Microscale Tools for Sample Preparation, Separation and Detection of Neuropeptides

ANDREAS DAHLIN
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Abstract

The analysis of low abundant biological molecules is often challenging due to their chemical properties, low concentration and limited sample volumes. Neuropeptides are one group of molecules that fits these criteria. Neuropeptides also play an important role in biological functions, which makes them extra interesting to analyze. A classic chemical analysis involves sampling, sample preparation, separation and detection. In this thesis, an enhanced solid supported microdialysis method was developed and used as a combined sampling- and preparation technique. In general, significantly increased extraction efficiency was obtained for all studied peptides. To be able to control the small sample volumes and to minimize the loss of neuropeptides because of unwanted adsorption onto surfaces, the subsequent analysis steps were miniaturized to a micro total analysis system (μ-TAS), which allowed sample pre-treatment, injection, separation, manipulation and detection.

In order to incorporate these analysis functions to a microchip, a novel microfabrication protocol was developed. This method facilitated three-dimensional structures to be fabricated without the need of clean room facilities.

The sample pre-treatment step was carried out by solid phase extraction from beads packed in the microchips. Femtomole levels of neuropeptides were detected from samples possessing the same properties as microdialysates. The developed injection system made it possible to conduct injections from a liquid chromatographic separation into a capillary electrophoresis channel, which facilitated for advanced multidimensional separations. An electrochemical sample manipulation system was also developed. In the last part, different electrospray emitter tip designs made directly from the edge of the microchip substrate were developed and evaluated. The emitters were proven to be comparable with conventional, capillary based emitters in stability, durability and dynamic flow range. Although additional developments remain, the analysis steps described in this thesis open a door to an integrated, on-line μ-TAS for neuropeptides analysis in complex biological samples.

Keywords: Neuropeptides, Microchip, Enhanced microdialysis, Poly(dimethylsiloxane) (PDMS), Electrospay ionization (ESI), Multidimensional separation, Electrochemical manipulation, Mass spectrometry (MS), Capillary electrophoresis (CE), Microdevice, Microfabrication, Micro total analysis system (μ-TAS)

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II A poly(dimethylsiloxane) based microchip for two-dimensional solid phase extraction-capillary electrophoresis with an integrated electrospray emitter tip Andreas P. Dahlin, Sara K. Bergström, Per E. Andrén, Karin E. Markides, Jonas Bergquist, *Submitted to Analytical Chemistry*.


VII Sample pre-treatment on a microchip with an integrated electrospray emitter Peter Lindberg, Andreas P. Dahlin, Sara K. Bergström, Sara Thorslund, Per E. Andrén, Fredrik Nikolajeff, Jonas Bergquist, Manuscript.

Permission to reprint the articles was kindly granted by the publishers.

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Paper I: Planned and performed all experiments except for the LC-MS part. Wrote the paper.
Paper II: Planned, performed the experiments with Sara Bergström. Wrote the paper.
Paper III: Planned, performed the experiments. Sara Bergström wrote the paper.
Paper IV: Planned the work, designed the microchip and wrote the microchip part.
Paper V: Conducted the MS experiments and wrote half of the paper.
Paper VI: Planned, performed all experiments except the electrochemical studies, wrote major parts.
Paper VII: Planned the project, conducted the MS experiments and wrote parts of the paper.

Paper not included in the thesis:

- Enhanced microdialysis of neuropeptides
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<tr>
<td>µ-TAS</td>
<td>Micro-total analysis system</td>
</tr>
<tr>
<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary electrophoresis</td>
</tr>
<tr>
<td>CRM</td>
<td>Charge residue model</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>dc</td>
<td>Direct current</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>FTICR</td>
<td>Fourier transform ion cyclotron resonance</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>i.d.</td>
<td>Inner diameter</td>
</tr>
<tr>
<td>IEM</td>
<td>Ion evaporation model</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>LHRH</td>
<td>Luteinizing Hormone-Releasing Hormone</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass-to-charge</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>PC</td>
<td>Polycarbonate</td>
</tr>
<tr>
<td>PDMS</td>
<td>Poly(dimethylsiloxane)</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly(methylmetacrylate)</td>
</tr>
<tr>
<td>RF</td>
<td>Radio frequency</td>
</tr>
<tr>
<td>RPLC</td>
<td>Reversed phase liquid chromatography</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>TOF</td>
<td>Time-of-flight</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>VIS</td>
<td>Visual/Visible</td>
</tr>
</tbody>
</table>
1. Introduction

The constant demand to better understand and to be able to control functions and processes in the nature is the driving force in research. For an analytical chemist, this means to develop methods and instrumentation that are able to measure molecules in different samples with different concentrations in a fast and accurate way. Today, the trends are heading towards complex biological samples in the sub-µL-volume ranges where the molecules of interest are found in nanomolar concentrations or lower. One group of molecules that has gained attention during the last few years is neuropeptides. Neuropeptides have been found to be responsible for a wide variety of functions in the body. Despite focused research, the area is still much unexplored and new neuropeptides are discovered on a regular basis. One reason for this is the analytical challenge neuropeptides provide. They are present in low concentration in complex biological samples; possessing wide inherent chemical diversity with respect to molecular size, charge and hydrophobicity. In order to be able to measure neuropeptides, sophisticated sampling, separation and detection techniques are required.

Among sampling techniques for neuropeptides, microdialysis is one of the most elegant and versatile techniques available. Microdialysis can be conducted in vivo in living animals with temporal resolution. Microdialysis also provides protein free samples\(^1\). However, microdialysis of neuropeptides is difficult to carry out due to the size and concentrations of the neuropeptides. A relative recovery of 25% is considered to be a successful yield\(^2\). However, this figure is more often less than 5%. With in vivo concentrations of neuropeptides in the femtomolar range\(^3\) and relative recoveries of less than 5%, pre-concentration steps and detectors with low detection limits must be used.

Classical pre-concentration methods include different extraction techniques, for example solid phase extraction (SPE) and separation techniques such as capillary electrophoresis (CE) and liquid chromatography (LC). CE and LC also provide separation between the neuropeptides, which is important for the subsequent detection. Several different detection techniques have been implemented\(^4\). However, electrospray ionization (ESI) coupled to mass spectrometry (MS) is becoming a more important detection technique due to its high selectivity, accuracy, speed, low detection limits and its ability to perform structural determination.
In order to reduce unwanted adsorption, one must control the surface chemistry and minimize the areas the sample comes in contact with. One way to do this is to strive towards an on-line miniaturized analytical platform, where sample pre-treatment, separation and detection can be conducted on one single device, i.e. a micro-total analysis system (µ-TAS) or more commonly referred to as a microchip.

Since the concept of µ-TAS was coined in 1990⁴ it has gained much attention and a whole new research field has evolved. Miniaturizing analysis methods to a microdevice have some apparent advantages, including reduced memory effects, reduced solvent demand, increased separation speed and efficiency⁴, possibilities to mass fabricate inexpensive and disposable devices⁴. However, intense research and development must be carried out in order to benefit from all these features.

This thesis deals with the possibilities to improve the microdialysis sampling of neuropeptides, and furthermore, with the possibilities to minimize the loss of sample by implement sample pre-treatment, advanced sample separation and electrospray ionization on a microchip platform. Different chip designs and materials are discussed as well as some future aspects.
2. Neuropeptides

Neuropeptides is the general name for a group of neuroactive peptides that are found in almost all tissues in the body but most predominately in the central nervous system and the gut. They are stored in the axon terminal of the neurons and are released upon an action potential from their respective cell body. Depending on the synapse and tissue, the neuropeptides may function as neurohormones, neuromodulators or neurotransmitters. Neuropeptides are built up by 3-100 amino acid residues resulting in much larger molecules compared to classical neurotransmitters, such as amines and amino acids. Consequently, neuropeptides contain more chemical information, which results in more recognition sites for the receptors and therefore higher binding affinities for neuropeptides compared to the classical neurotransmitters. This also allows neuropeptides to be present in lower concentrations in order to achieve neurotransmission.

As presented in Figure 1, neuropeptides are predominantly produced ribosomally in the cell body. The peptides are packed into large dense core vesicles and transported into axons and dendrites. The large dense core vesicles, which can contain classic transmitters, also contain processing enzymes, convertases, which release the bioactive peptides from the precursor. The release of peptides can occur from the cell soma, the dendrites and from the nerve endings. Neuropeptides are, in general, released outside the synapse under burst or high frequency firing compared to classic transmitters that are released into the synaptic cleft under low frequency. The classic transmitters receptors are found in the synaptic cleft whereas the peptide receptors, which are of G-protein-coupled type, are present in the cell soma, dendrites, axons and the nerve endings. The classic neurotransmitters also have a reuptake mechanism at both the cell and the vesicle membrane leading to transmission termination and recycling. Neuropeptides, in its turn, are broken down by extracellular peptidase and have to be replaced via axonal transport. Peptides are furthermore for slow and long-lasting response in contrast to classic neurotransmitters that have rapid and short lasting response times. This can be explained by the lower diffusion rate that the neuropeptides possess due to their size and bulkiness.
Neuropeptides often have multiple functions, for example substance P, which is involved in such functions as pain mediation, immune system activation and neurogenic inflammation. Decreased levels of substance P are found in Parkinson disease patients. The enkephalins that have structural similarities to morphine are one of three opiate peptides that play an important role in pain regulation. Increased levels of enkephalins may be observed at neurogenic pain. Somatostatin is a multifunctional inhibitor that inhibits the release and metabolism of many hormones, neuropeptides and neurotransmitters by opening the K+ channels to hyperpolarize the cell membranes. Somatostatin is, furthermore, involved in the maintenance of the adult and aging brain. Increased level of somatostatin in the blood plasma is a sign of endocrine tumors while a decreased level of somatostatin in the cerebrospinal fluid (CSF) is observed in patients with depression or dementia disorders. For more detailed information about neuropeptides and their biological role a number of reviews and books are recommended.
3. Microdialysis

The history of microdialysis goes back to 1966 when Bito et al.\textsuperscript{9} implanted semi permeable membrane sacs in the neck of dogs. They were able to measure free amino acids and other electrolytes in the extra cellular fluid of the brain and blood plasma. However, the sacs sampled the surrounding area by a static equilibrium and were not continuously perfused. In 1972, Delgado et al.\textsuperscript{10} performed the first \textit{in vivo} microdialysis experiment with a primitive microdialysis probe, which they called “dialytrode”. The probe permitted infusion and collection of samples from wake rhesus monkeys. The dialytrode itself was constructed by gluing two tubings together with one tip ending prior the other in a polysulfone bag, which acted as the membrane. The dialytrode was further refined to a microdialysis probe in 1974 by Ungerstedt and Pycock\textsuperscript{11}. Since then, microdialysis has been widely applied in neuroscience, pharmacokinetics and biotechnology. Today, microdialysis is an established technique that is conducted on routine basis in many laboratories all over the world.

3.1 Principles of microdialysis

The theory of microdialysis is based on the diffusion of molecules over a concentration gradient. This gradient is accomplished by implanting a microdialysis probe into the sampling area of interest, in which the probe then mimics the function of a capillary blood vessel. Usually, \textit{in vivo} microdialysis is carried out in the extra cellular fluid (ECF) space in the tissues\textsuperscript{1,12}. The basic microdialysis instrumental setup, which is presented in Figure 2, includes a syringe pump, a microdialysis probe and a collection and analysis part. Through the microdialysis probe, the syringe pump delivers a continuous flow of perfusate, i.e. a liquid that possesses the same properties in pH and ionic composition as the sampling area. Due to diffusion in both directions the system must equilibrate before experiments can be initiated. However, the membrane, which is permeable to water and small molecules, prevents molecules larger than its pore size from passing through the membrane. The membrane pore size is usually translated to a molecular cut-off, which typically is chosen from the 5-30 kDa range. The molecular cut-off effectively prevents large proteins and other macromolecules from entering the perfusate, resulting in clean and protein free dialysates, which can di-
directly be analyzed using CE or LC. It needs to be noted that this does not apply to ESI-MS detection. These properties, together with the ability to collect samples with temporal resolution and with minimal perturbation to the system of study, makes the technique very unique and versatile for many applications\textsuperscript{1,12}.

\textbf{Figure 2}. Schematic picture of the instrumental set-up for microdialysis experiments. The enlargement shows the basic principle of microdialysis where large molecules are excluded from entering the perfusate.

\subsection*{3.2 Estimation of the extraction efficiency}

Because the microdialysis probe is infused with a continuous flow of perfusate, a non-equilibrium state between the dialyzed sample and the surrounding sampling area will always be present. A difference in concentration of the analytes in the microdialysate compared to the sampling area will be
the result. This difference in concentration is called extraction efficiency or relative recovery and it is one of two common measurements of the efficiency of the microdialysis probe. The other measurement is the absolute recovery, which refers to the total amount of removed analytes from the perfusion medium instead.

Determining the extraction efficiency is considered to be a key issue of microdialysis, especially when in vivo studies are conducted. The reason for this is that the extraction efficiency is used to determine the concentration of analytes in the sampling matrix by Equation 1:

\[
E_d = \frac{C_{\text{Out}} - C_{\text{In}}}{C_{\text{In}}}
\]

where \(E_d\) is the extraction efficiency, \(C_{\text{Out}}\) is the concentration of the analytes in the dialysate, \(C_{\text{In}}\) is the analyte concentration in the sampling matrix, and \(C_{\text{In}}\) denotes the concentration of infused analyte. This parameter equals to zero if no analyte has been added to the perfusion fluid. As shown in Table 1, the extraction efficiency is dependent on many factors.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Typically experimental settings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>• 20 °C – 37 °C, 37 °C is recommended</td>
</tr>
<tr>
<td>Perfusion flow rate</td>
<td>• 0.5 µL/min – 5.0 µL/min</td>
</tr>
<tr>
<td>Diffusion coefficient</td>
<td>• Length: 0.1 – 2.0 mm</td>
</tr>
<tr>
<td>Membrane area</td>
<td>• Radius 200 – 500 µm</td>
</tr>
<tr>
<td>Membrane material</td>
<td>• Polyamide</td>
</tr>
<tr>
<td></td>
<td>• Polycarbonate</td>
</tr>
<tr>
<td></td>
<td>• Cuprophane</td>
</tr>
<tr>
<td></td>
<td>• Polyethersulphone</td>
</tr>
<tr>
<td>Membrane cut-off</td>
<td>• 5 kDa – 30 kDa</td>
</tr>
<tr>
<td>Sample composition / matrix</td>
<td>• Analytes e.g. peptides, aminoacids, monoamines</td>
</tr>
<tr>
<td></td>
<td>• Tissue, e.g. brain, liver, muscle</td>
</tr>
<tr>
<td>Perfusion medium composition</td>
<td>• Salt containing buffer e.g. Ringer’s solution or artificial CSF.</td>
</tr>
<tr>
<td></td>
<td>• pH 7.4</td>
</tr>
</tbody>
</table>

All the listed parameters are more or less based on the time the perfusate is allowed to equilibrate with the sampling area. A decrease in flow rate will increase the equilibrium time and hence increase the extraction efficiency. However, a low flow rate will affect the temporal resolution due to the inherent diffusion process but also since more time must be spent to collect sufficiently large sample volumes for further analysis. An increased mem-
brane surface area will also increase the extraction efficiency; this parameter is limited to the sampling matrix area and is therefore dependent on the application. The properties of the sample matrix affect the extraction efficiency because, before the analytes can diffuse through the membrane they must diffuse through the sampling matrix. High matrix tortuosity decreases the diffusion coefficient of the molecules. An increase in temperature will increase the diffusion coefficient with 1-2% per °C. Furthermore, the diffusion coefficient is also dependent on the molecular weight of the analyte in an inversely proportional relationship, which means that a large molecule will exhibit larger resistance than a small molecule, i.e. take longer time to be transported into the perfusate. The membrane molecular cut-off value (5-30 kDa) is determined during equilibrium conditions. Determining the cut-off value in this way means that the true values, during non-equilibrium conditions, are closer to 1-7 kDa, which in its turn results in difficulties to obtain high extraction efficiencies for molecules larger than 1 kDa1,13.

3.3 Enhanced microdialysis

To increase the extraction efficiency is especially important when the target analytes are neuropeptides. Neuropeptides are relatively large and bulky molecules with molecular masses ranging from 500 up to >5000 Da. They possess different hydrophobic and charged properties and are in vivo, present in low femtomolar to micromolar concentrations3. As discussed above, there are several ways to increase the extraction efficiency. One way is to increase the flux of analytes by increasing the concentration gradient across the membrane. This can be achieved through an increase in flow rate. However, increased flow rate produces lower extraction efficiency. Another way is to introduce mobile carriers with high affinity for the analyte of interest in the perfusion fluid16. Facilitated microdialysis or enhanced microdialysis have been applied with different carriers including proteins17-20, cyclodextrins21-23, lipids18,24,25, microbeads26 and particles27. In all cases an increase in extraction efficiency was reported. Facilitated transport using carriers combines diffusion with chemical affinity. Moreover, the carriers prevent unwanted adsorption of the analytes to the tubings in the microdialysis system. This peculiarity is in particular important for neuropeptides, which often are sticky by nature. In Paper I, a method able to transport a perfusate containing SPE particles through a microdialysis probe, in order to achieve higher extraction efficiencies was developed. After the microdialysis sampling, which was conducted in vitro, the peptides were eluted from the particles and quantified using LC-MS. The use of particles in the perfusion fluid compelled certain experimental modifications, so that a reproducible perfusate flow could be obtained. A linear microdialysis probe was used to avoid clogging of the particles and air bubbles were introduced with con-
trolled intervals to the perfusion flow in order to move the particles. The reason for these modifications depended on the used SPE particles, which were irregularly shaped with an average diameter of 100 µm. Notwithstanding initial difficulties, enhanced extraction efficiency were obtained.

Figure 3. Comparison of in vitro conducted microdialysis experiments of neuropeptides. The black bars represent enhanced microdialysis and the gray bars represent ordinary microdialysis. The error bars represent the relative standard error of the mean based on 6 and 4 measurements of solid supported microdialysis and normal microdialysis respectively.

As illustrated in Figure 3, the enhanced solid supported microdialysis mode gave considerably higher extraction efficiencies for most of the peptides. A 10 times higher extraction efficiency was obtained from the largest and least hydrophobic neuropeptide, luteinizing hormone releasing hormone (LHRH). The smallest peptide in the study, leucine-enkephalin did not give any significant change in extraction efficiency between the two microdialysis modes. The increased extraction efficiency is a combination of increased flux of analytes through the membrane and a decreased adsorption of analytes onto the tubings. It is, however, difficult to distinguish which effect actually contributed the most. It is clear, however, enhanced microdialysis increases the extraction efficiency for neuropeptides.
4. Separation Techniques

4.1 Reversed phase liquid chromatography

Reversed phase liquid chromatography (RPLC) is one of the most established separation techniques. The separation mechanism is based on the partitioning of the analytes between the stationary phase and the mobile phase, where the mobile phase is more polar compared to the stationary phase. The instrumentation used consists of a pump, injector, column and detector. Traditionally the LC column inner diameter (i.d.) has been 4.6 mm packed with 3 – 5 µm packing material facilitating flow rates in the mL/min range and µL injection volumes\textsuperscript{28}. However, demands of separation systems able to handle small sample volumes have led to the development of miniaturized LC systems with columns having an i.d. of 150 – 500 µm, providing flow rates in the 0.5 – 10 µL/min range and injection volumes of high nL\textsuperscript{28}. LC separations carried out in such columns are referred to as capillary-LC and were used in Papers I and III.

4.2 Capillary electrophoresis

Capillary electrophoresis is a separation technique that separates the molecules depending on their electrophoretic mobility, i.e. their charge and size. As depicted in Figure 4, the instrumentation of CE consists of two vials, a high voltage supply fitted with two electrodes, a detector, a data collection and analysis system and finally a fused silica capillary.

Basically, the fused silica capillary with an i.d. of < 100 µm is filled with separation buffer and put into two vials containing the same buffer. A high potential 5-60 kV is applied between the two vials, which cause an electric field inside the capillary. An electrical double layer will be formed on the capillary wall when ions from the buffer solution congregate at the opposite charged capillary surface. This double layer is the main foundation in the CE methodology, since it is responsible for the electroosmotic flow (EOF).
Figure 4. A schematic picture of the instrumental set-up for capillary electrophoresis. The enlargement shows the direction of the electrophoretic mobilities of the ions and the electroosmotic flow.

The EOF is constantly flowing towards one end of the capillary, in this case the cathodic end. The separation, however, is carried out according to the electrophoretic mobility of the analytes. Small and highly charged molecules will possess highest mobility and large molecules will possess lowest mobility. In the case described in Figure 4, negatively charged molecules will migrate towards the anode, while positively charged molecules will migrate towards the cathode and neutral molecules will be unaffected. However, the EOF, which in most cases is higher than the electrophoretic mobility of the molecules, will produce a net-flow towards the detector. The injections of sample into the capillary are either performed electrokinetically or hydrodynamically, where hydrodynamic methods include siphoning or the varying of an applied pressure. The injection volume is often in the low nanoliter or lower range\textsuperscript{39,30}.

A CE separation is fast with a high resolution and a very high efficiency. Several hundreds of thousands of theoretical plates per meter are routinely achieved. The high efficiency is due to the plug like flow profile, which is facilitated by the electrical double layer. CE is also a separation technique that has interest, due to its qualities in the field of miniaturization and in microchip applications. This will be discussed in more detail in Chapter 6.2.2.
4.3 Multidimensional separations

The term of true multidimensional separations originates from O’Farrell’s work on two-dimensional (2D) polyacrylamide electrophoresis (2D PAGE) in 1974\textsuperscript{31}. This is a well-known 2D separation technique, where the first dimension separates by isoelectric focusing and the second dimension separates by size. Giddings\textsuperscript{32} stated two criteria for the definition of true multidimensional separation. First of all, two or more separation mechanisms should be orthogonal of each other, i.e. possess different separation mechanisms. For example, the RPLC, which separates based on hydrophobicity, and CE, which separates according to the electrophoretic mobility, i.e. size and charge. Secondly, whenever two components are resolved in any of the separation steps, they should remain resolved during the whole process. These statements verify that the individual fractions obtained in the first separation have to be continuously fed into the second dimension, which is based on a completely different separation mechanism. In order to achieve true multidimensional separation, the sampling period of the second dimensions should be considerably shorter than the width of the peaks emerging from the first dimensional separation. As a consequence, the second dimension separation must be significantly faster than the first dimension since the time available between the injections is limited. Furthermore, one has to have knowledge about the elution profile in the first dimension to be able to sample representatively into the second dimension\textsuperscript{33}. Murphy et al.\textsuperscript{34} suggested that each peak in the first dimension should be sampled at least three times by the second dimension in order to achieve optimal resolution.

The major advantage in using a multidimensional separation system is that the peak capacity is largely increased. In an ideal orthogonal multidimensional separation, the peak capacity is approximately the product of the peak capacities of both dimensions\textsuperscript{32}. This means for instance that if two separation techniques with peak capacity of 100 each were combined, the 2D separation system would have a total peak capacity of 10,000. The true peak capacity is expected to be lower due to peak broadening in the second dimension and due to the natural difficulties experienced when coupling two divergent techniques\textsuperscript{35}.

Due to the large number of different kinds of multidimensional separation techniques, one may first divide them into 2D separations that separate in space, such as 2D gels, which separate analytes over a 2D planar surface, and 2D separations that separate over time, for example LC-CE\textsuperscript{35}. The 2D separation techniques that separate over time may also be divided in an off-line and an on-line mode. In the off-line mode, fractions are collected from the first dimension and manually injected in the second dimension, whereas in the on-line mode, samples are constantly injected into the second dimension from the first\textsuperscript{35,36}. The following Chapter focuses on on-line 2D LC-CE systems.
4.3.1 On-line LC-CE

As mentioned earlier, LC and CE are orthogonal techniques, which are suited for on-line coupling. Furthermore, the fast separation process of CE makes it ideal as a separation technique for the second dimension. Due to the dissimilarities between the techniques, some obvious obstacles when coupling LC to CE arise. When analyzing proteins and peptides it is preferable to use as low ion strength as possible in the LC separation in order to achieve stacking effects in the CE. However, a slow elution of LC and fast CE enables comprehensiveness without the need to adjust the ion strength. The elution volume in conventional LC is by order of magnitudes larger than the injection volume in the CE. This difference can be solved by incorporating a split in the LC effluent, however a loss in sensitivity and an increased band broadening will be the result. Another approach is to reduce the LC flow by using micro columns; in such cases on-line sampling solutions are essential. Since the CE separation should be as fast as possible, the injection time from the LC effluent into the CE dimension must be short and frequent.

The first comprehensive on-line LC-CE system was developed in 1990 by Bushey and Jorgenson. Several interface designs have thereafter been developed for coupling pump driven flows to CE, including transverse flow gating interfaces, optically gated interface, sample loop interfaces, two level cross interfaces. Among these designs, the transverse flow gate system has been most utilized. The injection system is based on a cross connection with the CE flow inlet placed opposite to the LC flow outlet. In the transverse channel a flow of CE electrolyte is flowing to prevent the LC effluent from entering the CE channel. Injections are carried out by shutting off the CE flow. A modified transverse flow gate system made in insulating elastomer poly(dimethylsiloxane) (PDMS) has been developed and further improved by Samskog and Bergström. The cross connection was made in two levels with a small contact area between the LC and CE channels. A slight overpressure was applied in the cross to provide fresh CE electrolyte to the CE separation and to assure independency of the LC and CE flows. This injection cross was further developed and will be described in more detail in Chapter 5.3.1.
5. Mass Spectrometry

A mass spectrometer is a powerful instrument that measures ions in gas phase according to their mass-to-charge (m/z) ratio. Figure 5 illustrates a typical mass spectrometer instrumentation including sample inlet, ion source, vacuum pumps, ion optics, mass analyzer, detector and a data handling system.

![Schematic picture of a typical MS instrumentation](image)

**Figure 5.** Schematic picture of a typical MS instrumentation, with ion source, ion optics, mass analyzer, detector and a data handling system

There are many variations of ion sources and mass analyzers. In this thesis electrospray ionization (ESI) has been used as ion source and time-of-flight (TOF), quadrupole, and Fourier transform ion cyclotron resonance (FTICR) mass spectrometers have been utilized as mass analyzers.

5.1 Electrospray ionization

After the pioneering work by Dole\textsuperscript{49,50} and Fenn\textsuperscript{51}, ESI has become one of the most important ion sources for large biomolecules and polymers. ESI is a gentle ionization technique that transfers ions in a liquid to gas phase, which
5.1.1 The mechanism of electrospray

The mechanism of electrospray has been described in several reviews\textsuperscript{53-58}. Briefly, it can be divided into three parts, the nebulization of a sample into electrically charged droplets, the evaporation of droplets resulting in liberation of ions and finally, the transportation of ions from atmospheric pressure into the vacuum of the mass spectrometer. The ESI process, depicted in Figure 6, is carried out by feeding a sample solution through a capillary tube. At the tip of the capillary, a high electrical potential, usually a few kV, is applied.

![Schematic picture of the electrospray process in positive ion mode.](image)

At a small distance from the tip end a counter plate with a lower potential is placed. Assuming positive ion mode, positive charges are pulled towards the liquid front and when the electrostatic force and surface tension balance perfectly, the liquid front protrudes into a cone known as the Taylor cone. At the apex of the Taylor cone the experienced electric field is at its maximum. With an additional increase in the electric field, droplets with net positive charges are formed since the surface tension in the sample solution become too low compared to the repulsion forces in the liquid. The positive charged
droplets are ejected towards the counter plate, where they shrink due to solvent evaporation and fission processes. Eventually only droplets containing single ions are present. Finally the solvent in the droplets will evaporate resulting in a gas phase ion from the sampled compound. This theory is also called charge residue model (CRM). In another theory, known as ion evaporation model (IEM) the phase where the ion evaporates into gas occurs directly from small highly charged droplets. Both theories may be valid simultaneously, but for gas phase macro-ions, the CRM theory is most likely to be the dominating process whereas the IEM theory seems to dominate for small surface-active ions.

5.2 Mass analyzers

The main task for a mass analyzer is to separate ions according to their $m/z$ ratio. There are a number of different mass analyzers able to accomplish this, including TOF, quadrupoles, ion traps, FTICR and sector instruments. The characteristics of a mass analyzer are usually described based on their resolving power, mass accuracy, mass range, linear dynamic range, speed, sensitivity, precision and compatibility with ionizer. No single mass analyzer is superior to another in all perspectives; instead, the various mass analyzers may be used as complements to each other. In this thesis, TOF, triple quadrupole and FTICR have been used.

5.2.1 Time-of-flight

TOF is a mass analyzer that accelerates ions in an electric field and measures the time it takes for the individual ions to travel a certain distance. A highly charged small ion will travel faster than a large ion. The TOF mass analyzer is by nature optimal for pulsed ionization techniques such as matrix assisted laser desorption/ionization. In order to couple a continuous ionization technique such as ESI, orthogonal acceleration TOF (oaTOF) is used. In oaTOF, the ions in the continuous ion beam are pulsed, in a right angle towards the detector. The pulse rate is determined by the time it takes for the heaviest molecule, after having been sampled, to reach the detector. The sampling frequency is in the kHz range, which results in acquisition rates of several spectra per seconds. Hence, oaTOFMS is the mass analyzer of choice for fast separation techniques such as CE. Additional advantages with oaTOF are the high mass range, the good mass accuracy and the good mass resolution. An oaTOFMS was used in Papers II, V-VII.
5.2.2 Quadrupole

The operation principle of the linear quadrupole mass filter is well known and has been and the most widely used mass analyzer for the last 30 years. A quadrupole mass filter consists of four rods or poles aligned parallel to each other. The mass selection is dependent on the ion motion arising from electric fields based on a direct current (dc) voltage and a radio frequency (RF). The ratio between RF and dc is used to control which masses are able to reach the detector. Ions with the selected \( m/z \) ratio will pass through the quadrupole and be collected at the detector while the unselected ions hit the rods. Single quadrupole instruments are still widely used but the main task for the quadrupole has been to act as a first stage in tandem mass spectrometers. Triple quadrupoles, quadrupole-TOF and quadrupole-ion traps are examples of this. In triple quadrupole, introduced by Yost and Enke in 1978, the first quadrupole acts as a mass filter for the precursor ion mass selection, the second is an RF-only quadrupole collision cell and the third quadrupole acts as mass filter for the product ion mass. Such an instrument was used in Papers I and IV. In Paper IV the MS/MS ability was utilized.

5.2.3 Fourier transform ion cyclotron resonance

In a FTICR, ions are trapped in an analyzer cell that is placed inside a magnet. The analyzer cell is oriented in a magnetic field, which is several Tesla strong. In this field two opposing pairs of plates are orthogonal to the direction of the magnetic field. FTICR separates ions according to their inherent cyclotron frequency, which they get when exposed to the magnetic field. The cyclotron frequency is inversely proportional to the mass to charge ratio of the ion and direct proportional to the magnetic field. Ions with lower \( m/z \) ratio will acquire higher cyclotron frequency compared to ions with higher \( m/z \) values. However, this motion does not provide a detectable signal. Therefore, packets of ions with given \( m/z \) need to be excited. This is accomplished by applying an oscillating RF electric field. When the RF frequency matches the cyclotron frequency of the ion packet, the ion packet will adsorb the energy and accelerate into a larger orbit radius without changing the ion cyclotron frequency. When the applied RF frequency is terminated the ion packets will continue to gyrate at this radius. Electrodes placed in the analyzer cell will conduct measurements and return a small alternating current. The amplitude of this current is proportional to the amounts of ions in the ion packet and the frequency is the cyclotron frequency i.e. \( m/z \) ratio. The alternating current is further Fourier transformed before a mass spectrum is displayed. The apparent advantage is that the packets of ions may be sampled multiple times since the detection system is not destructive. However, this requires a tremendously effective vacuum system. Moreover, a sophisticated data system is needed since FTICR provides large amounts of data due
to its unbeatable mass resolution and mass accuracy\textsuperscript{69-71} A 9.4 T; FTICR was used as mass analyzer in \textbf{Paper III}.

5.3 Interfacing separation techniques with ESI-MS

When interfacing separation techniques to MS via ESI, several important issues must be considered in order to achieve a compatible connection. The main issues are the flow rate and the solvent properties. It is also important to minimize dead volumes between the separation steps and the ESI in order to maintain the separation performance\textsuperscript{72,73}. The operational flow rates of CE and ESI are both in the \(\mu\text{L}/\text{min-nL}/\text{min-window}\) and do not pose a problem. Different solutions have been presented to solve the flow rate incompatibilities that previously existed between LC and ESI. A splitting tee has been employed before the ESI\textsuperscript{74}. Bruins et al.\textsuperscript{75} developed a pneumatically assisted interface i.e. ionspray, able to handle aqueous solutions and higher flow rates compared to pure electrospray. Hopfgartner et al.\textsuperscript{76} further extended the application of ionspray to handle flow rates up to 2 \(\text{mL}/\text{min}\) by adding a liquid shield between the ESI needle and the counter plate of the MS. Briefly, the pneumatic nebulizer is responsible for the aerosol formation and the electric field charges the droplet. The result of this is that ionspray can be operated at lower field strength and without the risk of electrical discharges ruin the ESI process. Furthermore, the degree of freedom in the positioning of the ESI emitter inside the ion source is larger compared to pure electrospray, resulting in an improved robustness. An ionspray interface was used in \textbf{Paper I} due to the operational flow rate from the high performance liquid chromatography (HPLC) column was 12 \(\mu\text{L}/\text{min}\). The most important issue of coupling LC to ESI is the development of robust and reliable miniaturized LC-systems operating in the \(\mu\text{L-nL}/\text{min}\) flow ranges.

The solvent composition in ESI resembles of the composition used in RPLC and CE. The solvent is water based and has an organic modifier, which decreases surface tension and enhances the spraying efficiency. Furthermore, a volatile electrolyte has to be added to both adjust the pH and to introduce electrolytes maintaining the spray and making the spray independent on the analyte concentration. In positive ESI mode, formate or acetate ions are often used in low mM concentrations\textsuperscript{58}.

A number of interface designs have been developed for LC-ESI and for CE-ESI. In general, two major approaches have been employed, which are sheathflow and sheathless interfaces. See Figure 7a and 7b.
Figure 7. a) Sheathflow and b) sheathless interfaces, two interfaces commonly used to interface liquid separation techniques with ESI-MS.

The sheathflow interface utilizes a sheath liquid that surrounds the separation capillary tip in order to stabilize the electrospray. The ESI potential is applied onto a stainless steel capillary, which stays in contact with the sheath liquid. Sheathflow interfaces have been one of the most used interface designs in CE applications\textsuperscript{64,72}. In CE-ESI, the addition of sheath liquid plays an important role since it makes the CE separation more compatible to ESI sources, which often operate optimally at flow rates of 1-5 µL/min. Since the sheath liquid dictates the spray performance, the buffer solution of the CE is negligible. However, increased background noise\textsuperscript{77}, shift in migration orders, and sample elution dilution are drawbacks with the sheath liquid design. Sheathflow interfaces were also the first interfaces developed for LC-ESI, however, the miniaturization of the LC system has led to increased usage of sheathless interfaces.

In the sheathless design\textsuperscript{74}, depicted in Figure 7b, the separation capillary is directly coupled to the ESI source. The ESI voltage is usually applied via an electrode inserted at the end of the separation capillary\textsuperscript{79,80} or via a conductive coating\textsuperscript{81-87} applied at the tip of the separation capillary. This design offers many advantages for instance, maximum sensitivity may be reached since no dilution effects exist due to added liquids. Therefore the choice of separation buffer is limited to be ESI compatible. The use of a conductive coating is advantageous since in theory, the separation can be carried out to the very end of the separation capillary resulting in no band broadening effects. The challenge has been to find a durable conducting coating able to withstand the harsh electrochemical environment present at the emitter tip. Numerous materials, including metals\textsuperscript{81,83}, conducting polymer\textsuperscript{84} and graphite\textsuperscript{85,87}, have been tested for this purpose. The materials were applied using
different methods, such as electroplating, gluing, evaporation and sputtering. In Paper III, graphite coated CE capillary\textsuperscript{86}, was used to facilitate sheathless ESI. A modification of this durable coating was further conducted and used in Papers II, IV-VII, and this will be discussed in more detail in Chapter 6.2.3.

5.3.1 On-line LC-CE-ESI-MS

Although extensive developments of LC-CE interfaces, only two of these have been coupled to mass spectrometry\textsuperscript{40,47}. Liquid separations coupled to mass spectrometry may be regarded as a multidimensional separation since the MS detects ions according to their mass to charge ratio\textsuperscript{88}, and hence provides an orthogonal separation dimension. LC-CE-MS would therefore be a true 3D separation and detection technique. As emphasized earlier, the second dimension separation must be much faster than the first one. The sampling rates must be high, at least 3 samplings from each peak in order to not lose resolution and be representative. The same criteria are thus valid for the third dimension. This requires a lot from the mass spectrometer, which has to be able to sample fast, i.e. fast scanning rate.

In Papers II and III, an LC-CE interface was developed and evaluated. The interface was fabricated in PDMS in a two level cross design with a small contact area as shown in Figure 8. In the lower level the flow from the first dimension was introduced while in the upper level a constant pressure driven flow of CE electrolyte was maintained. The CE flow was also pressurized in order to avoid leakage from the first dimension effluent into the second dimension separation. Injections were carried out by removing the applied overpressure.
Figure 8. Schematic picture of the LC-CE interface used in Papers II and III. The enlarged parts show the liquid flow during default mode and injection mode respectively.

The cross design and function were equal in both Papers but the separation dimension varied. In Paper II, which will be described in more detail in Chapter 6.2, a two-dimensional in-line SPE-CE-ESI microchip was developed. In Paper III a comprehensive RPLC-CE system coupled on-line to an ESI-FTICR-MS was developed. A limit of detection was calculated to be approximately 280 nM for studied peptides. The performance of the system was further evaluated by analyzing a tryptic digest of bovine serum albumin (BSA). Injections were carried out every 30 seconds into the CE from the LC flow. In total, 3126 unique peptide masses were detected in the mass spectrometer resulting in a sequence covering of 93 % (5 ppm mass measurement error).
6. Miniaturization

The vast developments in the field of microelectronics, where millions of electrical devices can be placed on one small microchip, have encouraged the miniaturization of microfluidic systems able to carry out advanced analytical analyses. These microfluidic systems, also known as lab-on-a-chip or µ-TAS, should ideally perform a complete, integrated and automated analysis of a target analyte in a complex sample matrix. The components of this microchip may include for instance sampling, sample pre-treatment, chemical manipulations, separations, detection and data analysis, i.e. have the functions of a complete analytical laboratory. The typical dimensions used for various steps range from few micrometers to several millimeters in length, and 0.1 – 100 µm in depth or height. These dimensions result in a microstructure miniaturized onto a few square centimeters.

While considering miniaturization and the µ-TAS concept, one might ask whether it is realistic or even possible to accomplish. The community which supports µ-TAS concept often refers to the fact that miniaturization is advantageous since low cost, disposable and advanced analytical devices may be constructed. These devices are able to analyze low sample volumes in a fast and efficient manner. It is debatable whether or not the µ-TAS concept is the ideal solution, but one thing is clear however. The field of µ-TAS will be developed as long as it is a need for improved analytical performance within miniaturization.

The first reported microchip was presented in the late seventies. On this silicon microchip, a complete gas chromatograph able to separate simple mixture of compounds in a very short time. This pioneering work was ahead of its time and only few developments were made in the field of µ-TAS. In 1990, Manz’s group presented a novel approach, in which an open-tubular liquid chromatograph with a conductometric detector was fabricated on one singular silicon chip. This was an important milestone, which led to the introduction of the µ-TAS concept. After these developments the field of µ-TAS has expanded rapidly and branched into many different areas, for example: clinical and forensic analysis, point-of care testing, molecular diagnostics, medical diagnostics and biological and chemical analysis.
6.1 Microfabrication methods and materials

There are mainly two types of materials, silicon and polymer, used as substrates in microchip devices. These materials have very different properties with the result that completely different fabrication methods must be employed. Silicon-based microchips that include glass, silicon, and quartz are the most commonly used materials in microchip. The reasons for this are their optical properties, well-explored surface chemistry and the extensively developed fabrication methods, adapted from the microelectronics industry. One obvious drawback using silica material is the high cost. Polymers have gained increased interest because of the, potentially lower production expenses compared with silicon material. Polymers consist of large amounts of monomers, often more than 1000 monomer units, which results in large macromolecules with molecular masses between 10 kDa and 100 kDa. The polymerization is initiated by the addition of a substrate or by a change in a physical parameter, for instance, temperature or light. The group of polymers can be divided into three subcategories based on their interconnection between the monomers in the polymer chain, which determines their molding behavior: duroplastics, thermoplastics and elastomeric polymers. In this thesis, thermoplastics and elastomeric polymers have been used as chip substrates and will therefore be discussed in more detail.

6.1.1 Thermoplastics

Thermoplastics belong to a large group of polymers that is more commonly known as plastics. Examples of thermoplastics are polyamide, also known as nylon, polycarbonate (PC), polypropylene, polystyrene, and polymethylmethacrylate (PMMA) also known as Plexiglas. Among these, PC and PMMA are the most commonly used thermoplastics in microfabrication. A common property for all thermoplastics is that they have a glass transition temperature $T_g$, which is the temperature when the hard plastics become fluidic, but not melted. This important characteristic is used in the microfabrication process. The temperature is increased above the $T_g$ and the solid and brittle material become plastic-viscous and can be molded. When the temperature decreases under $T_g$, the material once again solidifies. Two microfabrication approaches have been developed, direct techniques where each single device is fabricated separately, and molding techniques where the polymer material is replicated from a master structure. The commercial focus has been laid on the molding techniques due to the possibilities to mass fabricate large quantities of microstructures. The fabrication procedure, depicted in Figure 9, begins with the fabrication of a master structure. There are several master fabrication techniques optimized for microfluidic structures. It is beyond the scope of this thesis to further describe those techniques, but for more information, reviews are recommended. After the
fabrication of the master mold, the molding replication is conducted. There are mainly two molding techniques, hot embossing and microinjection molding. In both techniques, the polymer substrate is first heated above its glass transition temperature so it becomes liquid. In hot embossing, the structure is stamped into the polymer substrate, whereas in the injection molding technique, a structured mold is filled with the liquid polymer. The temperature is then decreased and two-dimensional microstructures are printed on the polymer wafer surface.

Figure 9. Fabrication procedure for thermoplastic microchips used in Paper V. The microstructure is etched in silicone, replicated in nickel and injection molded. The polymeric structures are then cut and bonded to a coverlid.

In order to complete the microchip, additional steps like for instance sealing the channels, drilling holes, channel surface modifications and metallization are usually needed\textsuperscript{102}. Among all steps, the sealing of the microchannels is the most difficult one. Surprisingly, this topic is also most often neglected in the literature. In Paper V, PMMA and PC microdevices were manufactured by injection molding using a nickel master. The devices were further diced into smaller pieces sealed with an unstructured PMMA or PC lid. A capillary was inserted at one end of the microchannel and an electrospray tip was shaped at the other end by milling, either by hand or by using milling equipment. Finally the tips were metallized in order to facilitate sheathless electrospray. Different emitter tip designs and conducting material were evaluated using visual inspections, electrochemical methods and mass spectrometry. The outlet of the channel was in many cases difficult to see, even when using scanning electron microscope. The reasons for this were that the channel dimensions at the end of the tip were tapered probably due to decreased rigidity during the tip sharpening. The chip substrate also tended to
smear out over the channel opening. However, the tip worked for electrospray even if the reproducibility was not optimal. The largest obstacle in this study was the sealing step, approximately a 20% success rate for both PC and PMMA was achieved.

6.1.2 Elastomeric polymers

The most important elastomeric polymer used in microfabrication is poly(dimethylsiloxane), (PDMS). PDMS is a silicone-based material, which has in qualities, such as being flexible and UV/VIS-transparent. It is also biocompatible and therefore suitable for protein and cell analysis. Furthermore, it is stable in the 40 – 95 ºC temperature range, which is the range where processes of biological material are conducted. PDMS is also cured at a relatively low temperature, approximately at 70 ºC, a feature that may facilitate the surface coating procedure. However, the most important advantage is the microfabrication simplicity PDMS offers. No clean-room facilities or sophisticated equipment is needed to obtain µm accurate microstructures. This makes PDMS very appealing for prototyping purposes, but also optimal for inexpensive mass production. Hence PDMS renders possibilities for many people to work in the area simultaneously, which significantly increase the speed of innovation\textsuperscript{103,106-108}. As stated by Quake and Scherer\textsuperscript{106}, the advances of PDMS microfabrication are analogous to the swift growth in software due to the personal computer development.

There are also some disadvantages with PDMS. Lee et al\textsuperscript{109}, considered three aspects of solvent compatibility; swelling properties, solute partitioning between the solvent and the PDMS material, and the dissolution of PDMS oligomers in a solvent. Among these three parameters, the swelling, due to organic solvent exposure, had the greatest influence. However, solvents and acids commonly used in LC, CE and ESI such as water, acetonitrile, methanol, formic acid and acetic acid did not swell the PDMS and are therefore highly compatible\textsuperscript{109}. Another property of PDMS that sometimes is considered a drawback is its hydrophobic surface, which is due to the methyl groups on the monomer as depicted in Figure 10.

\begin{figure}
\centering
\includegraphics[width=0.2\textwidth]{pdms_structure.png}
\caption{Chemical structure of PDMS}
\end{figure}
Different surface modification strategies have been developed. The purposes have been to hinder molecules like proteins and peptides to adsorb to the PDMS and to make the surface more electrophoretically compatible, i.e. increase the amount of charges on the surface. One way to modify the PDMS surface is to expose it to energy sources such as UV-light\(^{110,111}\), oxygen plasma\(^{112}\) and corona discharges\(^{113}\). One has shown that the hydrophobic surface turns more hydrophilic, probably due to formation of SiO\(_x\) groups at the expense of methyl groups (Si-CH\(_3\))\(^{111-113}\). Energy exposed PDMS-surfaces have larger resistance to adsorption of hydrophobic and negatively charged molecules than untreated PDMS surface. One may also modify the surface by chemical methods using covalent coatings, PDMS bulk modifications and dynamic coatings\(^{114,115}\). In the latter, the surface is modified using charged surfactants\(^{116,117}\) or polyelectrolyte multilayers\(^{118,119}\). In both cases, the modifying molecule contains a hydrophobic part and a charged part. A monolayer is created at the PDMS surface since the hydrophobic part of the molecule interacts with the hydrophobic PDMS surface. The charged part of the molecule thus creates a charge surface. Dynamic coatings are easy to apply and may withstand large pH-ranges and are also considerably more stable than energy exposed PDMS surfaces\(^{120}\). A dynamic coating was used in Papers II, III, VI were a cationic polyamine, PolyE-323\(^{121}\), was used in order to facilitate anodal EOF that is suitable for positive ESI, but also to minimize the peptide adsorption on the channel walls.

As emphasized earlier, the major advantage with PDMS is the microchip fabrication simplicity. The fabrication procedure is similar to the thermoplastic fabrication procedure. First, a master is fabricated, this master is thereafter used to replicate PDMS microstructures. Finally sealing and surface modifications are carried out to complete the microchip. The master fabrication is usually manufactured with the same methods used in the fabrication of masters for thermoplastic microchip. However, rapid prototyping methods for master fabrication for PDMS structures have been developed\(^{122}\). The largest gain when using PDMS is that the replication procedure is easily accommodated. A premixture of PDMS is simply cast over the master mold and allowed to polymerize, the PDMS wafer is then peeled off and a new replications may be conducted from the same mold. The sealing of two PDMS structures is also more straightforward. The surface of the two PDMS structures is exposed to energy and attached together. This creates an irreversible bond since the OH-groups produced from the energy exposure creates a covalent oxygen bond. The fabrication technique makes it possible to fabricate advanced microstructures with nanometer resolution. This technique was used in Paper VII since a precise grid structure had to be constructed. The grid was placed in the channel, which subsequently ended in an electrospray tip, which was fabricated directly from the PDMS substrate. In order to limit the thickness of the tip, a transparent film was used during the fabrication as a cover over the protruding tip.
Even if the sealing is more reliable when dealing with PDMS compared to thermoplastics, it still introduces a source of uncertainty to the microfabrication process. Furthermore, the replication process prevents to some extent fabrication of 3D structures. Chiou et al.\textsuperscript{123} presented a microfabrication method where wires were used to define the CE channel. In Papers II – IV and VI a similar microfabrication protocol was developed and utilized.

\textbf{Figure 11.} A nine-step fabrication procedure developed and utilized in Papers II - IV and VI.

The novel fabrication procedure facilitated 3D-constructions without the need of sealing in a nine-step procedure as seen in Figure 11. Metal wires and fused silica capillaries are inserted in the mold through drilled holes in the side modules of the mold. The aim of the wires and the capillaries is to
define the final channel pattern of the microchip. The method produces cylindrical channels, which facilitate no-dead volume connections to fused silica capillaries. This feature provides precise connection between capillary-based instrumentation and microchip technology, which is considered to be a major problem addressed in a review by Fredrickson et al.\textsuperscript{124} Furthermore, no clean room facilities are needed since no master mold has to be fabricated which makes the technique very accessible and inexpensive. It is also very easy to change and modify the final design of the chip, which makes the technique suitable for fast prototyping and evaluation of different interesting structures.

The fabrication technique may not produce very advanced chip layouts and it may no be suitable for mass fabrications since a somewhat craftsmanship is required to produce the microchips. Anyway, this interesting chip format fills up a gap between capillary-based technologies and chip-based technologies.

6.2 Incorporated microchip functions

Certain standard operation are involved in the \(\mu\)-TAS concept\textsuperscript{125}; sample pre-treatment\textsuperscript{126-128}, sample injection\textsuperscript{124,129}, reaction\textsuperscript{130}, separation and detection\textsuperscript{131,132}. These components are extensively developed in the macro scale. However in the microchip format these components have only recently started to be developed. In this thesis, sample pre-treatment, sample manipulation, separation and detection components have been investigated and will therefore be dealt with in more detail.

6.2.1 Sample pre-treatment on microchip

Sample pre-treatment is one of the most important steps in the analytical procedure. It is also the most overlooked step in chip-based analysis. The reason is that the apparent advantages gained when performing chip based sample pre-treatment are not as obvious as the benefits associated with other steps in the analytical procedure, for instance in separation\textsuperscript{128}. In a review by Lichtenberg et al.\textsuperscript{127} the sample pre-treatment on microchip was divided into four subcategories, biochemical sample pre-treatment, sample manipulation, separation of sample from sample matrix and sample pre-concentration.

Solid phase extraction is a well-developed technique for sample cleanup and sample pre-concentration. A target molecule is retained on a chromatographic stationary phase, and the unwanted sample matrix and other contaminants are removed by a washing solution. Finally, the target molecules are eluted in an appropriate solution. In order to be able to pack the microfluidic channel, some sort of a frit or a filter must be included in the channel to trap the beads. One popular and simple way to produce a frit has been to
restrict the height of the channel as illustrated in Figure 12a. This method has been employed to trap polystyrene beads and carry out immunoassays\textsuperscript{133}, to trap beads for desalting proteomic samples\textsuperscript{134}, and to construct packed columns for both SPE and electrochromatography analyses\textsuperscript{135-137}.

Figure 12b illustrates a fritless approach to trap beads. This approach is called the keystone effect\textsuperscript{138} and was demonstrated by Ceriotti et al.\textsuperscript{139}. The packing material is drawn from the larger channel towards the tapered channel by applying a pressure at the larger channel inlet. At the taper, the particles aggregate without a physical barrier or frit, due to an increased density. This means that particles can have a smaller diameter compared to the tapered channel. The first particles act as a keystone, which blocks the following particles and allows the segment to grow longer\textsuperscript{139}. This packing procedure was employed in Paper II for trapping 5 µm hyper cross-linked polystyrene beads. The major problem with the keystone approach is its instability. When an electric field is applied, the particles may be set in motion and travel to both ends of the channel\textsuperscript{139}. This problem was solved by decoupling the packed channel from the separation channel, both by designing a two level cross layout as described in Figure 8 and by letting the syringe pump, delivering the liquids through the SPE column, to be floating on the potential induced by the CE. The packing procedure was shown to be very easy to accommodate and therefore it is very appropriate for channel geometry evaluation purposes.

In the third approach, Figure 12c, a grid structure is incorporated in the channel\textsuperscript{140-142}. This approach was used in Paper VII. The advantages with a grid structure are its ability to obtain a small, stable packed column with
limited flow resistance\(^\text{140}\). The grid structure also facilitates a more plug-like flow compared to the height-restricted frit, which results in decreased dispersion effect of the eluted sample\(^\text{141}\).

There are only a few described packed microstructures with integrated ESI emitter\(^\text{138,143}\). Wang et al.\(^\text{138}\) used trypsin immobilized onto 40-60 µm beads, which were trapped in a microchannel using a height restrictor. At the outlet of the microchannel, a 3 cm long spray capillary was inserted to facilitate ESI. In another approach described by Yin et al.\(^\text{143}\), a microchip made in polyimide was packed and used for sample enrichment followed by a chromatographic separation. At the end of the separation channel an emitter tip was shaped directly from the microchip substrate in order to maintain sheathless electrospray. The main challenge when integrating a SPE function and an ESI on a microchip is the problem to remove the sample matrix from the microchip without contaminating the mass spectrometry. In Papers II and VII, two designs of integrated SPE-ESI microchip were developed and evaluated on a TOF-MS. In Paper II, as emphasised earlier, a two level, transverse injection layout was used in order to prevent the sample matrix from entering the CE channel and thus the MS. In Paper VII, the immediate spray onset of the emitter tip was utilized by only have the ESI potential switched on during sample elution from the beads. The beads used in both Papers II and VII had similar properties as the particles used in the perfusate in Paper I. The sample used in Papers II and VII was prepared with the aim to mimic a typical microdialysate sample concerning peptide concentration and salt content. In both Papers, femtomole levels of the neuropeptides could be detected, which is within the range of the in vivo neuropeptide concentration.

Sample manipulation involves modification of the analytes before or after a processing operation, e.g. separation. The sample manipulation is often carried out in order to enhance the performance of the detector either by improving the detection limit or the selectivity. Up to now the majority of the on-chip sample modifications have been done chemically, with the most common application being the labeling of molecules to enable fluorescence detection\(^\text{127}\). The manipulation procedure should ideally be fast and exhibit a high conversion efficiency. It should also be possible to carry out the procedure with minimal dead volume introduction. In order to fulfill these criteria, an efficient mixing between the sample and the derivatizing agent is needed. Mixing fluids in microstructures is a difficult assignment since the laminar flow profile only allows mixing due to diffusion. A micromixer structure is therefore needed\(^\text{130,144}\). In general, micromixers are divided into passive and active mixers. The passive mixer relies solely on diffusion or chaotic advection without the need of external energy, while active mixers use disturbances generated by an external field, e.g. pressure, temperature or electrohydrodynamics. The passive micromixers are most frequently utilized be-
cause of their robustness. However, they also need an advanced channel pattern to produce effective mixing, which may introduce dead volumes.

In Paper IV, an electrochemical sample manipulator coupled directly to ESI was integrated on a PDMS microchip. The integration of electrodes coiled around the microchannel was possible due to the developed fabrication procedure. The use of electrodes in microchip devices has previously been explored for electrochemical detection purposes. However, for on-chip sample manipulation coupled to ESI, only one approach has been developed previously. In the latter method, mass tags on proteins and peptides were generated by utilizing electrochemical reactions at the electrospray high voltage electrode. In this way it was possible to tag free cysteine residues in proteins and peptides with benzoquinones. In Paper IV, an array of electrodes was coiled around the microchannel, where the number of turns in the coils was used to vary and select the electrode area. One electrode in the array, which was placed 5 mm from the emitter tip, was used to oxidize dopamine prior to the detection of the products by ESI-MS. The conversion efficiency of the electrochemical cell was calculated to be 30% at a flow rate of 0.5 µL/min. The use of electrochemical reactions prior to ESI-MS has many advantages, such as the ability to simulate oxidative metabolism of drug candidates. Electrochemical cells coupled to ESI-MS also yield higher electrochemical conversion efficiencies for the ionization of neutral compounds compared to electrochemical manipulation based on the inherent electrochemical reactions in ESI process. The electrode array may also be used as a sample pre-treatment device for desalting and sample pre-concentration purposes as demonstrated by Bökman et al. Electrochemical cells may also be used as active micromixers, based on electrohydrodynamic force.

6.2.2 CE in microchip

As mentioned earlier, CE is a very interesting separation technique to miniaturize for a couple of good reasons. First, it is fairly straightforward to introduce CE-electrodes in microfluidic devices. Basically, two electrodes are inserted in two reservoirs separated from each other by a separation channel. Along the separation channel, an injection cross and a detection device are placed. The difficulties of coupling capillary-based instrumentation, e.g. pumps to the microdevice are therefore avoided. Furthermore, the EOF flow provides an excellent way of moving fluids in microdevices with low dispersion effects and it also minimizes the risk of introducing air bubbles. The most important advantage, however, is the possibility to obtain rapid, highly efficient and resolved separations.

Efficiency and speed are proportional to the applied voltage, and therefore, an increase in the CE voltage enhances the speed of the separation together with the efficiency. This is only true in ideal cases when diffusion is
the only source of band broadening. In fact, this is seldom the case since several other factors limit the efficiency as the voltage is increased, including Joule heating, adsorption sample overloading (electromigration dispersion), sample injection and detection29,30. The largest benefit when miniaturizing a CE channel is achieved by the suppression of Joule heating, since this parameter is proportional to the channel cross-area. The longitudinal diffusion is also reduced since the separation in microchip is often very rapid, as reports of sub millisecond separations verifies150. However, when decreasing the channel size, the surface area-to-volume ratio is increased which in its turn increases the chance of adsorption. Therefore, it is of crucial importance that the surface chemistry of the microdevice is controlled, especially when analyzing neuropeptides, which tend to adsorb onto hydrophobic materials.

The length of the separation channel on a microchip is limited to the size of the chip. This increases the demands on the injection procedure, since the injection plug is dependent on the total separation channel length for maintaining both the efficiency and the resolution. Three injection modes have been applied: electrokinetic injection151-155, pressure injection156,157 and pressure in conjunction with electrokinetic injection158. The injection solutions are based on tee-intersections151, double tee intersections152 and cross intersection153,154. The electrokinetic injection has been most employed due to its inherent compatibility with the CE-microchip. However it also exhibits a bias towards different analytes and is also very dependent on the surface of the channel wall159,160. These biases are decreased when pressure injections are carried out since they do not discriminate any molecules due to charge and electrophoretic mobility156,157.

Microchip based CE-separations were carried out in Papers II and III. In both studies, the channels were dynamically coated with PolyE-323 in order to facilitate anodal EOF, as stressed in previous Chapter. In Paper VI a 17 cm long fused silica capillary-PDMS-microchip hybrid was used. The hybrid comprised of an 11.5 cm long capillary inserted in a 5.5 cm long PDMS channel ending in an ESI emitter tip. Injections were carried out hydrodynamically and a separation between 5 neuropeptides was achieved within 1 minute. In Paper II, a novel, two level cross injection system was used, (described in Chapter 5.3.1). The injections into the CE channel were carried out by remove an overpressure giving reproducible injections with a RSD of 2 % for eleven consecutive injections. Which is comparable with hydrodynamically and electrokinetically injections made on chip where RSD of 0.4% for nine injections157 and 1.6 % for 16 injections155 respectively were reported. Complete separation between the six analyzed neuropeptides could not be obtained. Anyhow, the device functioned as an effective SPE device, which with optimization could be used for comprehensive 2D analyses.
6.2.3 ESI from microchip

Several important issues must be considered when implementing detection techniques to a microdevice. The detector must be fast, with low detection limits, and if possible, universal to many analytes\textsuperscript{131,132}. The most commonly used detection methods for microdevices are laser-induced fluorescence, electrochemical detection techniques and mass spectrometry\textsuperscript{131,161}. All these three techniques fulfill the demands of a successful detector. Mass spectrometry is the most versatile and universal technique due to both its low detection limit and its selectivity. Microdevices have in particular been coupled to mass spectrometry via ESI. The reason is that ESI is a continuous ionization technique, which is compatible with the low flow rates induced from a microchip. Moreover, the electrospay process becomes more efficient using lower flow rates, which results in lower detection limits\textsuperscript{56,57}. The coupling of microchip to MS via ESI poses the same and some additional challenges as when coupling conventional capillaries to MS via ESI. The additional challenges are caused by the microfabrication process, which impedes three-dimensional structures to be fabricated at the edge of the microdevices.

Since the first microfabricated ESI-devices, which were presented by Ramsey’s\textsuperscript{162} and Karger’s\textsuperscript{163} groups in 1997, the development of different emitter designs made in different materials has been intensive. The most straightforward approach is to spray directly from the outlet of the microchannel\textsuperscript{162-166}. Problems with unstable electrospray due to spreading of the spraying liquid at the hydrophilic glass substrate were showed in the early studies\textsuperscript{162,163}. A recently presented solution to this problem is the usage of more hydrophobic chip substrates\textsuperscript{164-166}. However, spraying directly from the edge of the chip is not suitable for CE separation, since the ESI potential must be applied upstream in the channel, which results in introduction of dead volume.

To overcome this problem, either a spray capillary or a needle has been attached at the exit of the microchannel for transferring the sample into the ESI source\textsuperscript{123,137,167-173}. This approach has proven to be robust and microchip CE separations have been carried out\textsuperscript{167,168}. Another approach to perform microchip CE before the ESI has been to connect the microchip to a spraying needle via a liquid junction\textsuperscript{174}. Despite the high sensitivity and robustness of these techniques, the drawback of these chips will always be the increased dead volume and subsequently, the decrease in usefulness for separation applications. Furthermore, this technique is not compatible with high density electrospay microchips, i.e. with multi-system fabrication on a single device\textsuperscript{175}.

Another method has been to shape an emitter tip directly from the microchip substrate. This has been done in PC\textsuperscript{176}, PMMA\textsuperscript{177,178}, polyimide\textsuperscript{143,179}, parylene\textsuperscript{180}, PDMS\textsuperscript{181-184} and the photoresist SU-8\textsuperscript{175}. Among these chips,
many designs have been developed, including open electrospray tip\textsuperscript{175,177,183}, planar two dimensional chip\textsuperscript{1176,177,179,181} and three-dimensional emitter tips shaped either manually\textsuperscript{178}, by laser ablations\textsuperscript{143} or by casting\textsuperscript{182}. The fluid movement has mainly been electro driven\textsuperscript{175-177,179,182}, although pump driven flows have been reported\textsuperscript{143,178,180,183}. Multiple ESI emitters have been reported\textsuperscript{180,184} on the same microchip and incorporated functions like desalting\textsuperscript{185,186}, chromatographic separation\textsuperscript{143} and isoelectric focusing assisted by a sheath gas and liquid electrospray\textsuperscript{176} have been implemented.

Notwithstanding one exception\textsuperscript{143}, the ESI potential has been applied via an electrode placed upstream in the microchannel and apart from one publication\textsuperscript{176}, no electrophoretic separation has been carried out prior to the ESI tip. As emphasized earlier, emitter tips with conductive coating eliminate the band broadening since it facilitate separation to the exit of the channel. However, in order to obtain efficient electrospray, the coating on the emitter tip must be sustainable. In Papers V-VII different conductive coated emitter tips directly shaped from the microchip substrate were developed and evaluated. As elucidated in Table 2 all evaluated emitter tips showed good stability and robustness. This is important in order to facilitate reliable EOF when CE is performed prior to the tip. The conducting layer durability can be compared to similar coating made on fused silica capillaries\textsuperscript{187}. The emitter tips evaluated in Paper V, were made in thermoplastics, which made the tip fabrication laborious. However, the thermoplastics gave less background signal compared to PDMS, even if the signal was largely suppressed when the PDMS microchips were allowed to cure for 48 h instead of 2 h. The emitter tip design used in Paper II, IV and VI was easiest to fabricate with an approximately yield of 95%. It also facilitated implementation of analytical functions to the chip. The tip evaluated in Paper VII allows advanced chip designs to be fabricated prior to the tip, which is interesting for further µ-TAS developments.
Table 2. Comparison of the three different microchip ESI emitters developed in Papers V-VII.

<table>
<thead>
<tr>
<th>Paper</th>
<th>V</th>
<th>II, IV and VI</th>
<th>VII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emitter Design</td>
<td><img src="image1" alt="Emitter V" /></td>
<td><img src="image2" alt="Emitter II, IV and VI" /></td>
<td><img src="image3" alt="Emitter VII" /></td>
</tr>
<tr>
<td>Chip material</td>
<td>PMMA and PC</td>
<td>PDMS</td>
<td>PDMS</td>
</tr>
<tr>
<td>Conductive layer</td>
<td>Gold - sputtered, Gold - glued, Graphite - glued</td>
<td>Graphite - glued</td>
<td>Graphite - glued</td>
</tr>
<tr>
<td>Channel outlet (µm)</td>
<td><img src="image4" alt="Channel V" /></td>
<td><img src="image5" alt="Channel II, IV and VI" /></td>
<td><img src="image6" alt="Channel VII" /></td>
</tr>
<tr>
<td>Flow delivery</td>
<td>Pump driven</td>
<td>Pump driven, Electro driven</td>
<td>Pump driven, Electro driven</td>
</tr>
<tr>
<td>Flow rates nL/min</td>
<td>700 - 2000</td>
<td>100 - 1000</td>
<td>100 - 2000</td>
</tr>
<tr>
<td>Stability (RSD)</td>
<td>3.5 % for 5 min</td>
<td>3.9 % for 30 min</td>
<td>5.8 % for 5 min</td>
</tr>
<tr>
<td>Durability (h)</td>
<td>Not tested</td>
<td>180</td>
<td>&gt; 800</td>
</tr>
<tr>
<td>Need for clean room</td>
<td>Yes</td>
<td>No</td>
<td>Yes, for master fabrication</td>
</tr>
<tr>
<td>Incorporated functions</td>
<td>Direct infusion</td>
<td>Direct infusion (VI), CE (VI), Electrochemical manipulation (IV), SPE-CE (II), FIA-CE (II)</td>
<td>Direct infusion, SPE</td>
</tr>
<tr>
<td>Spray picture</td>
<td><img src="image7" alt="Spray V" /></td>
<td><img src="image8" alt="Spray II, IV and VI" /></td>
<td><img src="image9" alt="Spray VII" /></td>
</tr>
</tbody>
</table>
7. Conclusions and future aspects

The thesis describes strategies for sampling, sample preparation, separation and detection of neuropeptides on a micro scale basis. I have chosen to summarize the thesis in Figure 13:

![Figure 13](image)

*Figure 13. A concluding picture of the presented Papers in the thesis.*

Figure 13 illustrates a microchip coupled to microdialysis sampling. The functions on the chip include sample pre-treatment, injection, separation,
manipulation and finally ESI-MS detection. Different microchip materials were also evaluated and a fabrication procedure for fast prototyping of PDMS chip was developed. Common in all Papers are that ESI-MS have been used as detector and apart from Paper IV, neuropeptides were used as model analytes. It should be noted that the developed techniques is not dedicated to the analysis of neuropeptides. Similar principles can be used to analyze other compounds for example protein. As seen in Figure 13, individual parts have been developed and tested. As a future study, a complete integrated chip with all functions is desirable. In order to reach that goal, developments in the solid supported microdialysis method and the CE separation is needed together with some general optimizations and developments.

The development of integrating instrumentations onto a chip has been intense. Micropumps, high voltage supplies and even mass spectrometers are under constant development. One day, maybe you will be able to get a complete integrated microchip including pumps, high voltage supplies and a mass spectrometer fitted onto a couple of square centimeters.
8. Acknowledgements

Genom åren som doktorand har jag lärt känna massor av människor som har hjälpt mig på olika sätt.


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Andreas Dahlin
Uppsala 26/4 - 2005
9. Summary in Swedish

9.1 Mikroskaliga verktyg för provpreparering, separation och detektion av neuropeptider


I artikel II och III så tillverkades ett hybridchip där chipdelen bestod utav det elastiska silikonbaserade materialet poly(dimetylsiloxane) (PDMS). Till dessa hade glaskapillärer fästs på ett sätt så att ingen bandreddning introducerades i övergången. Syftet med enheten var att skapa ett två-
dimensionellt separationssystem kopplat till mass spektrometri (MS) via elektrospray (ESI). Koppling mellan de tvåseparationsdimensionerna var konstruerad i en två nivåers korsdesign där de båda separationskanalerna stod i kontakt med varandra via ett litet hål. I artikel II utfördes den två-dimensionella separationen på ett 6×2×0.5 cm (längd, bredd, höjd) stort mikrochip. I den första dimensionen koncentrerades och tvättades en neuropeptidlösning innehållande fysiologiska saltkoncentrationer. Efter avsaltningen så injicerades provet direkt till en kapillärelektrofores (CE) kanal där en snabb separation utfördes innan det slutligen analyserades på en time-of-flight masspektrometer (TOF-MS). Enheten fungerade tillfredställande. Trots att inte fullständig separation kunde erhållas så kunde man ändå mäta femtomolmängder av neuropeptider. I artikel III så bestod den första separationsdimensionen av en vätskekromatografi (LC) och den andra av en CE. Dessa separationskolonner var externa från chippet och således kunde dessa hållas längre för att på så sätt maximera separationseffektiviterna. Utloppet på CE kanalen kopplades via ESI mot en Fourier transform ion cyclotron resonance masspektrometer (FTICR-MS). Prover av varierande komplexitet analyserades från enkla neuropeptidstandarder till tryptiskt fragmenterade digerat av humant cerebrospinalvätska och bovin serum albumin (BSA). Sekvenstäckningen för BSA beräknades till 93% med en feltolerans på 5 ppm.


I de sista tre artiklarna V-VII behandlas olika designer på elektrosprayspetsar tillverkade med olika metoder och material direktt ifrån kanten på mikrochippet. I artikel V, så testades spetsar från chip gjorda i termoplasterna poly(methylmetacrylate) (PMMA) och polycarbonat (PC). Olika designer på spetsarna testades liksom olika tekniker att tillverka dessa spetsar på. Eftersom elektrosprayspännningen applikerades på ett strömledande skikt på elektrosprayspetsarna så utvärderades även olika sådana strömledande material med avseende på dess mekaniska och kemiska hållbarhet.

I artikel VI så utvecklades, som nämnt ovan, en helt ny teknik för att fabricera tredimensionella mikrochip i ett steg. Kortfattat så använder man sig av en gjutform. I denna form kan man placera olika funktioner som det slutgiltiga chippet skall ha. Tunna metalltrådar sätts in, och kommer i det färdiga
chippet ha definierat kanalerna. Borrade hål i formen kommer att yttra sig som externa strukturer. Ohärdad PDMS tillsätts sedan i formen och den till-
låts att härda i låg temperatur under ett antal timmar. Därefter avlägsnas gjuftformen, metalltrådarna dras ut och ett färdigt mikrochip är resultatet. Fördelarna med denna tekniken är att man undkommer problemet med att sammanfoga två chipskivor (den mönstrade bottenskivan och ett lock) som annars är fallet vid konventionell chiptillverkning. Dessutom så erhålls cy-
lindriska kanaler som gör det möjligt att koppla glaskapillärer till chippet utan någon påtaglig dödvolym. Detta är av extra betydelse, då det medför att kapillärbaserade instrument kan sammankopplas med mikrochipteknologi på ett robust och reproducerbart sätt. De tillverkade chippen har vidare en kanal som mynnar ut i en elektroosprayspets. På denna spetsen limmades ett tunt lager av grafit för att möjliggöra en elektrisk kontakt mot ESI. Spetsens och dess grafitlager utvärderades med avseende på stabilitet och hållbarhet med de klassiska elektrokemiska mätmetoderna; cyklisk voltametri och chron-
amperometri. Detta arbete låg sedan till grund för artiklarna II-IV.

I artikel VII så tillverkades ett mikrochip i PDMS men med konventionel chipteknik, innebärande fotolitografi av en master, gjutning av chippillator och sammanfogning utav två plattor till ett slutgiltigt mikrochip. Även denna chipdesign mynnade ut i en elektroosprayspets vars yta var täckt med ett lager av grafit. Strax innan spetsen placerades en gallerstruktur som hade till uppgift att fungera som ett stopp för de avsaltningspartiklarna som chippet packades med. Dessa partiklar var av samma sort som de partiklarna som användes i artikel I. En saltinnehållande neuropeptidlösning avsaltades effektivt innan neuropeptiderna eluerades från avsaltnings-partiklarna och analyserades i en TOF-MS.

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