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Integrated Micro-Analytical Tools for Life Science

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Abstract
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Advances in life science require knowledge of active molecules in complex biological systems. These molecules are often only present for a certain time and at limited concentrations. Integrated micro-analytical tools for sampling, separation and mass spectrometric (MS) detection would meet these requests and are therefore continuously gaining interest. An on-line coupling of analytical functions provides shorter analysis time and less manual sample handling. In this thesis, improved compatibility of microdialysis sampling and multidimensional separations coupled to MS detection are developed and discussed.

Microdialysis was used *in vitro* for determination of the non-protein bound fraction of the drug ropivacaine. The sampling unit was coupled on-line to capillary column liquid chromatography (LC) followed by ultraviolet or MS detection. For MS detection, the system was extended with a desalting step and an addition of internal standard. A method for MS screening of microdialysates, collected *in vivo,* was also developed. The method involved sampling and measurements of the chemical pattern of molecules that generally are ignored in clinical investigations. Chemometric tools were used to extract the relevant information and to compare samples from stimulated and control tissues.

Complex samples often require separation in more than one dimension. On-line interfaces for sample transfer between LC and capillary electrophoresis (CE) were developed in soft poly(dimethylsiloxane) (PDMS). MS detection in the LC-CE system was optimised on frequent sampling of the CE peak or on high resolution in mass spectra using time-of-flight (TOF)MS or Fourier transform ion cyclotron resonance (FTICR)MS, respectively. Aspects on electrode positioning in the LC-CE interface led to development of an on-column CE electrode. A successful method for deactivation of the PDMS surface using a polyamine polymer was also developed. The systems were evaluated using peptides and proteins, molecules that are gaining increased attention in bioscience, and consequently also in chemical analysis.

Keywords: Microdialysis, Multidimensional separation, Liquid chromatography (LC), Capillary electrophoresis (CE), Electrospray ionisation (ESI), Mass spectrometry (MS), Microchip device, Free drug concentration, Screening of microdialysates, Pattern recognition

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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 Koivisto Pernilla, Bergström Sara K., Markides Karin E. (2001). Determination of free concentration of ropivacaine in plasma by packed capillary liquid chromatography: A comparison of ultrafiltration and microdialysis as sample preparation methods. *J. Microcol. Sep.* 13(5): 197-201

II Bergström Sara K., Markides Karin E. (2002). On-line coupling of microdialysis to packed capillary column liquid chromatography-tandem mass spectrometry demonstrated by measurement of free concentrations of ropivacaine and metabolite from spiked plasma samples. *J. Chromatogr. B* 775(1): 79-87

III Bergström Sara K., Goiny Michel, Danielsson Rolf, Ungerstedt Urban, Andersson Marit, Markides Karin E. (2005). Screening of microdialysates using on-line desalting and mass spectrometric detection. *(Submitted to J. Chromatogr. A)*

IV Samskog Jenny, Bergström Sara K., Jönsson Mats, Klett Oliver, Wetterhall Magnus, Markides Karin E. (2003). On-column polymer-imbedded graphite inlet electrode for capillary electrophoresis coupled on-line with flow injection analysis in a poly-(dimethylsiloxane) interface. *Electrophoresis* 24(11): 1723-1729

V Bergström Sara K., Samskog Jenny, Markides Karin E. (2003). Development of a poly(dimethylsiloxane) interface for on-line capillary column liquid chromatography-capillary electrophoresis coupled to sheathless electrospray ionization time-of-flight mass spectrometry. *Anal. Chem*. 75(20): 5461-5467

VI Bergström Sara K., Edenwall Niklas, Lavén Martin, Velikyan Irina, Långström Bengt, Markides Karin E. (2005). Polyamine deactivation of integrated poly(dimethylsiloxane) structures investigated by radionuclide imaging and capillary electrophoresis experiments. *Anal. Chem.* 77(3): 938-942

VII Bergström Sara K., Dahlin Andreas P., Ramström Margareta, Andersson Marit, Markides Karin E, Bergquist Jonas. (2005) A simplified multidimensional approach for analysis of complex biological samples: On-line LC-CE-MS. *(Submitted to Anal. Chem.)*

VIII Dahlin Andreas P., Bergström Sara K., Andrén Per E., Markides Karin E., Bergquist Jonas. (2005) Poly(dimethylsiloxane)-based microchip for two-dimensional solid-phase extraction-capillary electrophoresis with an integrated electrospray emitter tip. *Anal. Chem.* 77(16): 5356-5363.

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Related papers not included in the thesis:

- Lavén Martin, Wallenborg Susanne, Velikyan Irina, Bergström Sara, Djodjic Majda, Ljung Jenny, Berglund Oskar, Edenwall Niklas, Markides Karin E., Långström Bengt. (2004) Radionuclide imaging of miniaturized chemical analysis systems. *Anal. Chem*. 76(23): 7102-7108.

- Pettersson Dahlin Andreas, Wetterhall Magnus, Liljegren Gustav, Bergström Sara K., Andrén Per, Nyholm Leif, Markides Karin E., Bergquist Jonas. (2005) Capillary electrophoresis coupled to mass spectrometry from a polymer modified poly(dimethylsiloxane) microchip with an integrated graphite electrospray tip. *Analyst* 130(2): 193-199.

- Lindberg Peter, Dahlin Andreas P., Bergström Sara K., Thorslund Sara, Andrén Per E., Nikolajeff Fredrik, Bergquist Jonas. (2005) Sample pre-treatment on a microchip with integrated electrospray emitter. (*Submitted to Electrophoresis).*

Author contribution

In Paper I, I performed the microdialysis experimental work in my undergraduate project and took part in the discussion of the results. I was responsible for planning and carry out the experimental work and writing Papers II, III and V. In Paper III the microdialysis experiments were performed at Karolinska Institute together with Michel Goiny. In Papers IV and V, the experiments were made in equal part by Jenny Samskog and me, and in Papers VII and VIII by Andreas Dahlin and me. I wrote paper VII, while Andreas Dahlin wrote Paper VIII. In Paper VI, I was responsible for planning the work and writing the paper, the radionuclide imaging experiments were performed together with Niklas Edenwall and Martin Lavén, while I performed the capillary electrophoresis experiments.

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Abbreviations

µLC	Micro liquid chromatography
µTAS	Micro total analysis systems
2D	Two-dimensional
2D-PAGE	Two-dimensional polyacrylamid gel electrophoresis
APS	3-aminopropyl-trimethoxysilane
CE	Capillary electrophoresis
CZE	Capillary zone electrophoresis
EOF	Electroosmotic flow
ESI	Electrospray ionisation
FTICR	Fourier transform ion cyclotron resonance
FWHM	Full width at half maximum
HV	High voltage
i.d.	Internal diameter
LC	Liquid chromatography (i.e. high performance liquid chromatography)
m/z	Mass-to-charge
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MudPIT	Multidimensional protein identification technology
PDMS	Poly(dimethylsiloxane)
RPLC	Reversed-phase liquid chromatography
SIM	Single ion monitoring
SPE	Solid phase extraction
TOF	Time-of-flight
UV	Ultraviolet detection

1. Introduction to micro-analysis in life science

What are the questions that require answers within life science today? An answer to that is that we need to investigate processes in the body that help us understand the underlying reasons for different processes, e.g. diseases. We want to diagnose disorders at an early stage, and enable identification of the causes and not just relieve the symptoms. Consequently, we need more information than present today on the active, ongoing chemistry in the body.

The completion of the human genome sequence, at the turn of this millennium, has resulted in a number of new fields of discoveries related to life science. The gene products, e.g. proteins and peptides, and compounds related to their synthesis and degradation are nowadays common research areas.

There is thus a need for developing tools for characterisation of biomolecules in body fluids (or tissue), as a complement to clinical analyses performed today. To measure a single compound in a sample is not always enough; instead a more holistic biomarker pattern approach may be required to e.g. establish profiles of active compounds in biological systems[1]. This field is actually one of the most promising bases for developing diagnosis tests and drug candidates. It is also important to extract as much information as possible from a data matrix by advanced mathematical operations in the field of chemometrics[2]. Furthermore, there is a need for developing techniques adjusted to following a process or event in a living systems, e.g. in a patient during surgery. A frequent sampling procedure, e.g. by microdialysis (Chapter 4), preferably coupled on-line or performed in close vicinity to the analysis, is needed for following such a process[3]. If all analytical steps from sampling to detection are performed fast enough, it is possible to rapidly reveal complications and take measurements for recovering of the patient.

Technically, this implies development of methods that are well adapted not to disturb the studied process and that can preserve information through all steps of the analytical procedure. To achieve good temporal resolution and to register small changes, low sample volumes should be withdrawn, and the possibilities to further decrease the volume to concentrate the samples are thus limited. Samples related to life science are very complex; i.e. they consist of a large number of different components present at a wide

concentration range. This requires high-resolving separation systems combined with sensitive and selective detection techniques.

Electrospray ionisation (ESI) coupled to mass spectrometry (MS)[4] has become a popular detector for biomolecules. With this method, ionic compounds are released from liquid phase by an electric potential and gas-phase ions, transferred into the MS detector, are sorted with respect to their mass-to-charge ratio (Chapter 3). The result achieved is thus very informative. To avoid all components to reach the detector at the same time, highly efficient and selective separation systems are advantageous to use up front of the detection. Liquid chromatography (LC) and capillary electrophoresis (CE) meet these demands. In LC analytes are loaded onto a packed column, usually with high affinity for hydrophobic compounds (reversed phase (RP)LC). Elution is then performed with a liquid phase buffer, resulting in a chromatogram where the least retained compound appears first[5]. CE is generally performed in an electrolyte filled, non-packed, capillary (capillary zone electrophoresis (CZE)). A strong electric field over the capillary yields fast separation where small and highly charged ions (positively or negatively charged depending on polarity used in CE) generally reach the detector first[6]. Endogenous and foreign molecules are all interacting in living systems and knowledge about these interactions and distributions, at a molecular level, can only be realised when different disciplines join effort. This drives the research towards on-line coupling of these critical technologies in hyphenated systems.

This thesis deals with the integration of different micro-analytical tools. Methods for sampling, sample preparation and separations are integrated in on-line systems adapted to MS detection. Miniaturised methods are used in order to handle small sample volumes without severe dilution. They can be divided into two directions: the miniaturisation of conventional techniques, e.g. use of microcolumns (internal diameter [i.d.]<1 mm) instead of conventional columns (i.d. 4.6 m) in LC, and the micro total analysis system (μTAS) developments, where all analytical steps are combined on a centimetre sized chip device[7]. Micro liquid chromatography (μLC) is suitable for small sample volumes, and provides a more effective combination with MS[8]. This thesis describes developments within both these directions. Miniaturised conventional techniques (Papers I-III), sometimes in combination with micro-structured couplings (Papers IV-VII), as well as chip-based developments (Paper VIII), are included. The usefulness of systems are demonstrated by drug-protein binding studies (Papers I and II), screening for biomarkers (Paper III), separating complex samples, especially peptides and protein digests (Paper VII) and pre-concentration of low levels of neuropeptides (Paper VIII).

2. The aspect of time in chemical analysis

Time is a factor in chemical analysis that has not gained the focus that it merits. Time is of course inherent in all processes and it becomes important when there is an interest to follow a change in molecular composition, especially when it involves active fractions and non-active bound fractions (Chapter 6). In addition, time is critical in sampling and sample transfer when integrating different analytical steps. The aspect of time in chemical analysis is therefore much more than just to perform the analysis as fast as possible. The aspect of time is brought into this thesis in order to ...

...study robustness of analysis. Time aspects should be considered in all analytical steps to achieve repeatable results. An inherent process is equilibration, which is highly important for the robustness of systems. In this work, this has been important in microdialysis sampling (Papers I-III), when utilising LC gradients (Papers V and VII) and in pressure and flow adjustments (all Papers). Inappropriate equilibration time resulted in non-repeatable results. Time for equilibration should thus be included when considering the total analysis time. Another example of time adjustments for robust results is the data collecting frequency in the detector. To be able to characterise peaks in separations, at least 10 data points per chromatographic peak[5] are required. In this work, time adjustments in the detector have mostly been concerned for CE separations coupled to MS detection (Papers IV, V, VI, VIII). CE separations produce peak widths on the time scale of seconds and therefore demand highly frequent data collection. This is generally not a problem, when using ultraviolet (UV) detection, but for full scan MS detection, this must be considered (Chapter 3).

...follow chemical processes. On-line real time monitoring changes in chemical composition requires, again, consideration of the time aspect. Among the sampling tools that can be used for on-line measurements in life science analysis, microdialysis is shows promising features (Chapter 4). It can be used for monitoring biological processes and for establishing concentration profiles with time. The analysis time, compared to the time interval for sampling, must be related to how fast changes take place in the studied process. Samples must thus be collected with sufficient frequency to maintain the resolution of studied process. The same line of arguments can be applied to process industry, where the concept of process analytical technology (PAT)[9], is used for controlling manufacturing through timely

measurements (i.e. during processing) with the goal of ensuring final product quality.

...couple different techniques. The principle of maintaining resolution, achieved in a separation, is also important when adding further separation steps. To obtain high resolution in two-dimensional (2D) separations, it is suggested that peaks from the first dimension should be sampled at least three times into the second dimension[10]. For analysis of complex samples, "comprehensive" sampling, where equal percentages of all components are transferred into the second dimension, should be applied. In comparison, if focusing on a certain compound a "heart-cut" of the corresponding elution volume can be fractionated. When coupling e.g. LC on-line to CE (Papers V and VII) in a 2D separation, a fast CE separation is preferred to not increase the total separation time in comprehensive sampling. The faster CE, the more frequent sampling of the LC can be achieved, and the resolution achieved in the LC can then be maintained (Chapter 5).

...achieve powerful analytical systems. What capacity does a total analysis system have? That question can be answered by a number of defined parameters, e.g. the number of theoretical plates, the peak capacity of the system, the detection limit, the number of identified peaks, and so on. All these parameters should be related to the total analysis time, in order to correlate the capacity and the time aspect of analysis. A common goal today, is to perform faster separations by reducing the column i.d. and the size of the packing material to maintain the resolution. At the same time, the identification of analytes may relay on a selective detection method, e.g. tandem mass spectrometric (MS/MS) detection. A common challenge is then that the time allowed for MS investigations of analytes after the LC separation is limited. One solution to this problem is to decouple the LC and the MS and collect fractions that are analysed by enhanced MS scans[11]. This is, of course, not possible when analysis requires a real-time feedback of a chemical process.

Consequently, the aspect of time in chemical analysis is rather complex and all aspects should be considered and optimised to provide the best results, within a reasonable time, for each specific analytical method.

3. Mass spectrometric detection in liquid micro-flow systems

Liquid-based micro-flow techniques, e.g. μLC, CE and micro-chip separations, are techniques for handling small sample volumes of analytes at low concentrations. To detect an appropriate real-time signal of analytes eluting from such systems, a sensitive detection method that can handle a continuous liquid flow of analyte, is required. Mass spectrometric detection has become a powerful detection tool for biomolecules due to its ability to produce a selective response that corresponds to the mass-to-charge ratio (*m/z*) of the analytes. This facilitates structural elucidation and identification of molecules. In addition, a mass spectrometer may identify the presence of coeluting peaks when they are not fully resolved by preceding separations. A schematic illustration of a mass spectrometer is given in Figure 1. Since mass spectrometers sort ions in a vacuum chamber, analytes present in a liquid micro-flow need to be transferred into gas phase ions before entering the detector. There are different ways of producing gas phase ions from compounds in a continuous liquid flow. The most widely used method today, ESI, made its first breakthrough as a mass spectrometric ionisation technique from liquid solutions by Yamashita and Fenn in 1984[12]. This method has been used throughout the work presented in this thesis.

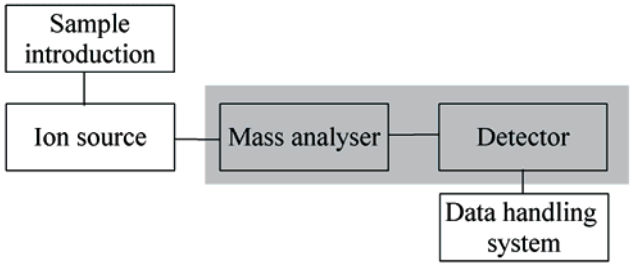

Figure 1. A schematic description of a mass spectrometer. The ionisation in the ion source is nowadays generally performed at atmospheric pressure while the mass analyser and commonly also the detector require vacuum (shadowed) conditions.

Most analytes, small and large, encountered in endogenous life science have the characteristics that allow them to be analysed by ESI. If analytes are uncharged or difficult to release as ions from liquid to gas phase,

atmospheric pressure chemical ionisation (APCI)[13,14,15,16] or atmospheric pressure photo ionisation (APPI)[17,18] are attractive alternatives. In APCI, the eluent is first nebulised and the analyte are transferred into gas-phase. Thereafter, a discharge process (usually by using a discharge needle) produces free electrons that initiate gas-phase ionisation of the analyte. In APPI, gas-phase ions are produced after vaporisation of the eluent, by interaction between a photon emitted by an UV source and analytes (often mediated through a reactive dopant, e.g. toluene or acetone). Both methods are thus especially useful for analytes not easily ionised in the liquid phase and therefore not completely amenable for ESI. Matrix effects are less pronounced in APCI and APPI than in ESI[16], and they can thus tolerate higher concentrations of buffer additives or even non-volatile buffers[19]. Large molecules, e.g. peptides and proteins can not be ionised in gas phase directly. Instead, they need the soft ionisation already in liquid phase.

Moreover, the separation in liquid phase can also be decoupled from the ionisation and volatilisation processes, e.g. by fractionate the eluate onto a matrix assisted laser desorption ionisation (MALDI)[20,21] plate. On the plate, the sample is mixed with a light-absorbing matrix and the ionisation takes place by irradiating the spot with a laser beam. MALDI has the advantage that it is more tolerant towards sample contaminants and typically produces singly charged protonated molecules. In the future, this ionisation technique will likely become a routinely used complementary alternative for separations coupled to on-line real time MS detection, since it allows on-site analyte manipulations after separation and possibilities to perform multiple MS analyses[22].

3.1 Electrospray as ionisation method in liquid micro-flow systems

The intrinsic nature of ESI provides a straightforward on-line interface between liquid phase separation systems and mass spectrometry. A high potential of 1-3 kV is generally applied to one side (usually the entering liquid) to produce an electric field between the ESI needle and the inlet of the mass spectrometer, as depicted in Figure 2. This also creates charged droplets from an elongating cone (the Taylor cone). The solvent in these droplets then evaporate and charged analytes are released as gas-phase ions, which are electrically accelerated into the MS[23,24,25]. ESI is a gentle ionisation method and is especially well suited for studying intact molecules, and even non-covalent complexes, with no or very low fragmentation.

The mechanism for generating gas-phase ion is still under investigation, but two theories have been proposed. First, the ion evaporation model (IEM) introduced by Iribarne and Thomson[26], assumes that gas-phase ions can be

ejected from the droplets before the solvent has completely evaporated. Second, the charge residue model (CRM), introduced by Dole et al.[27], proposes that solvent evaporate until a single ion is left in each droplet. Both theories may be valid at the same time, but the CRM theory is more likely valid for macro-molecules[28], whereas the IEM theory probably dominates for small surface-active ions[29].

To facilitate the evaporation, heat or different gas flows can be used, especially for high liquid flow-rates. It is important to chose volatile buffers and to ionise the analyte already in liquid phase. Salts and other non-wanted compounds in the sample should be removed to avoid signal reduction, i.e. ion suppression[30,31,32], and thereby lowering the sensitivity. Aspects on method development and desalting will be further discussed in chapters 4 and 5.

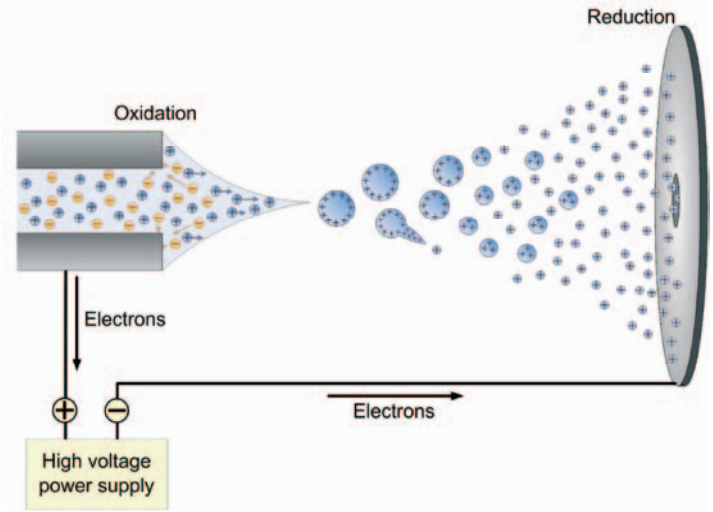

Figure 2. Schematics of the processes involved in positive ESI.
Picture by Andreas Dahlin.

ESI ion sources can accommodate flow-rates from nL/min to mL/min. The highest absolute sensitivity (greatest peak height for a fixed sample amount injected) is generally obtained with the lowest possible flow and smallest possible LC column i.d., due to high ionisation efficiency and less chromatographic dilution[33]. Packed capillary LC columns (Papers I, II, V and VIII), commonly have an optimal flow-rate of around 1 µL/min, which is directly compatible with pure ESI[33], also called sheathless ESI. In pure ESI, droplet formation is only due to the applied electrical field, i.e. not assisted by nebulising gas, heat or additional liquid buffer flow. The upper limit for pure ESI is 10-20 µL/min[15]. Also CE separations with high EOF

that provides a stable flow (3-400 nL/min) for sheathless ESI is nowadays easily combined with this ionisation technique (Papers IV, V, VI, and VIII).

As depicted in Figure 2, the ESI process involves redox reactions (generally reduction in negative mode and oxidation in positive mode, but may be altered if CE separations are preceding the ESI[34]) at the spray needle[25,35]. Since water, the dominating component in liquid buffers, is involved in redox reactions at relative low overpotentials, the most probable reaction in the ESI process is electrolysis of water. Such a reaction produces gas bubbles (O_2 or H_2) and implies that the spray needle material must withstand gas-bubble formation without loosing its electrical contact. Stainless steel material is the most generally used material in electrospray needles and it is not affected by gas bubbles. In practice, the liquid eluting from a micro liquid-flow system, either gets direct contact with the stainless steel (sheathless interface) or gets in contact with an assisting flow that in its turn has contact with the electrode (sheathflow interface). In Paper II a sheathless micro-ion-interface and in Paper III a turbo-ion-interface (both from Sciex) were used. Even though these commercially available low flow stainless steel interfaces are stable and have low dead volumes, they are not suitable for all liquid micro-flow applications. When a CE separation, with narrow electrophoretic peaks, is the last step before MS detection, it is more advantageous to use an electrode placed directly onto the CE column outlet (Papers IV, V, VII and VIII). Such electrodes have been produced in-house by tapering the fused silica capillaries to achieve a high electric field, and after that applying a conductive coating of gold[36] or graphite particles imbedded in a polymer of either polyimide[37] or polypropylene[38]. These emitters have shown high electrochemical stability and long life times when used in ESI.

Liquid based separations can also be performed in micro-chip systems, and their integration to ESI-MS has been done using various techniques[39]. Most important is to maintain the generic features of ESI, e.g. that a narrow base of the Taylor cone, to reduce dead volumes, is established if separations are preceding steps[39]. One approach has been to fabricate the emitter tip directly from the bulk material at the micro-chip channel outlet. This allows for separation to be the final step before ESI, compared to when the electric contact is applied upstream. This strategy was also used when the CE separation was performed in a soft polymeric poly(dimethylsiloxane) (PDMS) based microchip structure, as in Paper VIII. An in-structure ESI emitter based on conducting graphite[40] was fabricated directly at the edge of the micro-chip. Recently, a method using micro-fabricated templates for production of ESI emitter tips was also developed in our laboratory[41].

A final consideration on ESI-MS is that the ion intensities in the mass spectrum not directly correspond to the analyte composition in solution. The signal produced is dependent on ESI efficiency, which varies a lot with physico-chemical properties, e.g. surface activity, of the analyte ions, ion

sampling efficiency into the vacuum and ion transmission efficiency through ion optics and mass analysers[23,24].

3.2 Mass spectrometric analysers for biomolecules

In mass spectrometry, a number of available ionisation methods can be combined with a broad spectrum of mass analysers for recording ions. This makes the MS approach very flexible. The mass analysers commonly used today are quadrupole (Q)[42], time-of-flight (TOF)[43,44], ion trap[45], Fourier transform ion cyclotron resonance (FTICR)[46] and hybrids of these[47]. The flexibility not only lies in the fact that different analyser can be used, but also in the interchangeable way of spectra recording, e.g. the entire mass spectrum at once or one ion at a time, and a number of different MS/MS alternatives. As a comparison, using quadrupole instruments qualitative LC-MS requires the acquisition of the complete mass spectrum in each chromatographic LC data point, while quantitative applications probably need low detection limits and are preferably recorded using selected ion monitoring (SIM). Examples of what different analysers and scan modes can achieve in for a µLC peak eluting during 25 seconds are given in Table 1.

Table 1. *Comparison of different analysers used in µLC-MS, for a 25 s µLC peak.*

Mass analyser	Detection mode	Data collection frequency [points/s]	Resolution[*48] (m/Δm)	Sensitivity[48]
TOF	Full scan	10-20	$3\text{-}10\cdot10^3$	Constant with mass range, suitable for qualitative analysis.
Q	Full scan	1 (*m/z* region:1 000, 1 ms dwell time[**])	$4\text{-}6\cdot10^2$ (higher resolution decreases S/N[***])	Decreases with increased mass range.
Q	SIM	125 (200 ms dwell time[**])	$5\cdot10^2$	Detection limits comparable to TOF and FTICR, suitable for quantitative data.
FTICR	Full scan	0.1-0.2	$5\text{-}10\cdot10^5$	Constant with mass range, suitable for qualitative analysis

[*] Resolution = m/Δm, where Δm corresponds to full at width half maximum (FWHM).
[**] Dwell time = How long time a certain *m/z* value is measured.
[***] S/N = Signal to noise ratio.

Mass analysers have a variety of shapes, sizes and prices. The choice of a proper mass spectrometric detector requires understanding of the alternatives. The mass spectrometer should match the analytical needs.

Coupling to chromatographic or electrophoretic separations generally requires a mass spectrometer that can record mass spectra rapidly, such as ion trap, quadrupole or time-of-flight mass spectrometer. Again, the time aspect is crucial. For fast CE separation of peptides, a TOF analyser, which can achieve a full spectrum in 100-200 μs, is the preferable choice to (Papers IV, V, and VIII).

For selective detection or for studying ion/molecule fragmentation, e.g. for detection of low concentrations of drugs in biological matrices (Paper II), a triple quadrupole will be well suited. Among the different scan modes, Multiple Reaction Monitoring (MRM) is the most selective. The direct current (DC) voltage and radio frequency (RF) voltage applied to the electrodes in the triple quadrupole analyser are then adjusted to select a mother ion for each analyte in the first quadrupole, fragment it by collision with an inert gas, followed by detection of a certain fragment of the mother ion in the third quadrupole.

For applications where high mass resolution (definition in Table 1), typically 10^6 and mass accuracy on the ppm-level, are needed, the FTICRMS is a superior instrument, e.g. for identification of peptides in complex samples (Paper VII). It is thus possible to identify peptides in a narrow CE peak (Paper VII) by only 1-2 spectra.

The resolution of mass spectra may also be important when comparing mass spectra with chemometric tools (Paper III). In this process it is important to correlate peaks that correspond to a certain compound, even though the assigned *m/z*-value may vary between mass spectra. As a comparison, mass spectra were recorded by an ion trap and a quadrupole analyser in Paper III. In the ion trap, the spectrum gained had a mass resolution of around 3 000 FWHM, compared to the quadrupole where the corresponding figure was around 700. The ion trap spectra were thus easier to compare and to interpret.

Mass analysers can handle transmission of ions with *m/z* values up to a certain level, e.g. 3 000 for quadrupoles and up to 10^5-10^6 for FTICR and TOF analysers. Macromolecules of interest in life science, e.g. proteins, often have masses >10 kDa but is possible to analyse on a quadrupole due to multiple charged ions[49], when ESI is used. The developed method should be as simple and robust as possible. In most cases, analysis can be performed with unit mass resolution below *m/z* 600, and then a quadrupole or an ion-trap mass spectrometer, which are the two most common MS in laboratories, are good alternatives.

4. Integrated liquid micro-flow sampling

When following chemical processes, both at small and large scale, it is advantageous to use micro-flow systems for sampling of small volumes followed by on-line analysis. Such systems provide high frequent sampling, rapid feedback, facilitate sampling from hazardous areas and reduce manual sample handling.

Today, on-line real-time monitoring is becoming more and more important for *in-vivo* analysis[3]. A rapid screening of biological fluids bedside to the patient may provide valuable feedback concerning the patient's status during e.g. surgery and intensive care[3]. For this reason, there is an increased need for identifying biological markers that correspond to certain events or disorders in the body. Thereafter, analysis and detection methods for measuring these markers must be developed. The analysis can be more or less advanced, either as internal measurements via fixed sensors or as external measurements via on-line analytical techniques, e.g. separation systems. A sensor is generally small and is easier to incorporate into a stream of sample matrix than an analytical technique[50]. On the other hand, the need for simultaneous measurement of the change in concentrations of different compounds creates a demand for analytical techniques with high separation efficiency. In addition, an internal standard for background control can only be added to an external system. For small-scale processes, the sampled volume needs to be small, typically nL to a few μL, to avoid depletion of the sample source, which in turn implies use of a sensitive detection method, e.g. MS detection. Nowadays, the same strategy is used for quality assurance in process industry[9,51], and for monitoring of environmental processes[52].

The sampling procedure, including hardware and methodology, is probably the most critical step for on-line monitoring of change in a chemical pattern. At present, there is also a lack of suitable sampling systems. For life science, the sampling corresponds to how to collect a representative sample from the body and to transfer it into an analytical system. Shunts that perform splitting of a small part of a body fluid, e.g. whole blood[53], into an analyser have been described in the literature. This saves a considerable amount of time, but requires instrumentation that can handle such complex matrix without clogging or discrimination of certain compounds. Among the sampling tools that can be used for on-line monitoring in life science analysis, microdialysis is probably the most

promising. It allows continuous sampling of possible biomarkers from the body without withdrawal of body fluids and with the ability to add an internal standard. This makes it suitable for long-term monitoring and provides concentration profiles of analytes. Below, this technique is described in more detail.

4.1 Microdialysis sampling

Microdialysis is an analytical technique for local sampling of unbound molecules in extracellular fluid (ECF) in living tissue. The basic principle is to mimic the function of a capillary blood vessel. A probe containing a thin dialysis membrane, implanted into the body (*in vivo*) or immersed in a sample (*in vitro*), is perfused with a physiological fluid. During the sampling period, molecules diffuse back and forth over the membrane. The out-coming fluid, the dialysate, is analysed and reflects the variation in chemical composition at a local region. The principle is visualised in Figure 3. Reviews concerning microdialysis sampling give further details of the technique and its theory[54,55,56].

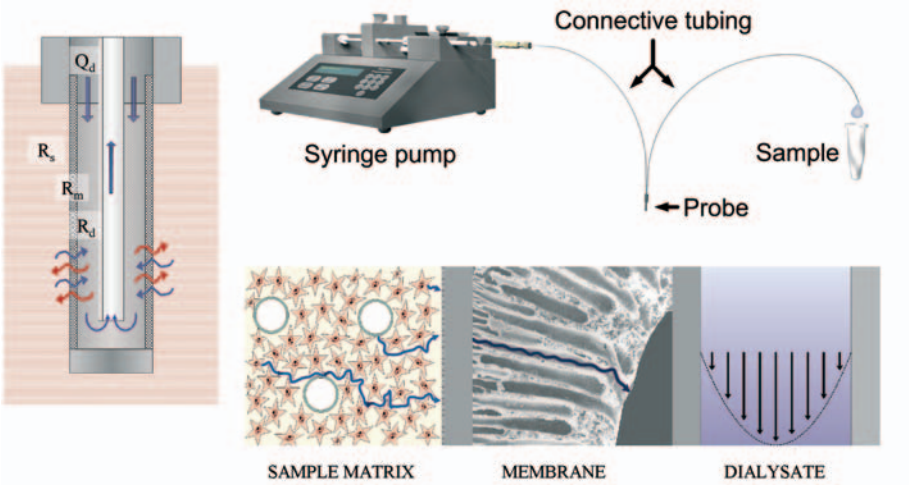

Figure 3. The principle of microdialysis. In microdialysis, a probe is perfused with a physiological salt solution (upper right). The probe consists of a hollow fibre membrane and connecting tubing, and compounds diffuse over the membrane to establish equilibrium between the perfusate and the surrounding fluid (left). Resistances from the sample matrix (R_s), the membrane (R_m) and the dialysate (R_d) restrict the recovery of a substance (lower right). *(Figures by Andreas Dahlin.)*

Microdialysis was developed from early versions of implanted "dialysis sacs"[57] and "dialytrodes"[58] to "microdialysis probes" with integrated hollow fibres[59,60] and has now been a tool in neuroscience for 30 years[61]. Today

microdialysis is widely applicable to *in vivo* sampling in tissue, organs and blood in both animals and humans[62] e.g. for sampling of peripheral markers[63], drug investigations[64,65], and cancer research[66]. The technique has also been useful in other areas such as in environmental research[67]. Furthermore, it is also useful as sample preparation technique *in vitro*, since large molecules such as proteins and lipids are excluded and only small molecules can selectively diffuse over the membrane. This makes the dialysate relatively clean and possible to directly inject into a number of analytical systems. This also implies that microdialysis is a tool designed for determining free drug concentrations in plasma samples (Papers I and II) [64,68], as further discussed in Chapter 6.

In microdialysis only a small part of the total amount of a compound is extracted from a local sampling area. One of the most important questions is how to relate the concentrations in the dialysate to the true concentrations outside the sampling probe. Commonly, the concept of extraction efficiency (E_d) (also called concentration recovery, relative recovery or just recovery) is used. The extraction efficiency is defined as the relationship between the amount recovered and the actual concentration, and can be related to different parameters as described in Equation 1[69,70]

$$E_d = \frac{C_{out} - C_{in}}{C_\infty - C_{in}} = 1 - \exp\left(- \frac{1}{Q_d (R_s + R_m + R_d)} \right) \qquad (1)$$

where C_{out} is the concentration of analyte in the dialysate, C_∞ the concentration in the sample matrix and C_{in} the concentration of analyte in the perfusate (usually zero). The extraction efficiency depends on Q_d, which is the flow rate of the perfusate, and on R_s, R_m and R_d, which are the transfer resistance through the sample space, the dialysis membrane and the dialysate, respectively, as depicted in Figure 3. The different resistances are dependent on, the distance for the molecule to diffuse (Δr), the effective diffusion coefficient in the media (D_{eff}), the surface area (S), and the volume fraction of the media (ϕ) as described in equation 2[70].

$$R = \frac{\Delta r}{D_{eff} S \phi} \qquad (2)$$

The different factors affecting each parameter in Equation 1 are summarised in Table 2.

Table 2. *Factors influencing the parameters that regulate relative recovery in microdialysis.*

Parameter	Factors
Q_d	Perfusion flow rate
R_s*	Diffusion of substance within sample medium Rate of metabolism Uptake of analyte into cells Extent of vascularisation Probe geometry Flow rate of sample
R_m	Diffusion of substance within the membrane Probe geometry Molecular weight cut-off value/pore size/volume fraction Chemical interaction; analyte and membrane
R_d	Diffusion of the substance in the perfusion medium compared to the perfusion flow rate Probe geometry Composition of the perfusate Chemical interaction; analyte and tubing

*For *in vivo* sampling in tissue, R_s is the dominating resistance but for *in vitro* sampling, utilising stirring, this parameter can be neglected.

As expressed in Table 2, many resistances are affected by probe geometry. In this work, in-house produced (Paper I), as well as commercial, microdialysis probes (Papers II and III) have been used *in vitro* (Papers I and II) and *in vivo* (Paper III). The advantage of in-house manufactured probes is their low cost, but more important, the versatility to construct probes for specific applications[64]. Probes of in-house construction were optimised with respect to probe design, membrane properties and connecting tubings in order to increase the recovery of the model drug ropivacaine. The parameters considered were membrane material (polyamide, polycarbonate, polyamidsulphone [20 kDa cut-off] were tested), perfusion flow rate (0.1-2.0 µL/min were tested), probe geometry and dialysis tubing. Unwanted surface adsorption of analyte to tubing is difficult to control, but was in these studies minimised by adjusting the dimensions to obtain a short contact time. The relative recovery increased, as expected, at lower flow rates, due to longer equilibration time. An acceptable recovery of 90 % of ropivacaine at a concentration of 0.2 µM was achieved using a 14-15 mm long, 0.5 µm i.d. polyamide membrane and a flow rate of 0.5 µL/min. This flow rate was also applied to a commercial probe with a polyamide membrane length of 20 mm (Paper II). This probe was of medical grade to enable possible *in vivo*

applications and had a reproducible recovery of 50 % of ropivacaine at 10-nM concentration.

In addition to the parameters in Table 2, temperature, perfusion liquid composition, pressure differentials, stirring and type of analyte will also influence the recovery. For many experiments, accurate calibration of the microdialysis probe is not necessary, e.g. for relative changes in concentration, but for absolute quantification of an analyte; an accurate recovery is needed. The recovery is also important when deciding which analytical system to use. In practice, the recovery is easily determined *in vitro* by comparing the dialysate concentration with a known sample concentration. Sometimes this value is used to estimate *in vivo* recovery, but generally more sophisticated methodologies, e.g. the no-net-flux or the retrodialysis method are used[54,56,69]. In the no-net-flux method, the analyte is added to the perfusate at different concentrations and its loss or gain is determined and in the retrodialysis method, an internal standard is added to the perfusate and its delivery is assumed equal to recovery of analyte. Among these methods, the no-net-flux method is considered most accurate even though it also takes a longer time to perform. In Paper III, an uncalibrated probe with a 10-mm long polyamide membrane was used *in vivo* for sampling in rat liver. Since the aim of this study was to screen for unspecified analytes, it was not possible to perform calibration of the probe. Instead, the recovery was considered as one of the parameters that contributed to the chemical pattern achieved.

4.2 On-line coupling of microdialysis to analytical techniques

The superior advantage of microdialysis sampling is the possibility to couple it on-line to an analytical system. An on-line coupling is, in one way, straightforward since proteins and other large compounds, which tend to clog the separation column, already are excluded from the microdialysate. On the other hand, the inherent high salt content in the microdialysis perfusate must be compatible with the analytical technique. This might require a desalting procedure. Microdialysis sampling coupled on-line to micro-separation techniques has been reviewed by Davies and Lunte[71] and the general principle for this is described in Figure 4.

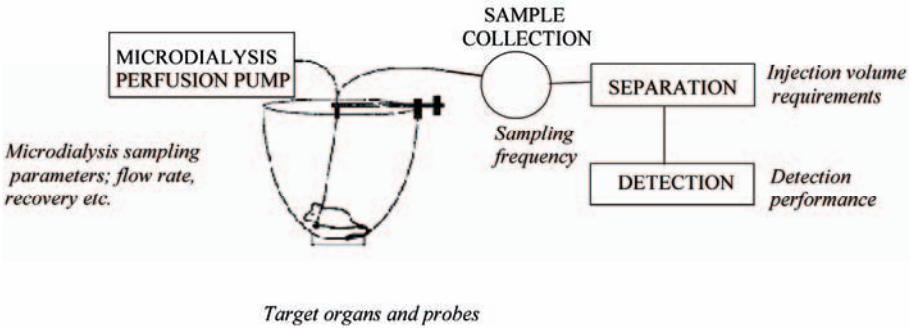

Figure 4. Schematic picture of microdialysis sampling coupled on-line to an analytical technique. The microdialysis probe can be implanted into the target organ, here symbolised by a rat, and samples are then collected in an unit coupled on-line to the following separation and detection steps.

Except when a continuous sensor/detector is used, the continuous flow of dialysate from the microdialysis probe has to be converted into discrete samples matching the analytical separation technique. Results from microdialysis studies thus represent "average" concentrations of compounds during the time interval sampled, commonly ranging from 5 to 20 minutes. How frequent samplings should be performed depends on the process under study. For example, synthesis of new proteins in the body takes around 4 h and then a sampling every 20th minute or less would probably be enough. On the other hand, fast processes, e.g. a nerve impulse taking place in a few milliseconds, could also be interesting to monitor, and hence the sampling frequency and subsequent analysis needs to be extremely fast to resolve such an event. The rate-limiting process in e.g. tissue sampling is mass transfer through the sample matrix. The temporal resolution of a microdialysis study is determined by a combination of the perfusion rate through the probe, the sample volume requirement and the speed of the analytical technique[64]. The on-line coupling may hamper the high resolution gained from the microdialysis if a large injection volume or a long analysis time is required. Methods used should thus be able to handle small sample volumes, typical nano- to microliters. As an example, a typical LC assay requires 5-10 µL samples for analysis, which corresponds to a temporal resolution in the microdialysis sampling of 5-10 minutes if a perfusion flow rate of 1 µL/min is employed. In addition, during the time required for transferring sample from the injection loop onto the column, chemically interesting information might be permanently lost, if not a dual-loop system is constructed. Increasing the flow rate in the microdialysis sampling is not an option, since that would lower the recovery. Instead, separation systems need to be adjusted to small sample volumes and fast analyses. Miniaturised chromatographic and electrophoretic systems, e.g. µLC (i.d. 50 µm-1 mm)[72,73,74], nano LC (i.d. 25-50 µm)[75,76] and rapid CE [77,78,79]separations are

commonly employed with microdialysis since they can handle small sample volumes without dilution[8]. The need for downscaled methods to integrate with microdialysis has resulted in fast chip-based sensors[80] and CE microchip systems[81].

In this work, microdialysis has been combined with LC columns of 0.2-mm i.d. (Papers I-III). The temporal resolution in the microdialysis sampling was not the critical point in these experiments since the experiments were performed *in vitro* and were not related to a progressing event. Sampling could be performed every 20 or 25th minute, which corresponded to the total analysis time since the next sample filled the loop while the previous was analysed (Papers I and II). The on-line approach was found to be very useful since it required less manual sample handling. In addition, higher recoveries and better reproducibility was achieved compared to off-line sampling (Paper II), as depicted in Figure 5. A prerequisite for robust on-line microdialysis sampling is that there is no backpressure from tubing or the analysis system that could cause fluid loss over the semi-permeable membrane[69]. In Paper I, it was necessary to minimise restrictions from tubings and filters in the LC injector.

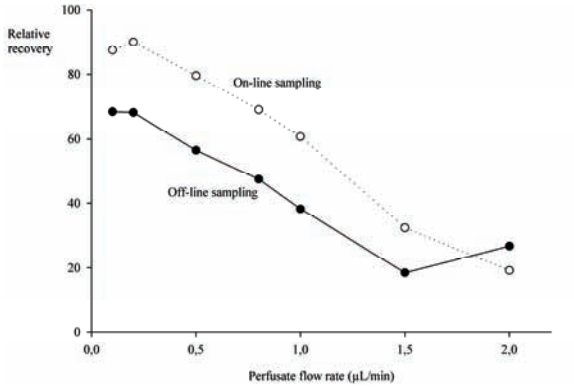

Figure 5. Comparison of off-line and on-line sampling of ropivacaine by microdialysis. Results are summarized from Paper II.

The concentration of analytes in microdialysates from biological samples will span over several orders of magnitude, i.e. from µM levels of e.g. glucose and amino acids to pM levels of e.g. neuropeptides and catecholamines. A low concentration in combination with a small sample volume requires a sensitive detection method; e.g. laser induced fluorescence (LIF)[77], electrochemical detection (ECD)[80] or MS detection[74,75,76]. MS detection provides a selective answer and can resolve coeluting, non-isobaric, peaks from a separation. An on-line coupling of microdialysis and MS detection is not straightforward since the perfusate, as mentioned in

Chapter 4.1, has a natural high salt content, which implies ion suppression in the mass spectrometric ionisation process[82]. Most on-line couplings of microdialysis to MS detection thus involve a desalting step, i.e. the samples are loaded onto a pre-column (Papers II and III) or directly onto a separation column[74,75,76] and washed with a liquid phase with low elution strength before the actual analysis. In this step, it is also possible to pre-concentrate the sample.

To control response variations in the ESI-process, and in the total analytical system, it is advantageous to use an internal standard. This might be difficult when coupling the microdialysate flow on-line to the analytical instrumentation, but with a coupled-column system it is possible to externally add standards. An additional valve, to which the internal standard is loaded, can be placed between the sample loop and the desalting pre-column/separation column (Paper II). In this way a constant amount of internal standard, preferably deuterated analyte (or by other means isotopically labelled), is incorporated in the analysis each time, which verifies the functionality of the system.

5. Integrated liquid micro-flow separations

Many analytical problems require more information than what a single separation step coupled to a conventional photometric detection, e.g. LC-UV, can provide. To increase the power of the system, separations are often integrated to MS detection[83]. This adds selectivity to the detection and may also provide structural information about the analytes. A single liquid separation step, e.g. LC or CE, can easily be coupled to ESI-MS detection. To further increase the resolving power of the system, several dimensions of separation can be incorporated, in multidimensional systems, where aliquots from the first separation/dimension are injected to the next separation/dimension. Performing this in an on-line system provides several possible advantages, e.g. automation, minimised sample loss, protection of analytes, decreased total analysis time, higher repeatability and increased sample throughput. Systems that combine multidimensional separations and MS detection thus become especially powerful. On the other hand, more complex equipment and more extensive method development are required for on-line combinations. For efficient systems, compatibility of buffers and solvents between the different separation steps, and between the final separation step and the ESI-MS detection, are needed. Matching of flow rates in the different steps also requires consideration, as well as, control of possible current leakage, which can be produced when integrating electrical potentials to an on-line system. Finally, the development of accurate and high frequent injections needs a lot of effort. The principle of a two-dimensional liquid separation, using coupled columns, is visualised in Figure 6.

Today, a number of on-line liquid based multidimensional separation systems have been reported, e.g. LC-LC, LC-CE and CE-CE systems[84,85,86,87,88]as a complement to the traditional techniques such as two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)[89]. The decreased off-line sample handling in the liquid based system is a real benefit compared to 2D-PAGE. Many applications of on-line systems have also been coupled to ESI-MS detection, mainly for proteome studies[90,91,92,93,94] but also in the pharmaceutical area[95].

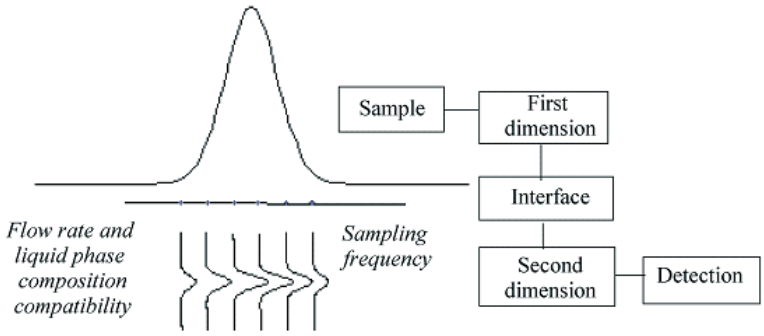

Figure 6. A schematic picture of a two dimensional separation using coupled columns. The sample is first injected onto the first column and an aliquot of the eluate from this column is then transferred via an interface to the second column, followed by subsequent detection. Compatibility of flow rates, liquid phase composition and sampling frequency is important to control.

5.1 Multidimensional liquid separations

There are mainly three main concepts of concern when constructing an on-line multidimensional separation system; resolution in separation*, analyte transfer between dimensions, and evaluation of the capacity of the system. The highest resolution is gained when there is no correlation between the combined separation mechanisms, i.e. the dimensions are orthogonal to each other. Some examples are the combinations of isoelectrical focusing and size exclusion in 2D-PAGE[89] or hydrophobicity and charge/size in LC and CE[85].

A critical point is also how to inject analytes from one dimension to the next. Different strategies and possibilities can be identified where all, or equal percentages of all, components (comprehensive) or selected components (heart-cut) of the first dimension is transferred into the second dimension. Qualifications for a comprehensive 2D-system[96,97,98], include fulfilment of the following requirements:

I Every part of the sample should be subjected to two different separation mechanisms.

II Equal percentage of all sample components should be subjected to two different separations and eventually reach the detector.

III The separation/resolution obtained in the first dimension should be essentially maintained.

* The resolution in separation (R_s) between two peaks is defined as the difference between the two retention times (t_R), divided by the arithmetic mean of the two peak widths (w)[5]: $R_s = 2(t_{R2}-t_{R1})/(w_1+w_2)$.

To maintain the resolution achieved in the first separation, sufficiently frequent sampling of the first dimension into the second dimension is required. As depicted in Figure 6, several samplings of the peak in the first dimension into the second dimension are required for accurate characterisation of the first separation. Sampling frequencies resulting in a minimum of 3-4 second dimension analyses per peak in the first dimension are recommended[10,97,99]. Sampling intervals substantially longer than the widths of the peaks emerging from the primary column will lead to loss in primary column resolution. Fast, or parallel, separations in the second dimension are thus required for comprehensive analysis. On the other hand, too high sampling frequencies may limit the time available for secondary separation and thereby also the total gain in resolution[97].

The enhanced performance of a multidimensional system can be described by dramatically increased resolving power, since the overall peak capacity (n_{tot}) is the product of the peak capacities for each individual resolving step (n_1, n_2,...), as described by equation 3[96]. This is seldom achieved due to random distribution of peaks in each dimension; the actual resolved number of compounds is substantially smaller[100].

$$n_{tot} = n_1 \cdot n_2 \cdot \ldots \qquad (3)$$

Peak capacity describes how many peaks that theoretically can be separated in a system of a certain resolution[101]. The calculated peak capacity (n_c) is often achieved by dividing the time for separation (L) by the average peak width (w) for each separation, as described in equation 4.

$$n_i = \frac{L_i}{w_i} \qquad (4)$$

RPLC with gradient elution, which is considered as the most powerful liquid chromatographic one-dimensional column technique, possesses a peak capacity of about 200[86,102] and a predicted maximum achievable peak capacity of 1 400-1 600[102]. The dominating two-dimensional system used today, planar 2D-PAGE, can ideally reach a peak capacity of around 10 000[103] and it is in practise capable of resolving around 2 000 proteins per gel. To reach these high capacity values by column separations is a real challenge, but it is important in order to compensate for the drawbacks of 2D-PAGE; limited analyte selection, contamination and background as well as automation problems. Other shortcomings, usually related to this technique, such as manual chemical handling, risk for contamination and staining with hazardous reagents are also diminished with column technology. Coupled LC-LC, LC-CE and CE-CE systems can thus be good

alternatives. A mathematical model predicts that current 2D-LC systems using gradient elution can achieve a peak capacity of 5 000, or 15 000 in 8 h[102]. On-line column based LC-LC approaches, e.g. ion exchange chromatography-RPLC have reported estimated peak capacities of ~500[104] to ~3 000 (the multidimensional protein identification technology [MudPIT] approach[105]). LC-CE systems resolve around 400 peaks (n_{tot} around 15 000) [106], and the LC-CE systems in this work have separation peak capacities of 48 and 100, Paper V and Paper VII, respectively. For on-line CE-CE, using capillary based separations of capillary zone electrophoresis (CZE) and capillary gel electrophoresis (CGE), peak capacity of 780 has been calculated[107]. For chip based CE-CE separations, combining micellar electrokinetic chromatography (MEKC) and CZE, an estimated peak capacity of 4 200 have been reported[108]. It is still true that further improvements in this area are needed if 2D-PAGE is to be challenged. On the other hand, many of the column-based alternatives can be coupled on-line to ESI-MS detection, which instantly provides identification or at least characterisation of compounds.

It is also possible to use MS detection as an additional separation technique[105,109], in the sense that multiple components can be selectively detected. It will then be questionable how to calculate the peak capacity for this system. Two different strategies are reported in the literature; a) to use the number of spectra acquired by the MS during an LC or CE peak[105] or b) to use the resolution in the mass spectra[†109]. The MudPIT approach, mentioned above, thus achieve a peak capacity of 23 000[105] using the a)-alternative, and an approach using FTICRMS detection achieved a peak capacity of several millions[109] using the b)-alternative. Depending on strategy, a large variation in calculated peak capacity (over six orders of magnitude) for one and the same system can be achieved (Paper VII). It is thus important to declare what strategy that is used when comparing systems.

System performance in terms of peak capacity should also be related to how long time it takes to gain a certain peak capacity. The concept of peak capacity per unit time was therefore introduced for a more relevant comparison (Paper V). As an example, the peak capacity of gradient RPLC increases when using more shallow gradients, i.e. introducing a longer analysis time[102]. The question is if the overall system performance really is improved. 2D-separations on chip[110,111,112], with relative high peak capacity achieved in a short time (5-10 min total time) may instead be more advantageous.

[†] The resolution in a mass spectrum is defined as mass of an ion peak (m) divided with its width at half maximum (FWHM); (Δm); $m/\Delta m$ [4].

In this thesis on integration of different on-line separation techniques, focus has been put on on-line LC-CE (Chapter 5.2) and its coupling to sheathless electrospray ionisation (Chapter 5.3). The following section therefore discusses aspects of such a system.

5.2 Aspects of on-line LC-CE-MS

When interfacing a pressure driven flow in LC with an electrical driven flow in CE, there are many parameters to consider, especially if subsequently integrating to sheathless ESI-MS detection. Many important parameters were already identified by Jorgenson and co-workers when developing the first LC-CE interface in the early 1990's[113] and the following "transverse flow gated" interface[114] and "optically gated" interface[115] during the 1990's. Table 3 summarises the parameters, requirements and approaches studied in Papers IV, V and VII, resulting in interfaces A, B and C, respectively, depicted in Figure 7.

Table 3. *Important parameters in development of an LC-CE interface with ESI-MS-detection.*

Parameters	Requirements	Solutions/Developments
Flows	Independent Controlled	Two-levelled interface with an independent CE electrolyte flow. Compatibility of flow rates and volumes.
Volumes	Low dead volumes	Direct connection of capillaries into the cast structures.
Material	Chemically inert Non-conducting Non-adsorbing	Elastomeric and insulating poly(dimethylsiloxane) modified with APS (B) or PolyE-323 (C).
Electrochemistry	Electrical contact Stable CE current	Polymer imbedded graphite electrode integrated on CE column (A,B) or outside the interface (C). Low current density in the interfaces.
Injections	Defined Reproducible	CE electrolyte on/off by a manual switch (B) or by a pressure controlled injection unit (C)
Buffers	ESI-compatible	Low ionic strength, organic modifier, volatile buffer components

The large difference in peak volume in LC and the injection volume in CE makes the on-line coupling more difficult than e.g. integrated LC-LC or CE-

CE. In addition, decoupling of the LC and CE flow is of major concern for a well performed interface. A flow of CE electrolyte is commonly used to provide fresh CE electrolyte and to facilitate distinct injections. Compared to the earlier developed interfaces[106,114], in which hundreds of µL/min of CE electrolyte was forced through the interface, about 2 µL/min was sufficient in the new designs. This improvement may be a result of the two-levelled design used in interfaces A, B and C.

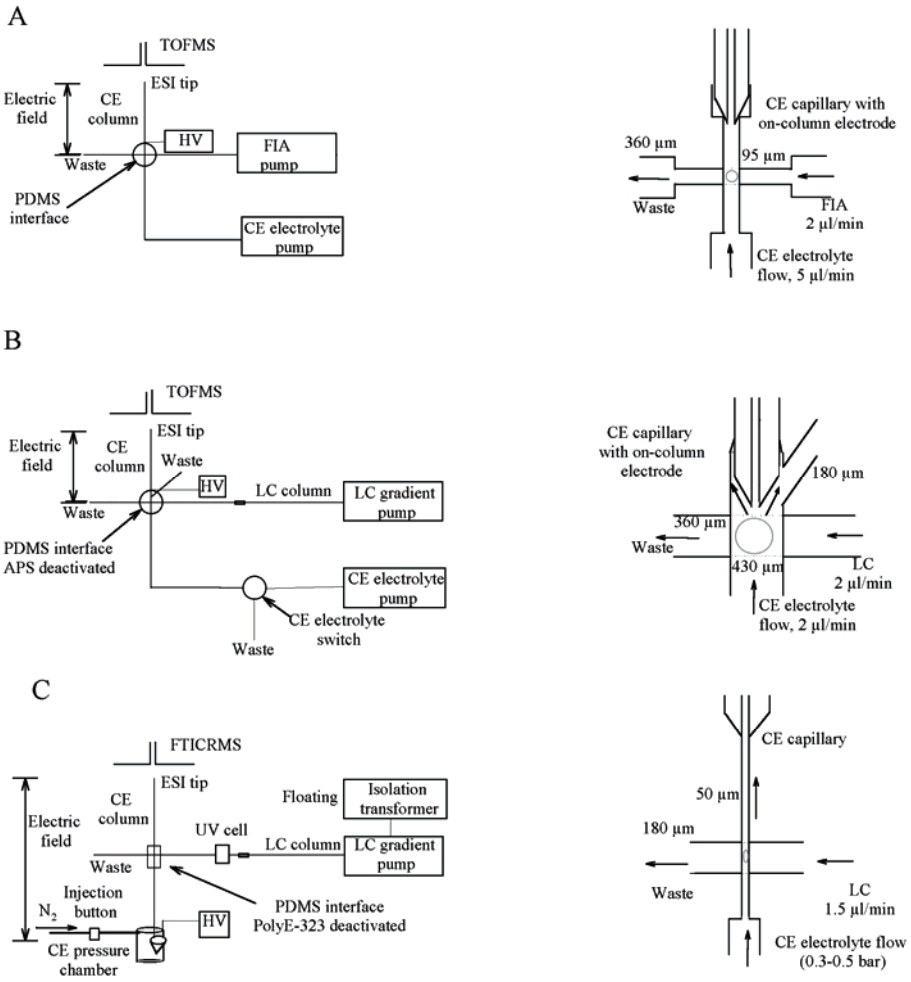

Figure 7. Comparison of the three developed LC-CE systems and interfaces (A-C, left) and the corresponding flow patterns within these interfaces (right). HV means high voltage.

The two-levelled design also simplifies the construction of the interfaces. Fused silica capillaries or metal wires of suitable dimensions were simply crossed on top of each other in a mould, after which unpolymerised PDMS was cast. When the PDMS had been cured in an oven, the templates were pulled out leaving a defined channel system with impressive repeatability of the opening hole between the two levels. This strategy was found to be very useful for fast production of new structures and has also been used for microchip production[40,41]. Fused silica capillaries were easily connected to the PDMS channels with a tight fit, providing low dead volume connections in the interfaces. Other groups have also reported combinations of hard fused silica technology and hard chip-structures[116], as well as the combination of LC and on-chip electrophoresis[117]. As an additional advantage, the PDMS material is an elastomeric and transparent material, which facilitates easy probing of problems. It is also compatible with the commonly used solvents in both LC and CE, and insulating against electrical current. As a drawback, this material is prone to adsorption of hydrophobic substances[118], a feature that is easier to control with previously reported interface materials; stainless steel[113,114,115] and transparent polycarbonate[106]. In a first attempt, the siloxane material was first modified using 3-aminopropyl-trimethoxysilane (APS)[119], a reagent used for silica surfaces, to reduce the adsorption (Paper V). This modification was not satisfying, since it still showed adsorption when injecting compounds like the basic peptide substance P. A new way of surface modification, using the cationic polymer PolyE-323[120], was thus investigated. This polymer was found, in radionuclide imaging and CE experiments, to substantially reduce the adsorption of peptide and proteins to the PDMS surface (Paper VI). In addition, this polymer was applied to both fused silica and PDMS surfaces in one and the same fairly simple coating procedure. PolyE-323 was utilised to reverse the electroosmotic flow (EOF) in the fused silica CE column, at the same time as it prevented adsorption of peptides to the PDMS interface (Papers VII, VIII) and to PDMS in micro-chip structures[40,41].

Another important aspect of on-line LC-CE is how the electrode is used at the coupling side of the CE and how the voltage is applied. The electrical contact has been applied to the LC-CE connections in different ways, most commonly by grounding the interface and by letting the whole interface act as an electrode[113,114,115]. An alternative is to use an on-column electrode for high voltage (HV) control in the interface (Papers IV, V). The electrode position relative to the inlet end of the CE column is then constant, and in addition, no additional entrance for a separate electrode to the interface is needed. The material of the on-column electrode must be stable, and not flake off, when exposed to high potentials. The developed electrode was, by electrochemical tests, found stable in both oxidative and reductive mode. Using an on-column electrode in a small electrolyte volume may cause electrolysis of water, involving gas bubble production. Gas bubbles disturb

the CE current if present in the column. This tendency was especially observed in positive mode, and in Paper V, a negative potential was used and the current density was reduced, by an extended electrode surface and by CE electrolytes with low ionic strengths. Another alternative was to apply the high voltage to a vial outside the interface (Papers VII, VIII). A conventional metal CE electrode was used, and the applied high voltage created an electric field over the total distance of the connecting capillary, the PDMS interface and the CE column. This approach provided a distinct point of high voltage in the interface, but decoupled the possible electrolysis of water from the LC-CE interface.

Injection from LC to CE is another important parameter to consider. Since the elution volume from the LC step mismatches the sample volume for CE, a restricted part of the LC effluent is commonly split and injected in the CE either electrokinetically[106,113,114] or by optically gated injections[115]. Automated injections in LC-CE have been reported by Jorgensen and co-workers[106,114] where a lower injection potential, compared to the separation potential, is used. A lower injection potential is required to not overload the CE column. To adequately sample the LC separation, the sampling frequency into the CE needs to be fast, e.g. for a 1 min LC peak, a CE separation of around 25 seconds is needed in order to sample the LC peak at least 3 times[97,99]. The injection from LC to CE became the limiting factor in the systems considered in this thesis and it was also found that the sampling frequency needs to be improved. Injections could not easily be performed in a fully automated manner and electrokinetic injections had to be performed at the selected separation potential. In an initial test, injections were performed using an overpressure in the waste line (Paper IV). More convenient was to use the CE electrolyte flow to regulate the injections while the high voltage was maintained (Paper V). This provided repeatable injections but the injection volume was still too large. Even more convenient and safe was to control the CE electrolyte by an overpressure regulated by a timer adjusted on/off switch (Papers VII, VIII). This further improved the repeatability, but the injections, had to be performed at the separation voltage. Peptides from digested bovine serum albumin (BSA) were used to test the transmission, in the sampling procedure, of analytes from LC to CE. Satisfying results were achieved when using the LC-CE-FTICRMS system, Figure 7C, (Paper VII), since 90 % sequence coverage was achieved.

Structures developed for LC-CE have nowadays also been found in other applications, e.g. coupling of RPLC to capillary electrophoresis immunoassays using the “flow gating” interface[121,122]and coupling of 2D-CE[123] using the two levelled cross design in PDMS developed in this work (Papers IV, V, VII).

New trends for on-line LC-CE are miniaturisation down to microchip structures, as well as its coupling to MS detection[124,125,126]. In this area, a flexible and multifunctional structure of PDMS has been constructed (Paper

VIII). The structure contained a similar cross-structure to that in Paper VII. In addition, it had the CE and a desalting solid phase extraction (SPE) bed incorporated in the microchip structure. The SPE bed consisted of 5 µm hyper cross-linked polystyrene beads, which were easy to pack into the structure and had a high loading capacity for peptides. The SPE beads were excellent for concentrating and desalting peptides in physiological salt solution, resembling e.g. the situation after microdialysis in brain tissue. At the end of the CE column, an integrated ESI-emitter was shaped directly within the microchip substrate in order to facilitate subsequent MS detection. On-chip CE separations have been adapted to ESI-MS[127,128], and also a few reports describe packed microstructures with integrated ESI emitter[129,130,131]. The combination of both is thus rather unique. The challenge when combining SPE and ESI-MS functions on a microchip is to remove the sample matrix from the washing step without contaminating the mass spectrometer. With the two-levelled design, sample matrix was diverted to waste in a separate plane from the liquid reaching the ESI-MS (Paper VIII). This was advantageous since optimisation of the CE separation and the ESI could be performed at the same time as the desalting step. In addition, the optimised conditions were maintained when injection plugs from the elution profile was transferred into the CE channel. Femtomole amounts, corresponding to a detection limit of 100 nM, of neuropeptides could be detected with this system.

5.3 Integrated separations coupled to MS detection

Today, a number of methods for determining pharmaceuticals and endogenous substances are developed for UV-detection. It is generally believed that an analysis based on MS detection, instead of UV, provides a more selective and sensitive result. Co-eluting peaks can be identified and detection of a specific ion will lower the detection limits. In contrast, a change to MS detection requires consideration of a) the ionisation compatibility of the buffers used for separation and b) the separation performance using an ionisation compatible buffer[132]. The result is often a compromise.

Liquid separations coupled to sheathless ESI-MS should be performed using volatile buffers, e.g. exchanging phosphate and borate buffers for ammonia acetate or formate, and to add an organic modifier, e.g. methanol or acetonitrile, to reduce surface tension, increase volatility and lower conductivity. Buffer concentrations of 10-20 mM[133] are suitable. It is also helpful if the analyte can be ionised already in the liquid buffer, e.g. by choosing a suitable pH for a protolyte or by association to an ionic compound[23]. Commonly used ion-pair agents in reversed phase LC, e.g. trifluoroacetic acid for separations of polar compounds can be difficult to

integrate in the system, but there are volatile alternatives that can be used instead[134]. Sheathflows can be useful for adding an organic modifier between the separation and the electrospray ionisation in order to optimise both processes. On the other hand, a sheath flow interface introduces dilution of the eluting compounds and adds complexity to the system.

All LC applications in this work have been performed using a volatile buffer of formic acid, at pH 3 and an ionic strength of 10 mM. This was used to ionise the model drug ropivacaine in the liquid phase before MS (Papers I and II). In the LC-CE systems, the LC liquid phase was generally based on acetic acid at low pH, for separation of peptides, and at low ionic strength to avoid introduction of salt into the CE-MS detection.

Buffers like phosphate and borate, at high pH and at relatively high ionic strengths, are often used in CE separations to achieve highly efficient and fast separations. Such buffers have earlier been used in on-line LC-CE systems[113,106]. Because of their non-volatile properties, they are difficult to use with MS. For peptide separations by CE-MS, the EOF is commonly reversed by applying a positive surface inside the capillary, in contrast to the negatively charged walls in unmodified fused silica capillaries. In this work, the EOF of the CE separation was reversed by the surface treatments APS (Paper V), or by PolyE-323 (Papers VII and VIII). A negative injection potential and a positive detection potential (same as the ESI potential) produced an EOF towards the MS detector. For on-line LC-CE, a CE electrolyte composition consisting of 10 mM acetic acid and acetonitrile was used for different reasons. First, this ESI-enhancing liquid composition provided an EOF sufficiently high for sheathless ESI and thereby also rapid separations. Secondly, the low pH yielded positively charged peptides already in solution, which facilitated the ionisation processes.

6. Towards an on-line monitoring of active chemistry and chemical pattern realising early diagnosis

6.1 Monitoring of free fraction in biological fluids

Today, the interest for monitoring the active chemistry, i.e. the effect-inducing compounds, in biological systems is dramatically increasing and in this discussion, the drug-protein binding is of significant importance. It is generally known that drugs bind to plasma proteins to different degrees and it is believed that only the unbound, free drug is pharmacologically active[135]. This non-covalent drug-protein complex has different binding sites as well as different time-constants for dissociation. The main plasma proteins involved in drug-protein binding are albumin and α_1-acid glycoprotein (AGP), but binding can also occur to other large biomolecules like lipoproteins. Parameters associated with drug binding to albumin and AGP are listed in Table 4[136,137].

Table 4. *Parameters for drugs binding to albumin and AGP.*

Protein	Molecular weight [Da]	Normal plasma concentration [g/L; µM]	Net charge at pH 7.4	Drugs attracted
Albumin	67 000	35-50; 500-700	Cationic	Weakly acidic
AGP	42 000	0.4-1.0; 10-20	Anionic	Basic drugs

Compared to drugs with low degree of protein binding, <50 %[136], highly bound drugs have a small distribution volume limited to the volume of plasma. They are also slowly eliminated from the body since the protein-bound fraction is usually not available for the kidneys. The drug-protein equilibrium can be altered in many ways, e.g. by other drugs competing for the same binding site[137,138] and alteration of the protein concentration by e.g. surgery and tissue injury[135], diseases, pregnancy or age[136]. Small changes in the degree of protein binding are especially relevant for highly bound drugs, since the corresponding change in free drug concentration will be relatively large and may significantly alter the pharmacological effect.

The question whether the total or the free drug concentration in a biological fluid or tissue should be measured depends on the situation. To

date, the commonly used procedure in pharmaceutical research is to measure the total concentration. This procedure gives a good estimation of the free and active concentration as long as the degree of protein binding is known and constant over the whole therapeutic range[136]. The free drug concentration is therefore always better to measure, especially when the protein concentrations are expected to vary or competitive mechanisms can be expected.

The basic principle for measuring free drug concentrations is a physical extraction of the free drug without disturbing the equilibrium. The most commonly used techniques today for measuring the free fraction are equilibrium dialysis[135,136]and ultrafiltration[135,136], but also microdialysis has proven to be a valid tool [63,68,139,140,141,142]. Other methods for free concentration measurements are described in reviews by Pacifici[143] et al. and Oravcová[144]. The model compound in Papers I and II, ropivacaine, has been studied by a number of methods including micro-ultrafiltration[145] (Paper I), microdialysis (Papers I and II), equilibrium dialysis[146], solid-phase microextraction[147], small-scale equilibrium dialysis[148] and supported liquid membrane extraction[149]. Sampling from biological equilibrium requires consideration of the time constant of the studied complex. Either the sampling should be a snapshot, faster than the time-constant for the binding, e.g. microdialysis and ultrafiltration or as a steady-state equilibrium of the free drug with an outer volume, e.g. equilibrium dialysis.

In Figure 8, the most important factors influencing the drug extraction are illustrated. Among these, the size of the pores in the membranes, which determine the cut-off value of how large molecules that can be sampled, is very important. In addition, it is necessary to control the chemical properties of adsorption to and pressure gradients over the membrane. Ideally the ion strength, the osmotic pressure, the temperature (37 ºC) and the pH (7.4) of the dialysis buffer should be as close as possible to those of the extracellular fluid studied.

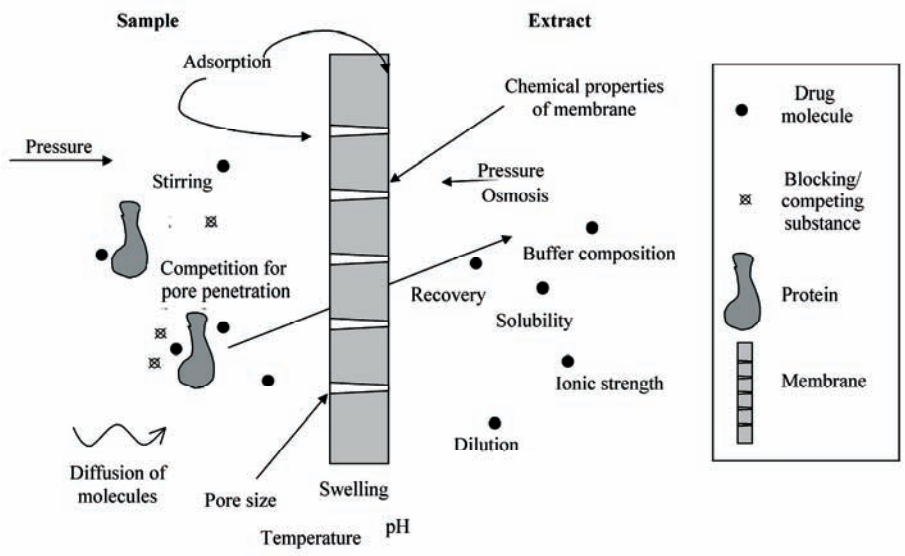

Figure 8. The extraction of the free concentration of a drug from the bound fraction is a physical separation, using a membrane. Examples of important parameters influencing this extraction are illustrated in the figure.

Microdialysis was in this work investigated as a tool to measure free drug concentrations in plasma samples (Papers I and II). The temperature was controlled by a block thermostat and the pH of the plasma sample was adjusted with e.g. acetic acid. Microdialysis was compared to ultrafiltration and was found to give equivalent results for the local anaesthetic ropivacaine (Paper I), as shown in Table 5. The experiments were performed with an on-line microdialysis-LC-UV system. The advantage of microdialysis was the possibility of on-line coupling to the analysis instrumentation, which reduced the manual sampling handling and improved the precision (Chapter 4.2). In addition, microdialysis methods employing probes of medical grades can, as shown by other research groups[150,151], be applied *in vivo* for determination of unbound compounds.

Table 5. *Comparison of ultrafiltration (UF) and microdialysis (MD) using UV and MS detection for determination of free concentrations of ropivacaine.*

Method	Analyte	Spiked concentration [μM]	Mean free fraction ± S.D*. [%]	Reference
UF-LC-UV	Ropivacaine	4.0	6.0±0.3	Paper I
MD-LC-UV	Ropivacaine	4.0	6.1±0.4	Paper I
MD-LC-MS	Ropivacaine	0.2	12±3	Paper II
MD-LC-MS	PPX	0.02	47±5	Paper II

*S.D. = Standard deviation of day-to-day measurements.

Microdialysis was further used for sampling low nM-levels of ropivacaine and was then coupled to mass spectrometric detection to obtain even more sensitive detection (Paper II). With this system it was also possible to quantify S-(pipecoloxylidide)hydrochloride (PPX), a metabolite of ropivacaine, which overlapped with the plasma matrix when UV detection was used. A deuterated internal standard compensated for possible ion suppression in the ESI. A limiting factor for the use of this technique is loss in precision of the microdialysis sampling at lower concentrations, see Table 5. The use of selective monitoring by multiple reaction monitoring in the mass spectrometer lowered the detection limit to 0.1 nM. The lower part of the therapeutic concentration range of ropivacaine was thus secured for measurements.

6.2 Screening for biomarkers in microdialysates

Another way of monitoring the effect-inducing compounds in biological systems is screening for markers that imply a certain activity or disorder in the body. Changes in the chemistry of tissues and organs can often appear before any clinical signs of complications become evident. The challenge is to identify markers that appear early in the progress of disease and measure them by timely analysis. These markers can thus be used for diagnosis and treatment in an early stage[1]. The screening strategy is a new paradigm in life science, and requirements in technical and methodological tools are still not completely defined.

In this work, the following issues have been considered: First, a set of events should be followed in order to compare the chemistry of a normal and an abnormal situation. The samples to study can be urine, blood or microdialysate from any part of the body. Secondly, the sample handling should be as simple as possible and only a small sample volume should be withdrawn, which then require micro-flow analysis techniques. Finally, new biomarkers are expected at low concentrations, requiring sensitivity and selectivity detection, e.g. MS detection.

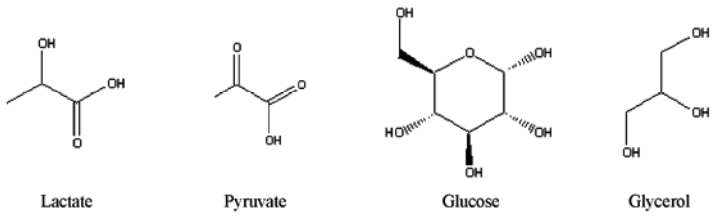

Figure 9. Examples of typically analysed compounds in clinical microdialysates.

Samples collected today for routine clinical investigations, e.g. urine and microdialysis samples posses an enormous unexplored potential. They are usually analysed with respect to a few well-known markers. As an example, commonly analysed markers of ischemia (local shortage of oxygen) and cell damage are glucose, lactate and pyruvate (reflecting carbohydrate metabolism), glycerol (reflecting lipid breakdown), urea, amino acids, purines and ions in microdialysates [152,153]. As depicted in Figure 9, all these compounds are small molecules (molecular weight <200 g/mol), highly polar markers present in the body at μM to mM levels. Beside these already well-known compounds, there is a potential of other biological markers present in the samples that can be explored and utilised for diagnosis and drug development.

Larger molecules are expected to be recovered in microdialysis to a relatively lower extent than those molecules depicted in Figure 9 but still, if they are present in the dialysate they should be detected if a sensitive detection method can be applied. MS analysis of microdialysates has been used for a number of applications and by different research groups[75,76,154,155,156], but then mainly for detection of specified analytes, usually peptides. A new strategy for searching biomarkers in microdialysis samples was presented in Paper III. A pre-concentrating/desalting step and subsequent MS detection was used to screen microdialysates collected from rat livers *in vivo*, before and after an induced stimulation. The analytical method limited the range of compounds that was detected; compounds must possess a hydrophobic character to be retained on the desalting C_{18}-column, and a *m/z* value in the chosen region of 200-1 100. These are totally different characters compared to the general analysed clinical markers, e.g. the amino acid tryptophan was not retained enough on the pre-column, but the peptide leucine-enkephalin was recovered (Paper III). By using the microdialysis technique, samples from control and stimulated local tissue could be collected in one and the same experiments since the stimulator (potassium or paracetamol) was added to the perfusate after the control samples had been secured. Paracetamol is known to be hepatotoxic[157] and deviations in ion composition, e.g. elevated potassium concentrations, have been shown to affect levels of neurotransmitters and drugs in the brain[158].

The results are commonly difficult to interpret, because the answer lacks specificity from the beginning. Instead, a fingerprint (or a pattern), of e.g. *m/z*-values in a mass spectrum, can be compared between control and patient groups. It is then possible to identify what *m/z*-values that differ between groups. If this methodology is clinically applied in the future, an abnormal pattern may be enough to make an early diagnosis of a disease. Eventually, variables may be related to a certain biologically active molecule.

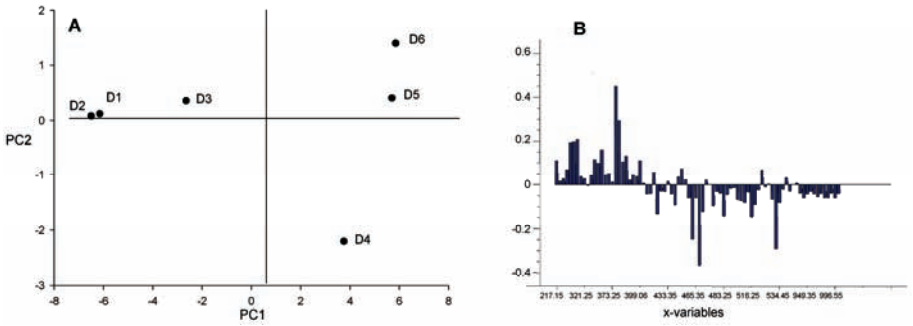

Figure 10. Example of a typical screening of a microdialysate sampling series in rat liver. A) PCA of dialysate 1-6 (D1-D6), where D1-D3 were controls and D4-D6 were taken after stimulation with potassium. B) A loading plot for PC1 correlated to A. Dialysates with positive PC1 values (D4-D6) possessed a mass spectrum with increased intensities in the lower *m/z* region.

The results have so far been focused on finding differences between different sample groups, i.e. before and after stimulation, by using the chemometric tools principal component analysis (PCA) or partial least square (PLS) [2,159]. Similar strategies for screening of biomarkers in other body fluids[160] e.g. urine have been reported[161,162163]. An example is shown in Figure 10, where it was possible to classify the dialysates into groups corresponding to before and after stimulation with potassium. Other experiments showed that it is possible to see an effect of the change of perfusate in the PCA, but this effect seems to decline since all other samples are gathered in one group. Further optimisation of microdialysis experiments, e.g. longer equilibration between surgery and start of sampling, longer sampling series and adding a separation step before MS detection are recommended for pursuing this methodology to need-driven applications like, for example, early diagnosis of inflammation.

7. Concluding remarks and future outlook

This thesis aims at providing novel analytical tools needed for further understanding of life science. It focuses on methods that can be used for determination of active molecules in biological matrices, e.g. plasma, microdialysates or cerebrospinal fluid.

Microdialysis and ultrafiltration are two equivalent tools for determining the free (not-protein bound) concentration of drugs in small plasma volumes. Microdialysis is advantageous since it can be coupled on-line to an analytical system, which enables higher recovery and better reproducibility due to the less manual sampling handling. The coupling of microdialysis to MS detection is very attractive, e.g. as a tool for screening of biomarkers, but requires specific consideration. Within future applications in early diagnosis of disease and organ damage, the presented methodology of combining microdialysis sampling with internal standard implementation, desalting, MS detection and multivariate data analysis is expected to be most useful.

The combination of conventional fused-silica capillary technology and the soft, polymeric PDMS material have, in this work, facilitated integration of orthogonal separations such as LC and CE in low dead-volume on-line systems. By combining the positive sides of both materials e.g. the inertness and robustness of fused-silica and the easy connection and transparency of PDMS, suitable couplings were developed. A two-leveled design introduced in this work enabled independent flow characterization. Aspects of high voltage connection and deactivation of the PDMS were crucial for the functionality of the developed devices.

The detection limits of developed methods have commonly been evaluated for peptides or for the model drug ropivacaine. The lower range of the therapeutic interval of ropivacaine was secured by the coupling to MS detection, where a detection limit of 0.1 nM was achieved. For LC-CE-MS separations of peptides detection limits around hundreds of nM were achieved in these early, prototype systems. Improved detection limits of three orders of magnitude, or more, would be required to further expand the detectable range of the most informative compounds in biological matrices.

Developments in pre-concentration, loading capacity and separation functionality for the developed systems would be the natural next steps for the systems concerned. Also, developments of algorithms to handle large amount of information would need robust and strategic data handling, especially in MS detection.

8. Acknowledgement

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9. Summary in Swedish :

Integrerade mikro-analytiska verktyg för bättre förståelse av biologiska processer

9.1 Introduktion till biologisk analys

Vad driver den biomedicinska forskningen framåt? Idag finns ett ständigt ökande intresse att förstå varför människokroppen reagerar på olika sätt i olika situationer, kanske framförallt i sjukdomsförlopp. Om det kan utvecklas nya sätt att tidigt i ett sjukdomsförlopp hitta markörer som gör att man snabbt kan sätta in behandling, så skulle det vara ett steg i rätt riktning. I dag behöver vi mera information om den aktiva kemin i kroppen, och för att nå dit behöver vi utveckla nya tekniker.

I slutet av förra millenniet kartlades det mänskliga genomet, dvs. den genetiska koden som finns i alla celler. Produkterna som bildas med hjälp av den genetiska koden, peptider och proteiner, samt deras nedbrytningsprodukter är numera högaktuella i bioanalys. Andra viktiga molekyler är läkemedelssubstanser. När läkemedel distribueras i kroppen sker ett antal reaktioner, t.ex. inbindning till proteiner, interaktioner med receptorer för att ge effekt, och tyvärr ibland också interaktion med andra receptorer som ger biverkningar. Många analytiska system utvecklas i dag för att kunna hantera kroppsegna och främmande molekyler.

I vissa fall är det inte tillräckligt att bara mäta och bestämma en viss molekyl. Istället tittar man på mönster av massor av substanser för att kunna skilja patienter från friska kontroller. Då behövs avancerade matematiska verktyg, det vill säga det analytiska kemister kallar kemometri[2]. I andra situationer är det viktigt att kunna följa ett förlopp i ett levande system. Då är det viktigt med analytiska verktyg som kan utföra frekvent provtagning och snabb analys, helst direktkopplat, för att kunna vidta åtgärder när eventuella komplikationer dyker upp[3].

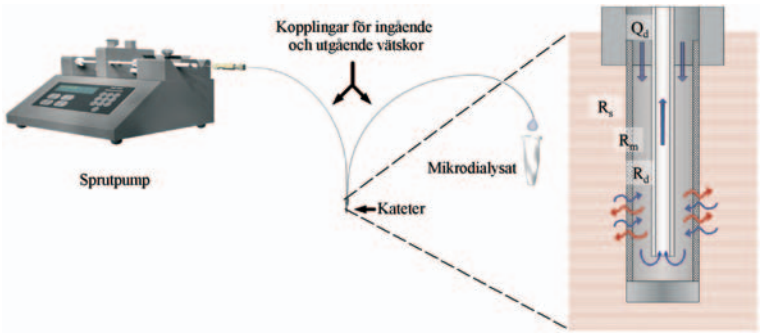
Tekniskt sett så behövs det metoder som klarar av att hantera små provvolymer och komplexa prov, innehållande substanser med stora skillnader i egenskaper och koncentration. I den här avhandlingen ges en

introduktion till mikrodialysprovtagning, miniatyriserade multidimensionella separationssystem och masspektrometrisk detektion. Arbetet har fram för allt varit inriktat på att koppla samman dessa i direktkopplade system.

9.2 Ett enkelt sätt for insamling av biologiska prover – *mikrodialysprovtagning*

Bland de provtagningstekniker som kan användas för direktkopplad mätning av biomarkörer i kroppen är mikrodialys en av de mest intressanta eftersom den kan samla prov kontinuerligt utan att föra bort vätska från kroppen.

Principen för mikrodialys är att efterlikna ett blodkärl. En kateter bestående av ett membran med små porer, med tillhörande ingående och utgående slangar, är kopplat till en pump. Genom katetern pumpas en vätska med fysiologisk sammansättning och under tiden diffunderar molekyler fram och tillbaka över membranet. Den utgående vätskan, dialysatet, analyseras och man får ett svar på hur den kemiska sammansättningen varierar i den lokala regionen där man placerar katetern. Hur stora molekyler som finns i dialysatet beror på storleken på membranets porer. Vanligtvis är dessa så små att proteiner och andra större molekyler inte kan transporteras igenom. Tekniken är välutvecklad och finns detaljerat beskriven i litteraturen[54,55,56].



Figur 11. Principen för mikrodialysprovtagning. *Figur av Andreas Dahlin.*

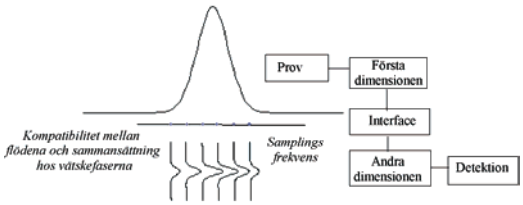
Idag används mikrodialys till provtagning *in vivo* i vävnad, organ och blodkärl i både djur och människor för ett antal olika applikationer t.ex. cancer forskning, samt bestämning av biomarkörer och läkemedel. Den stora fördelen med mikrodialys är således att den kan kopplas direkt till en analysteknik eftersom proteiner, som normalt brukar täppa igen analyssystem, skiljs bort under provtagningen. Detta innebär också att mikrodialys kan användas till provupparbetning *in vitro* eller till att bestämma fri läkemedelskoncentration i plasmaprov.

Används mikrodialys till att följa en pågående process, måste man beakta hur ofta prov ska samlas in i förhållande till hur snabbt förändringar sker i det studerade förloppet. För att inte förlora upplösning kan det krävas att prover tas in t.ex. var 10:e minut, och med ett flöde på 0.5 µl/min så innebär det provvolymer på 5 µl. Provhantering och analysmetoder måste därför vara speciellt anpassade till dessa små volymer.

I denna avhandling har mikrodialys använts till att bestämma fri koncentration (det som inte är bundet till proteiner i blodet) av läkemedlet ropivacaine (lokalbedövningsmedel) (Artikel I och II). Provtagningen skedde i plasmaprov och katetens utgående slang kopplades direkt till vätskekromatografi- (LC) systemet. Direktkopplingen av mikrodialys till LC och masspektrometrisk detektion har detaljstuderats och för ett funktionellt system behövs (1) minimering av mottryck i utgående kopplingar av katetern (Artikel I) och (2) avsaltning och tillsats av intern standard innan MS detektion (Artikel II). Dessutom har mikrodialys använts *in vivo*, där katetern var inopererad i en råttlever. Syftet var att utveckla en metod för att söka efter nya biomarkörer som ändrar i koncentration när man inducerar en förändring i systemet (Artikel III).

9.3 Hur man sorterar komponenter från en biologisk matris – *kolonnbaserade multidimensionella separationer*

I ett biologiskt prov kan man anta att det finns tusentals komponenter. Om man ska lyckas identifiera dessa måste de först sorteras på något sätt. Traditionellt används gelelektrofores[89]. Enkelt kan man säga att man gjuter en gel av akrylamid i en form och applicerar sedan sitt prov i ena änden. Man låter sedan molekylerna separera i två steg, först i en pH gradient för att bestämma dess isoelektriska punkt och sedan efter storlek. Detta kallas för tvådimensionell analys. Gelelektrofores har dock sina begränsningar, och ofta används istället kolonnbaserade, ofta miniatyriserade, alternativ t.ex. LC eller kapillärelektrofores (CE). I LC appliceras provet till en packad kolonnbädd, ofta med affinitet för hydrofoba (fettälskande) substanser, och elueras med en vattenbaserad buffert. Den minst hydrofoba substansen kommer att nå detektorn först. I CE fylls en kapillär (utan packning) med vattenbaserad elektrolytlösning. Efter att provet injicerats läggs en spänning över kapillären och man får en separation med avseende på storlek och laddning. För att eftersträva samma kapacitet som hos gelelektroforesen måste man kombinera flera kolonnbaserade separationer, och kan man göra det i direktkopplade system är det en stor fördel. Principen för detta är beskriven i Figur 12. De kombinerade separationerna ska ha olika separationsmekanismer för bästa effekt.



Figur 12. En schematisk bild av en tvådimensionell separation via kolonnbaserade tekniker. Prover appliceras först i den första kolonnen och konstant fraktion av det som kommer ut överförs sedan via ett gränssnitt till den andra kolonnen som följs av en detektor. Kompatibilitet mellan flödeshastigheter, vätskefassammansättning och frekvens i överförningen är speciellt viktigt att studera.

En kritisk punkt när man kombinerar olika separationstekniker är hur man för över en fraktion från den ena dimensionen till den andra. Här finns det olika strategier, t.ex. att ta den mest intressanta komponenten eller att kontinuerligt överföra en konstant fraktion till den andra dimensionen. Den senare varianten är intressant om man önskar bevara upplösningen ifrån den första separationen. Då krävs en högfrekvent överföring, såsom i Figur 12, där 6 fraktioner tas från en topp i första dimensionen.

I den här avhandlingen så har fokus lagts på direktkoppling av LC och CE, dess koppling till masspektrometrisk detektion. Gränssnitt i poly(dimetylsiloxan) för överföring av fraktioner från LC till CE har utvecklats (Artiklar IV-V, VII-VIII). Eftersom detta material är benäget till att adsorbera peptider och proteiner, har också en deaktiveringsmetod för ökad kompatibilitet utvecklats (Artikel VI).

9.4 Ett effektivt sätt att mäta biologiska substanser – *masspektrometrisk detektion*

För biologiska prover krävs ofta en detektor som kan mäta låga koncentrationer av aktuell substans. Masspektrometri har idag blivit en populär detektor eftersom den ger en selektiv respons som motsvarar en molekyls massa-till-laddnings-kvot (*m/z*-värde). Denna kvot kan kopplas till molekylens struktur och underlättar identifieringen av substansen.

En masspektrometer sorterar joner i en vakuumkammare. Det innebär att joner som befinner sig i ett vätskeflöde, t.ex. från en LC- eller CE-analys, måste överföras till joner i gasfas innan de kan behandlas och detekteras i masspektrometern. Det vanligaste sättet för detta är så kallad elektrosprayjonisation. Man lägger då en potential mellan vätskan och

ingången till masspektrometern. Detta gör att det bildas laddade droppar i vilka lösningsmedlet sedan avdunstar och till slut bildas fria joner i gasfas. Dessa joner kan sedan accelereras av pålagda potentialer in till masspektrometern. För att underlätta jonisationen krävs att buffertar med flyktiga komponenter används. Likaså bör provet avsaltas innan för att undvika att jonerna i saltlösningen konkurrerar om laddningen med de aktuella substanserna. Det är också fördelaktigt om analyterna är laddade redan i lösningen, t.ex. genom att pH-värdet justeras så att protolyter befinner sig i jonform. För flöden upp till 10 µl/min klarar den pålagda potentialen att skapa droppar av vätskan, men för högre flöden i till exempel. LC kan det behövas både nebuliseringsgas och värme.

När gasfasjonerna når själva massanalysatorn kan de sorteras på olika sätt, vanligen med hjälp av växlande elektriska fält, elektriska potentialer och magnetiska fält. Oavsett vad som används, så får man ett resulterande masspektrum med intensiteter för olika *m/z*-värden. Mera selektivt är att välja ut ett visst *m/z*-värde som motsvarar en intressant substans, och bara mäta på denna. Genom att man fokuserar masspektrometerns mätning enbart på detta kan man således mäta mycket låga koncentrationer. Detta, tillsammans med strukturinformation, gör mas spektrometri till ett mycket användbart verktyg vid analys av biologiska molekyler.

I denna avhandling har masspektrometri genomgående använts som detektionsmetod. Det innebär att alla använda analytiska system är anpassade till elektrosprayjonisation. Olika analysatorer och metoder har använts i syfte att (1) selektivt detektera låga halter av läkemedel (Artikel II), (2) jämförande analys av mikrodialysat (Artikel III) (3) snabb detektion av LC-CE separationer (Artiklar IV, V, VIII) samt (4) högupplösande detektion av LC-CE separation (Artikel VII).

10. References

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