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Development of methods in CE, CE-MS and MS/MS

Applications in Pharmaceutical, Biomedical and Forensic Sciences

BY

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ABSTRACT
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Capillary electrophoresis-mass spectrometry has been used successfully for the analysis of a wide range of analytes such as chiral local anaesthetics, sulphonated reactive dyes and endogenous neurotransmitters and neuropeptides.

The partial filling technique was used in CE-MS for chiral separation of bupivacaine and ropivacaine using the non-volatile selector β-cyclodextrin. By only partially filling the capillary with selector and using capillaries coated with polyacrylamide to suppress the electroosmotic flow, introduction of the selector into the mass spectrometer was avoided. An impurity of 0.25% of the R-enantiomer of ropivacaine in the S-form could be detected.

The partial filling technique was developed further using CE employing two different selectors in separate plugs in the capillary. This enhanced the separation efficiency and offered greater flexibility in controlling the separation.

By using transient-isotachophoresis (tITP)-CE-MS it was possible to concentrate and detect classical neurotransmitters and neuropeptides with masses ranging from 104 Da to 1642 Da. γ-Aminopropyltriethoxysilane coated capillaries were used to minimise adsorption of the peptides onto to capillary surface. Endogenous dopamine, glutamate, γ-aminobutyric acid (GABA), acetylcholine, methionine-enkephalin and substance P 1-7 were detected in the striatum of marmoset monkey.

Sulphonated dyes obtained from single textile fibres were analysed using CE-MS. Capillary electrophoresis was found to be a good way of removing the excess amounts of glucose present in the sample that would otherwise interfere with the electrospray ionisation.

Automatic function switching, originally developed for use together with liquid chromatography, was found to be a great method for acquiring MS/MS data when doing infusion experiments saving both time and sample without decreasing the quality of the MS/MS data. It was also found to be a more time efficient way than using the precursor ion scanning mode on the Q-TOF to obtain precursor ion data.

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Contents

1. Papers discussed

This thesis is based on the following papers, referred to by their Roman numerals:

I. Simultaneous separation and enantioresolution of racemic local anaesthetic drugs by capillary zone electrophoresis with Tween 20 and methyl-β-cyclodextrin as selectors, employing a double plug technique
Ahmad Amini, Emmy Jäverfalk, Salumeh Bastami and Douglas Westerlund, Electrophoresis, 20, 204-211 (1999).

II. Chiral Separations of Local Anaesthetics by a Capillary Electrophoresis/Partial Filling Technique Coupled On-line to Microelectrospray Mass spectrometry
Emmy Jäverfalk, Ahmad Amini, Douglas Westerlund and Per E. Andrén, J. Mass Spectrom., 33, 183-186 (1998).

III. Simultaneous analysis of endogenous neurotransmitters and neuropeptides in brain tissue using capillary electrophoresis-microelectrospray-tandem mass spectrometry
Emmy Jäverfalk-Hoyes, Ulf Bondesson, Douglas Westerlund, Per E. Andrén, Electrophoresis, 20, 1527-1532 (1999).

IV. Analysis of reactive dyes obtained from single textile fibres using capillary electrophoresis/time-of-flight mass spectrometry
Emmy Hoyes, Gary Harland and Simon Gaskell. *In manuscript.*

V. Automatic function switching and its usefulness in peptide and protein analysis using direct infusion microelectrospray-quadrupole time of flight spectrometry
Emmy Hoyes and Simon Gaskell. Rapid Comm. Mass. Spec. *Accepted.*

2. Introduction

Capillary electrophoresis (CE) was invented in 1967 by Hjertén who first performed free zone electrophoresis in tubes with a diameter of 1-3 mm [1]. Compared to the traditional gel electrophoresis the capillary version offers a number of advantages such as improved on-line detection capabilities, improved sensitivity, peak efficiency and analysis speed. The greatest improvement of the technique occurred in 1981 when Jorgensson and Lukacs first demonstrated the use of capillaries with an internal diameter (ID) of less than 100 μm[2]. This improved the separation efficiency of the technique and made it commercially available. The applications of CE range from biomolecules like peptides and proteins to drugs and small inorganic compounds.

The most common detection method for CE has been and still is UV-absorption, but in 1987 Olivares et al. performed experiments using mass spectrometric (MS) detection in conjunction with capillary electrophoresis.[3] The mass spectrometer is a highly selective and widely applicable detector for analytical purposes. The high sensitivity and the structural information that it can provide makes it well suited for coupling to separation techniques such as GC, LC and CE. The advantages of using MS-detection are that analytes are identified both by their differential separation and their molecular masses and/or fragmentation patterns. The invention of electrospray ionisation is the main reason why interfacing MS with separation techniques such as LC and CE has had a tremendous increase in popularity. Before electrospray ionisation it was difficult to transfer analytes in liquid phase at atmospheric pressure to desolvated ions in high vacuum. Although techniques such as fast electron bombardment (FAB), thermospray and liquid secondary ion mass spectrometry (LSIMS) had been developed earlier, no technique was as simple and versatile as electrospray.[4]

Since 1987 the technique has undergone tremendous development in both CE and MS instrumentation.[5] CE-MS interfaces are now commercially available, although they are still commonly constructed in house. Electrophoretic techniques, such as isotachophoresis[6-8], isoelectric focusing [9,10] and the use of selectors such as micelles or chiral compounds dissolved in the background electrolyte[11], are continuously being modified to enable mass spectrometric detection. CE-MS is also

combined with on-line sample preconcentration devices based on membranes [12,13] or derivatised silica particles [14-16] to increase the sensitivity of the analysis. It has also been demonstrated that techniques such as liquid-liquid electroextraction and electrodialysis also can be combined with CE-MS to improve sample loadability.[17,18]
As the limit of detection is constantly being improved, the use for CE-MS in peptide and protein analysis is on the increase, as demonstrated in the review articles by Aebersold [19] and Krylov *et al*. [20] However, CE-MS is still not used as commonly as LC-MS or GC-MS. More research is needed to improve and develop new methods to demonstrate the usefulness of CE-MS.

The aim of this thesis is to explore different aspects of CE-MS starting with pure CE and ending with pure MS. Each different kind of sample and technique presents its own special problem that has to be solved.

3. Aim of study

To investigate the double plug technique and it's usefulness for separation of enantiomers in mixtures of related compounds.

To develop a stable and sensitive CE-MS interface.

To demonstrate the usefulness of the partial filling technique in CE-MS using non-volatile buffer additives for chiral separation.

To show that CE-MS can be used for simultaneous analysis of endogenous neuropeptides and neurotransmitters in brain tissue.

To develop a method using CE-MS for forensic analysis of reactive dyes from single fibres.

To explore the benefits of using software techniques to do more efficient data acquisitions for the analysis of mixtures.

4. Capillary electrophoresis

4.1. Principles

Capillary electrophoresis is performed in narrow-bore capillaries with a typical inner diameter of 25-75 μm. The separation is based on the different mobilities of charged species in an electric field. The capillary, which is generally between 20-90 cm long, is filled with a background electrolyte, usually a buffer with a pH that is suitable for the separation of the compounds analysed. A sample is injected into the capillary either electrokinetically using voltage, or hydrodynamically using pressure or vacuum. The capillary ends are then placed in vials containing the electrolyte. When the separation voltage is switched on the compounds starts to migrate. The velocity (υ) of the migration is determined not only by the charge (z) and the size of the molecule but also by the electric field strength (E) and the viscosity (η) of the buffer, which is shown in equation 1, where e is the charge of a proton or electron and r is the radius of the solvated ion.

$$\upsilon=(z*e*E)/(6\pi r\eta) \quad (1)$$

The compounds are detected when they reach the other end of the capillary. The most common method of detection is UV, which is done while the compounds still are in the capillary by creating a detection window by removing a few millimetres of the polyimide coating. Other methods of detection include fluorescence, electrochemical and mass spectrometric ionisation techniques.

Capillary electrophoresis is a very efficient separation technique with peak efficiencies often exceeding 10^5 theoretical plates. The fundamental limit of the efficiency is the longitudinal diffusion of the sample zone, however there are a number of other factors such as Joule heating, injection length, adsorption, electrodispersion etc that can have a bigger impact on band broadening, but fortunately they are to some degree controllable. Techniques to minimise band broadening relevant to CE-MS are discussed further in chapter 6.

4.2. *Electroosmotic flow*

The velocity of an ion in CE is not only determined by its electrophoretic velocity, but also by the electroosmotic flow (EOF). The EOF is a flow of liquid inside the capillary, which is created by the negatively charged silanol groups on the walls of an untreated fused silica capillary. The negative surface attracts cationic species from the background electrolyte (BGE) and the static layer created is called the Stern layer. Another layer with more mobile cationic ions, the so-called diffuse layer is formed next to the Stern layer. When the voltage is applied the cations in the diffuse layer will migrate towards the cathode and drag the bulk liquid along as shown in Figure 1.

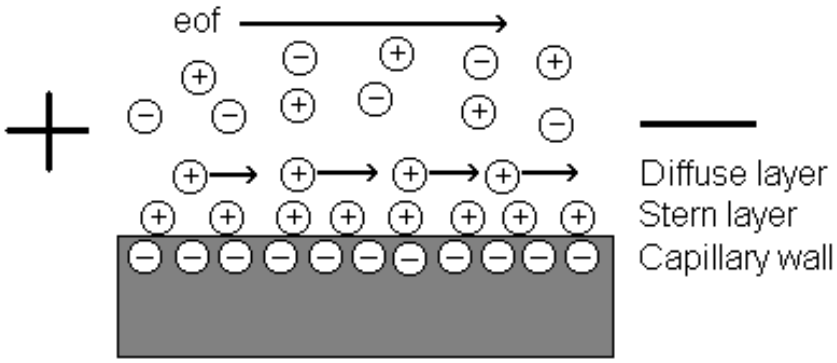


Figure 1. Principals of electroosmotic flow.

The velocity and direction of the electroosmosis can be affected by changing pH and ionic strength of the background electrolyte as well as by the addition of organic solvents, micelles, cyclodextrins or neutral polymers [21]. When electroosmosis is not desired, for instance when applying the partial filling technique, the surface of the capillary can be modified with e.g. polyvinylalcohol (PVA), cellulose or polyacrylamide as demonstrated in paper I and II. This covers the silanol groups and gives the surface a neutral character, which gives a negligible EOF. The coating can also be used for decreasing the adsorption of analytes to the capillary surface.

Another way of decreasing the adsorption of cationic analytes is to use a positively charged capillary surface. The surface will prevent adsorption by electrostatic repulsion of the analytes. A number of different coatings can be used for this, some reagents are dissolved in the background electrolyte or

just need to be rinsed through the capillaries before separation, others are covalently bound to the surface such as the aminopropyl coating used in paper III [22,23] or the increasingly popular MAPTAC coating [24].

4.3 Different electromigration techniques

There are a number of different ways of performing capillary electrophoresis. The most common way is straightforward capillary zone electrophoresis (CZE) which is performed without any added selectors to the background electrolyte in a continuous buffer system (paper IV). However, CZE can also be used with chiral selectors present in the electrolyte to achieve enantioseparation [paper I, II, 25,26]. Increased separation efficiency can be achieved by adding selectors such as micelles, which results in a form of CE called micellar electrokinetic chromatography (MEKC) [paper I, 27]. In MEKC the separation is not only determined by the charge and size of the ions but also by their hydrophobic properties, which makes it possible to separate neutral molecules using CE if charged micelles are used.

When separating large biopolymers such as DNA, where the charge increases evenly with the size of the molecule, it is necessary to use a gel that act like a molecular sieve to achieve a size-based separation. This is called capillary gel electrophoresis (CGE) [28] and can be compared to the traditional slab gel electrophoresis but offers a number of advantages such as speed, on-capillary detection and amenability to instrumental automation.

Capillary isotachophoresis (CITP) and capillary isoelectric focusing (CIEF) techniques can be used in CE to achieve a concentration as well as separation of the sample. Isotachophoresis, which was used in paper III, is discussed further in chapter 8.2. CIEF is a way of very efficiently separating and focusing peptides and proteins on the basis of their pI.[29] CIEF is performed by filling the capillary with a mixture of solutes and ampholytes. The vials contain an acidic solution at one end of the capillary and an alkaline solution at the other end. When the voltage is switched on a pH gradient will form and the analytes will migrate until they reach the pH that corresponds to their pI when they will stop. When the current reaches zero a steady state has been reached and the analytes can be mobilised by pressure to be detected.

5. Mass spectrometry

5.1. History

In 1898 Wilhem Wien, a German physicist, laid the foundations of mass spectrometry when he discovered that a beam of charged particles could be deflected by a magnetic field. The first mass spectrometer was not constructed until 15 years later when J J Thomson built his parabola spectrograph. It was with such a device he and F W Aston could demonstrate the presence of isotopes in 1919. Isotope analysis is just one field where mass spectrometry has become one of the main analysis techniques, other areas include impurity determination, analysis of unknown organic and inorganic samples, structure elucidation of complex organic substances and surface analysis, just to mention a few.

5.2. Principles

A mass spectrometer consists of five basic parts. The first part is a sample handling system where the sample can be introduced into the mass spectrometer for ionisation. This takes place in the ion-source where a beam of charged particles characteristic for the sample is created. The ion beam is then separated into its components according to their mass-to-charge ratio in the analyser and the ions detected by a detector, which is mostly of the electron multiplier type. The fifth essential component is a high vacuum system since the whole process after the creation of the ion beam to the detection takes place under increasingly higher vacuum, in the order of 10^{-4}-10^{-7} mbar.

5.3. Electrospray ionisation

Electrospray is the most widely used ionisation technique when performing CE-MS or LC-MS. The formation of ions from the liquid to the gas-phase is achieved by applying an electric field over the liquid phase to create charged droplets. These droplets are rapidly reduced in size by evaporation and disintegration due to the increasing charge density, and will eventually form gas phase ions. [4]

The electrospray technique is capable of producing multiply charged ions which has revolutionised the analysis of larger compounds, such as peptides

and proteins, using mass spectrometry. For example neurotensin, which was analysed in paper III has a molecular weight of 1673 Da but was detected as a triply charged ion at m/z 558. This becomes even more important when working with proteins since the number of charges increase with the mass of the molecule. Therefore a protein with a mass of 10-60 kDa will have 10-30 charges. This makes it possible to use conventional mass spectrometers capable of detecting ions in the mass-to-charge ratios of 10-2000 for analysis of large biopolymers.

5.4. Analysers

There are many different ion-sources and analysers and the combination of the techniques gives rise to a great number of different instruments that range from being very expensive to being unbelievably expensive. Common analysers that are commercially available are quadrupole, time-of-flight, ion traps and magnetic sector analysers. Below is an overview of the analysers that have been used in this thesis.

5.4.1 Quadrupole analyser

The quadrupole analyser consists of four cylindrical metal rods where the correct ratio of dc and radio frequency ac potentials can selectively transmit ions of a narrow mass-to-charge ratio that are detected at the other side of the rods. By increasing the voltages it is possible to vary the range of size of ions transmitted from around 10 m/z to up to 3000-4000 m/z in some in instruments. A full spectrum can generally be recorded in a second or even less. They are commonly called mass filters because of selective removal of all ions not within the desired m/z ratio.

Even though the instrument can scan across a wide m/z range it is advisable to only scan across the mass range that is of interest for that particular analysis. This improvement in duty cycle increases the sensitivity since time isn't spent recording ions of no interest. The narrower the mass range scanned the higher the sensitivity. The highest sensitivity is achieved when only a single m/z ratio is transmitted so called single ion monitoring (SIM). This also makes the data file associated with the analysis several order of magnitudes smaller, something that is very much appreciated when doing LC or CE-MS or dealing with large numbers of samples.

Structural information is obtained in tandem mass spectrometry using triple quadrupole instruments where three quadrupoles are coupled together (Figure 2).

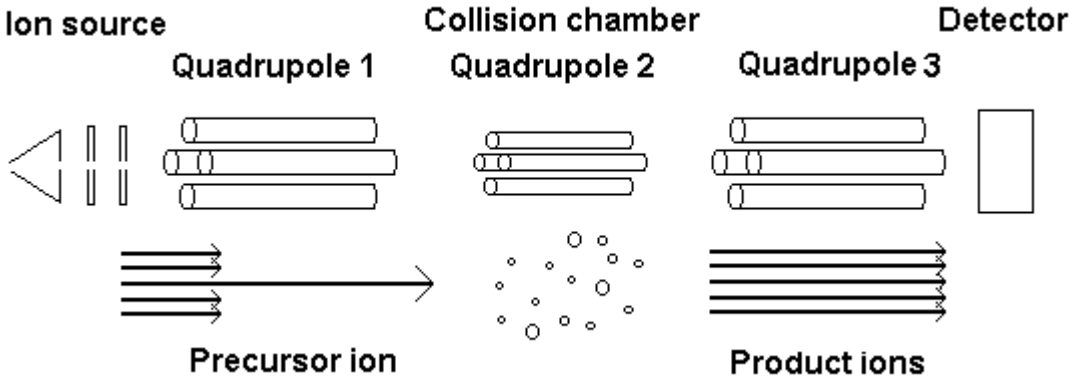


Figure 2. Principles of tandem mass spectrometry.

The first quadrupole isolates a single m/z species, the so called parent ion or precursor ion. The precursor ions are then accelerated into the second quadrupole where they hit a collision gas usually helium or argon. The precursor ion is fragmented upon the collision and the fragments, the daughter ions or product ions, are analysed in the usual way by the third quadrupole. This method is useful for chemical structure elucidation and for identification of analytes and has been used in paper II and III. If a sample has the right m/z ratio and also gives rise to one or more fragments with the predicted masses it is believed that the compound is identified. This was particularly important in paper III where biological samples containing hundreds of compounds sometimes with the same m/z ratio were analysed. To achieve the highest sensitivity the mass spectrometer was set in the multiple reaction monitoring mode (MRM) where 8 different m/z ratios corresponding to 4 neuropeptides and 4 neurotransmitters were selected for analysis. The first quadrupole was set to transmit these masses one after the other and after fragmentation their corresponding product ions were monitored spending 0.15 seconds on each one. MRM and SIM are good methods for improving sensitivity but requires that the mass/charge ratios of the analytes of interest are known. For unknowns and structural elucidation a wide mass range must be scanned to collect as much information of the ions present as possible.

The same method of looking only at the product ion was also used in Paper II even though there were no doubts of what compounds gave rise to which peaks. In this case the sole purpose was to achieve the highest possible sensitivity. It has often been discussed whether using MS/MS decreases or increases the sensitivity of the analysis. MS/MS can improve the detection limit of certain compounds by removing the chemical background and increasing the signal/noise ratio. If the first quadrupole is parked on a single m/z this means a 100% transmission or duty-cycle on this particular mass. If the scanning takes place over a wide m/z range on the third quadrupole the time spent on each m/z is rapidly decreasing with the increasing size of the detection window resulting in a decrease in transmission. If the third quadrupole also is parked to transmit only a single mass than the duty-cycle is going to be 100% here as well, resulting in no decrease in sensitivity. However, since all the background ions are filtered out by the first quadrupole the decrease in baseline noise is often quite substantial so that the net result is a greatly improved signal-to-noise ratio. The size of the detection window can be increased up to a certain point where the sensitivity improvement achieved by the decreased chemical noise is cancelled out by the decreasing transmission.

5.4.2 Time-Of-Flight analyser

The time-of-flight analyser is based on the time it takes for an ion to travel through an electric field. The bigger and the fewer charges the ion has the longer it takes to reach the detector. Linear TOF instruments have been used together with the pulsed ionisation technique of matrix-assisted laser desorption ionisation (MALDI) for a long time. A new generation of orthogonal TOF instruments allows TOF to be combined with continuos ion sources such as electrospray ionisation. The parallel nature of the orthogonal TOF analyser offers high resolution (typically 5000-10 000) which makes charge state recognition and identification of the ion based entirely on its molecular weight possible, but also superior sensitivity compared to the triple quadrupole when acquiring data over a wide mass range.

5.4.3 Quadrupole-Time-Of-Flight analyser

The quadrupole-time-of-flight analyser (Q-TOF) has become very popular as the field of proteomics is increasing. By coupling a quadrupole in front of the TOF analyser it possible to perform high sensitivity tandem mass spectrometry for structure elucidation.[30] The quadrupole isolates a

precursor ion, which is fragmented and the time-of-flight analyser records a spectrum showing the product ions. Peptide sequencing is one area where the instrument has proved to perform better than any other type, but it works equally well for the analysis of small pharmaceutical drugs.

6. Development of a CE-MS system

6.1. Instrumentation

Nowadays CE-instruments are often sold with an optional MS-interfacing kit. The contents of these kits vary but often you pay a lot of money for very little equipment. An ideal CE instrument for use with mass spectrometry should be able to cope with different detection techniques and be comparatively compact and should be able to be controlled without using a computer if desired.

The first thing to find out before attempting to connect your CE to an MS is to see how safe your CE is. The less safe the better. The power supply must be able to apply a potential on one of its electrodes without the need for a current being registered at the other. This means that the detection end of the capillary can be taken out of the outlet vial and connected to a CE-MS electrospray interface. If the power supply has a safety feature to prevent this type of dangerous experiment you will have to get a second power supply or use a different type of CE-instrument.

The next step is to try to get the detection end of the capillary out of the instrument while keeping the length of it as short as possible. This is often not a problem but keep safety in mind when doing it. Sometimes it might be necessary to drill a hole in one of the panels of the instrument. If the capillary can go through the UV detector on the way out of the instrument without drastically increasing the capillary length, it is a good idea to use it. The UV traces can be useful for trouble shooting in the beginning of the method development but is not essential.

To minimise band broadening it is recommended to have the level of the buffer surface at the same height as the outlet of the capillary in the electrospray interface to avoid siphoning. Generally this is not a problem unless you are using very wide capillaries. If a difference in height in

unavoidable it is preferable to have the CE at a higher level to avoid introducing air into the capillary at the detection end.[31]

6.2. Interfaces

There are a number of different kinds of interfaces for CE-ESI-MS. The most commonly used is the co-axial arrangement first developed by Smith [32] where a sheath liquid is used for maintaining the electrical contact necessary for the CE-separation and the electrospray. The CE-capillary is surrounded by another larger capillary, which provides the sheath liquid. Sometimes even a third capillary is added in which nebulising gas can be introduced.

Another approach is the liquid junction interface where a transport capillary, usually a spray needle, between the CE-capillary and the mass spectrometer is used. [33] The electrical contact is applied in the gap between the two capillaries, where additional make-up liquid also is introduced. In a comparison study between the liquid junction and the co-axial interfaces by Pleasance it was found that both methods provided efficient coupling of CE to MS, but the co-axial sheath flow interface was more robust and reproducible. [34]

A third type of interface is the sheath-less interface, where the tapered tip of the capillary is coated with an electrically conducting layer such as gold or graphite. Since electrospray is a concentration dependent technique, this type of interface is known to be the most sensitive, since no make-up liquid is necessary which may dilute the sample zone. Unfortunately it is also one of the more difficult ones to use since it can be difficult to get a stable spray and it is common that the gold layer wears off after some time of spraying. Also, the choice of CE-buffer is limited since there is no make-up liquid that can compensate for the poor electrospray properties of some CZE-buffers. However, many groups have reported successful use of the interface. [35,36] and easy methods such as the “fairy dust” technique of producing coated tips have been developed. [37,38]

Other approaches to the sheath-less interface are to use a metal wire acting as an electrode [39] or a metal union to provide the electrical contact to the CE-buffer. [40]

6.3. Home-made interface

The home-built interface used in the present studies resembles the co-axial sheath flow interface by Smith [32]. The electrospray probe consisted of a fused silica capillary surrounding the CE-capillary. The end of the outer capillary was tapered in a flame and ground by a cutting stone to obtain an opening just large enough for the tip of the CE-capillary. It is vital that the ends of both the outer capillary and the CE-capillary are smooth to obtain a stable electrospray. The CE-capillary protruded about 0.2-0.7 mm from the opening of the outer capillary. Depending on the length of the probe the stainless steel tee was situated either 20 cm (paper II) or 7 cm or less (paper III, IV) behind the tip. The tee provided the high voltage connection and the place for introducing the sheath liquid. The interface is shown schematically in Figure 3.

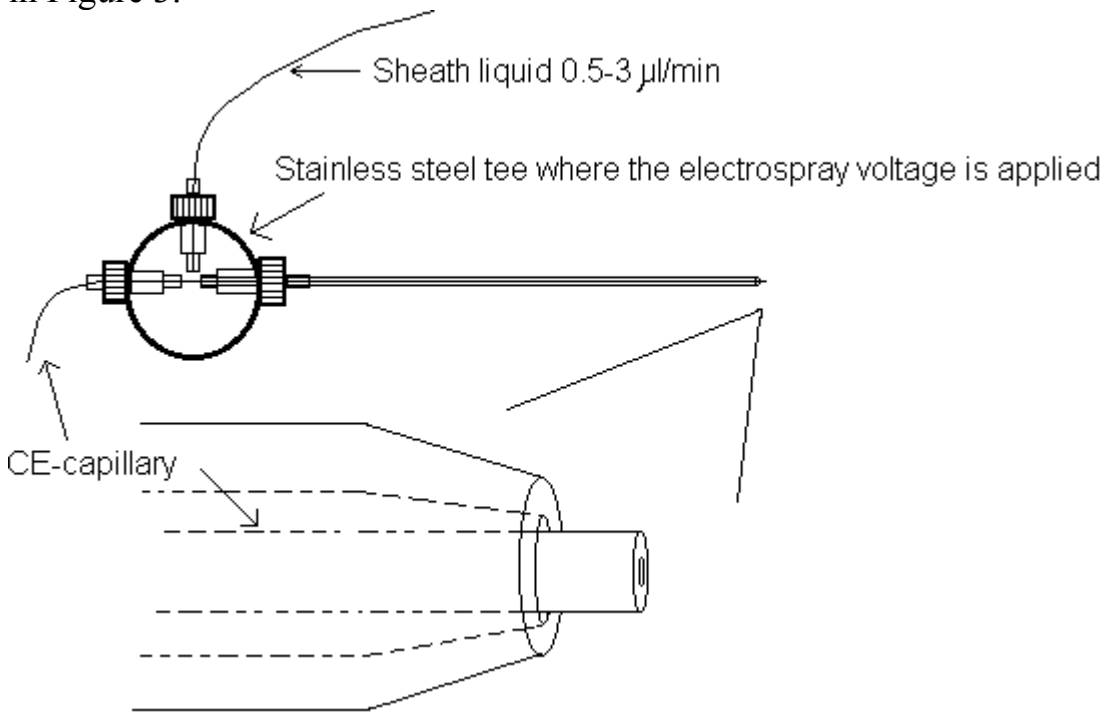


Figure 3. CE-MS interface

The sheath liquid in positive electrospray consisted of 50% methanol and 0.25% acetic acid and was delivered by a Harvard syringe pump. The sheath liquid flow rate was dependent on the flow rate (electroosmosis) from the CE. In paper II where a polyacrylamide coated capillary with no electroosmotic flow was used, the flow rate of the sheath liquid was 3 µl/min, while in paper III were the EOF was high, only 0.5 µl/min was necessary.

The positioning of the interface relative to the sampling capillary of the mass spectrometer, the electrospray voltage, sheath flow rate and positioning of the CE capillary inside the outer capillary are all parameters that are best tuned with the separation voltage switched on and the analyte of interest present in the background electrolyte. This is a good way of making sure that the highest sensitivity and stability is achieved since the tuning conditions resemble the actual analysis conditions as much as possible.

In experiments using a stainless steel capillary as the outer capillary both the sensitivity and stability of the electrospray was found to be satisfactory. The reason why a metal capillary was not used in most of the work in the present studies was that sometimes a corona discharge occurred which caused severe (and expensive!) damage to the mass spectrometer.

In my opinion the co-axial sheath flow interface is by far the easiest to work with. It is easily and quickly made from inexpensive materials, the same interface can be used for months and can often be used for different instruments without any modifications. It is more versatile than for example the sheath less type, since the sheath liquid can be used for improving the electrospray properties of the separation buffer by for example adding more organic solvent (paper II-IV) or changing the pH.(paper IV)

6.4. Electrolytes

Using mass spectrometry as a detection method means that the CE-system must be compatible with electrospray ionisation. The electrolytes used for the separation must be volatile since involatile salts such as phosphate crystallise during the ionisation process and quickly block the CE-capillary and the orifice of the mass spectrometer. The most common electrolytes for use with mass spectrometry are acetic acid, formic acid and ammonium acetate. It is advisable to use as low concentration of the electrolytes as possible without compromising the separation efficiency since high concentration of the background electrolyte tends to decrease the sensitivity[41] and could also contribute to band broadening due to Joule heating if the current is too high. In certain cases, when for instance negative ions are to be detected by the mass spectrometer and a negative electrospray voltage is employed, an electrolyte with a high conductivity can cause the potential at the electrospray tip to be dominated by the CE power supply rather than that of the electrospray. (paper IV) This can cause severe

problems with stability of the spray and sometimes make it impossible to obtain a spray at all. This can be avoided by using a more powerful power supply that is able to sink the current delivered by the CE to maintain the desired potential at the electrospray tip, or if that isn't feasible, reducing the conductivity of the electrolyte by lowering the concentration or adding more organic modifier can sometimes help. If non-volatile additives are used for improving the separation a technique such as partial filling of the capillary (paper I, II) must be employed to prevent the selectors reaching the end of the capillary and being sprayed into the mass spectrometer.

6.5. Capillaries

Due to the distance between the CE and the mass spectrometer, fused silica capillaries used for CE-MS are usually longer than for CE-UV. This may improve the separation but may also make adsorption of analytes to the capillary surface a bigger problem. Additives can be used to decrease the adsorption but will often interfere with the electrospray ionisation. The easiest way of decreasing the adsorption is to derivatise capillaries to make their walls less prone to attracting the analytes. (Paper I, II, III)

Capillaries with outer diameters of 190-220 μm rather than the standard 375 μm that most people use for CE-UV analysis were used in the this thesis. The advantage of using a thinner capillary is that the electrospray interface can be made smaller by using a narrower spraying capillary to deliver the sheath flow. The miniaturisation of the sprayer offers higher sensitivity and better stability.

Not all CE instruments are capable of using the thinner capillaries but usually the problems can be overcome by minor alterations. The CE system used in paper II-IV can easily be adapted to take the thinner capillaries by cutting a small hole in the vial cap to avoid the CE capillary bending and snapping while being introduced into the vial.

7. Chiral separations using the partial filling technique

7.1 Chiral separations

Chiral separation in CE was first demonstrated by Gassman et al. in 1985, using optically active copper (II) complex of L-histidine as chiral support electrolyte for the enantioseparation of a range of DL-dansyl amino acids. [42] Since then a wide number of stereoselective binding agents have been used as buffer additives in CE. Some examples are cyclodextrins [43,44], heparin[45], crown ethers[46], micellar forming agents[27,47], macrocyclic antibiotics[48] and proteins[49-52]. CE has proven to be a useful technique in enantioseparation because of its speed, efficiency and simplicity compared to liquid chromatography (LC).

Cyclodextrin is composed of β-D-glucopyranosyl units linked via 1-4 β-glucosidic linkages to form a hollow truncated cone structure and is one of the most widely used chiral selectors. It can be obtained as α,β and γ-cyclodextrin depending on how many sugar units that is enclosed in the ring (6-8 units) and with different modifications of the side groups. Methyl-β-cyclodextrin was used in papers I and II because of its ability to separate the enantiomers of local anaesthetic drugs, such as bupivacaine, ropivacaine, mepivacaine and prilocaine. The aromatic group of the compounds fit favourably in the cavity of the cyclodextrin molecule, while the side chains interact with the hydroxyl groups of the sugar units. Methyl-β-cyclodextrin showed higher affinity for the S-enantiomer of the local anaesthetics than the R-enantiomer.

7.2. The partial filling technique

Using selectors dissolved in the background electrolyte to enhance the separation can sometimes cause problems with the detection. UV-absorbing selectors such as proteins and macrocyclic antibiotics will interfere with the UV-detection and non-volatile selectors are not compatible with electrospray-MS. This can easily be avoided by using the partial filling technique [53-56]. Papers I and II demonstrate examples of further developments of this technique such as using double plugs (paper I) and using partial filling in conjunction with electrospray-MS for chiral separations (paper II).

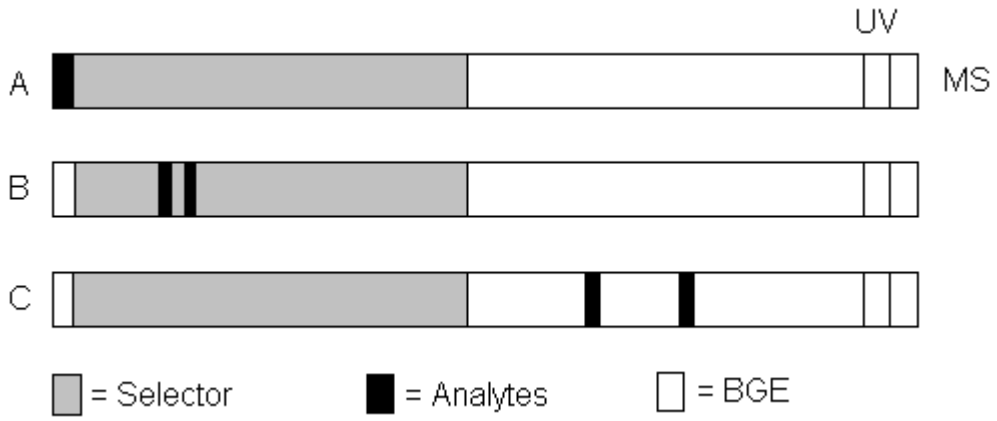


Figure 4. Principles of the partial filling technique.

Partial filling is performed in capillaries that have been coated to give a negligible electroosmotic flow. The capillary is first rinsed with neat electrolyte. Before the sample is injected the capillary is partially filled with electrolyte containing selector, Figure 4. During the separation the analytes migrate through the selector-plug where they are separated and when reaching the other side of the zone they can be detected without disturbances from the selector.

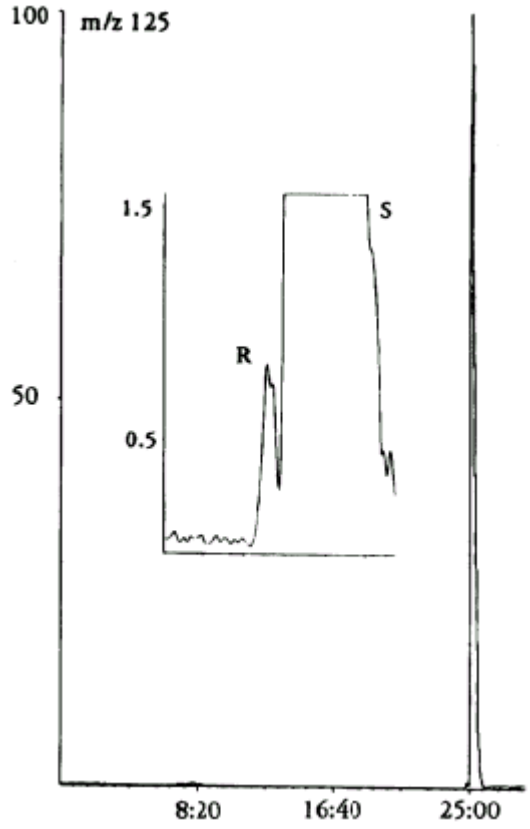


Figure 5 from paper II demonstrates a separation of the (R)- and (S)-enantiomers of ropivacaine, a drug that is given in pure (S) form. The (R)-enantiomer is present at 0.25% in the sample, which corresponds to an injected amount of 1.1 fmol (310 fg). Almost 90% of the length of the capillary were filled with 100 mg/ml methyl-β-cyclodextrin. The detection was performed by electrospray-MS/MS using a triple quadrupole instrument.

Figure 5. Separation of 0.25% of R-ropivacaine in S-ropivacaine using the partial filling technique-CE/MS.

As well as avoiding problems with detection the partial filling have other advantages. Compared to the normal method where the selector is dissolved in the entire electrolyte system the partial filling technique only uses a fraction of the amount, which is important when there is a limited amount of selector available or it is very expensive (paper II).

7.2.1 The double plug technique

The double plug technique is an extension of the partial filling technique, where two different selectors are used. Instead of mixing them and injecting them as one single plug they are injected separately in consecutive zones. The advantages of this procedure were investigated in paper I where a mixture of four chiral local anaesthetics, mepivacaine, ropivacaine, prilocaine and bupivacaine, were separated using methyl-β-cyclodextrin and the surfactant Tween 20. Methyl-β-cyclodextrin resolved the enantiomers but to achieve separation of all eight peaks it was necessary to use micelles. The first zone contained 38 mM or 76 mM methyl-β-cyclodextrin dissolved in BGE and the second contained 100 mM solution of Tween 20. The zone lengths were carefully varied until the separation shown in Figure 6 was achieved.

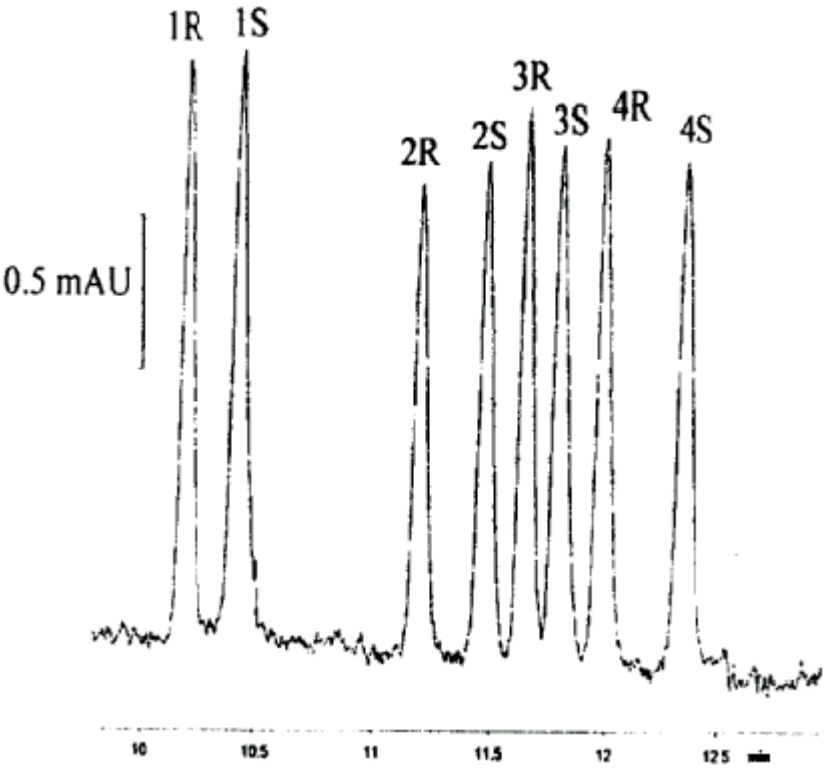


Figure 6. Separation of four local anaesthetics and their enantiomers using the double plug technique.

Not only does this method offer the possibility of easily controlling the effect of each selector on the separation, but the resolution is also improved by avoiding mixing the two selectors. Analytes do not have to compete with Tween 20 molecules for the binding sites in the cyclodextrin cavity.

8. Analysis of biological samples using CE-MS

8.1. Adsorption

The two main problems with using CE for the analysis of biological samples are adsorption and sensitivity. Adsorption of proteins and peptides to the fused silica surface of the capillary results in poor reproducibility with migration times increasing by several minutes after each experiment even after careful rinsing and conditioning of the capillary. It can also cause an already small peak to "disappear" completely because of severe band broadening. Generally it helps to lower the pH of the separation electrolyte to decrease the amount of negatively charged hydroxyl groups on the capillary surface, but sometimes that is not enough and other methods have to be used.

Common ways of decreasing the adsorption are by using high ionic strength buffer systems, high concentration of zwitterionic buffers or by dynamically changing the capillary surface by using cationic surfactants, however these methods are generally not compatible with MS detection.
A more suitable method of avoiding adsorption is to derivatise the capillary with polymers or cationic reagents like aminopropylsilane. [22]

It was found that using aminopropyl coated capillaries the adsorption dramatically decreased and the electroosmotic flow was reversed and increased compared to uncoated fused silica capillaries.

8.2. Isotachophoresis

One way to improve the detectability in CE is to increase the injection volume. This can be done by using isotachophoresis (ITP) which is performed in a discontinuous buffer system with a leading electrolyte and a terminating electrolyte with the sample located in between. To be focused the analytes must have mobilities that are higher than the ion of the same charge in the terminating electrolyte but lower than the leading electrolyte.

During the ITP the sample components will form a pattern of consecutive zones between the two electrolytes and can be concentrated up to a thousand times according to the Kohlraush function.[56]

A common approach is to use ITP for sample concentration followed by CZE for the final separation. This can be done in different ways, Stegehuis et al. has demonstrated the use of a two capillary set-up[57] and others are using a single capillary and applying a constant back pressure to keep the sample zone in the capillary during the ITP process, before the focused sample zone is pushed back and one of the buffers is exchanged to obtain a continuous buffer system suitable for CZE. [58-60] This method is not feasible when applying CE-MS since there is no outlet vial to supply the electrolyte needed for filling the capillary and moving the sample zone backwards in the capillary.

Another very simple approach is to dissolve the sample in a buffer that together with the background electrolyte will create an ITP system. This is called transient ITP[7,8,61] and was used in paper III. To achieve a transient-ITP system the sample was dissolved in ammonium acetate. The ammonium ions have a higher mobility than the analytes and therefore acted as a leading electrolyte. The background electrolyte was 50 mM acetic acid which acted as a terminating electrolyte due to the partly dissociated proton, which had a lower mobility than the analytes.

The transient-ITP system was compared to normal stacking or dissolving the sample in BGE. Stacking was achieved by dissolving the analytes in a solution with lower conductivity, which in this case was 5 mM acetic acid or water. The signal to noise ratio increased on average 9 times for a mixture of classical neurotransmitters and neuropeptides when comparing the result obtained using ammonium acetate with the ones with BGE, but then all compounds were not equally effected by the ITP focusing. For instance glutamate was not affected at all since it is not positively charged. Figure 7 shows the signal-to-noise ratios for some of the compounds when dissolved in different solutions.

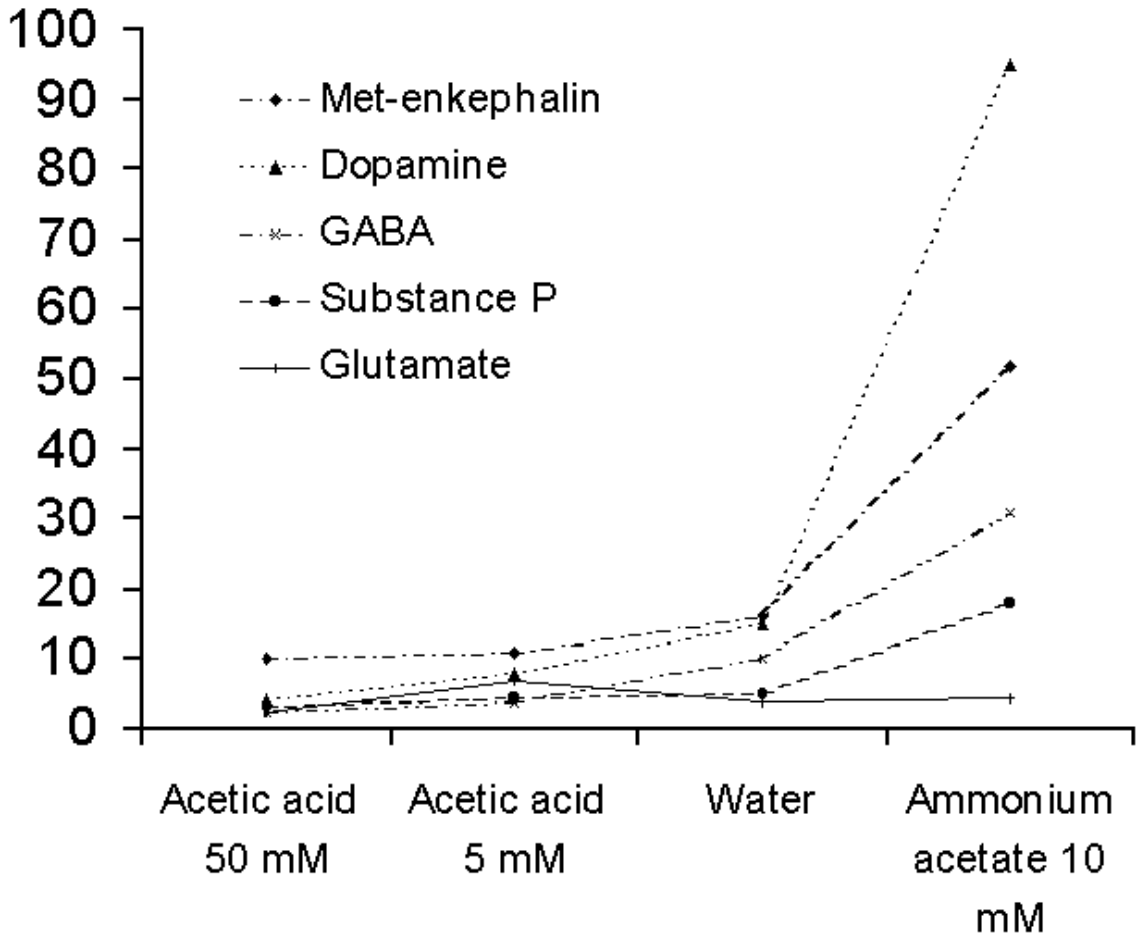


Figure 7. Signal-to-noise ratios for various analytes dissolved in four different solutions.

As paper III shows it was possible using transient-isotachophoresis to concentrate and analyse a mixture of a number of endogenous compounds with very different abundance and chemical character regarding structure and molecular weight (MW 103-1673). In a sample prepared from brain tissue from marmoset monkey neurotransmitters such as acetylcholine, GABA, glutamate and dopamine and the neuropeptides methionine-enkephalin and substance P 1-7 could be detected as the mass electropherogram shows in Figure 8. This was the first time 'classical' small molecule neurotransmitters were detected simultaneously as neuropeptides in the same analysis.

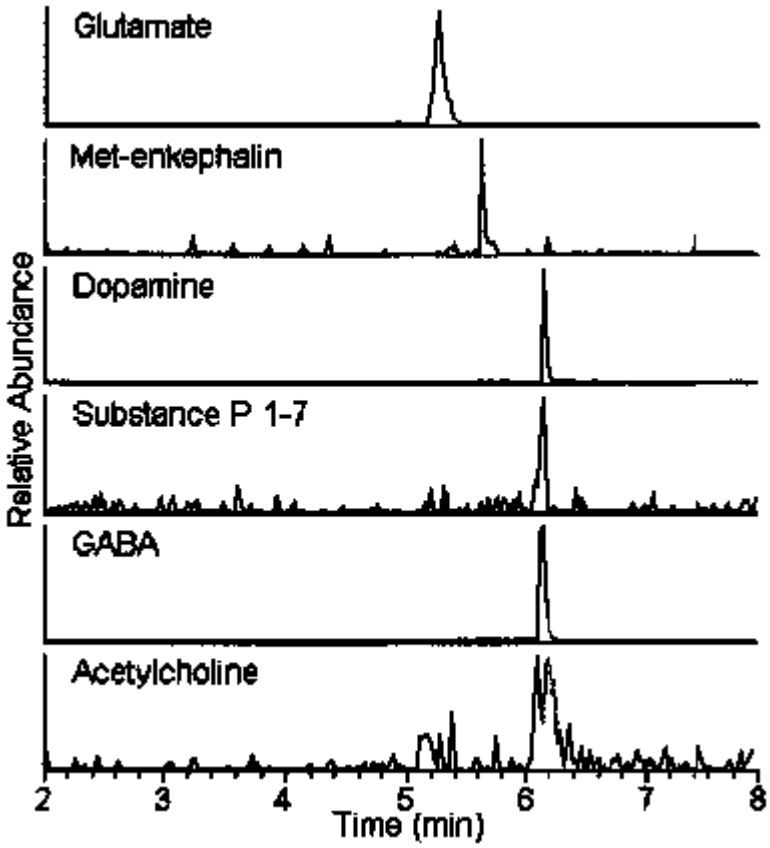


Figure 8. Single ion electropherograms of analysis of striatum from marmoset monkey. Sample dissolved in 10 mM ammonium acetate.

9. Analysis of sulphonated dyes obtained from single textile fibres

The aim of the work in paper IV was to develop a mass spectrometric method for analysing single cotton fibres that could be used for forensic purposes to establish if there has been a connection between a suspect and the evidence recovered at a crime scene. The most efficient way of releasing the dye that was covalently bound to the cotton fibre was to use cellulase enzyme to cleave the long polysaccharide into small pieces. This produced a solution of free dye coupled only to a few glucose units. The solution also contained very high amounts of glucose that made direct infusion into the mass spectrometer impossible. Experiments showed that the peaks were hardly visible above the baseline even though the sample solution had a strong pink colour. In preliminary experiments, LC, anion exchange cartridges and dialysis were used to remove the glucose prior to analysis but none proved satisfying. Capillary electrophoresis was found to be the most efficient method for sample clean up due to the chemical nature of the sulphonated dyes and is a technique that has been used by several groups for the analysis of dyestuffs. [62-64]

The most common way of separating negatively charged compounds by CE is to use alkaline buffers to increase the electroosmotic flow sufficiently to counteract their mobility away from the detector. Using 10 mM ammonium acetate at pH 9 with 10 % isopropanol gave a very nice separation of the dyes using UV detection. When connected to the mass spectrometer the high conductivity of the ammonium acetate caused the positive voltage applied at the inlet vial to dominate over the electrospray voltage since the power supply delivering the electrospray voltage was not able to sink the current. Instead of having a negative potential at the capillary tip to ionise the negatively charged analytes the potential was slightly positive causing the spray to disappear or be very unstable. This was more pronounced on the Mariner instrument than the Q-TOF probably due to differences in the power supplies providing the potential for the electrospray.

The problem mentioned above was easily solved by switching the separation voltage to negative and by using an acidic buffer (10 mM acetic acid) to suppress the electroosmotic flow. The electroosmosis in this case would flow away from the mass spectrometer. To make sure that no air was sucked into the capillary tip a pressure of 100 mbar was applied at the capillary inlet. Although slightly less efficient this system could easily separate the dye from the glucose in less than 10 minutes. An example of the analysis of Procion Red MX-5B obtained from a single fibre is shown in Figure 9.

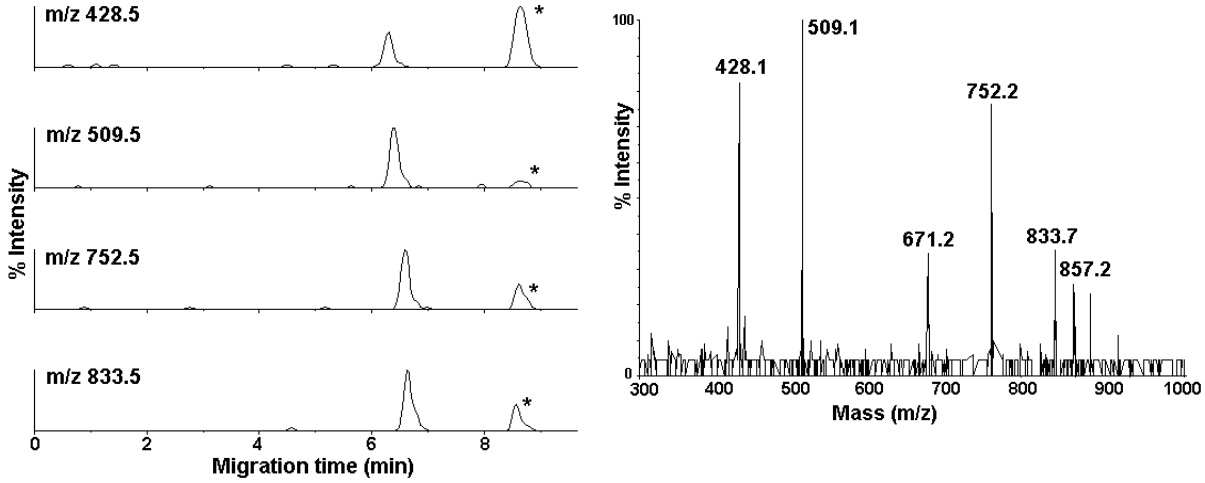


Figure 9. Mass electropherogram and summed spectrum from the analysis of Procion Red MX-5B obtained from a single fibre. Peaks marked (*) are due to an increase in baseline noise due to the high amounts of glucose eluting at that time.

The small amount of sample obtained from a single fibre (5 μL) was enough for several analyses using CE-MS as only approximately 50 nL was injected each time. The remaining microliters could be used for analysis using other methods such as MALDI-TOF-MS.

This method also demonstrates the usefulness of using a sheath flow since it makes it possible to adjust the pH to optimise electrospray efficiency. The reactive dyes are detected as negative ions by the mass spectrometer. To generate negatively charged ions in an efficient way the electrospray voltage is set at a negative potential and the analytes are usually dissolved in an alkaline solution. To change the pH at the electrospray tip, from an otherwise acidic CE-electrolyte, the sheath flow used contained 0.1 % w/v ammonium acetate in a solution of isopropanol and water (80:20, v/v). A high amount of organic solvent was also beneficial for achieving a stable and efficient spray.

10. Tandem mass spectrometry without separation using automatic function switching

When developing a new method for analysing a particular type of sample it is advisable to make the method as simple as possible. The mass spectrometer is a highly selective detector compared to for instance UV so sometimes there is no need for a complicated separation method before analysing the samples. Situations that demand separation of the sample are when the sample contains high amounts of salts or other compounds such as glucose in paper IV that would interfere with the ionisation process, analysis of chiral substances (paper II) or when the sample is too complex and needs to be purified through LC or CE. LC is also commonly used for pre-concentration of samples coupled on-line to a mass spectrometer. Clean-up and concentration steps can also be performed off-line to save valuable instrument time, and samples can then be infused by a loop-injection or by using a syringe pump at a flow rate 0.5 -1.0 μL/min into the mass spectrometer without having to wait for a separation or an equilibration before data from the sample can be collected.

A proteomics application often generates hundreds of samples, each containing a complicated mixture of digested protein. Tandem mass spectrometry is often used to sequence peptides to identify proteins and for studies of protein/peptide modifications. When dealing with such a large

number of complex samples the method of acquiring the data can dramatically affect the time it takes to analyse each sample, but can also be crucial when the amount of sample is very limited.

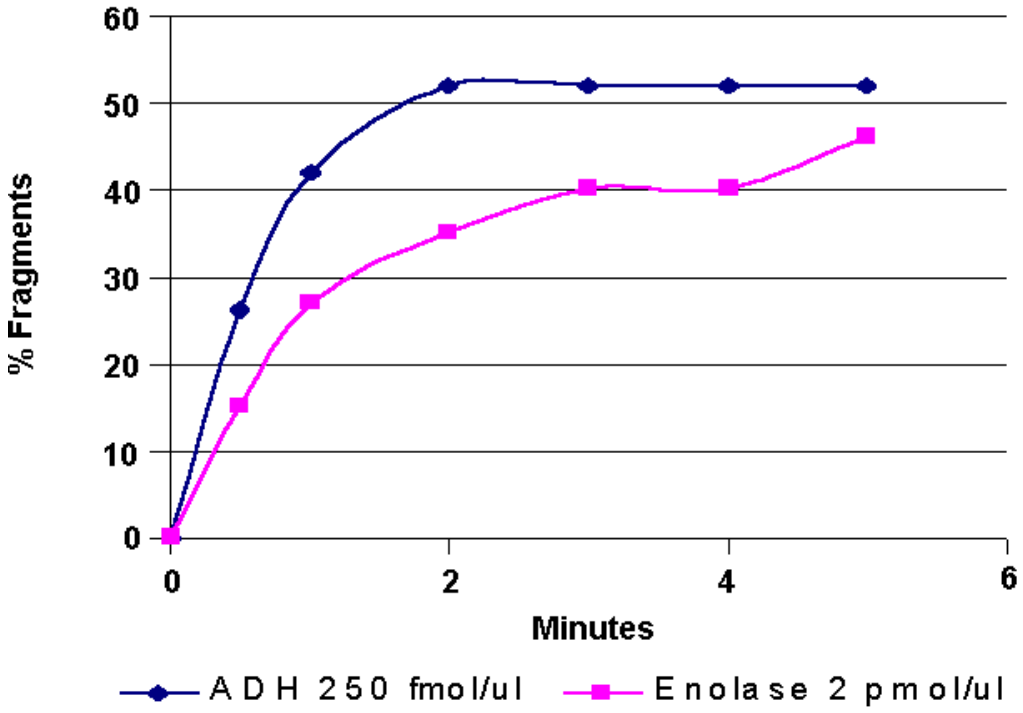
Product ion data from infusions of complicated sample mixtures can be collected either by manually choosing peaks present in the spectrum for MS/MS or automatically by using the automatic function switching feature present in most mass spectrometers' software. The automatic function switching feature was primarily developed for use with LC, when MS/MS had to be performed on peaks for which m/z were not known beforehand [65,66], but works equally well on simple infusions as demonstrated in paper V.

When performing automatic function switching the mass spectrometer first collects a spectrum over the full mass range, a so-called survey scan. The survey scans are used for detecting peaks that have intensity above a set threshold. These peaks are then selected for MS/MS according to their intensity starting with the most intense peak first. Product ion spectra are then collected for up to 8 peaks “simultaneously” during a set amount of time (typically 4-10 seconds) and by using different collision energies (up to 5 different energies) for each compound the fragmentation is generally improved. After finishing the MS/MS acquisition the mass spectrometer creates another survey scan and uses this spectrum to select the next peak/peaks to fragment. Any peaks that have been selected earlier are disregarded.

There are several parameters that can be optimised to make sure that the data is of highest possible quality and collected in the most efficient way. When acquiring in LC-MS/MS mode it is preferable not to fragment more than 1-2 peaks at a time and spend as short time on them as possible without compromising the sensitivity. While the mass spectrometer is executing MS/MS everything that elutes from the column will go undetected so it is advisable to acquire a new survey scan fairly often. The automatic function switching works best for LC-MS/MS when the sample is well separated.

There is much more freedom when automatic function switching is used for analysing samples that are continuously infused into the mass spectrometer. There is no need to do many survey scans since the spectrum will be the same during the whole analysis. The maximum number of peaks (in MassLynx it is 8) that can be selected for MS/MS at the same time, and if

needed, the time spent on each peak can be extended to achieve higher sensitivity. The mass spectrometer will perform MS/MS on the 8 peaks of the highest intensity in the spectrum and thereafter selecting the next 8 peaks and another 8 peaks after that. It takes only minutes until the peaks analysed are only slightly higher in intensity than the baseline noise. A useful application of this is the sequencing of peptides from protein digests where a large number of peaks have to be analysed in preferably as short time as possible. Figure 10 from paper V demonstrates how quickly the main peaks from two protein digests were analysed. Each peptide was fragmented using three different collision energies 22,28 and 35 V. When the MS/MS spectra were summed together the product ion spectrum contained good range of fragments which could be used for sequencing the peptide or for database



searching.

Figure 10. The proportion of all tryptic fragment ions selected for product ion scanning by the automatic function switching method after a total analysis time of five minutes.

Automatic function switching is not only highly preferable to manually doing MS/MS on complicated sample mixtures but can sometimes be used

instead of using the precursor ion scanning mode on a Q-TOF. The precursor ion scanning method was developed using triple quadrupole instruments for detection of for instance phosphate containing compounds [67,68]. The third quadrupole is set to transmit a specific mass characteristic for the fragmentation of a compound containing phosphate, while the first quadrupole scans across a wide mass range. The precursors that give rise to ions being registered by the detector are then said to contain phosphate. It is a very selective, fast and sensitive method when performed on a triple quadrupole mass spectrometer.

It is possible to perform precursor ion scanning on a Q-TOF but in a slightly different way. Instead of the third quadrupole the Q-TOF has a time-of-flight analyser, which will collect a full product ion spectrum for each mass transmitted by the quadrupole as it scans across the mass range. Every spectrum will contain much more information but the sensitivity will be lower compared to a triple quadrupole instrument. To achieve better sensitivity the quadrupole is scanned more slowly across the mass range of interest, spending typically one second on each m/z unit, which can give rise to very long analysis times if the mass range of interest is wide. Since the automatic function switching is basically collecting the same type of data but rather than doing it starting at one end of the spectrum and doing it in m/z steps, it selects the peaks according to their intensity. This means that it starts by acquiring data where the peaks are and is saving the area between the peaks to be analysed later if time permits. Doing it this way means that the time spent on each mass can be increased giving rise to higher sensitivity and at the same time shortening the overall analysis time.

11. Conclusions

Often when using mass spectrometry as a detection technique due to its tremendous selectivity a separation before introducing the sample might seem unnecessary. However as shown in this thesis the separation is frequently essential.

When analysing enantiomers of chiral compounds a separation prior to detection is always necessary, as the mass spectrometer is unable to distinguish between enantiomers since they have exactly the same mass and will fragment in identical ways. The partial filling technique used in paper II proved to be an excellent way of avoiding contamination of the electrospray source by the non-volatile selector necessary for the separation. As demonstrated in paper I the partial filling technique had other advantages such as improved separation when using more then one selector and low selector consumption when compared to traditional methods.

Paper IV demonstrated a different case where the sample of digested cotton fibres contained high amount of glucose, which prevented it from being infused directly into the mass spectrometer. Using capillary electrophoresis it was easy to separate the glucose from the dyestuffs used for colouring the cotton and a sample obtained from a single fibre could be successfully analysed.

Separation methods such as liquid chromatography and capillary electrophoresis can also be used for concentrating analytes present in a sample. This is shown in Paper III where transient-ITP was used to concentrate and separate very dilute samples containing a wide range of endogenous compounds present in the brain of a marmoset monkey.

The manufacturers of mass spectrometers have recognised the need for specially designed software for MS/MS data acquisitions when doing separation coupled on-line to mass spectrometry. The result of this has been the development of “automatic function switching” or “data dependent acquisition”. Paper V demonstrates that these methods of data acquisition can also be a great tool for standard infusion experiments saving both time and sample without decreasing the quality of the MS/MS data.

The important thing to bear in mind when faced with a new type of sample is to choose the easiest way of analysing it. Do I have to do a separation? If I

do, which is likely to be the easiest method? By using this approach it has been possible to analyse very challenging samples in a comparatively straightforward way without having to modify either the capillary electrophoresis instrument or the mass spectrometer to any greater extent. Simple, reliable methods that are easy to automate are going to make it possible to have CE-MS as a routine analysis in commercial and academic laboratories in the future.

12. Future studies

As demonstrated in this thesis CE-MS has a wide range of applications. The different types of compounds being analysed by CE-MS are steadily on the increase, with more and more people starting to use the technique. In just a few years it has developed from being a rare novelty performed in just a few labs around the world to being an accepted technique that is now commercially available.

The aim for future studies would be to reach even lower detection limits by either improving the electrospray interfacing between the CE and MS or by the use of different sample concentration devices. Quantification is another area that needs investigating if CE-MS is going to be widely used in commercial labs in the future.

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