

The column might be considered to be the most important part of a chromatographic system. It is known that the flow rate has a direct effect on the value of σ_{column}^2 . The flow rate thus has to be optimized to reduce the column band broadening. The flow rate and its effects on the band broadening are most often expressed as reduced flow velocity (v) and reduced plate height (h). The use of reduced parameters is preferred because they normalize the efficiency of the column. The reduced parameters take

into account the effects of column length, particle diameters and diffusion and thus make the comparison between different columns easier. In addition, h versus v can be fitted to one of several plate height equations (for instance Knox equation) and thereby be used to determine the important sources of band broadening. The optimum reduced velocity (v_o) for a given component can also be obtained where the lowest value of reduced plate height (h_m) is attained. It should be noted that flow rates used in LC often correspond to higher velocities than v_o to achieve faster separations.

The reduced velocity is defined as

$$v = \frac{ud_p}{D_m} \quad (8)$$

where D_m is the diffusion coefficient of the solute of interest in the mobile phase.

The reduced plate height is defined as

$$h = \frac{H}{d_p} \quad (9)$$

where H ($H = N/L_c$) is the height of a theoretical plate. The smaller value of h the better is the column.

Columns of 1 to 10 mm i.d. have very similar values for their minimum reduced plate height (h_m) and corresponding velocity (v_o) i.e. $h_m \approx 2$ and $v_o \approx 3$ and very close h vs. v curves [15]. In general for microcolumns with i.d. below 300 μm the reduced plate height decreases and the optimum reduced velocity increases with decreasing column i.d. [12,13]. This result is due to a reduction of the A and C terms in the Knox equation

$$h = Av^{0.33} + B/v + Cv \quad (10)$$

where the A, B and C are the flow dispersion, longitudinal diffusion, and the resistance to mass transfer terms respectively [16]. The reduction of A and C with decreasing column diameter is suggested to be caused by three factors. These factors are decreased by decreasing the mobile phase flow inhomogeneities as well as retention inhomogeneities within the column and finally diffusional relaxation (shorter time needed for solute molecules to diffuse from the middle of the column to the wall) [13]. The magnitude of the improvement (low ϕ and h_m and high v_o values) is dependent on the ratio of column to particle diameter.

2.2.2.2 Extracolumn band broadening

The geometry and the volume of the injection system, the detector, connecting tubing and the connecting fittings may all add to the extracolumn band broadening.

$$\sigma_{extracolumn}^2 = \sigma_{injector}^2 + \sigma_{connections}^2 + \sigma_{detector}^2 \quad (11)$$

It is generally accepted that the extracolumn band broadening may reduce the resolution (R) of a chromatographic separation by a maximum 5% [1,2]. It can be derived that

$$R = \frac{\Delta V}{\sqrt{\sigma_{total}^2}} = \frac{\Delta V}{1.05\sqrt{\sigma_{column}^2}} \quad (12)$$

where ΔV is the retention volume difference between two closely eluted peaks. Thus, the condition in Equation 6 and an acceptable resolution are satisfied when the extracolumn variance is

$$\sum \sigma_{extracolumn}^2 \leq 0.1025\sigma_{column}^2 \quad (13)$$

Table II: Extracolumn contributions to chromatographic band broadening

	Volumetric variance (σ^2)	Definitions
Plug injection [17]	$\frac{V_{inj}^2}{K^2} \quad (14)$	V_{inj} = injection volume K = a constant for each injection technique, theoretically is equal to $\sqrt{12}$ but experimentally is as low as 2
Connecting tube [18]	$\frac{\pi r_T^4 L_T F}{24 D_m} \quad (15)$	r_T = tube radius L_T = tube length D_m = solute diffusion in the mobile phase F = volumetric flow rate
Detector cell [18]	$\frac{V_D^2}{F^2} \quad (16)$ or $\frac{\pi r_D^4 L_D F}{24 D_m} \quad (17)$	V_D = cell volume F = volumetric flow rate r_D = tube radius L_D = tube length The detector cell is considered either as a mixing chamber (Equation 16) or as a connection tube (Equation 17)

In Table II equations that can be used for calculation of the individual extracolumn contributions are displayed.

A more common expression of band broadening for calculation purposes is the fraction of peak broadening (θ^2). The ratio of each individual variance to the column variance is defined as the fraction of peak broadening e.g. 1% peak broadening equals $\theta^2 = 0.01$ [15]. Equation 13 can therefore also be expressed as

$$\sum \theta_{extracolumn}^2 \leq 0.1025 \quad (18)$$

Decreasing the column internal diameters reduces the value of σ_{column}^2 . Thus, it is increasingly important to reduce the extracolumn volumes to preserve the separation efficiency in microcolumn LC.

In Paper I, the post-column band broadening effects of different configurations (A, B, C, D) was investigated (see Fig. 2 for further details). These configurations were constructed of either an integrated column or a separated column and emitter. These configurations were used for coupling a nano LC system to a time-of-flight (TOF) mass analyzer. Introduction of a separate emitter always increases the post-column band broadening effect due to passage of the column effluent through a capillary and unwanted dead volumes. However, a separate emitter has its advantages, see further discussion under section 3.1.1.5. The experiments were performed at 600 nL/min, which were shown to be well suited for the constructed emitters.

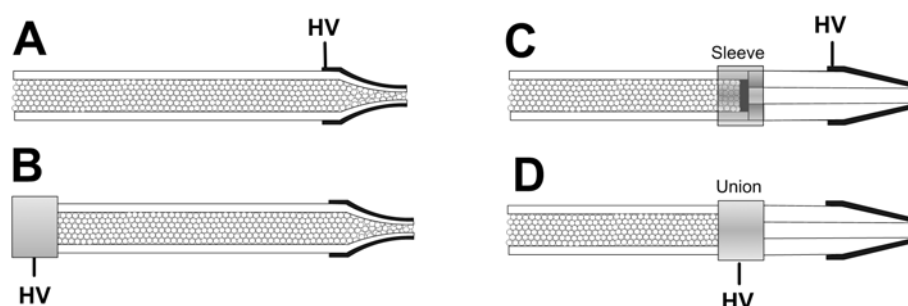


Figure 2: Schematic drawings of the different nano LC-column configurations used in Paper I. A and B represent columns with integrated emitters, while C and D represent separate columns and emitters. The electrospray high voltage (HV) was applied at the outlet end in configuration A and at inlet end of the column in configuration B. In C, the electrospray voltage was applied at the outlet end of the emitter while in D, the electrospray voltage was applied via a union between the column and emitter.

The pre-column band broadening was avoided by preparing the sample, a ten peptides standard mixture, in a weak solvent (acidified water) in

combination with a gradient elution. The performances of different configurations were measured by monitoring retention time, peak widths at half height, peak height, and peak area for the analytes. The primary outcome of the experiments was a large amount of data, which was overviewed by performing principal component analysis (PCA). The interpretation of data, especially peak widths at half height indicated that the efficiency of the LC system is rather independent of the configuration when using 95 μm i.d. columns, acetic mobile phase and standard peptides as a sample.

In Papers I, V and VI, a flow direction valve with an extern tee was used to avoid the dead volumes in the flow direction valve. The set-up used in Papers V and VI is shown in Fig. 3. The eluent from the column diverted from the mass spectrometer by the flow direction valve for the initial desalting step. The tee between the column and the transfer capillary to the ion source is connected to the flow direction valve. The inner diameter (250 μm) of the tubes connecting the tee to the waste via the flow direction valve was larger than the inner diameter (50 μm) of the fused-silica capillary connecting the tee to the ion source. Because of difference in inner diameter of the tubes the eluent in the initial desalting step was diverted from the mass spectrometer and directed into the waste.

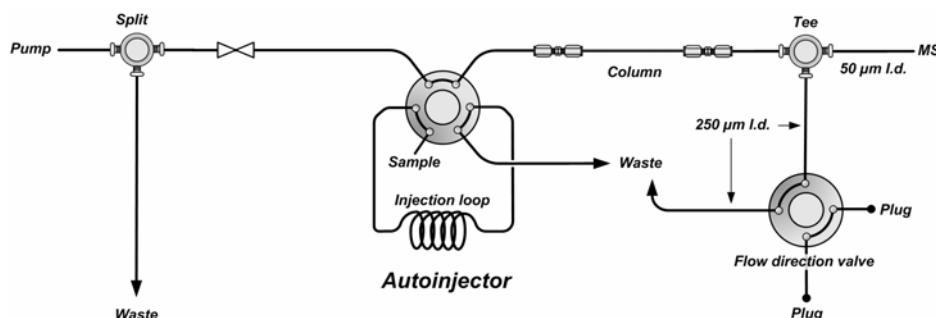


Figure 3: Experimental set-up of the LC-MS/MS system used in Papers V and VI. Column: 150 \times 0.5 mm packed with 3 μm C18 particles (ODS AQ, YMC), Capillary connecting the tee to the electrospray source: 50 μm i.d. Capillary connecting the tee to the waste: 250 μm i.d.

2.2.3 Injection volume

As the diameter of the column is decreased the amount of the sample necessary to perform an analysis also decreases with the use of a concentration sensitive detector. This is especially useful when the sample amount is restricted. There are two problems, however, which make exploitation of reduced sample volume difficult. Pre-treatment of complex samples are often required before injection onto the columns. Pre-treatment

of small amount of samples can be difficult or even impossible in some cases. The second problem is absence of injectors that are able to inject sample volumes below 20 nL.

The maximum concentration of the eluted peak can be expressed as

$$C_{peak} = \frac{C_{inj} V_{inj}}{V_R} \sqrt{\frac{N}{2\pi}} \quad (19)$$

where C_{inj} is concentration of the injected sample [17].

The injection of an equal volume into columns of different internal diameters (d_1 and d_2 where $d_1 < d_2$) increases the maximum concentration of the eluted peak (C_{peak}) for the miniaturized column. In the miniaturized column, the C_{peak} is increased by the inverse of the square of the column internal diameter (d_2^2/d_1^2). The miniaturized column should give the lowest detection limit with the use of a concentration sensitive detector if the efficiency of the LC system (N) is not changed. A simple calculation predicts an increase in C_{peak} by a factor of 100 when a column of 460 μm i.d. is used instead of a conventional column of 4.6 mm i.d. However, increasing the injection volume on a column with a given i.d. will also increase the injection system band broadening ($\sigma_{injector}^2$, see Equation 14). The use of so-called on-column focusing techniques can help to eliminate or at least reduce $\sigma_{injector}^2$ and overcome the limitations in the injection volume. In this thesis, on-column focusing techniques in combination with gradient elution were frequently used for decreasing the pre-column band broadening as well as increasing the pre-concentration of the analytes. Whenever it was possible, samples were dissolved or diluted with a solvent similar to the mobile phase with the weakest elution strength. In Papers V and VI pre-concentration of the analytes were enhanced by addition of an ion-pairing agent to the samples. Trichloroacetic acid was chosen due to its superior ion-pairing effects on all major metabolites of tryptophan (in kynurenine as well as in indole pathways) compare to other ion-pairing agent [19]. In addition, the packing material, ODS-AQ, used in these experiments allowed use of 100% water as a mobile phase without experiencing stationary phase collapse [20], which facilitates on-column focusing.

The injection of a large amount of sample may lead to undesirable change in separation due to column overloading. The overloading of the column is due to the mass of the sample rather than sample volume. This happens due to saturation of the stationary phase by components in the sample (not necessarily only with the analytes of interest). Overloading the column causes change in the peak shape and retention time of the analytes [21,22]. In addition, the continuous elution of matrix components from the overloaded LC column is considered to be the major factor contributing to signal suppression in LC-MS experiments [23-25]. The overloading of the

column and signal suppression caused by fast eluting components in a sample can be overcome by e.g. using 2D LC (LC-LC) [24,26].

2.3 Economical and environmental aspects

The use of microcolumns is to a certain extent hampered by the somewhat more complicated and expensive instrumentation. Although, recently better and less expensive instrumentation has become commercially available. It should be considered that higher initial cost could be compensated by decreasing consumption of chemicals and faster method development due to more freedom in changing column diameter or packing materials. The lower volumetric flow rate through a microcolumn would minimize consumption of chemicals like organic modifiers or ion pair reagents in the mobile-phases. Complete utilization of reduction in flow rate is, however, not always possible. The lower flow rates, $\mu\text{L}/\text{min}$ and nL/min , are mostly achieved by means of flow splitting. More dedicated LC pumps (either syringe or reciprocating) have also become commercially available to be used solely with microcolumns. These instruments are able to perform stable nL/min and $\mu\text{L}/\text{min}$ flow rates in isocratic and gradient mode of operation respectively without flow splitting. Newer conventional HPLC system can be run at typical flow rates of $100 \mu\text{L}/\text{min}$ or less in gradient mode. Although a flow splitter still must be used with these systems, the mobile phase consumption is far from what is demanded when using conventional LC systems [27].

Reproducible packing of microcolumns is relatively easy performed in-house [11,28,29] thus a range of columns for method development with a variety of packing material can be fabricated at only a fraction of the cost of conventional columns. All column used in this thesis has been packed in house.

3. Mass spectrometry

A mass spectrometer (MS) is an analytical instrument designed to separate charged species, according to their mass-to-charge ratios, and to record their intensities. A typical MS instrument comprises several parts: sample inlet, ion source, mass analyzer, detector and data handling system.

3.1 Ion sources

Initially in an MS analysis, a sample is introduced through a so-called inlet to an ion source. An ionization technique is necessary for transfer of analytes in a liquid phase into a mass analyzer, which operates under vacuum. There are several types of ion sources, which utilize different ionization techniques for creating charged species. The choice of ionization technique depends on type of analyte, the sample preparation, the eventual separation technique, the analytical demands and compatibility with the available mass analyzer. Ion sources working at atmospheric pressure are most commonly used for the on-line coupling of a liquid separation technique to a mass analyzer. Atmospheric pressure ionization (API) methods can handle relatively high sample flow rates and are easier to handle than ionization techniques working under low pressure. In addition, API methods are in general considered to be “soft” or gentle ionization i.e. transferring labile molecules into the gas phase as intact ions.

3.1.1 Electrospray ionization

Dole et al. reported the first use of an electrostatic sprayer for formation of ions at atmospheric pressure i.e. electrospray [30,31]. Electrospray ionization (ESI) is a technique for transformation of ions from a liquid into the gas phase. ESI is a simple method that operates at atmospheric pressure and usually at ambient temperature. ESI is probably the most gentle ionization technique and best suited for medium polar ionic compounds. In addition, ESI often produces multiple charged ions of large analytes such as peptides and proteins, which facilitates analysis of large analytes with a mass analyzer with a limited mass to charge working range. Fenn et al., reported multiple charge ion formation [32], which together with other findings was

awarded the Nobel Prize in chemistry 2002. Electrospray is becoming the most important ionization technique in use.

3.1.1.1 The mechanism of ESI

This section is intended to give a short overview of the mechanism of the ESI process. The principal mechanism of ESI is however under investigation and still not totally understood.

An electric field between the liquid in a thin capillary or needle and a counter electrode causes separation of ions in the liquid phase and formation of a double layer of the ions at the meniscus of the liquid. The electrostatic attraction of the ions in the outer layer of the liquid phase towards the counter electrode (with opposite charge) causes a bending of the liquid surface in a cone (so-called a Taylor cone [33]) or liquid jet form. Deformation of the liquid surface is counteracted by the surface tension of the liquid phase. At a certain voltage, the onset voltage [34] (see Table III), the surface tension is not able to hold the surface together, which result in the emission of charged droplets from the surface of the liquid phase. The formation of charged droplets will continue as a consequence of excess charge, which is provided either by removing or supplying electrons in an oxidation or reduction reaction. The initial charge as well as the size of the droplets depends on the spray conditions [35,36].

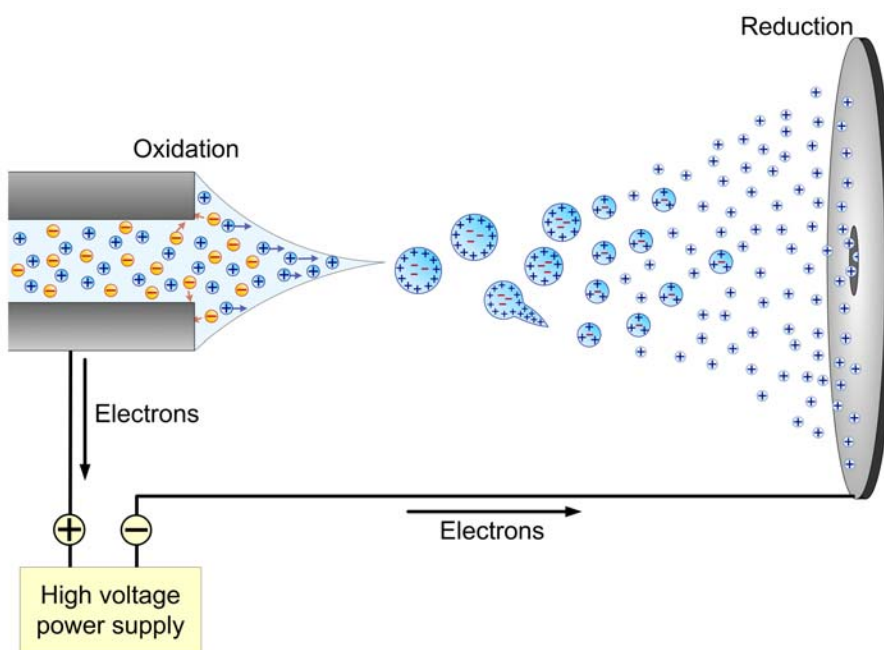


Figure 4. Schematics of electrospray set-up in positive mode.

Table III: Theoretical equations for the potential required for the onset of electrospray, the radius and the charge of the initial charged droplet, and charge of droplet at the Rayleigh stability limit [37]

Equations	Definitions
$V_{on} \approx \left(\frac{r_o \gamma \cos \theta}{2 \epsilon_0} \right)^{1/2} \ln(4d / r_o) \quad (20)$	<p>V_{on} = potential required for onset of electrospray r_o = outer radius of emitter tip γ = surface tension of solvent (0.0226, 0.030 and 0.073 N/m for CH₃OH, CH₃CN and H₂O respectively) θ = half-angle of Taylor cone apex (49.3°) ϵ_0 = permittivity of vacuum ($8.8 \times 10^{-12} \text{ J}^{-1} \text{ C}^2 \text{ m}^{-1}$) d = distance from emitter tip to counter electrode</p>
$R = \left(\frac{3 \epsilon \gamma^{1/2} V_f}{4 \pi \epsilon_0^{1/2} K E} \right)^{2/7} \quad (21)$	<p>R = radius of initial charged droplets ϵ = permittivity of the solvent V_f = volumetric flow rate K = conductivity of solution E = electric field</p>
$q = 0.5 \left(8 (\epsilon_0 \gamma R^3)^{1/2} \right) \quad (22)$	<p>q = charge of droplet</p>
$q_{Ry} = 8 \pi (\epsilon_0 \gamma R^3)^{1/2} \quad (23)$	<p>q_{Ry} = charge of droplet at the Rayleigh stability limit</p>

The charged droplets are subject to evaporation and deformation during their path towards the counter electrode. The solvent evaporation decreases the size of the droplets while the charge remains constant. Thus, the charge to volume ratio will increase. This increase will continue until conditions close to the Rayleigh stability limit are reached. At the Rayleigh stability limit, the electrostatic replication overcomes the force of the surface tension holding the droplets together and the droplets undergo coulombic fission. Coulombic fission of charged droplets emit smaller charged droplets (offspring droplets). The emissions of offspring droplets stabilize the parent charged droplets, which later can again undergo coulombic fission due to evaporation. This process continues until the residues of the parent droplets either are stripped of their excess charge or collided to the counter electrode. The offspring droplets have often a larger charge to volume ratios than their parent droplets. Therefore, this process is often called uneven fission of charged droplets [38]. The offspring droplets act as parent ions for the next generation of offspring droplets in the same fashion as their parent droplets. The uneven fission process can be described as a multiple extraction process

that strips mass and charge at the surface of the parent droplets to the offspring droplets.

The formation of gas phase ions from the very small offspring droplets (produced after several generations) is still under debate. There are two different theories regarding the formation of gas phase ions, namely the Charge Residue Model (CRM) and the Ion Evaporation Model (IEM). The CRM, introduced by Dole [30] and later extended by Röllgen [39] predicts that the recursive uneven fission continues until the last generation of the offspring droplets only contain one ion per droplet. The IEM, introduced by Iribarne and Thomson, assumes that ion emission to the gas phase (ion evaporation) occurs directly from the very small offspring droplets [40,41]. The distinction between these models in the details of gas phase ion formation has little if any influence on the use of ESI for LC-MS.

3.1.1.2 Modifications of electrospray ionization

The formation of a charged-aerosol is working well for solvents with low surface tensions at low flow rates. An increase of one, or both, of these parameters must be compensated by an increased electric field. However, increasing the electrical field between the electrospray tip and the counter electrode can give rise to unwanted electrical discharges, which can disturb the electrochemical reactions or damage the emitter and the power supply electronics.

The simple electrospray interface can be modified in various ways in order to make ESI more compatible with high/low flow rates and high surface tensions. In addition, changing the pH or hydrophobicity of the solvent can also increase the efficiency of ionization for certain analytes.

Post-column addition of modifier solution is one way to alter the chemical or physical properties of the sprayed solvent. Coaxial addition of a sheath liquid is often implemented after a separation step without introduction of any dead volume, thus retaining the effects on the separation efficiency. Smith et al. was the first to report the use of a sheath flow method in combination with capillary electrophoresis (CE) and ESI-MS [42]. Coaxial addition of a sheath flow may be considered for miniaturized LC without causing any band broadening effects. Post-column modification for LC-MS can often be performed through a tee connection e.g. to reduce suppression effects caused by the presence of trifluoroacetic acid [43].

The pneumatically assisted electrospray (Ionspray), introduced by Bruins et al., allows higher mobile phase flows than pure electrospray [44]. The application of Ionspray has been extended to flow rates up to 2 mL/min by adding a simple liquid shield between the spray and the ion sampling orifice [45]. Furthermore, Ionspray decrease the electric field required for the nebulization. It also makes the spray process less dependent of position of the interface inside the ion source and allows a larger distance between the spray capillary and its counter electrode. The lower electrical field, together

with a larger distance between the spray tip and counter electrode, prevents the occurrence of electrical discharges. This freedom of positioning the spray inside the ion source have been used to spray diagonal or at perpendicular angles with respect to the source axis. These configurations improve the stability of operation and prevent penetration of the droplets and contaminants into the mass analyzer.

The pneumatically assisted electrospray was used in Papers II-VI due to the relatively high flow rates, between 1 and 12 $\mu\text{L}/\text{min}$, in the experiments. The experiments in Paper I was performed without any nebulizer gas due to the relatively low flow rate (600 nL/min) that was employed. In Paper II an integrated column and emitter was used, which was almost identical to those (configuration A see Fig. 2) used in Paper I (with the exception of coating material). The continuous infusion experiments performed in Paper I, where a peptide signal was monitored for different flow rates and concentrations, showed that the flow rates 500 and 700 nL/min yielded the most stable signal (see Fig. 5). Utilization of nebulizer gas enables the use of a higher flow rate, 1 $\mu\text{L}/\text{min}$, without any problems.

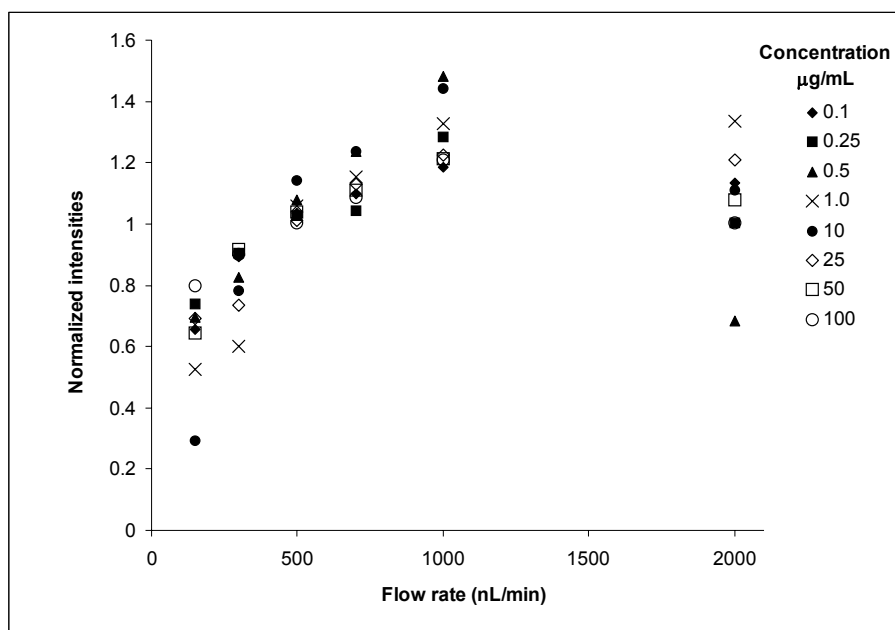


Figure 5: Influence of the flow rate on the response of the mass spectrometer, using continuous infusion of methionine-enkephaline at different concentrations ranging from 0.1 to 100 mg/mL. The normalized intensity is calculated as the signal divided by the average signal over all flow rates. For each the ESI potential and emitter position were optimized.

3.1.1.3 Electrospray as a concentration or mass flow sensitive device

Mass spectrometry is inherently a mass-flow sensitive detection technique [46]. However, depending on the ionization technique used, MS can show a concentration sensitive behavior. The signal height (S_{\max}) in a concentration sensitive detector is dependent on the concentration of the analyte in the peak maximum (C_{\max}) and is independent of the flow rate as:

$$S_{\max} \propto C_{\max} \quad (24)$$

On the other hand, the signal height in a mass flow sensitive detector is dependent on the concentration of the analyte in the peak maximum and the flow rate (F) as:

$$S_{\max} \propto C_{\max} F \quad (25)$$

The ESI-MS signal can be affected by changes in the sample flow rate and is therefore not considered as a pure concentration sensitive detector [45,47-49]. The effect of the flow rate is highly dependent on the interface characteristics. It has been shown that a pure ESI-MS can behave as a mass flow sensitive detector at low flow rates ($<0.4 \mu\text{Lmin}$) [49].

In Paper I, the relation between the flow rate and peak intensity was investigated for sheathless ESI-MS. A peptide signal was monitored for different flow rates and concentrations in continuous infusion experiments to establish the lowest flow rate where the MS still acts as a concentration sensitive detector (see Fig. 5). A sharp change from a mass flow to a concentration sensitive detector was not observed. However, the results clearly show a drop of the MS response at flow rates below 500 nL/min.

3.1.1.4 ESI response

The ESI response of an analyte will directly influence sensitivity, specificity and accuracy of a quantitative MS analysis. The ESI response of an analyte is also affected by a large number of variables (such as concentration and physical/chemical properties of the analyte and the solvent composition) as well as ion source design and experimental parameters (like flow rate). An understanding of the effects of these variables is important for LC-ESI-MS method development and trouble shooting. Cech and Enke recently published a review of different variables and their effects on the ESI process [50].

In general, high concentration of organic solvents and volatile buffers are suitable for API-MS. Organic solvents are helpful since they will accelerate solvent evaporation and decrease the surface tension (see Table III), which are considered to be beneficial for the ionization and nebulization processes. Different organic solvents may however have quite different effect on the

ionization efficiency for a given analyte [51]. Nonvolatile buffers and additives are, on the other hand, avoided in order to prevent accumulation of salts and plugging of the sample orifice as well as decrease in signal intensity. Petritis et al. have published a general guide for volatility of potential mobile phase/electrolyte additives [52]. However, It is well known that volatile additives e.g. ion-pairing agents may decrease the MS response [43,53-55].

The ESI response of equimolar amounts of different compounds can vary significantly even with identical solvents [56] which clearly shows the important effect of analyte properties. The properties of an analyte is largely effected by the solvent composition e.g. effect of pH on the charge as well as hydrophobicity of weak acids or bases. Ionization of weak bases is generally best achieved at low pH where the analyte is protonated. However, the protonated analyte ions in positive ion ESI have also been observed in solution where the pH was higher than the pK_a value of the analyte. A possible explanation can be the electrolytic oxidation of water ($2H_2O \rightarrow 4H^+ + 4e^- + O_2$) at the high voltage contact surface, which decreases the pH of the solution. Another possible explanation can be the previously mentioned uneven fission of mass and charge in the ESI process, which would change the pH in offspring droplets due to an accumulation of H^+ ions in the offspring droplets [57]. The opposite case has also been observed for weak acidic analytes in acidic solution in negative mode. The ESI response of an analyte is directly related to polarity and surface-activity of a compound and can be correlated with different accessible variables e.g. the retention time on a reversed phase liquid chromatography column (longer retention time \sim higher MS response) [58]. The aforementioned uneven fission would favor the ionization of the surface-active components [59] and at the same time suppress other, less surface-active, components in the solution (ion suppression). Surface-active substances (e.g. surfactants [60]) with an opposite charge to an analyte ion and salts [61] may also cause ion suppression. The analytes studied in Papers V and VI have relatively hydrophilic character, which added real challenges towards the ionization and quantification of these substances.

The magnitude of the ion suppression is highly dependent on various compounds in the sample matrix that are competing in the ionization process. Matrix suppression is used to describe the suppression of MS response for a given analyte by sample matrix [62]. This matrix suppression may be decreased at lower flow rate. Wilm and Mann (in experiments with unseparated peptide mixtures and a nanoES device) have shown that ESI at a flow rate of ~ 20 nL/min provides more freedom to choose the solvent as well as pH and a higher tolerance towards salt contamination [63]. Several factors may contribute to the higher ionization efficiency obtained at low flow rate. Formation of small and monodisperse charged droplets, which contain few analyte ions, compared to larger charged droplets and size

distribution at higher flow rates may be one possible explanation. Another factor is the enhanced charge to mass ratio of charged droplets compared to those obtained at higher flow rates. Wilm and Mann has compared the electrical current in their experiment using the nanoES device ($\sim 0.25 \mu\text{A}$) to the electrical current obtained in a conventional electrospray source ($\sim 1 \mu\text{A}$), while the flow rate was decreased about two orders of magnitudes, resulting in much larger charge to mass ratio for droplets at lower flow rate. In addition, the ion source operating at low flow rate can be placed closer to the orifice and vacuum system and the curtain gas can be decreased, which increases the portion of ions transferred. It should be noticed that electrical discharges could be avoided due to the lower potential needed for obtain electrospray at low flow rates (600-700 V in nanoES device compare to a few kV in conventional ESI). It is important to remember that the increase in the ionization efficiency at low flow rates does not necessary mean an enhancement of the obtained MS response. Ionization efficiency is defined as “the ratio of the number of ions formed to the number of electrons, photons, or particles that are used to produce ionization” [64].

Unfortunately it is not always possible to use optimal conditions for an analyte in the ESI. Sample preparation and liquid chromatography are often necessary steps before ionization in order to minimize matrix suppression effects. Mobile phases suitable for ESI are often used for LC in order to obtain compromised conditions for both LC and ESI. Post-column modification can sometimes be helpful to achieve optimal ESI-MS sensitivity e.g. by removing a nonvolatile ion pairing reagent [65]. Another option is hydrophilic interaction chromatography (HILIC) or reversed phase LC with more hydrophobic stationary phases such as porous graphitic carbon (PGC). The increase in organic solvent/water ratio in these methods can narrow the gap between best condition for both LC and ESI. Porous graphitic carbon has some unique features such as a very hydrophobic plane surface compared to e.g. octadecylsilane (ODS, i.e. C_{18}). These features of PGC have made it a complementary stationary phase for the separation of closely related substances [66] and separation of polar analytes [67,68]. In Paper III, the backward current, which causes long-term changes of PGC, was investigated (see below).

3.1.1.5 High voltage connection

In principal, the electrical voltage can be applied at any point before the ESI spraying tip, at the spraying tip, or even at the mass analyzer interface plate. A review of recent developments on interface constructions and the application of the high voltage needed to achieve ionization was published by Gelpí [69]. In this thesis, the electrical voltage was applied either at some point before the spraying tip or at the spraying tip. The third option, where the electrical voltage is applied at the mass analyzer interface plate and the

ESI emitter is grounded, could not be used in this thesis work due to the design of the mass analyzers in use.

An aforementioned appliance of a high voltage to an eluting liquid generates an electrical field between the liquid at the spray cone and the counter electrode providing the electrical current for the ionization process (excess charge). The production of the excess charge can be best described by regarding electrospray as a control current electrolytic cell, where the ion transport does not occur through the solution but through the gas phase [70]. The electrochemical reactions at the high voltage connection point may cause undesired (or desired) effects such as the oxidation or reduction of the analyte [71], alteration of the pH of the spray [72], corrosion or even destruction of the spray emitter [73] and introduction of metal ions (such as Fe^{2+}) due to the corrosion of the stainless steel capillary or union [70,74]. However, the current in the ESI ion source, can take an unwanted path, when there is a ground point at some point before the high voltage connection point i.e. through the eluting liquid as backward current. This ground point may be voluntarily inserted for safety reasons or otherwise be around e.g. somewhere in the chromatographic system. The backward current can then be much larger than the forward current passing through the gas phase [72] and may increase the electrochemical reactions and their effects. For example, the backward current can alter the pH of the solution, which for example causes a decrease of pH resulting in protein unfolding [72].

In addition, Liu et al. showed results, which may imply that the backward current causes oxidation of the analytes when a chromatographic system based on a fused silica capillary precolumn and column packed with C_{18} particles was used [75]. The site of oxidation was shown to be on, or near, the pre-column and was avoided by the introduction of a ground point between the precolumn and the electrospray capillary. The use of such a ground point is however not without risk since electrochemical reactions still take place at this ground point. The introduction of a ground point is applied in the majority of LC-ESI systems without noting oxidation of the analytes. Two possible explanations can be either the presence of other more easily oxidized materials such as iron or a limited reaction surface area available for oxidation of the analyte on the ground point. Another explanation may be that such oxidations are simply not considered.

In Paper II, the lifetime of gold-coated fused silica electrospray emitters based on vapor deposited adhesion layers of titanium was investigated. The Ti/Au coated emitters were manufactured to investigate the possibilities of producing durable ES emitters applicable in chip-based analytical devices. The long lifetime (500-700 hours) of the Ti/Au coated emitters facilitated the LC-MS experiments exemplified by nano LC-MS analysis of neuropeptides.

In Paper III, the backward current causing long-term changes of PGC was investigated. The observed effects were ascribed to the presence of redox reactions at both ends of the PGC column. This problem was also avoided by

the introduction of a ground point between the column and the electrospray emitter. The backward current can also prevent proper function of electrical devices, such as an autosampler, which was observed during the automation of an LC-system in Paper VI.

The main purpose of Paper I was to investigate the post-column band broadening effects of different LC-ESI-MS configurations (A, B, C, D see Fig. 2 for further details). These configurations were used for the coupling of a nano LC system to a TOF mass analyzer. These configurations are interesting due to different ways of applying the ESI voltage. The integrated system (A, B) should theoretically be the best way to avoid any band broadening effects. Configuration B failed in the experiments (separation of ten peptide standards) due to obtained lower peak intensity and instable electrospray performance. The lower response obtained with configuration B is probably due to the longer path between the spray tip and the electrical contact. This could not be compensated for, due to limitations for the potential applied by the mass spectrometer. There is also a correspondingly shorter path between the contact point and ground point (the injector), which leads to an increased backward current and possibly formation of bubbles at the contact caused by oxidation of water. Configuration A worked perfectly well for the separation of peptide standards. However, the use of a separate column and emitter (C, D) can still be motivated mainly due to the possibility to easily replace either the emitter or the column. Electrochemical reactions may cause undesired effects on the emitter due to corrosion or even destruction of the emitter, which was the subject of Paper II. Vanhoulst et al. showed that a configuration similar to D was sensitive to different mobile phases [76]. Thus, configuration C was chosen for further investigation, namely separation of tryptic digested proteins.

3.1.2 Atmospheric pressure chemical ionization and photo ionization

In work described in this thesis, ESI was used as the ion source. However, there are two other API methods, which should be mentioned; atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo ionization (APPI). These methods are complementary rather than rival to ESI. Both these techniques combine a heated pneumatically assisted nebulization with a gas phase ionization process. In APCI and APPI interfaces, the solution from the LC passes through a heated nebulizer into the ion source. The free molecules in the gas phase are ionized by charged species commonly generated around a charged needle (corona discharge needle) or energetic photons generated by an ultraviolet (UV) lamp in front of the nebulizer [77]. Both techniques seem to be most suitable for samples having a high gas phase basicity or acidity. Especially APCI is a mature technique and widely used for small, less-polar compounds (about 1000 u).

In general, APCI have shown to be mass flow sensitive at both low and high flows [78-80].

3.2 Mass analyzers and fragmentation

The separation/selection of the charged species in a mass analyzer depends on their mass to charge ratio (m/z) in vacuum, which is accomplished by applying electric or magnetic fields in their path through the mass analyzer. There are several different types of mass analyzers e.g. quadrupole, ion traps, time-of-flight (TOF), ion cyclotron resonance and sector instruments.

It is well known that ion sampling and transfer efficiency as well as detection efficiency of the mass analyzer, affects the MS response for a given analyte [81-86]. It is important to notice that different substances (despite optimization) can have quite different responses due to discrimination in transfer and detection by the mass analyzer.

Apart from separation/selection, the mass analyzer can also be used for fragmentation of the charged species. Fragmentation of charged species occurs usually in regions with relatively high pressure, due to collision between accelerating charged species and surrounding gas molecules. This kind of fragmentation is often called collision-induced-dissociation (CID) [87]. In-source and up-front CID are widely used for declustering by moderate acceleration of the charged species between the ion source (operating at atmospheric pressure) and vacuum inside the mass analyzer. However, unwanted in-source and up-front CID are a notable problem for small labile molecules [88]. A clear example of this phenomenon is tryptophan and its metabolites studied in Papers V and VI. The analytes with an NH_3 group readily will lose this group, which should not be a problem per se. The produced fragment ions can efficiently be transported into the mass analyzer. Unfortunately the ions produced after loosing the NH_3 group in this study are not suitable for the MS/MS analysis due to formation of several product ions instead of preferably one product ion in the MS/MS process. CID can be preformed in a more controllable fashion by MS/MS techniques where parent ions, collision energies, collision gas and collision gas pressures can be selected and optimized. There are two main categories of instruments for such MS/MS experiments [89]. The first category utilizes mass analyzers in tandem (triple quadrupoles and sector instruments). The second category of MS/MS instruments is capable of storing selected parent ions, which later are fragmented during a selected time (ion cyclotron resonance and quadrupole ion trap instruments). The MS/MS fragmentation is often called tandem MS, which can be tandem in space (the first category) or tandem in time (the second category).

In this thesis work, a triple quadrupole (MS and MS/MS) and a TOF (MS) instrument were used.

3.3 Detectors

Several types of commercially available detectors are used in MS instruments. The electron multiplier is the detector of choice for most routine MS experiments. The ions are detected after mass selection in a mass analyzer by converting the detector-surface collision energy of the ions into emitted electrons. The emitted electrons are then sensed with various charge detectors. Electron multipliers are in general rugged, reliable and are capable of providing high current gains at nanosecond response times [90]. However, electron multipliers have a limited linear response due to the saturation effects of the detector. Performing analysis on the isotope of an analyte can sometimes solve the problem of saturation. In Papers V and VI, tryptophan, kynurenine and kynurenic acid were simultaneously quantified. The concentrations of these analytes varied over about three orders of magnitude. The relatively high concentration of tryptophan compared to the other analytes implied a saturation of the detector. Thus, the second most abundant isotope of tryptophan was selected for quantification and saturation was consequently avoided.

4. Quantitative aspects

This section is intended to give a short overview of the quantification of small endogenous molecules such as tryptophan and its metabolites or peptides in body fluids by LC-MS and LC-MS/MS. The same principles can be applied to quantification of other endogenous compounds such as proteins or exogenous drugs and drug metabolites in biological samples.

4.1 General considerations in quantitative analysis

Although, the main objective of this section is the theory and practice of developing a quantitative LC-MS method, some general guidelines regarding the long-term use of the method cannot be avoided. The reliability of the obtained results from a quantitative analysis is depended on the skill of personnel performing the analysis, the equipment used and the analysis method. The apparatus test and calibration at specific intervals ensure that the instrumentation fulfills its intended function. Validation is a procedure to ensure reliability of results obtained from an analytical method. The combination of these two quality measures (apparatus test and validation) is referred to as system suitability test. The suitability test is performed in order to guarantee that the method meets the demands made on the current instrumentation at that instant [91-95].

Validation results are expressed in terms of acceptable precision and accuracy. There seems to be no official guidelines for method validation especially for endogenous compounds in biological matrices. However, there are some general guidelines published in the literature [96-98] for method validation performed in the pharmaceutical industry. Method validation is often expressed by the determination of the following measures for the quantification of an analyte in a given matrix.

Precision: A measure of reproducibility of several results obtained with identical samples by the same method (does not measure if the results are correct).

Accuracy: A measure of closeness of the obtained results to their true values.

Sensitivity: The ability to analyze samples with low concentration of analyte. Limit of detection (LOD) and limit of quantification (LOQ) are

often used to measure the sensitivity of a method [14]. The use of sensitivity as term for “having a low limit of detection” is a common source of confusion because it is also defined as the slope of the calibration graph [99].

Selectivity (or Specificity): The ability of an analytical method to find and quantify the analyte of interest in the presence of other components.

4.2 Modes of acquiring LC-MS data

Sensitivity for an analyte is generally best when only one ion is formed from the analyte. Thus, the lowest detection limits are obtained when multimer formation, cluster formation and fragmentation of “the target ion” (e.g. protonated analyte or in-source fragment ion) is reduced to a minimum by optimization of solvent composition and instrumental settings. The sensitivity and selectivity of mass analyzers that use scanning for selecting ions, i.e. quadrupoles, are also affected by the ion-monitoring mode used during acquisition. Limiting the scan range to one or a few ions are known as selected ion monitoring (SIM) in MS and as multiple reaction monitoring (MRM) in tandem MS. Reducing the scan range increases the sensitivity of the method and the speed of data recording. Other advantages are simplicity and low demands on the data handling system. For quantification purposes SIM or MRM are most often used. Using MRM combines sensitivity and specificity especially in analysis of complex samples due to the decrease of the chemical noise by fragmentation of the selected ion. The specificity of MRM can be improved further by observing three or more fragmented ions. In addition to the detection of several ions, the specificity of a method can be improved by considering the retention time and the ratio of the fragmented ions [100]. The dwell time (the time spent counting ions per selected ion) has also a large effect on signal-to-noise ratio (S/N), longer dwell time increases the value of S/N. However, at least six points per peak in an LC-MS chromatogram must be acquired to fulfill the Nyquist theorem [101]. For quantification purposes, 20 data points are sufficient to quantify peaks by their area or heights [102]. In practice, peak area quantification needs much fewer data points than peak height quantification.

4.3 Quantification methods

Quantification analysis in general relates the response of the analyte to its concentration in a sample. This relationship is performed with some kind of calibration (comparison to known amounts of analyte or an internal standard). A calibration (standard) curve can often be used for graphic presentation of the relationship between the obtained response of an analyte and its known concentration.

However, quantitative measures such as normalized peak area are also used to get an estimation of the amount of component of interest in comparison with the other components in a sample without any calibration. The responses in the obtained chromatogram, as the integrated peak areas, are then divided by the sum of the peak areas. The main disadvantage of this method is the variation in response factors for different components. Normalized peak area is often used in situations when no standard is available.

The main methods of choice for quantification are external standard calibration, internal standard calibration and the method of standard addition. These methods are often utilized to interpret results obtained with quadrupole instruments operating in SIM or MRM mode. The relationship between the concentration of an analyte and its mass response is obtained by univariate regression. Performing full-scan on a quadrupole instrument (when the low sensitivity is not an issue) or using a TOF mass analyzer provides additional information about the analyte response like different fragments of the analytes or decrease in MS response due to coeluting compounds. Alternative methods like multivariate regression can use this information and improve the reliability of the quantification results [103]. Application of multivariate regression models like partial least squares regression (PLS) and multiway methods like parallel factor analysis (PARAFAC) to direct sampling MS clearly shows the benefit of these methods [104].

4.3.1 External standard calibration

In external standard calibration, the detector response can be described as a function of analyte concentration:

$$\text{response}_{\text{analyte}} = f(c_{\text{analyte}}) \quad (26)$$

The obtained function should have zero intercept i.e. $f(0) = 0$ (see Fig. 6). The calibration is performed by analysis of known concentrations of analyte (standards). The term “external standard calibration” is used because the standards are analyzed in separate chromatographic runs from those of the unknown samples. External standard calibration is a simple method but should only be used, when low levels of errors due to sample preparation and small instrumental performance variations is expected. In addition, in LC-MS analysis ion suppression effects on the MS response of the analytes must be considered. Appropriate separation and selective sample preparation can reduce ion suppression effects. Preparation of standards in the sample matrix can also take into account the matrix suppression effects.

A useful method to evaluate matrix effects is post-column infusion of an analyte into the ion source [24,25,105,106]. The injection of blank matrix

can reveal the extent as well as the profile of the matrix suppression effects. Absence of blank matrix for endogenous compounds in biological samples make this method less useful but it can still give an estimation of matrix suppression in the vicinity of the eluting analyte. Determination of the post sample preparation recovery is another way to reveal matrix suppression. Spiking the blank sample after the sample preparation step(s) reveals the loss of intensity caused by the matrix compounds. The lack of a blank matrix should not be any problem in this case, since relative MS responses (from the sample and the corresponding spiked sample) can still reveal the matrix suppression effects. Naturally the low recovery in the spiked matrix may be caused by the sample preparation step if spiked a priori. Comparing the peaks area obtained by spiked sample before and after sample preparation can be used for evaluation of the recovery [105,107].

Another problem can be the low precision of the obtained results. On average LC-APCI-MS is shown to provide less precise results than LC-UV (UV is considered to be a stable detector) [108]. Although these problems have been met with APCI-MS, similar results are often obtained using other ionization techniques such as ESI. The precision of the result also depends on the properties of the analyte, solvent composition and coeluting compounds.

During the method development for quantification of tryptophan and its metabolites in plasma (Paper V), external calibration was first performed. Post-column infusion of the analytes as well as the recovery test indicated presence of matrix effects on the analyte responses caused by the injected plasma sample.

Systematic errors caused by matrix effects and random variation may be avoided by applying internal standard calibration.

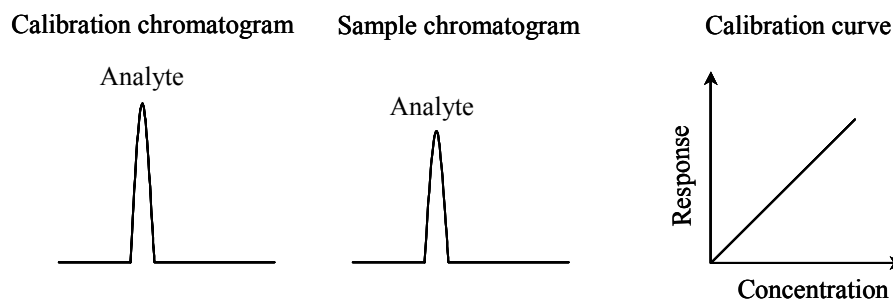


Figure 6: External standard calibration method for quantitative analysis.

4.3.2 Internal standard calibration

Another method for calibration is addition of an internal standard (IS) at constant level in both standards and unknown samples. The detector response ratio of analyte to internal standard can then be described as a function of analyte concentration:

$$\text{response}_{\text{analyte}}/\text{response}_{\text{IS}} = f(c_{\text{analyte}}) \quad (27)$$

The obtained function should have zero intercept (see Fig. 7). The introduction of an IS can reduce errors due to sample preparation, instrumental performance variations and ion suppression. A proper choice of internal standard is the determining factor in reducing errors.

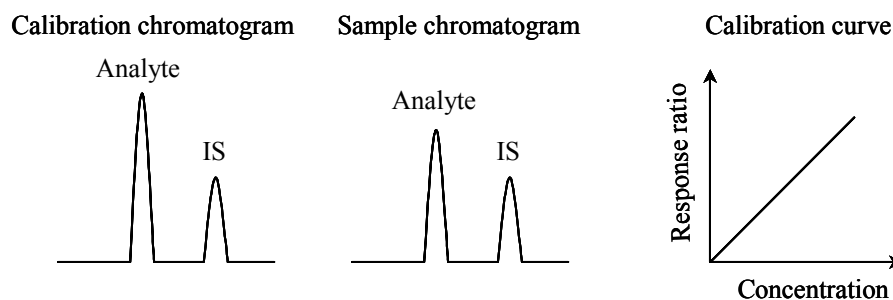


Figure 7: Internal standard calibration method for quantitative analysis.

4.3.2.1 Choice of internal standard

The following properties are often desired when an IS is chosen for LC-MS:

- Not present in the sample
- Similar physical/chemical behavior to the analyte (especially if the IS is added into the sample before sample preparation)
- Similar retention (time) as the analyte, preferably it should co-elute with the analyte
- If co-eluting with the analyte it should not have any common ions with the analyte under MS or MS/MS mode
- Not contaminated with analyte (highly pure)
- Adequately stable
- Not reactive e.g. with the sample compounds or the mobile phase
- Similar concentration as the analyte

Stable isotope-labeled (^2H , ^{13}C , ^{15}N , ^{18}O) analogues are most suitable as internal standards in LC-MS [109]. The isotopic cluster for the analyte must

be considered before choosing an IS. For small molecules, the mass difference between the IS and the analyte should have a minimum of 3 m/z in order to avoid disturbance from naturally occurring isotopic peaks. These internal standards behave almost identically to the analyte in sample preparation, separation and ionization processes. Although isotope-labeled internal standards are widely used in LC-MS experiments, there are some limitations that have been addressed [110]. Impurity of isotope-labeled IS in form of the analyte (even as low as 0.1%) can contribute significantly to the response of the analyte at low concentration. In addition, in some cases, deuterated labeled internal standards have inadequate stability e.g. isotope exchange occurs during the extraction. Furthermore, isotope-labeled components are expensive and are not available for all analytes. An alternative is to use a structural analog, which are used in the majority of cases. Structural analogs can mimic an analyte, but only compensate for variable ionization due to matrix components when simple sample preparations or little chromatographic separation are implemented [107]. Another problem can be the low precision of the obtained responses for the structural analog internal standard and the analyte, which can reduce the reliability of this method. The responses obtained from the structural analog internal standard and the analyte are more or less independent quantities therefore random variation and matrix effects may have a considerable effect on the $\text{response}_{\text{analyte}}/\text{response}_{\text{IS}}$ ratio. The situation is quite different when a stable isotope-labeled internal standard is used. The analyte and internal standard in this case are considered to be dependent quantities.

An internal standard is in general introduced in a sample in two ways, prior to the sample work-up (surrogate IS) or prior to analysis (volumetric IS) [111]. Surrogate internal standards can to a certain degree address errors in the sample preparation (e.g. degradation or adsorptive losses of an analyte), instrumental errors (e.g. injection volume) and quantitative errors attributed to ion suppression. Volumetric internal standards can address instrumental errors and quantitative errors attributed to ion suppression with various success. Post column introduction of an IS can also be performed to address quantitative errors attributed to ion suppression only [112]. In any case, the added amount of the internal standard should be well above the LOQ without causing ion suppression.

In Paper IV, internal standard calibration was used to determine the relative efficiency of different microdialysis methods for six endogenous neuropeptides. The solid supported enhanced microdialysis for analysis of neuropeptides were compared with conventional microdialysis. The samples were collected in fractions and, after initial sample preparation steps, spiked with a structural analog peptide, kemptide. After vacuum drying, the samples were resolved in a formic acid buffer in order to make them suitable for capillary liquid chromatography mass spectrometry (LC-MS). The results

indicated that the solid supported method gave, in general, higher relative recoveries.

Once again during the method development for quantification of tryptophan and its metabolites in plasma (Paper V) internal standard calibration was first tested for quantification. The internal standard was 3-nitro-L-tyrosine which Widner et al. have used for quantification of tryptophan and kynurenine by LC-UV [113,114]. Unfortunately, this approach was not successful probably due to one or a combination of the following reasons: Different matrix effects on the internal standard compared to the analytes, the large concentration range (3 orders of magnitude) between the analytes and the low precision of the results. However, the use of stable isotope-labeled internal standards was not an option due to lack of commercially available analogs for all analytes. In absence of a suitable internal standard a method of standard addition calibration was developed.

4.3.3 Method of standard addition

An ideal calibration is performed by preparation of standards in a blank matrix. However, in some cases, it is difficult to obtain suitable blank matrices e.g. for endogenous compounds in body fluids. In the literature, spiked levels of 25-100% of the expected analyte concentration in the sample are recommended [14]. In the method of standard addition, the detector response can be described as a function of the analyte concentration:

$$\text{response}_{\text{analyte}} = f(c_{\text{analyte}}) \quad (28)$$

The obtained function must be linear and the lowest calibration point is the unspiked sample (see Fig. 8).

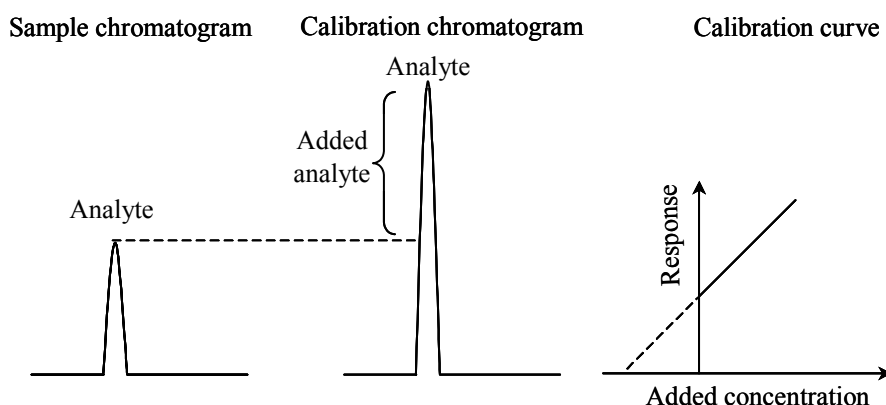


Figure 8: Method of standard addition for quantitative analysis.

Extrapolation of the calibration plot provides the original concentration of the analyte. The method of standard addition can be combined with the internal standard method. Since the method of standard addition is an extrapolation method, it is generally considered to be less precise than interpolation methods. In addition, it requires more sample than the other calibration methods.

A typical example can be found in Papers V and VI, where three endogenous compounds (tryptophan, kynurenine and kynurenic acid) were quantified in plasma by the method of standard addition. In this method, the sample is spiked with a known amount of analyte. In Paper V we spiked the samples with 50% and 100% while in Paper VI only 100% of the expected sample concentration was used.

4.3.4 General consideration for using various calibration methods

The relatively narrow linear range is a problem when LC-MS is utilized for quantification purposes. APCI has often a wider linear range than ESI [115,116]. By using nonlinear calibration, the full dynamic range rather than the linear dynamic range of the obtained MS response can be used to obtain a calibration curve. Non-linear and spline functions (calibration curve made up of a series of linked section of functions) are possible approaches [99]. However, the question of propagation of errors requires considerable attention [111].

4.3.4.1 Estimation of errors

It is always necessary to consider possible errors in a calibration. The random error in a calibration is often calculated and presented together with an obtained result as a confidence interval. Confidence intervals give estimated precision of a measurement. A more realistic estimation of the precision, can be obtained by applying weighted regression, instead of non-weighted regression [43]. Another source of errors of calibration are systematic errors that affect the accuracy of results. Systematic errors cause deviation of a calibration curve in form of the intercept (so-called constant-systematic error) or the slope (so-called proportional-systematic error) or both of them from their true values [117]. Constant-systematic error reveals itself as an intercept (either negative or positive sign) differing from zero. Thus, this type of error cannot be detected in the method of standard addition where the method inherently has an intercept. Use of specific methods like MS/MS (as in Papers V and VI) decreases or eliminates interferences from coeluting compounds, thus decreasing the error for the obtained intercept. Proportional-systematic deviation either decreases or increases the true slope of the calibration plot. Systematic errors can be detected by the

determination of a recovery function [117] or, if possible, by comparing with well established existing methods [118].

4.3.4.2 Limits of detection and quantification

Limit of detection (LOD) and limit of quantification (LOQ) are often used to express the sensitivity of a quantification method. There are a number of different definitions in terms of signal-to-noise ratio for LOD ($S/N = 2-5$). The definition recommended in IUPAC is “the limit of detection is the sample concentration which gives a signal equal to the blank signal plus three times the standard deviation of the blank” [118]. Similar definition in term of signal-to-noise ratio for LOQ is often used ($S/N = 10$ or greater). There are also recommendations for calculation of LOD and LOQ by using the propagation of errors approach or the confidence intervals, which consider the effect of random errors on the slope or the intercept [99,118,119].

In Papers V and VI, LOD and LOQ are defined as concentration of the analytes corresponding to $S/N = 3$ and $S/N = 10$, respectively, where N was defined as the standard deviation of the noise in vicinity of the detected peaks (due to the absence of a representative blank).

4.4 Role of ionization technique

Although, ESI is the dominating ionization techniques, the use of APCI has shown to have several advantages, especially for quantification purposes of relatively small analytes. Several groups have reported elimination of the matrix effects when APCI was used instead of ESI in LC-MS/MS experiments [110,120,121] probably due to the absence or less severe ionization suppression effects in the gas phase [106]. In addition, APCI has often a wider linear range than ESI, as mention above. However, APCI is best suited for less-polar compounds [122].

During method development for tryptophan, kynurenine and kynurenic acid (Paper V), APCI was also tested. The LOQ ($S/N = 10$) was from 0.3 to 3 μM for all analytes, which were two orders of magnitude higher than the expected concentration of kynurenic acid in plasma. The higher LOQ values obtained for the analytes in APCI compared with the ESI result obtained in Paper V (10 nM) can mainly be explained by ionic and high polar characters of the analytes.

Post-column split of the mobile phase and performing ESI at submicroliter per minute flow can reduce matrix effects [123]. Another option is nano LC-MS especially when the amount of sample is limited.

Another issue is the contamination of ion optics due to accumulation of salts (from nonvolatile additives and sample matrix) that may affect the precision of the obtained results as well as the intensity of MS responses.

Cleaning of the ion source and mass analyzer at specific intervals ensures that the instrumentation fulfills its intended function [124].

4.5 Sample preparation of biological samples

The sample preparation is often the bottleneck of an analytical method. The complexity of biological samples often demands for the introduction of a sample preparation step before the LC-MS or MS/MS analysis. The extent and type of sample preparation can vary due to properties of the analytes and the sample as well as the separation method, the ionization technique and the analytical demands. Sample preparation of a biological fluid for the analysis of small substances are used for several reasons, namely to remove unwanted compounds in the sample matrix e.g. proteins and salts and even for enrichment of the analytes. Direct injection of a biological sample with high contents of proteins can clog the column. In addition, salts (and other compounds or contaminants e.g. plasticizer) can cause systematic errors in the obtained result due to their effect on the ionization efficiency in ESI [106]. Sample preparation conditions must be considered due to risk of degradation or adsorption of an analyte. There are a number of different sample preparation methods for small substances such as protein precipitation (even called plasma crash), liquid-liquid extraction and solid phase extraction (SPE). These methods have shown to have different success in decreasing matrix suppression effects in ESI [125]. More selective sample preparation methods and chromatographic separations with high resolution have been used for decreasing the matrix suppression [110]. However, using extensive sample preparation methods and chromatographic separation is not always successful even with less complex samples such as saliva [62]. The chemical properties of the analytes rather than the sample preparation method seem to play a more important role in matrix suppression effects in ESI i.e. the most polar compound is the most sensitive to matrix suppression [126]. Another approach is to accept a minimum matrix suppression effect and use an internal standard or the method of standard addition or using alternative ionization method such as APCI, as described earlier.

In Papers V and VI, protein precipitation was used for sample preparation. Proteins precipitation is a fast and easy sample pretreatment. The precipitation of proteins can be preformed by addition of protein denaturing solvents (such as strong acids, heavy metal salts and organic solvents miscible with water) to the sample [127], enzymatic digestion and heating the sample. The effects of the denaturing solvent on the chromatographic system and separation performance (such as peak shape and retention time) as well as on the analytes must be considered [128]. For example, a strong acid or base may damage the column or cause degradation of the analytes. This may be avoided by neutralization of the sample after

sample preparation. Organic solvents (at higher concentration than the mobile phase) and heavy metals often have negative effects on the chromatographic performance. Injection of an organic solvent into the chromatographic system can be avoided e.g. by drying and resolving the sample in a more suitable solvent. However, drying and resolving samples often decrease the total recovery.

5. Endogenous substances analyzed and their biological role

5.1 Neuropeptides

Neurotransmitter is the general name for a group of molecules that are used by the central and peripheral nervous system to transfer chemical messages within and between individual cells. The neurotransmitters are stored in the axon terminals until the terminal receives an action potential from their respective cell body. The neurotransmitters are then released into the extracellular space and the chemical messages are transferred [129]. Peptides are one major group of neurotransmitters, which are responsible for a variety of functions e.g. mediation of pain (substance P), reducing pain (enkephalines), regulation of water balance (vasopressin) and regulation of blood pressure (neurotensin) [130]. Neuropeptides are in vivo, often present in μM -pM concentrations. The neuropeptides are relatively large and bulky molecules with a mass of 500 Da – 5000 Da. They also possess a wide variety of inherent chemical and physical properties expressed in hydrophobicity and charge. The analysis of neuropeptides is challenging due to the rapid neurotransmission processes, their low concentration and the fact that extracellular environment of the tissue is both physically and chemically complex. Neuropeptides are however important neurotransmitters responsible for many functions in the body and are therefore highly interesting molecules to monitor. In Paper IV, a solid supported enhanced microdialysis for analysis of neuropeptides were compared with conventional microdialysis. The results indicated that the solid supported method gave, in general, higher relative recoveries.

5.2 Tryptophan and its metabolites

L-tryptophan is one of ten essential amino acids and plays an important role in protein synthesis and as precursor of many biologically active substances such as serotonin (see Fig. 9). Tryptophan is predominantly metabolized in the kynurenine pathway [131]. Degradation of tryptophan by

a cytokine induced indoleamine-2,3-dioxygenase (IDO) to formyl kynurenine seems to enhance when the cellular immune system is activated [113,114]. The kynurenine pathway metabolites also seems to be involved in the pathogenesis of several neurological disorders such as depression and anxiety [132], schizophrenia [133], multiple sclerosis, Alzheimer's disease [134] and Parkinson's disease [135,136]. Thus the possibility to monitor the metabolic route of tryptophan is becoming increasingly important.

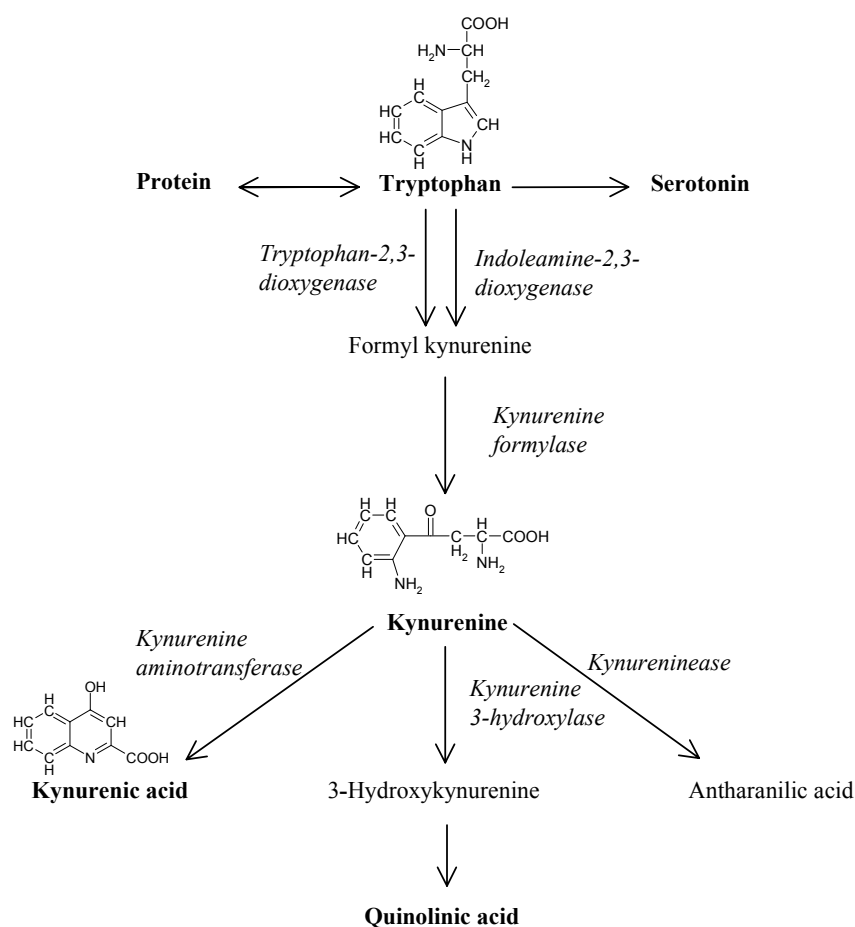


Figure 9: Abbreviated pathway of tryptophan metabolism showing pathways to protein, serotonin and kynurenine.

The subjects of Paper VI, was patients with multiple sclerosis. The plasma samples collected from the patients were monitored for changes in the tryptophan metabolism during 24 hours. Multiple sclerosis is an autoimmune disease, where inflammation and axonal loss are occurring at the same time [137,138]. At the beginning multiple sclerosis is characterized by the activated immune system, but also neurodegeneration is present in the

early stages of the disease. Activated immune competent cells are more easily crossing the blood-brain barrier, taking part in several immune processes in this compartment. Changes in the tryptophan metabolism influenced the regulation of the T cell activity in an animal model of multiple sclerosis [139]. Until 3-hydroxykynurenine and quinolinic acid exert neurotoxic properties, kynurenic acid antagonizes excitotoxic neuronal death [140]. In Paper VI we report the levels of tryptophan metabolites in 8 healthy, 16 multiple sclerosis patients naive to beta-IFNs (with at least two relapses in two years) and 11 patients receiving long-term beta-IFN, presuming that in multiple sclerosis patients, especially in the beta-IFN-naive group, the metabolism of tryptophan is preferentially directed along the neurotoxic kynurenine metabolic route. According to the data in healthy controls, tryptophan metabolism might have a circadian rhythm, if we consider the changes in the kynurenine/tryptophan ratio. The increases of the kynurenine/tryptophan ratio in multiple sclerosis patients receiving beta-IFN for the first time indicate the induction of IDO by beta-IFN as described earlier [141]. Long-term beta-IFN administration have not changed the kynurenine/tryptophan quotient, the patients were lacking the circadian rhythm observed in the healthy and IFN-naive multiple sclerosis group, which might be due to a compensatory mechanism.

The most important enzyme in the kynurenic pathway, IDO is activated through participants of the immune system. Due to its presence in inflammatory and neurodegenerative processes it could be a link in the immune mediated neurodegeneration in multiple sclerosis.

6. Concluding remarks

Liquid chromatography-mass spectrometry is a powerful technique for analyzing biological samples of both small and large molecules. The ionization process is preferably performed by API techniques specially ESI due to its unique features.

The introduction of ESI has contributed strongly to miniaturization of LC due to the possibility or even advantages of performing ESI at low flow rates. The strongest argument for utilizing a miniaturized LC system is probably the limited amount of samples.

The optimum utilization of miniaturization of LC-MS requires understanding of the potential pitfalls and issues that the technique is tampered by.

Miniaturized LC systems should not be used beyond its capability by injection of very large volumes of sample, which is a strong temptation in trace analysis. Overloading of LC columns has negative effects on the chromatographic performance as well as the ESI response of analytes.

Exploitation of MS as an orthogonal separation dimension to LC should not be performed without regarding the effects of “unseen” sample matrix. Thus, understanding of the API process is essential for the optimum exploitation of LC-MS.

Hydrophilic compounds have especially low ionization efficiency and high sensitivity to the sample matrix, which ongoing research shows, can be improved by decreasing the flow rate. Further improvement of miniaturized LC-ESI systems as well as mass analyzers (in form of increasing ion transport efficiency) may help to overcome these problems.

In this thesis, issues such as control of flow rate, band broadening effects and backward electrical current have been discussed and some suggestions have been presented for improving the design of miniaturized LC-ESI systems. In addition, miniaturized LC systems were used for qualitative and quantitative analysis of endogenous compounds.

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8. Utveckling av tekniker och metoder för kvantitativ analys av endogena substanser med mikrokolonn vätskekromatografi sammankopplad med masspektrometri

Vätskekromatografi sammankopplad med masspektrometri (MS) är en kraftfull teknik för analys av bl.a. endogena (kroppsegna) substanser i biologisk forskning. Joniseringsprocessen sker i stor utsträckning med hjälp av tekniker som arbetar under atmosfärstryck. Bland dessa tekniker är elektroprayjonisering den mest använda på grund av dess enastående egenskaper att t.ex. skapa flerladdade joner. Införandet av elektropray har starkt bidragit till en utveckling mot vätskekromatografi i miniatyrformat.

I denna avhandling har flera aspekter som rör designen av ett vätskekromatografisystem och elektropraykoppling i miniatyrformat undersökts. Dessa system har använts för både kvalitativ and kvantitativ analys av små endogena substanser, peptider och enzymspjälkade proteiner.

Olika typer av elektropraykoppling har jämförts med avseende på deras prestanda inom miniatyriserad vätskekromatografi-MS. Elektropraykopplingarna är antingen integrerade i vätskekromatografisystem eller som fungerande separata delar. Jämförelserna utfördes med gradientseparation och utvärderades mha en blandning av neuropeptider. Resultaten antyder att effektiviteten av ett vätskekromatografisystem är oberoende av elektropraykopplingarna, åtminstone då systemet är uppbyggt av kapillärer med den testade innerdiameter. Dödvolymer i elektropraykopplingarna var således mycket liten.

Silikakapillärer, belagda med guld ovanpå ett lager av ångutfällt titan, har tillverkats för att undersöka möjligheten att uppnå en ledande beläggning med lång livstid. Elektroprayspetsens långa livstid talar för användning av denna typ av spetsar vid kopplingar mellan vätskekromatografi i miniatyrformat och MS. Detta har exemplifierats genom separationer av neuropeptider med vätskekromatografi-MS.

Den pålagda spänningen över elektroprayspetsen har negativ påverkan på vätskekromatografiska separationer där en kapillärkolonn med poröst grafitiserat kol som stationärfas används. Denna störning beror på att det elektiska fältet har en långvarig inverkan på det ledande packnings-

materialets ytegenskaper. Denna inverkan kunde undvikas genom att en jordpunkt mellan kolonnen och elektrospayspetsen infördes.

Mikrodialys är en mycket viktig provtagningsmetod som används för att filtera ut analyter ur tex levande vävnad. Metoden bygger på att ett jämviktsförhållande uppnås mellan den extracellulär vätskan i vävnader och provtagningsvätskan i mikrodialysproben. När man vill studera neuropeptider och andra större endogena substanser har man tidigare haft problem med utbytet, varför införandet av en pseudostationärfas i provtagningsvätskan har utarbetats. Denna anrikade mikrodialysmetoden har jämförts med konventionell teknik vid analys av neuropeptider. Skillnaden mellan dessa metoder har kvantitativt utvärderats med kapillär-vätskekromatografi-MS. Mikrodialysmetoden med pseudostationärfas gav generellt högre utbyte.

Till sist har en standardadditionsmetod utvecklats för att bestämma totalhalten av tryptofan och två av dess huvudmetaboliter i human plasma mha kapillär vätskekromatografi-MS. Analyterna är polära varför responsen påverkas i större utsträckning av matriseffekter än vad fallet skulle vara för mindre polära substanser i elektrospayprocessen. Detta beror på de polära analyternas låga ytaktivitet vilket i sig orsakar diskriminering under joniseringsprocessen. En standardadditionsmetod har använts för kvantitativ analys för att kunna ta hänsyn till dessa matriseffekter. Kvantitativ analys av tryptofan och dess metaboliter är ett viktigt redskap i studier av t.ex. neurologiska sjukdomar. Ämnesomsättning av tryptofan sker i stor utsträckning via kynureninmetaboliseringsvägen till bl.a. kynurenin och kynureninsyra. Den utvecklade LC-MS metoden har applicerats vid en klinisk studie där multipel skleros patienter har genomgått behandling med cytokiner (Interferon Beta 1a och 1b). Resultaten visar att tryptofanmetabolismen påverkas vid denna behandling, troligen via interaktion med enzymet indolamin-2,3-dioxygenas (IDO).

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