New electromigration-driven enrichment techniques for peptidomics and metabolomics

Petrus Wilhelmus Lindenburg

New electromigration-driven enrichment techniques for peptidomics and metabolomics

PROEFSCHRIFT

Ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof. mr. P.F. van der Heijden
volgens besluit van het College voor Promoties
te verdedigen op dinsdag 5 juni 2012
klokke 15.00 uur

door

Petrus Wilhelmus Lindenburg

geboren te Schiedam in 1979

Promotiecommissie

Promotor:	prof. dr. Thomas Hankemeier
Overige leden:	prof. dr. M. Danhof Leiden University, the Netherlands
	prof. dr. J. van der Greef Leiden University, the Netherlands
	prof. dr. A. IJzerman Leiden University, the Netherlands
	prof. dr. M.-.L. Riekkola University of Helsinki, Finland
	prof. dr. R. Shimmo Tallinn University, Estonia

Het in dit proefschrift beschreven onderzoek is financieel ondersteund door TNO (the Systems Biology Program)

Printed and bound by CPI Koninklijke Wöhrmann

ISBN 9789074538787

There is a crack in everything…that's how the light gets in.

Leonard Cohen, 1992

Voor Maartje

Contents

Chapter 1

General introduction and scope

1. Systems biology, peptidomics and metabolomics

Over the past two centuries, medical science has been mostly based on reductionism, meaning the dividing of complex problems into smaller, simpler units. The human body was viewed as a collection of components, and the component most likely responsible for a certain condition was singled out as drug target. This approach has been successful in dealing with several widespread diseases, but it ignores the important role of interactions between system components. After the Human Genome Project was completed, it became clear that most phenotypic defects, such as cancer, asthma or atherosclerosis, cannot be explained by a single genetic mutation. Without a system-level understanding of the functioning of genes, the benefits of the information that the Human Genome Project generated cannot be fully exploited. In popular words, the forest cannot be explained by studying the trees individually [1]. The realisation that reductionism is not sufficient to achieve breakthroughs in health care is further strengthened by the fact that the number of drugs annually allowed on the market by the Food and Drug Administration has continued to decrease, while the costs that have to be made before a drug is allowed on the market have increased [2]. Medical systems biology offers a holistic approach, which not only takes interactions between genes into account, but also interactions between proteins and metabolites. Systems biology studies biology as an integrated system of genetic, protein, peptide, metabolite, cellular and pathway events that are in flux and interdependent. This offers an opportunity to overcome the limitations that the medical sciences and pharmaceutical industry currently face [3].

Peptidomics has been defined as the comprehensive qualitative and quantitative analysis of all peptides of a given biological system at a definite point in time. The concentration of peptides reflects detailed information about the physiological status of an organism [4]. The metabolome has been defined as the complete set of metabolites and/or low molecular weight intermediates, which is context dependent, varying according to the physiology, developmental or pathological state of the cell, tissue, organ or organism. Metabolomics is the analysis of the individual metabolites in an organism [5], i.e. the metabolome. Information of the concentration behaviour of peptides and metabolites in time is essential to be able to study a system. The importance of metabolomics has been demonstrated elegantly in yeast cultures. Yeast strains with so-called silent mutations cannot be distinguished based on their growth curves, but metabolomics analysis clearly showed different metabolic profiles that were caused by these mutations. Based on these results, 'silent' genes that influence

metabolic pathways could be identified [6].

2. Practical systems biology

Systems biology is the integration of chemistry, mathematics, biology and medicine with biostatistics and bioinformatics [3]. To conduct systems biology experiments, comprehensive sets of biological data should be obtained. After measurement, the data are pretreated, annotated with the help of a database, visualised using multivariate statistical methods (Fig. 1.1) and biologically interpreted. This workflow may result in the discovery of a set of biomarkers that are indicative (either they play a direct role in the pathways, or they correlate to them) for the presence of a disease or drug-based intervention [7].

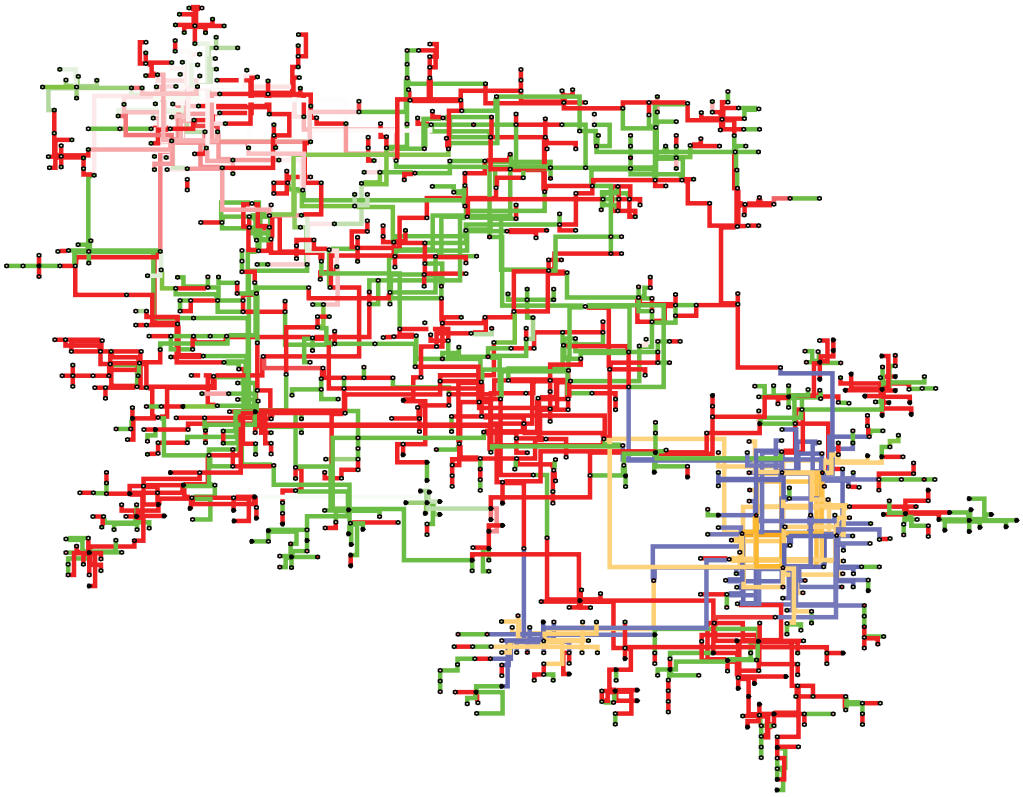


Figure 1.1 Example of a visualised biological system based on experimental data obtained from adipose tissue. It represents a correlation network based on transcript levels, protein concentrations and metabolite concentrations, determined with RNA-assays, LC-MS and GC-MS, respectively (kindly provided by Aram Adourian, BG Medicine Inc.).

One of the main technological challenges with respect to practical systems biology is the development of sensitive tools for identifying and quantifying the concentrations, fluxes and interactions of various types of molecules, at high spatial and time resolution [8]. This challenge can be summarised into three requirements for analytical method development.

Requirement 1: The analytical method/technique should provide a wide concentration range with a low detection limit

Low abundant metabolites are often highly relevant for the functioning of a system, and in consequence thereof, should be measured. The peptidome and metabolome extend over an estimated 7-9 magnitude of concentration (pmol/L - mol/L) [7, 9]. Next, methods with low detection limits allow for smaller sample size, which is especially beneficial when limited sample volume can be obtained, as for example is often the case in time-resolved studies [10].

The International Union for Pure and Applied Chemistry (IUPAC) defines the limit of detection (LOD) as the concentration, C_L, or the quantity, q_L, derived from the smallest measure, x_L, that can be detected with reasonable certainty for a given analytical procedure. The value of x_L is given by the Equation 1:

1) $$x_L = \bar{x}_{bi} + ks_{bi}$$

where $\bar{x}_{bi}$ is the mean of the blank measures, s_{bi} is the standard deviation of the blank measures, and k is a numerical factor chosen according to the confidence level desired [11]. For the LOD, a value of 3 is mostly used for k, which means that the smallest peak that can be detected with certainty has a signal-to-noise ratio of 3. Attempts have been made to define a limit of quantitation (LOQ), which is regarded as the lower limit for precise quantitative measurements. A value of 10 is suggested for k, but this has not been widely adapted by the scientific community [12].

Requirement 2: The total analysis time should be short

A thorough systems biology study involves analysis of large sample series. To cope with this number of analyses, the total analysis time, including sample pretreatment and data processing, should be as short as possible.

Requirement 3: The analytical method should allow the analysis of a wide range of chemical entities

Peptides and, to an even larger extent, metabolites range greatly in size and polarity, which offer major challenges to the analytical techniques that are used to carry out metabolomic studies.

3. Current analytical techniques applied in quantitative peptide and metabolite profiling

The major objective of quantitative peptidomics and metabolomics is to measure the concentration of as many endogenous compounds as possible in a biological sample [4, 5, 13, 14]. Currently, peptide analysis is mainly carried out with liquid chromatography (LC) coupled to mass spectrometry (MS) and sometimes with capillary electrophoresis (CE) coupled to MS [15]. Often, metabolite analysis is carried out with nuclear magnetic resonance (NMR) spectrometry, gas chromatography coupled to MS (GC-MS), LC-MS, and sometimes with CE-MS [13]. However, no single analytical method is currently capable of detecting all peptides and/or metabolites in a biological sample [16]. Every sample pretreatment will result in the loss of some classes of compounds, the number of possible separated peaks is usually lower than the number of compounds present, and there are no universal detectors allowing for detection of all compounds in all concentrations. Therefore, combinations of analytical techniques, including sample pretreatment, should be explored to increase total coverage of the sample composition and to enhance selectivity.

3.1 Detection

As mentioned in the previous paragraph, two detection methods are generally used: NMR (mostly metabolites) and MS (peptides as well as metabolites) [14].

NMR is widely applied in metabolomics studies, because it requires minimal sample pretreatment and is an unbiased, non-destructive and robust technology that allows for the determination of absolute concentrations [13, 14, 17]. However, the sensitivity of NMR is low, and therefore NMR is not well suited for quantitative analysis. Moreover, several chemical moieties, such as sulphates, cannot be detected with NMR [13, 14].

MS is sensitive, selective and can be used to obtain structural information of compounds, especially when ultra-high resolution MS and fragmentation techniques are used (MS^n, MS-

MS) [16, 18]. For these reasons, MS can be considered as the detection method of choice in peptidomics and metabolomics research.

MS analysis requires ions in the gas phase. Several ionisation techniques have been developed to achieve this, the most important being electrospray ionisation (ESI). A major drawback of all commonly used ionisation techniques is the fact that the response factor may be affected by the composition of the sample. The simultaneous ionisation of multiple compounds causes unpredictable response signals, and may even cause compounds to remain undetected. This process is called ion suppression (IS). Due to the occurrence of IS, the sensitivity of MS is affected and, moreover, compounds cannot be quantified reliably. Therefore, direct-infusion MS (DI-MS), in which a sample is infused into the MS without prior separation, has limited applicability [14]. The ideal solution to avoid ion suppression would be to let analytes reach the MS one by one. To this end, separation methods are performed prior to transfer to the MS detector. The two main separation methods that are being employed are gas chromatography (GC) [18] and liquid chromatography (LC) [16, 19], while the role of capillary electrophoresis (CE) is expanding [13].

3.2 Separation techniques in peptidomics and metabolomics

3.2.1 Gas chromatography

Since peptides are non-volatile and thermostabile, while derivatisation procedures to overcome this are very cumbersome in the case of peptides, GC is not suitable for peptide analysis. However, GC-MS is a valuable technique in the metabolomics field. It is capable of identifying and quantifying hundreds of metabolites in one sample [20]. Initially, GC-MS has been used mostly to obtain metabolic profiles of microorganisms and plants, but currently an increasing amount of applications to mammalian samples are reported. The resolving power of GC has been further enhanced by the development of GC x GC techniques. A drawback of GC is the relatively long separation time and the laborious sample pretreatment it requires, often including a time-consuming two-step derivatisation procedure to enhance volatility and thermostability of polar and medium polar metabolites [14].

3.2.2 Liquid chromatography

As in GC, the separation mechanism of LC is based on selective distribution of analytes between the mobile and the stationary phase. LC-MS is an attractive technique for producing metabolic and peptidomic fingerprints, since it is robust, sensitive and selective.

Moreover, normally no chemical derivatisation is required. LC has already been shown to be applicable to a wide range of peptides [15] as well as metabolites [14]. Recent developments of LC in peptidomics focus on improved separation, for example by the development of multidimensional separation platforms (i.e. LC x LC-MS), and improved LOD (nanoLC). Innovations in LC-based metabolomics developments aim at improving LOD (capillary LC, or μLC), efficiency (ultra-performance LC, UPLC) and selectivity (new column materials, such as materials that allow for hydrophilic interaction chromatography (HILIC)) [14, 16].

3.2.3 Capillary electrophoresis

CE is based on a separation mechanism that is completely different from the separation mechanism of chromatography. It is based on differences in electrophoretic mobility of charged compounds in an electric field. In principle, compounds are separated based on their mass-to-charge ratio. All peptides and many important metabolite classes, such as for example fatty acids, keto acids, acyl phosphates, phospholipids, acylcarnitines, amino acids, coenzyme A derivatives and nucleotides [21] are charged compounds or can be charged by choosing a suitable pH, and are therefore susceptible to CE. Many examples of the application of CE-MS to peptidomics and metabolomics can be found in literature [22-25]. While LC requires expensive columns and large amounts of solvents, CE requires only a cheap fused silica capillary and a minimal small amount of solvent. The separation efficiency of CE is unmatched. On top of this, CE can handle sample volumes as small as a few nL. An aspect that has delayed the growing interest in CE-MS, is the lack of robustness of the hyphenation of CE to MS, but significant progress is being made in this area [26]. Several capillary electrophoretic separation techniques have been developed; the most important being capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE) and micellar electrokinetic chromatography (MEKC). Moreover, on-line analyte enrichment is possible with various stacking methods, capillary isoelectric focusing (cIEF) and capillary isotachophoresis (cITP).

As electromigration-based techniques applied to peptides and metabolites are the main topic of this thesis, section 4 is devoted to introducing the theory of electrophoresis and its most common methods and applications.

3.3 Sample pretreatment

3.3.1 Conventional sample pretreatment techniques

In peptide analysis, sample pretreatment often involves ultrafiltration to remove proteins and SPE to remove salts [27]. For untargeted metabolic profiling, sample pretreatment should be as minimal as possible in order to avoid the exclusion of metabolites classes [13]. Often, sample pretreatment for untargeted metabolomics consists only of diluting the sample. However, the high salt and, in plasma and serum, protein contents present in biological matrices can interfere with both separation and detection. Therefore, some sample pretreatment is often carried out prior to analysis, to remove both proteins and salts. To remove proteins, protein precipitation with organic solvents is commonly applied [7, 29], while for the desalting of samples, reversed phase solid phase extraction (RP-SPE) is mostly used [7]. When a specific set of metabolites is studied, a more selective approach can be used, for example by using alternative SPE materials such as ion exchange materials [28].

3.3.2 Electromigration-based sample pretreatment

In chromatography-based sample pretreatment, the injection of more sample volume (bulk) into analytical systems can be attractive to achieve lower LOD values. However, this approach is limited, since it usually also increases the amount of contaminants and bulk components that may disturb sample pretreatment, separation and detection. Instead of increasing the injected sample *volume* and along with it, the amount of injected contaminants, only the injected *amount* of analytes should be increased. Under well-chosen experimental conditions, electromigration is capable of offering this selectivity, leaving neutral components and, depending on the polarity of the electric field, either cations or anions behind.

Another strong argument for using electromigration-based sample pretreatment is the fact that chromatography and electrophoresis can complement each other, since they are based on different mechanisms, as mentioned above. The results shown in for example [30] and [31] underline the complementary nature of electromigration-based techniques to chromatography-based techniques. In [30], human urine of male and female subjects was analysed with CE-MS and LC-MS. The results were analysed with principal component analysis (PCA) plotting and with both separation techniques, male and female subjects could be distinguished. When the classifying features were studied and compared, it was revealed that different features were responsible for the gender classification. In [31], it was shown that when electrodialysis was used as sample pretreatment, other peptides in synovial fluid are found than when the sample pretreatment consisted of ultrafiltration, SPE, and subsequent freeze-drying.

The potential of electromigration-based sample pretreatment of large volumes is not yet fully exploited by the analytical community, while in the field of CE-based techniques it is already common practice to use electrophoretic mechanisms to selectively and rapidly concentrate analytes [32-34]. Electromigration-based sample pretreatment is especially suitable for trace analysis, because it offers removal of bulk components and selective concentration of trace compounds in one single step [35].

For targeted analysis, preparative isotachophoresis and isoelectric focusing have been used [36]. Zone electrophoresis sample treatment (ZEST) was presented in the late 80s and early 90s. A special valve was developed and coupled on-line to LC. With this valve, several pharmaceuticals could be isolated within 15 min from rather large biological samples (20 μL), based on their electrophoretic migration velocities. [37-39]. Despite the promising results that were presented, no recent literature can be found on ZEST.

More recent examples of electromigration-based sample pretreatment techniques mostly employ field-amplified transport across phase boundaries and membranes, the most important being electro-membrane extraction (EME), electrodialysis (ED) and electroextraction (EE) [35].

In ED, charged analytes migrate from one solution into another through a semi-permeable membrane under the influence of an electric field [31, 40, 41]. To our knowledge, ED is the only electromigration-based method that has been used as sample pretreatment procedure for metabolite or peptide profiling. In [31], the feasibility of ED for fast and selective sample pretreatment method for the profiling of low-abundant peptides in synovial fluid was demonstrated. After ED, the extracted peptides were analysed with nanoLC-MS and several new peptides were found compared to conventional sample pretreatment (ultrafiltration combined with RP-SPE).

In electro-membrane extraction (EME), an organic solvent is immobilised in the pores of a probe consisting of polymeric material and serves, upon application of an electric field, to enhance the extraction rate when the probe is immersed in a donor solution. As a result, extraction is faster and the enrichment is typically one order of magnitude. Extraction takes place from an aqueous sample, via the immobilised organic solvent, into another aqueous acceptor solvent. This promising method has been applied successfully to the analysis of several pharmaceuticals in plasma and urine [35, 39, 42], and also peptides spiked to plasma [43], but not to metabolomics. In EME of peptides, however, the structure of the peptide (i.e. polarity and charge) influences the extraction efficiency strongly (some peptides were, due to their structure, not extracted at all) [44], making the method suitable for selective

(targeted) analysis, but less for peptidomics. The extraction of peptides spiked to plasma showed moderate recoveries of 25% to 43% and endogenous levels could not be detected [43]. However, EME has been studied well, its feasibility has been demonstrated and it might become a useful tool for analyte isolation in the future.

Electroextraction (EE) is electromigration-assisted liquid-liquid extraction, where analytes migrate from the donor solvent into the immiscible acceptor solvent when an electric field is applied [45]. The mechanism of EE and its application to peptides and metabolites in biological samples is discussed and presented in detail in chapter 3, 4, and 5 of this thesis.

4. Introduction in electrophoresis

Electrophoresis is the movement of charged species due to attraction or repulsion in an electric field. It was introduced as a separation technique by Arne Tiselius when he demonstrated 'the moving-boundary method of studying the electrophoresis of proteins' [46]. He performed electrophoretic experiments on protein mixtures that were in a tube between buffer solutions and showed that the sample components migrated in a direction and that the migration rate was determined by the mobility of the component. Later, he was awarded the Nobel Prize for this work.

The electrophoretic velocity of an ion is described as [47, 48]:

2) $$v_{ep} = \mu_{ep} E$$

where v_{ep} is ion velocity, μ_{ep} the electrophoretic mobility and E the electric field strength. The electric field strength is described as [47, 48]:

3) $$E = \frac{V}{L_{tot}}$$

where V is the applied voltage and L_{tot} the length over which V is applied.
The electrophoretic mobility is the balance between the attracting electric force and the opposing frictional force.

The electric force is described as [47, 48]:

4) $$F_E = zeE$$

where z is the number of elemental charges on the ion and e the elemental charge. The frictional force for a spherical ion is described by Stokes' Law [47, 48]:

5) $$F_F = -6\pi\eta r v_{ep}$$

where η is the dynamic viscosity, r the Stokes radius of the ion and v_{ep} the ion velocity. At the start of an electrophoresis experiment, the ions accelerate to reach a constant speed within a short time. At constant speed, the electric force and frictional force will be in equal and opposite [47, 48]:

6) $$zeE = 6\pi\eta r v_{ep}$$

When Equation 2 is substituted into Equation 7, μ_{ep} can be defined as [47]:

7) $$\mu = \frac{ze}{6\pi\eta r}$$

In practice, this means that smaller ions with more elementary charges migrate faster.

4.1 Capillary electrophoresis

When electrophoretic experiments are carried out in free solution, as was the case in Tiselius' experiments, thermal diffusion and convection have a detrimental effect on the separation efficiency. To deal with this, electrophoresis in anti-convective media such as agarose and polyacrylamide gels was developed. Currently, gel electrophoresis is common practice in biochemical laboratories and it is the most used form of electrophoresis, despite the low separation efficiency, long analysis time and laborious procedures that are difficult to automate. Furthermore, gel electrophoresis is only suitable for large biomolecules (proteins,

DNA).

By performing electrophoresis in a capillary, high performance fast separations can be carried out, thanks to the very high electric field strengths that can be applied. A common experimental condition is the application of 30 kV on a capillary with a length of 50 cm, resulting in an electric field strength of 60 kV/m. Because of the very small inner diameter of a capillary (50-100 μm), only low currents (10-250 μA) are generated. Therefore, limited heat generation takes place, even at very high voltages. The little heat that is generated is easily dissipated to the environment because of the high surface-to-volume ratio of a capillary. As a consequence, the influence of thermal diffusion and convection is limited, and high performance electrophoresis can be carried out in free solution.

Capillary electrophoresis was pioneered in the 1960s by Hjertén [49], who performed separations in 1 mm quartz tubes that were coated with methyl cellulose and immersed in a water bath to dissipate Joule heating. The quartz allowed for UV detection. In the 1970s, Mikkers *et al* performed CE in a narrow-bore PTFE (Teflon) tube (0.2 mm ID, 0.35 mm OD), which was the first high performance CE experiment [50]. In the 1980s, CE in open, tubular glass capillaries (75 μm ID, 550 μm OD) developed by Jorgenson and Lukacs [51], resulting in the commercially employed technique that CE is nowadays. From then on, CE received increasing attention, resulting in the development of several CE-based separation and on-line concentration methods, and commercial CE equipment. A basic CE set-up consists of a capillary filled with background electrolyte, a high voltage supply, buffer vials and two electrodes [47]. Usually, the capillary is made of fused silica. In aqueous solutions, the silanol groups of the capillary wall can (depending on the pH) become deprotonated, which results in a negatively charged wall. The negatively charged wall attracts cations from the solution, which form a layer. As soon as the separation voltage is applied, the layer of cations starts migrating, dragging the rest of the bulk liquid with it. The resulting flow is called electro-osmotic flow (EOF). The flow profile of EOF is typically flat, which is the main reason that, in spite of the fact that a bulk flow exists, very high separation efficiencies can be achieved with CE in comparison with LC, where hydrodynamic flow leads to a parabolic flow profile [47].

Detection in CE initially usually took place using spectrophotometry, via a detection window in the capillary. Nowadays, robust CE-MS hyphenation has been achieved [26]. The most important CE separation methods are capillary zone electrophoresis (CZE), capillary electrochromatography (CEC), capillary gel electrophoresis (CGE) and micellar electrokinetic chromatography (MEKC). Moreover, CE can be used for on-line analyte

concentration. On-line concentration methods are based on smart buffer choice, which induces field-amplified or chemically induced sample stacking. These processes can be employed to concentrate analytes on-line and in conjunction with a consecutive separation. Two CE methods can be used to concentrate and separate analytes simultaneously, namely capillary isoelectric focusing (cIEF) and capillary isotachophoresis (cITP).

4.2 CE separation techniques

4.2.1 Capillary zone electrophoresis

In CZE, the most common CE form, analytes are separated based on their different electrophoretic mobilities after they are injected into a capillary that is filled with background electrolyte and a separation voltage is applied. Thanks to the presence of EOF, both cations and anions can be analysed in one run. Neutral compounds cannot be separated with CZE [47].

Non-aqueous capillary electrophoresis (NACE) is increasingly gaining interest. In NACE, the background electrolyte consists of an organic solvent. As a rule, non-aqueous solvents are less conductive than aqueous solvents. Therefore, low currents exist in NACE and hardly any Joule heating is produced. As a consequence, higher electric field strengths as well as wider bore capillaries can be used. Due to higher electric field strengths, separation efficiency can be enhanced, while wider bore capillaries enable the injection of more sample volume and so offer lower LOD values. Moreover, separation selectivity can be easily manipulated with various optional organic solvents. Since organic solvents are mostly volatile and have a low surface tension, which is beneficial for electrospray, NACE-MS will probably be applied more often in the near future [52]. Currently, relatively few bio-analytical applications of NACE have been reported [53, 54], but given the high potential that has been shown so far, this may well change in the coming decade.

4.2.2 Capillary electrochromatography

CEC is a hybrid separation method of CZE and chromatography. Separation takes place in a capillary that is packed or coated with a stationary phase. The separation principle of CEC is based on differences in electrophoretic mobility and differences in affinity for the stationary phase. As a consequence, CEC is capable of separating ionic as well as neutral compounds. The neutral components are only separated due to difference in their affinity for the stationary phase. When the analytes are transported towards the detector by EOF,

very sharp peaks can be obtained [47].

4.2.3 Capillary gel electrophoresis

CGE is almost uniquely applied to DNA analysis. DNA molecules of different size have similar mass-to-charge ratios, since each extra building block also introduces an extra charge. Therefore, their electrophoretic mobilities are similar, and conventional CZE does not suffice for good separation. By carrying out the separation in a capillary filled with cross-linked polyacrylamide, analytes can be separated based on their size. The separation speed of CGE is around five times faster than slab gel electrophoresis and can be carried out in automated multiplex mode. The invention of CGE and the development of multiplexed CGE with stabile gel matrices and multiple-capillary detection have significantly accelerated the completion of the sequencing of the Human Genome Project [55], which is still one of the most remarkable successes of CE.

4.2.4 Micellar electrokinetic chromatography

MEKC is, like CEC, capable of separating neutral analytes. This is achieved by adding an ionic surfactant to the background electrolyte in a concentration that is above its critical micelle concentration. The micelles serve as a pseudo-stationary phase and analytes will be distributed between the micelles and the aqueous buffer. Often, sodium dodecyl sulphate (SDS) micelles are employed. When an electric field is applied, an injected sample will migrate towards the anode due to EOF, while the anionic SDS micelles migrate towards the cathode. Separation of the neutral analytes then takes place according to differences in selective distribution between the buffer and the micelles [56]. With MEKC, highly efficient separations can be achieved, but its main drawback is the cumbersome coupling to MS, due to the presence of high levels of surfactants. With the partial filling approach, MEKC has been successfully coupled to MS [55]. There is also evidence that MEKC can be coupled directly to MS with atmospheric pressure chemical ionisation (APCI) [57, 58].

4.3 On-line sample concentration

Electrophoresis is a process of ion fluxes, and by carefully selecting the experimental conditions, an analyte flux can be generated and on-line preconcentration can be performed, leading to an enhanced LOD of the method. CE injections that allow for good consecutive separation typically are around 1% of the capillary volume, which is in the low nL range. For example, 1% of the volume of a conventionally dimensioned capillary (75 μm ID x 1

m length) means only 20 nL sample is used. Due to the low volume loadability of CE, the determination of (very) low abundant compounds is often problematic. Moreover, relevant biological compounds often occur in very low concentrations. In LC-based analysis, a simple solution for this is to inject more sample volume, but this often compromises separation or leads to fouling of the system. To achieve LOD values, an analyte flux instead of bulk flow should be generated. The nature of electromigration and the capillary format offers excellent possibilities for on-line sample preconcentration by carefully selecting suitable buffers or electrolytes. Below, the most commonly used on-line sample concentration techniques are discussed briefly, divided in stacking techniques (field-amplified stacking (FASS) and chemically-induced stacking) and techniques that concentrate and separate simultaneously (cIEF and cITP).

In chapter 2 of this thesis, the potential of cITP-MS is explored and the development of a highly effective special type of FASS is studied and applied for the first time in bioanalysis, namely electroextraction, is shown in chapter 3, 4 and 5.

4.3.1 Field-amplified sample stacking

FASS relies on a conductivity difference between the sample and the running buffer. The work on electroextraction, which is described in this thesis in chapter 3, 4 and 5, is also a form of FASS.

When the injected sample has a lower conductivity than the background electrolyte, a higher electric field strength exists over the sample plug than over the rest of the capillary, according to Ohm's law. Equation 2 states that the electrophoretic velocity (v) is proportional to the electric field strength (E). Therefore, the sample molecules will migrate faster in the sample zone than in the buffer zone. As soon as the sample molecules migrate out of the sample zone, into the buffer zone, they slow down and are stacked [32].

4.3.2 Chemically induced sample stacking

Analytes can be stacked on the boundary between sample and background by chemically changing their electrophoretic velocities. The most common ways of achieving this are dynamic pH junction and sweeping [34]. In a dynamic pH junction, the pH of the background electrolyte is chosen such that the analyte becomes neutral when it enters it. As a consequence, the electrophoretic velocity of the analyte drops to zero and the analytes are concentrated [34]. In sweeping, a pseudo-stationary phase as used in MEKC is injected after the sample plug. When the pseudo-stationary phase migrates through the sample zone

it picks up and concentrates the analytes, i.e. it 'sweeps' the analytes into a very sharp zone. The concentration gain is dependent on the affinity of the analyte to the pseudo-stationary phase [34].

4.4 Simultaneous concentration and separation

Two CE techniques are capable of separating and concentrating analytes in one step, namely cIEF and cITP. In both methods, analytes are concentrated in so-called self-sharpening zones that are adjacent to each other.

4.4.1 cIEF

cIEF is only suitable for ampholytic compounds and specifically applied in peptide and protein analysis. In a cIEF experiment, a pH gradient is created in the capillary with the help of carrier ampholytes. An analyte electromigrates through the pH gradient until it reaches the zone where the pH is equal to its pI value; in this zone it becomes electrically neutral and ceases to migrate further. Due to this mechanism, analytes are separated according to their pI value and simultaneously concentrated in their pH zones. The analyte zones are self-correcting; when an analyte molecule diffuses from its zone, it enters another pH, gets charged and migrates back [59].

4.4.2 cITP

In cITP, the sample is injected between two different electrolytes: the leading electrolyte (LE) and a terminating electrolyte (TE). The choice of electrolytes is based on the electrophoretic mobility of the analytes: the LE contains an ion species with a higher electrophoretic mobility and the TE contains an ion species with a lower electrophoretic mobility than the analytes. When an electric field is applied in this system, a steady-state will be formed which results in the formation of 'a train' of analyte zones that all migrate with equal speed. The analytes are arranged according to their electrophoretic mobilities [60]. According to Kohlrausch's regulation function (Equation 8), the concentration of ion a (the analyte) is dependent on the concentration of the preceding ion l (the leading ion) [47].

8) $$C_a = C_l \frac{\mu_a(\mu_l + \mu_r)}{\mu_l(\mu_a + \mu_r)}$$

In this equation, C_a and C_l are the molar analyte and LE concentrations, respectively, and μ_a, μ_r and μ_l are the electrophoretic mobilities, respectively, of the analyte *a*, counter

ion *r* and the leading ion *l*. The consequence of this process is that analytes that occur in concentrations below the LE concentration will be concentrated and compounds that occur in concentrations above the LE concentration will be diluted. For this reason, cITP has a high dynamic range and is very suitable for analysis of trace compounds in complex samples. Each analyte zone has its own electric field strength value, which results in self-sharpening zones. When an analyte diffuses out of its own specific zone, it will be delayed or sped up back to its own zone due to the lower or higher electric field strength it meets in the adjacent zone. In an ITP experiment, either cations or anions can be separated in one experiment and the analyte zones are being formed directly adjacent to each other. In other words, they are not physically separated and an isotachopherogram does not contain peaks, but a series of block signals. At the borders of the analyte zones, a mixed zone exists that contains both analytes. In case of trace compounds, analyte zones become so narrow that during detection, it is impossible to detect them separately; they are mixed [61]. To separate the adjacent zones, cITP is often combined with CZE. In this combination, cITP is transient. In transient ITP (tITP), the capillary is filled with a LE zone, a sample zone, a short TE zone and again a LE zone, respectively. In the first instant, tITP is taking place, but within a short time, the TE zone is overtaken by the LE that was injected behind it. Then, the cITP system has become a CZE system and the analytes are separated [62]. A disadvantage of this process is that the zones lose their self-sharpening ability. Especially very narrow ITP zones will suffer from band-broadening, which partly cancels out the concentrating ITP effect and results in less favourable LOD values.

An alternative approach is the use of spacer molecules, which will be focused between the analytes [63]. In this way, analyte zones can be separated physically without losing the power of the ITP process. In chapter 2 of this thesis, the feasibility of this approach for plasma peptides is explored in order to couple ITP of peptides directly to MS detection.

5. Scope and outline of the thesis

The paradigm shift from reductionism towards systems biology demands innovations from analytical chemistry. A good systems biology approach demands comprehensive knowledge of the identity and concentration of all metabolites and peptides, including low-abundant ones, in a multitude of (small) samples. The research fields of peptidomics and metabolomics require from analytical techniques improvements in LOD, speed and suitability to measure a wide range of analytes.

The goal of this thesis is to demonstrate that electrophoretic on-line concentration procedures meet these three requirements. Electromigration is capable of enhancing selective analyte migration. Selective analyte migration can be used to increase the amount of analyte that is injected into an analytical system, while it leaves the sample volume with the contaminants behind. In consequence, the injection of contaminants and bulk components that may disturb sample pretreatment, separation and detection can be avoided and LOD values can be lowered without the problems associated with direct injection of larger sample volumes.

The work that is presented in this thesis is focused on fast electrophoresis-based LOD improvement of the analysis of peptides and/or metabolites in plasma and urine.

In **Chapter 2**, the use of carrier ampholytes as spacer molecules to couple cITP of peptides directly to MS is explored. In this way, the isotachophoretic peptide zones can be separated from each other without losing ITP conditions, in this way enabling cITP-MS analysis with low LOD values of pure, highly concentrated trace peptides.

The remainder of the thesis is devoted to the demonstration of electroextraction (EE) as on-line electrophoretic concentration technique for sensitive, fast and comprehensive peptide and metabolite profiling. In **Chapter 3**, to demonstrate the ability of EE to quickly extract a wide range of peptides from complex samples and concentrate them, a capillary EE (cEE) method is developed, its potential for coupling to LC-MS is demonstrated, and the resulting cEE-LC-MS system was applied to protein digests and endogenous peptides in urine. To further increase analyte enrichment factors and speed, an improved new large volume cEE set-up that is capable of extracting larger volumes within a shorter time span is shown in **Chapter 4**. Furthermore, its on-line coupling to LC-MS and its applicability to plasma peptide analysis is studied. In **Chapter 5**, to demonstrate the comprehensiveness and versatility of EE, the capacity of large volume cEE to extract metabolites from untreated urine was studied. In addition, the potential of combining large volume cEE with CZE-MS was explored.

6. References

[1] Ahn, C.A., Tewari, M., Poon, C-H., Phillips, R.S. *PLoS Med,* **2006***, 3 (6),* 709-713

[2] Van der Greef, J., McBurney, R.N., *Nat Rev Drug Discov,* **2005***, 4,* 961-967

[3] Van der Greef, J., Stroobant, P., Van der Heijden, R., *Curr Opin Chem Biol,* **2004***, 8,* 559-565

[4] Schulz-Knappe, P., Schrader, M., Zucht, H-D., *Comb Chem High Throughput Screen,* **2005***, 8,* 697-704

[5] Griffin, J.L., *Philos Trans R Soc Lond B Biol Sci,* **2004** *359, 1446,* 857-71

[6] Raamsdonk, L.M., Teusink, B., Broadhurst, D., Zhang, N., Hayes, A., Walsh, M.C., Berden, J.A., Brindle, K.M., Kell, D.B., Rowland, J.J., Westerhoff, H.V., Van Dam, K., Oliver, S.G., *Nat Biotechnol,* **2001***, 19,* 45-50

[7] Dunn, W.B., Ellis, D.I., *Trends Analyt Chem,* **2005***, 24, 4,* 285-294

[8] Aderem, A., *Cell,* **2005***, 121,* 511-513

[9] Anderson, N.L., Anderson, N.G., *Mol Cell Proteomics,* **2002***, 1,* 845-867

[10] Van der Greef, J., Martin, S., Adourian, A., Plasterer, T., Verheij, E.R., McBurney, R.N., *J Proteome Res,* **2007***, 6,* 1540-1559

[11] McNaught, A.D., Wilkinson, A., Nic, M., Jirat, J., Kosata, B., Jenkins, A., *IUPAC. Compendium of Chemical Terminology*, **2006,** *2nd edition (the "Gold Book") Blackwell Scientific Publications, Oxford. XML on-line corrected version: http://goldbook.iupac.org (website consulted at 25 April 2012)*

[12] Miller, J.N., Miller, J.C., *Statistics and chemometrics for analytical chemistry*, **2000**, 4th edition, Prentice Hall, 121-122

[13] Ramautar, R., Somsen, G.W., De Jong, G.J., *Electrophoresis,* **2009***, 30,* 276-291

[14] Xiayan, L., Legido-Quigley, C., *Electrophoresis,* **2008***, 29,* 3724-3736

[15] Boonen,, K., Landuyt, B., Baggerman, G., Husson, S.J., Huybrechts, J., Schoofs, L., *J Sep Sci,* **2008***, 31,* 427-445

[16] Allwood, J.W., Goodacre, R., *Phytochem Anal* **2010***, 21,* 33-47

[17] Beckonert, O., Keun, H.C., Ebbels, T.M.D., Bundy, J., Holmes, E., Lindon, J.C., Nicholson, J.K., *Nat Protoc,* **2007***, 2, 11,* 2692-2703

[18] Zhang, X., Wei, D., Yap, Y., Li, L., Guo, S., Chen, F., *Mass Spectrom Rev,* **2007***, 26,* 403-431

[19] Wilson, I.D., Plumb, R., Granger, J., Major, H., Williams, R., Lenz, E.M., *J Chromatogr B,* **2005***, 817,* 67-76

[20] Lisec, J., Schauer, N, Kopka, J., Willmitzer, L., Fernie, A.R., *Nat Protoc,* **2006***, 1, 1,* 387-396

[21] Wishhart *et al*, *Nucleic Acids Res,* **2009***, 37,* D603-D610

[22] Soga, T., Ohashi, Y., Ueno, Y., Naraoka, H., Tomita, M., Nishioka, T., *J Proteome Res,* **2003***, 2,*

488-494

[23] Hirayama, A., Kami, K., Sugimoto, M., Sugawara, M., Toki, N., Onozuka, H., Kinoshita, T., Saito, N., Ochiai, A., Tomita, M., Esumi, H., Soga, T., *Cancer Res* **2009**, *69, 11,* 4918-4925

[24] Sugimoto, M., Wong, D.T., Hirayama, A., Soga, T., Tomita, M., *Metabolomics,* **2010**, *6,* 78-95

[25] Schiffer, E., Mischak, H., Novak, J., *Proteomics,* **2006**, *6,* 5615-5627

[26] Hommerson, P., Khan, A.M., De Jong, G.J., Somsen, G.W., *Mass Spectrom Rev,* **2011**, *30, 6,* 1096-1120

[27] Aristoteli, L.P., Molloy, M.P., Baker, M.S., *J. Proteome Res,* **2007**, *6,* 571-581

[28] Griffiths, W.J., Sjövall, J., *Biochem Biophys Res Commun,* **2010**, *396,* 80-84

[29] Villas-Bôas, S.G., Højer-Pedersen, J., Åkesson, M., Smedsgaard, J., Nielsen, J., *Yeast,* **2005**, *22,* 1155-1169

[30] Ramautar, R., Nevedomskaya, E., Mayboroda, O.A., Deelder, A.M., Wilson, I.D., Gika, H.G., Theodoridis, G.A., Somsen, G.W., De Jong, G.J., *Mol Biosyst,* **2011**, *7,* 194-199

[31] Kamphorst, J.J., Tjaden, U.R., Van der Heijden, R., De Groot, J., Van der Greef, J., Hankemeier, T., *Electrophoresis,* **2009**, *30,* 2284-2292

[32] Chien, R-L., Burgi, D.S., *J Chromatogr,* **1991**, *559,* 141-152

[33] Britz-Kibbin, P., Terabe, S., *J Chromatogr A,* **2003**, *1000,* 917-934

[34] Breadmore, B.C., Thabano, J.R.E., Dawod, M., Kazarian, A.A., Quirino, J.P., Guijt, R.M., *Electrophoresis,* **2009**, *30,* 230-248

[35] Kubáň, P., Šlampová, A., Boček, P., *Electrophoresis,* **2010**, *31,* 768-785

[36] Bier, M., *Electrophoresis,* **1998**, *19,* 1057-1063

[37] Kok, W. Th., Hupe, K.P., Frei, R.W., *J Chromatogr A,* **1988**, *436,* 421-427

[38] Debets, A.J.J., Hupe, K.-P., Brinkman, U.A.Th., Kok, W.Th., *Chromatographia,* **1990**, *29, 5/6,* 217-222

[39] Debets, A.J.J., Schoutsen, T.P., Kok, W.Th., Hupe, K.-P., Brinkman, U.A.Th., *Chromatographia,* **1992**, *34, 11/12,* 581-588

[40] Brewster, J.D., Piotrowski, E.G., *J Chromatogr,* **1991**, *585,* 213-217

[41] Buscher, B.A.P., Tjaden, U.R., Van der Greef, J., *J Chromatogr A,* **1997**, *788,* 165-172

[42] Pedersen-Bjergaard, S., Rasmussen, K.E., *J Chromatogr A,* **2006**, *1109,* 183-190

[43] Balchen, M., Halvorsen, T.G., Reubsaet, L., Pedersen-Bjergaard, S., *J Chromatogr A,* **2009**, *1216,* 6900-6905

[44] Balchen, M., Reubsaet, L., Pedersen-Bjergaard, S., *J Chromatogr A,* **2008**, *1194,* 143-149

[45] Stichlmair, J., Schmidt, J., Proplesch, R., *Chem Eng Sci,* **1992**, *47, 12,* 3015-3022

[46] Tiselius, A., *Trans. Faraday Soc,* **1937**, *33,* 524-531

[47] Mazereeuw, M., Tjaden, U.R., *Capillaire Elektroforese, Chromatografie in de Praktijk, Ten Hagen Stam*, december **1996**, 1-88

[48] Riekkola, M.-L., Jönsson, J. Å, Smith, R.M., *Pure Appl Chem*, **2004**, *76 (2)*, 443-451

[49] Hjertén, S., *Chromatogr Rev,* **1967***, 9,* 122-219

[50] Mikkers, F.E.P., Everaerts, F.M., Verheggen, Th.P.E.M., *J Chromatogr A,* **1979***, 169,* 11-20

[51] Jorgenson, J.W., Lukacs, K.D., *Anal Chem,* **1981***, 53,* 1298-1302

[52] Riekkola, M.-L., Jussila, M, Porras, S.P., Valkó, I.E., *J Chromatogr A,* **2000***, 892,* 155-170

[53] Lai, E.P.C., Feng, S.Y., *J Chromatogr B,* **2006***, 843,* 94-99

[54] Sasajima, Y., Lim, L.W., Takeuchi, T., Suenami, K., Sato, K., Takekoshi, Y., *J Chromatogr A,* ***2010****, 1217,* 7598-7604

[55] Karger, B.L., Guttman, A., *Electrophoresis,* **2009***, 30,* s196-s202

[56] Terabe, S., *Chem Rec,* **2008***, 8,* 291-301

[57] Takada, Y., Sakairi, M., Koizumi, H., *Anal Chem,* **1995***, 67,* 1474-1476

[58] Yang, L., Lee, C.S., *J Chromatogr A,* **1997***, 780,* 207-218

[59] Shimura, K., *Electrophoresis,* **2009***, 30,* 11-28

[60] Petr, J., Maier, V., Horáková, J., Ševcík, J., Stránský, Z., *J Sep Sci,* **2006***, 29,* 2705-2715

[61] Peterson, Z.D., Bowerbank, C.R., Collins, D.C., Graves, S.W., Lee, M.L., *J Chromatogr A,* **2003***, 992,* 169-179

[62] Timerbaev, A.R., Hirokawa, T., *Electrophoresis,* **2006***, 27,* 323-340

[63] Chartogne, A., Reeuwijk, B., Hofte, B., Van der Heijden, R., Tjaden, U.R., Van der Greef, J., *J Chromatogr A,* **2002***, 959,* 289-298

Chapter 2

Potential of capillary isotachophoresis coupled to mass spectrometry of peptides using spacer molecules

Based on
Lindenburg, P.W., Tjaden, U.R., Van der Greef, J., Hankemeier, T.
Potential of capillary isotachophoresis mass spectrometry of peptides using spacer molecules.
In preparation for submission.

Abstract

Capillary isotachophoresis (cITP) is an electrophoretic separation technique that is able to concentrate analytes during separation, in consequence improving the sample concentration detection limits. In health sciences, the importance of peptides is widely recognised, since changes in body fluid peptide profiles often reflect disturbed homeostasis or disease. The analysis of peptides is complicated, since peptides typically occur in very low concentrations. In conventional cITP coupled to mass spectrometry (MS), trace peptides are focused in adjacent bands. This causes several adjacent peptides to enter the MS all at once, resulting in multiple peptide mass spectra and signal discrimination due to ion suppression. In this paper, we explore the use of spacer molecules to overcome this complication of cITP-MS. Carrier ampholytes (CA), which are normally used to generate pH gradients in (C)IEF, can act as spacer compounds in cITP. In this way the peptide bands are separated from each other, making MS analysis of pure peptides possible. As an example the analysis of a set of 6 model peptides as well as a tryptic digest of bovine serum albumine (BSA) is reported. Finally, some recommendations for spacer compounds in cITP are done.

1. Introduction

Qualitative and quantitative information about the concentration of trace compounds such as peptides, for example in plasma, is highly important for health sciences, since they often are markers for diseases [1]. Counting all proteins and peptides of all variations and origins, the plasma proteome is believed to contain as much as 1 million molecule species. Their physiological range spans from 35-40 mg/mL in the case of the protein serum albumin and 0-5 pg/mL in the case of the peptide interleukin 6 [2]. The role of peptides in important biological processes such as inflammatory response, tumour biology and endocrine processes has been established [3], so there is a great demand for knowledge about human peptides and their functions. Progress is mainly limited due to the lack of techniques that enable a comprehensive peptide analysis [4], especially at very low concentrations where many relevant peptides occur (pg/mL). Nowadays, (nano)LC and CZE coupled to MS are the two most common techniques used for peptide analysis. However, both approaches have limitations regarding their dynamic range and limit of detection (LOD).

A benefit of CE-based systems is the simplicity of the system, i.e. a disturbed CE-system is repaired by just replacing the capillary, which is cheap and not time-consuming. On the contrary, troubleshooting in a disturbed nano-LC system can be complex [5], time-consuming and maintenance costs can become high.

CZE coupled to electrospray ionisation MS (ESI-MS) offers high separation power, high mass sensitivity and structural information about the peptides, but due to the low loadability of CZE this technique suffers from high sample concentration LOD values. For this reason and for the fact that CZE is just as chromatography in essence a diluting separation technique, very low-abundant peptides are not detected. This can be partly overcome by using appropriate sample pretreatment, making the overall analytical process more complex.

However, cITP offers a way to increase the analyte concentrations during separation. Thanks to a discontinuous electrolyte system consisting of a leading electrolyte (LE) and a terminating electrolyte (TE), in cITP the ultimate migration velocity of the analyte ions is the same in the whole capillary. The choice for leading and terminating electrolytes depends on the mobilities of the analytes that are to be studied; i.e. a suitable 'isotachophoretic window' should be created. In the sample plug, which is injected between LE and TE, analytes are arranged according to their electrophoretic mobilities. As a result, the peptides are separated from each other. The capillary can be completely filled with sample, implying

an improvement in detectability via extended loadability.

According to Kohlrausch's regulation function (Equation 1), the concentration of ion a (the analyte) is dependent on the concentration of the preceding ion l (the leading ion).

1) $$C_a = C_l \frac{\mu_a(\mu_l + \mu_r)}{\mu_l(\mu_a + \mu_r)}$$

In this equation, C_a and C_l are the molar analyte and LE concentrations, respectively, and μ_a, μ_r and μ_l are the electrophoretic mobilities of the analyte *a*, counter ion *r* and the leading ion *l*, respectively. The effect of this process is concentration of sample compounds with concentrations below the LE concentration, while on the other hand sample compounds present at concentrations higher than LE will be diluted. As a consequence, the dyamic range of cITP-MS is increased compared to LC- or CZE-MS, enabling detection of low as well as the high abundant compounds in one single analysis. As stated above, the concentrations of the peptidome span a dynamic range of 10 orders of magnitude [2]. The concentration factor that can be achieved using cITP can be as high as 10^6 times [6]. In addition to the concentrating effect, the diffusion of analytes is counter-acted, since the analyte bands are self-sharpening as they have each their own electric field strengths. And finally, cITP can aid in sample clean-up by choosing suitable TE and LE so that interfering matrix compounds having higher or lower electrophoretic mobilities than the peptides investigated can be excluded from the separation. In literature a clear explanation of ITP is given by Holloway and Trautschold [7] and Petr *et al* [8].

The detection method of choice for peptides (and proteins) nowadays is MS [9]. Using MS, mass information as well as structural information about peptides can be obtained, enabling quick identification of peptides and discovery of new peptide species. A drawback of MS is the fact that, when more compounds are present at the same time during ESI, ion suppression compromises the linear response of the analytes, which makes reliable quantitation problematic [10].

Coupling cITP with MS is not as straightforward as for example CZE-MS or LC-MS, since in the isotachophoretic process the analytes are focused in directly adjacent zones instead of physically separated zones. This creates mixed zones at the boundaries between the analyte zones [11], which was already noted in the same time frame as the invention of ESI in 1989 [12]. In these mixed zones, ion suppression takes place and as a consequence quantitative determinations are affected, especially in the case of low analyte concentrations, when the zones are prone to collapse and mix fully [13]. This is a severe drawback of cITP-

MS for trace analysis with small sample volumes. According to Equation 1, the very low-abundant peptides are strongly concentrated, resulting in very narrow bands. These narrow bands will enter the MS at virtually the same time. As a consequence, the separation is lost and therefore ion suppression between the analytes may occur during ESI, making reliable quantitation cumbersome [13].

For these reasons, the direct coupling of cITP with MS has not often been utilised yet and instead cITP-MS has mostly been applied in combination with CZE [14], via directly coupled cITP-CZE [15,16,17] or via transient isotachophoresis coupled to CZE (tITP-CZE) [18,19,20, 21]. Direct comparison of analyses of the plasma levels of the peptides angiotensin II and gonadorelin with tITP-CZE-UV and LC-UV has already shown that tITP-CZE-UV is superior to LC-UV in terms of concentration LOD [22]. In [23], preparative tITP-CZE and strong cation exchange solid phase extraction (SCX-SPE) as first dimension in a twodimensional shotgun proteomics set-up were compared. The second dimension was nano-reversed phase liquid chromatography coupled to MS in both cases. Analysis of glioblastoma-derived cancer stem cells demonstrated that tITP-CZE achieved superior performance in total peptide, distinct peptide and distinct protein identifications. Moreover, its analytical reproducibility was good. The authors conclude that with the help of tITP-CZE multidimensional proteomics platforms can mine deeper into the proteome and the technology is expected to serve as a critical component in the overall toolset required for biomarker discovery in proteomics.

From a CZE perspective, the combination with cITP means an improvement, but from the cITP perspective the combination with CZE means sacrificing two of its major benefits, i.e. the self-correcting ability of the analyte zones and the high loadability. It is desirable for higher resolution as well as the LOD that the isotachophoretic process is maintained until the sample is infused into the MS, because, CZE starts as soon as the zone-sharpening isotachophoretic process is aborted, implying that zone broadening by diffusion inherent to CZE occurs. To be able to perform cITP coupled directly to MS detection, we explored the use of spacer molecules. These will be positioned between the different isotachophoretic peptide zones, preventing that several peptide bands enter the MS at the same time. To demonstrate this approach, experiments with carrier ampholytes (CA) as spacer compounds were carried out.

CA are mixtures of many amphoteric molecule species that have slightly different isoelectric points. Originally, they were designed to generate pH-gradients in IEF and consisted of N- methylated or -ethylated oligoamines that have reacted with acrylic acid [24]. The

commercially available CA used in this research consisted of the products of a copolymerisation reaction of amines, amino acids and dipeptides in epichlorohydrin [25]. Since CA are complex mixtures of molecules, a wide range of electrophoretic mobilities is present. In a cITP separation of peptides with CA present, both should behave electrophoretically in a comparable way, in order to let the CA end up as spacers between the peptides. Since hundreds of CA species are present, the chance that bands are being positioned between the peptides is expected to be high. For cITP-MS of proteins, it has already been shown that CA act as spacer molecules between the protein zones [26], while for cITP-UV the use of CA as spacer compounds has been shown for the analysis of lipoproteins [27]. Inorganic acids, organic acids and amino acids were used as spacer molecules in cITP-UV analysis of humic acids [28], while 2-chloropropionic, glyoxylic and levulinic acids were used as spacers in cITP of ribonucleotides [29]. Moreover, a procedure has been described to choose suitable amino acids and dipeptides as spacers for cITP of various anionic species via a calculation that takes isotachophoretic steady-state parameters based on the ionic mobilities and pK values of the separated compounds and migration order into account [30].

However, CA are known to disturb MS detection. For example, in cIEF 0.2% CA added to the sample causes a loss in peptide MS signal of 15-45% [31]. In cITP, this effect is expected to be less present or even absent because the peptides and CA are better separated from each other and, more importantly, according to Kohlrausch' regulation function the CA concentration will never exceed the LE concentration. As a result, when a typical LE concentration (5-10 mM) is used, the maximum CA concentration that is reached during ITP should have a limited influence on the ionisation efficiency of the co-introduced peptides. Adding a higher amount of CA will not affect their concentration in the various ITP bands, only their bands will be broader.

In this study, different from the approaches described in literature so far, we focus on the direct coupling of cITP to MS with the help of CA as spacer molecules. This is demonstrated with peptides as model compounds. Furthermore, the potential of this approach for bio-analysis is investigated with the cITP-MS analysis of a digest of BSA.

2. Materials and methods

2.1 Equipment

cITP was carried out in untreated fused silica capillaries using a programmable PrinCE capillary electrophoresis system (Prince Technologies, Emmen, The Netherlands). Fused silica

capillaries (ID 75 μm, OD 320 μm) were purchased from Bester (Bester B.V., Amstelveen, The Netherlands). Elutions from solid phase extractions were freeze-dried in a Zibus Vaco I freeze-dryer (Zirbus Technology, Bad Grund, Germany) under vacuum that was applied with an Alcatel 2008A vacuum pump (Alcatel Vacuum Products, Hingham, MA, USA). Mass spectrometric experiments were carried out on a Bruker Daltonics MicrOTOF mass spectrometer (Bruker Daltonics, Bremen, Germany) using a CE-MS electrospray interface supplied by Agilent Technologies (Amstelveen, The Netherlands).

2.2 Chemicals

All reagents were of analytical grade or higher. A Millipore Q-guard water purifying system (Billerica, MA, USA) was used to obtain pure water, ACN was obtained from Biosolve (Valkenswaard, The Netherlands), formic acid from Acros Organics (Bridgewater, NJ, USA), ammonium acetate, ε-aminocaproic acid, BSA and trypsin from Sigma (St. Louis, MO, USA), acetic acid and sodium hydroxide from JT Baker (Philipsburg, NJ, USA) and the model peptides from Genscript Corporation (Piscataway, NJ, USA). The CA mixture (Pharmalyte 3-10) was purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden).

2.3 Capillary electrophoresis

Prior to every cITP experiment, the separation capillary (length 100 cm) was conditioned by subsequently flushing for 15 min with 1 M NaOH, 15 min with 0.1 M NaOH and 15 min with LE. The LE consisted of 5 mM ammonium acetate and the TE of 10 mM ε-aminocaproic acid. LE, TE and sample were adjusted to pH 3 with acetic acid [18,19]. Hence, the leading ion was NH_4^+ and protonated ε-aminocaproic acid functioned as the terminating ion. Hydrodynamic injection was applied to fill the capillary with the electrolytes and sample.

Prior to loading a sample for cITP analysis, CA were added to the sample. A typical cITP procedure consisted of filling the capillary with LE, loading a sample plug and finally injecting a TE plug. The sample plug as well as the TE plug comprised roughly 5 % of the capillary volume, corresponding with around 0.22 μL. Since the BSA peptides occurred in low concentrations in the sample, a larger amount of sample was injected in order to obtain broader, better detectable BSA peptide zones: 25% of the capillary was filled (1.1 μL). To avoid the sample to enter the MS before separation (by electro-osmotic flow and the pulling

effect of the electrospray) was completed, a higher backpressure than in the experiments with model peptides was used. The counter pressure was generated by a hydrostatic pressure (standard difference in height of inlet and outlet of 35 cm (inlet lower) combined with a negative pressure (±11 mbar in the case of experiments with model peptides and -20 mbar in the case of BSA digest) at the capillary inlet. While applying counter pressure a separation voltage of 24 kV was applied. After 60 min of counter pressure, the sample was allowed to enter the MS by applying 50 mbar pressure at the capillary inlet, while maintaining the ITP voltage.

cIEF-experiments were carried out with 10 mM NaOH as anolyte and 1% FA as catholyte in the same capillaries as were used for cITP. After flushing the capillary with the anolyte, a sample plug consisting of the model peptides and 2% of CA was injected hydrodynamically, followed by catholyte. Then, the analytes and CA were allowed to focus for 30 min before the sample was transferred to the MS using a hydrodynamic pressure of 50 mbar generated by the injector, while maintaining the IEF voltage.

CZE was carried out with 1% acetic acid as background electrolyte. The capillary was filled for ±1% with sample, after which high voltage and 50 mbar hydrodynamic pressure were applied for separation and transfer to the MS.

2.4 Mass spectrometry

The coupling of cITP to the MS was based on a pre-fabricated CE coupling sheath-liquid assisted grounded spray source delivered by Agilent Technologies (Agilent Technologies, Amstelveen, The Netherlands). The sheath liquid consisted of a 50/50 mixture of ACN and 0.2% formic acid and the sheath liquid flow rate was set at 3 µL/min.

Identification of peptides was based on matching of the known masses of the target peptides with the mass information obtained with the MS. To decrease noise levels, all ion traces were processed with Gaussian smoothing (value 4.03) using the software delivered with the MicrOTOF MS by Bruker Daltonics (Bruker Daltonics MicrOTOF DataAnalysis version 3.3). Mass spectra were processed with background subtraction (using an MS spectrum taken from the TE without any other added component) to remove some neutral contaminants in the TE that were detected throughout the whole sample.

2.5 BSA digestion and sample clean-up

BSA was digested using sequence grade trypsin. To improve digestion, the protein

was pretreated with *S*-methyl methanethiosulfonate (MMTS) and tris(2-carboxyethyl) phosphine (TCEP) to break and reduce disulfide bonds, respectively. The digested proteins were purified by SPE using TopTip© pipette tips (Glygen Corp., Maryland, USA) filled with a small amount of Poros RP-2 SPE material. In brief, the material was wetted with ACN, then equilibrated with 0.1% formic acid after which the sample (acidified to pH 3 with formic acid) was applied. Then, the bound analytes were washed with 0.1% formic acid, after which elution was achieved with a 60/40 mixture of ACN and 0.1% formic acid. Next, the eluent was freeze-dried and resuspended in 10 mM TE. The proteomic tool Peptide Mass on www.expasy.ch was used to forecast masses of peptides after digestion.

3. Results and discussion

3.1 Proof-of-principle using model peptides

First, an experiment was done to confirm that the chosen LE and TE were suitable for the cITP of peptides. This was done by using two LE concentrations, 5 mM and 20 mM, in a cITP separation of the 3 model peptides. When using 20 mM, the peptide zones were higher and narrower than with 5 mM LE (Fig. 2.1), which is in agreement with ITP theory. Therefore, the combination of protonated ε-aminocaproic acid as terminating ion and NH_4^+ as leading ion appeared to be suitable for cITP of peptides. Moreover, a direct comparison of CZE with ITP was conducted, which showed considerably higher peptide zones when using the two-buffer system consisting of the leader ammonium acetate and the terminating ion protonated ε-aminocaproic acid (data not shown), while the initial concentrations of the injected samples were equal. On top of this, a series of experiments has been carried out in which the sample loading was varied. These experiments resulted in isotachopherograms with peptide zones of different widths, but of equal concentration (zone heigths) for the different sample loadings (data not shown). According to the ion concentration regulating principle of Kohlrausch (Equation 1) an increase of analyte amount results in broader zones, but not in higher concentrations. Moreover, the current profile was typical for ITP, starting at ± 12.5 μA and decreasing to reach a stable plateau at ± 5 μA after 30 min. Based on the results of these experiments, it can be concluded that cITP takes place.

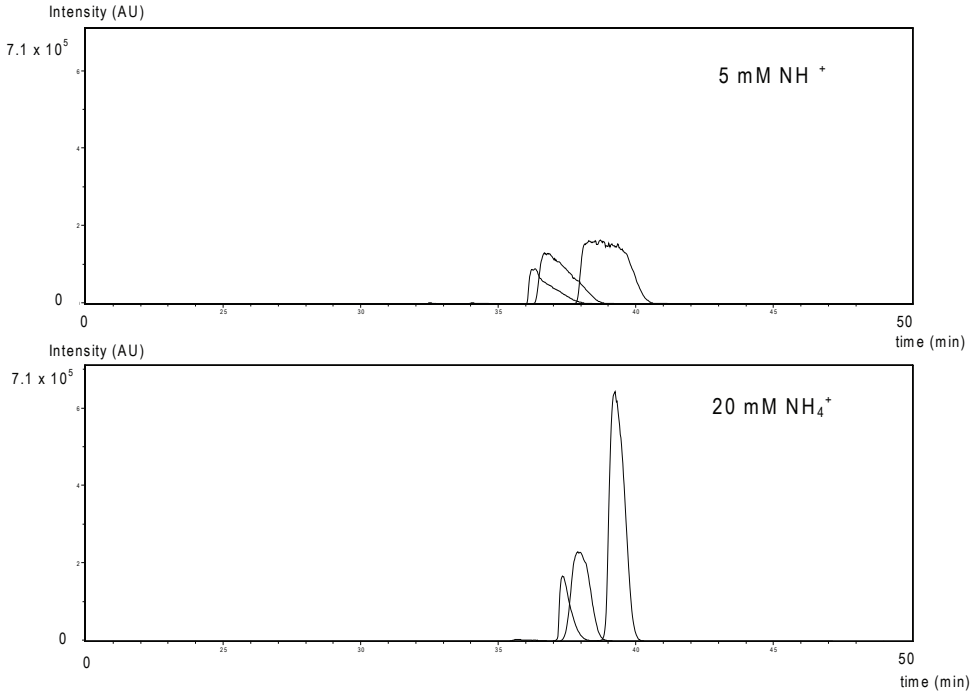


Figure 2.1 Comparison of cITP separations of, in migration order, bradykinin (1-5), bradykinin (1-6) and angiotensin 2 (3-8) with 5 mM and 20 mM NH_4^+ as LE.

Next, the influence of CA on the isotachophoretic behaviour of a set of 6 model peptides (Table 2.1) was examined by comparing the isotachopherograms as obtained with different concentrations of CA (Fig. 2.2). The concentration of the model peptides was 62.5 µg/mL, corresponding to a molar concentration range of 48-110 µM and 10-25 pmol per injected peptide. As expected, the addition of CA to an academic mixture of model peptides improved the separation of the peptides (Fig. 2.2).

Table 2.1 The analysed model peptides, their physicochemical properties and migration orders in cIEF, CZE and cITP.

PEPTIDE DETAILS				**MIGRATION ORDER**		
Name	**Sequence**	**$[M + H]^+$**	**p*I***	**cIEF**	**CZE**	**cITP**
Thymopentin	RKDVY	680.76	8.88	4	1	1
Bradykinin	RPPGFSPFR	1061.20	11.97	1	2	2
Angiotensin 1	DRVYIHPFHL	1297.48	7.38	5	3	3
Bradykinin (1-5)	RPPGF	573.66	10.00	2/3	4/5	4
Bradykinin (1-6)	RPPGFS	660.80	10.00	2/3	4/5	5
Angiotensin 2 (3-8)	VYIHPF	775.90	7.19	6	6	6

Without CA, thymopentin, bradykinin and angiotensin I were not separated, while in the presence of 1% CA they were. When 10% CA was present, the peptide zones were more separated compared to the presence of 1% carrier ampholyte. With only one exception, the mass spectra of all peptides (Fig. 2.2 A-G) revealed that each zone contained only one peptide. For example, whereas without CA the model peptides bradykinin and thymopentin were not separated at all, after addition of 1 or 10% CA, separated single peptide zones were observed. Two peptides could hardly be separated at all, with or without CA, i.e. bradykinin (1-5) and bradykinin (1-6) (Fig. 2.2 F). These peptides differ by only one amino acid (see Table 2.1) and as a consequence apparently have almost the same electrophoretic mobility. Adding CA did not have a spacing effect between the bradykinin (1-5)/bradykinin (1-6) zone and the angiotensin II 3-8 zone. Apparently the CA mix did not contain compounds with the required electrophoretic mobilities to act as spacer between these peptides. In the mass spectra of the isotachopherogram it can be seen that most CA are migrating ahead of the peptides (Fig. 2.2 A). Between bradykinin and thymopentin, only one CA species is observed (Fig. 2.2 C), demonstrating that the majority of the electrophoretic mobilities present in the CA mixture are not in the range of the electrophoretic mobilities of the peptides. In the absence of CA, some peptide zones are not adjacent, because some contaminants present in the academic mixture or in the buffer were concentrated too. These contaminants were not detected by the MS, possibly because their masses were outside the tuning range of the MS (300-1500 m/z).

It is reasonable to suspect that the addition of CA to the peptide mixture results in IEF rather than ITP, since CA were originally designed for IEF separations. To exclude this possibility, a cIEF experiment was performed, which resulted, as expected, in a separation according to the iso-electric points of the peptides (Table 2.1). The peak order in CZE corresponded with the electrophoretic mobilities of the peptides (Table 2.1), excluding the option of a hybrid IEF-ITP separation too. Moreover, in IEF the principle of Kohlrausch does not apply due to the absence of leading and terminating ion zones. This was supported by a comparison between the isotachopherograms with 1% CA and 10% (Fig. 2.2); when more CA were present, the zones were further apart.

In IEF separations, ion concentrations are not regulated by the LE concentration which in practice often results in peptide or protein precipitation at their pI during focusing because too high concentrations are generated [31], instead of the broader zones with regulated concentrations that would be generated in cITP.

Comparison of the intensities of the peptide isotachopherograms shows a decrease in

intensity and differences in releative zone heights between the peptides when more CA are added. This is due to the nature of the CA mixture, which will be discussed in the next section.

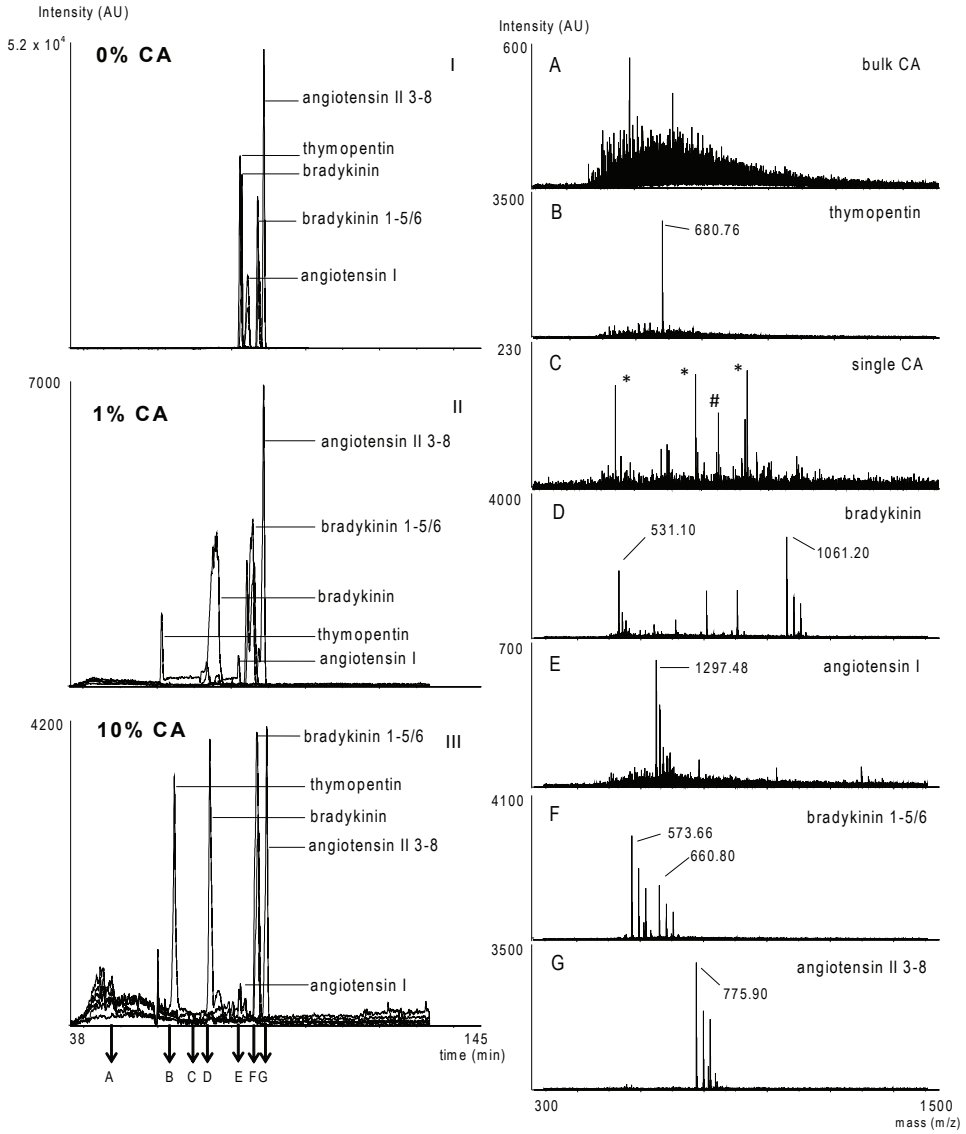


Figure 2.2 Reconstructed ion cITP-MS electropherograms of model peptides and corresponding mass spectra. (I) no CA added, (II) 1% CA added, (III) 10% added to sample. (A-G) MS spectra of zones in III. *) = TE background contamination, #) = CA. Peptide masses are indicated; most masses have 2 sodium adducts. AU means arbitrary units.

3.2 CA as spacers in cIPT-MS of complex biosamples

The initial concentration of BSA before digestion was 4.9 μM. After digestion, RP-SPE and freeze-drying, the peptides were diluted 10 times with TE so that the final concentration was 490 nM in the case of complete recovery. This corresponds to around 500 fmol injected BSA digest.

In Fig. 2.3, the results of the analysis of the BSA peptides without CA are shown. Since the concentration of the peptides is low, they are concentrated in adjacent narrow zones that collapse easily [13] and in consequence enter the MS virtually simultaneously in one plug. In a mass spectrum taken from this plug, it can be observed that many peptides are present indeed.

After adding 1% CA to the sample, the peptides are more separated and spread in the resulting isotachopherogram. In Fig. 2.4 it can be seen that five peptides are fully separated and single-peptide mass spectra could be acquired, of which four were BSA peptides and one was an unknown compound, most probably a trypsin peptide, keratin peptide or a BSA peptide that originated from hydrolysis instead of trypsin digestion. The same five peptides were also detected in the bulk of co-migrating peptides when no CA were added. So far, only these five separated peptides were found. When 10% CA was present during cITP, no peptides could be observed at all (data not shown). It should be noted that since lower backpressure was used during these experiments, migration times and zone lengths in Fig. 2.3 and 2.4 were increased in comparison with Fig. 2.2..

A big disadvantage of the CA mixture used in this study was that the composition of the mixture is not exactly known, as it is a commercial product, meaning that the production and product composition is proprietary and not disclosed. This is actually also true for other commercial CA mixtures. However, a recent series of studies have provided more insight into the composition of commercial CA mixtures [33]. The CA were separated by preparative iso-electric focusing using a Rotofor device and the composition of the obtained fractions was characterised using MS. It appeared that, Pharmalyte pH 2.5-10.5 consists of hundreds of compounds in the mass range of 150-1179. However, it was also discovered that a significant part of the CA in Pharmalyte mixtures were poorly functioning as CA. For Pharmalyte 8-10.5 this was estimated to be as much as 50% of all the detected 'CA'. This was concluded from the fact that no focusing took place, since a continuous distribution along the entire pH gradient in the Rotofor was observed [33]. The CA mixture that was used in our study, Pharmalyte 3-10, also contained as many as hundreds of molecule species. Given the migration behaviour of ampholytic molecules in a pH-gradient with an applied

electric field, the fact that some components were not migrating at all can only be explained by the conclusion that these molecules were neutral. These findings can most likely explain the poor results that were obtained from the BSA digest analysis in our study. Since the sample is confined between the LE and TE zone and due to the very low EOF at this (low) pH, the neutral components of the CA mixture will be inseparable from the peptides by electrophoresis once they are mixed with the sample. These neutral CA cause a high background as well as ion suppression.

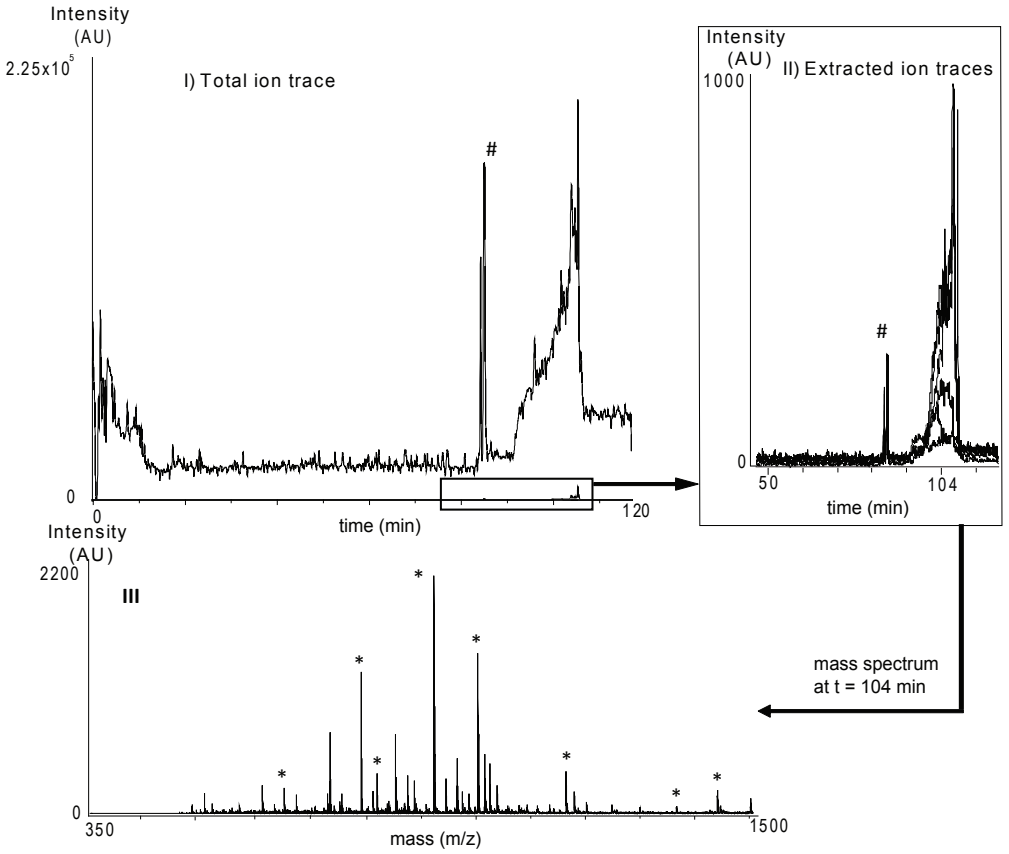


Figure 2.3 cITP-MS of BSA peptides without CA: (I) total ion count isotachopherogram, (II) reconstructed ion isotachopherograms of selected peptides, (III) mass spectrum of the highest peptide signal at 104 min; masses due to BSA peptide are marked (*), (#) indicates an artefact.

In the analysis of academic mixtures of model peptides they already caused a severe signal decrease, from 5.2 x 10^4 AU for angiotensin II 3-8 when no CA were added, to 4.2 x 10^3 AU when 10% of CA were added (Fig. 2.2). This negative influence of impurities in the CA

mixture was not equal for every zone, since the relative zone height differed between 0, 1 and 10% added CA, as can be observed in Fig. 2.2.

To infuse the sample into the MS, some hydrodynamic pressure had to be used, due to the aforementioned very low EOF, which inevitably caused some zone broadening. When academic mixtures with relatively high analyte concentrations were injected, broad sotachophoretic peptide zones were obtained, that are less susceptible for disturbing effects

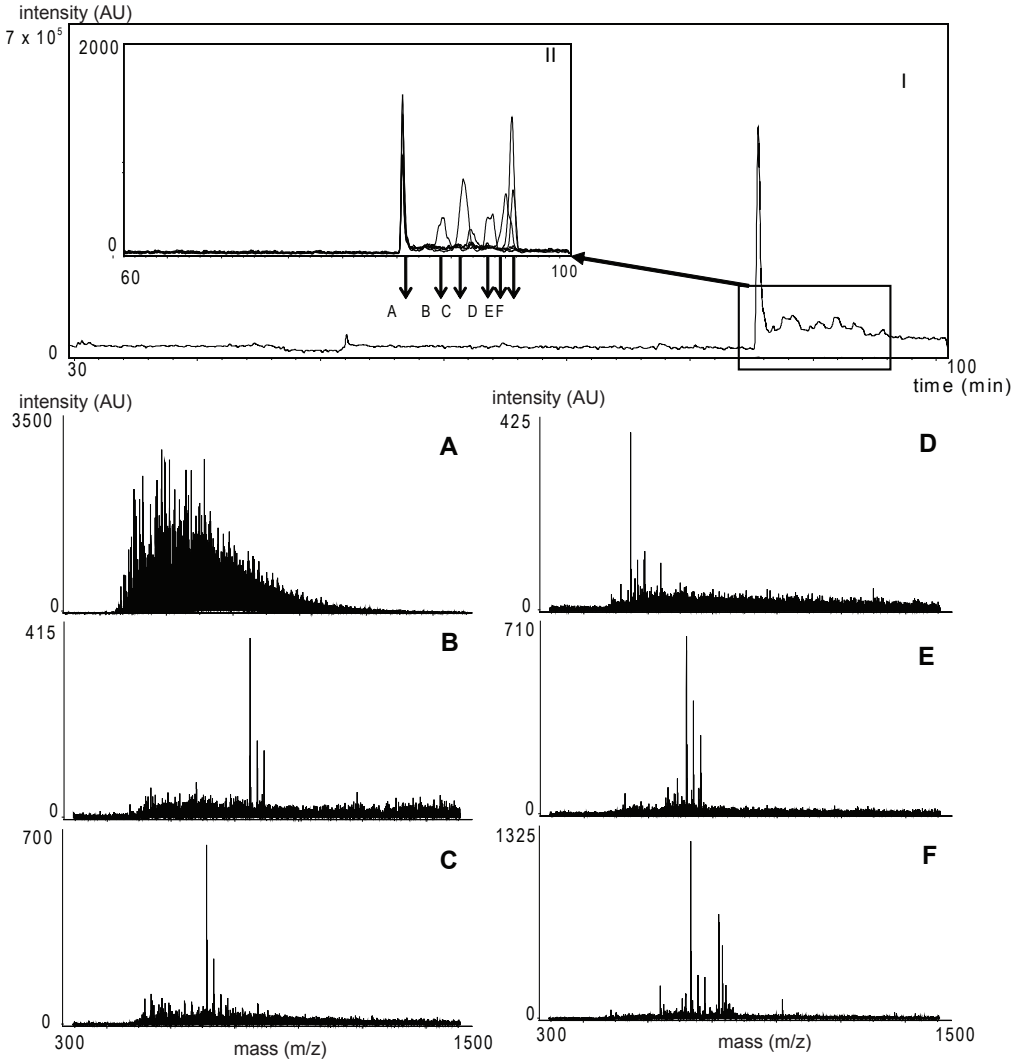


Figure 2.4 cITP -MS of BSA peptides containing 1% CA: I) total ion count isotachopherogram, II) reconstructed ion isotachopherograms of selected peptides, (A) mass spectrum of CA, mass spectra of BSA peptides (B) LSQKFPK, (C) SEIAHR, (D) VASLR, mass spectra of (E) unknown molecule species and (F) mass BSA peptide FGERALK and an unknown molecule species.

due to zone broadening. However, when the peptide zones are narrower, as was the case with the BSA digest, a little zone broadening already causes a severe decrease of signal. In combination with a high background this results in a poor bioanalytical performance.

We have demonstrated that it is possible to physically separate peptide analyte zones by mixing the sample with spacer molecules, making a consecutive zone broadening step such as CZE unnecesary. However, due to the severe contamination in the CA mixture, the overall performance of the developed cITP-MS method was not satisfactory yet, and just a few peptides were detected in a complex biosample. To obtain better results, more suitable spacer compounds should be developed. This spacer compound mixture should meet two main criteria. First, such a mixture should contain a large and continuous gradient of electrophoretic mobilities that are close to peptide electrophoretic mobilities in order have a maximal chance of spacers between the peptide zones. Secondly, the spacer compounds should not interfere with the ionisation of the peptides during ESI to avoid ion suppression of the peptide signals. This can be achieved by choosing compounds that are more polar and hydrophilic than peptides. Polar compounds have a lower surface activity and therefore tend to be on the inner side of the spray droplets during ESI and therefore they ionise worse and cause less ion suppression [34].

4. Concluding remarks

In this paper, we explored the use of spacer compounds to couple cITP directly to MS. We have shown that it is possible to physically separate and detect peptide analyte zones in cITP-MS after when spacer molecules are added to the sample before injection. With this approach, we were able to detect sub-micromolar peptide concentrations in a complex sample, in single-analyte mass spectra. However, especially in the analysis of the more complex sample with lower peptide concentrations we encountered a severe drawback of CA as spacer molecules: CA mixtures appeared to be ill-defined mixtures with a lot of disturbing contaminations present. To be able to detect sub-nanomolar peptide concentrations, i.e. for bio-analysis, there is a demand for more suitable spacer compounds. Future research will be dedicated to the development of alternative spacer compounds.

5. References

[1] Schulte, I., Tammen, H., Sell, H., Schulz-Knappe, P., *Expert Rev Mol Diagn,* **2005**, *5,* 145-157

[2] Leigh Anderson, N., Anderson, N.G., *Mol Cell Proteomics,* **2002**, *1,* 845-867

[3] Aristoteli, L.P., Molloy, M.P., Baker, M.S., *J Proteome Res,* **2007**, *6,* 571-581

[4] Schrader, M., Schulz-Knappe, P., *Trends Biotechnol,* **2001**, *19,* s55-s60

[5] Ishihama, Y., *J Chromatogr A,* **2005** *1067,* 73-83

[6] Kvasnička, F., Jaroš, M., Gaš, B., *J Chromatogr A,* **2001**, *916,* 131-142

[7] Holloway, C.J., Trautschold, I., *Fresen J Anal Chem,* **1982**, *311,* 81-93

[8] Petr, J., Maier, V., Horáková, J., Ševcík, J., Stránský, Z., *J Sep Sci,* **2006**, *29,* 2705-2715

[9] Aebersold, R., Mann, M., *Nature,* **2003**, *422,* 198-207

[10] Cech, N.B., Enke, C.G., *Anal Chem,* **2000**, *72,* 2717-2723

[11] Smith, R.D., Loo, J.A., Barinaga, C.J., Edmonds, C.G., Udseth, H.R., *J Chromatogr A,* **1989**, *480,* 211-232

[12] Fenn, J.B., Mann, M., Meng, C.K., Wong, S.K., Whitehouse C.M., *Science,* **1989**, *246*, 64-71

[13] Peterson, Z.D., Bowerbank, C.R., Collins, D.C., Graves, S.W., Lee, M.L., *J Chromatogr A,* **2003**, *992,* 169-179

[14] Klampfl, C.W., *Electrophoresis,* **2006**, *27*, 3-34

[15] Hamoudavá, R., Pospíšilová, M., Spilková, J., *Electrophoresis,* **2006**, *27,* 4820-4826

[16] Hernández, M., Aguilar, C., Borrull, F., Calull, M., *J Chromatogr B,* **2002**, *772,* 163-172

[17] Gysler, J., Mazereeuw, M., Helk, B., Heitzmann, M., Jaehde, U., Schunack, W., Tjaden, U.R., Van der Greef, J., *J Chromatogr A,* **1999**, *841,* 63-73

[18] Larsson, M., Lutz, E.S.M., *Electrophoresis,* **2000**, *21,* 2859-2865

[19] Witte, D.T., Någård, S., Larsson, M., *J Chromatogr A,* **1994**, *681,* 155-166

[20] Toussaint, B., Hubert, Ph., Tjaden, U.R., Van der Greef, J., *J Chromatogr A,* **2000**, *871,* 173-180

[21] An, Y., Cooper, J.W., Balgley, B.M., Lee, C.S., *Electrophoresis,* **2006**, *27,* 3599-3608

[22] Waterval, J.C.M., Krabbe, H., Teeuwsen, J., Bult, A., Lingeman, H., Underberg W.J.M., *Electrophoresis,* **1999**, *20,* 2909-2916

[23] Fang, X., Balgley, B.M., Wang, W., Park, D.M., Lee, C.S., *Electrophoresis,* **2009**, *30,* 4063-4070

[24] Vesterberg, O., *Acta Chem Scand,* **1969**, *23,* 2653-2666

[25] Vesterberg, O., Ann. N. Y., *Acad Sci,* **1973**, *209,* 23-33

[26] Chartogne, A., Reeuwijk, B., Hofte, B., Van der Heijden, R., Tjaden, U.R., Van der Greef, J., *J Chromatogr A,* **2002**, *959,* 289-298

[27] Inano, K., Tezuka, S., Miida, T., Okada, M., *Ann Clin Biochem* **2000**, *37,* 708-716

[28] Nagyová, I., Kaniansky, D., *J Chromatogr A,* **2001**, *916,* 191-200

[29] Chen, S., Lee, M.L., *J Microcolumn Sep,* **1998***, 10,* 423-430

[30] Marák, J., Nagyová, I., Kaninasky, D., *J Chromatogr A,* **2003***, 1018,* 233-249

[31] Storms, H.F., Van der Heijden, R., Tjaden, U.R., Van der Greef, J., *Electrophoresis,* **2004**, *25,* 3461-3467

[32] Liu, X., Sosic, Z., Krul, I.S., *J Chromatogr A,* **1996***, 735,* 165-190

[33] Righetti, P.G., Simó, C., Sebastiano, R., Cittero, A., *Electrophoresis* **2007***, 28,* 1488-1494

[34] Cech, N.B., Enke, C.G., *Anal Chem,* **2001**, *73*, 4632-4639

Chapter 3

On-line capillary liquid-liquid electroextraction of peptides as fast pre-concentration prior to LC-MS

Based on
Lindenburg P.W., Seitzinger R., Tempels F.W.A., Tjaden U.R., Van der Greef, J., Hankemeier, T.
On-line capillary liquid-liquid electroextraction of peptides as fast pre-concentration prior to LC-MS.
Electrophoresis, **2010**, *31,* 3903-3912.

Abstract

In this research paper we show that capillary electroextraction (cEE) is capable of fast on-line peptide concentration and that it can be coupled on-line to LC-MS to result in a fast and sensitive method. EE takes place when an electric field is applied in a two-phase liquid-liquid system. Sample ions in the organic phase migrate very fast into the aqueous phase and are concentrated in a small zone. In this work, cEE of peptides is developed and coupled on-line to LC-MS via a switching valve. Comparison of 10 min of cEE-LC-MS with a conventional LC-MS injection showed more than 100-fold increased peak heights. Good calibration curves of five model peptides in the range of 0.05 - 5 μM were obtained. The linearity was good (R^2 values between 0.984 and 0.996) and relative standard deviations ranged from 5 % at the highest to 25 % at the lowest concentration (n=3). The LOD of bradykinin, angiotensin I converting enzyme inhibitor and angiotensin I were in the low nM range. Analysis of a tryptic digest of eight model proteins resulted in more than 170 peptides, without bias for pI or hydrophilicity. Urine analysis is demonstrated, resulting in a LOD around 0.04 μM urine for tryptic cytochrome C peptides spiked to urine and an increase of 42% in the number of chromatographic peaks compared to conventional LC-MS. In summary, cEE-LC-MS is a fast electrophoresis-driven sample preconcentration technique that is quantitative, able to extract a wide peptide range and applicable to bioanalysis.

1. Introduction

For several decades it is known that applying an electric field in solvent extraction procedures enhances mass transfer from one phase into the other. This process is sometimes referred to as two-phase electrophoresis [1-5], but more conveniently called 'electroextraction' (EE), a term that was first proposed in the early 1990s by Stichlmair et al [6]. Initially, EE has been developed as a purification technique in the field of chemical engineering, to enhance product yields [1-5, 7, 8]. However, around a decade ago Van der Vlis *et al.* adapted liquid-liquid EE for analytical purposes, using ethyl acetate (EtOAc) as organic phase and performing EE of test solutions in capillaries (cEE) [9-11]. Despite the promising results that were presented in these publications, analytical cEE has not been developed further since and no bio-analytical applications have been published. Recently, the potential of EE and related techniques received renewed attention via review papers on electrochemically modulated extraction methods [12] and electric field-amplified transport across phase boundaries [13].

When an electric field is applied in a two-phase liquid-liquid system that consists of a low conductive organic phase and a high conductive aqueous phase, charged compounds that are in the organic phase will migrate fast towards the aqueous phase. As soon as the aqueous phase is entered, migration speed decreases dramatically, causing analyte concentration. EE offers enhanced selectivity, as either cations or anions are extracted, while neutral compounds will largely remain behind since they only migrate to the aqueous phase by passive diffusion. In this set-up, only the electric field on the low-conductivity side of the interface will be of importance for ion migration [8]. To achieve a sample concentration effect, the analytes should therefore dissolve in the organic phase. A prerequisite of the organic phase is that it has some conductivity to enable ion transfer. Pure EtOAc hardly conducts current and therefore is not suitable for EE. However, when saturated, EtOAc contains 3.5% (v/v) water, which allows the presence of ions. The solubility of the small protein egg white lysozyme has been reported to be good (10 mg/mL) in EtOAc, [14] and some enzyme activities are even enhanced in an organic environment [15].

The major difference between cEE and conventional field amplified sample stacking (FASS) [16-19] is, thanks to the using of two immiscible liquid phases, the presence of a liquid-liquid interface. The liquid-liquid interface limits the influence of thermal currents caused by convection. When only one phase is present, as in FASS, thermal currents caused by convection can occur that frustrate the concentrating effect, while in a two-phase

system convection is constrained to a single phase by a physical border [6]. Therefore, zone broadening during EE is limited [6, 8]. Moreover, since very high electric fields strengths occur in the organic phase, EE is fast.

In this research paper, the cEE process was characterised by studying the current behaviour using ammonium as model cation and crystal violet for visualisation. Then, cEE of peptides was developed and coupled to LC-MS. The resulting cEE-LC-MS procedure was successfully applied to the analysis of a tryptic digest of several model proteins and urine. To our best knowledge, the application of cEE to biomolecules in complex biological samples has not reported before in literature.

2. Experimental

2.1 Chemicals

All reagents were of analytical grade or higher. A Millipore Q-guard water purifying system (Billerica, MA, USA) was used to obtain pure water. Acetonitrile (ACN), EtOAc and formic acid (FA) were obtained from Biosolve (Valkenswaard, The Netherlands), ammonium acetate, ammonium bicarbonate, crystal violet, model proteins, proteomics grade trypsin, dithiothreitol, iodoacetic acid and glycine from Sigma (St. Louis, MO, USA), acetic acid and sodium hydroxide from JT Baker (Philipsburg, NJ, USA) and the model peptides (bradykinin 1-5, mass 572; bradykinin 1-6, mass 659; angiotensin I converting enzyme inhibitor (alias [Des-Pro2]-Bradykinin), mass 963; thymopentin, mass 679; bradykinin, mass 1060; angiotensin I, mass 1296 and substance P, mass 1347) from Genscript Corporation (Piscataway, NJ, USA).

2.2 Equipment and techniques

2.2.1 First EE set-up

To study the EE process, experiments have been carried out in a custom-made EE device (Fig. 3.1A). This device consisted of a vial (height: 108 mm, ID: 15 mm, material: polychlorotrifluorethylene (PCTFE), thickness 3 mm) with a conical bottom (angle 28˚) with two threaded inlets where a platinum electrode and a capillary were fixed with finger-tight nuts (Standard Head Fitting for 360 µM OD tubing, Upchurch Scientific, Oak Harbor, WA, USA). The capillary (OD: 400 µm, ID: 320 µm, material: fused silica) served to inject the aqueous phase below the organic phase using a 500 µL micro syringe (Kloehn

Ltd., Las Vegas, NV, USA). The bottom electrode was grounded. The conical bottom enabled extraction into a small aqueous phase volume. The electric circuit was closed with a gold coated anode (to minimise reduction/oxidation reactions) with a flat circled end contacting exactly the whole surface of the organic phase, ensuring all ions to be exposed to the electric field. High voltage was applied with a Spellman High Voltage Power Supply (Spellman, Hauppauge, NY, USA). Electric current was measured with a simple multimeter (Voltcraft, Oldenzaal, The Netherlands) and conductivity with a conductivity meter from Hanna Instruments (type HI 8733, Woonsocket, RI, USA).

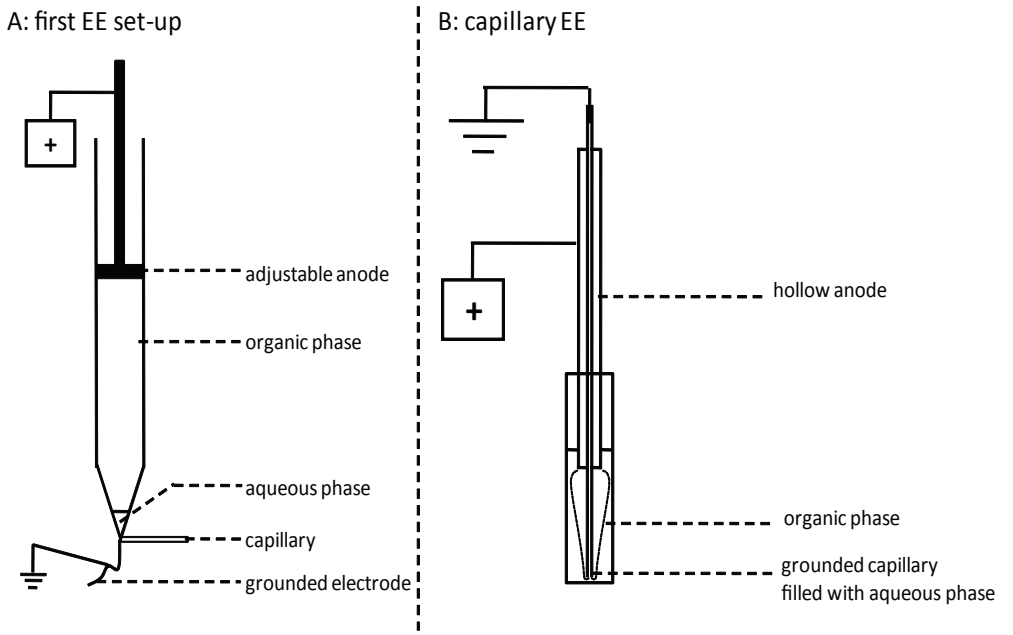


Figure 3.1 A) Schematic set-up of EE in the laboratory made device. B) Sample introduction set-up of capillary EE. The dotted line indicates the boundary of the effective extraction volume

Samples taken prior to and after EE were freeze-dried in a Zirbus Vaco I freeze-dryer (Zirbus Technology, Bad Grund, Germany) under vacuum that was applied with an Alcatel 2008A vacuum pump (Alcatel Vacuum Products, Hingham, MA, USA).

2.2.2 cEE-MS

cEE was carried out in an Agilent Technologies 1600 series CE apparatus (Agilent Technologies, Santa Clara, CA, USA). An untreated fused silica capillary (65 cm, 75 μm ID)

was filled with aqueous phase and dipped via a hollow electrode in the organic phase that contained the charged analytes. The organic phase (50 µL) was in a flat-bottom glass insert vial of 30.75 x 5 mm (Micro-insert, Bester BV, Amsterdam, The Netherlands). The capillary outlet was grounded via the spray source, while the organic phase was in contact with the anode. No height difference between capillary inlet and outlet was present. The capillary inlet was located close to the vial bottom (inner diameter 3 mm) and the hollow electrode tip close to the liquid surface (Fig. 3.1B), so the electric field was present throughout a maximal part of the organic phase. The extraction volume was estimated to be 14 µL (a cone with a length of 6 mm and a base diameter of 3 mm) by was visually observing an extraction of crystal violet, which showed depletion in the expected area. MS experiments were carried out on a Bruker Daltonics MicrOTOF MS (Bruker Daltonics, Bremen, Germany). Coupling of cEE to direct infusion (DI) was achieved using a sheath-liquid assisted grounded spray source supplied by Agilent Technologies (part number G160760001). The sheath liquid (50%/50% (v/v) ACN and 0.2% FA, 3 µL/min) served to close the electric circuit and for good spray. Further spray settings were: end-plate offset -529 V, capillary voltage -1762 V, nebuliser gas pressure 0.4 bar, dry gas flow 4.0 L/min and dry temperature 200 °C. The recorded mass range was 200-1200 m/z.

2.2.3 Liquid chromatography

LC was carried out with an Agilent Technologies 1200 series µHPLC apparatus, equipped with a ZORBAX SB-C18 (5 µm, 150 x 0.5 mm) capillary HPLC column, also purchased from Agilent Technologies. A 20 µL/min gradient elution was employed (solvent A: 0.1% FA in water, solvent B: 0.08% FA in ACN), starting at 0% B and ending after 30 min at 35%, unless otherwise stated. Next, the column was conditioned for 5 min with 95% solvent B and 5 min with 100% solvent A. On each day, a blank run was performed first.

2.2.4 Interface for coupling cEE and LC

Coupling of EE with LC was facilitated with a bio-compatible 6-port 2-way switching valve (Cheminert® HPLC injection valve model C2-1246, Valco Instruments, Houston, TX, USA). The rotor consisted of polyaryletherketone (PAEK). In this switching valve, the liquid has no contact with metal parts, preventing short circuiting and unsafe working conditions. The valve was switched manually and therefore a polytetrafluorethene (PTFE) handle was used.

2.2.5 On-line cEE-LC-MS

Coupling of cEE-LC to the time-of-flight (TOF) MS detector was realised with a conventional electrospray needle that was provided with TOF-MS (Bruker Daltonics) part number EN8389). The spray settings were as described in section 2.2, except nebuliser gas pressure (1.0 bar) and dry gas flow (8.0 L/min) to cope with the higher flow rate. In the case of cEE-LC-MS of digested model proteins, a linear triple quad - ion cyclotron resonance - Fourier transform - mass spectrometer (LTQ-ICR-FT-MS) (Thermo, San Jose, CA, USA) served as detector, to facilitate peptide identification. LC-MS was achieved with a conventional electrospray device delivered by the manufacturer. Spray settings for LTQ-ICR-FT-MS were: sheath gas flow 15 L/min, source high voltage 3 kV, capillary temperature 200 °C, capillary voltage 30 V and tube lens voltage 155 V. During analysis, the MS continuously performed scan cycles in which first a high-resolution (R = 100 000) full scan (200-1200 *m/z*) in profile mode was acquired by the FT-MS, after which MS/MS spectra were recorded in centroid mode by the LTQ for the 3 most intense ions (isolation width, 4 *m/z*; normalised collision energy, 35%). Dynamic exclusion was enabled (repeat count, 1; repeat duration, 30 s; exclusion list size, 500; exclusion duration, 180 s; relative exclusion mass width, 5 ppm) as was charge state screening (q = 1-4).

2.2.6 Data analysis/protein identification

Identification of model peptides was based on matching of the known masses with the obtained mass information. When MS/MS was performed, a database search was performed with the software application Bioworks (version 3.3), using the following settings for peptide identification: $\Delta CN > 0.100$, Xcorr versus charge state $1 \geq 1.80$, $2 \geq 2.50$, $3 \geq 3.50$, peptide probability < 0.5 and $\Delta ppm < 5.0$.

2.3 Sample pretreatment

First, a model peptide mixture was diluted twice with 1 mM acetic acid and acidified to pH 3 with FA to ensure cationic peptides. Then, EtOAc was saturated with the sample, by adding as much as possible without allowing a two-phase system to form. Then, it contained 2.2 % (v/v). The maximal percentage of 3.5% (v/v) was not reached, most likely because some water was already present.

Cytochrome C (horse and human), hemoglobin (human), ribonuclease A (pancreatic bovine), lysozym (chicken) and bovine serum albumine were digested using sequence grade trypsin. Per protein, 100 µg was digested with 1 µg trypsin in 200 µL 100mM ammonium bicarbonate (pH 8.5). Prior to digestion (12 hours at 37 °C), the proteins were reduced with

dithiothreitol (4.5 mM) and alkylated with iodoacetic acid (10 mM), as prescribed in [20]. After adding FA (2% v/v sample concentration), the digested proteins were purified by SPE using TopTip© pipette tips (Glygen Corp., Maryland, US) filled with a small amount of Poros Reversed Phase (RP)-2 SPE material. The material was wetted with ACN, equilibrated with 2% aqueous FA and the sample was applied. The trapped peptides were washed with 2% aqueous FA and elution was achieved with 50 µL 70/30 mixture of ACN and 2% aqueous FA. Next, 6.25 µL of the eluent was mixed with 110 µL anhydrous EtOAc. 70% ACN is better miscible with pure EtOAc (5.6%) than water (2.2%). No precipitation was observed.

Freshly voided urine samples were obtained from adult, healthy volunteers (age 18 to 29), pooled, acidified with FA (2%), divided in aliquots and stored at -80 ˚C until analysis.

3. Results and discussion

3.1 Description of the electroextraction process

At first, the EE process was characterised using the laboratory-built device (Fig. 3.1A). EE is a process of ion fluxes, and therefore the EE process can be followed by monitoring the current. The organic phase consisted of 11 mL EtOAc saturated with 10 mM ammonium acetate which was first adjusted to pH 3 with FA. After saturation, the EtOAc contained 0.2 mM ammonium acetate and 0.55 nM FA. The aqueous phase consisted of 100 µL 10 mM ammonium acetate (adjusted to pH 3 with FA). These phases have a large difference in conductivity (0.3 µS/cm and 1150 µS/cm were measured in the donor and acceptor phase, respectively). Electric field strengths of 40 kV/m (2.5 kV over 6.25 cm), 24 kV/m (1.5 kV) or 16 kV/m (1 kV) were applied for 10 min at similar pH, temperature and phase ratios.

Ammonium was used as model cation to study EE, for practical reasons (available in large amounts, enabling measurable currents), and crystal violet (0.5 µM) was added to the donor phase for visualisation of the EE experiment. Due to their very low concentrations FA and crystal violet do not contribute significantly to the measured current. Dissociation constants of neutral acids and cationic acids can shift in different solvents. However, for ammonium this shift is not dramatic in various studied organic solvents such as dimethyl sulphoxide and propylene carbonate [21], so it can be assumed that ammonia will be protonated and therefore present as ammonium in EtOAc under these experimental conditions. In Fig. 3.2A, the current profiles are shown.

Initially, the current decreased unpredictably, but after the first minute, the current

decreased exponentially (established by fitting with exponential trend lines) until a stable low current (I_{rest}) was reached. Increasing the field strength resulted in a faster current decrease. It was observed that the crystal violet immediately started to migrate into the aqueous phase, giving rise to a sharp zone and a growing depleted zone at the cathode side in the organic phase. Within several minutes, the whole organic phase was depleted into a sharp aqueous zone. It was observed that the speed with which the depleted zone was formed decreased over time, while simultaneously the current decreased exponentially (Fig. 3.2A). In the depleted zone, the removal of ions has caused a lower conductivity and therefore a higher electric field strength. Since the applied voltage is constant, the electric field strength that is over the organic region that still contains ions will be lowered. As the depleted region grows, the electric field strength in the non-depleted zone is decreasing. As a consequence of this changing electric field distribution in the EE system, EE takes place in a non-linear manner, namely, as shown in Fig. 3.2, exponentially. In Fig. 3.3, the change of electric field strength in the different zones is depicted schematically. At the start of EE (I), a uniform electric field strength is present in the organic phase. As EE proceeds (II, III, IV), a depleted region with a high electric field strength grows. Simultaneously, a slightly decreasing electric field strength is present in the aqueous zone, due the increasing amount of ions. Since the conductivity difference between the aqueous phase and the organic phase is so large, the small change in the aqueous phase has no significant influence on the situation in the organic phase.

We assumed that the exponential current decrease that was observed can be described as:

1) $$I(t) = I_0 . e^{-\beta t} + I_{rest}$$

In this equation $I(t)$ is the current as function of time t, I_0 the starting current and I_{rest} the current that is let through by saturated EtOAc, and β represents the exponential coefficient with which the current decreases during EE, therefore called the extraction rate. As can be observed in Fig. 3.2A, I_{rest} reached a constant value. To determine values of β with different electric field strengths, $I(t)$ was differentiated and the differentiated current $I'(t)$, i.e. dI/dt, of the current profiles between 1 and 10 min was plotted versus $I(t) - I_{rest}$. The resulting slopes are the β values, since the differentiated current can be expressed as

2) $$I'(t) = -\beta I(t)$$

Since EE is a process of depletion, β is negative. The larger the absolute value of β, the higher the extraction rate. As can be observed in Fig. 3.2B, rather good linear curves are

obtained (R^2 values 0.99, 0.94 and 0.77 for 40, 24 and 16 kV/m respectively). Increasing the applied electric field strength increases the extraction rate. However, when electric field strengths above 40 kV/m were applied in this EE set-up, visible instabilities (fluid motion and droplet formation) occurred at the liquid-liquid interface, while the current became instable and EE failed, which has also been reported in literature [7, 8].

A comparison between different ionic strengths in the aqueous phase showed that I_0 as well as the exponential factor β by which the current decreases were not influenced (data not shown). The conductivity difference between the aqueous phase and the organic phase is so large, that a 10–fold change in the ionic strength of the aqueous phase has no measurable effect. In consequence, EE is robust towards ionic strength variations in the acceptor phase, in contrast to field-amplified sample stacking (FASS). When cEE is coupled to LC, the ionic strength of the mobile phase (which also serves as aqueous acceptor phase in cEE) does not affect the performance of the EE process.

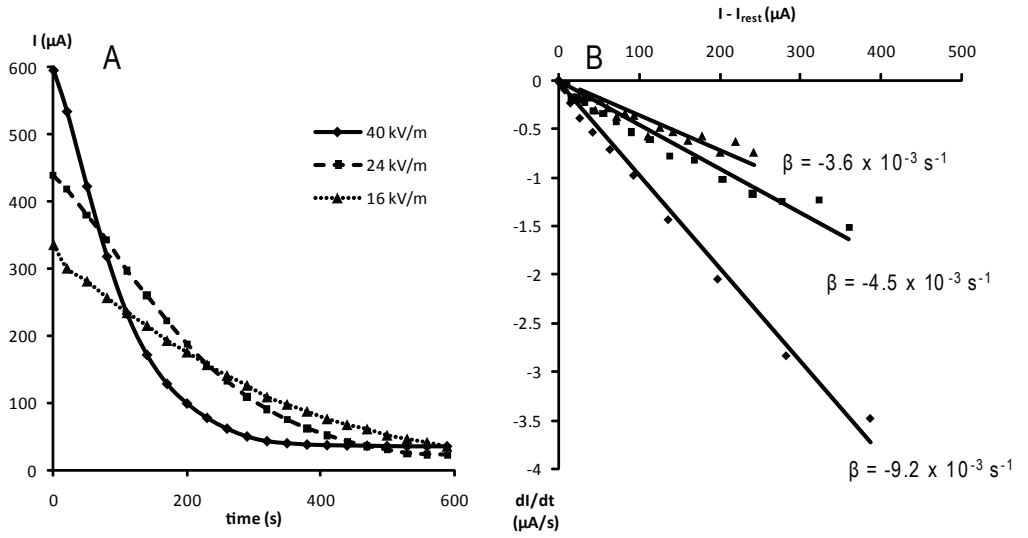


Figure 3.2 A) current profiles (n=3) of EE performed with 3 different electric field strengths B) dI/dt plotted against $I-I_{rest}$ of the current profiles shown in part A, to determine β values.

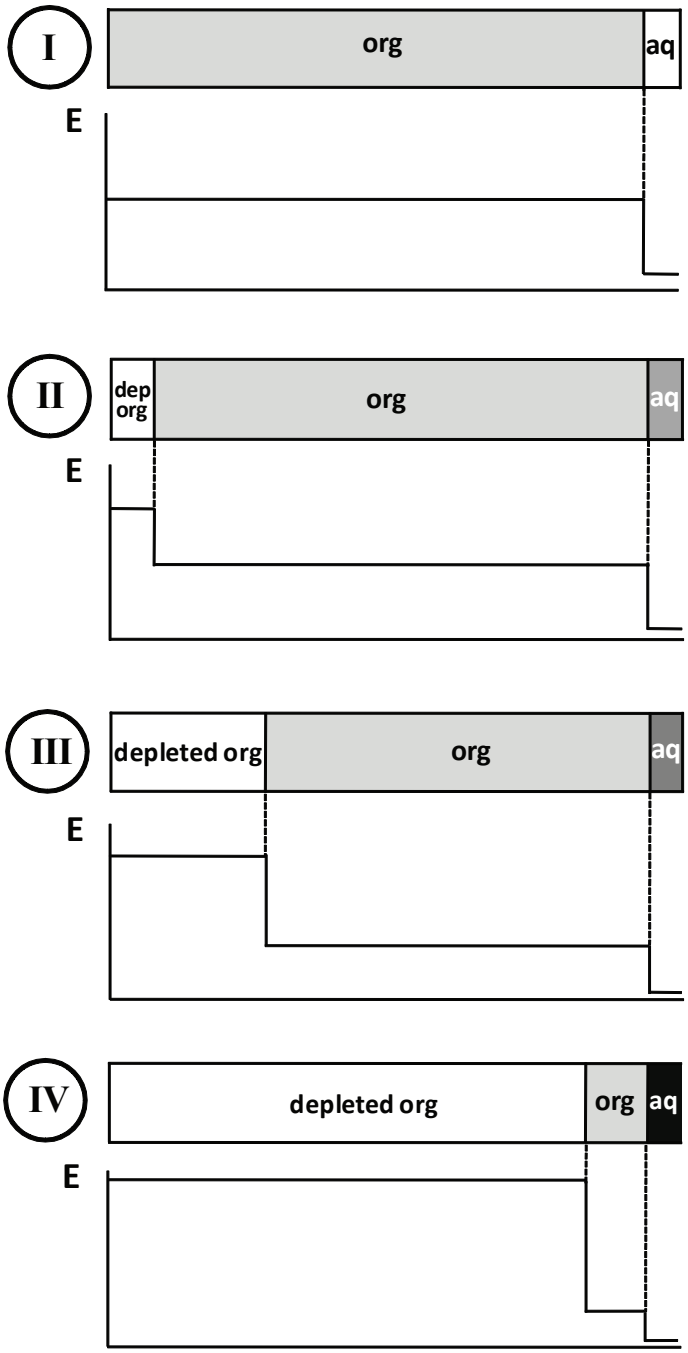


Figure 3.3 Schematic drawing of the changes in electric field strength during EE in the different zones. I, II, II and IV depict different time points in the process. During EE, the electric field strength in the organic zone decreases, while it increases in the depleted organic zone.

3.2 cEE of model peptides

A CE apparatus enables the handling of very small volumes and well-controlled high voltage and pressure application. Moreover, in a capillary, much higher electric field strengths can be used, since heat is dissipated more efficiently. This makes cEE an attractive option as selective sample injection method, as has been described before [9, 10]. On top of this, the influence of disturbances at the liquid-liquid interface can be expected to be smaller, since the area of the liquid-liquid interface is much smaller [7]. This partly compensates the fact that performing EE with a smaller area of the liquid-liquid interface is not advantageous for the speed of cEE. Moreover, much higher electric field strengths can be realised when using the CE apparatus. When 15 kV is applied, the electric field strength between the liquid-liquid interface and the electrode tip can be as high as 1.36×10^3 kV/m, which is more than 30 times higher than in the large-scale EE experiments.

A very high electric field strength exists in a short plug of low-conductive organic phase at the inlet of a capillary that is for the rest filled with higher conductive aqueous solution. The shorter the plug length and the larger conductivity difference between the zones will be, the larger the electric field strength will be, as was described by Chien and Burgi [17], who determined the optimal conditions for in-capillary sample concentration using field amplification.

Based on the above-mentioned aspects, a cEE procedure was developed. Theoretically, cEE speed is maximal when the liquid-liquid interface is exactly at the inlet of the capillary. However, due to interface instabilities (as described above) as well as EOF mismatch [18] it is difficult to fix the interface position at an exact location in the capillary. When the interface was too close to the inlet, cEE appeared to be unreliable. Therefore, a small aliquot of EtOAc was injected (approx. 2 cm, 3% of the capillary volume, i.e. ~88 nL). During cEE, this zone was slowly pushed back into the vial, using back-pressure, which also served to counter-effect the influence of the low EOF that still existed at pH 3. Optimisation of back-pressure, extraction time and extraction voltage resulted in the following injection scheme, analogous to Van der Vlis *et al* [9]: after a small EtOAc plug was injected by pressure (25 mbar) into the capillary, the first minute of cEE was carried out without back-pressure to let some more EtOAc enter the capillary by EOF. Then, a back-pressure of 25 mbar was applied during the remaining 9 min of the cEE procedure, to slowly push out the EtOAc plug without losing the concentrated sample zone. During EE, 15 kV was applied and the current profiles were monitored. Between measurements, the fused silica capillary wall was reconditioned by flushing 5 min with 0.1 M NaOH and 10 min with deionised water.

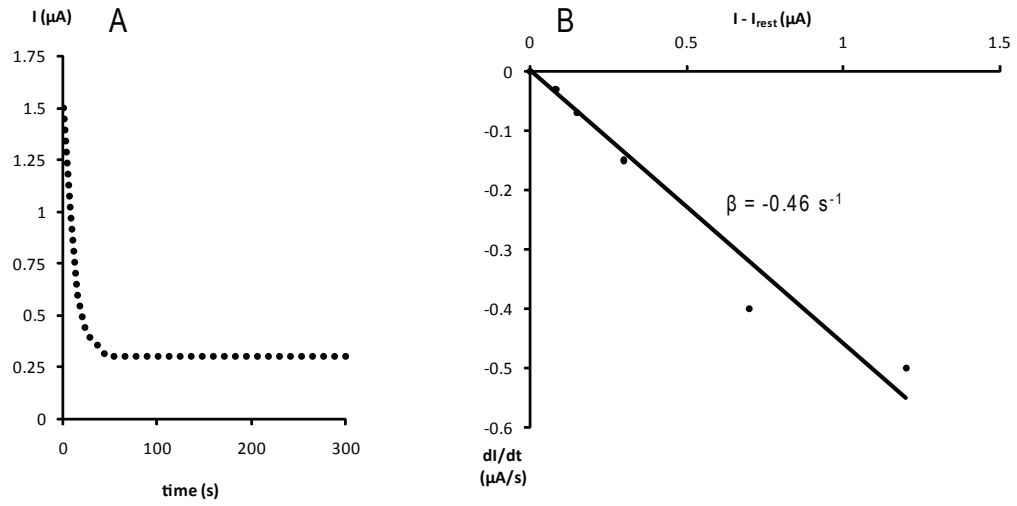


Figure 3. 4 A) cEE current profile of the extraction of 0.5 x 10^{-3} M ammonium (n=3) B) dI/dt plotted against $I-I_{rest}$ of the current profile shown in part A, to determine β values.

To study whether the cEE process is similar to the large-scale EE experiment discussed in the previous section, current behaviour during the extraction of 0.5 x 10^{-3} mol/L ammonium in EtOAc was monitored. Again, the current decreased exponentially (Fig. 3.4A), but much faster; the extraction rate β was much higher (Fig. 3.4B) compared to the first EE set-up. In Fig. 3.4A, it can be seen that during this period the current is stable and cEE is taking place without being disturbed. The current profiles were found to be reproducible.

Three consecutive cEE cycles of bradykinin, angiotensin I, bradykinin 1-5 and substance P (concentration in EtOAc 2.1 – 5 nM) were carried out without refilling or shaking of the sample vial. Since peptides have lower electrophoretic mobilities than ammonium, longer extraction times are needed to achieve peptide depletion, hence an extraction time of 10 min was chosen. After each cEE cycle, the sample zone was flushed to the MS using 50 mbar, for DI-MS analysis. After the first cEE, more than 96% of each peptide was extracted, while a consecutive extraction resulted in a 1 – 3% fraction, and a third consecutive extraction less than 0.1% (Table 3.1); percentages were calculated in relation to the sum of the peak areas of all three experiments. The total volume in the vial was 50 µL, but the electric field was not present throughout this whole volume (as discussed in section 2.2.2). This was confirmed by experiments in which a larger sample volume was used: peptide peak areas did not increase. On top of this, after mixing of the electroextracted sample, a consecutive cEE experiment

resulted again in high peptide peaks (data not shown). When the effective extraction volume is depleted, only peptides that slowly diffuse into the electric field zone are extracted. This is only a small amount of peptide in one experiment, because diffusion is a slow process. Since only a portion of the organic phase is analysed in the cEE set-up, it is complicated to obtain correct recovery data. Altering the shape or position of the electrodes to ensure that the electric field is present throughout the whole volume would be a solution, however, in the present experimental set-up using a commercial CE autosampler this is difficult to realise. Therefore, recovery was determined using the EE device described in section 2.2.1, where a sample of organic phase (100 µL) could easily be sampled before and after EE (10 mL EtOAc, 100 µL 10 mM ammonium acetate adjusted to pH 3 with FA as aqueous phase, 2.1 – 5 nM peptides, 10 min, 5 kV). The samples were evaporated, reconstituted in 100 µL 50%/50% (v/v) ACN/0.2% FA and analysed with DI-MS at a flow rate of 3 µL/min. Recoveries (based on measured ion intensities) were 77% for bradykinin, 90% for angiotensin 1, 80% for bradykinin 1-5 and 92% for substance P.

Table 3.1 Result of cEE of 4 model peptides. Percentages were calculated relative to the sum of the peak areas of the three consecutive experiments. In column of the 1st and 2nd cEE, the standard deviations are given (n = 3).

	peak area (%)		
peptide	**1st EE**	**2nd EE**	**3rd EE**
bradykinin	98.0 ± 1.4	2.0 ± 1.4	< 0.1
angiotensin I	98.8 ± 1.2	1.2 ± 1.2	< 0.1
bradykinin 1-5	97.7 ± 1.2	2.3 ± 1.2	< 0.1
substance P	96.6 ± 2.8	3.4 ± 2.8	< 0.1

3.3 Coupling of cEE to LC-MS

In [11], a cEE needle device was developed to couple cEE to LC. However, a switching valve offers a less complicated solution. A 6-port 2-way switching valve with an electrically isolated rotor and stator (see experimental section) was used (Fig. 3.5). This valve model has proven to be useful as interface for coupling LC-type systems to CE in several designs [22-24]. The cEE-LC-MS method includes (i) the cEE step; the valve is in the load position, the sample loop connects the inlet and outlet of the CE system; (ii) the sample transfer step; after cEE, the sample is flushed into the sample loop by applying pressure (~1000 mbar). The optimal flush time was determined to be 45 s. A rather large sample loop (5 µL) was chosen

to give the operator a suitable time window to manually switch the valve; (iii) the injection step; the valve is switched into the inject position and the sample is injected into the LC column. In future work, manual switching can be exchanged with automated switching. Band broadening due to the large sample loop volume and application of hydrodynamic pressure was overcome by starting the LC gradient with 1 min 100% 0.1% FA (solvent A), to allow for peptide trapping on the front of the column.

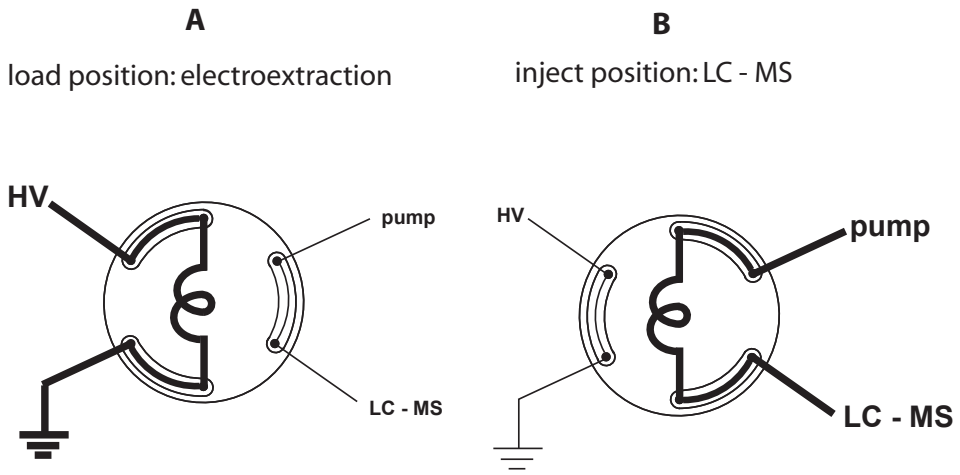


Figure 3.5 Schematic drawing of the coupling of cEE to LC. The bold line indicates the active part of the system. In part A, cEE of the analytes takes place, in part B, the concentrated analyte plug is injected into the LC-MS system

An LC-MS run and a cEE-LC-MS run of a test mixture of 5 model peptides (bradykinin 1-6, bradykinin 1-5, ACIE-bradykinin, bradykinin and angiotensin 1) were compared (Fig. 3.6). In the LC-MS run, 0.1 µL sample (5 µM per peptide in 70% ACN / 30% 0.1% FA) was injected, the recommended maximum for this column when the sample is not in solvent A (0.1% FA), as is the case after RP-SPE sample pretreatment. cEE-LC-MS resulted in 100 times increased peak areas using the same starting peptide concentrations, requiring only 10 min longer analysis time. Peak widths were not impaired and the relative peak heights basically remained the same. The LC injection volume was 0.1 µL, while cEE depletes maximal 14 µL (section 3.1). The peak heights were about 100 times increased, indicating approximately 70% depletion of 14 µL. However, in section 3.2, we showed that nearly complete depletion takes place, so apparently the volume where the electric field is present is somewhat smaller, i.e. about 10 µL, than expected.

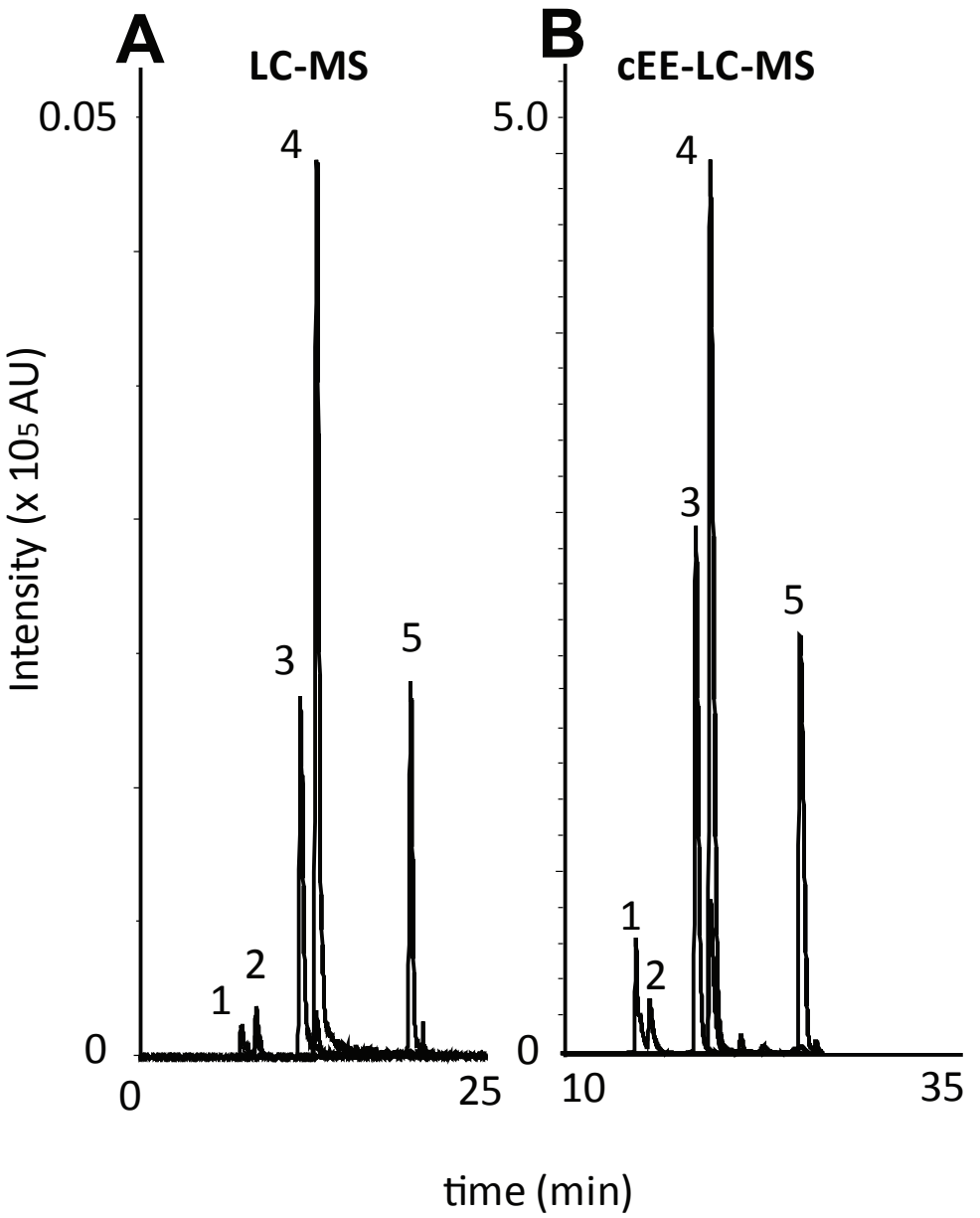


Figure 3.6 Typical example of the concentrating effect of EE. A) chromatogram resulting from conventional LC-MS and B) chromatogram resulting from cEE-LC-MS. When EE was performed, base peak heights were increased ~2 orders of magnitude compared to conventional LC-MS. 1) bradykinin 1-5, 2) bradykinin 1-6, 3) angiotensin I converting enzyme inhibitor , 4) bradykinin, 5) angiotensin I (AU = arbitrary units).

Calibration curves of electroextraction of 5 peptides from EtOAc were generated, containing 5 points (n = 3). As internal standard, thymopentin was used, while the concentrations of the 5 other peptides were varied between 0.05 μM and 5 μM in EtOAc, corresponding with injected sample amounts between 0.01 and 5 pmol. These levels are within biological range [25]. The linearity of cEE-LC-MS after internal standard correction is excellent (R^2 values between 0.984 – 0.996), the curve intercepts are significantly through zero (P values < 0.05) and the repeatability of the calibration curves is good (relative standard deviation of the curve slope between 0.73 and 12.0%) (Table 3.2). The relative standard deviations of the experimental values ranged between 4.6% at the highest concentration to 25.0% at the lowest concentration; the overall mean relative standard deviation of all the measurements was 14.7%. At the trace levels from 0.05 μM in EtOAc and below, reproducibility decreased dramatically. However, at 20 nM, bradykinin, angiotensin I converting enzyme inhibitor and angiotensin I were still detectable with a signal-to-noise ratio above 10. In consequence, for these peptides the LOD is estimated to be around 3 nM.

Table 3.2 Regression data of the calibration curves of 5 model peptide academic standards, P < 0.05 indicates intercept is through zero (n = 3).

Peptide	R^2	slope ± rel. st. dev (x 10^6 AU)	P-value intercept
bradykinin	0.996	15.5 ± 0.1%	0.00026
bradykinin 1-5	0.990	0.87 ± 7.9%	0.00026
bradykinin 1-6	0.984	1.03 ± 12.3%	0.000023
angiotensin I converting enzyme inhibitor	0.991	7.09 ± 1.9%	0.00031
angiotensin 1	0.988	27.5 ± 4.6%	0.0002

3.4 Applications

cEE can serve as a fast and simple interface between SPE and LC. Normally, RP-SPE eluents are freeze-dried and reconstituted in water, which is labour-intensive, time-consuming and may also introduce an additional error to quantitative results due to sample loss by evaporation, adsorption or reconstitution problems. cEE only requires mixing of the eluent with EtOAc and is capable of concentrating the analytes in a very small volume (< 1 μL) that can be handled accordingly. Moreover, as mentioned in section 2.3, the

eluent, containing 70% ACN, is better miscible with EtOAc than water (~5.5% instead of 2.2%), enabling a larger sample amount to be introduced for cEE. When attempting the first cEE experiments with biological samples, low recoveries were obtained. Probably, the peptides are not entirely in solution in cationic form in the EtOAc phase, possibly due to a precipitation reaction with matrix components. Possibly, adding extra components to the EtOAc will offer a remedy, this has our future attention.

3.4.1 Protein digests

A model protein digestion mixture containing human cytochrome C, horse cytochrome C, human haemoglobin, chicken lysozyme, bovine ribonuclease A and bovine serum albumin was analysed. Of each protein, the initial amount (prior to SPE), was 12.5 µg, corresponding with 0.18 - 1.0 nmol. The amount of digested protein present in one cEE experiment was maximal 0.023 - 0.13 nmol. Since the effective extraction volume was ~ 10 µL, the amount of protein that was actually extracted was maximal 2.1 - 12 pmol.

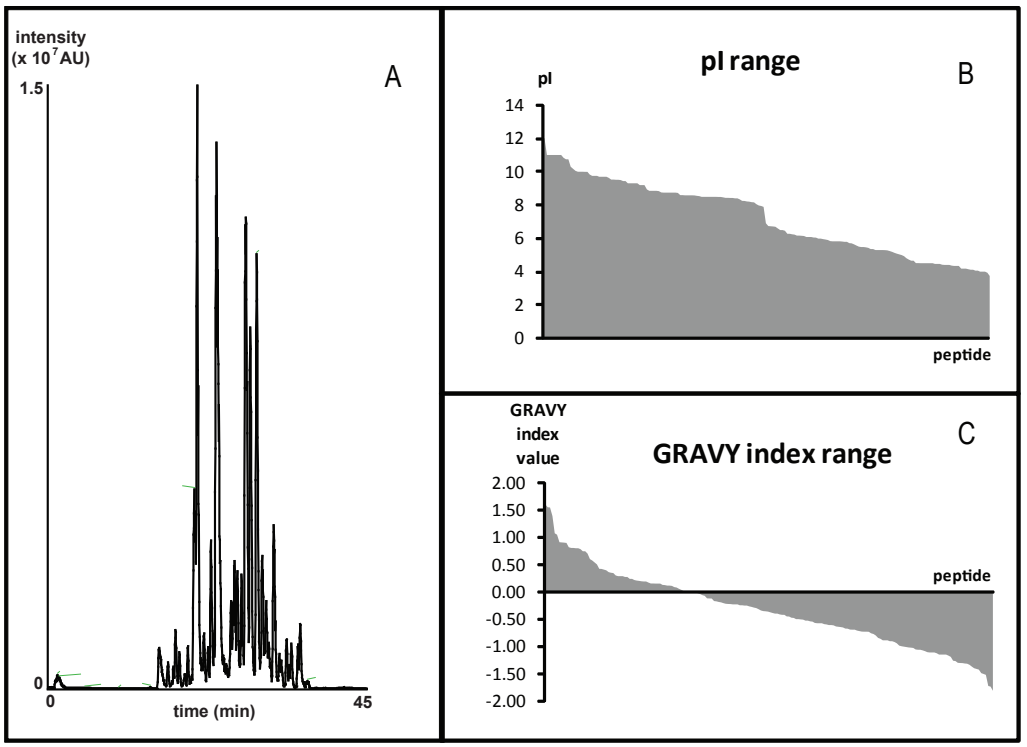


Figure 3.7 A) cEE-LC-ICR-FT-MS chromatogram of a model protein digest. Gradient: 0 to 50% B in 50 min. B) pI range of all extracted peptides and C) GRAVY index range of all extracted peptides

More than 170 peptides were extracted, separated and identified in one single run. The found peptide masses ranged from 500 to 4200 Da, 5 to 39 amino acids. Protein coverage was satisfactory: between 48% and 94 %, average 65%. As can be observed in Fig. 3.7, peptides with pI values ranging from 4 to 12 were extracted. The GRAVY index is a measure for the hydrophobicity of a protein, where lower values represent more hydrophilic peptides [26]. In Fig. 3.7, it can be observed that peptides above and below 0 were extracted. Studying the peptides that were not found back in this experiment (hence the protein coverage percentages below 100), revealed no different pI and GRAVY index range and pattern (data not shown).

3.4.2 Urine analysis

Fig. 3.8 shows a direct comparison between LC-MS and cEE-LC-MS of an SPE eluent (70% ACN) of urine spiked with 2 model peptides and cytochrome C digestion (0.5 µmol/L). In the LC-MS run, the eluent was diluted (5.5 %, i.e. 18 times) with water leading to the same analyte start concentrations as when cEE-LC-MS was performed. In a 1 µL LC-MS injection an aliquot of 0.2 µl urine was injected. In total, 96 peaks were detected, the highest being 1.35 x 10^4 AU (Fig. 3.8A). However, after cEE-LC-MS, a larger aliquot of approximately 3 µl urine could be injected and the peaks were around 15 times higher (the highest being much higher; 25 x 10^4 AU) and as much as 136 peaks were found (Fig. 3.8B), corresponding to an increase of 42% of the number of peaks. Probably, not all peaks represent peptides, but also a variety of endogenous metabolites.

To emphasise the potential of cEE-LC-MS in the field of peptidomics, a brief feasibility study of the extraction of peptides from urine was carried out. To aliquots of 200 µL, different amounts of horse cytochrome C digestion were added, resulting, assuming complete digestion, in peptide concentrations of 10 µM to 0.2 µM. Angiotensin I (2 µM) served as internal standard. The most abundant tryptic peptide, TGPNLHGLFGR, m/z 1169.62, could be detected even at the lowest concentration of 0.2 µM (2.5 µg/L) with a signal-to-noise ratio of ~50, corresponding to an LOD of this peptide around 0.015 µM. LOD values reported for LC-MS of peptides using similar columns and gradients, including evaporation of the SPE eluent, but larger injected sample aliquots, are between 0.065 and 1 µM [25]. The calibration curve (n = 3) showed excellent linearity (R^2 > 0.99), good reproducibility (relative standard deviation 14 %) and a recovery (of the whole method, thus including RP-SPE) of 56%. Moreover, several endogenous peptides were found, of which one could be identified to be bradykinin, which is a peptide known to occur in urine in a concentration between 10 and 35 nM [27].

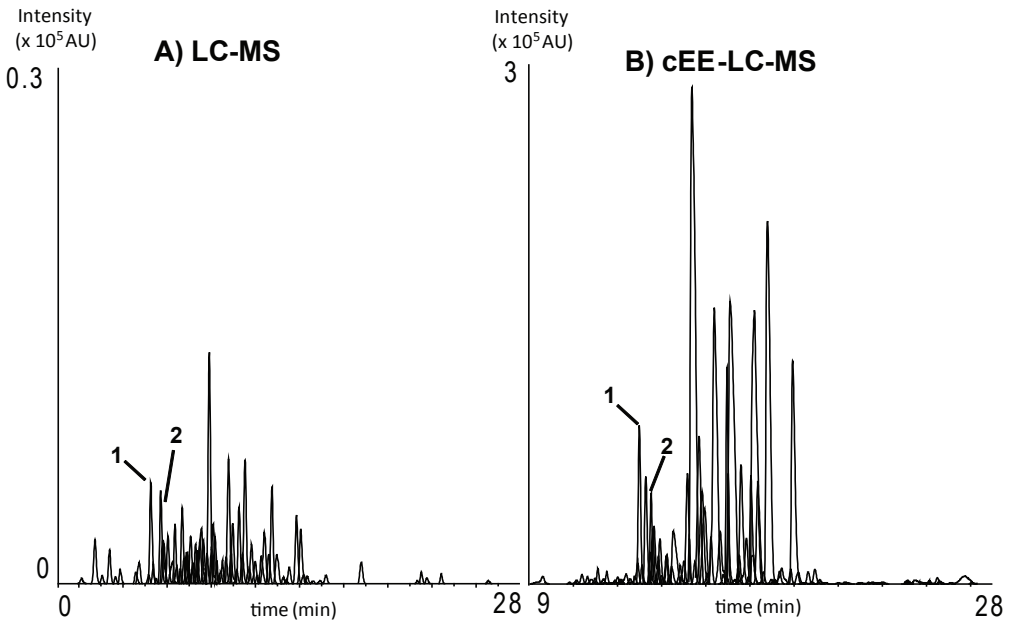


Figure 3.8 Extracted ion chromatograms of all peaks resulting from A) LC-MS with 1 µL injection, B) capillary cEE-LC-TOF-MS of urine spiked with cytochrome C digestion and internal standards after off-line SPE 1) cytochrome C peptide TGPNLHGLFGR, 2) angiotensin I. Other peaks are other cytochrome C fragments, putative endogenous peptides or other metabolites. Please note the difference of one order of magnitude between the y-axes. AU stands for arbitrary units.

In the cEE-LC-MS procedure used, an aliquot of approximately 3 µL urine is injected. Alternatively to cEE-LC-MS, the SPE-eluent (containing 70% ACN) can be injected directly. If the same urine aliquot as in the cEE-LC-MS experiments is then to be injected in a LC-MS system, 1 µL SPE eluent should be injected. An attempt to do this showed that this is not feasible (data not shown). This is because the elution strength of the SPE eluent (70% ACN) resulted in severely impaired separation on the used column (0.5 x 200 mm). It is possible to inject a higher volume of SPE eluent after it is sufficiently diluted with water, but this will not allow injecting an aliquot of 3 µL urine into this µLC system, where maximal 8 µL can be injected. One alternative would be evaporation of the eluent, but this results in the above mentioned disadvantages. Another alternative is to inject 3 µL untreated urine directly into the LC-MS system, but then high levels of contaminants are introduced that will interfere with separation and detection, leading to poor analysis and fouling of the systems.

4. Concluding remarks

The process of EE has been characterised and it has been demonstrated that the developed EE protocol is applicable to systems with different physical dimensions. The potential of cEE of peptides has been demonstrated using a set of model peptides. The peptides were concentrated two orders of magnitude from the organic into the aqueous phase. Moreover, the on-line coupling of cEE with LC-MS via a valve interface allows concentration, separation and detection of the concentrated analyte plug and minimises sample handling after, e.g., an SPE step. Linear response of the on-line cEE-LC-MS set-up has been obtained for model peptides. The analysis of a protein digest showed no bias to pI value or hydrophobicity. Furthermore, cEE of peptides was applied to a biological matrix. cEE can serve as a simple and fast interface between SPE and LC. Instead of evaporation and reconstitution of an SPE eluent, it can be mixed with EtOAc and injected into the LC system. In combination with an SPE step good LOD values, comparable to current published LC-MS methods (at low nM level), could be obtained for endogenous peptides in urine,. On-line coupling of SPE to LC via cEE has our future attention, as has an improved design of the cEE set-up to increase the effective extraction volume and improving reproducibility. In addition, we will optimise the SPE step. By enhancing the loadability of the method, i.e. enlarging the extraction volume, lower LOD values can be reached. We aim at the analysis of an entire SPE eluent in one experiment. For improvement of reproducibility, it seems a logical step to ensure that the effective extraction volume equals the total present organic phase, since turbulence of the organic phase and diffusion do not affect the extraction result anymore.

Next to peptides, metabolites have been detected in urine, suggesting that the EE approach is also suited for the analysis of metabolites.

5 . References

1 Marando, M. A., Clark, W. M., *Sep Sci Technol,* **1993**, *28*, 1561-1577

2 Zhai, S. L., Luo, G. S., Liu, J. G., *Sep Purif Technol,* **2001**, *21*, 197-203

3 Zhai, S. L., Luo, G. S., Liu, J. G., *Chem Eng J,* **2001**, *83*, 55-59

4 Theos, C. W., Clark, W. M., *Appl Biochem and Biotechnol,* **1995**, *54*, 143-157

5 Luo, G. S., Shan, X. Y., Qi, X., Lu, Y. C., *Sep Purif Technol,* **2004**, *38*, 265-271

6 Stichlmair, J., Schmidt, J., Proplesch, R., *Chem Eng J,* **1992**, *47*, 3015-3022

7 Bailes, P. J., *Ind Eng Chem Process Des Dev,* **1981**, *20*, 564-570

8 Scott, T. C., *Sep Purif Methods,* **1989**, *18*, 65-109

9 Van der Vlis, E., Mazereeuw, M., Tjaden, U. R., Irth, H., Van der Greef, J., *J Chromatogr A,* **1994**, *687*, 333-341

10 Van der Vlis, E., Mazereeuw, M., Tjaden, U. R., Irth, H., Van der Greef, J., *J Chromatogr A,* **1995**, *712*, 227-234

11 Van der Vlis, E., Mazereeuw, M., Tjaden, U. R., Irth, H., Van der Greef, J., *J Chromatogr A,* **1996**, *741*, 13-21

12 Collins, C. J., Arrigan, D. W. M., *Anal Bioanal Chem,* **2009**, *393*, 835-845

13 Kubáň, P., Šlampová, A., Boček, P., *Electrophoresis,* **2010**, *31*, 768-785

14 Chin, J. T., Wheeler, S. L., Klibanov, A. M., *Biotechnol Bioeng,* **1994**, *44*, 140-145

15 Klibanov, A. M., *Nature,* **2001**, *409*, 241-246

16 Mikkers, F. E. P., Everaerts, F. M., Verheggen, T. P. E. M., *J. Chromatogr,* **1979**, *169*, 11-20

17 Chien, R. L., Burgi, D. S., *Anal Chem,* **1992**, *64*, A489-A496

18 Urbanek, M., Krivankova, L., Bocek, P., *Electrophoresis,* **2003**, *24*, 466-485

19 Quirino, J. P., Terabe, S., J Chromatogr A, **2000**, 902, 119-135

20 Matsudaira, P., A Practical Guide to Protein and Peptide Purification for Microsequencing, **1993**, 2nd edition, Academic Press, p. 48

21 Porras, S. P., Riekkola, M. L., Kenndler, E., *Electrophoresis,* **2003**, *24*, 1485-1498

22 Tempels, F. W. A., Underberg, W. J. M., Somsen, G. W., de Jong, G. J., *Electrophoresis,* **2007**, *28*, 319-1326

23 Puig, P., Tempels, F. W. A., Somsen, G. W., de Jong, G. J., *Electrophoresis,* **2008**, *29*, 1339-1346

24 Ponton, L. M., Evans, C. E., *Anal Chem,* **2001**, *73*, 1974-1978

25 Van den Broek, I., Sparidans, R. W., Schellens, J. H. M., Beijnen, J. H., *J Chromatogr B,* **2008**, *872*, 1-22

26 Kyte, J., Doolittle, R. F., *J Mol Biol,* **1982**, *157*, 105-132

[27] Hial, V., Keiser, H. R., Pisano, J. J., *Biochem Pharmacol,* **1976**, *25*, 2499-2503

Chapter 4

On-line large-volume capillary electroextraction coupled to LC-MS to improve detection limits of peptides

Based on
Lindenburg, P.W., Tempels, F.W.A., Tjaden, U.R., Van der Greef, J., Hankemeier, T.
On-line large-volume capillary electroextraction coupled to LC-MS to improve detection limits of peptides.
Accepted for publication in *J Chrom A.*

Abstract

In this research-paper we demonstrate a new electroextraction (EE) set-up and its on-line coupling with LC-MS. EE takes place in a two-phase liquid-liquid system, consisting of an aqueous and an organic phase, where an applied electric field causes ions to be extracted from one phase into the other, to be concentrated close after the liquid-liquid interface.

The extraction takes place in a wide-bore capillary that is connected to a 2-way 10-port switching valve, which serves to couple capillary EE (cEE) with LC-MS. In this set-up, volumes as high as 100 µL can be extracted, which is a ten times larger volume than has been reported, earlier, when cEE took place from a vial into a capillary. Moreover, it is faster. First, the feasibility of this set-up was studied using the cationic purple dye crystal violet. Then the method was coupled to LC-MS and large volume cEE of several model peptides was optimised. Both liquid phases, the extraction voltage and the extraction time were optimised and it was found that the addition of trifluoroacetic acid (TFA) improved extraction dramatically. Moreover, the presence of some ACN at the liquid-liquid interfaces improved cEE. The whole procedure was automated and could be used routinely. Calibration curves of 5 test peptides in water have been constructed, resulting in good repeatability, good linearity and LOD values between 0.5 and 10 nM.

Finally, the method was applied to plasma analysis and calibration curves of the relevant plasma peptides angiotensin 1 and 2 as well as the 3-8 fragment of angiotensin 2 (angiotensin 2 (3-8)) were constructed which had good linearity and repeatability; LOD values were 10-50 nM. Analysis of unspiked plasma allowed the detection of about 60 putative endogenous peptides, underlining the great potential of EE as on-line sample concentrating technique. On-line large volume cEE-LC-MS allows for enrichment, separation and detection of plasma peptides from large sample volumes, minimises sample handling and can be an important step in full automation of analytical procedures.

1. Introduction

The quickly erupting field of systems biology and metabolomics research is putting analytical methods for major challenges. In order to be able to characterise a biological system and come to a greater understanding of the system, data should be collected on as many relevant biological components as possible, preferably with one analytical method [1]. A complicating factor is the fact that many biologically relevant compounds occur at trace levels, as is for example the case for many peptides [2]. Therefore, to be able to acquire relevant metabolomics data, limits of detection (LOD) should be improved.

An option to improve the LOD is to use electroextraction (EE) in the sample pretreatment procedure. EE takes place in a two-phase liquid-liquid system, consisting of an aqueous and an organic phase, where, under the proper conditions, an applied electric field causes ions to be extracted from one phase into the other, to be concentrated just after the liquid-liquid interface [3]. At the start, the analyte ions are in the organic phase and since the conductivity of this phase is much lower than in the aqueous phase, a very high electric field strength exists there that causes these analytes to migrate very fast into the aqueous phase. Since the electric field strength that is present in the aqueous phase is very low, the analytes migrate with a very low speed. As a consequence, they are concentrated as they reach the aqueous phase. A crucial requirement of the organic phase is that it contains some water, in order to hydrate ions, enabling the presence of ions; for example ethyl acetate (EtOAc) can contain approximately 2.5% water and is therefore suitable for EE experiments. Initially, EE was developed to enhance product yields in industrial-scale solvent-solvent extractions [4-10]. In the 1990s, analytical EE was developed, coupled to CZE and LC and demonstrated with simple test solutions [11-13]. Recently, we reported capillary electroextraction (cEE) coupled to LC-MS to improve the LOD of peptides in complex samples [14]. Within 10 min, peptide peaks in the resulting chromatograms could be increased ~100 times by performing on-line cEE as sample preconcentration compared to a conventional sample injection of 0.1 μL. However, we believed that the enrichment factor could be higher, without increasing the extraction time but rather decreasing it.

The enrichment factor is mainly dependent on the volume of organic phase where an electric field is present. In the earlier cEE set-up, this volume was between the capillary inlet and the tip of the electrode, and was around 10 μL, the capillary tip being close to the bottom of the sample vial and the electrode tip being close the liquid surface [14]. This extraction volume was not fixed, but just a part of the total sample volume. On top of this,

the aqueous phase was in the capillary, above the organic phase, while the organic phase had a lower density. Dipping the capillary, with the plug close to its end, in and out vials during the measurement frequently resulted in sample plug loss, introducing unreliability in the cEE set-up. With an increased extraction volume, higher enrichment factors and therefore better LOD values should be achievable. In literature, modified electrode configurations to improve injection amounts in electrokinetic injection in CZE have been described [15, 16], but these solutions require complicated alterations of the CE apparatus and/or sample vials and increase the risk of sample carry-over.

In the approach presented in this work, large volume cEE takes place in a wide-bore capillary (internal diameter of 1 mm). The liquid-liquid interface is located just in front of a valve with a sample loop. After cEE, the concentrated sample is pushed into a sample loop by shortly applying hydrodynamic pressure. The use of wide-bore capillaries in CE is not favourable due to extensive Joule heating, but in this case the large zone of low-conductive organic phase limits the current and as a consequence heat generation. Therefore, a high electric field strength could be applied.

The large volume EE set-up was evaluated by performing experiments on solutions comprising several test peptides. The main system components were optimised, namely the composition of the organic and aqueous phases, the extraction voltage and the extraction time. It was found that adding a counter ion to the organic phase dramatically improved the EE of peptides. Acetic acid, formic acid (FA) and TFA were studied and it was found that TFA improved peptide extraction best. First, the composition of the aqueous and organic phase, the extraction time and the extraction voltage was optimised for several test peptides in water. Then the method was transferred to plasma analysis and successfully validated.

2. Experimental

2.1 Chemicals

All reagents were of analytical grade or higher. A Millipore Q-guard water purifying system (Billerica, MA, USA) was used to obtain pure water. ACN, EtOAc, methanol, iso-propanol, dimethyl sulphoxide (DMSO), TFA and FA were obtained from Biosolve (Valkenswaard, The Netherlands), acetic acid from JT Baker (Philipsburg, NJ, USA) and the test peptides (bradykinin (1-5), mass 572; bradykinin (1-6), mass 660; ACIE-bradykinin, mass 964; bradykinin, mass 1060; angiotensin 2, mass 1296, angiotensin 1, mass 1046; and substance P, mass 1347) from Genscript Corporation (Piscataway, NJ, USA). A protease

inhibitor cocktail (product number P8340) and crystal violet are from Sigma Aldrich (St. Louis, MO, USA).

2.2 Equipment and Techniques

2.2.1 Large volume cEE set-up

Voltage and pressure application during cEE experiments was achieved with an Agilent Technologies (Santa Clara, CA, USA) 1600 series CE apparatus, using LC Certified 12x32mm screw thread autosampler vials with black 8 mm white silicone/red PTFE screw caps from Grace (Deerfield, IL, USA) and tubing and capillaries were purchased from Inacom Instruments (Overberg, the Netherlands). The 10-port 2-way switching valve with polyaryletherketone (PAEK) stator including actuator (Cheminert® HPLC injection valve model C2H-2340) was purchased at Valco Instruments (Houston, TX, USA). This valve is bio-compatible and the liquid has no contact with metal parts, preventing short circuiting and unsafe working conditions. For injection of the aqueous and organic phases, the flush function of the CE apparatus was used.

In Fig. 4.1 all the major components are depicted and in section 3.1 the procedure is described. During the whole procedure, the start signals of the CE apparatus is controlling the other components.

2.2.2 Mass spectrometry

Mass spectrometric experiments were carried out on a Bruker Daltonics MicrOTOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Spray settings were: end-plate offset -529 V, capillary voltage -2000 V, nebuliser gas pressure 0.4 bar, dry gas flow 4.0 L/min and dry temperature 200 °C. Identification of the test peptides took place by searching for the known peptide masses.

2.2.3 Liquid Chromatography

LC was carried out with an Agilent Technologies 1200 series µHPLC apparatus, equipped with a ZORBAX SB-C18 (5 µm, 150 x 0.5 mm) capillary HPLC column, also purchased from Agilent. A 20 µL/min gradient elution was employed with 0.1 % FA in water as solvent A and 0.08% FA in ACN as solvent B, starting at 0% B and ending after 30 min at 35%. In plasma analysis, the gradient ended after 40 min. Post conditioning concerned 5 min 95% solvent B and 5 min 100% solvent A. On measurement days, a blank run was performed first.

2.3 Sample pretreatment

Plasma (EDTA, citrated, and heparin) was purchased from Richmond Pharmacology (London, Great-Britain. Ultracentrifugation took place in Microcon mass centrifugal filter devices (MW cut-off 30 kDa; Millipore, Billerica, MA, USA) using an Eppendorf 5415R centrifuge (Eppendorf AG, Hamburg, Germany) and for SPE an Oasis HLB µElution plate was used (30 µm, Waters Corporation, Milford, MI, USA).

To break interactions between peptides and plasma proteins [17], plasma (300 µL) was 5 times diluted with 5% DMSO. Then the sample was ultracentrifuged (mass cut-off 30 kDa, 14000g, 4°C). After 50 min, 100 µL 5% DMSO was added and the sample was centrifuged for another 50 min. The filtrate was acidified with TFA (final concentration 1%), after which RP-SPE was carried out. The SPE material was wetted with ACN, equilibrated with 1% TFA, after which the sample was applied. Elution took place with 75 µL 100% ACN, which was directly mixed with 450 µL EtOAc for consecutive large volume cEE, by pipetting up and down. In one cEE experiment, 100 µL EtOAc is extracted, representing a plasma aliquot of 66.7 µL.

In the first instance, it was noticed that the peptide spikes, especially bradykinin, were degraded, resulting in unsatisfactory results. After adding a protease inhibitor mix (Sigma Aldrich, product number P8340 (St. Louis, MO, USA)), most of the degradation was avoided and results improved significantly.

3. Results and discussion

3.1 Description of the EE-LC-MS procedure

In Fig. 4.1, a scheme of the switching valve and the 4 main steps of the EE-LC-MS procedure are depicted. When the flush function of the CE apparatus is used, a pressure of approximately 950 mbar is applied on the inlet vial. This function was chosen to be able to quickly fill the large-volume EE capillary (part B in Fig. 4.1). The flow was restricted via the inlet capillary (part A in Fig. 4.1). The EE capillary, valve and outlet capillary had such a high inner diameter that virtual no back pressure was generated. By choosing optimal dimensions for the inlet capillary, a controlled flow was generated that allowed for precise injections within a reasonable time span. It was found that an inlet capillary with a length of 25 cm and an ID of 75 µm delivered just the right flow to obtain reasonable injection times (less than 3 min). To investigate whether this injection method was reliable, fixed-time injections were carried out. The vials were weighed prior and after each injection period, to

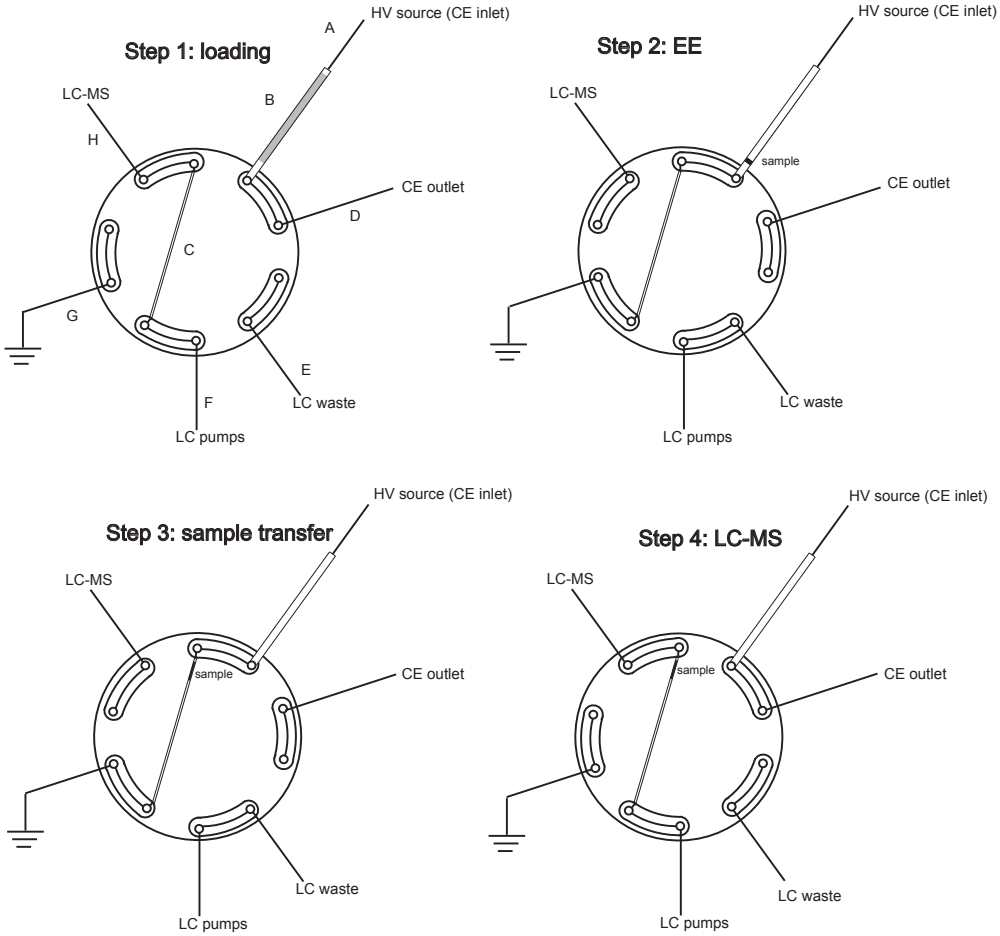


Figure 4.1 Valve set-up for interfacing large volume cEE with LC. A) inlet capillary (fused silica, L = 25 cm, OD = 365 μm, ID = 75 μm), B) EE capillary (PEEK, L = 20 cm, OD = 1.59 mm, ID = 1 mm), C) sample loop (PEEK, L = 20 cm, OD = 1.59 mm, ID = 0.5 mm) D) outlet capillary (fused silica, L = 75 cm, OD = 365 μm, ID = 200 μm) E) LC waste (PEEK, L = 100 cm, OD = 1.59 mm, ID = 0.5 mm), F) LC pump tubing (PEEK, L = 30 cm, OD = 1.59 mm, ID = 50 μm), G) tubing to earth (PEEK, L = 20 cm, OD = 1.59 mm, ID = 0.5 mm). H) tubing to LC-MS (PEEK, L = 20 cm, OD = 1.59 mm, ID = 50 μm) Step 1: situation after all phases have been loaded. The gray zone in the EE-capillary depicts the organic phase with analytes. Before EE starts, the valve is switched. Step 2: situation after EE is finished; the small black zone depicts the concentrated analytes. Step 3: situation after the sample has been transferred into the sample loop by applying pressure. After sample transfer, the valve is switched back. Step 4: situation where the sample zone is being injected into the LC-MS system.

be able to determine the injection volume.

During these experiments it was noticed that room temperature fluctuations significantly

influenced the injection volume; this is because temperature significantly influence the viscosity. This is undesirable, because when the location of the liquid-liquid interface is not well-controlled, the sample may not reach the sample loop completely or, on the other hand, be (partly) pushed beyond the loop. Therefore, the auto-sampler of the CE apparatus was cooled with streaming tap water, which controlled the temperature to 16.0-16.3 °C. Then, the injection method appeared to be highly repeatable for both the organic (40.5 ± 0.23 µL/min) and the aqueous phase (18.8 ± 0.054 µL/min). In summary, the location of the liquid-liquid interface and therefore also the injection volume could be controlled very accurately. During step 1 of the cEE-LC-MS procedure (Fig. 4.1), the EE capillary (B) was filled with the aqueous and organic phases via the inlet capillary (A). First, 0.1% FA in water was injected, which was the mobile phase used in LC. Then, EtOAc, containing the analytes, was injected and finally the second aqueous phase, containing 5% FA. Optimisation of the composition of the liquid phases will be discussed in section 3.3.2.1 and 3.3.2.2. After the loading step was finished, the valve was switched and cEE started (step 2). During cEE, the electrical circuit was grounded via an external vial containing 0.1% FA. As soon as the positive voltage was applied, the cationic analytes migrating towards the ground electrode (earth) were extracted and concentrated into a small zone. At the beginning of each measurement day, the tubing to earth (part G in Fig. 4.1) was flushed with 0.1 % FA to ensure good connection with earth. When cEE was finished, the sample zone was transferred into the sample loop using hydrodynamic pressure (step 3) after which the valve was switched back and the sample was injected in the LC-MS system and consecutive LC-MS analysis took place (step 4). The whole large volume cEE-LC-MS procedure was automated and was able to run without human interference for at least 18 hours, performing 24 experiments in a row.

The location of the liquid-liquid interface in the system is of crucial importance for the success of the experiments. In the first instance, it was attempted to carry out cEE in the port-to-port volume (internal loop volume) of the valve. However, this appeared to result in current leakage through the valve, due to the very low conductivity of the large EtOAc zone. Therefore, ideally, the interface is just in front of the valve. Then, after cEE, only a short pressure application step is needed to push the sample into the sample loop.

At first, a series of feasibility experiments was carried out in which 0.5 µM crystal violet was extracted from EtOAc into 0.1 % FA in a transparent EE capillary (PTFE, 20 cm x 0.8 mm) to be able to visually follow the progress of cEE (Fig. 4.2). On top of this, the current behaviour during cEE was monitored. The purpose of these experiments was to globally characterise how the liquid-liquid interface behaved and to study the cEE process

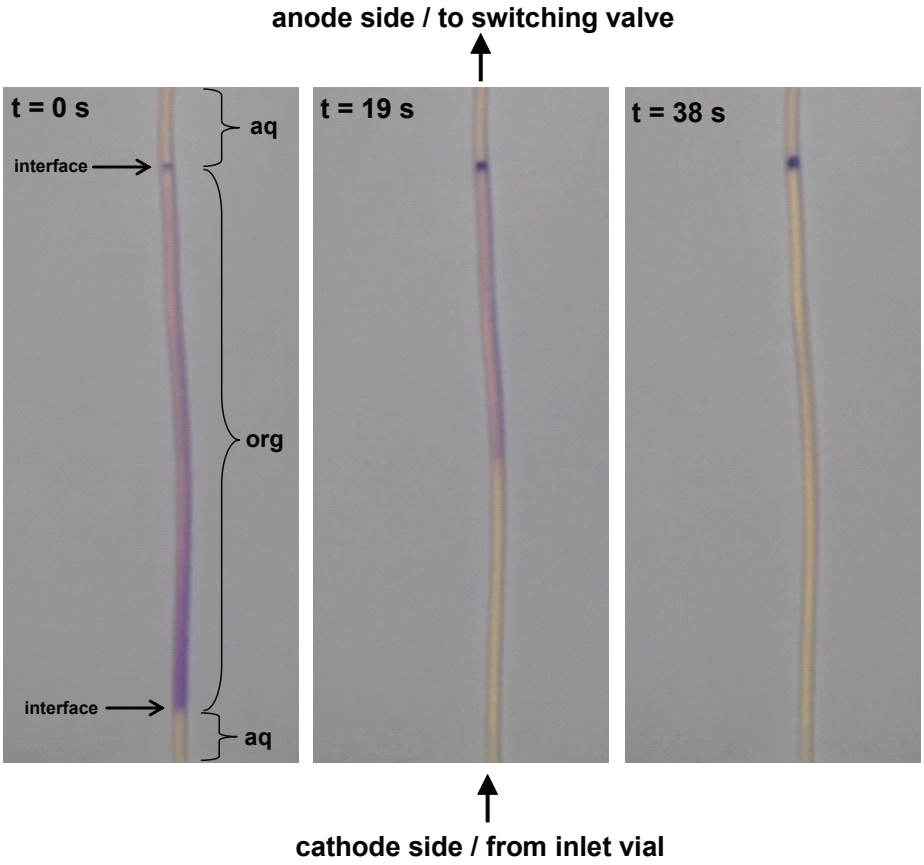


Figure 4.2 Video stills of cEE of the purple dye crystal violet in a 0.8 mm ID PTFE capillary. The organic phase consisted of EtOAc containing 0.5 µM crystal violet; the aqueous phase at the anode side of 0.1% FA and the aqueous phase at the cathode side of 5% FA.

It was observed that when a voltage higher than 1 kV was immediately applied, the cEE process was halted due to bubble formation. When the voltage was kept at 1 kV or lower during the whole experiment, extraction times became unfavourably long (> 10 min). However, it was found that when the voltage was increased gradually after applying a start voltage of 1kV no bubble formation occurred and complete extraction could be achieved within a minute. As shown in [14], the current is maximal at the start of cEE, after which it decreases exponentially. When the extraction voltage is low at the start of cEE, excessive Joule heating which results in bubble formation can be avoided.

It was also observed that during the loading procedure water droplets sometimes occurred in the organic phase. Despite the fact that these artefacts in the organic phase did not prevent cEE from taking place, it took longer and the current profile became unpredictable. However, it was found that when some ACN, which is miscible with both water and EtOAc, was injected before and after the organic phase, no water droplets occurred and cEE performance was significantly improved. The effect of injecting similar amounts of methanol, ethanol and isopropanol at the liquid-liquid interfaces were studied as well and showed similar effects. The volume of ACN that was injected was varied as well. When 1 µL ACN was used, cEE stability and speed was observed to be optimal (data not shown). Apparently, the presence of a small quantity of polar organic solvent at the interface that is soluble in both liquid phases is beneficial for cEE. The explanation for these observations is a subject for ongoing research.

It was also observed that increasing the FA concentration of the aqueous phase increased the extraction speed dramatically. This can be explained by the larger electric field strength that will be in the organic phase when the conductivity of the aqueous phase increases.

3.2 Application to peptides

3.2.1 System alterations

After the feasibility experiments, the method was coupled to LC-MS and the extraction of 5 test peptides was optimised. In these experiments, a PEEK capillary (20 cm x 1 mm ID) was used instead of the 20 cm x 0.8 mm ID PTFE capillary mentioned in the previous section, to increase the extraction volume. PTFE tubing of 1 mm ID appeared to be too difficult to connect properly due to the softness of the material; therefore a PEEK capillary was used. The organic sample volume was 100 µL for this set-up. Chromatographic peak area was used as measure for the extraction efficiency. The loading procedure in all experiments was as follows: 1) 10 min 950 mbar with 0.1% FA 2) 24 s 50 mbar ACN 3) 2.66 min 950 mbar EtOAc with analytes (= 100 µL) 4) 24 s 50 mbar ACN 5) 3.2 min 950 mbar with 5% FA. Then, the cEE process was started. After cEE was finished, the generated sample plug was pushed into the injection loop with 0.95 min 950 mbar, after which the valve was switched and the sample was injected into the LC-MS system. The resulting chromatograms showed good separation, an example of which can be seen in Fig. 4.4.

3.2.2 Organic phase optimisation

When only test peptides were present in the EtOAc, almost no current was observed

and the resulting chromatogram contained only low peptide peaks. This is not surprising, since there is a zone with very low conductivity with a length of 12.7 cm present that acts as insulator. To improve extraction, the conductivity of the EtOAc zone was increased. The addition of a small amount of pure FA (p*Ka* 3.77), acetic acid (p*Ka* 4.76) or TFA (p*Ka* 0.23) was investigated. First, a series of experiments (n=3, EE time 4 min, EE voltage gradient 1 to 15 kV) was carried out with 0.1% or 1% of one of the three acids present in the organic phase. From these experiments it became clear that, while all three acids caused an increased, comparable current, TFA enhanced the peptide extraction by far the best (Fig. 4.3A). Moreover, 1% acid had a greater effect than 0.1% in the case of all three studied acids. TFA is the strongest acid of the three acids studied and an acidic environment is obviously favourable for peptides to be present in the cationic form. However, more research will be required to assign the effects of adding acids.

Next, the TFA concentration was optimised. A series of experiments (n = 3, equal EE conditions) was carried out in which the amount of TFA was varied (0.2% to 1.8% in steps of 0.2%). The results of these experiments indicated that the TFA concentration was not of great influence on the peak area (data not shown). At the highest TFA concentration the peptide peak areas were slightly larger, while the overall relative standard deviation improved also slightly. When TFA concentrations above 1.8% were used, the current became so high that too much Joule heating took place leading to bubble formation and consequently decreased cEE performance or even failure of experiments. In the rest of the experiments, 1.8% TFA was present in the organic phase.

3.2.3 Aqueous phase optimisation

The aqueous phase into which the peptides were extracted had to be injected into the LC. To avoid sample solvent related problems during LC separation, this aqueous phase was chosen to be mobile phase A, i.e. 0.1% FA, and not further optimised.

The composition of the aqueous phase at the other side of the organic phase, however, was optimised. In cEE experiments with crystal violet, it was noticed that when a higher FA concentration was used, cEE proceeded faster (see section 3.1). Since the conductivity of the aqueous phase increases at increased FA percentage, the electric field strength decreases. This causes a higher electric field strength in the organic phase and therefore faster ion migration in the organic phase. In a series of experiments (n = 3, EE time 4 min, EE voltage 15 kV, 1.8% TFA in organic phase), the FA concentration was varied and the relative peak area increased from 0.1% to 2% FA after which it remained rather constant with a slight optimum at 5% FA (Fig. 4.3B).

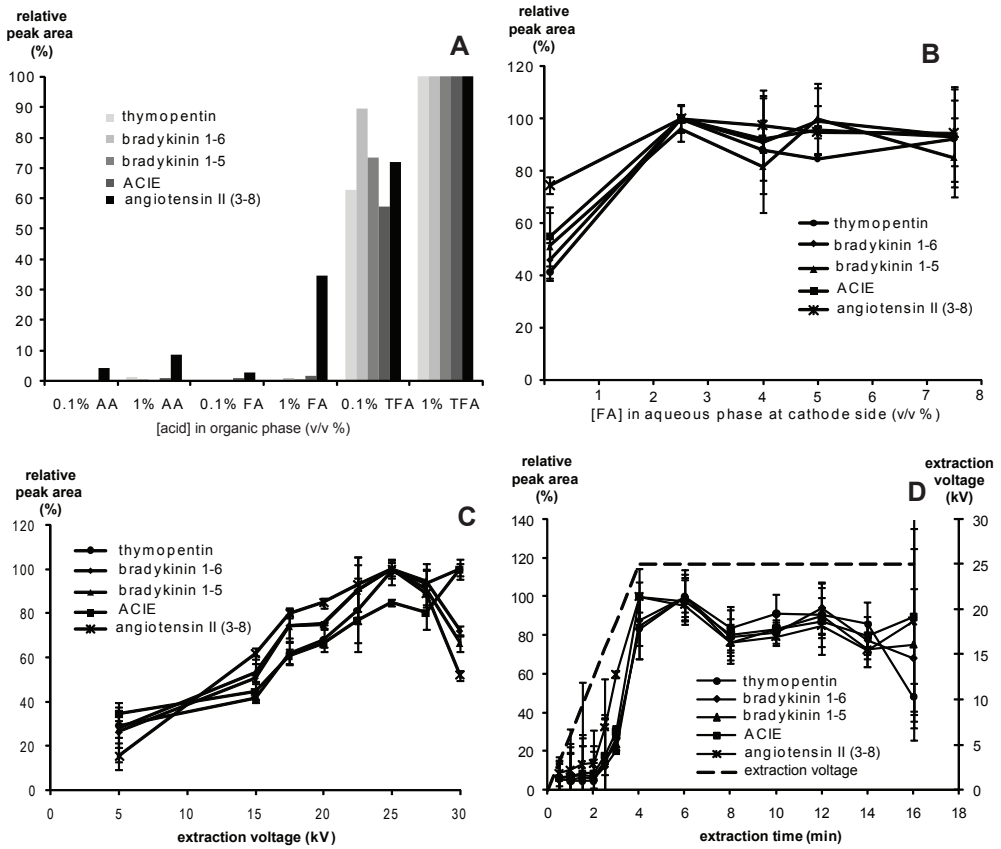


Figure 4.3 A) influence of presence of two concentration levels of acetic acid (AA), FA (FA) and TFA (TFA) on the extraction of 5 test peptides from EtOAc, B) optimisation of FA in aqueous phase C) optimisation of extraction voltage D) optimisation of extraction time. Relative peak area is calculated by setting the highest obtained value at 100%.

3.2.4 Extraction voltage and time optimisation

During several series of experiments, all carried out in triplicate, the optimal HV and extraction time were determined (other conditions: 1.8% TFA in organic phase, 5% FA the aqueous phase at the inlet side, 0.1 % FA at the outlet side). It was studied how fast the HV could be increased and how long it should be applied after it reached its maximum. As can be seen in Fig. 4.3C, the optimal HV appeared to be 25 kV. The optimal time to increase the HV from 0 to 25 kV was 4 min, i.e. 6.25 kV/min. When faster HV gradients were applied, current profiles became instable and lower peaks were obtained (data not shown). When the

maximal HV of 25 kV was reached, prolonging the extraction time did not increase peptide extraction (Fig. 4.3D). All peptides showed the same pattern in these voltage and time optimisation series of experiments, indicating that differences in electrophoretic mobility have no influence on the extraction success at this timescale.

In summary, the optimal cEE voltage procedure was to start at 0 kV and increase with 6.25 kV/min to the maximum voltage, being 25 kV.

3.2.5 Enrichment effect

To determine the enrichment factor that the optimised cEE procedure can achieve, chromatographic peak areas resulting from large volume cEE-LC-MS were compared with conventional 0.1 µL LC-MS injections (the maximal injection volume advised by the column manufacturer when the sample is not in water) of the sample. The starting concentration of the peptides was equal in cEE-LC-MS and LC-MS experiments. In Fig. 4.4, a comparison between a conventional LC-MS injection of 0.1 µL EtOAc and a cEE-LC-MS injection of 100 µL EtOAc containing peptides is shown. Based on this comparison, enrichment factors could be determined to be 570 times for thymopentin, 840 times for bradykinin (1-6), 990 times for bradykinin (1-5), 830 times for ACIE and 590 times for angiotensin 2 (3-8).

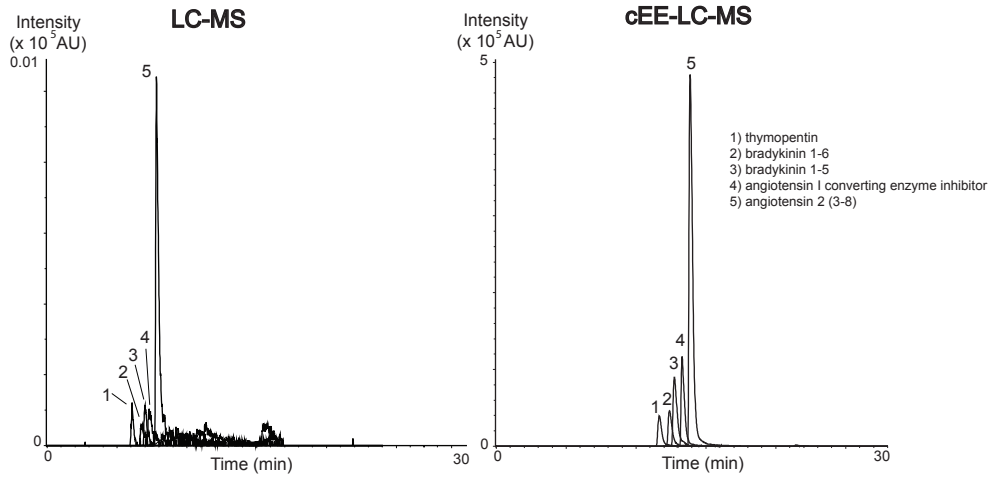


Figure 4.4 Comparison of chromatograms obtained from LC and large volume cEE-LC-MS analysis. After an EE step of only 4 min, peaks are considerably higher. Injection volume in LC was 0.1 µL EtOAc; extracted organic phase in EE was 100 µL. The initial peptide concentration was equal.

This is a significant improvement with respect to the previously described cEE method, where enrichment factors of ~100 were obtained [14]. Whereas the maximum possible enrichment factor is 1000 times, the obtained enrichment factors are lower. During the

optimisation, it was shown that prolonging the extraction time does not result in increased peptide peak areas (Fig. 4.3D). A possible explanation for the sub-maximal enrichment factors is peptide loss due to wall adsorption, a phenomenon that has been reported previously in literature [18, 19].

3.2.6 Linearity and repeatability

When constructing calibration curves, it was noticed that at low analyte concentrations, the peak area of the internal standard (bradykinin) decreased, even though the concentration of it was equal in all calibration samples. This affected the linearity of the calibration curves (data not shown). As mentioned in section 3.3.2.4, a probable reason is adsorption of the peptides to the wall. As a remedy, 2 μM substance P was added to all samples to suppress analyte and internal standard adsorption by competition. This resulted in equal internal standard peak areas in all calibration measurements. Satisfactory calibration curves (range 0.2 - 1000 nM in water) of the 5 test peptides could be constructed (n=3), of which the regression data is shown in Table 4.1. Regression analysis showed that the curves had no significant intercept with the y-axis ($P < 0.05$ at a confidence level of 95%), which indicated that the lines could be drawn through origin. The repeatability and linearity were good and LOD values were between 0.5 and 10 nM

Table 4.1 Analytical performance characteristics of cEE-LC-MS analysis of test solutions of model peptides

peptide	slope ± stdev	standard error	# data points	correlation coefficent	range (uM)	LOD (nM)
thymopentin	0.56 ± 0.072	0.034	18	0.985	0.01-1	10
bradykinin 1-6	0.43 ± 0.022	0.0094	21	0.998	0.005-1	5
bradykinin 1-5	0.77 ± 0.041	0.017	21	0.997	0.005-1	5
ACIE	1.22 ± 0.067	0.025	30	0.997	0.0005-1	0.5
angiotension 2 (3-8)	4.39 ± 0.28	0.1	30	0.996	0.0005-1	0.5

3.3 Application to plasma analysis

3.3.1 Sample pretreatment prior to plasma analysis

When untreated, i.e. when no SPE or ultrafiltration was applied and plasma was directly mixed with EtOAc prior to cEE, low peptide recovery was obtained and the EE process was unreliable. Therefore, plasma samples were deproteinated with ultrafiltration and desalted with RP-SPE. The trapped peptides were eluted from the cartridge with ACN. Instead of evaporating the SPE eluent and reconstitution in water before injection into the RP-

LC system, which is common practice in peptide analysis [20, 21], the eluent was mixed with EtOAc by pipetting up and down and cEE was performed. This required less sample handling; therefore it is less labour-intensive, less susceptible to experimental errors and easier to automate. Test runs with EDTA, heparinated and citrated plasma showed similar results and EDTA plasma was chosen for all further experiments.

3.3.2 Quantitative aspects

Contrary to test mixtures, a multitude of charged compounds can be expected to be present in plasma analysis, even after desalting. This will likely influence the EE process and therefore, cEE voltage and time were re-optimised. It was found that above 15 kV, the liquid-liquid interface became instable and that when 15 kV was used as maximal voltage, the extraction time had to be prolonged with 2 min (data not shown). In summary, the voltage was increased in 4 min from 0 to 15 kV (3.75 kV/min) and then 15 kV was maintained for 2 more min. Under these circumstances, similar enrichment factors (500-1000 times) as with the academical mixtures were achieved (data not shown).

The recovery of the ultrafiltration and SPE procedure was studied. Large volume cEE-LC-MS peak areas resulting from samples spiked prior to and after ultrafiltration and SPE were compared. The recoveries were determined to be 87 ± 2% for angiotensin 2 (3-8), 82 ± 17% for angiotensin 1, 70 ± 10% for bradykinin, 62 ± 11% for angiotensin 2, and lower than 5% for thymopentin, bradykinin (1-5) and bradykinin (1-6) (n=3). The low recoveries obtained for both thymopentin, bradykinin (1-5) and bradykinin (1-6) fragments are not caused by cEE (as shown in section 3.3.2.4, large volume cEE of these peptides is highly feasible) but can be explained by the fact that the SPE conditions were not optimal for these rather hydrophilic peptides.

Then, calibration curves of the relevant plasma peptides angiotensin 1 and 2 as well as angiotensin 2 (3-8) spiked to plasma were constructed, using bradykinin as internal standard, and measured in triplicate (Fig. 4.5). Regression analysis showed that the curves had no significant intercept with the y-axis ($P < 0.05$ at a confidence level of 95%), which indicated that the lines could be drawn through origin. Linearity was good to excellent, R^2 0.990 - 0.998, and mean relative standard deviation of the slope satisfactory between 1 % and 18%. LOD values were 10-50 nM (Table 4.2).

Large volume cEE-LC-MS experiments performed on unspiked plasma resulted in around 60 putative endogenous peptides (Fig. 4.6).

Table 4.2 Analytical performance characteristics of large volume cEE-LC-MS of three peptides spiked to plasma

peptide	slope ± stdev	standard error	# data points	correlation efficient	range (uM)	LOD (nM)
angiotensin 2	6.36 ± 0.086	0.31	18	0.991	0.005-1	10
angiotension 2 (3-8)	0.18 ± 0.00054	0.03	18	0.994	0.05-5	50
angiotensin 1	0.76 ± 0.10	0.23	18	0.984	0.05-5	50

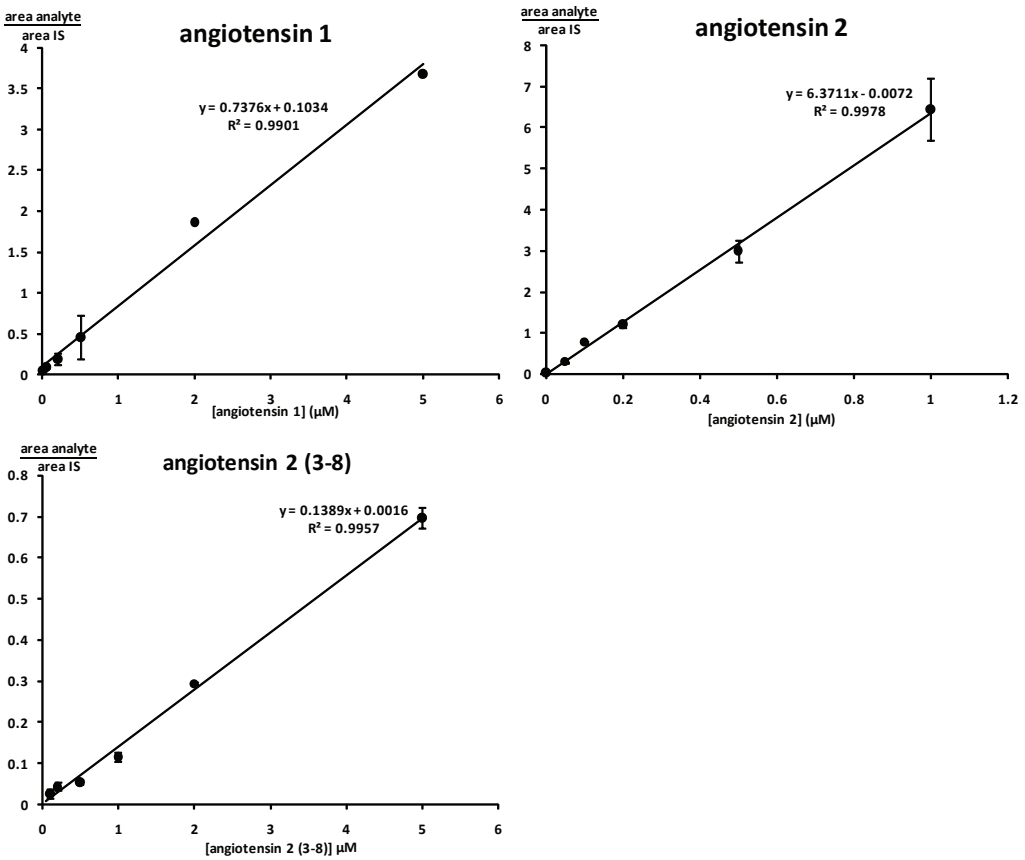


Figure 4.5 Calibration curves and data of angiotensin 1, angiotensin 2 and angiotensin 2 (3-8) in plasma (n = 3).

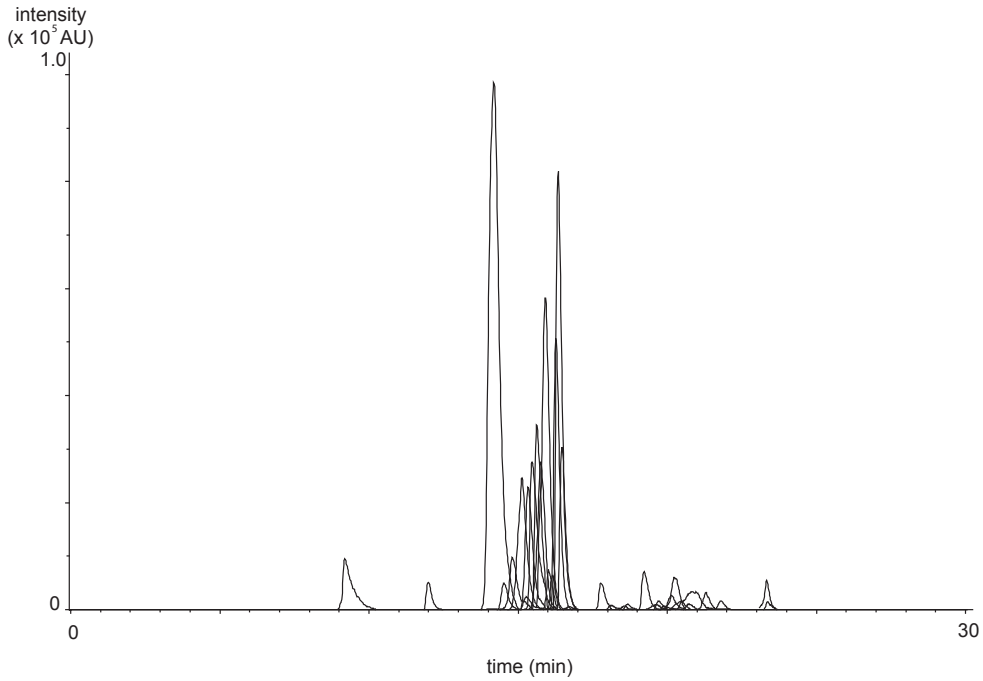


Figure 4.6 Large volume cEE-LC-MS chromatogram of unspiked EDTA plasma. The injected plasma aliquot was 67 μL.

4. Conclusions

We described the set up, optimisation and biological application of large volume cEE-LC-MS of peptides. The method presented in this chapter is capable of on-line enrichment of peptides within 4 min, resulting in significantly increased peak heights compared to conventional LC injection. Enrichment factors were between 570 and 990 times when compared with a conventional LC injection. In comparison with the previously described cEE set-up [14], the method is capable of extracting larger volumes, resulting in significantly higher enrichment factors, and it is faster. The addition of TFA improved EE dramatically. The whole procedure has been automated and could be used routinely for plasma. Linearity and repeatability were excellent between 0.2 nM and 1000 nM. The described method is fully automated and minimises sample handling after SPE (i.e. evaporation and reconstitution steps). Therefore, it can be an important step in full automation of analytical procedures. Future research will be devoted to coupling of SPE directly to large volume cEE, to coupling of large volume cEE to other separation methods than RP-HPLC and to analysis of other compounds than peptides.

5. References

1 Van der Greef, J., Stroobant, P., van der Heijden, R., *Curr Opin Chem Biol,* **2004**, *8,* 559-565

2 Tammen, H., Peck, A., Budde, P., Zucht, H-D., *Expert Rev Mol Diagn*, **2007**, *7(5)*, 605-613

3 Stichlmair, J., Schmidt, J., Proplesch, R., *Chem Eng J,* **1992**, *47*, 3015-3022

4 Marando, M. A., Clark, W. M., *Sep Sci Technol,* **1993**, *28*, 1561-1577

5 Zhai, S. L., Luo, G. S., Liu, J. G., *Sep Purif Technol,* **2001**, *21*, 197-203

6 Zhai, S. L., Luo, G. S., Liu, J. G., *Chem Eng J,* **2001**, *83*, 55-59

7 Theos, C. W., Clark, W. M., *Appl Biochem and Biotechnol,* **1995**, *54*, 143-157

8 Luo, G. S., Shan, X. Y., Qi, X., Lu, Y. C., *Sep Purif Technol,* **2004**, *38*, 265-271

9 Bailes, P. J., *Ind Eng Chem Process Des Dev,* **1981**, *20*, 564-570

10 Scott, T. C., *Sep Purif Methods,* **1989**, *18*, 65-109

11 Van der Vlis, E., Mazereeuw, M., Tjaden, U. R., Irth, H., Van der Greef, J., *J Chromatogr A,* **1994**, *687*, 333-341

12 Van der Vlis, E., Mazereeuw, M., Tjaden, U. R., Irth, H., Van der Greef, J., *J Chromatogr A,* **1995**, *712*, 227-234

13 Van der Vlis, E., Mazereeuw, M., Tjaden, U. R., Irth, H., Van der Greef, J., *J Chromatogr A,* **1996**, *741*, 13-21

14 Lindenburg P.W., Seitzinger R., Tempels F.W.A., Tjaden U.R., Van der Greef, J., Hankemeier, T., *Electrophoresis*, **2010**, *31,* 3903-3912

15 Hirokawa, T., Koshimidzu, E., Xu, Z., Electrophoresis, **2008**, *29,* 3786-3793

16 Xu, Z., Koshimidzu, E., Hirokawa, T., Electrophoresis, **2009**, *30,* 3534-3539

17 Van Midwoud, P. M., Rieux, L., Bischoff, R., Verpoorte, E., Niederländer, H. A. G., *J Proteome Res*, **2007**, *6*, 781-791

18 Kašička, V., *Electrophoresis*, **2008**, *29,* 179-206

19 **Rodriguez, I.,Li, S. F. Y.,** *Anal Chim Acta,* **1999**, *383 (1-2)* ,1-26

20 Lövgren, U., Johansson, S., Jensen, L. S., Ekström C., Carlshaf, A., *J Pharm Biomed Anal,* **2010***, 53(3),* 537-545

21 Tian, Y., Zhou, Y., Elliott, S., Aebersold, R., Zhang, H., *Nat Protoc,* **2006**, *2,* 334-339

Chapter 5

Feasibility of electroextraction as versatile sample concentrating pretreatment for fast analysis of low abundant urine metabolites and its application to acylcarnitines

Based on
Lindenburg P.W., Tjaden U.R., Van der Greef, J., Hankemeier, T.
Feasibilityof electroextraction as versatile sample concentrating pretreatment forfast analysis of low abundant urine metabolites and its application to acylcarnitines.
Submitted to *Electrophoresis.*

Abstract

In this work we demonstrate the applicability of electroextraction (EE) to urine metabolites. To investigate which urine metabolite classes are susceptible to EE, off-line EE experiments were carried out with a prototype device, in which urine metabolites were electroextracted from ethyl acetate (EtOAc) into water. The obtained extracts were examined with direct infusion MS and the results demonstrated that several compound classes could be extracted, amongst which amino acids and acylcarnitines. Acylcarnitines were selected for evaluation of the performance of EE. For this, the EE set-up was adapted to capillary EE (cEE) to be able to analyze large urine sample series, and it was coupled on-line to LC-MS. cEE-LC-MS of acylcarnitines was optimised and characterised. The recovery, linearity, repeatability and limit of detection of the cEE-LC-MS method was good to excellent. To demonstrate the versatility of EE for sample preparation in analytical procedures, extracts were injected into a CZE-MS system, resulting in detection of the acylcarnitines along with more than 100 presumed metabolite peaks. The results presented here indicate that EE can be used as a fast sample preconcentration technique of low abundant urine metabolites, in combination with both LC and CZE.

1. Introduction

Metabolomics studies typically involve the measurement of large sample sets. To be able to gain more insight into important metabolic processes, as many metabolites as possible should be measured, including the low-abundant ones that often are of high biological relevance. Therefore, fast analytical techniques are required that are capable of measuring many low-abundant compounds.

Sample pretreatment procedures that are based on electromigration are very promising, because they meet the requirements mentioned above in several ways. First, a considerable part of the metabolome consists of compounds that are or can be charged and so are suitable for an electrophoretic approach, such as fatty acids, carbohydrates, nucleotides, amino acids and acylcarnitines [1]. Furthermore, electromigration can be used to concentrate analytes. Despite the fact that this approach is well-known in CE-based techniques [2-4], relatively few examples of the application of electromigration-based sample pretreatment to large sample volumes can be found in literature. One example is electrodialysis, which has been applied as a fast and selective sample pretreatment method to LC-MS analysis of low-abundant peptides in synovial fluid [5]. Other examples of electromigration-based sample pretreatment techniques that have been applied to complex samples are preparative isotachophoresis [6-8] and isoelectric focusing [8].

Another electromigration-based analyte preconcentration technique is electroextraction (EE), which is capable of concentrating analytes two-three orders of magnitude within several minutes, resulting in improved LOD [9, 10]. EE takes place when an electric field is applied to a two-phase liquid-liquid system consisting of an aqueous and an organic phase. Ions in the organic phase are subjected to a very high electric field strength, due to the very low conductivity. As a consequence, they migrate at full speed towards the liquid-liquid interface, to be concentrated in the aqueous phase directly after they pass the interface, because the electric field strength is so much lower there [9, 10]. Initially, EE has been developed as a purification technique in the field of chemical engineering, to enhance product yields resulting from solvent extraction [11-16]. However, EE can also be used to enhance the sample aliquot that is injected into analytical methods. In the 1990s Van der Vlis *et al.* adapted EE for analytical purposes, using EtOAc as organic phase and performing EE of test solutions in capillaries (cEE) [17-19]. Recently, we showed the successful application of cEE coupled to LC-MS to the analysis of peptides in urine [9]. Then, we further improved cEE to large volume cEE and showed extraction of plasma peptides from large sample volumes

(up to 100 µL) coupled to LC-MS [10].

So far, cEE has been reported for some β-agonists, leukotrienes and catecholamines from test mixtures [17-19] as well as cEE of peptides in urine and plasma [9, 10]. The goal of this research paper is to demonstrate that cEE is applicable to a wider range of compound classes in complex samples than has been demonstrated so far. Therefore, the performance of EE as sample pretreatment of human urine samples was investigated. In a quantitative screening experiment using a prototype EE device (Fig. 5.1), it was investigated which metabolite classes are susceptible to EE. Due to its biological relevance [20], in further experiments the focus was put on analysis of acylcarnitines. The performance of EE of acylcarnitines in urine was evaluated with consecutive on-line LC-MS analysis. On-line large volume cEE as described in [10] was optimised, then calibration curves of 4 acylcarnitines spiked to urine were generated and endogenous levels were determined by the standard addition method.

Finally, to assess how versatile EE is as a generic sample pretreatment technique, EE was coupled off-line to CZE-MS instead of LC-MS.

2. Experimental

2.1 Chemicals

All reagents were of analytical grade or higher. A Millipore Q-guard water purifying system (Billerica, MA, USA) was used to obtain pure water. Acetonitrile (ACN), EtOAc, formic acid (FA), ammonium hydroxide, sodium hydroxide and TFA were obtained from Biosolve (Valkenswaard, The Netherlands). The acyl-L-carnitine standards were from Sigma (St. Louis, MO, USA).

2.2 Sample pretreatment

L-acylcarnitine stock solutions of 5 mM were prepared in 1:1 water/methanol (v/v), and were stored at -20° C. Stock solutions of 0.5 mM and 0.05 mM were made by dilution with 1:1 water/methanol (v/v). Calibration solutions were made by adding various amounts of the stock solutions to water or urine. Urine was collected from 3 healthy volunteers, pooled, centrifuged and stored at -80° C in aliquots of 1 mL. This urine was used in all experiments.

2.3 Equipment and techniques

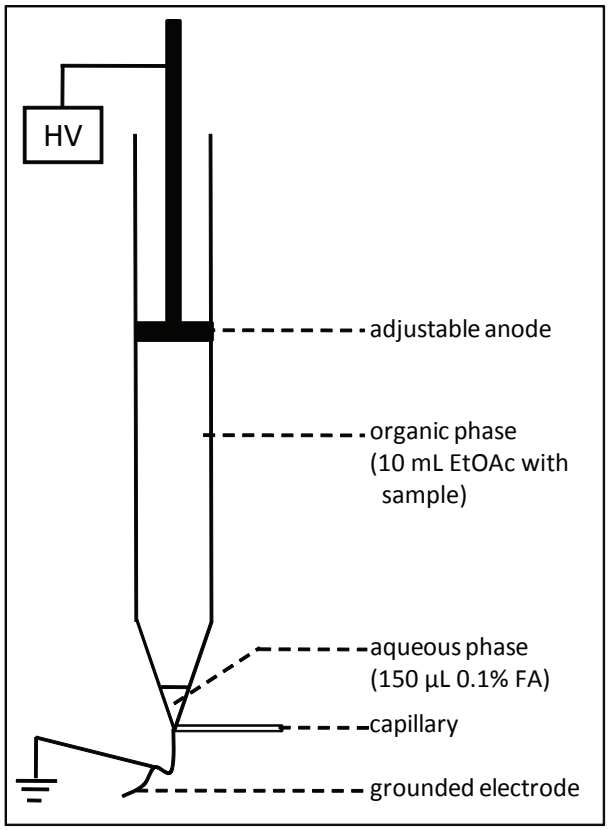


Figure 5.1 Schematic drawing of the off-line EE prototype device

2.3.1 Screening experiment with off-line EE set-up

The prototype EE device (Fig. 5.1) has been described in detail in [9]. In short, the device consisted of a polychlorotrifluorethylene (KelF) vial with a pointed bottom, where a capillary and a platinum grounding electrode were located. The capillary served to inject and remove aqueous phase underneath the organic phase, in the pointed bottom of the device. The electric circuit was closed by using a piston-like gold coated electrode as anode fitting exactly in the vial, thus contacting exactly the whole surface of the organic phase to ensure that all charged molecules in the organic phase were in the electric field. High voltage (HV) was applied with a Spellman HV Power Supply (Spellman, Hauppauge, New York, USA). Urine was brought to 2% FA and 20 µL was added to 10 mL EtOAc. The sample was centrifuged 5 min at 15000 g to remove insoluble particles, after which EE was performed on the supernatant at 2.5 kV for 10 min, with 150 µL 0.1% FA as aqueous acceptor phase.

The extracts were infused into a linear triple quad - ion cyclotron resonance - Fourier transform - mass spectrometer (LTQ-FTICR-MS) (Thermo Fisher Scientific, San Jose, CA, USA) to obtain accurate mass values of the extracted compounds. Direct infusion mass spectra were measured, at a resolution of 100000. To get an impression of which metabolite classes were extracted, the most intense recorded masses (mass range 50-300 m/z) were matched with a database (www.hmdb.ca, [1]), taking 0.06 Dalton as maximal mass difference for identification.

2.3.2 On-line large volume cEE-LC-MS set-up

The on-line large volume cEE-LC-MS set-up (Fig. 5.2) has been described in detail recently [10]. In short, aqueous and organic phases were injected into a capillary (part B in Fig. 5.2) and the location of the interface was chosen to be just in front of the valve (step 1). Then, the valve was switched and the extraction voltage was applied. When cEE is finished (step 2), the concentrated sample plug was transferred into the sample loop (step 3) by applying pressure, after which the valve was switched back to inject the sample into the LC-MS system (step 4). Voltage and pressure application during cEE experiments were done with an Agilent Technologies (Santa Clara, CA, USA) 1600 series CE apparatus. The start signal of the CE apparatus was used as a trigger pulse for the valve, LC and MS.

LC was carried out with an Agilent Technologies 1200 series μHPLC apparatus, equipped with a ZORBAX SB-C18 capillary HPLC column (5 μm, 150 x 0.5 mm; Agilent Technologies). A 20 μL/min gradient elution was employed with 0.1 % FA in water as solvent A and 0.1% FA in ACN as solvent B and consisted of 5 steps: 1) 1 min 100% A, 2) decrease to 20% in 14 min 3) 6 min at 20% A, 4) 5 min at 0% A for cleaning and 5) 5 min at 100% A for reconditioning. On each measurement day, a blank run was performed first.

Detection was achieved with a Bruker Daltonics MicrOTOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Mass spectra from the time-of-flight MS (TOF-MS) were internally calibrated using a sodium formate adduct plug (10 mM NaOH, 0.1 FA in 1:1 methanol/water) that was infused (using a Pharmacia LKB HPLC Pump 250 (Uppsala, Sweden)) at the end of every chromatogram via the switching valve on the MS.

2.3.3 CZE-MS

Experimental conditions of CZE experiments were based on [21] and were carried out using an Agilent Technologies 1600 series CE apparatus. An untreated fused capillary was used (length 80 cm, ID 75 μm) and hydrodynamic injection was employed (2s, 50 mbar). Separation took place in 0.8% FA as background electrolyte under a separation voltage of 30

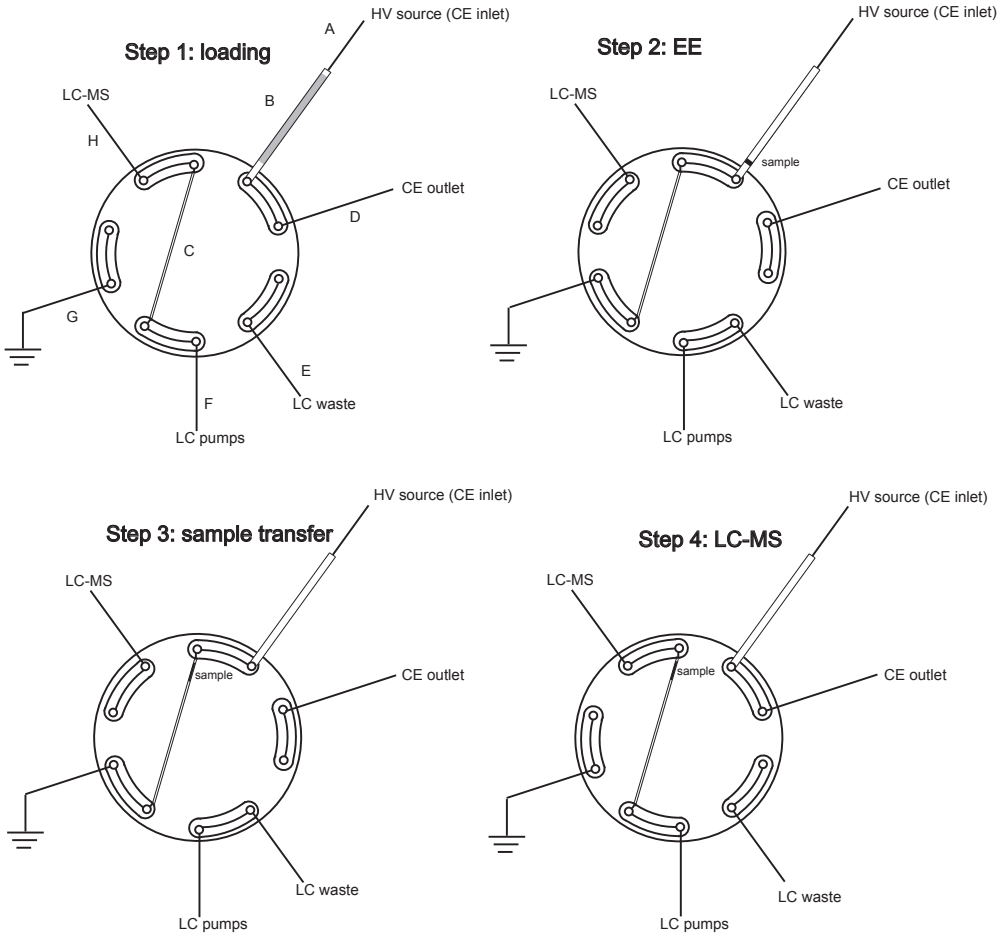


Figure 5.2 Valve set-up for interfacing large volume cEE with LC. A) inlet capillary (fused silica, L = 25 cm, OD = 365 µm, ID = 75 µm), B) EE capillary (PEEK, L = 20 cm, OD = 1.59 mm, ID = 1 mm), C) sample loop (PEEK, L = 20 cm, OD = 1.59 mm, ID = 0.5 mm) D) outlet capillary (fused silica, L = 75 cm, OD = 365 µm, ID = 200 µm) E) LC waste (PEEK, L = 100 cm, OD = 1.59 mm, ID = 0.5 mm), F) LC pump tubing (PEEK, L = 30 cm, OD = 1.59 mm, ID = 50 µm), G) tubing to earth (PEEK, L = 20 cm, OD = 1.59 mm, ID = 0.5 mm). H) tubing to LC-MS (PEEK, L = 20 cm, OD = 1.59 mm, ID = 50 µm) Step 1: situation after all phases have been loaded. The gray zone in the EE-capillary depicts the organic phase with analytes. Before EE starts, the valve is switched. Step 2: situation after EE is finished; the small black zone depicts the concentrated analytes. Step 3: situation after the sample has been transferred into the sample loop by applying pressure. After sample transfer, the valve is switched back. Step 4: situation where the sample zone is being injected into the LC-MS system.

kV. Between measurements, the capillary was reconditioned by flushing 5 min with distilled water, 5 min with 1 M ammonium hydroxide and 5 min with background electrolyte. The CZE system was coupled to an Agilent Technologies 6530 QTOF-MS, using a CE-MS

sheath-liquid assisted sprayer which was also from Agilent Technologies (part number 1607-60001). The sheath liquid consisted of 50/50% methanol/ 0.1% acetic acid in distilled water and was delivered with an Agilent Technologies 1100 series pump at a rate of 10 μL/ min.

3. Results and discussion

3.1 Off-line EE

The purpose of this screening experiment was to quickly get a rough idea which metabolite classes in urine are susceptible to EE. For this, electroextracts from urine were infused into an LTQ-FTICR-MS for accurate mass determination and, using the human metabolome database, 49 masses could be putatively attributed to metabolites that were electroextracted into the aqueous phase. The number of putatively identified metabolites can be expected to be higher, since the mass spectra contained hundreds of peaks. However, the purpose of this experiment was, as mentioned above, to obtain a rough impression of which metabolite classes can be electroextracted from urine. Therefore, no further effort was made to annotate all the mass peaks. Definitive identification of the metabolites requires MS/MS of MS^n analysis of the observed masses, which is beyond the scope of this study.

Many of the supposed identified metabolites were amino acids and acylcarnitines (Table 5.1). To optimise the cEE procedure, to validate with cEE-LC-MS and to demonstrate the potential of the cEE approach, acylcarnitines were chosen as metabolite class of interest. Acylcarnitines (carnitine esters) are conjugates of carnitine and fatty acids that can function as biomarkers for various inborn metabolism errors; many genetic and acquired disorders affect the acylcarnitine levels in biological fluids [20].

The prototype device is useful for a quick experiment, but for the analysis of a large series of samples, the set-up of the method has to be automated and coupled on-line. Therefore, an on-line large volume cEE set-up was optimised for acylcarnitines and coupled to LC-MS.

3.2 On-line large volume cEE of acylcarnitines in urine

The cEE-LC-MS set-up that has proven its potential for peptides [10] was now optimised for acylcarnitines. In a series of experiments, the extraction voltage and TFA concentration (0, 0.1, 0.5, 1, 1.5 and 2%) were optimised using test solutions of EtOAc that was saturated with acylcarnitines in water. The injected volume of organic phase extracted was 100 μL. The extraction procedure was as follows: in 4 min the voltage was gradually increased from

1 kV to the maximum voltage (1, 15 or 30 kV), after which the maximum voltage was maintained for 2 additional min. As can be seen in Fig. 5.3A, 15 kV was sufficient and it was therefore selected for subsequent experiments.

Table 5.1 List of classes of putatively assigned compounds detected in urine by off-line EE/DI-LTQ-FT-MS compounds classes and detected number of compounds per class

compound class	# detected analytes
Amino acids	19
Acylcarnitines	6
Amino ketones	3
Acyl glycines	2
Amino alcohols	2
Aromatic acids	2
Fatty acids	2
Nucleosides	2
Aliphatic amines	1
Catecholamines	1
Cyclic amines	1
Dicarboxylic acids	1
Dipeptides	1
Hydroxy acids	1
Indoles	1
Keto acids	1
Peptides	1
Polyphenols	1
Purines	1
Total	49

Apart from a blank, 5 different TFA concentrations were studied (Fig. 5.3B). In [10], it was found that the addition of TFA to the organic phase improved cEE of peptides dramatically. Between 0.1% and 2% TFA, the results were satisfactory for all acylcarnitines, except for stearoylcarnitine. The unpredictable results of stearoylcarnitine are possibly due to its low solubility in water. However, when no TFA was added, peak areas were significantly lower and not reproducible. There were no marked differences between 0.5%, 1% and 1.5% TFA. The lowest suitable concentration, 0.5% TFA, was chosen for the remainder of the experiments to avoid risk of excessive Joule heating caused by the increased conductivity. As the results obtained for HV and TFA optimisation were very similar to the results obtained in peptide analysis [10], the same extraction time and aqueous phase composition were selected.

Summarising, the final cEE procedure was a HV gradient of 4 min from 1 to 15 kV,

followed by 2 more min of 15 kV. The TFA concentration was 0.5%, the aqueous phase at the cathode side 5% FA and at the anodic side 0.1% FA (i.e. the LC mobile phase).

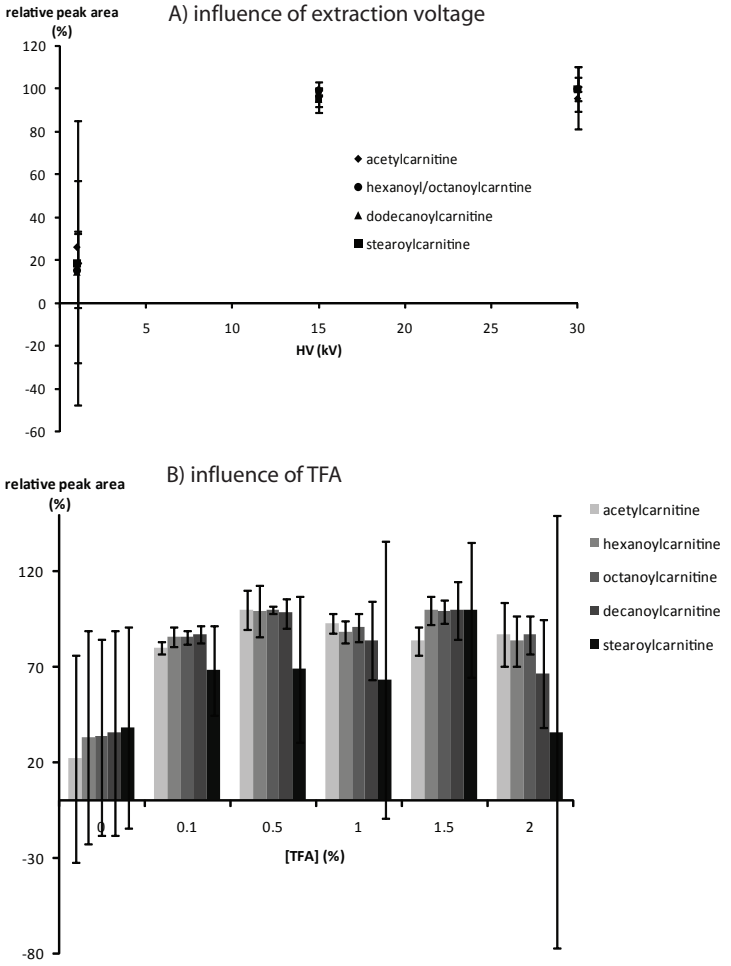


Figure 5.3 Optimisation of A) extraction voltage and B) TFA concentration. Relative peak area was calculated per acylcarnitine, by setting the highest obtained peak area per acylcarnitine to 100%

Peak areas of chromatograms resulting from on-line large volume cEE-LC-MS (100 µL) and LC-MS (0.1 µL) of EtOAc saturated with acidified urine were compared (Fig. 5.4) and it was found that the peak area of hexanoylcarnitine was increased 970 times, decanoylcarnitine 920 times, octanoylcarnitine 790 times, stearoylcarnitine 640 times and acetylcarnitine 175 times. Based on the corresponding volume ratios, the enhancement can

amount to maximally 1000 times, which demonstrates that the cEE of hexanoylcarnitine and decanoylcarnitine is virtually complete, while some loss of octanoylcarnitine and stearoylcarnitine occurs. The low recovery of acetylcarnitine might be explained by the fact that it elutes from LC with the void volume in 100 % solvent A (0.1% FA), which makes it difficult to compare the conventional LC injection with the cEE-LC injection, due to possible ion suppression by matrix compounds.

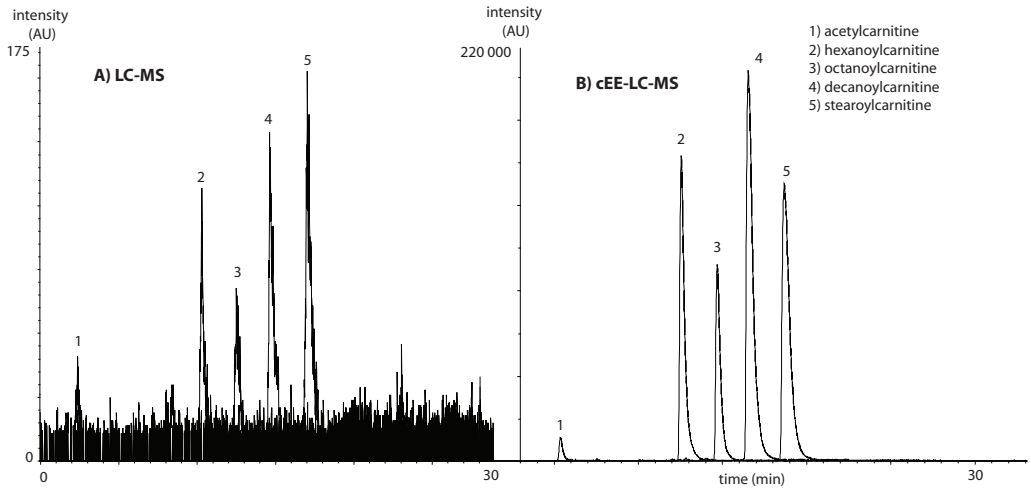


Figure 5.4 Comparison of chromatograms obtained from LC and cEE-LC-MS analysis of acylcarnitines spiked to urine. After a cEE step of only 6 min, peaks are considerably higher. Injection volume in LC was 0.1 µL; extracted organic phase in cEE was 100 µL. The initial acylcarnitine concentration in urine was equal (octanoylcarnitine 20 µM, other acylcarnitines 50 µM).

After optimisation experiments with acylcarnitine test solutions, acylcarnitines spiked to urine were analyzed. In one single run, an aliquot of 2 µL urine was analyzed, i.e. 100 µL EtOAc saturated with urine was electroextracted. Sample pretreatment of the urine only required acidification with TFA, mixing with EtOAc and centrifugation prior to transfer into an autosampler vial for subsequent cEE-LC-MS analysis. In Fig. 5.5, the calibration curves obtained for acetylcarnitine, hexanoylcarnitine, decanoylcarnitine and stearoylcarnitine spiked to urine are shown. For the calibration curve of acetylcarnitine, a separate series of measurements was carried out, because its high endogenous concentration required a different calibration range and internal standard concentration. Octanoylcarnitine was used as internal standard (20 µM in the case of acetylcarnitine, 2 µM in the remainder). Concentrations for construction of the calibration curves were 0, 1, 2, 5, 10, 20, and 50

μM in the case of acetylcarnitine and 0, 0.05, 0.1, 0.2, 0.5, 1, 2, and 5 μM in the other acylcarnitines.

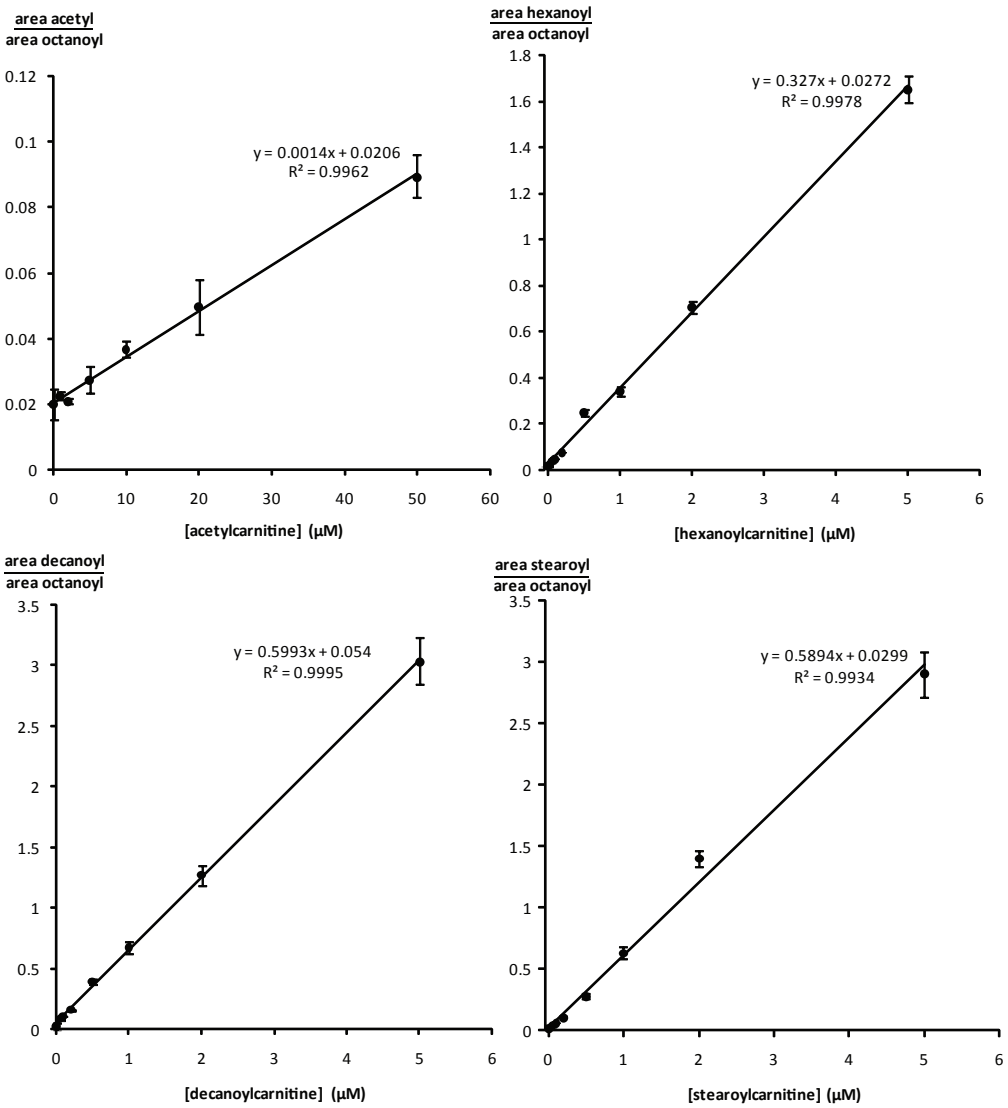


Figure 5.5 cEE-LC-MS calibration curves of acetylcarnitine, hexanoylcarnitine, decanoylcarnitine and stearoylcarnitine in urine

Repeatability (characterised by the analysis of three calibration sample sets) and linearity are excellent in the whole range (n = 3). In the blank urine samples, acetylcarnitine, hexanoylcarnitine and stearoylcarnitine peaks were detected (Fig. 5.6). The signal-to-noise

ratio of the endogenous peaks was 25-112, which was sufficient for quantification, and the endogenous concentrations that were found are given in Table 5.2. Based on the signal-to noise ratios of the endogenous peaks, the LOD could be estimated to be 0.18 μM for acetylcarnitine, 0.008 μM for hexanoylcarnitine and 0.007 μM for stearoylcarnitine, corresponding to on-column injected amounts of 1 around pmol, 50 amol and 20 amol (based on the injection volume of 2 μL). In the case of hexanoylcarnitine and stearoylcarnitine, these values are roughly one order of magnitude more favourable than methods described in the literature [21, 22], describing sample pretreatment involving multiple steps. In [23], acylcarnitines were also observed in urine with LC-MS. When taking into account the different column dimensions (2 mm in [23], 0.5 mm here), the injected aliquot of urine is increased 16 times. An advantage of on-line large volume cEE is the minimal sample handling; after having mixed the urine with EtOAc, centrifugation and transferred it to an autosampler, the whole analysis takes place without further human interference. In consequence, the method is not labour-intensive and no errors and sample losses inevitably associated with each extra sample handling, such as evaporation and reconstitution [21, 22], are introduced. For example, in [22], urine was first diluted 5 times with water, then acidified with HCl (1 M) and finally subjected to strong cation exchange SPE. The eluent was evaporated to dryness, reconstituted in 1 mL methanol, re-evaporated and then reconstituted in methanol-water, prior to injection into the LC-MS system. In [21], a study was made of SPE as well as LLE as sample pretreatment prior to CZE-MS. In SPE, urine was evaporated to dryness, reconstituted in methanol, centrifuged and applied to a silica gel column. The eluent was again evaporated to dryness and reconstituted prior to injection. In LLE, 500 μL EtOAc/ACN (9:1) was used to extract acylcarnitines from 500 μL urine. The organic layer was evaporated and the residue was reconstituted in 200 μL 1.5% FA in methanol, which was used for injection.

Table 5.2 validation results for cEE-LC-MS of several acylcarnitines in urine

Analyte	R^2 value	Mean relative standard deviation (%)	Between day mean relative standard deviation (%)	Endogenous concentration (μM)	S/N ratio endogenous peak	Recovery (%)
acetyl	0.9962	11.3	11.8	14.7	112	52
hexanoyl	0.9978	5.6	7.3	0.083	23	84
decanoyl	0.9995	4.7	7.2	-	-	96
stearoyl	0.9934	7.4	10.8	0.051	23	90

(*: concentrations obtained from standard addition calibration curves of Fig. 5.5)

The recovery of cEE-LC-MS was calculated by determining the ratio of the slopes of the calibration curves from urine and water and expressed as percentages. The results (Table 5.2) indicate that cEE of hexanoylcarnitine, decanoylcarnitine and stearoylcarnitine are affected only to a limited extent by the urine matrix, or hardly at all (decanoylcarnitine). The recovery of acetylcarnitine is rather low; this may be explained, as mentioned above, by the fact that it co-elutes in the void volume with various other compounds in LC, leading to less favourable electrospray conditions (ion suppression).

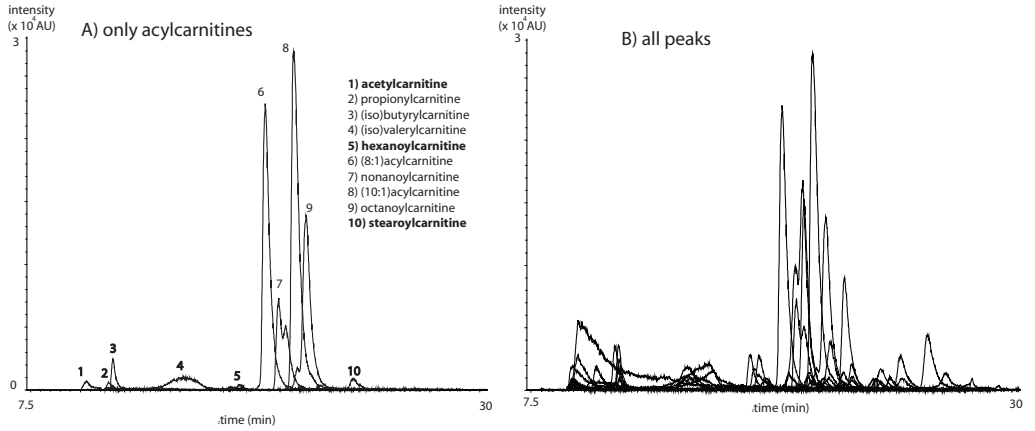


Figure 5.6 cEE-LC-MS chromatogram of A) reconstructed ion chromatograms of endogenous acylcarnitines, except for the added spike octanoyl carnitine, in urine; acylcarnitines mentioned in bold are identified by adding standards, other acetyl carnitines are putative; (8:1) means the acyl group contains 8 C atoms and one double bond, 10:1 means 10 C atoms and one double bond; and B) reconstructed ion chromatograms of all detected metabolite peaks

In total, more than 60 peaks have been detected in cEE-LC-MS of urine with the internal standard octanoylcarnitine as the only spike. Of these, 17 masses could be putatively annotated using the accurate mass and elemental composition (determined with the SmartFormula function of the Bruker Daltonics DataAnalysis software) in combination with the Human Metabolome Database (www.hmdb.ca) and a literature search (see references in Table 5.3). Interestingly, apart from the three acylcarnitines already described, strong indications of the presence of five more endogenous acylcarnitines were found. In Fig. 5.6A, a reconstructed cEE-LC-MS chromatogram with only the acylcarnitines peaks is shown and in Fig. 5.6B, all peaks are shown. To be conclusive about the identity of the 5 acylcarnitines not used for validation, standards of these acylcarnitines should be added. The retention

order of these acylcarnitines is supporting the identification; it corresponds well with the expectations based on their polarity and with what has been reported previously [22]. To elucidate the exact structure of (iso)butyrylcarnitine, (iso)valerylcarnitine, octenoylcarnitine (8:1 carnitine) and decenoylcarnitine (10:1), MS/MS work should be done. In the Human Metabolome Database and in [24] only 9-decenoylcarnitine and 2-octenoylcarnitine are mentioned, indicating that these are the found acylcarnitine species.

In [22], it is stated that a typical LC-MS chromatogram (150 x 2.0 mm C_8 column) of healthy urine shows an acetylcarnitine peak, and depending on the urine concentration, also propionylcarnitine and (iso-)valerylcarnitine can be observed. In [21], only acetylcarnitine could be quantified with CZE-MS, the other acylcarnitine being detectable but could not be quantified. With the cEE-LC-MS method developed, combining the electrophoretic with the chromatographic approach, 7 acylcarnitines peaks that were high enough for quantitation were found and of three of them, trace concentrations could actually be determined in only 2 µL urine.

Table 5.3 Putatively identified metabolites in 2 µL urine, analyzed with cEE-LC-MS

Obtained MW (M+H [1+])	Database MW (M+H [1+])	MW difference (Da)	RT (min)	Compound	Reference
155.07	155.07	0	9.3	Hydroxytyrosol	25
114.07	114.07	0	9.8	Creatinine	26
144.1	144.1	0	9.8	Proline betaine	27
204.12	204.12	0	10.3	L-Acetylcarnitine	24
229.16	229.15	0.01	10.6	L-leucyl-L-proline	28
229.16	229.15	0.01	10.6	L-isoleucyl-L-proline	28
165.06	165.05	0.01	11.2	Phenylpyruvic acid	29
165.06	165.05	0.01	11.2	m-Coumaric acid	30
218.13	218.13	0	11.4	Propionylcarnitine	24
232.16	232.15	0.01	11.6	(Iso)butyryl-L-carnitine	24
188.08	188.07	0.01	14.8	Indoleacrylic acid	31
205.1	205.1	0	14.8	L-Tryptophan	28
246.17	246.17	0	15.2	(Iso)valerylcarnitine	24
130.06	130.05	0.01	16	Pyroglutamic acid	32
195.09	195.09	0	16.1	Caffeine	33
260.19	260.19	0	17.6	L-Hexanoylcarnitine	24
286.2	286.2	0	19.2	2-Octenoylcarnitine	24
302.23	302.23	0	20.7	Nonanoylcarnitine	24
314.23	314.23	0	21.3	9-Decenoylcarnitine	24
129.06	129.07	0	22.5	Dihydrothymine	34
344.22	344.28	0.06	23.6	Stearoylcarnitine	24

3.3 EE as sample pretreatment prior to CZE

The combination of EE with LC offers selectivity, since the electrophoretic as well as the chromatographic mechanism is employed during analysis. For example, when EE is

coupled to RP-LC, as is carried out in [10] and in this work, the system is suitable for the analysis of charged, rather apolar compounds, such as peptides, and acylcarnitines. The charge of the analytes makes them susceptible to the electric field strength that is employed during EE, while their apolar character ensures retention on the RP column. For a more comprehensive (i.e. less selective and therefore less discriminative) approach desirable in metabolomics analysis, the combination of EE with CZE is attractive, since both techniques are electromigration techniques and all compounds that are feasible in EE should be feasible in CZE, regardless their polarity.

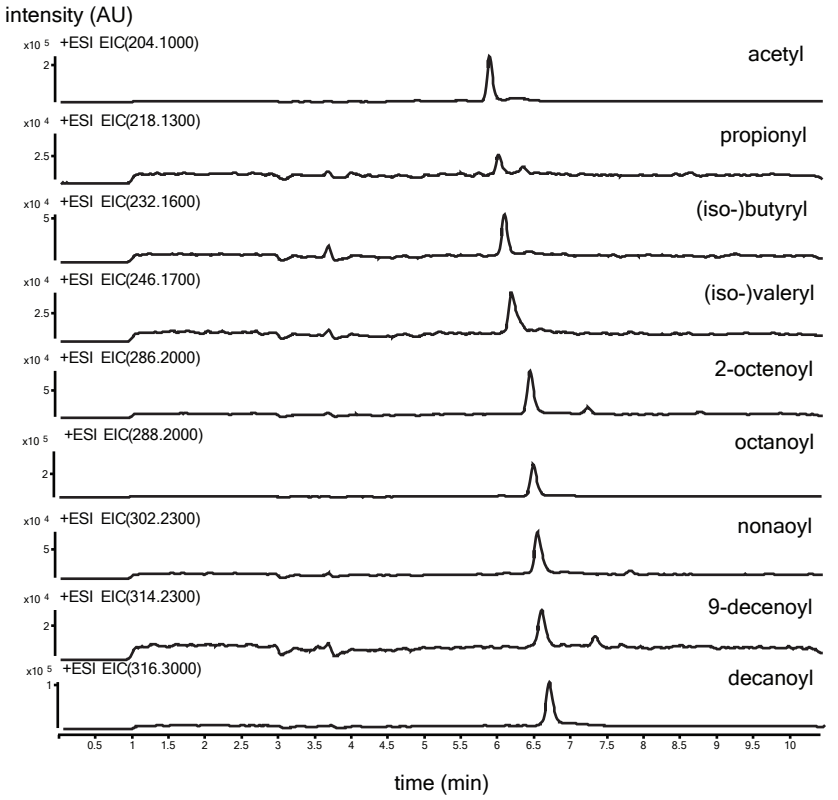


Figure 5.7 Results from off-line EE-CZE-MS. Acetylcarnitine, octanoylcarnitine and decanoylcarnitine were spiked to urine (2 μM each) prior to EE, the other detected acylcarnitines are endogenous.

To explore the applicability of EE prior to CZE, an off-line EE experiment of urine spiked with acetylcarnitine, octanoylcarnitine and decanoylcarnitine (2 μM each) prior to EE, was carried out, after which the extract was injected into a CZE-MS system. The resulting

electropherogram (Fig. 5.7) showed good separation of all endogenous acylcarnitines that were found using on-line cEE-LC-MS, except for hexanoylcarnitine, which was not present. These first results indicate that the high salt concentration in urine does not have a negative effect on the CZE separation, a well-known complication in CZE analysis of biological samples [35]. Possibly, the salt precipitates when the urine is mixed with EtOAc. Future experiments are directed at investigating this process. Moreover, almost twice as many peaks were observed as with cEE-LC-MS, as a matter of fact more than 100. The combination of large volume EE with CZE has great potential and the direct on-line coupling of EE to CZE has our current attention.

4. Conclusions

The intention of this study was to demonstrate the great potential of EE in metabolite analysis. Our results indicate that electromigration-based sample pretreatment techniques for bio-analysis deserve more attention. We successfully demonstrated the applicability of EE as sample pretreatment of low-abundant urine metabolites in urine. Also, we showed that many compounds, belonging to several classes, can be extracted from untreated urine samples with EE. Using acylcarnitines as an example in on-line cEE-LC-MS, it was shown that EE is linear, sensitive and has a high recovery. In aliquots of only 2 μL urine, 10 endogenous acylcarnitines could be found. Three of them were quantified with the standard addition method. In an off-line EE/CZE-MS experiment, the same acylcarnitines were found, along with around 100 other putative metabolites. This demonstrates that EE is a versatile technique and that combining EE with CZE, has great potential. Further work will be directed to the on-line coupling of large volume cEE to CZE-MS and identification of all extracted metabolites.

5. Ackowledgements

Jeroen van Vonderen is acknowledged for his help with the CZE experiments.

6. References

1 Galperin, M.Y., Cochrane G.R., *Nucleic Acids Res*, **2009**, *37*, D1-D4

2 Chien, R-L., Burgi, D.S., *J Chromatogr,* **1991**, *559,* 141-152

3 Britz-Kibbin, P., Terabe, S., *J Chromatogr A*, **2003**, *1000,* 917-934

4 Breadmore, B.C., Thabano, J.R.E., Dawod, M., Kazarian, A.A., Quirino, J.P., Guijt, R.M., *Electrophoresis,* **2009**, *30,* 230-248

5 Kamphorst, J.J., Tjaden, U.R., Van der Heijden, R., De Groot, J., Van der Greef, J., Hankemeier, T., *Electrophoresis*, **2009**, *30*, 2284-2292

6 Hutta, M., Kaniansky, D., Kovalčíková, E., Marák. J., Chalányová, M., Madajová V., Šimuničová, E., *J Chromatogr A*, **1995**, *689*, 123-133

7 Wang, T., Qin, Y., He, H., Lv, J., Fan, Y., *J Chromatogr A*, **2011**, *1218*, 185-189

8 Bier, M., *Electrophoresis*, **1998**, 19, 1057-1063

9 Lindenburg P.W., Seitzinger R., Tempels F.W.A., Tjaden U.R., Van der Greef, J., Hankemeier, T. *Electrophoresis,* **2010**, *31*, 3903-3912

10 Lindenburg, P.W., Tempels, F.W.A., Tjaden, U.R., Van der Greef, J., Hankemeier, T. *On-line large-volume electroextraction coupled to LC-MS to improve determination limits of peptides*. Accepted for publication in J Chromatogr A

11 Zhai, S. L., Luo, G. S., Liu, J. G., *Sep Purif Technol,* **2001**, *21*, 197-203

12 Zhai, S. L., Luo, G. S., Liu, J. G., *Chem Eng J,* **2001**, *83*, 55-59

13 Theos, C. W., Clark, W. M., *Appl Biochem and Biotechnol,* **1995**, *54*, 143-157

14 Luo, G. S., Shan, X. Y., Qi, X., Lu, Y. C., *Sep Purif Technol,* **2004**, *38*, 265-271

15 Bailes, P. J., *Ind Eng Chem Process Des Dev,* **1981**, *20*, 564-570

16 Scott, T. C., *Sep Purif Methods,* **1989**, *18*, 65-109

17 Van der Vlis, E., Mazereeuw, M., Tjaden, U. R., Irth, H., Van der Greef, J., *J Chromatogr A,* **1994**, *687*, 333-341

18 Van der Vlis, E., Mazereeuw, M., Tjaden, U. R., Irth, H., Van der Greef, J., *J Chromatogr A,* **1995**, *712*, 227-234

19 Van der Vlis, E., Mazereeuw, M., Tjaden, U. R., Irth, H., Van der Greef, J., *J Chromatogr A,* **1996**, *741*, 13-21

20 Longo, N., di San Filippo, C.A., Pasquali, M., *Am J Med Genet Part C Med Genet,* **2006***, 142c,* 77-85

21 Heinig, K., Henion, J., *J Chromatogr B*, **1999**, *735,* 171-188

22 Vernez, L., Hopfgartner, G., Wenk M., Krähenbühl., *J Chromatogr A,* **2003***, 984,* 203-213

[23] Kloet, F.M., Tempels, F.W.A., Ismail, N., Van der Heijden, R., Kapser, P.T., Rojas-Cherto, M., Van Doorn, R., Spijksma, G., Koek, M., Van der Greef, J., Mäkinen, V.P., Forsblom., Holthöfer, H., Groop, P.H., Reijmers, T.H., Hankemeier, T., *Metabolomics*, **2012**, *8(1)*, 109-119

[24] Montgomery, J. A., Mamer, O. A., *Anal Biochem*, **1989**, *176,* 85-95

[25] Miró-Casas, E., Farré Albaladejo, M., Covas, M-I., Ortuño Rodriguez J., Menoyo Colomer, E., Lamuela Raventós, R. M., De la Torre, R., *Anal Biochem*, **2001**, *296*, 63-72

[26] Clark, L. C., Thompson, H. L., *Anal Chem*, **1949**, *21(10)*, 1218-1221

[27] Chambers, S. T., Kunin, C. M., *J Clin Invest*, **1987**, *79,* 731-737

[28] Guo, K., Li, L., *Anal Chem,* **2009**, *81,* 3919-3932

[29] Lee, S. H., Kim, S. O., Chung, B. C., *J Chromatogr B Biomed Sci Appl,* **1998**, *719 (1-2)*, 1-7

[30] Ito, H., Gonthier, M-P., Manach, C., Morand, C., Mennen, L., Rémésy, C., Scalbert, A., *Br J Nutr*, **2005**, *94,* 500-509

[31] Greenberg, A. J., Ketcham, R., *J Pharm Sci*, **1978**, *4,* 478-480

[32] Bishop, M. J., Crow, B. S., Kovalcik, K. D., George, J., Bralley, J. A., *J Chromatogr B,* **2007**, *848,* 303-310

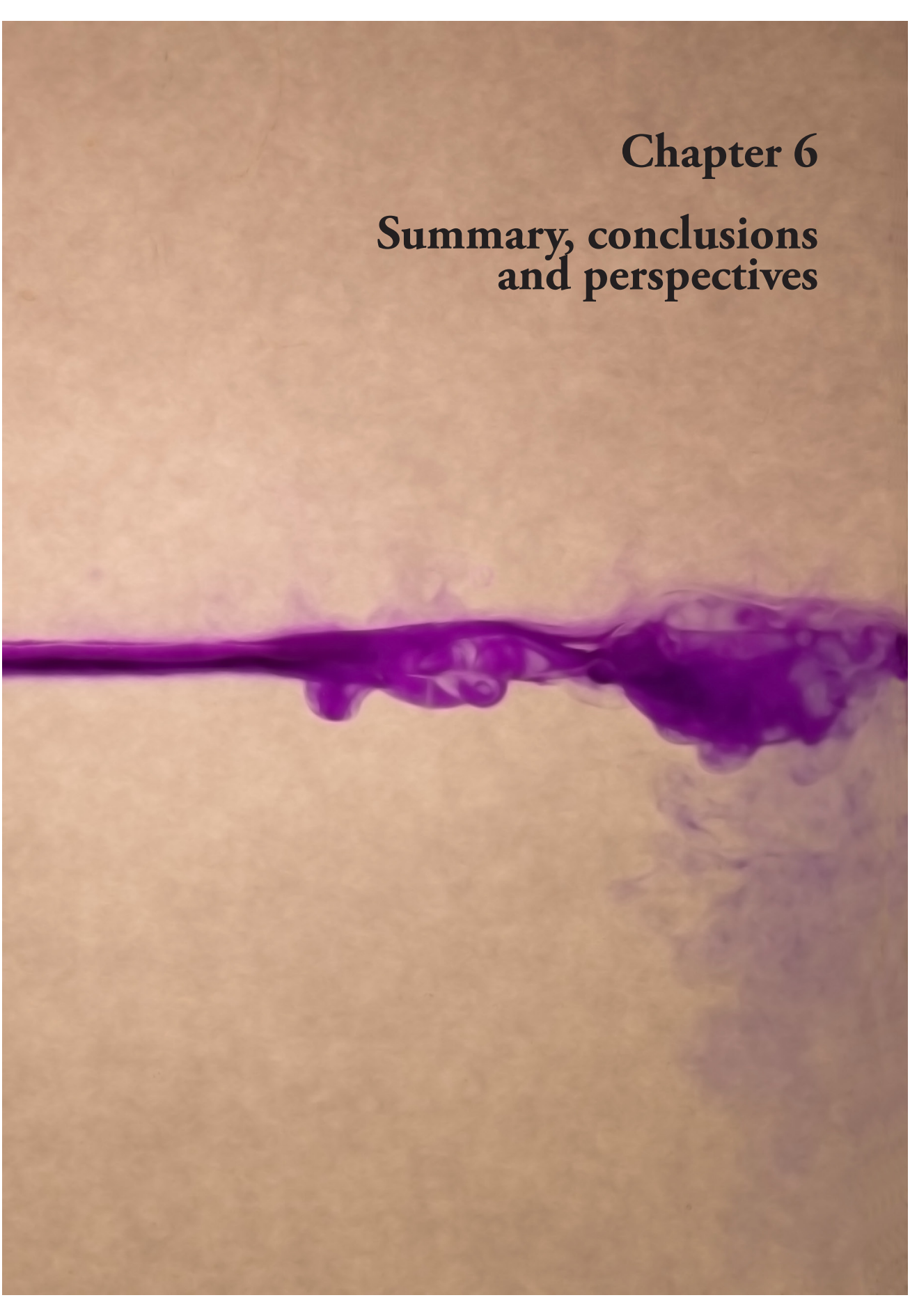
[33] Zydroń, M., Baranowski, J, Baranowska, I., *J Sep Sci*, **2004**, *27,* 1166-1172

[34] Hofmann, U., Schwab, M., Seefried, S., Marx, C., Zanger, U. M., Eichelbaum, M., Mürdter, T. E., *J Chromatogr B*, **2003**, *791*, 371-380

[35] Veraart, J.R., Lingeman, H., Brinkman, U.A.Th., *J Chromatogr A*, **1999**, *856,* 483-514

Chapter 6

Summary, conclusions and perspectives



The paradigm shift from reductionism towards systems thinking has stimulated the field of analytical chemistry to develop bio-analytical techniques that are faster, more comprehensive, and have lower detection limits.

In this thesis, it is demonstrated that electrophoretic methods can be exploited to achieve these developments. For this, carefully chosen electrolyte systems were used, leading to conditions that were suitable for isotachophoresis (ITP) or electroextraction (EE). In such a suitable electrolyte system, an analyte flux can be generated by an electric field. This analyte flux can be utilised to carry out on-line preconcentration of the analytes. In addition, in ITP the analytes are simultaneously separated. All peptides and a large part of the known metabolites are charged or can become charged by varying pH; consequently, migration of these compounds can be manipulated by application of an electric field.

In **Chapter 2**, the potential of capillary isotachophoresis (cITP) coupled to mass spectrometry (MS) of peptides using spacer molecules was explored as a strategy to detect peptides one by one. cITP-MS combines the concentrating power of cITP with the selective and sensitive detection power of MS. In principle, cITP-MS has great potential for the analysis of low-abundant peptides and metabolites, due to its ability to concentrate and separate analytes simultaneously. However, a major limitation has to be overcome, namely the mixing of ITP zones during transfer to MS: as soon as ITP conditions are abandoned, the concentrating effect, which is the key characteristic of ITP, is diminished and the isotachophoretic zones start to collapse and mix. When the isotachophoretic zones are very narrow, as is the case with low-abundant analytes, the collapsing of these zones results in zones that contain multiple peptides. Proof-of-principle experiments are described in which spacer compounds are added to a peptide sample prior to cITP separation. As expected, the peptides are physically separated by the spacer molecules and enter the MS one by one instead of all at once, as happened when no spacer molecules were added.

During the study, it was noticed that the spacer compound mixture that was used (carrier ampholytes (CA)) needed improving. CA mixtures were originally developed to generate stable pH gradients in isoelectric focusing (IEF). However, the currently available CA mixtures have not been well defined and contain high levels of neutral contaminations which are present in all ITP zones, frustrating MS detection and resulting in less favourable detection limits or even no analyte detection at all. A solution for this could be to clean up the CA mixture prior to use, for example by performing weak cation exchange solid phase extraction. A more elegant solution would be to develop a well-defined spacer compound mixture dedicated to ITP use and of which the exact composition is known, including the

electrophoretic mobilities of all the individual spacers. It is interesting to note that during the period IEF was invented, the theoretic concept of IEF was introduced first, and practical IEF was only possible after a chemist took the challenge of synthesizing appropriate CA [1]. In the case of ITP, suitable spacer compounds should possess electrophoretic mobilities in the range of the analytes, they should cause no ion suppression and they should be volatile in order not to clog the spray needle. In proteomics, transient ITP-CZE has been successfully used to concentrate and fractionate complex peptide samples prior to LC-MS analysis. Direct coupling of cITP to MS, using spacer molecules, will result in an analytical method which has improved detection limits, which covers a high dynamic range, is easy to set up, uncomplicated, not labour-intensive and cheap.

The second electrophoretic approach that was studied in this thesis is electroextraction (EE) **(Chapters 3-5)**. It was demonstrated that EE is a fast sample preconcentration technique that is quantitative, applicable to peptidomics and metabolomics, easy to automate, easy to hyphenate with LC-MS, and able to deal with large sample volumes (10-100 µL, with the possibility to further enlarge the volume).

With EE, sample molecules can be extracted rapidly into a small volume. Therefore, the amount of sample injected into an analytical separation can be increased without increasing the injected sample volume. Increasing the sample volume to achieve lower detection limits is often undesirable, since large amounts of contaminations are injected along with the compounds of interest. EE also offers selectivity; in one extraction either cations or anions are extracted and all other compounds, including neutrals, remain largely behind, since they only slowly passively migrate by diffusion into the acceptor phase.

To carry out EE, the charged sample molecules should be dissolved in a low-conductive (organic) donor phase that is immiscible with the (aqueous) acceptor phase, which has a high conductivity. When a high voltage is applied over such a liquid-liquid system, a very high electric field strength will be present in the low-conductive phase, causing the charged compounds that are present there to migrate very fast towards the acceptor phase. In the highly conductive acceptor phase, a very low electric field strength exists and the charged compounds are slowed down as they enter; therefore, they are concentrated just after they have passed the liquid-liquid interface.

In this thesis, two on-line set-ups for EE-LC-MS were successfully demonstrated: capillary EE (cEE) (**Chapter 3**) and large volume cEE (**Chapter 4 and 5**). The main differences between cEE and large volume cEE were the positioning of the liquid phases and the volume of organic donor phase that could be extracted.

In cEE (**Chapter 3**), the aqueous acceptor phase was positioned in a capillary and the organic donor phase in a sample vial, in which the grounded capillary was immersed as well as an electrode. When an electric field strength was applied, approximately 10 µL organic phase could be depleted from cationic analytes, which migrated into the aqueous phase in the capillary. The collected sample plug was transferred to an LC-MS system via a switching valve. A peptide enrichment factor of around 100 times in comparison to a conventional reversed phase LC injection of the organic donor phase was achieved, demonstrating the capacity of EE to increase the loadability of LC-MS. With this set-up, it was shown for the first time that EE is suitable for enrichment of biomolecules (peptides) from a biological matrix (urine).

In **Chapter 4 and 5** cEE-LC-MS was further improved, so that it could extract larger sample volumes (100 µl instead of 10 µL, e.g. large volume cEE-LC-MS) in order to achieve higher enrichment factors (and therefore improve detection limits). Moreover, the repeatability of EE was improved. These improvements were achieved by performing EE in a large bore capillary, in which the whole extraction volume of organic phase is injected. Then, extraction from this large, well-defined and controlled organic sample volume took place. Peptide enrichment factors were improved to approximately three orders of magnitude and it was demonstrated that the method is also suitable for also acylcarnitines. With on-line large volume cEE-LC-MS, peptides in plasma as well as low abundant metabolites in urine were determined.

In this thesis, large volume cEE was used to hyphenate SPE off-line with reversed phase LC-MS. Normally, SPE extracts have to be evaporated to dryness and reconstituted prior to reversed phase LC-MS in order to be able to inject a sample amount large enough. Both evaporation and reconstitution are labour intensive, result in sample loss and introduce errors in the measurement. In this thesis, it is demonstrated that due to the implementation of cEE, these steps can be eliminated. The construction of a fully automated, closed analytical system in which SPE is coupled on-line to large volume cEE-LC-MS is foreseeable. Such a system minimises experimental errors, sample loss and labour, since no human interference is required after filling the autosampler with the appropriate samples. For full hyphenation of SPE to LC-MS via large volume cEE, the organic solvent should be able to elute the compounds of interest from the SPE material as well as to serve as a donor solvent in EE.

In **Chapter 5**, the combination of large volume cEE with CZE-MS for the analysis of low abundant urine metabolites, using acylcarnitines as example, is demonstrated and shown to be promising. The on-line coupling with CZE is attractive, since large volume

cEE can overcome a major limitation of CZE, namely its low volume loadability. To obtain a reliable on-line coupling of large volume cEE to CZE, a dedicated valve could for example be developed.

In all the EE work described in this thesis, only ethyl acetate (EtOAc) was used as organic donor solvent. EtOAc is a suitable solvent, since it can form a two-phase system with water and it can contain some (up to 3.5%) water to enable the presence of ions. The range of compounds that can be extracted (in terms of polarity, for example) can be further expanded by using other organic solvents or solvent mixtures.

The EE process lends itself very well to microfluidic applications. In an EE-chip, very small volumes can be extracted and the use of high voltage equipment is unnecessary since in these small dimensions high field strengths can already be achieved with low voltages. When on-chip EE is successfully developed, the consecutive development of small, easy-to-operate EE equipment coupled on-line to analytical separations is foreseeable.

To summarise, we have demonstrated in this thesis that electromigration-based sample pretreatment techniques for bio-analysis have great potential, showing cITP and EE as examples. It can be expected that such techniques will be implemented in analytical instrumentation in the near future. Both cITP and cEE can be expected to contribute to an increased coverage of the peptidome and the metabolome, which will result in the discovery of new biomarkers. Therefore, cITP as well as cEE have great potential to become new additions to the repertoire of the modern analytical chemist.

References

1 Vesterberg, O., Svensson, H., *Acta Chem Scand,* **1966**, *20*, 820-834

Nederlandse samenvatting

De verschuiving van reductionisme naar systeemdenken heeft het vakgebied van de analytische chemie gestimuleerd om bioanalytische technieken te ontwikkelen die snel een breed scala aan componenten die in lage concentraties voorkomen kunnen meten.

In dit proefschrift wordt gedemonstreerd dat elektroforetische methoden gebruikt kunnen worden om deze doelen te bereiken. Daartoe werden zorgvuldig gekozen elektrolietensystemen gebruikt, die leiden tot omstandigheden waarin isotachoforese of elektroextractie kan plaatsvinden. In 'een dergelijk elektrolietensysteem kan een flux van analieten gegenereerd worden door een elektrisch veld aan te leggen. Omdat alle peptiden en een groot deel van de metabolieten in geladen toestand kunnen worden gebracht door de pH te varieren, kunnen ze deel uit maken van deze analietenflux. Dankzij deze flux kunnen dus zowel peptiden als metabolieten worden geconcentreerd en, in het geval van isotachoforese, ook gescheiden worden.

In **Hoofdstuk 2** wordt het gebruik van zogeheten 'spacer-moleculen' om capillaire isotachoforese van peptiden aan massaspectrometrie te koppelen verkend. De koppeling van capillaire isotachoforese aan massaspectrometrie combineert de kracht van capillaire elektroforese, namelijk het concentrerende effect, met de kracht van massaspectrometrie, namelijk de selectieve en gevoelige detectie. In principe is deze combinatie om bovengenoemde redenen zeer geschikt voor de analyse van lage concentraties peptiden en metabolieten.

Echter, de directe koppeling van isotachoforese aan massaspectrometrie is momenteel slechts beperkt succesvol. Dit komt omdat, zodra de experimentele omstandigheden die ervoor zorgen dat isotachoforese plaatsvindt verlaten worden, de scheiding en de concentrering die bewerkstelligd waren gedeeltelijk teniet worden gedaan. Hierdoor raken de analieten deels weer met elkaar vermengd. Wanneer de analietzones zeer smal zijn, zoals het geval is wanneer zeer lage concentraties geanalyseerd worden, leidt dit er zelfs toe dat zij weer geheel vermengd raken, terwijl het concentrerende effect van isotachoforese ook grotendeels verloren gaat.

In dit proefschrift wordt getoond dat de toevoeging van spacer-moleculen aan het monster, voordat het geanalyseerd wordt, het vermengen van de analieten (in dit geval peptiden) tijdens het overbrengen naar de massaspectrometer kan voorkomen. Zoals verwacht werden de peptiden fysiek van elkaar gescheiden doordat spacer-moleculen zich tussen de analietzones situeerden tijdens de isotachoforetische focussering. Dit resulteerde in verschillende opeenvolgende massaspectra met steeds één peptide, in plaats van massaspectra waarin alle peptiden tegelijk voorkomen (zoals waargenomen wanneer

geen spacer-moleculen waren toegevoegd). Gedurende deze studie werd opgemerkt dat het gebruikte mengsel spacer-moleculen verbetering behoeft. Het gebruikte mengsel bestond uit amfolieten die normaliter worden gebruikt in experimenten waarin gewerkt wordt met isoëlektrisch focusseren. De exacte samenstelling van dit mengsel is onbekend en het bevat een hoog gehalte vervuilingen die massaspectrometrische detectie negatief beïnvloeden vanwege ionensuppressie. Een mogelijk oplossing voor dit probleem is het opschonen van het spacer-molecuulmengsel voordat het wordt toegevoegd aan het monster. Eleganter zou het zijn om een speciaal voor deze toepassing geschikt mengsel van spacer-moleculen te ontwikkelen. Deze spacer-moleculen moeten over elektroforetische mobiliteiten beschikken die in hetzelfde bereik liggen als de mobiliteiten van de te analyseren componenten. Daarnaast moeten zij geen ionensuppressie veroorzaken bij introductie in de massaspectrometer en ze moeten vluchtig zijn, zodat ze de naald waarmee ze worden verneveld niet verstoppen.

De directe koppeling van capillaire isotachoforese aan massaspectrometrie met gebruik van spacer-moleculen zal resulteren in een weinig bewerkelijke, goedkope, eenvoudig op te zetten analytische methode met verbeterde detectielimieten en een groot dynamisch bereik.

De tweede op elektroforese gebaseerde benadering die in dit proefschrift (**Hoofdstuk 3-5**) werd bestudeerd is elektroextractie. Er werd aangetoond dat elektroextractie een snelle, kwantitatieve techniek is die analieten concentreert. Elektroextractie is toepasbaar op complexe monsters die peptiden en/of metabolieten bevatten, eenvoudig te automatiseren, eenvoudig te koppelen aan vloeistofchromatografie en massaspectrometrie en in staat om grote volumes geïnjecteerd monster te extraheren (10-100 µL, met de mogelijkheid dit verder te vergroten).

Door middel van elektroextractie kunnen analieten snel naar een klein volume worden geëxtraheerd. Hierdoor kan de hoeveelheid analiet die geïnjecteerd wordt in een analytisch scheidingssysteem vergroot worden zonder dat het geïnjecteerde monstervolume vergroot wordt. Het vergroten van het geïnjecteerde monstervolume om lagere detectielimieten te verkrijgen is vaak ongewenst, omdat, samen met de analieten, ook grote hoeveelheden vervuilingen geïnjecteerd worden.

Elektroextractie biedt ook selectiviteit omdat in hetzelfde experiment óf kationen óf anionen worden geëxtraheerd, terwijl neutrale componenten grotendeels achterblijven omdat zij slechts door middel van diffusie naar de acceptorfase migreren.

Om een elektroextractie uit te voeren moeten de analieten opgelost zijn in een (organische) donorfase met lage geleidbaarheid, die niet mengt met een (waterige) acceptorfase met hoge geleidbaarheid. Als een hoog voltage over dit vloeistof-vloeistof systeem wordt aangelegd, zal

er een zeer hoge veldsterkte over de donorfase en een zeer lage veldsterkte over de acceptorfase ontstaan. Het gevolg hiervan is dat ionen die zich in de donorfase bevinden zeer snel naar de acceptorfase zullen migreren. Door de lage elektrische veldsterkte in de acceptorfase zal de migratiesnelheid van de ionen, zodra ze deze fase intreden, zeer sterk dalen. Dit heeft tot gevolg dat deze ionen, zodra ze het grensvlak tussen donor- en acceptorfase gepasseerd zijn, sterk geconcentreerd worden.

In dit proefschrift worden twee succesvolle on-line opstellingen gepresenteerd die elektroextractie koppelen aan vloeistofchromatografie en massaspectrometrie: capillaire elektroextractie (**Hoofdstuk 3**) en groot-volume capillaire elektroextractie (**Hoofdstuk 4 en 5**). De belangrijkste verschillen tussen beide opstellingen liggen in de positionering van de vloeistoffases en het extractievolume.

In capillaire elektroextractie (**Hoofdstuk 3**) bevinden de waterige acceptorfase zich in een capillair en de organische donorfase in een monstervaatje. Het ene uiteinde van het capillair bevindt zich in de organische fase, het andere uiteinde is geaard. Daarnaast bevindt zich ook een elektrode in de organische fase. Zodra een elektrisch veld werd aangelegd, migreerden de ionen vanuit de organische donorfase de waterige acceptorfase in om daar geconcentreerd te worden. Op deze manier konden alle analieten uit ongeveer 10 µL organische fase geëxtraheerd worden. De aldus verzamelde analietenzone werd via een schakelkraan geïnjecteerd in een vloeistofchromatografie-massaspectrometrie systeem. Directe vergelijking met een conventionele injectie in een dergelijk systeem liet zien dat de peptiden ongeveer 100 keer verrijkt konden worden met behulp van elektroextractie en dat dus de belaadbaarheid van vloeistofchromatografie met deze factor vergroot was. Experimenten met urinemonsters resulteerden in de constatering dat elektroextractie geschikt is voor de extractie van peptiden uit complexe biologische monsters.

In **Hoofdstuk 4 en 5** werd het systeem verder verbeterd zodat grotere volumes organische donorfase geëxtraheerd konden worden. Door het extractievolume te vergroten van 10 µL naar 100 µL kon meer verrijking en daardoor dus betere detectielimieten bereikt worden. Ook kon de herhaalbaarheid van elektroextractie verbeterd worden. Deze verbeteringen werden bewerkstelligd door de elektroextractie te laten plaatsvinden in een capillair met grote diameter waarin het te extraheren volume organische fase geïnjecteerd werd. In dit capillair vond elektroextractie vanuit een goed gedefinieerd volume donorfase plaats, resulterend in verrijkingsfactoren van peptiden van rond de 1000. Met deze opstelling zijn peptiden en metabolietensporen in zowel bloedplasma als urine bepaald.

Groot-volume elektroextractie kan goed worden gecombineerd worden met vaste-stof-extractie. Gewoonlijk wordt het eluens dat de analieten bevat na vaste-stof-extractie gevriesdroogd en heropgelost voordat het in een vloeistofchromatografiesysteem geïnjecteerd kan worden. Zowel het vriesdrogen als het heroplossen zijn bewerkelijke stappen, die bovendien experimentele fouten in de analyse introduceren en verlies van analiet opleveren. In dit proefschrift wordt gedemonstreerd dat deze stappen weg gelaten kunnen worden door het eluens te vermengen met een geschikte donorfase zodat er direct elektroextractie kan worden uitgevoerd. Het valt te voorzien dat een volledig gesloten analytisch system ontwikkeld zal worden, waarin vaste-stof-extractie via elektroextractie gekoppeld is aan vloeistofchromatografie met massaspectrometrische detectie. In zo'n systeem zullen experimentele fouten tot een minimum beperkt zijn, evenals het verlies van analiet. Daarnaast zal de gebruiker na het inladen van de monsters geen bemoeienis meer hoeven te hebben met de analyse. Om vaste-stof-extractie direct on-line met elektroextractie te koppelen moet het eluens dat gebruikt wordt om de analieten van de extractiekolom te elueren ook geschikt zijn om te dienen als organische donorfase in elektroextractie te dienen.

In **Hoofdstuk 5** wordt de combinatie van elektroextractie met capillaire zone elektroforese gedemonstreerd veelbelovend te zijn. De on-line koppeling van elektroextractie met capillaire zone elektroforese is aantrekkelijk, omdat elektroextractie een belangrijke beperking van capillaire zone elektroforese, namelijk de lage belaadbaarheid ervan, kan verhelpen. Om elektroextractie on-line te koppelen zou bijvoorbeeld een daartoe speciaal geschikte schakelkraan ontwikkeld kunnen worden.

Samenvattend kan gesteld worden dat we in dit proefschrift, aan de hand van capillaire isotachoforese en elektroextractie, gedemonstreerd hebben dat de toepassing van op elektromigratie gebaseerde monstervoorbewerkingstechnieken in de bioanalyse grote potentie heeft. Het ligt in de lijn der verwachting dat zulke technieken in de nabije toekomst meer toegepast gaan worden in analytische instrumentatie. Van zowel capillaire isotachoforese als van elektroextractie kan verwacht worden dat zij de kennis van de in biologische monsters aanwezige peptiden en metabolieten zal vergroten, wat kan resulteren in de ontdekking van nieuwe biomarkers. Om deze redenen hebben capillaire isotachoforese en elektroextractie grote kans om nieuwe toevoegingen aan het repertoire van de moderne analytisch chemicus te worden.

Curriculum Vitae

Peter Lindenburg werd geboren op 23 oktober 1979 in Schiedam. In 1998 behaalde hij het VWO diploma aan Scholengemeenschap Spieringshoek Schiedam. In hetzelfde jaar startte hij zijn studie Biologie aan de Universiteit van Leiden. In 2000 startte hij daarnaast met de studie Bio-Farmaceutische Wetenschappen, eveneens aan de Universiteit van Leiden. Gedurende zijn studietijd liep hij stage bij de Division of Analytical Biosciences (Leiden/Amsterdam Centre for Drug Research, Universiteit van Leiden) en het Laboratory of Analytical Chemistry (University of Helsinki). In 2005 studeerde hij af in de Bio-Farmaceutische Wetenschappen en in 2006 in de Biologie.

Van 2006 tot en met 2010 werkte hij aan zijn promotieonderzoek bij de Division of Analytical Biosciences (Leiden/Amsterdam Centre for Drug Research, Universiteit van Leiden), waarvan de resultaten zijn beschreven in dit proefschrift. Daarna trad hij aldaar in dienst als onderzoeker en docent.

List of publications

Open tubular capillary electrochromatography: technique for oxidation and interaction studies on human low-density lipoproteins.
Kuldvee R., d'Ulivo L., Yohannes G., Lindenburg P.W., Laine M., Oörni K., Kovanen P., Riekkola M.L.
Anal Chem. **2006**, *78(8)*, 2665-71.

Schmitt-Kopplin (ed): Capillary Electrophoresis: Methods in Molecular Biology Series.
Lindenburg P.W., Hankemeier, T.
Mol Biotech, **2008**, 1 (384), 90

On-line capillary liquid-liquid electroextraction of peptides as fast pre-concentration prior to LC-MS.
Lindenburg P.W., Seitzinger R., Tempels F.W.A., Tjaden U.R., Van der Greef, J., Hankemeier, T.,
Electrophoresis, **2010**, *31,* 3903-3912.

On-line large-volume capillary electroextraction coupled to LC-MS to improve detection limits of peptides.
Lindenburg, P.W., Tempels, F.W.A., Tjaden, U.R., Van der Greef, J., Hankemeier, T.,
Accepted for publication in J Chrom A.

Feasibility of electroextraction as versatile sample concentrating pretreatment for fast analysis of low abundant urine metabolites and its application to acylcarnitines.
Lindenburg P.W., Tjaden U.R., Van der Greef, J., Hankemeier, T.,
Submitted to Electrophoresis.

Potential of capillary isotachophoresis mass spectrometry of peptides using spacer molecules.
Lindenburg, P.W., Tjaden, U.R., Van der Greef, J., Hankemeier, T.,
In preparation for publication.