New electromigration-driven enrichment techniques for peptidomics and metabolomics
Lindenburg, P.W.

Citation
Lindenburg, P. W. (2012, June 5). *New electromigration-driven enrichment techniques for peptidomics and metabolomics*. Retrieved from https://hdl.handle.net/1887/19049

Cover Page

Universiteit Leiden

Author: Lindenburg, Petrus Wilhelmus
Title: New electromigration-driven enrichment techniques for peptidomics and metabolomics
Date: 2012-06-05

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].
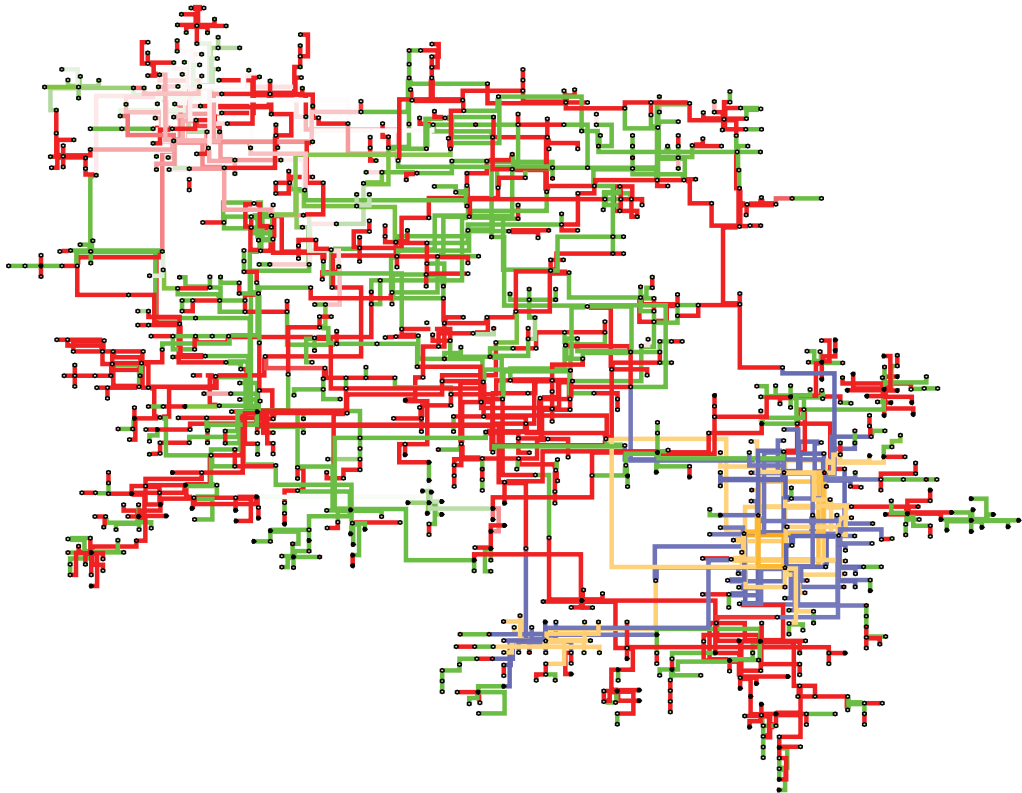

Figure 1.1 Example of a visualised biological system based on experimental data obtained from adipose tissue. It represents a correlation network based on transcript levels, protein concentrations and metabolite concentrations, determined with RNA-assays, LC-MS and GC-MS, respectively (kindly provided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

1

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) $$x_L = \bar{x}_{bi} + k s_{bi}$$

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

1

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) $E = \dfrac{V}{L_{tot}}$

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]:

$$4) \qquad F_E = zeE$$

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]:

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

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]:

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

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

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

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,

1

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 stable 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

1

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.

1

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

1