Isoelectric Focusing:
Sample Pretreatment – Separation – Hyphenation

Linda Silvertand

Isoelectric Focusing:
Sample Pretreatment – Separation – Hyphenation

Isoelectrisch Focusseren: Monstervoorbewerking - Scheiding - Koppeling

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 23 september 2009 des middags te 2.30 uur

door

Linda Henriette Hermina Silvertand

geboren op 21 juni 1979

te Heerlen

Promotor: prof. dr. G.J. de Jong

Co-promotor: dr. W.P. van Bennekom

Table of Contents

Chapter 1

Introduction

1.1   Project goals

The IOP Genomics project 'Proteomics on a chip for monitoring auto-immune diseases' was initiated by three universities: University of Twente, University of Utrecht and the University of Nijmegen, with the goal of developing separation and identification strategies for biomarkers (*e.g.* proteins and peptides) in highly complex biological matrices. Several industrial partners were also involved in the project and contributed scientifically as well as financially. The project was funded by the Netherlands Technology Foundation, STW.

'Proteomics on a chip'

The goal of the total project was: '… to develop an integrated "proteomics on a chip" device which comprises two successive on-chip separations and solid-phase affinity binding combined with Surface Plasmon Resonance (SPR*) imaging' [1]. The intent was to use this device for finding new biomarkers and to develop new diagnostic applications for auto-immune diseases in general, with emphasis on rheumatoid arthritis. To achieve this goal, each of the participating universities contributed with their own area of expertise (Fig. 1.1):
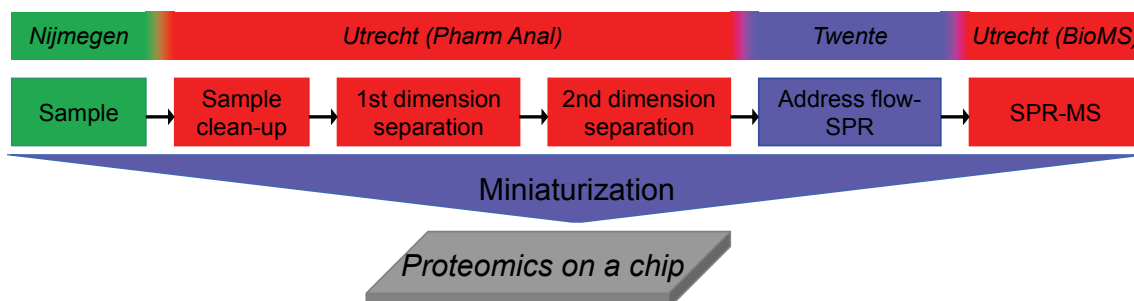
Figure 1.1. Schematic overview of contributions of the different academic partners: University of Nijmegen (green), University of Utrecht (red) and University of Nijmegen (blue) to achieve "Proteomics on a chip".

- The University of Nijmegen (prof. dr. G.J.M. Pruijn) focuses on the pathogenesis of rheumatoid arthritis and other auto-immune diseases. Studying disease mechanisms, supplying and testing the relevant recombinant antigens, auto-antibodies and patient sera with the iSPR technology is their part in the project.

- The Pharmaceutical Analysis group (prof. dr. G.J. de Jong and dr. W.P. van Bennekom) of the University of Utrecht is specialised in sample pretreatment,

*) On page 165 is a list of abbreviations

sensing and separation techniques. This expertise is to be used for developing clean-up and separation procedures and the coupling to sensing techniques.

- The Biomolecular Mass Spectrometry group (prof. dr. A.J. Heck), was responsible for the last step in the project: mass spectrometric confirmation and identification and the hyphenation of SPR to MS.

- The Biochip group (University of Twente, dr. ir. R.B.M. Schasfoort) has to integrate the developments of the other groups on microchip format and is responsible for the address-flow interface to iSPR.

Project goals: clean-up, separation and hyphenation

For the sample clean-up and separation part a literature study was carried out on how to approach the problem of isolating possible protein biomarkers from a highly complex matrix. From this survey, the project goals for this thesis were formulated.

In proteomic applications, patient samples, like blood, synovial fluid, organ tissue, etc. often need to be analysed *e.g.* in the search for signalling proteins or marker proteins. The biological matrices are highly complex and contain next to the compound of interest a large number of substances like salts, lipids, small molecules, cells, *etc.,* which can interfere with the separation and analysis of the biomarkers. In order to remove these compounds from the sample (reducing the sample complexity), an appropriate sample pretreatment procedure should be selected. There are several ways to remove salts, lipids and other components from the sample *e.g.* solid-phase extraction (SPE), dialysis, ultracentifugation or electrophoretic techniques. Furthermore, different (selective) isolation strategies are available like (immuno) affinity columns for LC as well as CE, immobilized metal-affinity columns (IMAC), lectin-affinity columns, etc. Although all these strategies may be very effective, electrophoretic techniques were selected for further investigation due to the ease of miniaturization and available knowledge:

- Isotachophoresis (ITP) in a column-coupled set-up is a method providing preconcentration and efficient salt removal.

- Capillary zone electrophoresis (CZE) is suitable for selective removal of compounds and separation of proteins and peptides.

- Isoelectric focusing (IEF) offers preconcentration of the proteins in combination with a high peak capacity. IEF has already proven to be effective

as first separation strategy in one of the major proteomic approaches as *e.g.* 2D gel electrophoresis.

After sample pretreatment, the remaining sample is further analyzed. The most common standard techniques for proteomics are:

- 2-Dimensional gel electrophoresis (2D-GE). The sample is first separated with IEF in a gel matrix on basis of the p*I* of the molecules. Then the compounds are separated on size (gel electrophoresis or SDS-PAGE). This results in a 2D plot and a very high peak capacity. After 2D-GE the compounds in a spot can be isolated, digested and analyzed with LC-MS/MS as described below. The main disadvantages of this approach are the long analysis times and the labour-intensity, although more samples can be analysed at the same time.

- Liquid chromatography (LC) coupled to mass spectrometry (MS). The proteins or peptides are chromatographically separated on *e.g.* charge (ion exchange chromatography (IXC)), size (size exclusion chromatography (SEC)) or hydrophobicity (reversed-phase chromatography). A second separation dimension is often added and proteins can be digested before or after an LC step. The last dimension is coupled to an MS or MS/MS for identification of the digested proteins via a peptide-database.

2D-GE and LC-MS/MS can also be coupled, where usually digestion of the proteins is performed prior to LC-MS/MS. This combination is extremely powerful: combining the separation efficiency of 2D-GE with the possibility to identify the peptides of the isolated protein with LC-MS/MS.

Next to these standard proteomics approaches, capillary electrophoresis (CE) techniques become increasingly popular. Although it is still a niche method, it can offer some advantages over these approaches:

- Compared to 2D-GE, CE is faster, no staining and destaining is required and CE can be automated.

- Although organic modifier free LC methods are available (SEC, IXC), in general, CE is more biocompatible (water-based) than LC. With biocompatible buffers, the 3D structure remains intact. Because an interaction step (SPR) follows the separation procedure (Fig. 1.1) this is an initial requirement.

- CE is easily implemented on a microfluidic device. The only requirements for CE separations are channels, buffer reservoirs and electrodes. The microfabrication technology is the expertise field of the Biochip group. LC on the other hand is more complicated to implement on chip due to the requirement of moving microparts, pumps and the implementation of stationary phases in the chip. However, the HPLC-MS-chip recently designed by Agilent has already proven to be successful.

One of the major drawbacks of CE is the unfavourable sensitivity; due to the small path length of the detection window (25-200 µm) in combination with the low injection volumes (nL), relatively high concentrations are required to obtain a signal. This also stresses the importance of a separation method that concentrates the compounds before or during separation. Also, CE is often not as reproducible and robust as LC. The main focus was on the study of electrophoretic techniques and the optimization of sensitivity and reproducibility.

CE is a versatile technique and there are several CE modes to choose from:

- CZE: Capillary zone electrophoresis

- CIEF: Capillary isoelectric focusing

- CITP: Capillary isotachophoresis

- CGE: Capillary gel electrophoresis

- MEKC: Micellar electrokinetic chromatography

- CEC: Capillary electrochromatography

- IACE: Immunoaffinity CE

A combination of two CE modes can also be used (Chapters 3 and 6). From the beginning of the project, an IEF-based approach was chosen as the main separation technique because of its high peak capacity, high separation efficiency, high loadability, high biocompatibility, a wide pI and molecular weight range and preconcentration capacity. Although the majority of the CE techniques fits to one or more of these requirements, cIEF seems to meet the requirements for protein separation best. Furthermore, the first separation mode in 2D-GE is an IEF step and also responsible for the high peak capacity of this system. However, the coupling of cIEF with MS is difficult because of the effect of the

buffer additives on the protein ionization in MS and the necessity of mobilizing the separated compounds towards the MS.

Next to the investigation of several sample pretreatment procedures (ITP, CZE and IEF) and IEF as separation strategy, another major goal of this thesis is the hyphenation of (c)IEF to other detection methods than UV. The most obvious is the coupling to MS for mimicking 2D-GE (separation on p*I* followed by Mw separation) and gaining structural information. Next to that, the possibilities of combining IEF and SPR are presented in this thesis, since SPR is to be implemented as sensing technique in the project device.

In order to study and compare the different sample pretreatment, separation and hyphenation techniques, only mixtures of standard proteins were used. Next to spiked biological samples, salts and interfering proteins were added to standard protein mixtures to study biological problems. However, no real proteomic research was performed.

1.2   Introduction to (bio)analytical techniques

The basic principles of the (bio)analytical methods described in this thesis are outlined.

Capillary electrophoresis

Electrophoresis is the migration of charged compounds in a fluid under the influence of an electrical field. The velocity of that migration depends primarily on:

- The charge and size of a compound

- The fluid (aqueous buffer, pH, ionic strength, viscosity, etc.) in which the analyte migrates. This fluid is called the background electrolyte or BGE

- The applied electrical field strength

If two compounds differ in velocity, they can be separated with electrophoresis. Since the BGE and the electrical field are equal for both compounds, the separation is solely dependent on size and charge.

Electrophoresis can be performed in a gel (Chapter 5), or in a free solution as in free flow electrophoresis (FFE, *e.g.* [2]) or in a capillary (capillary electrophoresis

or CE, Chapters 3, 4 and 6). The setup for a CE apparatus is depicted in Figure 1.2. The only requirements are a high voltage power supply, a capillary, 2 vials containing BGE and 2 electrodes, all in contact to create a closed electrical circuit. For rinsing the capillary and injecting a sample, a pressure system is used. To monitor the separation, a detection device (UV, fluorescence, conductivity, mass spectrometry) is added, as well as a PC with software for data acquisition
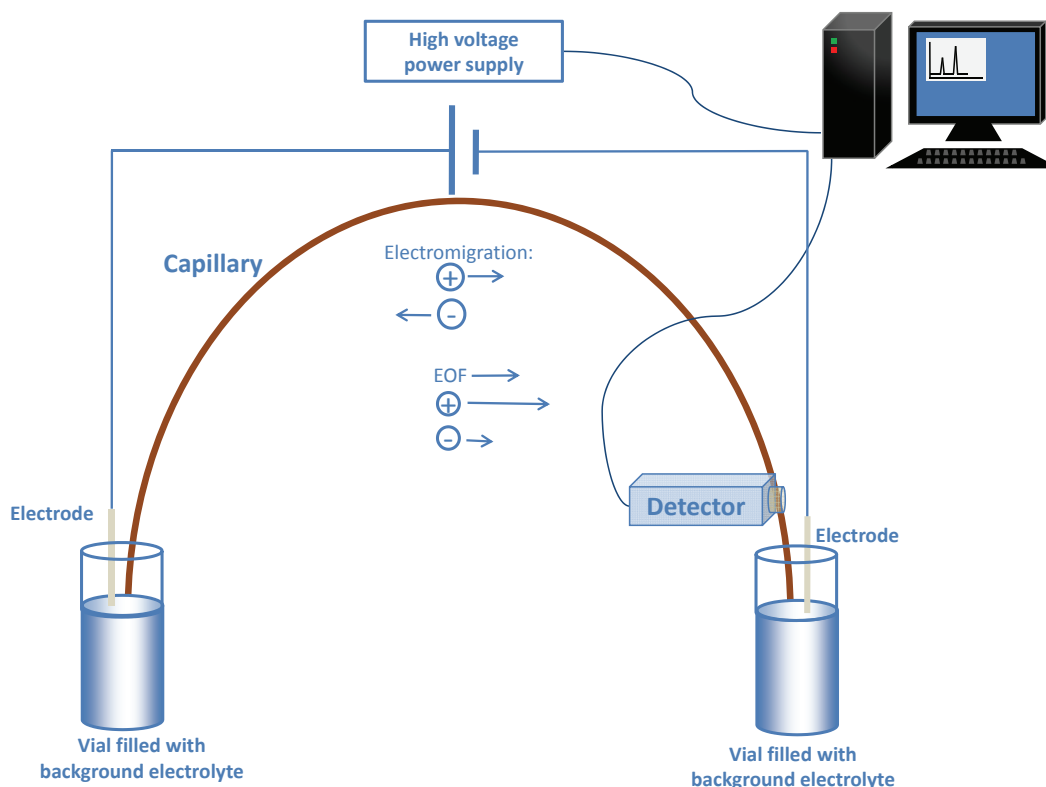
Figure 1.2. The basic setup of a capillary electrophoresis system.

and analysis. The very first experiments with glass tubes filled with migrating charged compounds in a buffer under the influence of an electrical field were carried out and reported as early as 1856 [3-5]. A few years later papers leading to the Kohlrausch theory were published. This theory describes all electrophoretic migration principles [6-9]. However, Kendall was the first to suggest to use this electrophoretic migration phenomenon or 'ionic migration' as a separation technique in 1928 [10], followed by Tiselius who described his 'moving boundary' theory for the separation of proteins in a U-tube in 1937 [11]. However, it took nearly 25 years before the first electrophoretic experiments in small diameter glass capillaries were performed [12]. Capillary electrophoresis (CE) had many advantages over gel and paper electrophoresis: less Joule heating,

automation, on-line detection, high-speed analysis and quantification. The diameter of the separation capillary was reduced from 3 mm in 1967 [13] to real capillary formats of 75 μm in 1981 by Jorgenson and co-workers [14], who therefore is considered the founding father of modern CE. From the early seventies, different (non-CZE) CE modes were described like isotachophoresis (ITP) in 1971, which was soon followed by capillary electrochromatography (CEC) in 1974 [15], capillary gel electrophoresis (CGE) in 1983 [16], micellar electrokinetic chromatography (MEKC) in 1984 [17] and capillary isoelectric focusing (cIEF) in 1985 [18]. The decrease of the diameter is unfavourable for detection, which is the major drawback of CE. The first UV detector for CE was developed in 1970 [19] and in the eighties more detection techniques for CE were introduced: fluorescence in 1981 [20], laser induced fluorescence in 1985 [21], chemiluminescence [22], raman spectroscopy in 1988 [23], radioactivity in 1989 [24] and mass spectrometry in 1987 [25]. Electrochemical detection was developed in 1987 [26] and nuclear magnetic resonance hyphenation was accomplished in 1994 [27].

In the nineties, CE becomes a more and more popular technique. Although it suffers from disadvantages like the above mentioned high UV-detection limits (due to small optical path length in combination with the low injection volume), low reproducibility, upscaling difficulties, *etc.*, CE has some advantages as well: high speed analysis, high efficiency, biocompatibility and low consumption of reagents. However, LC remains more popular due to the higher sensitivity, the head start of a few decades, implementation in educational programs and the higher robustness. CE is becoming more interesting in the application fields where LC suffers from limitations: small organic and inorganic substances, enantiomeric separations, large (bio)molecules (proteins) and DNA analysis. The elucidation of the human genome is the biggest success story in the history of CE.

Capillary zone electrophoresis

Charged compounds will migrate under the influence of an electrical field in a conducting fluid. This process is called electromigration and the compounds migrate with a velocity described by Equation 1.1.

$$v_{em} = \frac{\mu_{em}}{E} = \frac{q}{6\pi\eta r}$$

(Eqn. 1.1)

Where v_{em} is the electrophoretic velocity of the compound (in cm/s), μ_{em} the electrophoretic mobility (in cm^2/(V*s)), E is the electrical field strength (in V/cm), q the net charge, η is the viscosity of the BGE and r is the radius of the compounds (including the hydration shell).
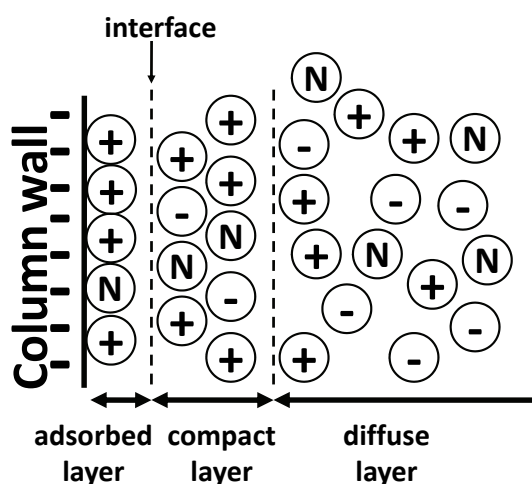
Figure 1.3. Schematic representation of the electrical double layer [28].
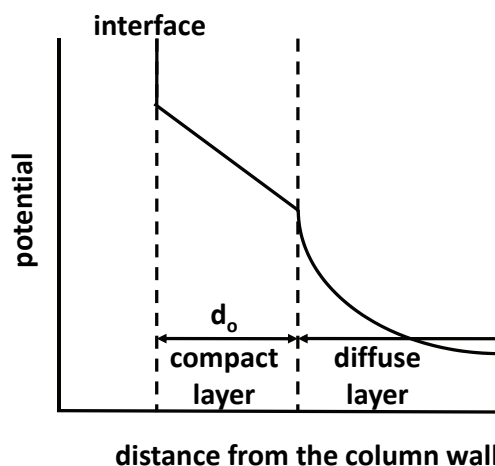
Figure 1.4. Zeta potential versus distance from the capillary wall [28].

As can be seen, the migration velocity is directly proportional to the charge-to-size ratio of the analytes. A larger molecule has a lower velocity and a highly charged compound has a higher velocity. This also indicates that compounds with opposite charges will migrate away from each other (to the oppositely charged electrodes). However, the negatively charged silanol groups in bare fused silica capillaries create an additional driving force (the electroosmotic flow or EOF) that causes the compounds to move in one direction and pass a detector (Fig. 1.2). The negatively charged Si-O$^-$ groups of the silica wall attract positively charged ions from the BGE. Compounds close to the wall are electrostatically adsorbed and cannot move (adsorbed or Stern layer [29] in Fig. 1.3). Charged ions further away create a compact layer that is mainly positively charged (compact or Gouy-Chapman layer [30, 31]). The potential at the plane of shear between these two layers is called the zeta potential (ζ) and the decrease of this potential over the distance from the capillary wall is depicted in Figure 1.4. When a voltage is applied, the ions in the compact layer (mainly positively charged), will migrate to the negatively charged electrode (cathode). Since the ions are solvated by water, the fluid is mobilized, thus creating an electroosmotic pump in the direction of one of the electrodes. The EOF has a flat flow profile, characterized by small peakwidths, in contrast to the laminar flow profile created by pressure

(Poiseuille). The velocity of this EOF pump is described by Smoluchowski in 1903 [32] (Eqn. 1.2):

$$v_{EOF} = \frac{\varepsilon \zeta E}{\eta}$$

(Eqn. 1.2)

With v_{EOF} being the velocity of the EOF (in cm/s), ε the dielectric constant of the BGE, ζ the zeta potential (in V), E the applied electrical field strength (in V/cm) and η the viscosity of the BGE.

The velocity of a compound in a certain system can be described as:

$$v_{total} = v_{em} + v_{EOF} = \frac{q}{6\pi\eta r} + \frac{\varepsilon \zeta E}{\eta}$$

(Eqn. 1.3)

All changes made to a system (changes in buffer pH, ionic strength, *etc.*) have influence on one or more parameters in Eqn. 1.3. With this equation, the effect of most changes made to the CE system can be predicted.

Capillary isoelectric focusing

In capillary isoelectric focusing (cIEF, Fig. 1.5), a pH gradient is created inside the capillary. The inlet vial is filled with an acidic BGE, supplying the hydrogen ions, and the outlet vial is filled with an alkaline BGE, supplying the hydroxide ions.
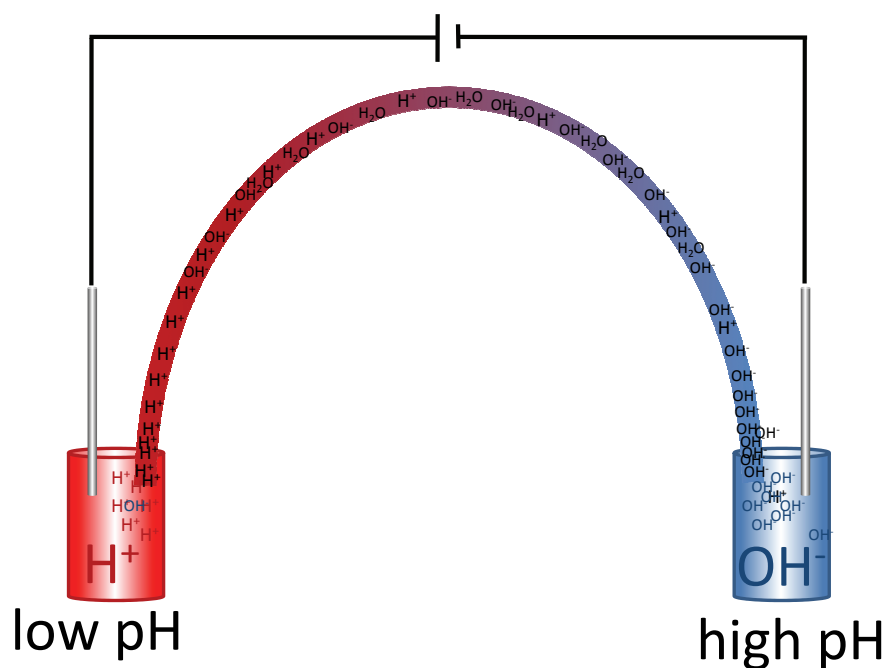

Figure 1.5. Schematic overview of a cIEF setup.

When a voltage is applied, the hydrogen ions migrate to the negatively charged electrode (cathode), while the hydroxide ions migrate to the positively charged electrode (anode). This will create a pH gradient inside the capillary. This system is very sensitive to changes like temperature, salts or injection of a sample. The buffer capacity of the pH gradient is low, especially between 5 and 8. Vesterberg was the first to synthesize compounds that stabilize this pH gradient and thereby paved the way for cIEF [33-35]: carrier ampholytes (carry the pH gradient and the current).These carrier ampholytes consist of 600-1200 compounds (depending on the brands used [36]) and buffer the local pH, allowing a stable pH gradient. When amphoteric analytes (proteins, peptides and carrier ampholytes) are introduced in this system and an electrical field is applied, these compounds will migrate to the electrode of opposite charge, until their nett charge is zero (p*I* matches the local pH). A steady state is reached: as soon as the compounds diffuse out of their zone, they acquire a charge and are forced back into their pH =p*I* zone. This auto-regulating steady-state mechanism is called focusing and besides separation also concentration of compounds in their zone is accomplished. After the focusing process, the separated compounds need to be mobilized towards a detector, usually a UV or MS detector. This can be performed by:

- Electroosmotic mobilization. The focusing and mobilization step occur simultaneously. In contrast to the other two procedures, an EOF is needed to accomplish mobilization [37-40]. Bare fused silica capillaries or charged coated capillaries are therefore often used, with the addition of a cellulose additive in the BGE.

- Electrophoretic mobilization (chemical or salt mobilization). After focusing one of the buffer reservoirs is replaced by a buffer vial containing additional salt or zwitterions [18, 40]. These ions compete with H^+ or OH^- to enter the capillary and cause the local pH to increase or decrease. As a result, the pH gradient shifts and the analytes acquire a charge. Since a voltage difference is applied over the capillary ends, the analytes will migrate to their new p*I*=pH zone.

- Hydrodynamic mobilization. The content of the capillary is mobilized by applying a pressure on the in- or outlet, while maintaining the electrical field [41, 42]. This is the mobilization method used throughout this thesis.

The efficiency of the cIEF separation is described by the cIEF resolving power (Equation 1.4)[43].

$$\Delta \text{p}I = 3 \sqrt{\frac{D(d\text{pH}/d\text{x})}{E(d\mu/p\text{H})}}$$ (Eqn. 1.4)

Where $\Delta \text{p}I$ is the resolving power, D the diffusion coefficient, dpH/dx the pH gradient over the capillary length, E the electrical field strength and $d\mu/d$pH is the dependence of mobility on pH. All changes made to the system affect one or more of these parameters and the effect can be predicted with this equation.

Isotachophoresis

Isotachophoresis (ITP) is an electrophoretic separation technique which is mainly used to preconcentrate compounds (stacking) before injection into a second separation system [44], as is applied in Chapter 6.

Isotachophoresis means 'electrophoresis at uniform speed', and in contrast to CZE, two different buffers are required to perform ITP: the leading electrolyte (LE) and the terminating electrolyte (TE). The sample is injected between the LE and the TE (Fig. 1.6). The LE contains an ion with a high mobility (higher than the highest mobility of the sample ions, usually Cl⁻ for anionic ITP), while the TE contains an ion with a low mobility *e.g* .citrate (lower than the lowest mobility ion in the sample) [45].
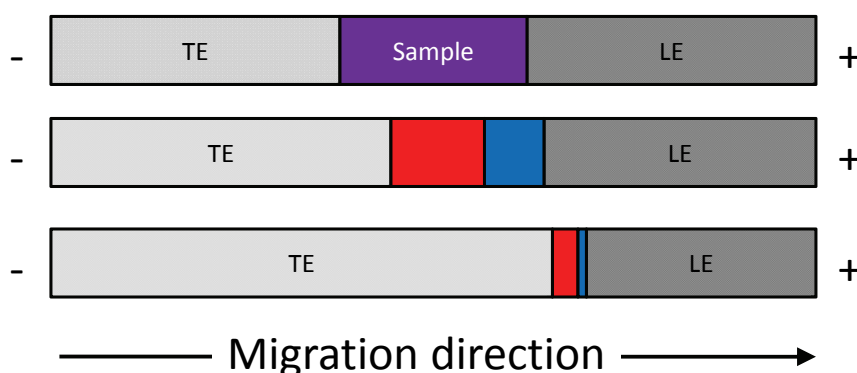
Figure 1.6. The principle of isotachophoresis.

When applying an electrical field, the velocity of the compounds can be described by Eqn. 1.1. Since in ITP usually the current (I) is kept constant, the electrical field (E) is described by Ohm's Law ([46], Eqn. 1.5) and proportional to the resistance (R) in a zone, or inversely proportional to the conductivity (κ), which in turn is dependent on the mobility and the concentration in a zone.

$$E = IR \qquad \text{with } R = \frac{1}{\kappa} \qquad \text{(Eqn. 1.5)}$$

Since the compounds will start to migrate according to their mobility, individual sample zones are created, but since κ changes, the applied electrical field and therefore the velocity of the compounds changes as well. LE with a high mobility ion, which is also highly conductive, will acquire a reduced electrical field (Eqn. 1.5) and a reduced velocity (Eqn. 1.1). The low mobility ion in the TE will acquire a high velocity. At steady state, the velocities become equal (hence the name isotachophoresis), or

$$v_{ITP} = \frac{\mu_{LE}}{E_{LE}} = \frac{\mu_{S1}}{E_{S1}} = \frac{\mu_{S2}}{E_{S2}} = \frac{\mu_{TE}}{E_{TE}} \qquad \text{(Eqn. 1.6)}$$

When compounds from one zone should diffuse into an adjacent zone, they are exposed to a different electrical field and are forced back into their own zone. Next to the separation, the compounds are also concentrated in this zone as described by Kohlrausch [9] in Eqn. 1.7.

$$\frac{C_{LE}}{C_s} = \frac{q_s \mu_{LE} \left(\mu_s + \mu_{CI} \right)}{q_{LE} \mu_s \left(\mu_{LE} + \mu_{CI} \right)} \qquad \text{(Eqn. 1.7)}$$

Assuming equal electrical charges (q), the concentration of the sample ion (C_s) cannot exceed the concentration of the leading ion (C_{LE}) since $\mu_{LE} > \mu_S$ (μ_{CI} is the mobility of the counterion).

Gel isoelectric focusing and off-gel electrophoresis

Gel isoelectric focusing uses the same principle as cIEF, but instead of a capillary, the separation is carried out in a gel matrix, typically polyacrylamide (PAA) or agarose. Furthermore, while in cIEF carrier ampholytes are used to stabilize the pH, in gel-IEF acrylamidobuffers are polymerized in the gel [47] (immobilized pH gradient or IPG) to create a fixed pH gradient [48]. Usually carrier ampholytes are added to the system to improve the separation efficiency [49], but for subsequent bioaffinity studies these carrier ampholytes might interfere (Chapter 5).

Off-gel electrophoresis (OGE) [50] is also based on gel-IEF. In both gel-IEF and OGE, the IEF separation is performed in a pH gradient gel. However, in OGE, the compounds diffuse in the solution above the gel upon reaching their pH=pI zone, as depicted in Figure 1.7. First, the wells are filled with buffer and sample. When an electrical field is applied, amphoteric compounds start migrating through the gel. In the wells, the analytes diffuse into the free solution above the gel, but due to the electrical field they are forced to migrate through the gel to adjacent wells. When the analytes reach the pH zone where their nett charge is zero, they will stop migrating and equilibrium is established between the gel and the solution. Advantage of this technique over standard gel IEF is that the analytes do not need to be stained and/or cut out for further analysis; they can simply be pipetted out of the wells. However, the high cIEF resolution is lost due to fractionation. This device can be used for *e.g.* protein purification and separation [51], fractionation [50] or desalting [52] but also for standard gel electrophoresis (Chapter 5).
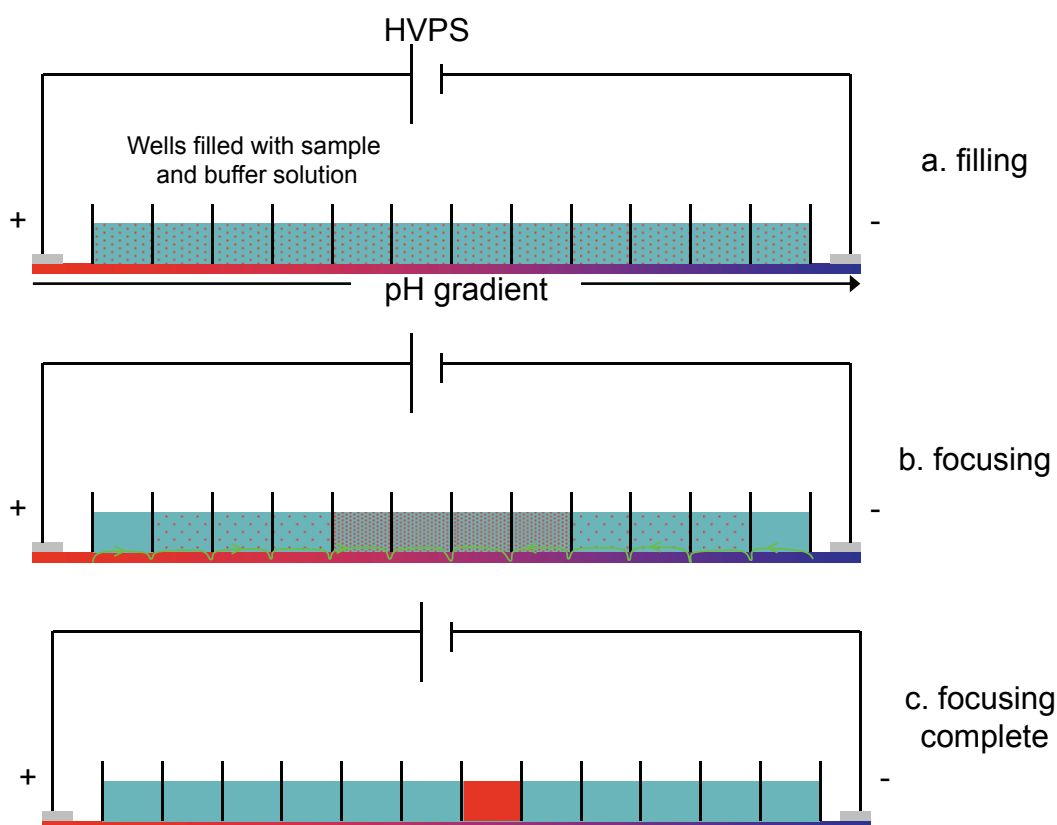
Figure 1.7. The principle of OGE.

Mass spectrometry

The first mass spectrometry (MS) experiments were described by Thomson in 1897 [53] when he studied the existence and properties of positive ions. Aston used electrostatic and magnetic fields to separate isotopes by mass and focus them on a photographic plate in 1919 [54]. Subsequently, MS underwent large improvements to become one of the major analytical techniques as it is in (bio)chemical labs today.

In MS, the mass of a molecule is determined by measuring the relative mass-to-charge ratio (or *m/z*) of its ions. This implies that before compounds can be analysed, they need to be ionized first. Over the years, several ionization techniques have been developed, *e.g.* electron ionization [55, 56], chemical ionization [57], field desorption ionization [58], atmospheric pressure ionization [59], matrix assisted desorption ionization [60, 61], desorption electrospray ionization [62]. But the most used ionization techniques are electrospray ionization (ESI, [63, 64]) and matrix assisted laser desorption (MALDI, [65, 66]). These two techniques have been indispensable to the analysis of biomolecules [67, 68].

After ionization, the molecules are introduced in a mass analyzer. Like with ionization techniques, several mass analyzers have been developed over the last 100 years: double focusing magnetic sector analyzers [69, 70], time-of-flight analyzers (TOF, [71]), ion-cyclotron-resonance analyzers ([72, 73]), quadrupoles ([74, 75]) inductively coupled plasma analyzers [76] and most recently the orbitrap [77]. Tandem mass spectrometry (MS/MS) is very useful for providing structural information on compounds. In MS/MS, two mass analyzers are coupled. The first analyzer is used to select a certain precursor ion, which is then fragmented in a collision-induced dissociation (CID [78]) cell. The fragments are analyzed by the second MS. By linking the product ions to the precursor ion, the structure of the initial compound can be elucidated. MS/MS is also very useful in the analysis of compounds in complex mixtures, since only the *m/z* value of the precursor ion (or even in combination with the main product ion) can be selected for further MS analysis.

The coupling of MS to separation techniques dates back to 1956, when Gohlke first presented the coupling of gas chromatography to a TOF MS [79, 80], and the first reports on coupling CE with MS were reported more than 30 years later [25,

81]. However, the most important hyphenation of MS in bioanalysis of drugs and proteomics research is the coupling to LC. The coupling of LC to MS was established by different groups already in the 1970s [82-85], However, it took until the 1980s before LC was coupled to soft ionization techniques like ESI [86] and MALDI [87]. These hyphenations heralded a new era of LC-MS as one of the major analytical techniques in protein analysis and proteomics [88, 89].

Since this thesis only describes the use of MALDI-TOF MS, the principle of this technique is presented here.

Matrix assisted laser desorption ionization-time of flight MS

MALDI-TOF consists of a MALDI ionization source combined with a TOF analyzer as depicted in Fig. 1.8. In MALDI, sample, matrix and an acid in a volatile solvent are brought on a stainless-steel plate, which is dried and inserted in the ion source. The matrix consists of low-molecular-weight compounds that have an absorption maximum at the wavelength of the laser. In the ionsource, the sample mixture is bombarded by photons of a pulsed laser. The matrix molecules absorb the energy of the laser and are released into the gas phase together with the analytes.
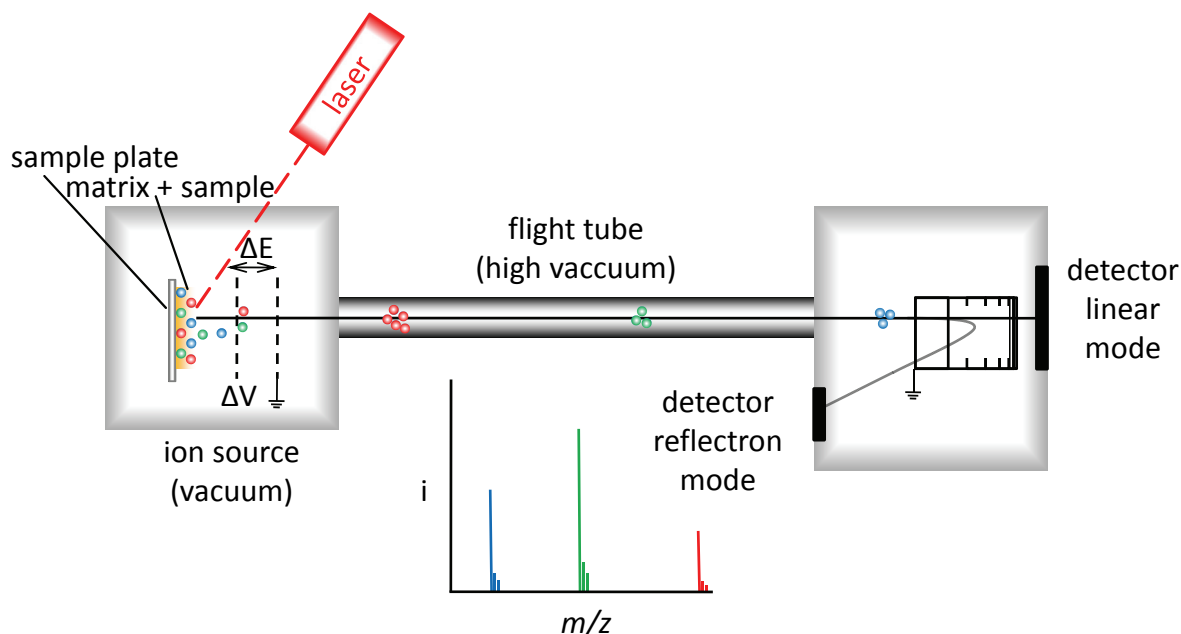
Figure 1.8 Schematic representation of a MALDI-TOF MS.

Charged matrix molecules transfer the charge onto the analyte molecules and enter a strong electrical field in which they are accelerated. A variable voltage grid generates a pulsed voltage which gives all the compounds that were

released by one pulse of the laser the same kinetic energy from one point. Since $U_{kin}=\frac{1}{2}mv^2$, the compounds with a low molecular-weight acquire a larger velocity, travel through the flight tube faster and reach the detector first. By recording the flight time (time of flight or TOF), the *m/z* can be determined.

Surface plasmon resonance

In surface plasmon resonance (or SPR), excitation of surface plasmons (free electrons) at an interface of two materials of different refractive index is used to measure changes at the surface of one of these materials. These plasmons were first observed by Wood in 1902 [90] and in 1968 both Otto [91] and Kretschmann and Raether [92] realised that a drop in reflectivity of *p*-polarized light from a metal film on a support material was caused by the excitation of these surface plasmons. But it was Liedberg in 1983 who first performed biomolecular interactions with an SPR based sensor [93].
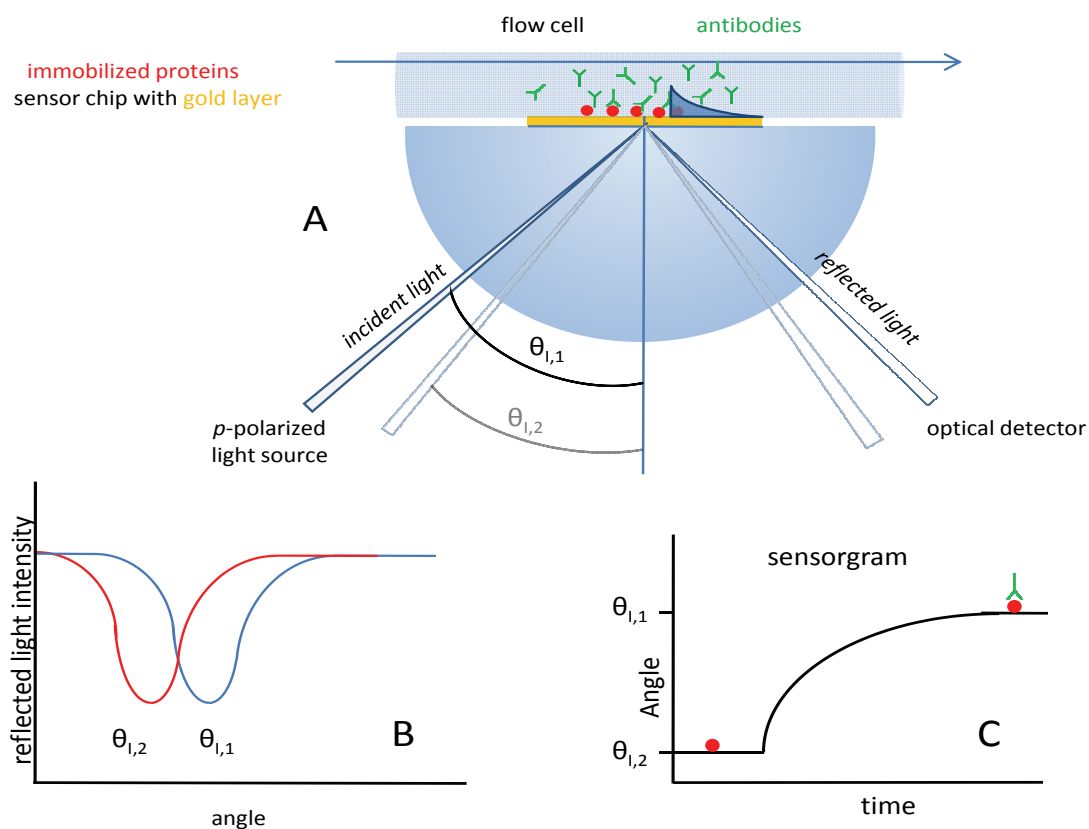
Figure 1.9. Schematic overview of SPR.

Figure 1.9A describes the most frequently used Kretschmann configuration. A *p*-polarized light source focuses the light through the prism and sensor chip (same dielectric material) on a metal film, in this case a thin gold film.The metal reflects

this light and the intensity is monitored by an optical detector. When changing the angle of incidence (Fig. 1.9A), a dip in light intensity is observed (Fig 1.9B). This dip is caused by free electrons in the metal-dielectric interface (plasmons) that interact with the photons in the evanescent field and start oscillating (resonance). The angle at which this resonance occurs is called the resonance or SPR angle. The SPR angle depends on the refractive index of the glass medium at one side of the gold film and the medium on the other side of the gold film *i.e.* the buffer inside the flow cell in Fig. 1.9A. The intensity of the signal is strongly dependent on the distance from the gold layer. Therefore changes in the vicinity of the gold layer can be measured (up to ½λ). Therefore, SPR is very popular for studying biomolecular interactions and kinetic models. One of the compounds of interest is covalently immobilized on the surface (proteins), while the other component is flushed over the surface (antibodies). Interaction at the surface leads to a shift in SPR angle, which is monitored in time (sensorgram or Fig. 1.9C).

In contrast to SPR, in imaging SPR (iSPR) instruments the entire sensor surface is illuminated by a broad beam polarized light source. The reflected light is monitored by a CCD camera. Whole surface imaging allows for monitoring multiple interactions in real time increasing the throughput. This iSPR set-up is used in Chapter 5.

1.3   Outline of the thesis

The three major goals of the research presented in this thesis are:

- Investigation of electrophoretic sample pretreatment strategies.

- Study and optimization of capillary isoelectric focusing for separation of proteins.

- Hyphenation of isoelectric focusing to MS and iSPR.

Chapter 2 gives an overview of the developments in cIEF over the past years including method improvements, new theoretical insights and applications.

Chapter 3 describes the effect of several BGE additives on cIEF-UV repeatability and linearity and on protein signal intensity in MALDI-TOF MS.

Chapter 4 describes the hyphenation of cIEF to MALDI-TOF MS via a spotting device. The development of the system as well as the effects of focusing and

spotting times on separation efficiency are shown. A 3D plot is constructed from cIEF and MS data and the applicability is demonstrated for a degraded biopharmaceutical compound (glucagon).

Chapter 5 is dedicated to the coupling of gel-IEF to iSPR via pressure blotting. The development of the system as well as the influence of the biological matrix on IEF separation and transfer efficiency is discussed. The applicability is demonstrated by the analysis of synovial fluid spiked with a known RA biomarker, *i.e.* citrullinated fibrinogen.

Chapter 6 describes the use of the IonChip for salt removal from a protein sample prior to CZE separation (ITP-CZE mode) and the removal of interfering proteins from a peptide sample with subsequent CZE separation (CZE-CZE mode).

Chapter 7 gives a general conclusion and recommendations for future research. Adjustments to the different systems and devices are proposed.

References

[1]   Schasfoort, R. B. M., van Bennekom, W. P., Pruijn, G. J. M., University of Twente, University of Utrecht, University of Nijmegen 2004.

[2]   Kohlheyer, D., Eijkel, J. C., Schlautmann, S., van den Berg, A., Schasfoort, R. B., *Anal Chem* 2008, *80*, 4111-4118.

[3]   Wiedemann, G., *Pogg Ann* 1856, *99*, 197.

[4]   Wiedemann, G., *Pogg Ann* 1858, *104*, 166.

[5]   Buff, H., *Ann Chem Pharm* 1858, *105*, 168.

[6]   Lodge, O., *Brit Ass Advan Sci Rep* 1886, *56*, 389.

[7]   Whetham, W. C. D., *Phil Trans Roy Soc London (Ser A)* 1895, *184*, 337.

[8]   Whetham, W. C. D., *Phil Trans Roy Soc London (Ser A)* 1895, *186*, 507.

[9]   Kohlrausch, F., *Ann Phys (Leipzig)* 1897, *62*, 209.

[10]   Kendall, J., *Science* 1928, *67*, 163-167.

[11]   Tiselius, A., *Trans Faraday Soc* 1937, *33*, 524.

[12]   Konstantinov, B. P., Oshurkova, O. V., *Dokl Akad Nauk SSSR* 1963, *148*, 1110.

[13]   Hjerten, S., *Chromatogr Rev* 1967, *9*, 122-219.

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

[15]   Pretorius, V., Hopkins, B. J., Schieke, J. D. A., *J. Chromatogr. A* 1974, *99*, 23.

[16]   Hjerten, S., *J. Chromatogr.* 1983, *270*, 1.

[17]   Terabe, S., Otsuka, K., Ichikawa, K., Tsuchiya, A., Ando, T., *Anal Chem* 1984, *56*, 111.

[18]   Hjerten, S., Zhu, M., *J. Chrom A* 1985, *346*, 265-270.

[19]   Arlinger, L., Routs, R. J., *Sci Tools* 1970, *17*, 21

[20]   Jorgenson, J. W., Lukacs, K. D., *Clin Chem* 1981, *27*, 1551-1553.

[21]   Gassmann, E., Kuo, J. E., Zare, R. N., *Science* 1985, *230*, 813-814.

[22]   Dadoo, R., Colon, L. A., Zare, R. N., *HRC & CC* 1992, *15*, 133.

[23]   Chen, C. Y., Morris, M. D., *Appl Spectrosc.* 1988, *42*, 515.

[24]   Pentony, S. L., Zare, R. N., Quint, J. F., *Anal Chem* 1989, *61*, 1642.

[25]   Olivares, J. A., Nguyen, N. T., Yonker, C. R., Smith, R. D., *Anal Chem* 1987, *59*, 1230.

[26]   Wallingford, R. A., Ewing, A. G., *Anal Chem* 1987, *59*, 1762.

[27]   Wu, N., Peck, T. L., Webb, A. G., Magin, R. L., Sweedler, J. V., *J Am Chem Soc* 1994, *116*, 7929.

[28]   Salomon, K., Burgi, D. S., Helmer, J. C., *J. Chromatogr. A* 1991, *559*, 69-80.

[29]   Stern, O., *Z Electrochem* 1924, *30*, 508.

[30]   Gouy, G., *J Phys* 1910, *4*, 457.

[31]   Chapman, D. L., *Phil Mag* 1913, *25*, 475.

[32]   Von Smoluchowski, M., *Bull Int Acad Sci Cracovie* 1903, 184.

[33]   Vesterberg, O., *Acta Chem Scand* 1969, *16*, 2653-2666.

[34]   Vesterberg, O., *Sci Tools* 1969, *16*, 24-27.

[35]   Vesterberg, O. A. Y., Sweden 1969.

[36]   Righetti, P. G., Simo, C., Sebastiano, R., Citterio, A., *Electrophoresis* 2007, *28*, 3799-3810.

[37]   Mazzeo, J. R., Krull, I. S., *Anal Chem* 1991, *63*, 2852-2857.

[38]   Mazzeo, J. R., Martineau, J. A., Krull, I. S., *Anal Biochem* 1993, *208*, 323-329.

[39]   Thormann, W., Caslavska, J., Molteni, S., Chmelik, J., *J Chromatogr* 1992, *589*, 321-327.

[40]   Hjerten, S., Liao, J. L., Yao, K. Q., *J Chromatogr* 1987, *387*, 127-138.

[41]   Chen, S. M., Wiktorowicz, J. E., *Anal Biochem* 1992, *206*, 84-90.

[42]   Huang, T. L., Shieh, P. C. H., Cooke, N., *Chromatographia* 1994, *39*, 543.

[43]   Righetti, P. G., *Isoelectric focusing: Theory, methodology and applications*, Elsevier biomedical press 1983, p. 386.

[44]   Gebauer, P., Bocek, P., *Electrophoresis* 1997, *18*, 2154-2161.

[45]   Martin, A. J., Everaerts, F. M., *Anal Chim Acta* 1967, *38*, 233-237.

[46]   Ohm, G. S., *Die galvanische Kette, mathematisch bearbeitet*, Berlin 1827, p. 254.

[47]   Gasparic, V., Bjellqvist, B., Rosengren, A., Sweden 1975.

[48]   Bjellqvist, B., Ek, K., Righetti, P. G., Gianazza, E., Gorg, A., Westermeier, R., Postel, W., *J Biochem Biophys Methods* 1982, *6*, 317-339.

[49]   Altland, K., Rossmann, U., *Electrophoresis* 1985, *6*, 314.

[50]   Michel, P. E., Reymond, F., Arnaud, I. L., Josserand, J., Girault, H. H., Rossier, J. S., *Electrophoresis* 2003, *24*, 3-11.

[51]   Ros, A., Faupel, M., Mees, H., Oostrum, J., Ferrigno, R., Reymond, F., Michel, P., Rossier, J. S., Girault, H. H., *Proteomics* 2002, *2*, 151-156.

[52]   Arnaud, I. L., Josserand, J., Jensen, H., Lion, N., Roussel, C., Girault, H. H., *Electrophoresis* 2005, *26*, 1650-1658.

[53]   Thomson, J. J., *Phil Mag* 1897, *44*, 293.

[54]   Aston, F. W., *Phil Mag* 1919, *38*, 707.

[55]   Smyth, H. D., *Proc Roy Soc A* 1922, *102*, 283.

[56]   Smyth, H. D., *Phys Rev* 1925, *25*, 452.

[57]   Munson, M. S. B., Field, F. H., *J Am Chem Soc* 1966, *88*, 2621.

[58]   Beckey, H. D., *Research/Development* 1969, *20*, 26.

[59]   Carroll, D. I., Dzidic, I., Stillwell, R. N., Haegele, K. D., Horning, E. C., *Anal Chem* 1975, *47*, 2369.

[60]   Liu, L. K., Busch, K. L., Cooks, R. G., *Anal Chem* 1981, *53*, 109.

[61]   Barber, M., Bordoli, R. S., Sedgwick, R. D., Tyler, A. N., *Nature* 1981, *239*, 270.

[62]   Takats, Z., Wiseman, J. M., Gologan, B., Cooks, R. G., *Science* 2004, *306*, 471.

[63]   Dole, M., Mack, L. L., Hines, R. L., Mobley, R. C., Ferguson, L. D., Alice, M. B., *Journal of Chemical Physics* 1968, *49*, 2240.

[64]   M. Yamashita, Fenn, J. B., *J Phys Chem* 1984, *88*, 4451.

[65]   Karas, M., Bachmann, D., Hillenkamp, F., *Anal Chem* 1985, *57*, 2935.

[66]   Tanaka, K., Ido, Y., Akita, S., Yoshida, Y., Yoshida, T., *Proceedings of the Second Japan-China Joint Symposium on Mass Spectrometry* 1987, 185.

[67]   Tanaka, K., Waki, H., Ido, Y., Akita, S., Yoshida, Y., Yoshida, T., *Rapid Commun Mass Spectrom* 1988, *2*, 151.

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

[69]   Mattauch, J., Herzog, R., *Zeitschrift fur Physik A* 1934, *89*, 786.

[70]   Johnson, E. G., Nier, A. O., *Phys. Rev* 1953, *91*, 10.

[71]   Stephens, W., *Bull Am Phys Soc* 1946, *21*, 22.

[72]   Hipple, J. A., Sommer, H., Thomas, H. A., *Phys Rev* 1949, *76*, 1877.

[73]   Comisarow, M. B., Marshall, A. G., *Chem Phys Lett* 1974, *25*, 282.

[74]   Paul, W., Steinwedel, H., *Z. Naturforschung* 1953, *8*, 448.

[75]   Yost, R. A., Enke, C. G., *Journal of the American Chemical Society* 1978, *100*, 2274.

[76]   Houk, R. S., Fassel, V. A., Flesch, G. D., Svec, H. J., Gray, A. L., C.E., T., *Anal Chem* 1980, *52*, 2283.

[77]   Makarov, A., *Anal Chem* 2000, *72*, 1156.

[78]   Jennings, K. R., *Int J Mass Spec Ion Physics* 1968, *1*, 227.

[79]   Golhke, R. S., McLafferty, F., Wiley, B., *Bendix corporation* 1956.

[80]   Golhke, R. S., *Anal Chem* 1959, *31*, 759.

[81]   Smith, R. D., Olivares, J. A., Nguyen, N. T., Udseth, H. R., *Anal Chem* 1988, *60*, 436.

[82]   Tal'roze, V. L., Skurat, V. E., Gorodetskii, I. G., Zolotai, N. B., *Russ J Phys Chem* 1972, *46*, 456.

[83]   Lovins, R. E., Craig, J., Thomas, F., McKinney, C., *Anal Biochem* 1972, *47*, 539-549.

[84]   Scott, R. P. W., Scott, C. G., Munroe, M., Hess Jr., J., *J Chromatogr* 1974, *99*, 395.

[85]   Horning, E. C., Carroll, D. I., Dzidic, I., Haegele, K. D., Horning, M. G., Stillwell, R. N., *J Chromatogr* 1974, *99*, 13.

[86]   Yamashita, M., J.B., F., *J Phys Chem* 1984, *88*, 4451.

[87]   Huber, J. F. K., Dzido, T., Heresch, F., *J Chromatogr* 1983, *271*, 27.

[88]   Covey, T. R., Bonner, R. F., Shushan, B. I., Henion, J., R.K., B., *Rapid Commun Mass Spectrom* 1988,

2, 249.

[89]   Wang, A. P. L., Li, L., *Anal Chem* 1992, *64*, 769.

[90]   Wood, R. W., *Philos Mag* 1902, *4*, 396.

[91]   Otto, A., *Zeitschrift fur Physik A* 1968, *216*, 396-410.

[92]   Kretschmann, E., Raether, H., *Zeitschrift Fur Naturforschung Part A-Astrophysik Physik Und Physikalische Chemie* 1968, *A 23*, 2135-2136.

[93]   Liedberg, B., Nylander, C., Lunström, I., *Sensors and Actuators* 1983, *4*, 299-304.

Chapter 2

Recent advances in capillary isoelectric focusing 2003-2007

L.H.H. Silvertand, J. Sastre Toraño, W.P.van Bennekom, G.J.de Jong, J Chromatogr A 1204 (2008) 157.

Abstract

The developments in capillary isoelectric focusing (cIEF) over the period 2003 to 2007 are reviewed. With the focus on technological aspects, cIEF papers published in the fields of methodology, new techniques, detection, multidimensional systems, miniaturization and applications are summarized. The methodology section covers recent research in ampholytes composition, detergents and other additives, carrier ampholyte free cIEF, coatings and other capillary modifications. In the section on new systems adjustments to the technique (e.g. dynamic IEF), different applications of cIEF (e.g. as injection system) and new devices are reported. Systems focusing on whole column imaging, fluorescence and chemiluminescence detection and coupling to mass spectrometers are discussed in the section on detection. Interfacing cIEF with MS via RPLC systems and hyphenation of cIEF with capillary electro-chromatography and other capillary electrophoresis modes are also summarized. Papers focusing on miniaturization are reviewed in the section on microfluidic devices. The section on applications will show analysis of biopharmaceutical compounds and isolated proteins for metabolomic studies. For the analysis of complex biological matrices, generally multidimensional systems are needed, which are mentioned throughout this review.

2.1   Introduction

The moment of conception for isoelectric focusing (IEF) was in the early sixties, when Vesterberg, under the supervision of Svensson, found a procedure to synthesize a wide range of carrier ampholytes [1-6]. With several ups and downs, IEF developed into a more preparative technique. Labour started in the early seventies with the first descendant: the polyacrylamide gel-based IEF (gIEF). Still, it took nearly 15 years before a second sibling saw daylight. The capillary format of isoelectric focusing (cIEF) was born around 1985 when Hjertén and Zhu decided to "adapt the equipment for high-performance electrophoresis to isoelectric focusing" [7]. And while gIEF in combination with mass spectrometry (MS) developed into one of the principal techniques used for protein analysis and later on in proteomics, cIEF has always lived in the shadow, but is now gradually coming of age. In the early days the focus was on figuring out the principles and solving the childhood diseases and shifted via new methodologies and one-

dimensional applications to integration in multidimensional platforms and miniaturized systems. The majority of problems in cIEF has been overcome and the (bio)analytical world is now seeing the benefits of cIEF for protein analysis: high resolution, speed, concentrating factor and the possibility of integration in automated (miniaturized) multidimensional platforms. Next to its utilization for the analysis of pharmaceutical proteins, the emerging 'proteomics' platform as an application field has contributed to the popularity of cIEF as well. This gradual shift is also observed in the time-line of publications. In cIEF-specific reviews the focus has shifted from the principles of cIEF via technological aspects towards proteomic and biopharmaceutical applications [8-19]. The last review dedicated to cIEF was published by Kilár [16] in 2003, but a number of cIEF papers from 2003 on has also been discussed in other reviews on various subjects like capillary electrophoresis (CE) in general [20-23], the role of CE in proteomics [24-36] and peptidomics [29,30,37-39], CE-MS coupling [25,29,31,32,34] and papers on specific clinical applications like hemoglobin [40], transferrin [41] or somatropin analysis [42].

Gel IEF and chromatofocusing are beyond the scope of this review, as well as two fairly new non-capillary (non)-gel isoelectric focusing techniques *i.e.* free flow IEF (FFIEF) and off-gel electrophoresis (OGE). This review focuses on papers published in the last four years (2003-2007) dealing with the capillary format of isoelectric focusing and will mainly discuss technological aspects : methodology, new techniques, detection systems, multidimensional platforms, miniaturization and applications.

2.2 Methodology

Carrier ampholytes

Although in the past much attention has been paid to the conductivity and buffering power of carrier ampholytes (CAs), the group of Righetti recently investigated various properties of different narrow-cut CA blends in a series of papers in Electrophoresis [43-48], including an overview [48]. Narrow-cut (2-3 pH units) CA blends of the four major brands (Pharmalytes, Servalyte, Ampholine and Bio-Lyte) were fractionated into 20 fractions with the Rotofor [49] and injected onto a capillary zone electrophoresis-electrospray ionization-ion-trap-MS (CZE-ESI-ion-trap MS) system. Linearity (pH of the fractions), mass

distribution, polydispersity and focusing properties were investigated. Their findings were as much surprising as they were clarifying. In the acidic range (up to pH 7) all blends contained high numbers of amphoteric compounds with good focusing properties (compounds present in only 1 to 3 Rotofor fractions were denoted as 'good' CAs) as can be seen in Table 2.1. In the alkaline range, however, the majority of the CAs does not only fail to focus, but the number of species per pH unit was low, due a low variety of pK_a values of the aminogroups. These factors lead to a decline in the quality of the pH gradient in that range and are possible explanations for the problems encountered when analyzing proteins in this pH region. Furthermore, the measured pH range covers usually more than the claimed cut. Exception is Ampholine 3.5-5, where measured pH values start at a pH of 3.8. Servalyte generally contains in all pH cuts the highest number of isoforms. This also explains that Servalytes form the p*H* gradient with the best linearity approach. Pharmalytes possess the highest percentage of 'good' CAs. The authors of this series of papers make a few remarks about their results *(e.g.* not all Rotofor fractions were analyzed), and they correctly state that the alkaline region of CA pH gradients can be improved by new synthesis routes. Unfortunately, the broad range pH gradients (3-10) were still not evaluated.

Mosher and Thormann used these new findings of the group of Righetti to adjust their 'High resolution computer simulation of the dynamics of IEF' [50] and came up with a more realistic approach to predict the outcome of cIEF: including unequal differences in pK_a and mobility of the 140 CAs in their model. In this model, at least two of the experimentally observed phenomena occurring in cIEF were simulated; the conductance gap (caused by a large ΔpK_a in certain parts of the pH gradient) and cathodic drift (caused by the unequal mobilities of the cationic and anionic species of the CAs). The cathodic drift phenomenon was further investigated in a very recent paper by the same group [51]. Lalwani and co-workers [52-54] added two new carrier ampholytes series to the list that can be used in pH-biased isoelectric trapping (IET in which a compound is added to keep the protein charged at pH=p*I* to increase solubility). These single-component CAs are either diamino sulfates (5.7 < p*I* < 9.0 [52,53]) or quaternary ammonium dicarboxylic acids (1.5 < p*I* < 4.3 [54]). However, these compounds do not cover the entire 3-10 pH range. North *et al*. [55] have synthesized UV-absorbing and fluorescent CAs in the pH-range 3-10. Their attempts to tag commercial CA blends (*e.g.* Pharmalyte and IsoLyte) failed: in the pH 6-8 region

the absorbance was higher than in the acidic and alkaline region. Their PEHA-based tagged CAs, however, showed a more balanced UV absorbance and fluorescence over the entire pH range.

Table 2.1 Composition of different brands of ampholytes [43-48].

	Ampholine	Servalyte	Bio-Lyte	Pharmalytes
Claimed pH cut	3.5-5	2-4	3-5	2.5-5
pH range	3.8-7	2-6	3.8-7	2-6
No. Mr species	105	277	84	245
No. isoforms	446	1201	383	857
Mr range	205-965	204-929	216-965	203-857
% 'good' CA	~70	~65	~55	~72
Claimed pH cut	4-6.5	4-6	4.6	4-6.5
pH range	3.8-7.8	3.6-6.9	4.0-6.4	3.6-7.5
No. Mr species	80	199	66	217
No. isoforms	325	1302	436	812
Mr range	203-893	204-907	388-835	150-1179
% 'good' CA	~50	~34	~20	~66
Claimed pH cut	6-8	6-8	6-8	5-8
pH range	5-8	5.3-9.5	4.8-8	5.4-8
No. Mr species	80	126	62	123
No. isoforms	326	703	237	476
Mr range	216-979	240-785	341-1048	221-992
% 'good' CA	~46	~26	~17	~45
Claimed pH cut	7-9	7-9	8-10	8-10.5
pH range	6.1-9.0	4.7-8.8	8.5-12.2	7.5-10.3
No. Mr species	29	65	43	58
No. isoforms	85	306	136	102
Mr range	210-870	290-760	205-495	200-725
% 'good' CA	~25	~35	~25	~ 50

Silvertand et al. [56] systematically tested the influence of broad range ampholytes blends, different classes and concentrations of detergents and viscosity increasing agents on linearity (p*I vs.* migration time), resolution, repeatability (for peak area as well as migration time) and background signal in cIEF-UV. Moreover, protein signal suppression of these compounds in matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF-MS) was studied. Their main finding is that compounds that increase efficiency and repeatability in cIEF (*e.g.* ampholytes, detergents and cellulose derivatives) cause a severe signal suppression in MALDI-TOF-MS. However, the amount of these interfering substances can be dosed by manipulating the spotting time and speed.

Poitevin *et al.* [57] and Busnel *et al.* [58] evaluated the performance of two cIEF systems after optimizing focusing and mobilization parameters: aqueous cIEF with a hydroxypropylcellulose-coated capillary (superior EOF suppression compared to polyvinylalcohol) and their glycerol-water system with a bare fused-silica capillary. In the latter system, it was found that glycerol stabilizes proteins, enhances solubility of hydrophobic proteins, decreases EOF and minimizes dispersion [58]. This glycerol system performed better for the separation of myoglobin isoforms of different origin. Furthermore, narrow pH cut ampholytes blends were added to increase the resolution of β-lactoglobulin A and B in these systems. Surprisingly, Pharmalytes were inferior to Ampholine and Beckman 3-10 ampholyte for parameters tested in this study, while in the studies performed by the group of Righetti [43-47] and Silvertand *et al.* [56], Pharmalytes were superior.
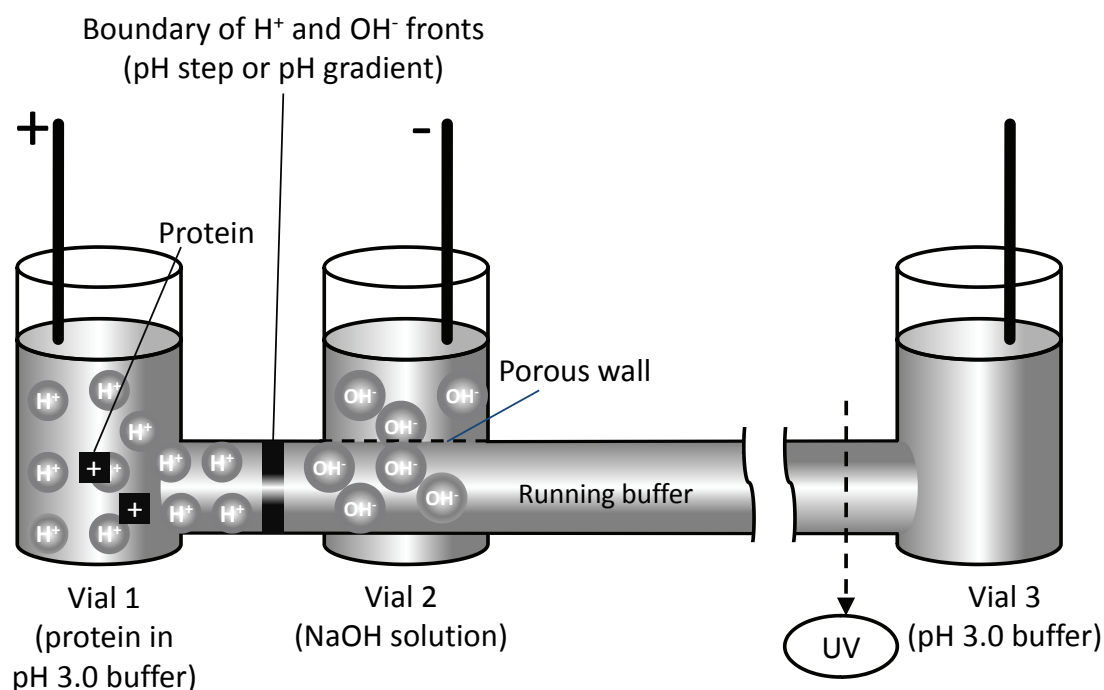
Figure 2.1. Experimental setup for CAF-cIEF sample injection for CZE. Compounds are concentrated during the isoelectric focusing process between vials 1 and 2. For CZE, the anolyte and catholyte solutions are replaced with buffer solution and a voltage difference is applied between vials 1 and 3 [60].

Carrier ampholyte free cIEF (CAF-cIEF) was coupled to an ESI-ion-trap by Storms *et al.* [59]. In this CAF-cIEF approach, peptides (mixture of five digested proteins) were used to create a pH gradient. However, the buffering capacity and the smoothness of the pH gradient were inferior to commercial CA blends. No linear correlation between the p*I* of the peptides and the migration time was observed.

Furthermore, peak-broadening and loss of resolution were reported. Low concentrations (0.2%) of CAs were added, merely as p*I*-spacers, and increased efficiency. Wu *et al*. [60,61] and Budilova *et al*. [62] both performed CAF-cIEF as a sample concentration step for CZE. Wu *et al.* performed cIEF-CZE in a single capillary by porosifying a short part of the capillary with HF. After injection in the cIEF system, the alkaline solution in vial 2 was replaced with the same buffer as in vials 1 and 3 and an electric field was applied between vials 1 and 3, thereby injecting the sample into a CZE system (Figure 2.1). Without ampholytes a sharp pH step was created (neutralization reaction boundary) in which the compounds were concentrated. Concentration factors up to 150 times were obtained. Continuous injection of tryptic peptides led to concentration factors of 1400-7700 and were estimated to be 8 to 45 fold higher than for conventional cIEF.

Coatings

In the past four years, a number of reviews on capillary coatings in CE has been published [33,63-65] covering the full spectrum of coatings used in CE. For cIEF usually only the neutral, hydrophilic polymers (like polyvinylalcohol (PVA), polyacrylamide (PAA) or cellulose derivatives) are used to minimize protein-wall interaction and the electroosmotic flow (EOF). Graf and Wätzig [66] proved that the adsorption of proteins and clusters to the coated capillary wall is the main cause of the often encountered bad repeatability and not the instability of the coating. Coating of the wall reduces the interaction but cannot avoid adsorption, especially at pH values around p*I*.

Covalently bound coatings in combination with a low amount of dynamic coating in the sample and the electrolytes are often used. Poitevin *et al*. [57] found hydroxypropylcellulose (HPC) to be more effective in reducing the EOF in cIEF than PVA (96% *vs.* 76% reduction at pH 2). Palm *et al*. [67] used cIEF to compare two other neutral polymers: poly(methyl methacrylate) (PMMA) and poly(vinylpyrrolidone) (PVP). The latter performed best in terms of long-term stability (100 *vs.* 30 runs) and reproducibility, while the coating procedure for PMMA was considerably faster (40 min *vs*. 2 days). Gao and Liu [68] improved the coating procedure for cross-linked PAA and found this coating to be more resistant to degradation under alkaline conditions than the linear variant of the polymer. A plasma-polymerization procedure was used by Tsai *et al*. [69] to deposit hexa(dimethylsiloxane) (HDMS) and acetonitrile on a Tempax glass

microchip. Separation efficiency of these two coatings was compared to results obtained with PAA-coated and plain glass chips. The resolution obtained with the plasma-polymerized HDMS (PDMS) was comparable to the PAA-coated microfluidic device. A different coating procedure for PDMS was used by Horká et al. [70], who bound PDMS to a bare fused-silica capillary via a sol-gel process which was used for the separation of microorganisms using cIEF (Figure 2.2).
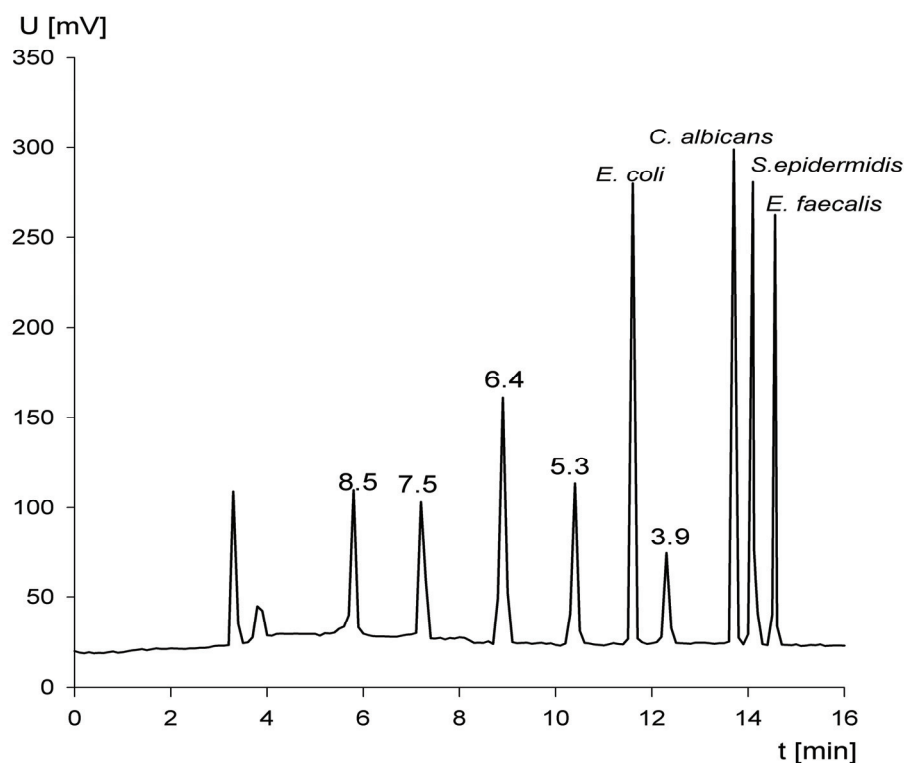
Figure 2.2. cIEF (pH gradient 3-10) of microorganisms (4 x 10^9 cell/mL in physiological saline solution) and low molecular weight markers (25mg/mL) on a sol-gel-PDMS coated capillary with UV detection (280 nm) [70].

Knittle *et al*. [71] focused a homogenous mixture of proteins and fluorescent peptide markers (calibration) with a PAA-acrylbenzophenone coated vinyl capillary. Via photo-immobilization, the separated compounds were covalently bound to this surface. Fluorescently and horseradish peroxidase (HRP) labeled antibodies were flushed through the capillary and bound to the immobilized proteins.Both fluorescence as well as chemiluminescence (SuperSignal West femto) detection were carried out. Chemiluminescence was slightly more sensitive, but better peak resolution, ease of use and better chemical stability were observed with fluorescence detection.

Although, as mentioned above, a combination of covalent and dynamic coatings is most popular, dynamic coatings alone are also used. Usually, the polymer which modifies the capillary surface is dissolved in the electrolyte and/or the sample, which makes the coating procedure less labor-intensive than coating covalently. Horká and co-workers added poly(ethylene glycol) (PEG) for the analysis of microorganisms in the pH range 2-5 [72] and 3-10 [73] with a bare fused-silica capillary using UV detection. Besides reducing the EOF, this system was superior in the acidic range to their own PAA sol-gel coated capillary with respect to resolution [70]. Horká and co-workers used a pyrenebutanoate-PEG (PB-PEG) modified coating for fluorescence detection of PB-PEG labeled microorganisms and proteins [74-76]. The pI values of the labeled microorganisms were similar to the native compounds.

Yeung *et al.* [77] added zwitterionic sulfobetaines to the sample. Only two of the compounds were effective in suppressing the EOF: in the acidic and neutral pH range hexadecyldimethyl(3-sulfopropyl)ammoniumhydroxide) and in the alkaline and neutral pH range Rewoteric AM CAS U (3-(3-cocoamido-propyl)-dimethylammonium-2-hydroxypropan-sulfonate, manufactured by Tego Chemie Service GmbH, Essen, Germany). These two compounds offer a better repeatability and resolution than the also tested bare fused-silica and dodecyldimethyl(3-sulfopropyl)ammoniumhydroxide coating. However, adding sulfobetaines increases solubility of the proteins and this may also play a role in the improved repeatability [56].

Instead of modifying the bare fused-silica wall surface with polymers, it would be more efficient and stable to use capillaries of these polymers. However, bare fused-silica offers some critical advantages over these materials like UV transparency and a very effective extrusion process which creates a smooth inner glass surface. However, Teflon offers a good alternative as shown by Maheshwari *et al*. [78], but it should be taken into account that the pI of proteins shifts when they are in contact with a surface like glass or Teflon.

Avoiding the problem of stable pH gradients and coatings, Yang *et al*. [79] created an immobilized pH gradient monolith (M-IPG) by synthesizing first a monolith and subsequently attaching CAs (Ampholine) covalently. Compounds were mobilized either hydrodynamically or chemically (replacing catholyte with anolyte solution). The immobilized CAs were able to maintain a relatively linear pH gradient in the

capillary for over 100 runs. Since the buffer is CA-free, the system can be used with MS and with lower UV detection wavelengths.

Special systems

Besides the chemical improvements to cIEF mentioned above, technical aspects of cIEF have also been further studied and optimized.
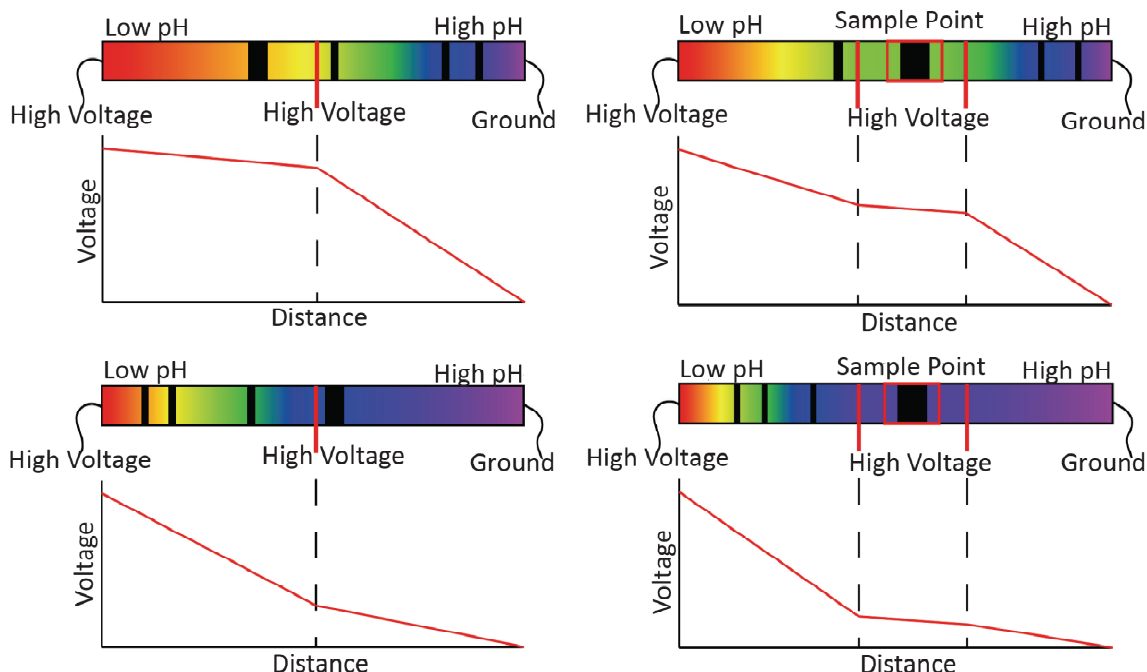
Figure 2.3. The principle of dynamic IEF. The electric fields and pH gradient in a three (left) and a four electrode system (right) with high voltages (a, upper graphs) and low voltages (b, lower graphs) on the middle electrode(s) [80].

In the group of Tolley, dynamic IEF (dIEF) was created [80]. This system, depicted in Fig. 2.3, is brilliant in its simplicity and uses multiple voltage gradients within one capillary to manipulate the electric field and thereby the local pH gradient. Between the outer electrodes the standard pH gradient is created, but the slope is regulated by the electrode(s) in between, so analytes can be moved and isolated to the window (or valve, located between the two middle electrodes) without loss of resolution. Furthermore, the selected compounds can be injected into a second system like CZE, LC or MS. This method offers the possibility of manipulating the linearity and slope of the pH gradient, which has always been a problem in cIEF, especially for accurate determination of p*I* values. Wu and Huang [81], however, circumvented this problem by using the peak position relative to two internal standards instead of the more common approach of

determining the correlation coefficient of migration times of several markers *vs.* p*I* values. Although it is more accurate to assume linearity over a smaller pH range, there is still no guarantee that the calculated p*I* is the true p*I* of the protein.

Huang *et al.* [82] studied transitional isoelectric focusing processes, *i.e.* the transitional double peak effect during focusing (two zones consisting of the same compound are formed at opposite ends of the capillary and migrate towards each other during focusing to create one peak) and the logarithmic increase in focusing time with increasing CA concentration.

A different problem in relation to the pH gradient is the separation of extremely acidic and alkaline proteins. Instead of whole column injection and subsequent focusing, Yang *et al.* [83] injected a plug of extremely alkaline or acidic proteins at the inlet of a pre-established pH gradient. Since the p*I* values of the proteins were outside the pH range of the gradient, the compounds migrated through the capillary to the opposite electrode. During this so-called pH gradient driven electrophoresis (PGDE), the charge-to-mass ratio constantly changes and the proteins will be separated and forced into narrow zones. Compared to CZE, lower limits of detection can be obtained due to the higher sample volume and concentrating effect during mobilization. Furthermore, when performing PGDE in combination with cIEF (in both anodic and cathodic direction), proteins with p*I* values in the entire pH range can be separated.

Like in the system of Wu *et al.* [60,61], Chen *et al.* [84] injected the sample electrokinetically from one reservoir. After focusing, the analytes were hydrodynamically mobilized to a UV detector instead of injected into a second CE mode. A disadvantage of electrokinetic injection is the injection bias, *i.e.* the injected amount is not the same for each compound.

2.3   Detection

Whole column imaging

UV

The mostly used detection method in cIEF is fixed point UV detection. Although very reliable, mobilization of the focused proteins to the detector is necessary, which leads to a decrease in separation efficiency. In whole column imaging

(WCI), detection takes place over the entire length of the column and high resolution is preserved since mobilization is not necessary. Resolution is determined by the focusing efficiency and the size of the detection array (typically a CCD camera). WCI also decreases analysis time considerably. However, for coupling to other detection systems like MS, mobilization is required. Wu and Pawliszyn developed a whole column imaging system specially designed to perform rapid cIEF [85], which was further developed and is now commercialized by Convergent Bioscience (Toronto, Canada) under the name iCE 280 Analyzer.
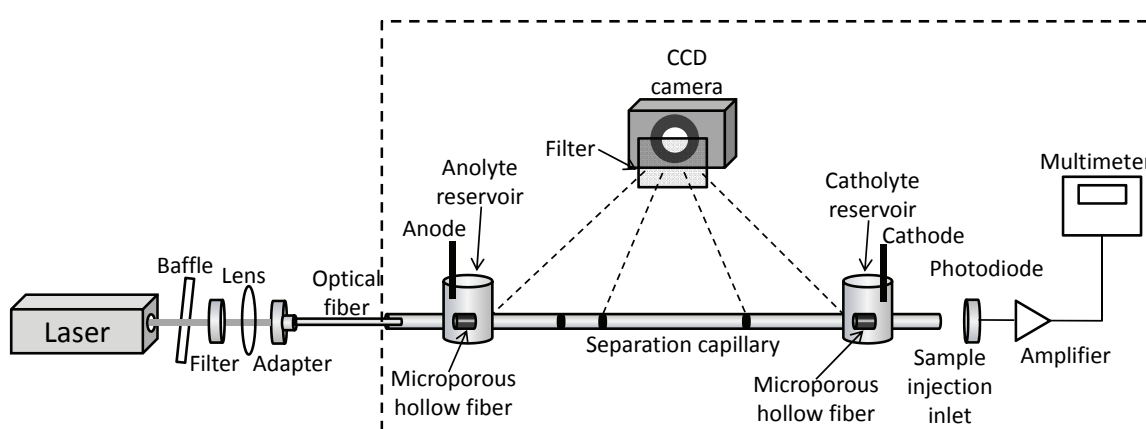
Figure 2.4. Schematic setup of the cIEF-LIF-WCI system [94].

The original UV setup, which is similar to the fluorescence setup shown in Figure 2.4, consists of in- and outlet capillaries which are connected to the separation capillary (5 cm, 100 µm ID, typically fluorocarbon coated) via hollow fiber membranes that allow the content of the capillary to be in contact with the electrolyte solutions (vials). After whole column injection, an electrical field is applied between these anolyte and catholyte solutions and focusing occurs. The whole process is recorded every 40s by a CCD camera which collects the transmitted light that has passed through the capillary, although the imaging interval can be as short as 10s. This UV-WCI-cIEF system has proven its applicability over the last four years in methodological cIEF studies [81], coupling to SPME [86], protein analysis [87-92] as well as p*I* determination of micro-organisms [91,93].

Fluorescence

To improve the detection limit of the WCI system, an argon ion laser was placed axially to the separation capillary and a CCD camera imaged the fluorescence.

This laser induced fluorescence whole column imaging (LIF-WCI) was first described by Liu and Pawliszyn (Figure 4) [94]. This group extensively used this system to analyze labeled proteins [90,94-96], cells [91], and microorganisms [96,97] (an example is shown in Figure 2.5), for interaction studies [95-99] and coupling to SPME [100].
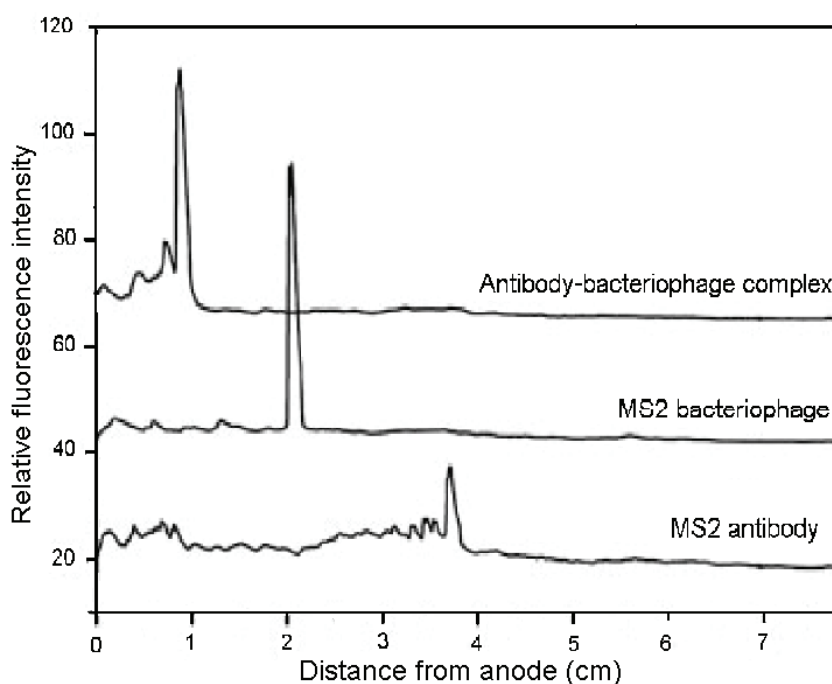
Figure 2.5. cIEF-LIF-WCI electropherograms (3-10 pH gradient) of NanoOrange labeled anti-MS2 IgG (10µg/mL), MS2 bacteriophage (1.25 x 10⁵ pfu/mL) and antibody-bacteriophage complex (10µg/mL anti-MS2 IgG plus 1.25 x 10⁵ pfu/mL MS2 bacteriophage) [97].

A quite different system for LIF-WCI was presented by Ren *et al*. [101], who placed the separation channel of a microfluidic PDMS/glass chip over an array of organic light emitting diodes (OLEDs). During focusing, these lined OLEDs were programmed to light up after each other and only excite the compounds in the channel directly above. The fluorescence was detected by a photon multiplier tube (PMT). The signal intensity and time were recorded and related to the position in the channel. The simplicity of the system and absence of mechanical parts, lenses and optical fibers in combination with the higher sensitivity of the single point PMT detection compared to a CCD camera, makes this system especially suited for miniaturization. The principle of this OLED system was demonstrated with a R-phycoerythrin sample. A different approach to microchip cIEF-LIF-WCI was presented by Herr *et al*. [102], who imaged the cross-interface of two orthogonally placed channels with a CCD camera. cIEF was performed in

one of the channels and the separated compounds were electrophoretically mobilized to the catholyte. When a compound of interest reached the cross interface, a voltage difference was placed over the electrodes of the second channel, thereby electrokinetically injecting the analytes into a CZE system. This 2D system was estimated to have a peak capacity of approximately 1300.In the previously described system of Knittle et al. [71] in which labeled antibodies were flushed through a capillary coated with cIEF separated proteins, three lasers of different wavelengths were used to perform fluorescence detection. Instead of moving the laser beam over the capillary, the capillary was moved along its axis to allow scanning of the whole column. An array of up to 60 parallel capillaries with WCI-LIF detection was devised by Mao and Zhang [103]. The capillaries were assembled on a plastic holder, polyimide was stripped and RPLC prefractionated liver cancer tissue samples were injected onto this cIEF-LIF-WCI system, resulting in an immense amount of data and a total peak capacity of approximately 18,000.

Fluorescence

As mentioned above, Horká *et al.* [70,74-76] used fluorescence detection to increase sensitivity of their system for the analysis of microorganisms and proteins. The capillary wall was coated with PB-PEG, which was also used as fluorescent label for the compounds. In the same group, Kostal *et al.* [104] developed a system in which the Teflon AF (DuPont) coated capillary not only acted as the separation channel, but also as a liquid core waveguide. This coated capillary is attached via a PMMA T-split interface to an optical fiber which is connected to a CCD camera. Excitation of the labeled proteins is accomplished with an argon ion laser. This setup minimizes detection of background scatter and offers the possibility of miniaturization. Unfortunately the sensitivity does not exceed that of the currently existing LIF detectors (10^{-9} M compared to 10^{-18} M for the sophisticated LIF detectors [104]).

Mass spectrometry

Several attempts have been made to couple cIEF to a mass spectrometer, which is usually done via an ESI or MALDI ionization source. A review of Wehr [105] discusses the problems and possible solutions when hyphenating cIEF with MS

until 2004. Setups combining cIEF via an RPLC system to MS will be discussed in the section on multidimensional systems.

Electrospray ionization (ESI)

Although ESI offers the possibility of coupling cIEF on-line to the MS, it suffers more from the signal suppression compared to MALDI, the other commonly used technique with cIEF. Storms *et al*. [59] already fathomed this phenomenon and eliminated the ampholytes from the cIEF system, which led to transient IEF and was characterized by the lack of correlation between the elution time and theoretical p*I* of the peptides. In proteomic approaches, 159 proteins from a digested periplasmatic protein extract (*E. coli*) were identified with this system [106]. Furthermore, this setup was used as an analytical tool in proteolytic [18]O labeling differential display studies [107].

Simpson *et al*. [108] used cIEF-ESI-FTICR-MS for the comparison of two different bottom-up proteomic approaches: SEC-RPLC-FTICR-MS and RPLC-cIEF-FTICR-MS. In both setups, the first dimension was used for fractionation of the *Shewanella oneidensis* cell lysate. In the second dimension these fractions were on-line analyzed with either RPLC-MS or cIEF-MS. The authors ascribe the lower protein coverage of the RPLC-cIEF-FTICR-MS system to the lower recovery of the prefractionation step (RPLC) and the lower amount of sample used for the cIEF-MS experiments. However, in the cIEF-MS setup, 0.5% ampholytes, which causes signal suppression, were used, which may also be part of the explanation for the lower amount of intact proteins found back in the cIEF-MS approach.

A validation study for the analysis of proteins with cIEF-MS was presented by Kuroda *et al*. [109]. A cIEF-ESI-TOF-MS setup was used for the determination of linearity, precision and accuracy of human transferrin. Although the data suggest that cIEF-MS can be used for quantitave analysis, not enough experiments were carried out to get really convincing results.

Matrix-assisted laser desorption ionization (MALDI)

Conventional cIEF was coupled with MALDI-TOF-MS for the analysis of human blood serum by Crowley and Hayes [110]. Using reverse polarity cIEF, the proteins were separated and deposited on a MALDI target plate via a spotting device, with ammonium hydroxide as sheath liquid catholyte. A total amount of 160 peaks were identified. However, conditions were not optimal since relatively

high ampholyte concentrations (2.5%) and low sampling rates (1 spot *per* 2 min) were used. Therefore the amount of proteins found is expected to be higher in a more optimal setup.

Yu *et al.* [111] fractionated a rat liver tissue extract with RPLC and subsequently separated the fractions with cIEF before deposition on MALDI target plate especially designed for collection, washing and digestion. A hydrophobic C18 layer coated with SE-30 allowed adsorption of the proteins on the plate, while the ampholytes could be washed off, resulting in less signal suppression. Furthermore, digestion was performed by depositing a trypsin solution on the spots, thereby allowing protein identification. The result of analyzing rat liver tissue with this setup is shown in Fig. 2.6, where 376 proteins were identified. Mok *et al.* [112] presented a pseudo-closed PMMA microfluidic device for cIEF-MALDI-TOF-MS.
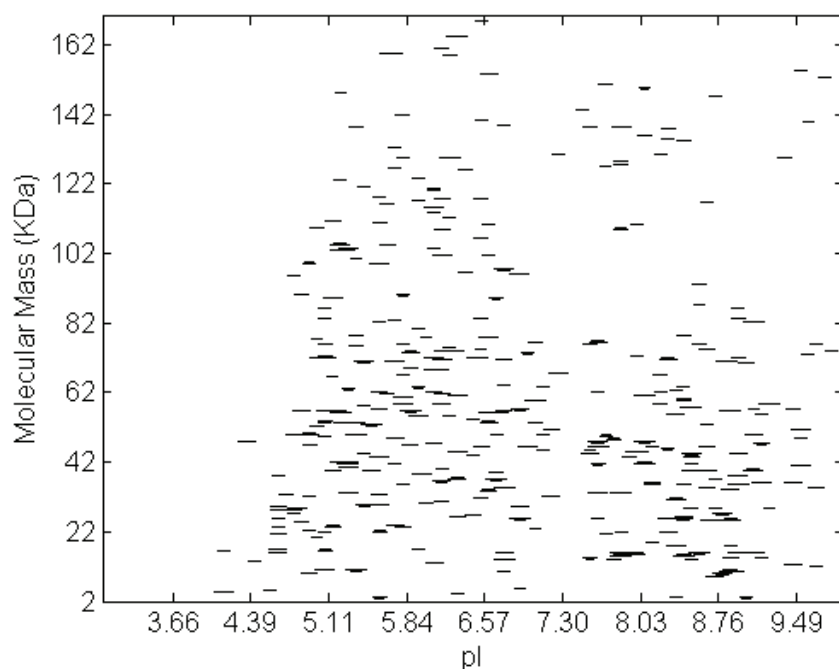
Figure 2.6. Reconstructed 2D map of rat liver tissue extract after RPLC-cIEF-MALDI-TOF analysis with on-plate digestion [111].

This microfluidic device, consisting of only an open separation channel and two reservoirs (for the anolyte and catholyte solution, and the electrodes), was used for cIEF separation. After focusing, the voltage was maintained until the solvent was evaporated. Subsequently, the MALDI matrix was deposited on the channel, the device was placed directly inside the MALDI-source and the complete

separation channel was scanned, performing WCI-MALDI-TOF-MS. Guo *et al.* [113] continued these experiments and tested five different polymer resins for the production of this UV-embossed microfluidic chip.Polyester chips gave the best results with respect to signal intensity and signal-to-noise ratio while the EOF was negligible. Furthermore, matrix deposition was improved by electrospraying the matrix onto the channel. This resulted in a decrease of dispersion of the proteins in the matrix. Unfortunately, no reconstructed cIEF electropherogram of these improvements was presented.
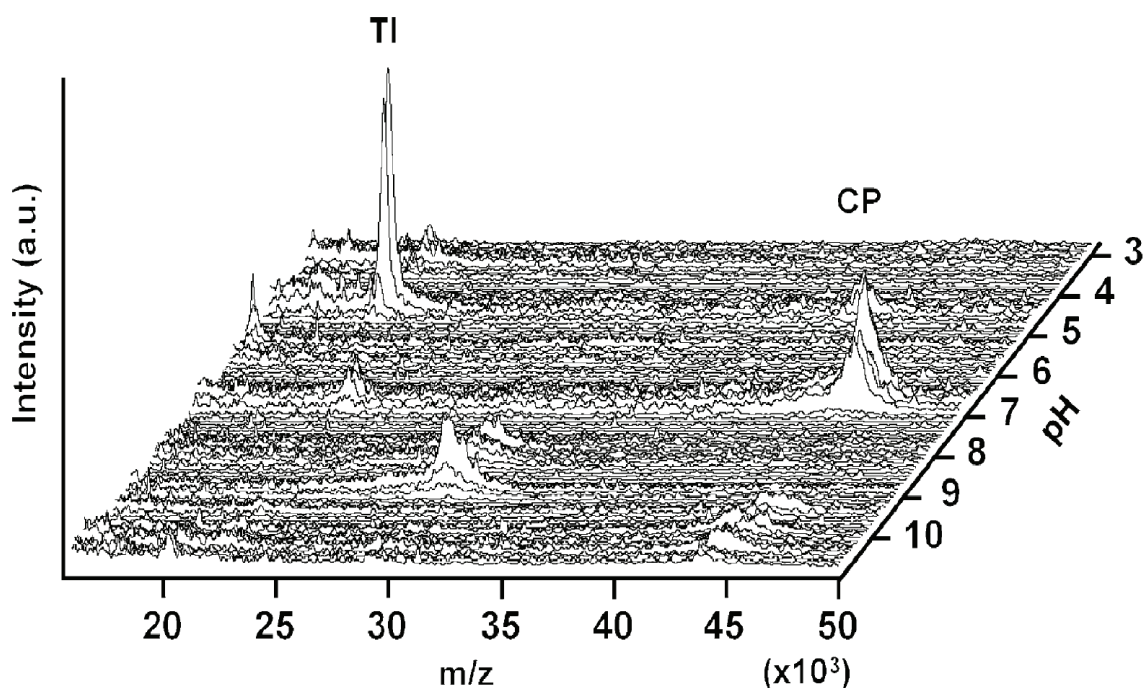
Figure 2.7. Reconstructed 2D map of creatinin phosphokinase (CP) and trypsine inhibitor (TI) separated with a microfluidic cIEF device coupled to MALDI-TOF-MS [114].

Fujita *et al.* [114] presented a different, but not less creative approach to couple cIEF to MALDI-TOF-MS. They performed cIEF in a microfluidic device with a removable resin tape on top. After focusing, the microchip was immediately frozen, the tape removed and the separated proteins freeze-dried before depositing the matrix solution and performing MALDI-TOF-MS. In 70 min, a complete 2D map of creatinine protease and trypsin inhibitor was produced (Fig. 2.7).

2.4 Multidimensional systems

cIEF-LC

Several attempts have been made to remove the ampholytes entering the MS after cIEF separation as described in the sections on special systems and MS. A different but elegant approach is to place a reversed-phase liquid chromatography (RPLC) system between the cIEF and MS systems. Although RPLC of proteins remains complicated, placing such a RPLC system in a cIEF setup introduces an additional separation dimension, offering the possibility of removing ampholytes and other interfering components thus injecting a fully compatible buffer into the MS. Furthermore, compared to 2D-PAGE, this would be a highly sensitive and high throughput approach for proteomics. Zhou *et al.* [115-117] produced such a cIEF-RPLC-MS system as is shown in Figure 2.8.
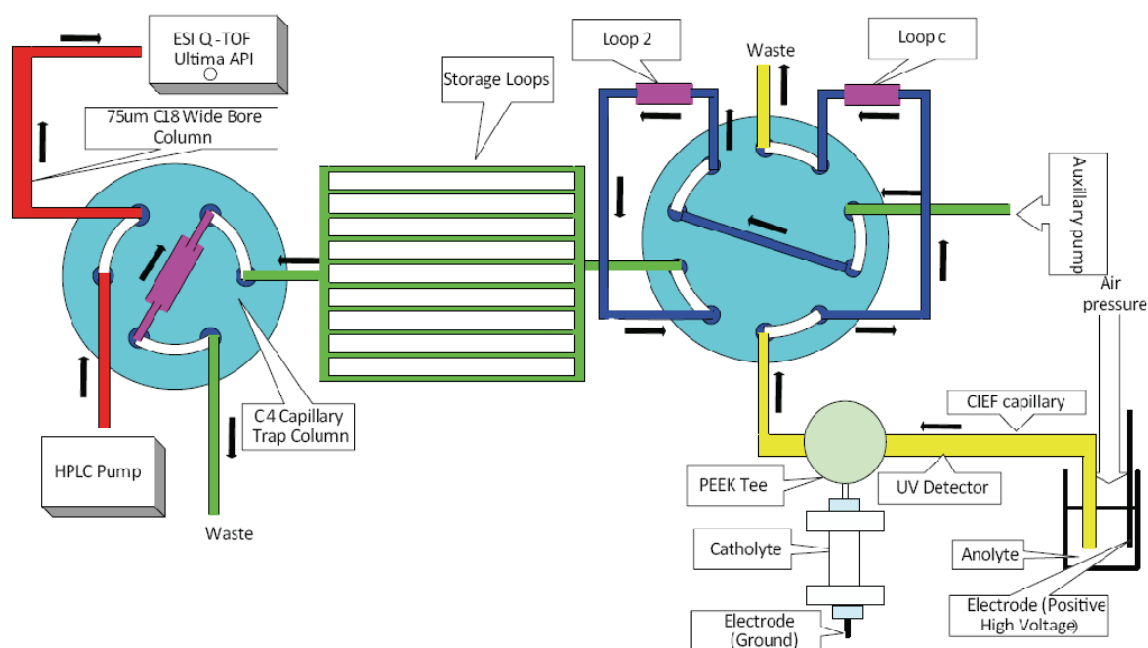
Figure 2.8. Setup of the cIEF-RPLC-ESI-Q-TOF system [117].

cIEF is performed between the anolyte and catholyte vial, whereafter the compounds are slowly mobilized via the T-split interface to the first valve. By frequently switching this valve, the sample is fractionated and transferred to the storage loops. Subsequently the fractions are mobilized to a trapping column present in the second valve which traps the proteins and washes out the ampholytes. Before injection into an ESI-Q-TOF-MS, proteins are further

separated on a RPLC separation column. This system was initially applied to the analysis and characterization of a standard protein mixture [115] but they have also shown its applicability for the analysis of yeast proteins [116] as well as for analysis of a *Chlorobium tepidum* extract [117].A similar system was developed in the group of Lee and Balgley [118] for the separation of a digest of a *Saccharomyces cerevisiae* extract. The difference between the two systems is that Lee and Balgley did not use a T-split interface, but directly placed the first valve in the cIEF system. Other applications of this group using this or a slightly modified system include the analysis of yeast proteins [119,120], micro dissected tumor tissue [121-123] and human saliva [124]. A review on the coupling of cIEF with RPLC-MS has also recently been published by Wang *et al.* [18].

Although the use of RPLC after the cIEF step removes the ampholytes, Kang and Moon [125] introduced an additional step into the system: hollow fiber flow field-flow fractionation (HFF-FFF). The cIEF system was directly coupled to this HFF-FFF step via a valve with a sample loop which collected the separated proteins before injection into the HFF-FFF system. This last step not only removed the ampholytes but also separated the proteins according to molecular weight. After the FFF step, the compounds were collected, digested and injected into a RPLC-MS/MS system, resulting in the identification of 114 proteins from a human urinary sample.

Instead of an RPLC step following the cIEF separation, RPLC can also be used as a prefractionation method in front of cIEF as already discussed in the sections on detection [103,126] and ESI [108].

cIEF-CEC

As an alternative to complicated setups in cIEF-RPLC platforms with rather long runtimes, the 2D microchip systems which are not yet fully applicable to proteomics applications and to setups combining cIEF with ampholyte-incompatible techniques, capillary electrochromatography (CEC) was hyphenated with cIEF via a nanoinjector valve as described by Zhang and El Rassi [127]. CEC offers the advantages of both CE and LC with regard to separation efficiency as well as compatibility with MS. The relatively simple setup, *i.e.* two capillaries interfaced with a valve, resulted in a theoretical peak capacity of approximately

55 thousand. Unfortunately, the authors did not present a full 2D map of a biological sample to demonstrate this peak capacity.

cIEF-CE

Besides the multidimensional microfluidic devices, that will be discussed in the section on microfluidic devices of this chapter, Mohan *et al*. [128] developed an on-line cIEF-CZE/ITP-ESI-FTICR-MS system for the analysis of the *Shewanella oneidensis* proteome. Coupling of the CE modes was accomplished with a simple dialysis membrane, removing the ampholytes before injection into the second dimension. They attributed the high proteome coverage (26.5% or 1174 identified proteins, a 2D map is shown in Figure 2.9) to the high sensitivity of the system: concentration in cIEF and ITP and high resolution and mass accuracy of the FTICR-MS.
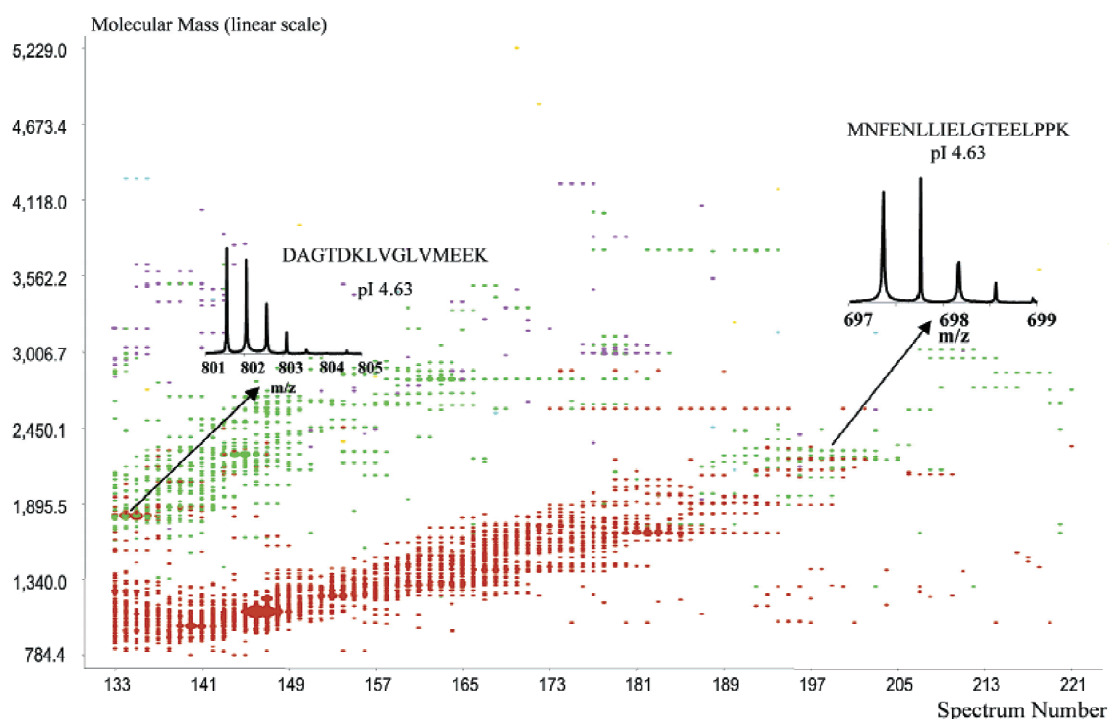
Figure 2.9. Reconstructed 2D map of the third cIEF fraction of a Shewanella oneidensis digest with cIEF-ITP/CZE-ESI-FTICR-MS analysis. The different colours represent different charge states: +2, red; +3, green; and +4, pink. The mass spectra of te peptides are depicted as inserts [128].

cIEF was also interfaced via a dialysis membrane with CGE by Yang *et al*. [129] for the separation of model proteins. The same system was also used for coupling to CZE in the analysis of ribonuclease [130]. Different approaches for interfacing cIEF to other techniques were used by Liu *et al*. who used a dialysis hollow-fiber

interface to hyphen cIEF and capillary non-gel sieving electrophoresis (cNGSE) [131] and an etched porous interface for cIEF-CZE [132]. cIEF-cNGSE resembles 2D-PAGE since the proteins are first separated with cIEF, moved to the hollow-fiber interface where they are complexed with sodium dodecylsulphate (SDS) before injection into a sieving matrix, which is in this case a dextran solution. The applicability was demonstrated for hemoglobin and a mixture of proteins excreted from rat lung cancer cells. However, as the authors conclude, identification of the compounds with MS, as next step common in 2D-PAGE, was unfortunately not possible due to the SDS.The on-column etched porous interface was prepared by treating a 5 mm polyimide-stripped piece of the capillary with HF. The relative simple preparation, but mainly the zero dead volume makes this interface more advantageously than *e.g.* valves or crosses. Liu and Pawliszyn [86] also used a hollow fiber microdialysis membrane to couple solid-phase microextraction (SPME) to cIEF-WCI, which was discussed in the section on whole column imaging. Model proteins desorbed from the SPME surface were electrophoretically moved into the microdialysis membrane chamber (18 kDa cut-off filter). Compounds larger than the pore size of the filter were trapped in this middle vial, which was also used as the inlet vial for the cIEF/CZE experiments. A similar setup was used to perform SPME-cIEF-LIF-whole column imaging detection (WCI) [100].

2.5   Microfluidic devices

Some of the microfluidic devices incorporating cIEF have already been mentioned in the sections on coatings [69] and detection [101,102,112-114]. A valve for pressed mobilization in a PDMS device was designed by Guillo et al. [133]. The microchip consists of two compartments: the separation channels and an area for valves. Applying a vacuum opens the valve, sucks the PDMS into the valve seats thereby allowing the valve to fill with fluid (diaphragm or membrane pumping). Flow rates and linearity of the flow rate were similar to conventional cIEF, thereby enabling the use of a single point (UV or MS) detector with hydrodynamic mobilization instead of either whole chip imaging or EOF mobilization. A PAA-coated PDMS microfluidic device for the integration of separation techniques with usually incompatible buffers was designed by Wang *et al.* [134]. In this paper, cIEF was coupled via some pressure-controlled microfluidic valves to a second dimension (high ionic strength) CZE or CGE. As can

be seen from Figure 2.10, the two separation channels are interconnected. This piece of common channel serves as separation channel for both modes and is the injection part of the second dimension. Fluid control is brought about by the two valve systems (1-2 and 3-4), which close the separation channel underneath when pressure is applied, thereby either performing IEF (1-2 closed), sample isolation (all four closed) or the second dimension separation step (3-4 closed).
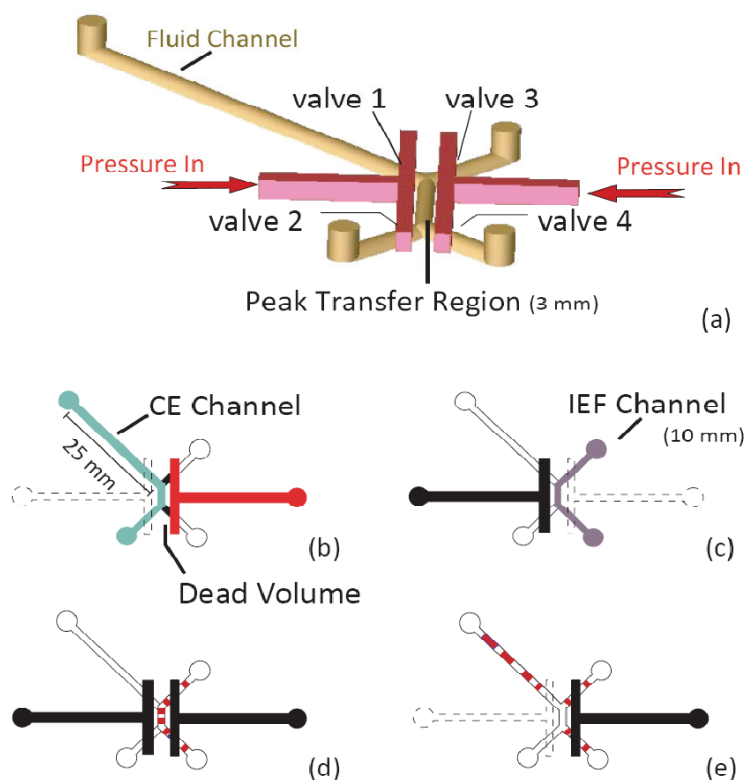
Figure 2.10. Schematic overview of the microfluidic PDMS 2D IEF separation system: (a) perspective view; (b) closing valve 3-4 while loading CE buffer; (c) closing valve 1-2 while loading IEF ampholytes mixture; (d) all valves closed for isolation of target compounds; (e) opening of valve 1-2 for injection of isolated compounds into second dimension CE system [134].

The flexibility of the PDMS in combination with pressure eliminates the use of moving mechanical parts. The combination IEF-CGE was also implemented on microchip by Griebel *et al*. [135]. In this setup, however, the first dimension consisted of an immobilized pH gradient (IPG) strip. The second dimension was orthogonally placed under the IEF cavity and comprised 300 parallel channels for performing CGE. The two dimensions were connected via a small opening, which acted as an injector to the second dimension. The injection into the second dimension was the most challenging part and the authors simulated the effect of different injection methods on parameters like peak broadening. Unfortunately, no experimental data were presented. Thormann *et al*. [136] developed a model to examine the effect of different capillary or channel materials on EOF for focusing as well as mobilization during cIEF. For materials with pH dependent surface charges (*e.g.* PMMA, PDMS and bare fused silica), the EOF decreased

with time during focusing. A steady state was reached where electrophoretic transport and electroosmotic zone displacement are opposite but equal. The process of reaching this steady state is predicted to be twice as long for PMMA and PDMS in comparison with fused silica. For chemical mobilization, a polybrene-polyvinyl(sulphonate) coating seems to be favorable since the EOF is independent of the p*H* and is fast enough to guarantee that the entire gradient passes the detector.

2.6   Applications

cIEF is especially advantageous in proteomics due to the preconcentration during focusing, the relatively mild conditions, the high peak capacity and the broad p*I* range over which proteins can be separated. However, for the analysis of real biological samples, cIEF alone is not sufficient and a second dimension separation is often necessary. These systems have already been described in previous sections, therefore the use of cIEF for the analysis cell lysates [91,106,108,116,117,119,128], tissue and matrix proteomics [83,103,110,111, 120-125,127,137], or interaction studies [99,138-141] is further presented in Table 2.2 and will not be discussed in this section. Next to that, the applicability of new systems mentioned throughout this paper is usually demonstrated by standard protein mixtures [58,142], or microorganisms in the case of the previously mentioned PEG coated capillaries used by Horká *et al* [70,72,73,75,76, 91,143,144]. These applications can also be found in Table 2.2. This table gives an overview of the cIEF papers published in the past four years and has been systematically ordered by application topic.

Table 2. Applications of cIEF (2003-2007).

Protein mixtures			
Standard protein mixture	cIEF-UV MALDI-TOF-MS	Effect ampholytes, detergents, cellulose derivatives	[56]
Standard protein mixture	cIEF-UV	cIEF in glycerol/water mixtures	[57]
Hydrophobic proteins	cIEF-UV	cIEF in glycerol/water mixtures	[58]
Digest of standard proteins	cIEF-ESI-Ion-trap-MS	CA free cIEF autofocusing of peptides	[59]
Standard protein mixture	cIEF-CZE, UV	CA free cIEF	[60]
Standard protein mixture	cIEF-UV	Continuous mode CA free cIEF for	[62]

		large volume injection	
Standard protein mixture	cIEF-UV	Study on reproducibility and protein	[66]
		absorption	
Standard protein mixture	cIEF-UV	PVP vs. PMMA coating	[67]
Standard protein mixture	cIEF-UV	HPC vs. CPA coating	[68]
Standard protein mixture	Microchip cIEF	Plasma-polymerized ACN vs. PDMS	[69]
		coating in microfluidic device	
Labelled proteins	cIEF-LIF-WCI and cIEF-chemiluminescence	Photo-immobilization of proteins to capillary wall after cIEF separation	[71]
Standard protein mixture	cIEF-UV	Zwitterionic surfactant coating	[77]
Labelled protein mixture	Dynamic IEF, Flu and MS		[80]
Standard protein mixture	cIEF-UV-WCI	Study of relative peak positions	[81]
Standard protein mixture	cIEF-UV-WCI	Study of transitional IEF processes	[82]
Extremely acidic and alkaline proteins	cIEF-UV		[83]
Standard protein mixture	cIEF-CZE-UV	CA free on bare fused-silica capillary	[61]
Standard protein mixture	cIEF-CZE-UV		[132]
Standard protein mixture	SPME-cIEF-UV-WCI and SPME-CZE-UV-WCI		[86]
Standard protein mixture	SPME-cIEF-LIF-WCI		[100]
Standard protein mixture	cIEF-WCI(UV)	Analysis of IET preseparated protein fractions	[87]
Standard protein mixture	cIEF-UV-WCI	Analysis of IET preseparated protein fractions	[88]
Standard protein mixture	cIEF-UV-WCI	Analysis of IET preseparated protein fractions	[89]
Standard protein mixture	cIEF-UV-WCI	Protein characterization	[90]
Standard protein mixture	cIEF-LIF-LCW-WCI		[94]
Standard protein mixture	cIEF-LIF-WCI		[96]
Standard protein mixture	cIEF-OLED-PMT-WCI	PMMA microfluidic device	[101]
Standard protein mixture	cIEF-CZE-LIF-CCD		[102]
Labelled standard protein mixture	cIEF-LIF-LCW-CCD		[104]
Digested protein mixture	cIEF-ESI Ion-trap MS/MS	Transient cIEF	[106]
Digested protein mixture	cIEF-ESI Iontrap MS/MS	Transient cIEF for proteolytic ^{18}O incorporation studies	[107]
Protein mixture	cIEF-ESI-TOF-MS		[109]
Standard protein mixture	cIEF-MALDI-TOF-MS	Microfluidic device	[112]
Standard protein mixture	cIEF-MALDI-TOF-MS	Microfluidic device	[113]
Standard protein mixture	cIEF-RPLC-ESI-Q-TOF-MS		[115]
Standard protein mixture	cIEF-CGE-UV		[129]
Ribonuclease solution	cIEF-CZE-UV		[130]
Aminoacid mixture	cIEF-LIF	Conventional and microchip	[133]
Standard protein mixture	cIEF-CZE and cIEF-CGE with fluorescence imaging	Microfluidic device	[134]
Standard protein mixture	cIEF-CGE with	PMMA microfluidic device	[135]

	fluorescence imaging	with on gel IEF	
Interaction studies			
MS2 virus and related antibodies	cIEF-WCI (UV and FLU)		[97]
Reactions of proteins	cIEF-WCI (UV and Flu)		[98]
Protein–DNA interactions	cIEF-WCI		[99]
Thermal stability and phospholipide protein interaction	cIEF-UV-WCI		[138]
BSA–tryptophan interaction	cIEF-UV-WCI		[139]
Phospholipid–protein interaction	cIEF-UV-WCI		[140]
PrPc antigen–antibody interaction study	cIEF-UV		[141]
Biopharmaceuticals			
Pharmaceutical formulations	cIEF-UV-WCI		[146]
rHUG-CSF	cIEF-UV		[145,143]
Isolated compounds			
F5cYs-MP-PEG(2000)-DPSE protein-lipopolymer conjugates	cIEF-WCI(UV)		[92]
G-proteins	cIEF and cIEF-WCI		[95]
CP and TI	cIEF-MALDI-TOF-MS	Microfluidic device	[114]
Isoforms transferrin	cIEF-UV		[137]
PTM cellobiohrolase	cIEF-UV		[147]
Carbamylated birch poll isoforms Bet v1a	cIEF-UV		[148]
Alfa-1-acidic glycoprotein	cIEF-UV		[149]
Myosin light chain phosphorylation	cIEF-LIF		[150]
Myosin light chain phosphorylation	cIEF-LIF		[151]
Hb and Hb Iowa	cIEF-UV		[152]
Antithrombin III	cIEF-UV		[153]
Biological matrices			
Hb and rat lung cancer cells excreted	cIEF-cNGSE-UV		[131]
Human blood serum	cIEF-MALDI-TOF-MS	Off-line coupling	[110]
Rat liver tissue extract	RPLC-cIEF-MALDI-TOFMS	On-plate digestion	[111]
Microdissected tumor tissue	cIEF-RPLC-ESI-ion-trap-MS	MS/MS	[121]
Microdissected tumor tissue (hydrophobic proteins)	cIEF-RPLC-ESI-ion-trap-MS	MS/MS	[122]
Microdissected tumor tissue	cIEF-RPLC-ESI-ion-trap-MS	MS/MS	[123]
Human saliva	cIEF-RPLC-ESI-ion-trap-MS	MS/MS	[124]
Human urine	cIEF-HFF-FF-UV	cIEF-HFF1FF followed by off-line	[125]
Human serum	cIEF-CEC-UV	RPLC-MS/MS	[127]
Cell lysates			
Velet antler extract	M-IPG-WCI	Monolithic ampholine-	[79]

		immobilized pH gradient column	
S. Cerevisiae digest	cIEF-UV	Continuous electrokinetic injection	[84]
Plant growth-promoting rhizobacteria lysate	cIEF-WCI (UV and Flu)		[91]
Shewanella oneidensis digest	cIEF-ESI-FTICR-MS	Lysate separated with RPLC and	[108]
		fractions digested	
Yeast enzyme concentrate	cIEF-RPLC-ESI-Q-TOF-MS		[116]
Chlorobium tepidum extract	cIEF-RPLC-ESI-Q-TOF-MS		[117]
Saccharomyces cerevisiae digest	cIEF-RPLC-ESI-ion-trap-MS		[118]
Yeast proteins	cIEF-RPLC-ESI-ion-trap-MS		[119]
Yeast proteins	cIEF-RPLC-ESI-ion-trap-MS		[120]
Yeast cytosol and BSA tryptic digest	RPLC-cIEF-LIF-WCI		[126]
Shewanella oneidensis digest	cIEF-ITP/CZE-ESI-FTICR		[128]
Microorganisms			
E. coli, C. albicans, S. epidermidis, E. faecalis	cIEF-UV cIEF-Flu	Sol–gel PDMS capillaries	[70]
E. coli CCM 3954, different Candida species, CCM 8223, P. vulgaris, K. pneumoniae, St. aureus CCM 3953, S. agalactiae CCM 6187, E faecalis CCM 4224 and S. epidermidis CCM 4418	cIEF-UV	PEG coated capillary	[72]
S. cerevisiae CCM 8191, E. coli CCM 3954, different candida species, S. aureus, S. agalactiae CCM 6187, E. faecalis CCM 4224, S. epidermidis CCM 4418 and S. maltophilia	cIEF-UV	PEG coated capillary	[73]
Pseudomonas species, Agrobacterium tumefaciens, including A. vitis, Xanthomonas arboricola pv. juglandis, X. campestris pv. Zinniae, and Curtobacterium sp.	cIEF-Flu	Pyrenebutanoate-PEG coated capillary	[74]
Candida species, Geotrichum candidum, S. cerevisiae, T. asahii and Y. lipolytica	cIEF-Flu	Pyrenebutanoate-PEG coated capillary	[75]
E. coli CCM 3954, S. epidermidis CCM 4418, P. vulgaris, E. faecalis CCM 4224, S.maltophilia. Strains of Candida species, and S. cerevisiae	cIEF-Flu	Pyrenebutanoate-PEGcoated capillary	[76]
Plant growth-promoting rhizobacteria	cIEF-WCI(UV and Flu)		[91]

Norovirus like particles	cIEF-WCI(UV)		[93]
E. coli, S. epidermidis, C. albicans, bacteriophage Phi X 174	cIEF-UV and fluorescence	Pyrenebutanoate-PEG coated capillary	[143]
Biofilm-positive and negative S. epidermidis strains	cIEF-UV	PEG coated capillary	[144]
Other studies			
UV absorbing and fluorescent CAs	IET ,cIEF-WCI		[55]
Humic acids in soil	cIEF-UV		[154]

The use of conventional cIEF-UV setups has not been mentioned above. The major application of these systems, and for cIEF in general, are pharmaceutical proteins. Although not many papers on this subject have been published over the past few years, biopharmaceutical and biotechnological companies are steadily implementing cIEF-UV (either with WCI or not). Zhou *et al*. [145] characterized recombinant human granulocyte colony stimulating factor by using the Beckman cIEF kit. They found three peaks for this protein with cIEF (Fig. 2.11), whereas only one peak was observed in CZE experiments demonstrating the power of cIEF for purity control.

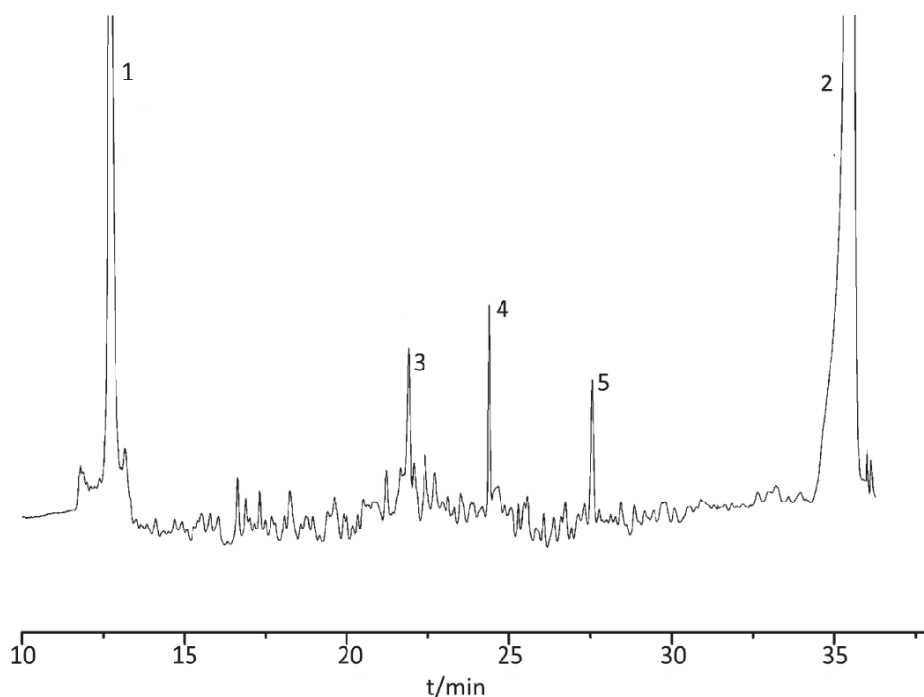*Figure 2.11. CIEF electropherogram of non-glucosylated rHuG-CSF in bulk sample. Peaks 1 and 2 are protein marker peaks (respectively ribonuclease A pI = 9.45 and CCK flanking peptide pI = 2.75). Peaks 3-5 represent non-glycosylated rHuG-CSF [145].*

The use of high-throughput cIEF devices like the iCE280 Analyzer is very successful in the biopharmaceutical industry for the determination of identity, heterogeneity and purity of proteins. Li *et al.* [146] used this device for the determination of p*I* and charge-related species (isoforms) in formulation development of protein therapeutics (glycosylated (IgG), non-glycosylated (Genotropin) and pegylated (PegIntron)) and found a high reproducibility for both p*I* as well as peak area of the tested compounds.A slightly different field displaying interest for cIEF, is the field of proteins isolated from biological matrices for the purpose of characterizing metabolites and biomarkers as is shown by Sandra *et al.* [147] for the study of glycoforms and phosphorylated forms of cellobiohydrolase 1 from *Trichoderma reesei*. cIEF was besides MS, nano-LC and CZE one of the techniques used and proved to be powerful in separating phospho-isoforms. Birch pollen allergen Bet v 1a was characterized with CZE and cIEF by Kronsteiner *et al.* [148]. They optimized a cIEF-UV system with a combination of broad-range and narrow-cut ampholytes for optimal resolution between carbamylated isoforms. Ampholytes were also mixed by Lacunza *et al.* [149] who developed a cIEF-UV method for the analysis of different forms of α-1-acid glycoprotein (AGP). Furthermore, the effect of different wall coatings (commercially available PVA and N-CHO coated), salt, focusing times and type of mobilization were evaluated, resulting in a reproducible analysis method for these rather acidic compounds. Typical electrophorograms for standard AGP as well as AGP from ovarian cancer are depicted in Fig. 2.12.
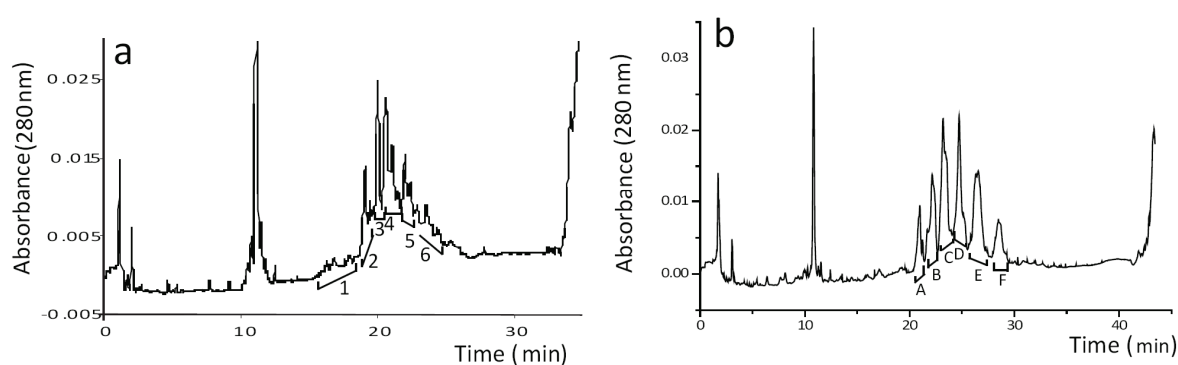

Figure 2.12. cIEF electropherograms of AGP standard (a) and AGP from ovary cancer cell sample (b). The reference trypsin inhibitor peak is marked with an arrow [149].

Betgovargez *et al.* [137] performed cIEF on a fraction of human plasma in search for isoforms. This fraction was obtained by chromatofocusing and was also analyzed with SDS-CGE. Both approaches led to the conclusion that the

transferrin present in the fraction had multiple isoforms. To determine the importance of myosin light chain phosphorylation in the muscle motion and contraction, a cIEF-LIF method for the determination of myosin light chain as well as its (multiple) phosphorylated forms was developed by Shiraishi *et al.* [150,151]. cIEF-LIF was found to be linear over the range of 50 amol-150 fmol and 3000 times more sensitive than the other methods tested (PAGE with western blotting, phosphorylation with [γ-^{32}P]ATP followed by both Cerenkov counting and SDS-PAGE, autoradiography and scanning densitometry). In a case report on a rare blood disease by Somjee *et al.* [152] an unknown hemoglobin variant was found next to Hb C. This unknown variant was later identified by DNA sequence analysis of the beta-globin gene as the extremely rare Hb Iowa. Kremser et al. [153] used cIEF next to other methods to characterize different isoforms of glycoprotein antithrombin III. p*I* determination offered similar results as gel IEF but quantification of gel isolated proteins was only carried out with CZE instead of cIEF. A different sample matrix*, i.e.* soil, was analyzed with cIEF for the determination of humic acids by Kovacs [154] with improved resolution and a higher number of fractions eluting from cIEF.

2.7 Conclusion

Looking at the progress of cIEF over the past years, we can see that although a number of the mysteries of cIEF has been solved, the study and optimization of the technique is still ongoing. Latest breakthrough in that area is the unravelment of the ampholyte composition. Besides that, cIEF is not only being used for separation, but cIEF as a sample pretreatment or preconcentration step for a second separation system like CZE is also becoming more popular. On the detection part of cIEF, the attempts of hyphenation with mass spectrometry are steadily ongoing. Whole column imaging, however, has made the technique very attractive in GLP and GMP controlled environments since it considerably increases repeatability by eliminating the need for mobilization.

For the applications, cIEF has established itself in two main areas. In the proteomics field, cIEF is still considered a high potential technique. However, real biological samples are often too complex to be analyzed by cIEF alone. Multidimensional analysis platforms, either conventional or miniaturized, are necessary. Gradually the challenges of coupling more systems are being solved, especially cIEF-LC-MS systems show a high degree of applicability in this area.

Besides the proteomics field, cIEF is very interesting for the pharmaceutical industry. With the increasing production of biotechnologically produced pharmaceutical proteins, reliable and high throughput cIEF systems like the iCE 280 are often applied. cIEF is gaining more and more fans besides the happy few who have been focusing on (and believing in) this technique since its birth.

References

[1]   H. Svensson, Acta Chem Scand 15 (1961) 325.

[2]   H. Svensson, Acta Chem Scand 16 (1962) 456.

[3]   H. Svensson, Acta Chem Scand 1(Suppl.) (1962) 132.

[4]   O. Vesterberg, T. Flatmark, Acta Chem Scand 20 (1966) 1497.

[5]   O. Vesterberg, Acta Chem Scand 21 (1967) 206.

[6]   O. Vesterberg, H. Svensson, Acta chem Scand 20 (1966) 820.

[7]   S. Hjertén, M.D. Zhu, J. Chromatogr. A 346 (1985) 265.

[8]   J.R. Mazzeo, I.S. Krull, Biotechniques 10 (1991) 638.

[9]   X. Liu, Z. Sosic, I.S. Krull, J. Chromatogr. A. 735 (1996) 165.

[10]   T.J. Pritchett, Electrophoresis 17 (1996) 1195.

[11]   P.G. Righetti, A. Bossi, C. Gelfi, J. Capillary Electrophor. 4 (1997) 47.

[12]   P.G. Righetti, C. Gelfi, M. Conti, J. Chromatogr. B 699 (1997) 91.

[13]   R. Rodriguez-Diaz, T. Wehr, M.D. Zhu, Electrophoresis 18 (1997) 2134.

[14]   X.H. Fang, C. Tragas, J.Q. Wu, Q.L. Mao, J. Pawliszyn, Electrophoresis 19 (1998) 2290.

[15]   K. Shimura, Electrophoresis 23 (2002) 3847.

[16]   F. Kilár, Electrophoresis 24 (2003) 3908.

[17]   P.G. Righetti, J. Chromatogr. A. 1037 (2004) 491.

[18]   Y.J. Wang, B.M. Balgley, C.S. Lee, Expert Rev. Proteomics 2 (2005) 659.

[19]   P.G. Righetti, Electrophoresis 27 (2006) 923.

[20]   P.G. Righetti, J. Chromatogr. A 1079 (2005) 24.

[21]   V. Dolník, S.R. Liu, J. Sep. Sci. 28 (2005) 1994.

[22]   H. Wätzig, S. Gunter, Clin. Chem. Lab. Med. 41 (2003) 724.

[23]   F.N. Lamari, N.K. Karamanos, Chromatographia 58 (2003) 349.

[24]   P.G. Righetti, Electrophoresis 25 (2004) 2111.

[25]   R. Haselberg, G.J. de Jong, G.W. Somsen, J. Chromatogr. A 1159 (2007) 81.

[26]   K. Ahrer, A. Jungbauer, J. Chromatogr. B .841 (2006) 110.

[27]   T. Manabe, J. Chromatogr. B 787 (2003) 29.

[28]   P.G. Righetti, A. Castagna, F. Antonucci, C. Piubelli, D. Cecconi, N. Campostrini, G. Zanusso, S. Monaco, Clin. Chem. Lab. Med. 41 (2003) 425.

[29]   M.R. Monton, S. Terabe, Anal. Sci. 21 (2005) 5.

[30]   M.R. Monton, S. Terabe, J. Chromatogr B 841 (2006) 88.

[31]   D.C. Simpson, R.D. Smith, Electrophoresis 26 (2005) 1291.

[32]   J. Hernandez-Borges, C. Neusüss, A. Cifuentes, M. Pelzing, Electrophoresis 25 (2004) 2257.

[33]   K. Hutterer, V. Dolník, Electrophoresis 24 (2003) 3998.

[34]   M. Moini, Anal. Bioanal. Chem. 373 (2002) 466.

[35]   S. Ma, Dev. Biol. (Basel) 122 (2005) 49.

[36]   V. Dolník, Electrophoresis 27 (2006) 126.

[37]   B.A. Fogarty, N.A. Lacher, S.M. Lunte, Methods Mol. Biol. 339 (2006) 159.

[38]   V. Kašička, Electrophoresis 24 (2003) 4013.

[39]   V. Kašička, Electrophoresis 27 (2006) 142.

[40]   M. Jenkins, S. Ratnaike, Clin. Chem. Lab. Med. 41 (2003) 747.

[41]   B. Wuyts, J.R. Delanghe, Clin. Chem. Lab. Med. 41 (2003) 739.

[42]   A. Bayol, A. Bristow, E. Charton, M. Girard, P. Jongen, Pharmeuropa Bio. 2004 (2004) 35.

[43]   R. Sebastiano, C. Simó, M.E. Mendieta, P. Antonioli, A. Citterio, A. Cifuentes, G. Peltre, P.G. Righetti, Electrophoresis 27 (2006) 3919.

[44]   C. Simó, M.E. Mendieta, P. Antonioli, R. Sebastiano, A. Citterio, A. Cifuentes, G. Peltre, P.G. Righetti, Electrophoresis 27 (2006) 4849.

[45]   C. Simó, M.E. Mendieta, P. Antonioli, R. Sebastiano, A. Citterio, A. Cifuentes, P.G. Righetti, Electrophoresis 28 (2007) 715.

[46]   C. Simó, A. Citterio, P.G. Righetti, Electrophoresis 28 (2007) 1488.

[47]   C. Simó, A. Citterio, P.G. Righetti, Electrophoresis 28 (2007) 3156.

[48]   P.G. Righetti, C. Simó, R. Sebastiano, A. Citterio, Electrophoresis 28 (2007) 3799.

[49]   N.B. Egen, M. Bliss, M. Mayersohn, S.M. Owens, L. Arnold, M. Bier, Anal Biochem 172 (1988) 488.

[50]   R.A. Mosher, W. Thormann, Electrophoresis 29 (2008) 1036.

[51]   W. Thormann, R.A. Mosher, Electrophoresis 29 (2008) 1676.

[52]   S. Lalwani, G. Vigh, Electrophoresis 26 (2005) 3.

[53]   S. Lalwani, E. Tutu, G. Vigh, Electrophoresis 26 (2005) 2503.

[54]   S. Lalwani, E. Tutu, G. Vigh, Electrophoresis 26 (2005) 2047.

[55]   R. North, A. Hwang, S. Lalwani, E. Shave, G. Vigh, J. Chromatogr. A 1130 (2006) 232.

[56]   L.H.H. Silvertand, J. Sastre Toraño, G.J. De Jong, W.P. Van Bennekom, Electrophoresis 29 (2008) 1985.

[57]   M. Poitevin, A. Morin, J.M. Busnel, S. Descroix, M.C. Hennion, G. Peltre, J. Chromatogr. A 1155 (2007) 230.

[58]   J.M. Busnel, A. Varenne, S. Descroix, G. Peltre, Y. Gohon, P. Gareil, Electrophoresis 26 (2005) 3369.

[59]   H.F. Storms, R. van der Heijden, U.R. Tjaden, J. van der Greef, Electrophoresis 25 (2004) 3461.

[60]   X.Z. Wu, L.H. Zhang, K. Onoda, Electrophoresis 26 (2005) 563.

[61]   L.H. Zhang, C.J. Zhang, X.Z. Wu, Anal. Sci. 22 (2006) 1039.

[62]   J. Budilova, J. Pazourek, P. Krasensky, J. Pospichal, J. Sep. Sci. 29 (2006) 1613.

[63]   E.A. Doherty, R.J. Meagher, M.N. Albarghouthi, A.E. Barron, Electrophoresis 24 (2003) 34.

[64]   V. Dolník, Electrophoresis 25 (2004) 3589.

[65]   C.A. Lucy, A.M. Macdonald, M.D. Gulcev, J. Chromatogr. A (2007).

[66]   M. Graf, H. Wätzig, Electrophoresis 25 (2004) 2959.

[67]   A. Palm, M. Zaragoza-Sundqvist, G. Marko-Varga, J. Sep. Sci. 27 (2004) 124.

[68]   L. Gao, S.R. Liu, Anal. Chem. 76 (2004) 7179.

[69]   S.W. Tsai, M. Loughran, A. Hiratsuka, K. Yano, I. Karube, Analyst 128 (2003) 237.

[70]   M. Horká, J. Planeta, F. Ružicka, K. Šlais, Electrophoresis 24 (2003) 1383.

[71]   J.E. Knittle, D. Roach, P.B. Horn, K.O. Voss, Anal. Chem. 79 (2007) 4978.

[72]   M. Horká, F. Ružicka, V. Hola, K. Šlais, Anal. Bioanal. Chem. 385 (2006) 840.

[73]   M. Horká, F. Ružicka, J. Horky, V. Hola, K. Šlais, J. Chromatogr. B. 841 (2006) 152.

[74]   M. Horká, J. Horky, H. Matouskova, K. Šlais, Anal. Chem. 79 (2007) 9539.

[75]   M. Horká, F. Ružicka, V. Hola, K. Šlais, Electrophoresis 28 (2007) 2300.

[76]   M. Horká, F. Ružicka, J. Horky, V. Hola, K. Šlais, Anal. Chem. 78 (2006) 8438.

[77]   K.K.C. Yeung, K.K. Atwal, H.X. Zhang, Analyst 128 (2003) 566.

[78]   R. Maheshwari, R. Bhavani, A. Dhathathreyan, J. Colloid Interface Sci. 293 (2006) 500.

[79]   C. Yang, G.J. Zhu, L.H. Zhang, W.B. Zhang, Y.K. Zhang, Electrophoresis 25 (2004) 1729.

[80]   R. Montgomery, X.G. Jia, L. Tolley, Anal. Chem. 78 (2006) 6511.

[81]   J.Q. Wu, T.M. Huang, Electrophoresis 27 (2006) 3584.

[82]   T.M. Huang, Z. Liu, J. Pawliszyn, Anal. Bioanal. Chem. 382 (2005) 783.

[83]   C. Yang, W.B. Zhang, J. Zhang, J.C. Duan, Y.K. Zhang, J. Sep. Sci. 28 (2005) 78.

[84]   J.Z. Chen, J. Gao, C.S. Lee, J. Proteome Res. 2 (2003) 249.

[85]   J.Q. Wu, J. Pawliszyn, Anal Chem 64 (1992) 219.

[86]   Z. Liu, J. Pawliszyn, Analyst 131 (2006) 522.

[87]   E. Shave, G. Vigh, Electrophoresis 25 (2004) 381.

[88]   E. Shave, G. Vigh, Electrophoresis 28 (2007) 2291.

[89]   E. Shave, G. Vigh, J Chromatogr A 1036 (2004) 3.

[90]   Z. Liu, T. Lemma, J. Pawliszyn, J. Proteome Res. 5 (2006) 1246.

[91]   Z. Liu, S.S. Wu, J. Pawliszyn, J. Chromatogr. A 1140 (2007) 213.

[92]   D.F. Nellis, S.L. Giardina, G.M. Janini, S.R. Shenoy, J.D. Marks, R. Tsai, D.C. Drummond, K. Hong, J.W. Park, T.F. Ouellette, S.C. Perkins, D.B. Kirpotin, Biotechnol Progr 21 (2005) 221.

[93]   L. Goodridge, C. Goodridge, J. Wu, M. Griffiths, J. Pawliszyn, Anal. Chem. 76 (2004) 48.

[94]   Z. Liu, J. Pawliszyn, Anal. Chem. 75 (2003) 4887.

[95]   J.M. Cunliffe, Z. Liu, J. Pawliszyn, R.T. Kennedy, Electrophoresis 25 (2004) 2319.

[96]   Z. Liu, J. Pawliszyn, Anal. Biochem. 336 (2005) 94.

[97]   Z. Liu, J. Pawliszyn, Electrophoresis 26 (2005) 556.

[98]   Z. Liu, J. Pawliszyn, J. Proteome Res. 3 (2004) 567.

[99]   Z. Liu, A.P. Drabovich, S.N. Krylov, J. Pawliszyn, Anal. Chem. 79 (2007) 1097.

[100]   Z. Liu, J. Pawliszyn, Anal. Chem. 77 (2005) 165.

[101]   K.N. Ren, Q.L. Liang, B. Yao, G.O. Luo, L.D. Wang, Y. Gao, Y.M. Wang, Y. Qiu, Lab Chip 7 (2007) 1574.

[102]   A.E. Herr, J.I. Molho, K.A. Drouvalakis, J.C. Mikkelsen, P.J. Utz, J.G. Santiago, T.W. Kenny, Anal. Chem. 75 (2003) 1180.

[103]   Y. Mao, Y. Li, X. Zhang, Proteomics 6 (2006) 420.

[104]   V. Kostal, M. Zeisbergerova, K. Šlais, V. Kahle, J. Chromatogr. A 1081 (2005) 36.

[105]   T. Wehr, LC GC North America 22 (2004) 998.

[106]   H.F. Storms, R. van der Heijden, U.R. Tjaden, J. van der Greef, J. Chromatogr. B 824 (2005) 189.

[107]   H.F. Storms, R. van der Heijden, U.R. Tjaden, J. van der Greef, Rapid Comm. Mass Spectrom. 20 (2006) 3491.

[108]   D.C. Simpson, S. Ahn, L. Paša-Tolić, B. Bogdanov, H.M. Mottaz, A.N. Vilkov, G.A. Anderson, M.S. Lipton, R.D. Smith, Electrophoresis 27 (2006) 2722.

[109]   Y. Kuroda, H. Yukinaga, M. Kitano, T. Noguchi, M. Nemati, A. Shibukawa, T. Nakagawa, K. Matsuzaki, J. Pharm. Biomed. Anal. 37 (2005) 423.

[110]   T.A. Crowley, M.A. Hayes, Proteomics 5 (2005) 3798.

[111]   W.J. Yu, Y. Li, C.H. Deng, X.M. Zhang, Electrophoresis 27 (2006) 2100.

[112]   M.L.S. Mok, L. Hua, J.B.C. Phua, M.K.T. Wee, N.S.K. Sze, Analyst 129 (2004) 109.

[113]   X. Guo, M.B. Chan-Park, S.F. Yoon, J.H. Chun, L. Hua, N.S.K. Sze, Anal. Chem. 78 (2006) 3249.

[114]   M. Fujita, W. Hattori, T. Sano, M. Baba, H. Someya, K. Miyazaki, K. Kamijo, K. Takahashi, H. Kawaura, J. Chromatogr. A 1111 (2006) 200.

[115]   F. Zhou, M.V. Johnston, Anal. Chem. 76 (2004) 2734.

[116]   F. Zhou, M.V. Johnston, Electrophoresis 26 (2005) 1383.

[117]   F. Zhou, T.E. Hanson, M.V. Johnston, Anal. Chem. 79 (2007) 7145.

[118]   J.Z. Chen, B.M. Balgley, D.L. DeVoe, C.S. Lee, Anal. Chem. 75 (2003) 3145.

[119]   Y.J. Wang, B.M. Balgley, P.A. Rudnick, E.L. Evans, D.L. DeVoe, C.S. Lee, J. Proteome Res. 4 (2005) 36.

[120]   W.J. Wang, T. Guo, T. Song, C.S. Lee, B.M. Balgley, Proteomics 7 (2007) 1178.

[121]   Y.J. Wang, P.A. Rudnick, E.L. Evans, J. Li, Z.P. Zhuang, D.L. DeVoe, C.S. Lee, B.M. Balgley, Anal. Chem. 77 (2005) 6549.

[122]   W.J. Wang, T. Guo, P.A. Rudnick, T. Song, J. Li, Z.P. Zhuang, W.X. Zheng, D.L. DeVoe, C.S. Lee, B.M. Balgley, Anal. Chem. 79 (2007) 1002.

[123]   T. Guo, W.J. Wang, P.A. Rudnick, T. Song, J. Li, Z.P. Zhuang, R.J. Weil, D.L. DeVoe, C.S. Lee, B.M. Balgley, J. Histochem. Cytochem. 55 (2007) 763.

[124]   T. Guo, P.A. Rudnick, W.J. Wang, C.S. Lee, D.L. DeVoe, B.M. Balgley, J. Proteome Res. 5 (2006) 1469.

[125]   D. Kang, M.H. Moon, Anal. Chem. 78 (2006) 5789.

[126]   Y. Mao, X.M. Zhang, Electrophoresis 24 (2003) 3289.

[127]   M.Q. Zhang, Z. El Rassi, J. Proteome Res. 5 (2006) 2001.

[128]   D. Mohan, L. Paša-Tolić, C.D. Masselon, N. Tolić, B. Bogdanov, K.K. Hixson, R.D. Smith, C.S. Lee, Anal. Chem. 75 (2003) 4432.

[129]   C. Yang, H.C. Liu, Q. Yang, L.Y. Zhang, W.B. Zhang, Y.K. Zhang, Anal. Chem. 75 (2003) 215.

[130]   C. Yang, L.Y. Zhang, H.C. Liu, W.B. Zhang, Y.K. Zhang, J. Chromatogr. A 1018 (2003) 97.

[131]   H.C. Liu, C. Yang, Q. Yang, W.B. Zhang, Y.K. Zhang, J. Chromatogr. B. 817 (2005) 119.

[132]   H. Liu, L. Zhang, G. Zhu, W. Zhang, Y. Zhang, Anal. Chem. 76 (2004) 6506.

[133]   C. Guillo, J.M. Karlinsey, J.P. Landers, Lab Chip 7 (2007) 112.

[134]   Y.C. Wang, M.N. Choi, J.Y. Han, Anal. Chem. 76 (2004) 4426.

[135]   A. Griebel, S. Rund, F. Schonfeld, W. Dorner, R. Konrad, S. Hardt, Lab Chip 4 (2004) 18.

[136]   W. Thormann, J. Caslavska, R.A. Mosher, J. Chromatogr. A 1155 (2007) 154.

[137]   E. Betgovargez, V. Knudson, M.H. Simonian, J. Biomol. Tech. 16 (2005) 306.

[138]   T. Bo, J. Pawliszyn, J. Sep. Sci. 29 (2006) 1018.

[139]   T. Bo, J. Pawliszyn, J. Chromatogr. A 1105 (2006) 25.

[140]   T. Bo, J. Pawliszyn, Electrophoresis 27 (2006) 852.

[141]   G.D. Li, X.J. Zhou, Y.H. Wang, A. El-Shafey, N.H.L. Chiu, I.S. Krull, J. Chromatogr. A 1053 (2004) 253.

[142]   J. Wu, X.Z. Wu, T. Huang, J. Pawliszyn, Methods Mol. Biol. 276 (2004) 229.

[143]   M. Horká, O. Kubicek, F. Ružicka, V. Hola, I. Malinovska, K. Šlais, J. Chromatogr. A 1155 (2007) 164.

[144]   F. Ružicka, M. Horká, V. Hola, M. Votavá, J. Microbiol. Methods 68 (2007) 530.

[145]   G.H. Zhou, G.A. Luo, G.Q. Sun, Y.C. Cao, X.D. Zhang, X. Zhang, J. Pharm. Biomed. Anal. 35 (2004) 425.

[146]   N. Li, K. Kessler, L. Bass, D. Zeng, J. Pharm. Biomed. Anal. 43 (2007) 963.

[147]   K. Sandra, I. Stals, P. Sandra, M. Claeyssens, J. Van Beeumen, B. Devreese, J. Chromatogr. A 1058 (2004) 263.

[148]   B. Kronsteiner, H. Malissa, Jr., H. Stutz, Electrophoresis 28 (2007) 2241.

[149]   I. Lacunza, J.C. Diez-Masa, M. de Frutos, Electrophoresis 28 (2007) 1204.

[150]   M. Shiraishi, R. Loutzenhiser, M.P. Walsh, Biophys J 86 (2004) 390a.

[151]   M. Shiraishi, R.D. Loutzenhiser, M.P. Walsh, Electrophoresis 26 (2005) 571.

[152]   S. Somjee, L.C. Yu, A.F. Hagar, J.M. Hempe, Hemoglobin 28 (2004) 7.

[153]   L. Kremser, A. Bruckner, A. Heger, T. Grunert, A. Buchacher, D. Josic, M. Allmaier, A. Rizzi, Electrophoresis 24 (2003) 4282.

[154]   P. Kovacs, J. Posta, Microchem. J. 79 (2005) 49.

Chapter 3

Improved repeatability and MALDI-TOF MS compatibility in cIEF

Abstract

Low repeatability of migration time, peak area and linearity (pI versus mobilization time) is a problem often encountered in cIEF and is mainly caused by protein precipitation and protein-wall interactions. In order to study the influence of these phenomena, the effect of different classes of additives on repeatability of migration time, peak area and linearity of a mixture of 7 model proteins has been investigated. Moreover, the influence of these additives on protein signal suppression in MALDI-TOF MS has been studied. The optimal ampholyte blend (stabilizes pH gradient) to be used depends on the selected UV detection wavelength. All tested ampholyte blends show a significant and comparable signal suppression in MS. The best detergent (to prevent precipitation and wall interaction) should be determined for each sample individually, but generally polyethylene oxide and zwitterionic detergents show good repeatability for migration time (RSD < 4.5%) and peak area (majority < 10%). The RSD of R^2 is < 1.3% for the hydrophilic protein mixture. However, these components cause severe signal suppression in MS. Therefore glucoside detergents should preferably be used for MS coupling. Viscosity-increasing agents (for hydrodynamic wall coating and to minimize diffusion) in particular cellulose derivatives, give good repeatability for migration times (RSD < 4.5% at lower concentrations), peak area (except for high concentration methylcellulose and hydroxyethylcellulose all within 7.5%), and correlation (pI vs. migration time), but severe signal suppression is observed in MALDI-TOF MS. Overall, cIEF repeatability and linearity can significantly be improved by adding the appropriate components. However, when the system is coupled to a MALDI-TOF MS, compromises have to be made between high repeatability and linearity on one hand and MS signal intensity on the other.

3.1 Introduction

After years of focusing on the elucidation of the human genome (genomics), the interest has shifted to the analysis of proteins, *i.e.* to proteomics. Although liquid chromatography (LC), surface plasmon resonance (SPR), immunoassays and capillary electrophoresis (CE) perform well in the study of proteins and peptides, the major technique for separating proteins in biological samples is still 2D-GE, in which the proteins are separated according to p*I* (IEF) followed by a separation

based on mass (SDS-PAGE). Although 2D-GE possesses unequalled power to resolve proteins, it is time-consuming and laborious. The need for automated, reproducible and high-throughput protein separation techniques is pressing. Therefore, CE techniques that can separate a wide variety of proteins within a single run and in a relative short time, are appealing. One of the CE techniques that has regained interest over the past years, is capillary isoelectric focusing (cIEF) [1-7]. cIEF combines the advantages of IEF (excellent resolution, high peak capacity and concentrating the compounds during analysis) with the possibility of automation and a significant reduction in analysis time.

In cIEF a fused-silica capillary is filled with the sample and carrier ampholytes (CAs) and the capillary ends are immersed in an acidic (anolyte) and alkaline (catholyte) solution. When applying a potential difference, a pH gradient is formed which is stabilized by the CAs. Proteins will migrate to the zone where their p*I* matches the local pH and will be concentrated (focusing). After completion of the focusing process, the proteins are mobilized to a detector, typically UV or MS. Mobilization is usually accomplished by adding components to the in- or outlet vials (chemical mobilization) or by applying a pressure difference between the ends (hydrodynamic mobilization). However, the silanol groups of the bare fused-silica wall should be shielded by coating the inner capillary with a neutral, hydrophilic polymer like polyacrylamide [8, 9], polyvinylalcohol [8, 10] or a cellulose derivative [8, 10, 11].

Despite the high peak capacity, analysis speed and automation, cIEF suffers from irreproducible migration times and peak areas, protein precipitation, protein-wall interaction and intolerance to higher salt concentrations (>10 mM) [1, 12-15]. Irreproducibility may be caused by many factors, but coating instability and protein-wall interactions seem to be the main causes [13]. These phenomena can be minimized by adding neutral, hydrophilic polymers like cellulose derivatives, which act as a dynamic wall coating thereby reducing the protein-wall interactions. An additional effect of the use of these polymers is the increase in viscosity, which decreases diffusion and peak broadening. Solubilizing agents like detergents prevent protein-protein and protein-wall interactions, thereby reducing protein precipitation and increasing repeatability. A number of papers has been published dealing with method development [5, 16-19] and optimization of sample handling [8, 16, 20-22] (*e.g.* effect of salts, use of detergents). However, a systematic study comparing the effects of ampholytes,

solubilizing agents and viscosity increasing agents on repeatability, correlation (p*I* *vs.* migration time) and resolution has not been published yet.

The coupling of cIEF with MS, as reported in [4-6, 21, 23-28], might be a very powerful tool in proteomics and is comparable to 2D-GE, since in both systems proteins are separated according to p*I* in the first dimension and *Mr* in the second dimension. However, for an efficient hyphenation, the effect of the cIEF additives in MS should be studied, since these compounds may have a negative influence on *e.g.* the efficiency of the ion formation in MS. Matrix-assisted-laser-desorption-ionisation time-of-flight mass spectrometry (MALDI-TOF MS) tolerates relatively high amounts of these additives. In MALDI, the protein mixture is spotted on a stainless steel target plate and the compounds are separated on base of their *m/z* ratio.

In this paper the effect of different concentrations and brands of carrier ampholytes, detergents and polymers on cIEF repeatability, separation efficiency and the main detection signals (UV and MS) is presented. The UV background signal as well as signal suppressing effects in MALDI of three commercially available brands of ampholytes have been determined. Furthermore, the effect of several solubilizing and viscosity-increasing agents on repeatability of migration time, peak area and peak height, on the correlation of p*I* *vs.* migration time (expressed as the correlation coefficient or R^2) as well as on signal suppression in MALDI has been studied.

3.2 Materials and methods

Materials

Pharmalyte™ (36% (${}^{w}/_{v}$), pH 3-10, copolymerisation of glycine, glycylglycine, various amines and epichlorohydrin) and Ampholine (for electrophoresis, pH 3.5-9.5, result of reaction of aliphatic oligoamines and acrylic acids) were purchased from Amersham Bioscience (Piscataway, NJ, USA). High Resolution (HR) ampholyte (~40% (${}^{w}/_{v}$), pH 3-10, mixture of numerous polyamino and polycarboxylic acids) was obtained from Sigma-Aldrich (Steinheim, Germany). Urea (ACS reagent, ≥ 99.5%), thiourea (reagent plus grade, ≥ 99.0%), CHAPSO (3-([3-cholamidopropyl]dimethylammonio)-2-hydroxy-1-propanesulfonate, for electrophoresis), SB3-12 (3-(N,N-dimethyldodecylammonio)propanesulfonate or

lauryl sulfobetaine, ≥ 98.0% (TLC)), Brij$^®$ 35 (polyoxyethylene(23) lauryl ether), Igepal® CA-630 (octylphenyl-polyethylene glycol or Nonidet™ P-40), DDM (n-dodecyl-ß-D-maltoside or lauryl-β-D-maltoside, ≥ 98.0% (GC)) and n-octyl glucoside (octyl β-D-glucopyranoside, ≥ 98.0% (GC)) were acquired from Sigma-Aldrich. Tween 20 (for synthesis) was obtained from Merck KgaA (Darmstadt, Germany). Hydroxypropylmethylcellulose (HPMC; ~ 4000 cP, 2% (w/v) in H_2O, 20°C), hydroxyethylcellulose (HEC, medium viscosity), methylhydroxyethyl-cellulose (MHEC; ~ 15,000-20,500 cP, 2% ($^w/_v$) in H2O, 20°C), methylcellulose (MC; ~ 400 cp, 2% (w/v) in H_2O, 20°C), dextran (Mr ~ 2,000,000), polyacrylamide (PAA; Mr ~ 10,000), sucrose, sorbitol and glycerol were purchased from Sigma-Aldrich. HPLC gradient grade acetonitrile (ACN) was purchased from Biosolve (Valkenswaard, The Netherlands). High purity water (Ultrapure > 18.2 MΩ*cm) was obtained from a Sartorius Arium 611 system (Sartorius AG, Göttingen, Germany). Proteins (see Table 3.1), phosphoric acid (85%), trifluoroacetic acid (>99%), sinapic acid (>99%), TEMED (~99%) and sodium hydroxide were purchased from Sigma-Aldrich.

Table 3.1. Specifications of the proteins used in this study.

#	Protein (abbreviation)	p*I*	Mr (kDa)	Source
1	Ribonuclease XII A (RNAse)	9.6	13.7	Bovine pancreas
2	Myoglobin (Myo)	7.3 and 6.8*	17.6	Equine skeletal muscle
3	Carbonic anhydrase II (CAII)	5.9	29	Bovine erytrhocytes
4	ß-Lactoglobulin B (LGB)	5.3	18.276	Bovine milk
5	ß-Lactoglobulin A (LGA)	5.15	18.363	Bovine milk
6	Glucose Oxidase II (GO)	4.2	160	Aspergillus niger
7	CCK precursor fragment 107-115 (CCK)	3.67 [44]	1.074	Human

*) Major (Myo I or 2a, pI 7.3) and minor (Myo II or 2b, pI 6.8) component.

Apparatus and methods

cIEF separations were performed with a Beckman P/ACE MDQ capillary electrophoresis system using Karat 32 Version 7.0 acquisition and processing software (Beckman Coulter, Fullerton, CA, USA), equipped with a UV detector (280nm, 8 Hz, unless indicated otherwise) and an eCAP neutral capillary (50 µm ID, 40.2 cm total length, Beckman Coulter). Prior to each run the capillary was rinsed with 10 mM phosphoric acid, water and a gel. This gel was used to

precondition the capillary and it consisted of half the concentration viscosity-increasing agent and the same concentration detergent as used in the sample. Subsequently, the capillary was filled with sample (RNAse (90 µg/mL), Myo, CAII, LGB (all at 10 µg/mL), LGA (16 µg/mL), GO (20 µg/mL), CCK (8 µg/mL), Pharmalyte^{TM} (1% $^w/_v$), TEMED (0.1% $^v/_v$), HPMC (0.5% $^w/_v$) and Tween 20 (1% $^w/_v$), unless indicated otherwise). The inlet vial (anolyte) was filled with 10 mM phosphoric acid in 0.5% HPMC, while the outlet vial (catholyte) was filled with 20 mM sodium hydroxide. Focusing was performed for 12-15 min at 20 kV and 22°C followed by mobilization at 20kV, 22°C and 0.5-1.0 psi air pressure.

In order to compare the viscosity-increasing agents in a more objective way, concentrations were chosen to give a similar viscosity as 0.5% HPMC at 1 psi and 22°C: a 50 µm ID bare fused-silica capillary was filled with 0.5% HPMC and a plug of formamide was injected at the inlet side. A pressure (1 psi) was applied and the time for the formamide plug to cover the distance to the detection window was recorded. For the other viscosity-increasing agents, the same procedure was carried out at different concentrations and the appropriate concentrations were selected for further experiments.

MALDI-TOF MS experiments were performed with an Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA). A protein (RNAse, Myo, CAII, LGA or LGB) was mixed with the additives, and diluted 1:10 with MALDI matrix (5 mg/mL sinapic acid in 80% $^v/_v$ ACN with 0.1% $^v/_v$ TFA), 0.75 µL of the mixture was manually spotted on a stainless-steel MALDI target plate. The *m/z* recording range was set to 5,000-30,000 with a focus *m/z* of 17,000 in linear mode. MALDI-TOF spectra were analyzed using Data Explorer software Version 3.0 (Applied Biosystems).

3.3 Results and discussion

Ampholytes

When performing cIEF, the sample is usually premixed with CAs. These CAs, a blend of zwitterionic compounds that stabilize the pH gradient, should possess a good buffering capacity and conductivity. They are required to 'carry' the pH and current, hence the term carrier ampholytes [7]. Furthermore, to form an almost continuous pH gradient, at least 20 different CAs per pH unit are required [29].

Buffering power, conductivity, mass distribution and linearity (measured vs. theoretical pH) of ampholyte blends have been investigated before [30-36]. Therefore, in this paper we will focus on the influence of three different blends of CAs on the detection: UV background absorbance and signal suppression in MALDI. Figure 3.1 depicts the UV absorbance of three CA blends, with (black trace) and without (grey trace) the protein mixture.
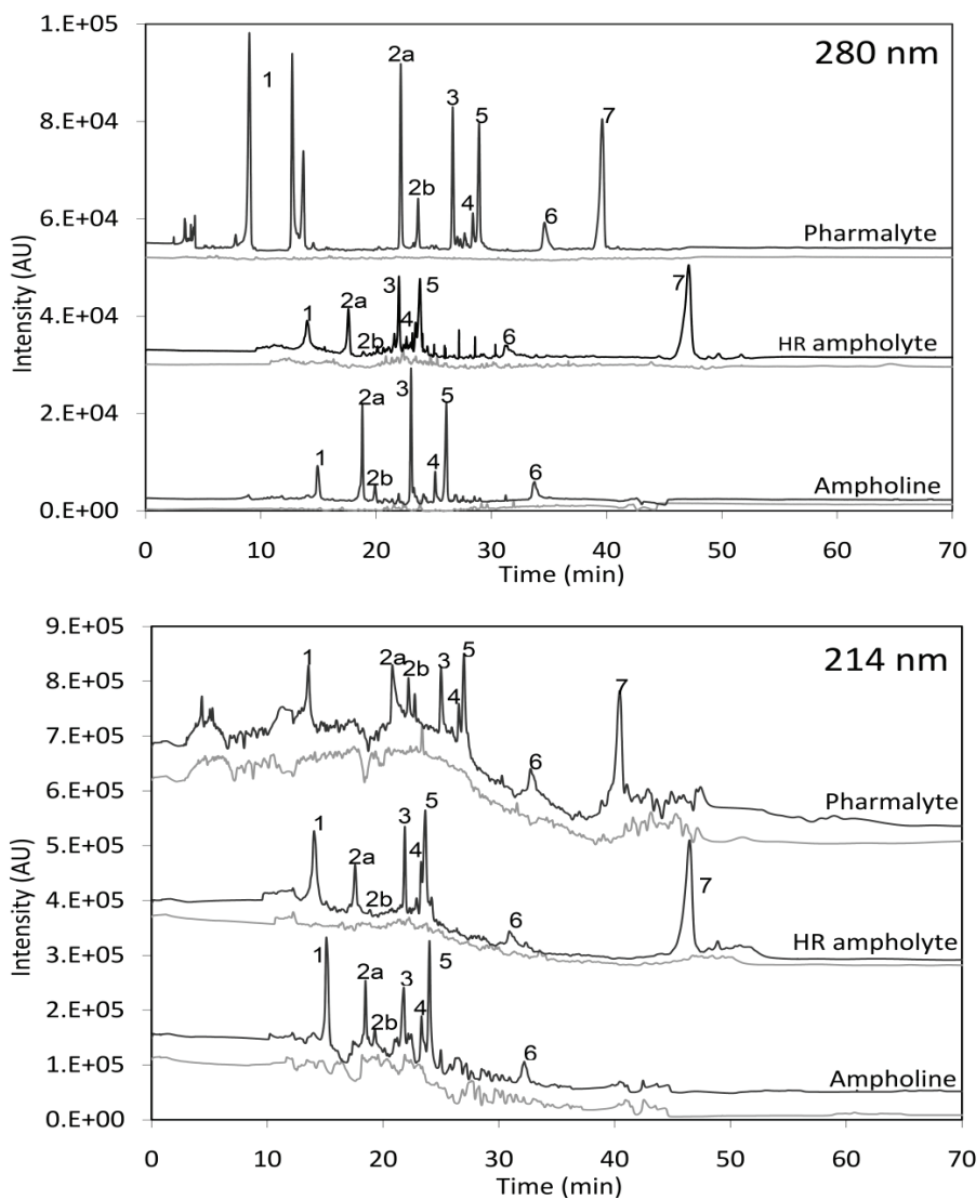
Figure 3.1. UV traces at λ=214nm (upper graph) and 280nm (lower graph) of Ampholine, High resolution ampholyte and Pharmalyte™, with (black trace) and without (grey trace) the protein mixture (all at a final concentration of 1% ʷ/ᵥ). On the x-axis, focusing time (12 min) and mobilization time is plotted. For clarity, the traces are stacked on the y-axis. The multiple peaks for RNase are due to the focusing process since RNase passes the detection window several times before focusing is complete.

Table 3.2. Protein peak height ratios (214nm/280nm, n=3), backgroundsignal ratios (214nm/280nm, n=3), resolution (Rs) and correlation (pI vs. migration time, R^2) of the model protein mixture analysed with three different ampholyte blends.

	Pharmalyte[TM]	HR ampholyte	Ampholine
Background	~100	~40	~30
RNase	5.6	23	33
Myo I	2.7	9.5	8.2
Myo II	6.9	11	8.3
CAII	4.1	9.7	3.7
LGB	9.5	9.6	17
LGA	6.3	12	12
GO	7.4	10	11
CCK	7.2	11	-
Rs [*1)] (214 nm)	1.4	0.89	2.7
Rs [*1)] (280 nm)	2.0	0.50	2.7
R^2 (214 nm)	0.9139	0.6996	0.8330[*2)]
R^2 (280 nm)	0.9661	0.7042	0.8942[*2)]

[*1)] *Resolution (Rs) was calculated from the migration times (t_{migr}, in minutes) and peak width at 50% height ($W_{1/2}$) of LGA and LGB according to the following equations:*

$Rs = t_{migr\,LGA} - t_{migr\,LGB} / \frac{1}{2}*(W_{B\,LGA} + W_{B\,LGB})$

with $W_B = 4*W_{1/2}/2.35$

[*2)] *Since CCK is not detected, it is not included in the calculations for R^2*

At 214 nm, where peptide bonds show absorbance, the ampholyte background signal is approximately 100 (Pharmalyte[TM]), 40 (HR Ampholyte) and 30 (Ampholine) times higher than at 280 nm, where the tryptophan and tyrosine residues absorb light. When performing cIEF-UV, resolution depends on the number of CAs focused between two analytes. Since the blends contain different amounts of ampholytes [31, 32, 36], resolution can be increased by mixing these CA blends [37], or by mixing blends with a broad and narrow pH range [10, 38, 39]. If there are sufficient CAs present between the 2 analytes, increase of CA concentration can also be used. However, a higher CA concentration will increase the background signal and, moreover, increase the initial current, causing joule heating, zone broadening, increased gas bubble formation, and protein precipitation (causing "spikes").

As can be seen in Fig. 3.1, CCK is not detected when using the Ampholine blend. Due to the pI value of CCK and the smaller pH range (3.5-9.5) of Ampholine, CCK can apparently not be focused in this pH range and migrates to the anode vial. The migration time of CCK is therefore not included in the calculation of R^2. Although theoretically the pH gradient cannot be linear, Pharmalyte[TM] gives the best approximation of a linear pH gradient.

Furthermore, when performing cIEF-MS, adding CAs can suppress the signal of proteins in the MS. The averaged (n=5) relative intensity of the protein signal in the MS is plotted versus the total amount of CAs (in µg) per spot. RNAse, CAII, Myo, LGA and LGB are tested, but only the data of RNAse (high proton affinity in MALDI) and LGA (low proton affinity) are shown in Figure 3.2.
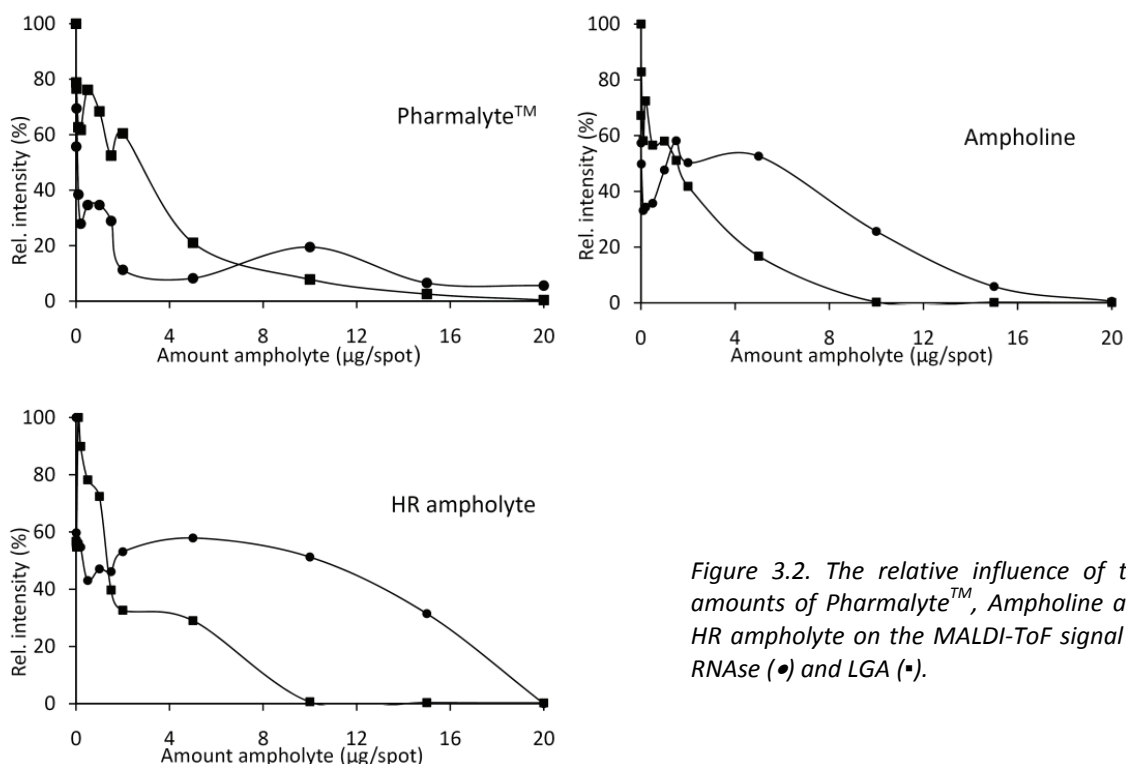

Figure 3.2. The relative influence of the amounts of Pharmalyte^{TM}, Ampholine and HR ampholyte on the MALDI-ToF signal of RNAse (•) and LGA (▪).

The total amount of protein *per* spot is constant (100 ng for RNAse and 50 ng for LGA), whereas the amount of CAs varies from 0 to 20 µg/spot. Although the amount of signal suppression is different for each protein, the general trend is that the MS signal significantly decreases with increasing CA amount of up to 4 µg/spot. All CA blends have a comparable effect on the MS signal and overall, the amount should not exceed 2 µg/spot, where still a signal of 20-40% of the signal intensity without ampholytes is observed. For the interpretation of results, several aspects should be taken into account. First, although some proteins are still observed at concentrations higher than 10 µg/spot, it must be noted that usually other components like detergents and viscosity-increasing agents, *i.e.* other compounds causing signal suppression, are added to the sample as well (see sections on solubilizing agents and viscosity increasing agents). Second, the protein-CA mixtures were prepared and then manually spotted on a MALDI target plate. When performing cIEF-MALDI-TOF, only the CAs that are focused near the protein, will be on that specific spot and will be responsible for the

signal suppression of the protein. Third, when performing on-line cIEF-MALDI-TOF, the cIEF outlet will be coupled to a spotting device and the total amount of ampholyte on the spot depends on the spot time and the mobilization velocity. In these experiments, the protein amount (50 ng for LGA) on the spot corresponds to a sample containing ~50 µg/mL LGA in a 40.2 cm x 50 µm ID capillary (volume about 1 µL), where the protein is spotted as one peak. The ampholyte amount is varied in these experiments. An amount of 0.5 µg Pharmalyte^{TM}/spot in Fig. 3.2 corresponds to a total concentration of about 1%^{w}/$_v$ Pharmalyte^{TM} in the cIEF sample when depositing 50 nL on a spot. When spotting 10 nL (by decreasing the mobilization flow speed or spot time) of the same cIEF sample, the amount on the spot is equivalent to 0.1 µg/spot in Fig. 3.2 and while the same amount of protein is spotted (one peak), the signal intensity has increased from 35% to 70%. However, decreasing the spotting time will also result in distribution of the protein over several spots, thereby decreasing the amount of protein *per* spot and decreasing the signal intensity. This implies that there is an optimal spotting time, which is related to the required resolution and the peak width at base, where the amount of ampholytes is low compared to the protein amount, since in theory only ampholytes with the exact same p*I* focus in the same zone as the protein.

Overall, Pharmalyte^{TM} is the best option for UV detection at 280 nm, while HR ampholytes perform best at 214 nm. Furthermore, the ionisation efficiency of proteins in MALDI decreases with increasing concentrations of ampholytes. By adjusting spot time and mobilization velocity, the amount of interfering substances can be tuned, depending on the required sensitivity and resolution. For concentrations generally used in cIEF (0.2-1%^{w}/$_v$), the MS signal of the proteins, although significantly reduced, may often be sufficient.

Solubilizing agents

One of the major advantages of cIEF is the concentrating effect of proteins during focusing, thereby improving the LOD. However, this concentrating effect is, in combination with the decrease in solubility at pH=p*I*, one of the reasons why proteins tend to precipitate or aggregate during cIEF. Precipitation and aggregation can lead to signal spikes (aggregates passing the detector), clogging of the capillary and poor migration-time and peak-area repeatability. Adding solubilizing agents (*e.g.* detergents, CAs, urea, *etc*.) prevents the interaction of

proteins with other proteins (aggregation) and with the wall (adsorption). The problem with solubilizers is that they can change the 3-D protein structure. Furthermore, there seems to be no single universal compound that can keep all proteins of a complex biological sample in solution. Therefore, the best type of solubilizer to be used should be determined for each sample. The compounds that will be tested here are commonly used in gel and capillary electrophoresis [40, 41]: chaotropic reagents and detergents. Compounds that are not neutral in the pH range 3-10, cannot be used in our study, since they will migrate to (one of) the compartments during focusing. Therefore only zwitterionic and neutral detergents, at concentrations commonly used in CE and gel electrophoresis, are included in these experiments.

As a measure for repeatability the relative standard deviation (RSD) of the migration time, peak area and peak height of each compound was determined. For information on the correlation of the pI versus the migration time, the R^2 was calculated. It should be mentioned that although the R^2 is often used as a parameter to express linearity of the pH gradient, it is intended to describe the relative peak position. Linearity of the pH gradient, as mentioned before, cannot be calculated from 7 proteins over a 3-10 gradient, since the pH profile between these markers is not taken into account. Table 3.3 shows the RSD of the migration time and the correlation coefficient (R^2) of the 7 proteins for the solubilizing compounds, while Table 3.4 depicts the RSD of the peak area and the resolution. Peak height results are not included in these tables since they are similar to the peak area results. PEO (neutral) and zwitterionic detergents show low RSDs for migration time (0.30-4.5%) and peak area (0.80-17%). R^2 is higher for these compounds compared to the glucoside and chaotropic reagents, which indicates that PEO and zwitterionic detergents approach a linear pH gradient best for the mixture of hydrophilic proteins used. For the glucoside detergents, DDM gives the best fit (R^2 = 0.9458), but RSDs are high (7.1-18% for migration time and 4.3-23% for peak area). n-Octyl glucoside on the other hand, exhibits a good migration time repeatability (0.45-1.9%) but the correlation (R^2 = 0.7444) and peak area repeatability (4.3-34%) are very poor.

Table 3.3. RSD (in %) of migration times of proteins and correlation (pI vs. migration time) for different solubilizing agents.

	Conc. ($^w/_v$%)	n	RNAse	Myo I	Myo II	CAII	LGB	LGA	GO	CCK	R^2
PEO detergents											
Brij 35	1.00	4	0.30	1.4	0.85	1.2	1.3	1.3	1.5	2.3	0.9572
Igepal	0.30	5	0.67	1.7	1.8	2.1	2.1	2.1	2.4	2.7	0.9606
Tween 20	1.00	10	0.87	1.1	1.2	1.4	1.6	1.6	1.8	2.5	0.9704
Glucoside detergents											
DDM	0.10	5	7.1	10	12	12	13	13	15	18	0.9458
n-Octyl glucoside	0.10	4	1.5	1.4	1.5	1.7	0.65	0.65	1.9	2.2	0.7444
Zwitterionic detergents											
CHAPSO	3.00	5	1.13	0.45	0.41	0.44	0.45	0.52	0.52	0.48	0.9660
SB3-12	1.00	10	1.0	1.0	1.3	1.7	1.9	1.9	2.6	4.5	0.9665
Chaotropic reagents											
Sorbitol	10.0	5	8.7	6.8	6.7	6.8	6.9	6.7	6.6	7.8	0.9682
8M Urea		3	1.5	1.5	1.6	1.5	1.6	1.7	-	-	0.7677
2M Urea		3	0.83	0.25	0.10	0.17	0.30	0.35	1.0	1.3	0.7250

Table 3.4. RSD (in %) of peak areas and resolution of proteins for different solubilizing agents.

	Conc. ($^w/_v$%)	n	RNAse	Myo I	Myo II	CAII	LGB	LGA	GO	CCK	Rs [1)]
PEO detergents											
Brij 35	1.00	4	6.6	2.1	5.4	2.7	7.7	1.9	6.8	6.5	2.1
Igepal CA 630	0.30	5	11	3.8	7.9	3.9	3.7	4.6	6.4	5.8	2.0
Tween 20	1.00	10	5.8	4.9	6.8	4.1	5.4	8.4	8.9	5.7	1.6
Glucoside detergents											
DDM	0.10	5	10	9.0	4.3	11	7.9	23	20	17	1.7
n-Octyl glucoside	0.10	4	4.3	7.1	34	7.3	20	18	10	4.5	1.6
Zwitterionic detergents											
CHAPSO	3.00	5	0.80	1.8	2.7	2.3	8.7	3.5	2.8	2.4	1.4
SB3-12	1.00	10	6.9	9.7	9.3	2.4	9.9	9.1	2.5	17	0.44
Chaotropic reagents											
Sorbitol	10.0	5	7.6	14	10	4.9	10	11	11	12	2.1
8M Urea		3	10	5.7	5.4	5.2	5.5	10	-	-	1.2
2M Urea		3	6.8	5.3	9.3	4.0	4.6	6.3	29	14	0.93

When using urea, the correlation decreases with increasing concentration, while some of the proteins cannot be observed at 2M and 8M, probably due to (partial) denaturation and/or aggregation (which is accompanied by a change in current profile). Best resolution (LGA-LGB) is also obtained with the PEO detergents. And although SB3-12 performs well with respect to repeatability and correlation, resolution (0.44) is disappointing. Taking all aspects (repeatability, correlation and resolution) into account, PEO detergents perform best for our hydrophilic protein mixture when using UV detection. However, when looking at signal suppression in MALDI, as shown in Fig. 3.3, these PEO detergents already exhibit severe signal suppression at low amounts (0.1-0.2 µg/spot).
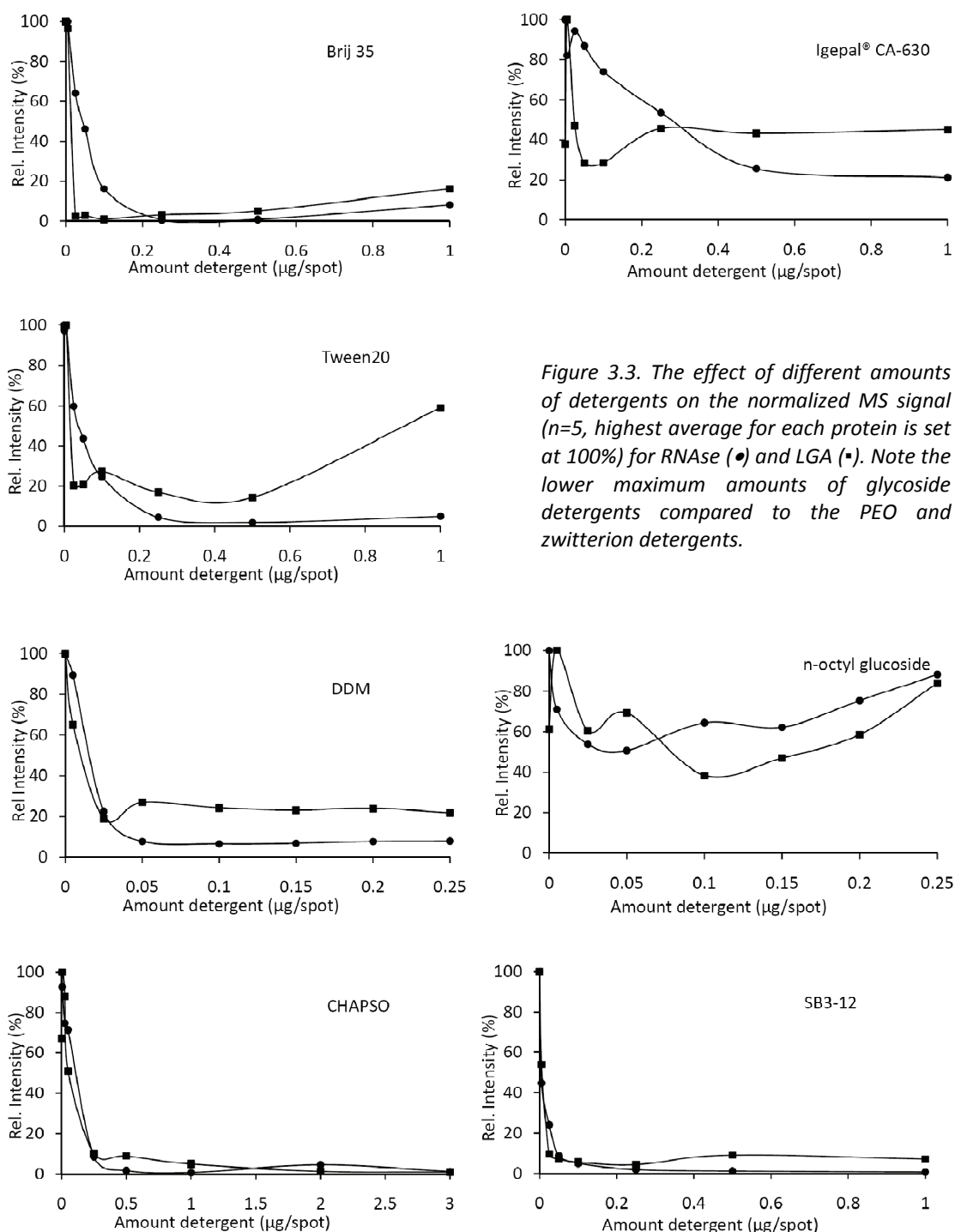
Figure 3.3. The effect of different amounts of detergents on the normalized MS signal (n=5, highest average for each protein is set at 100%) for RNAse (•) and LGA (▪). Note the lower maximum amounts of glycoside detergents compared to the PEO and zwitterion detergents.

With the zwitterions, especially SB3-12, the decrease in signal is even larger. For the glycoside detergents, the amounts per spot are 4-10 times lower than the other detergents, due to solubility limitations. DDM reduces the protein signal, but to a constant level of ~20% of the original signal in the range of 0.05-0.25 µg/spot. n-Octyl glucoside on the other hand, does not seem to have a significant effect on the protein signal at these amounts. An explanation for the relatively

low signal suppression of n-octyl glucoside might be that the proteins are distributed more evenly over the MALDI-spot, opposing the formation of sweet spots thereby increasing the repeatability and intensity of the signal as already described for Tween 80 [42]. Furthermore, as with the CAs, the amount of detergent on the spot can be adjusted by modifying the spot time and mobilization velocity. In Fig. 3.3, an amount of 0.1 µg/spot Tween 20 corresponds to 0.2%^{w}/$_v$ Tween 20 in the cIEF sample when spotting 50nL. So when spotting relatively small amounts (typically 10-50 nL), concentrations of up to 1% Tween 20 in the sample, which is common in cIEF, can be used. However, at these Tween 20 concentrations, a substantial signal reduction (up to 70%) still occurs. Overall, when performing cIEF-UV, the PEO exhibit the lowest variation in migration time and peak area and highest resolution and correlation coefficient of p*I* versus migration time. On the other hand, these compounds cause high suppression of protein signal in MALDI, whereas *n*-octyl glucoside hardly affects the signal.Viscosity-increasing agentsA different approach in preventing capillary wall adsorption is the addition of polymers, like cellulose derivatives or dextran, to the sample. During focusing the analyte is subject to two opposing phenomena: electromigration, forcing the analytes into their pH=p*I* zone, and diffusion, driving the compounds out of their focusing zone to a lower concentration, thereby causing the analytes to acquire a charge. Addition of polymers decreases diffusion and the ζ-potential by shielding charged groups on the capillary wall. This minimizes EOF and prevents interaction of the proteins with the charged wall, which is beneficial for both the repeatability of migration time and peak area. The RSD of the migration time and the correlation (p*I* vs. migration time) (Table 3.5) as well as the peak area repeatability and resolution (Table 3.6) were studied for 4 cellulose derivatives and dextran. Tween 20 (25%^{w}/$_v$), sorbitol (70% ^{w}/$_v$), glycerol (63% ^{w}/$_v$), sucrose (65% ^{w}/$_v$) or polyacrylamide (35% ^{w}/$_v$) were also tested, but peaks could not be observed (UV detection) in our sample. However, these results do not imply that these compounds can not be used for other samples like *e.g.* hydrophobic proteins [22]. As can be seen in Tables 3.5 and 3.6, all cellulose compounds perform well with respect to migration time RSD (all within 13%, but most <5%), peak area RSD (all within 14%, but majority <5%) and resolution (all >1.2). The cellulose compounds (except MHEC) perform better at lower concentrations for most parameters studied, which is beneficial for coupling to MS. Overall, best results

are observed for HPMC. Therefore we prefer to use HPMC when performing cIEF-UV.

Table 3.5. RSD (in %) of migration times of proteins and R2 for different viscosity-increasing agents.

	Conc. (^{w}/$_v$%)	n	RNAse	Myo I	Myo II	CAII	LGB	LGA	GO	CCK	R^2
HPMC	0.5	5	0.46	2.7	2.4	2.2	2.4	2.3	1.8	1.8	0.9460
	0.25	7	0.12	0.14	0.17	0.22	0.29	0.25	0.29	0.45	0.9558
HEC	1.2	5	5.0	4.7	4.2	3.9	3.6	3.6	3.3	8.5	0.7677
	0.6	5	4.5	3.3	3.2	4.4	3.2	3.1	3.1	2.5	0.8926
MHEC	0.3	5	2.6	3.9	4.1	4.3	4.5	4.6	6.1	7.0	0.9825
	0.15	4	1.8	1.4	1.4	1.5	1.5	1.5	1.5	2.0	0.8634
MC	0.8	4	13	8.9	8.3	6.8	5.9	5.7	3.2	4.2	0.7790
	0.4	4	0.24	0.54	0.60	0.64	0.64	0.63	0.63	0.88	0.9680
Dextran	5.0	4	1.5	0.96	0.87	0.74	0.72	0.79	0.78	0.80	0.9860
	2.5	5	0.76	0.50	0.51	0.45	0.41	0.39	0.81	2.1	0.6911

Table 3.6. RSD (in %) of peak areas of proteins and resolution (LGA-LGB) for different viscosity-increasing agents.

	Conc. (^{w}/$_v$%)	n	RNAse	Myo I	Myo II	CAII	LGB	LGA	GO	CCK	Rs
HPMC	0.5	5	1.8	1.7	4.5	6.9	6.2	2.3	3.2	3.1	2.2
	0.25	7	4.9	1.6	5.3	2.2	4.4	3.6	4.9	4.0	3.1
HEC	1.2	5	11	8.6	18	4.4	2.8	3.7	2.8	14	1.5
	0.6	5	2.2	4.9	4.3	2.3	2.3	4.7	3.6	3.9	1.3
MHEC	0.3	5	5.6	4.3	3.8	6.5	6.8	7.2	14	7.4	1.3
	0.15	4	3.8	3.6	5.8	1.4	3.6	2.8	3.5	4.4	-
MC	0.8	5	11	9.8	31	9.8	10	12	4.4	14	1.4
	0.4	4	2.7	2.5	2.5	1.6	4.2	4.4	3.6	4.8	1.2
Dextran	5.0	5	4.1	3.7	3.0	3.2	7.1	7.0	5.6	6.0	2.3
	2.5	5	5.8	6.5	6.3	4.0	2.7	5.2	4.1	4.9	1.2

Fig.3.4 shows the relative MS signal intensity for RNAse and LGA with an increasing amount of polymer on the spot. As can be seen, for dextran the lowest suppression of the MALDI-TOF signal was found. However, all the cellulose components show more signal suppression, in particular the methyl cellulose components. MC and HPMC already exhibit signal suppression at a very low concentration; 0.025 µg/spot (corresponding to 0.25% when depositing 10 nL) polymer causes a 45-90% drop in signal. For the ethylcellulose compounds, the decrease is either more gradual (MHEC) or the signal remains at 20-50% of its initial value (HEC). A possible explanation could be that due to the more hydrophilic character of the HEC-polymers, the proteins exhibit less interaction with these components. However, polymer chain length, gel formation, or other polymer properties may also have an influence.
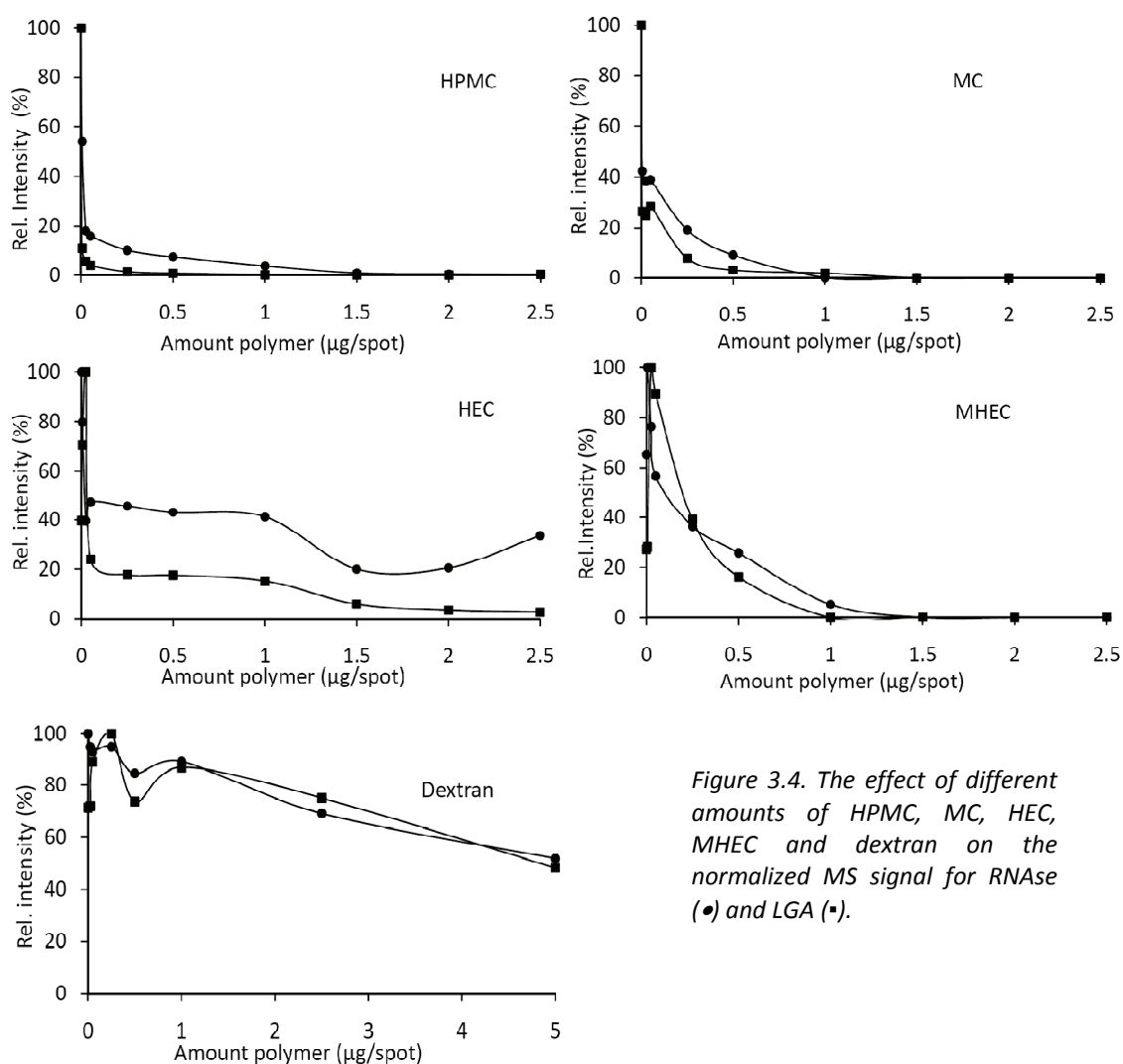
Figure 3.4. The effect of different amounts of HPMC, MC, HEC, MHEC and dextran on the normalized MS signal for RNAse (•) and LGA (▪).

Furthermore, the MALDI matrix consists of 80% ACN, which can cause the polymers to precipitate, irreversibly including the proteins in this network. These proteins will not be volatilized and detected in the MS.

For concentrations normally used in cIEF, all cellulose derivatives exhibit good repeatability, resolution and correlation, but show severe signal suppression, whereas dextran has only a small influence on the protein signal in MS.

3.4   Concluding remarks

This paper gives a systematic overview of the effect of CAs, solubilizers and viscosity-increasing agents on repeatability, resolution and linearity with UV detection and signal suppression in MALDI-TOF MS. When using UV detection, a system that provides the highest repeatability (migration time and peak area), resolution and correlation (p*I* and migration time) can be used.For our test

mixture of 7 hydrophilic proteins, excellent results for repeatability, resolution and correlation were obtained with 1% $^w/_v$ Pharmalyte^{TM}, 1% $^w/_v$ Tween 20 and 0.5% $^w/_v$ HPMC (Fig. 3.5).
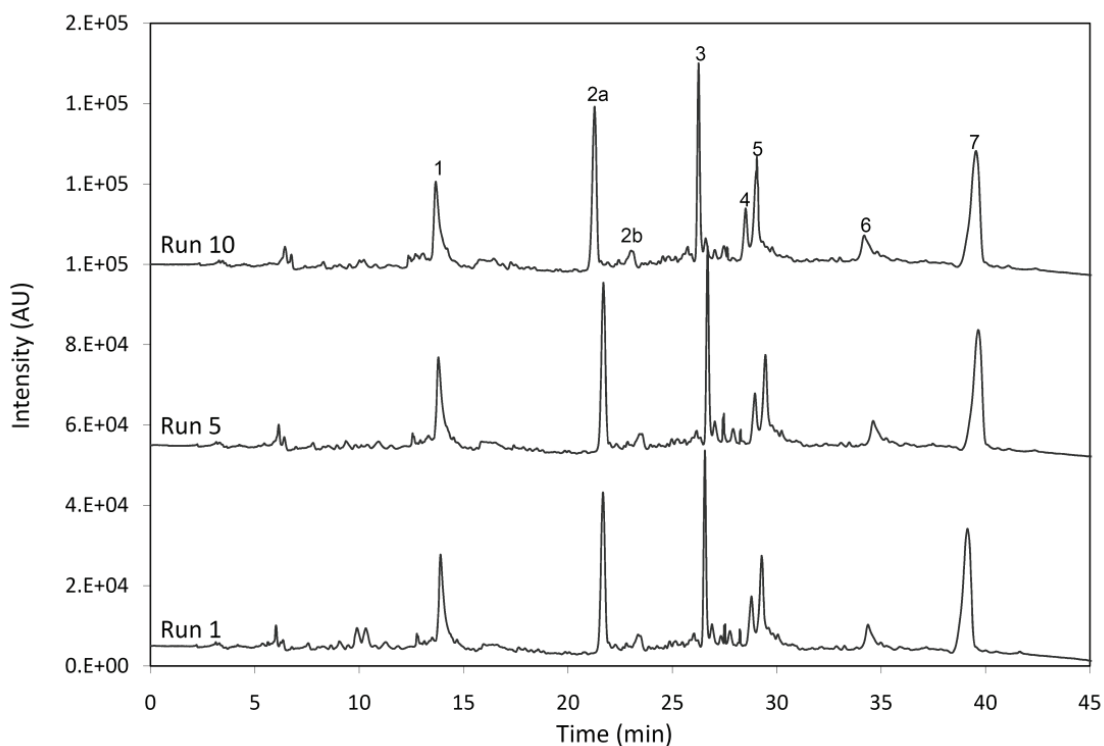

Figure 3.5. Typical electropherograms of a 7 protein sample with 1% Pharmalyte^{TM}, 0.5% HPMC and 1% tween 20. Runs 1, 5 and 10 are shown. For clarity, traces 5 and 10 are stacked on the y-axis.

However, this does not imply that for other mixtures and real biological samples this will be the optimal method. The compounds that should be added to the sample and to the electrolyte solutions should be determined for each sample. Furthermore, other parameters, like type of capillary wall-coating and focusing and mobilization method, play a major part in cIEF-UV analysis. The cIEF-UV conditions presented in this paper are only a guideline for choosing the right blend of ampholyte, detergent and viscosity increasing agent. However, compared to UV, the MS protein signal is more sensitive to other compounds present in the sample. Therefore it is not always possible to select a system with the highest resolution, repeatability or linearity. This paper shows that, in order to get the highest signal in MS (to obtain the lowest LOD), compromises have to be made. Although the blend of ampholyte used is not important for the MS signal, the concentration of ampholyte is critical. Increasing the concentration will improve cIEF resolution, but reduces the protein signal in MS. Comparable

results are observed with viscosity increasing agents. PEO and zwitterionic detergents, although significantly improving repeatability and linearity in UV, suppress the protein signal in the MS. Glucoside detergents, preferably *n*-octyl glucoside, should be used when performing cIEF-MS. But this would be unfavourable for cIEF performance. It should, however, be noted that all the MS results presented here only apply to MALDI, which is less sensitive to matrix effects than other ionisation methods [43]. Suppression effects are expected to be different in other ionisation sources.

Furthermore, the amount of interfering substance (ampholyte, detergent or polymer) can be tuned (decreased) by adjusting the spotting time and volume. This can be accomplished by decreasing the volume that is spotted. On the other hand, when the spotted volume is too small, the amount of protein per spot also decreases, which will lead to lower sensitivity. Peak width should therefore be as low as possible, so the amount of interfering substances is relatively low compared to the amount of protein on the spot. Decreasing peak width can in turn be accomplished by adding ampholytes, detergents and viscosity increasing agents.

References

[1]   Liu, X., Sosic, Z., Krull, I. S., J Chromatogr A 1996, 735, 165-190.

[2]   Righetti, P. G., Gelfi, C., Conti, M., J Chromatogr B Biomed Sci Appl 1997, 699, 91-104.

[3]   Rodriguez-Diaz, R., Wehr, T., Zhu, M., Electrophoresis 1997, 18, 2134-2144.

[4]   Shimura, K., Electrophoresis 2002, 23, 3847-3857.

[5]   Kilar, F., Electrophoresis 2003, 24, 3908-3916.

[6]   Wang, Y., Balgley, B. M., Lee, C. S., Expert Rev Proteomics 2005, 2, 659-667.

[7]   Righetti, P. G., Electrophoresis 2006, 27, 923-938.

[8]   Shen, Y., Xiang, F., Veenstra, T. D., Fung, E. N., Smith, R. D., Anal Chem 1999, 71, 5348-5353.

[9]   Gao, L., Liu, S., Anal Chem 2004, 76, 7179-7186.

[10]   Poitevin, M., Morin, A., Busnel, J. M., Descroix, S., et al., J Chromatogr A 2007, 1155, 230-236.

[11]   Shen, Y., Berger, S. J., Anderson, G. A., Smith, R. D., Anal Chem 2000, 72, 2154-2159.

[12]   Righetti, P. G., Bossi, A., Gelfi, C., J Capillary Electrophor 1997, 4, 47-59.

[13]   Graf, M., Wätzig, H., Electrophoresis 2004, 25, 2959-2964.

[14]   Conti, M., Galassi, M., Bossi, A., Righetti, P. G., J. Chromatogr. A 1997, 757, 237-245.

[15]   Lopez-Soto-Yarritu, P., Diez-Masa, J. C., Cifuentes, A., de Frutos, M., J Chromatogr A 2002, 968, 221-228.

[16]   Manabe, T., Iwasaki, A., Miyamoto, H., Electrophoresis 1997, 18, 1159-1165.

[17]   Manabe, T., Miyamoto, H., Iwasaki, A., Electrophoresis 1997, 18, 92-97.

[18]   Das, C., Fan, Z. H., Electrophoresis 2006, 27, 3619-3626.

[19]   Mosher, R. A., Thormann, W., Electrophoresis 2002, 23, 1803-1814.

[20]   Zhu, M., Rodriguez, R., Wehr, T., J Chromatogr A 1991, 559, 479-488.

[21]   Dolnik, V., Electrophoresis 2006, 27, 126-141.

[22]   Busnel, J. M., Varenne, A., Descroix, S., Peltre, G., et al., Electrophoresis 2005, 26, 3369-3379.

[23]   Simpson, D. C., Smith, R. D., Electrophoresis 2005, 26, 1291-1305.

[24]   Monton, M. R., Terabe, S., Anal Sci 2005, 21, 5-13.

[25]   Hernandez-Borges, J., Neusüss, C., Cifuentes, A., Pelzing, M., Electrophoresis 2004, 25, 2257-2281.

[26]   Hille, J. M., Freed, A. L., Wätzig, H., Electrophoresis 2001, 22, 4035-4052.

[27]   Shen, Y., Smith, R. D., Electrophoresis 2002, 23, 3106-3124.

[28]   Moini, M., Anal Bioanal Chem 2002, 373, 466-480.

[29]   Almgren, M., Chem. Scr. 1971, 1, 69-75.

[30]   Stoyanov, A. V., Das, C., Fredrickson, C. K., Fan, Z. H., Electrophoresis 2005, 26, 473-479.

[31]   Sebastiano, R., Simo, C., Mendieta, M. E., Antonioli, P., et al., Electrophoresis 2006, 27, 3919-3934.

[32]   Simo, C., Mendieta, M. E., Antonioli, P., Sebastiano, R., et al., Electrophoresis 2006, 27, 4849-4858.

[33]   Simo, C., Mendieta, M. E., Antonioli, P., Sebastiano, R., et al., Electrophoresis 2007.

[34]   Righetti, P. G., J Chromatogr 1980, 190, 275-282.

[35]   Righetti, P. G., Pagani, M., Gianazza, E., J Chromatogr 1975, 109, 341-356.

[36]   Simo, C., Citterio, A., Righetti, P. G., Electrophoresis 2007, 28, 1488-1494.

[37]   Mohan, D., Lee, C. S., J Chromatogr A 2002, 979, 271-276.

[38]   Li, N., Kessler, K., Bass, L., Zeng, D., J Pharm Biomed Anal 2007, 43, 963-972.

[39]   Kronsteiner, B., Malissa, H., Jr., Stutz, H., Electrophoresis 2007, 28, 2241-2251.

[40]   in: Von Jagow, G., Link, T. A., Schaegger, H. (Eds.), Membrane protein purification and crystallization: A practical guide, Academic Press, San Diego, CA, USA

1994, pp. 10-16.

[41]   Berkelman, T., Brubacher, M. G., Chang, H., BioRadiations 2004, 114, 30-32.

[42]   Brinkworth, C. S., Bourne, D. J., J Am Soc Mass Spectrom 2007, 18, 102-105.

[43]   Pan, C., Xu, S., Zhou, H., Fu, Y., et al., Anal Bioanal Chem 2007, 387, 193-204.

[44]   Shimura, K., Wang, Z., Matsumoto, H., Kasai, K., Electrophoresis 2000, 21, 603-610.

Chapter 4

Characterization of cIEF-MALDI-TOF MS for protein analysis

L.H.H. Silvertand, J. Sastre Toraño, G.J. de Jong, W.P. van Bennekom, Electrophoresis 30 (2009) 1828.

Abstract

This paper describes the hyphenation of cIEF and MALDI-TOF MS via a fractionation or spotting device. After focusing in cIEF the compounds are hydrodynamically mobilized and deposited on a MALDI target plate using a sheath liquid interface, which provides the catholyte solution and the electrical ground. From previous experiments, sample conditions that resulted in a high resolution in cIEF and acceptable protein signal intensity in MS were selected [Silvertand et al., Electrophoresis, 2008, 29, 1985-1996]. Besides the mixture of test proteins, the sample solution contains 1% Pharmalyte, 0.3% HEC and 0.1% Tween 20 and is used for both optimization as well as characterization of the cIEF-MALDI-TOF MS system. Hyphenation problems encountered are mainly due to transfer of the liquid from the needle to the MALDI target plate and are solved by choosing the proper sheath catholyte (200 mM NH_4OH in 50% MeOH with 0.1% Tween 20). MS electropherograms were reconstructed by plotting the intensities of the m/z values corresponding to the proteins versus migration time (related to spot number). Reproducibility, peak width and signal intensity for different focusing and spotting (fractionation) times were calculated using these reconstructed MS electropherograms as well as the UV-electropherograms. The best results were obtained with focusing time of 75 min (no under- or overfocusing) and a spotting time of 5 s (highest protein signal intensity in MS). The applicability of the system is demonstrated by the analysis of a biopharmaceutical (glucagon) and its deamidation product.

4.1 Introduction

Over the past 20 years capillary isoelectric focusing (cIEF) has proven to be a fast, high-resolution, p*I*-based technique for the separation of amphoteric compounds, *e.g.* proteins and peptides. In cIEF, ampholytes are added to the sample to create a stable pH gradient inside a capillary. Charged proteins/peptides migrate under the influence of an electrical field to the pH where their nett charge is zero (at pH=p*I*), leading to the separation of the analytes and a local increase in concentration. Upon completion of this focusing process, the sample is mobilized and the substances can be detected by various techniques, *e.g.* a single point fixed UV detector. Although cIEF-UV provides information on p*I*, little or no structural information is obtained. Furthermore,

proteins and peptides, which do not contain tryptophan or tyrosine residues, will not be observed at 280 nm. The added ampholytes interfere in the detection at 214 nm, therefore 280 nm is often the preferred wavelength in cIEF. For gaining structural information, a technique like MS, is therefore required.

In 1995, ten years after its introduction by Hjertén and Zhu [1], cIEF was hyphenated with mass spectrometry. Tang *et al*. [2] accomplished the coupling to electrospray ionisation (ESI) MS and Foret *et al*. [3] hyphenated cIEF with MALDI-TOF MS. cIEF-MS provides the advantages of cIEF and the high mass accuracy and additional structural information of MS, depending on the type of mass analyzer used. The information on the analytes obtained with cIEF-MS is often compared to 2D-GE, where the first separation is also p*I*-based (IEF) and in the second dimension the compounds are separated on mass. However, 2D-GE is more labour intensive, time consuming and still poorly automated compared to cIEF-MS. In contrast to these advantages of cIEF-MS, the major problems in hyphenation are the presence of ampholytes (signal suppression in MS) and the zone broadening from cIEF to MS. Tang *et al*. [2, 4] showed that for cIEF-ESI MS, ampholytes concentrations of more than 0.5% give significant signal suppression and considerably impair sensitivity. However, the number of cIEF-MALDI papers is by far outnumbered by the amount of papers on the coupling of cIEF to ESI (recently reviewed in [5-7]). Probably the on-line analysis and higher sampling rate are the reasons for the popularity of ESI over MALDI. On the other hand, MALDI is more tolerant to additives like ampholytes and offers the possibility of re-analysis and selection of specific spots for further analysis (MS/MS). Therefore, MALDI is chosen for the experiments described in this paper, although it was shown recently [8] that these additives also significantly decrease the protein signal in MALDI-TOF MS.

cIEF can be hyphenated to MALDI-TOF MS in several ways: fraction collection with subsequent off-line transfer of these fractions to a target plate [3, 9], direct spotting of the capillary eluent onto the target plate [10-13] or separation in microchip channels with the microchip serving as a target plate [14-16]. The direct spotting of the eluent on the plate, which is also used for the present experiments, is less labour intensive than fraction collection, but easier to perform than the microchip formats and is therefore our first choice. cIEF is usually coupled to MALDI-TOF MS via a spotting device. Upon completion of the focusing process, the separated proteins are hydrodynamically mobilized to a UV

and MS detector in series. The capillary outlet is inserted in a hollow needle. This needle also delivers catholyte to the system. The catholyte solution serves as the electrical ground, provides volume for droplet deposition and is the alkaline solution necessary to stabilize the pH gradient. After spotting directly on the MALDI target plate and adding MALDI-matrix, the plate is analyzed with MALDI-TOF. The transfer of the compounds from the cIEF to the MS introduces band broadening (interfaces, mixing (with sheath liquid) and diffusion) which lead to loss of the high resolution obtained in cIEF for the MS analysis.

This paper describes the optimization of interfacing cIEF to MALDI-TOF MS via a spotting device. In contrast to other cIEF-MALDI papers, all the parts and interfaces are standard commercially available. The influence of focusing and spotting time on several parameters is investigated with a standard mixture of intact proteins. The results will be presented in reconstructed electropherograms. To demonstrate the applicability of this cIEF-MS system, the degradation of a biopharmaceutical compound (glucagon) in a formulation (GlucaGen) is shown.

4.2   Materials and method

Materials

Pharmalyte^{TM} (36% ($^w/_v$) pH 3-10, copolymer of glycine, glycylglycine, various amines and epichlorohydrin) was purchased from Amersham Bioscience (Piscataway, NJ, USA). Tween 20 (for synthesis) was obtained from Merck KGaA (Darmstadt, Germany). Hydroxyethylcellulose (HEC, medium viscosity), phosphoric acid (85%), trifluoroacetic acid (>99%), sinapic acid (>99%), TEMED (~99%), sodium hydroxide and all proteins (see Table 4.1) were purchased from Sigma-Aldrich. GlucaGen (Novo Nordisk) was kindly donated by Drs L.J.F. Silvertand and Drs F.N. Sikkes (Apotheek Born, Born, The Netherlands). HPLC gradient grade acetonitrile (ACN) and methanol (MeOH) were purchased from Biosolve (Valkenswaard, The Netherlands). High purity water (Ultrapure, >18.2 MΩ cm) was obtained from a Millipore Synergy UV water purification system (Millipore, Bedford, MA, USA).

Apparatus and methods

cIEF separations were performed with a PrinCE 500 dual-lift capillary electrophoresis system using Dax Data Acquisition and Analysis Software version 6.0 (Prince Technologies, Emmen, The Netherlands), equipped with a Knauer K-2501 UV detector set at 280 nm (Knauer GmbH, Berlin, Germany) and an eCAP neutral capillary (50 µm ID, 40.2 cm total length, Beckman Coulter, Fullerton, CA, USA). Prior to each run the capillary was rinsed first with 10 mM phosphoric acid in 0.3% HEC, then with purified water and finally with 0.1 % Tween 20 in 0.3% HEC. Subsequently, the capillary was filled with Sample 1 (RNAse (90 µg/mL), Myo, CAII, LGB and Lac (all at 10 µg/mL), Pharmalyte^{TM} (1% $^w/_v$), TEMED (0.1% $^v/_v$), HEC (0.3% $^w/_v$) and Tween 20 (0.1% $^w/_v$)), or with Sample 2 (= Sample 1, but LGB and Lac are replaced with LGA (16 µg/mL) and CCK (8 µg/mL)). The inlet vial (anolyte) was filled with 10 mM phosphoric acid in 0.3% HEC. The capillary outlet was inserted in the hollow needle (electrically grounded) of the CE-interface of an LC Packings Probot Microfraction Collector (Dionex, Amsterdam, The Netherlands) (see also Fig. 4.1). Sheath liquid (200 mM ammonium hydroxide in 50% MeOH and 0.01% Tween 20) was delivered through this needle *via* the syringe of the Probot Spotter and was set to deposit 0.5 µL sheath liquid *per* spot throughout the entire run.

Table 4.1. Specifications of the proteins used in this study.

Protein (abbreviation)	pI	Mw	CAS	Source
Ribonuclease XII A (RNAse)	9.6	13.7 kDa	9001-99-4	Bovine pancreas
Myoglobin (Myo)	7.3, 6.8*	17.6 kDa	100684-32-0	Equine skeletal muscle
Carbonic anhydrase II (CAII)	5.9	29 kDa	9001-03-0	Bovine erythrocytes
ß-Lactoglobulin B (LGB)	5.3	18,276 Da	9066-45-9	Bovine milk
ß-Lactoglobulin A (LGA)	5.15	18,363 Da	50863-92-8	Bovine milk
A-Lactalbumin (Lac)	4.5	14,175 Da	9051-29-0	Bovine milk
CCK precursor fragment 107-115 (CCK)	3.67	1074.1 Da	198483-37-3	Human

) Major (Myo I, pI 7.3) and minor (Myo II, pI 6.8) component.

During focusing the capillary outlet and needle were submerged in the outlet vial (catholyte: 200 mM ammonium hydroxide in 50% MeOH). Focusing was performed at 20 kV at room temperature for 15 to 90 min.After focusing, the catholyte waste vial was removed and the separated proteins were mobilized at 20kV and 100 mbar air pressure towards an ABI stainless steel MALDI target plate (Applied Biosystems, Framingham, MA, USA) with 192+6 predefined spots. Upon

completion of the spotting procedure, 0.75 µL MALDI matrix (5 mg/mL sinapic acid in 80% $^v/_v$ ACN with 0.1% $^v/_v$ TFA) was added per spot and dried.MALDI-TOF MS experiments were performed with an Applied Biosystems 4700 Proteomics Analyzer. The *m/z* recording range was set to 5,000-40,000 with a focus *m/z* of 17,000 in linear mode. MALDI-TOF spectra were analyzed using Data Explorer software Version 3.0 (Applied Biosystems).

4.3 Results and discussion

In a previous paper [8] we examined off-line the influence of compounds that are usually added to a cIEF sample to improve repeatability (ampholytes, detergents and viscosity increasing agents) on the signal suppression of proteins in the MALDI-TOF-MS. The compounds providing a relatively high repeatability and resolution in cIEF were selected for these experiments: Pharmalytes, Tween 20 and HEC [8]. At deposition of the capillary eluent on the MALDI target plate during 1 min, a spotting time that is often used, it was calculated that 1%$^w/_v$ of ampholytes reduced the protein MS signal with approximately 50%, while 0.3% HEC decreased the signal intensity with about 60%. In order to maintain high resolution in cIEF, the concentration of additives was relatively high, while the spotting time was kept to a maximum of 60 s to minimize loss of signal. The amount of Tween 20 was relatively low, *i.e.* 0.1% $^w/_v$, resulting in a reduction of 50% signal intensity. These sample additives were selected for the coupling of cIEF to MALDI-TOF MS and for the experiments described here, unless otherwise indicated.

Optimization of the system

To provide a pH gradient in the capillary, the outlet of a cIEF capillary is usually immersed in an alkaline solution. However, from the outlet in this setup the content of the capillary is deposited on a MALDI-target plate and the alkaline solution is administered to this system *via* a sheath liquid (Fig. 4.1). The capillary is inserted in a hollow needle. The tip of this capillary is positioned ca 0.5 mm below the tip of the needle. The sheath liquid or catholyte is flushed through the needle and is in contact with the content of the capillary. Furthermore, the catholyte is mixed with the separated sample just prior to deposition. By grounding the needle, the electrical circuit is closed. In a standard cIEF setup, the

catholyte usually is NaOH (10 mM, pH 12.3). However, when using this relatively high concentration of NaOH, sodium adducts of the proteins are formed upon deposition which are observed in the MALDI-TOF MS. Therefore, the more volatile NH_4OH (200 mM, pH 11.3) is used. NH_4OH is removed as NH_3 gas from the MALDI target plate after deposition. However, when using NH_4OH instead of NaOH a slight decrease of cIEF resolution is observed. To improve spot-drying, an organic modifier is added to the sheath liquid. ACN, which is present in the MALDI matrix, is preferred. However, when adding ACN to the anolyte di- and trimers of the protein are observed in the MS (data not shown), whereas with the addition of MeOH less of these protein-polymers were detected. Therefore 50% ($^v/_v$) MeOH is added to the sheath liquid.
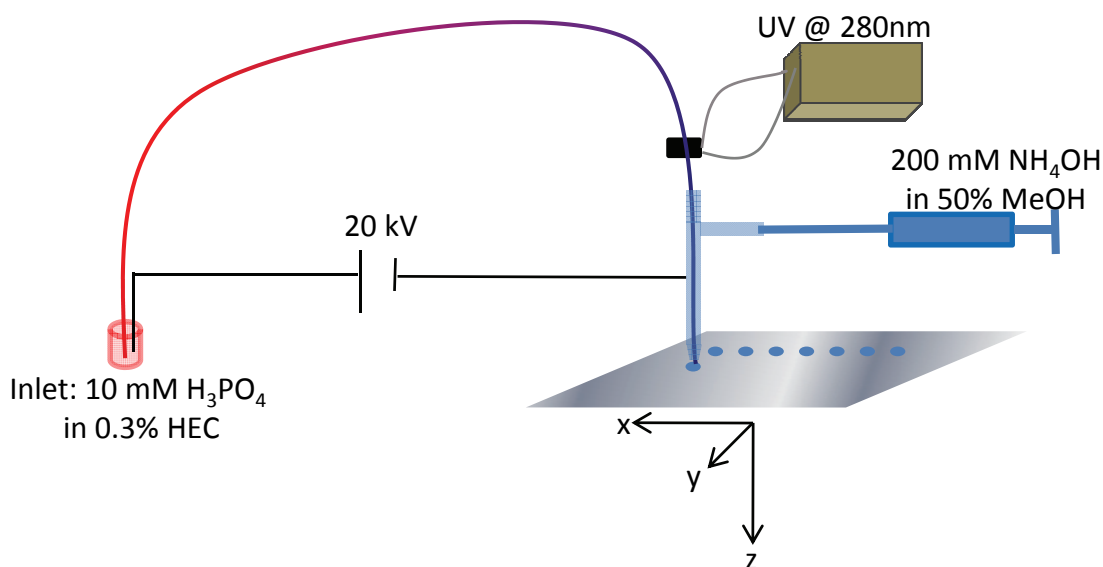
Figure 4.1. Schematic set-up of the cIEF-UV-MALDI-TOF MS system used.

The major practical problem of droplet deposition is the adhesion of catholyte-eluent droplets onto the needle instead of to the plate. Although the distance between the capillary outlet and the plate is kept to a minimum, this problem is persistent. Applying a Teflon film or a hydrophobic layer (*e.g.* oil) onto the tip of the needle only increased the repeatability of the droplet's contact to the needle, but not the adhesion to the target plate. Prewetting the spots on the plate with water to increase hydrophilicity of the contact surface does not improve adhesion to the plate either. If the needle is carefully cleaned with water and MeOH prior to each run, in combination with adding MeOH to the sheath liquid, droplets are formed on the plate instead of adhering to the needle. Adding a

small amount of Tween 20 (0.01%) to the sheath liquid reduces surface tension and leads to the formation of more repeatable droplets, and furthermore enhances an even crystallization of the compounds in the spots [17].

Focusing and mobilization

When performing cIEF-UV, focusing is assumed to be complete when the current is approximately 10% of its initial value [18]. However, overfocusing (focusing time too long) increases the risk of protein precipitation, which leads to spikes (when protein aggregates are passing the detector), irreproducible migration times (adhesion of compounds to the wall) and clogging of the capillary. The optimal focusing time depends on a variety of parameters, including viscosity of the medium, the kind and concentration of additives (in particular ampholytes), capillary length, *etc*. [19]. Therefore the optimal focusing time should preferably be determined for each system and each sample individually. In our study, different focusing times are tested: 15, 30, 45, 60, 75 and 90 min. Fig. 4.2 shows the UV electropherograms as well as the reconstructed MALDI-TOF MS electropherograms for 30, 75 and 90 min for Sample 1. For comparison, the migration time of myoglobin major component is set to 10 min for all (reconstructed) electropherograms (Figs. 4.2 and 4.3 and Table 4.2). Furthermore, the fractions were spotted onto the MALDI-target plate every 5 s in these experiments. For the lower focusing times (up to 45 min) double peaks (proteins zones forming at both capillary ends which migrate towards each other until they merge at the pI of the protein) are observed for some proteins. Especially for the proteins with high pI (*e.g.* RNAse, pI 9.6), which remain a shorter time under the influence of the electrical focusing field, this so-called transitional double-peak phenomenon is observed [20]. This is an indication that the focusing process is not complete. A too long focusing time (overfocusing) usually gives visible spikes. However, these spikes are observed in all UV electropherograms, even at 15 min. Therefore, a different explanation has to be found for protein aggregation or precipitation, since in our previous research spikes were seldom observed with cIEF-UV for this shorter focusing time [8]. In the current setup, however, 37% more sample was injected due to a longer capillary. Besides the higher concentration in the pI zone, the amount of detergent (added to keep proteins in solution) is now reduced from 1 %^{w}/$_v$ to 0.1 %^{w}/$_v$ Tween 20.
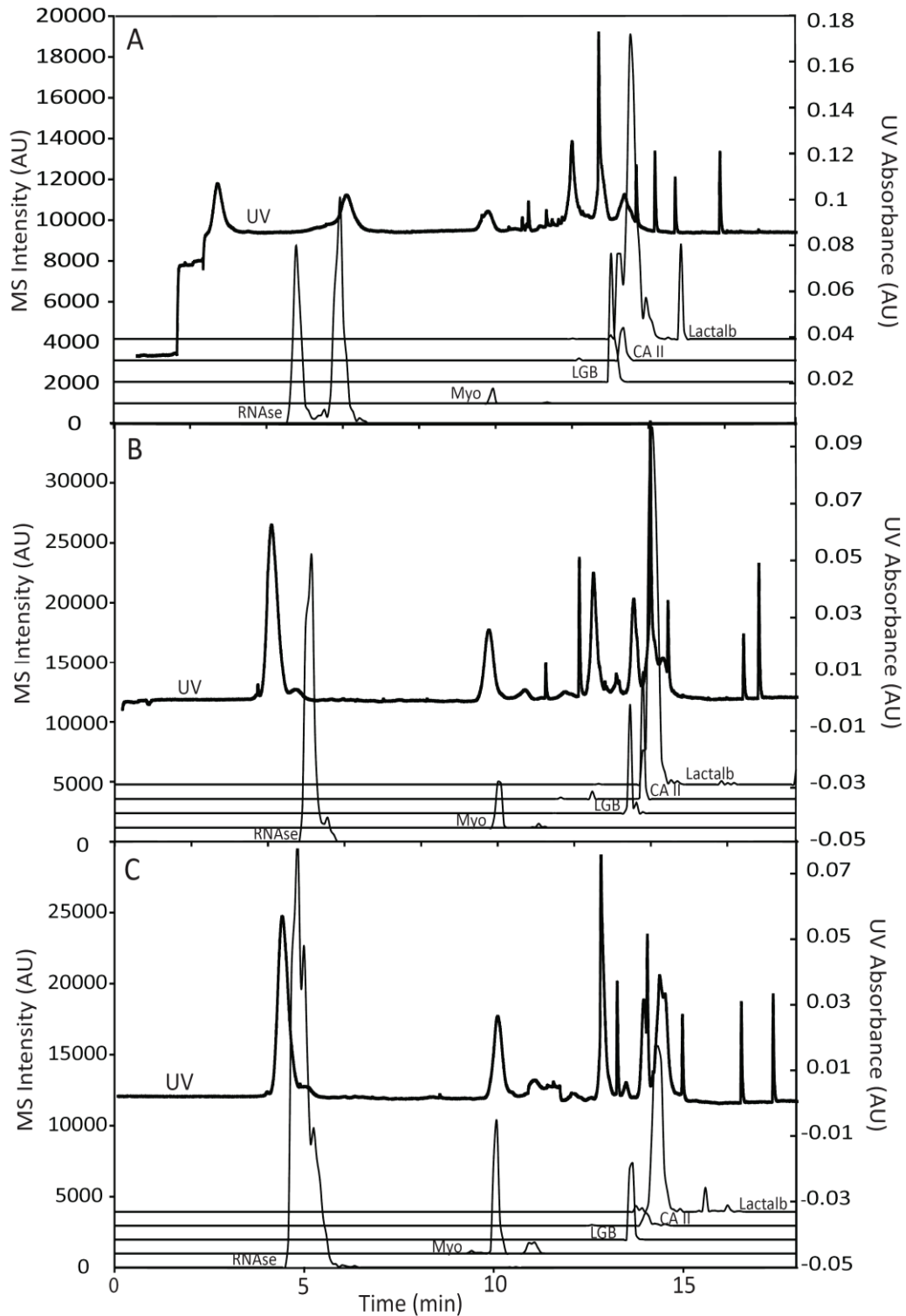
Figure 4.2. cIEF-UV-MALDI-TOF MS results; UV electropherograms (bold trace, right y axis) and reconstructed MS electropherograms (normal traces, left y-axis) are displayed for different focusing times (A, 30 min; B, 75 min and C, 90 min) for Sample 1. The MS-electropherograms were reconstructed by selecting the corresponding intensities of the specific m/z values of the proteins and plotting these data versus migration times.
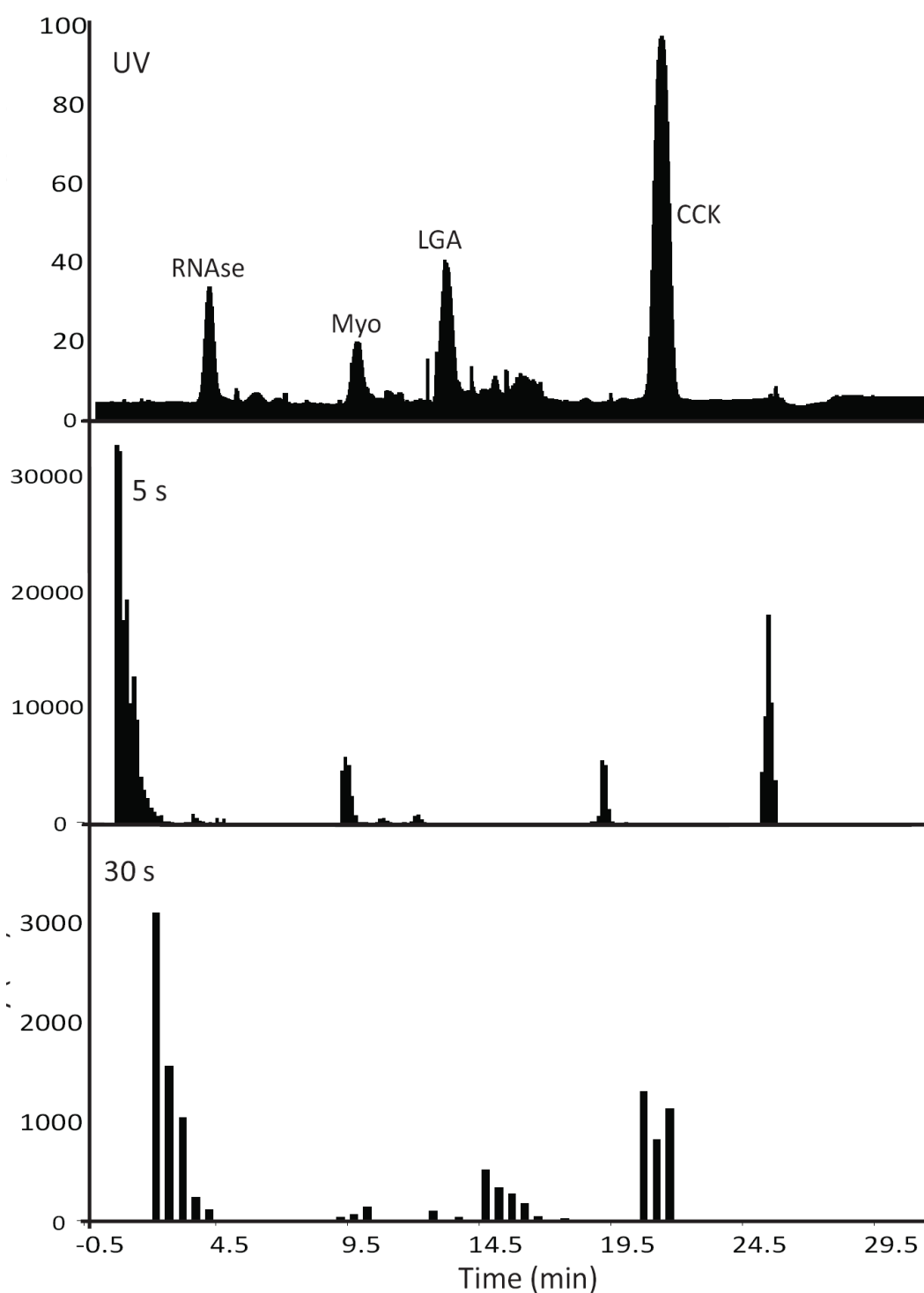
Figure 4.3. Reconstructed MS and UV electropherograms of cIEF-UV-MALDI-TOF MS of Sample 2 for different spotting times (C, 30 s and B, 5 s) and a typical UV trace (A). The MS-electropherograms were reconstructed by selecting the corresponding intensities of the specific m/z values of the proteins and plotting these data versus migration times.

This reduces the amount of signal suppression in MS, but on the other hand increases the probability of aggregation and precipitation. The focusing time is

inversely proportional to the electrophoretic velocity of the compounds in the capillary, which is described by Eqn. 4.1 [21]:

$$U_E(x) \sim \frac{d\mu_E}{d\text{pH}} E \frac{d\text{pH}}{dx}$$
(Eqn 4.1)

In which $U_E(x)$ is the electrophoretic velocity at any given point x in the capillary, $d\mu_E/d(\text{pH})$ the change in electrophoretic mobility over the pH, E the electric field strength and $d(\text{pH})/dx$ is the pH gradient in the capillary. With a longer capillary, the pH gradient decreases, reducing the overall velocity and increasing the focusing time. Since the overall charge of the protein is lower as it migrates towards pH=pI, $d\mu_E/d(\text{pH})$ decreases during the focusing process, which increases the focusing time even more. The third factor in this equation, electrical field strength (E) is also decreased since the same voltage difference is applied over a longer capillary. All three factors are due to a longer capillary and contribute to an increased focusing time compared to the results obtained previously [8]. Overall the optimal focusing time for these experiments was found to be 60 to 75 min. Next to the double peaks, a slight compression of migration times is observed between the UV and corresponding MS data. This is probably due to a combination of factors, *e.g.* the use of hydrodynamic mobilization, the use of a voltage while mobilizing (increasing the time the separated sample is under the influence of an electric field, which can cause slight compression of the pH gradient), *etc*.

Table 4.2 presents the average migration times with corresponding RSD values for UV and MS data. Data of RNase data at 15 and 30 min could not be calculated due to the double peaks, for both UV and MS data.

Table 4.2. Migration times and RSD calculations for cIEF-UV and cIEF-MS data.

	RNAse	Myo maj	Myo min	CA II	LGB	Lactalb
pI	9.5	7.3	6.8	5.9	5.3	4.5
UV						
Average (min)	4.96	10.0	10.9	12.5	13.4	13.9
RSD (%)	4.91	normalized	0.25	0.54	1.41	0.92
MS						
Average (min)	5.14	10.0	11.2	13.9	13.4	14.0
RSD (%)	4.95	normalized	2.32	4.88	3.85	3.97

As can be seen from Table 4.2, the relative standard deviation of all migration times after normalization was within 5%, which indicates that the cIEF-MALDI-TOF MS system described in this paper shows a good repeatability. Contrary to what was expected, the peak widths (Fig. 4.2) are approximately equal in UV and MS. Since the data sampling frequency in UV is much higher than in MS and more factors causing zone broadening are introduced in MS (transfer), the peak width in UV should be considerably lower compared to MS. However, the observed peak broadening is caused by the relatively large UV detector cell (1.2 mm). Furthermore, proteins are subject to signal suppression in cIEF-MS caused by the ampholytes, detergents and cellulose derivatives that are added [8]. Therefore the proteins are only observed in MS when a certain amount is present on the spot. Peaks in the reconstructed MS electropherograms are cut-off below this threshold. Since only the 'tip of the iceberg' is seen, the observed peak width in MS is therefore lower than the real peak width for the MS data.

Spotting time

Upon completion of focusing, the separated proteins have to be mobilized towards the UV detector and the MALDI target plate. Amongst the various possibilities of mobilization, hydrodynamic mobilization is the method of choice here, since it introduces a stable hydrodynamic flow towards the MALDI target plate and offers a high degree of repeatability. Hydrodynamic mobilization introduces zone broadening, which is counterbalanced by applying a voltage difference over the capillary during mobilization. Different spotting times (60, 30, 15 and 5 s) are tested to determine the effect of spotting time on resolution, selectivity and protein signal intensity for Sample 2. The reconstructed MS and UV electropherograms are shown in Fig. 4.3. Peak width, selectivity and MS signal intensity are plotted against different spotting times in Fig. 4.4. As can be seen in both figures, lower spotting times obviously lead to more data points per peak in the reconstructed MS electropherograms and therefore to better peak shapes. From Fig. 4.4a it can be concluded that selectivity does not change significantly with spotting time. When combining these results with Fig. 4.4b, where a decrease in spotting time leads to an overall decrease in peak width, it is concluded that peak resolution in cIEF-MS increases with decreasing spotting times. For a spotting time of 5 s, the peak width in cIEF-MS is comparable to the peak width in cIEF-UV. Furthermore, when decreasing the spotting time, more

components are detected, *e.g.* CA II.This trend is also observed in Fig. 4.4c, where it is shown that the decrease in spotting time increases the MS protein signal. In contrast to what was expected, a *decrease* in absolute amount of protein present per spot, gives an *increased* signal intensity in MALDI-TOF MS.
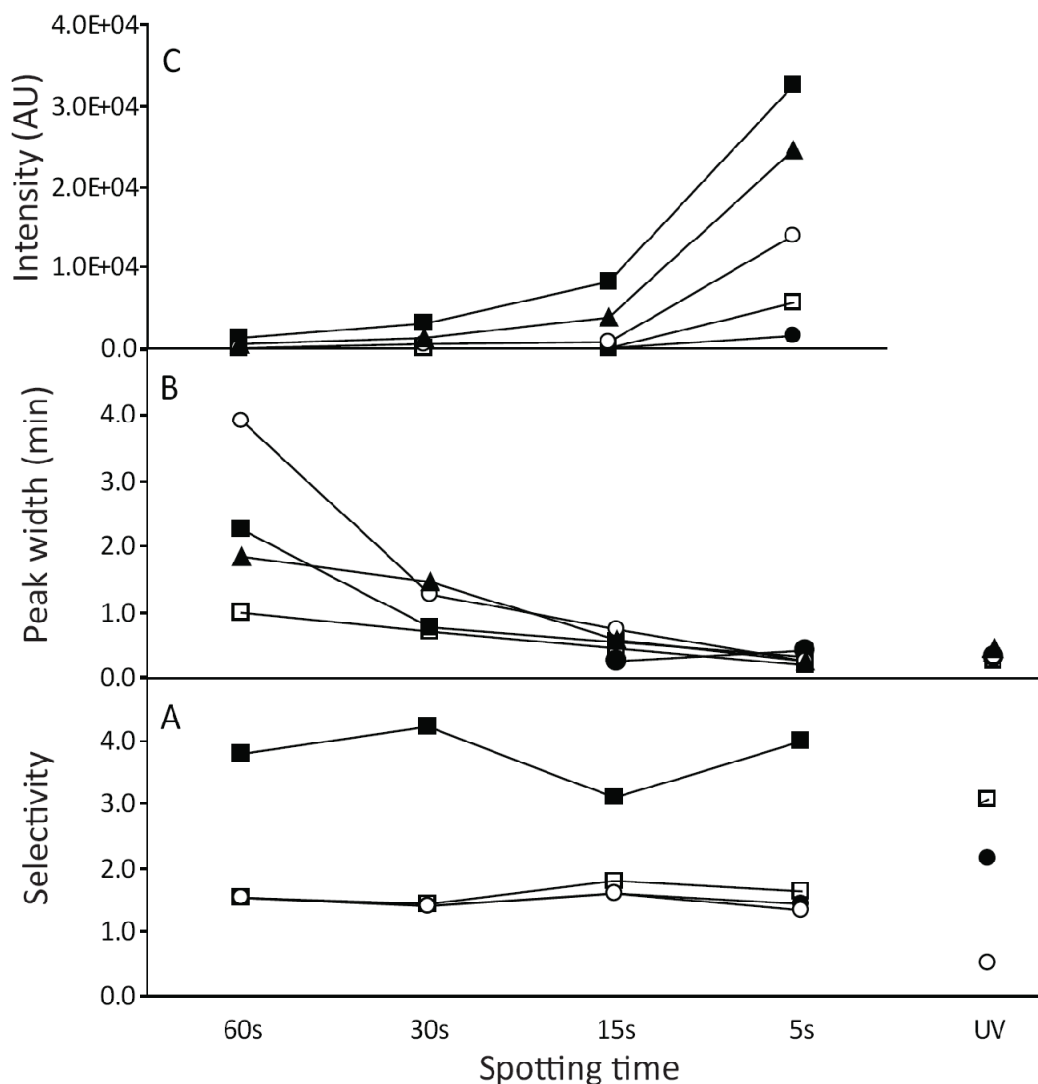

Figure 4.4. MS signal intensity (C), peak width (B) and selectivity (A) versus spotting time and UV signal for Sample 2 (RNAse (■); Myo (□); CAII (●); LGA (○) and CCK (▲) in B and C and RNAse-Myo (■); Myo-LGA (□); LGA-CCK (●) and Myo-CCK (○) in figure A). Focusing time was set to 75 min.

This observation can easily be explained by the fact that with decreasing spotting time, besides a decrease in absolute protein amount, there is also a decrease in cIEF additives (ampholytes, detergents, cellulose derivatives), which enhances the signal suppression in MS. From this we can conclude that the effect of additives on the protein signal intensity is much higher than the effect of the protein concentration itself. The decreasing amount of proteins and additives *per*

spot will lead to a higher protein signal intensity *per* spot. However, at a certain spotting time, the amount of protein *per* spot will be the main factor influencing the signal intensity in MS.
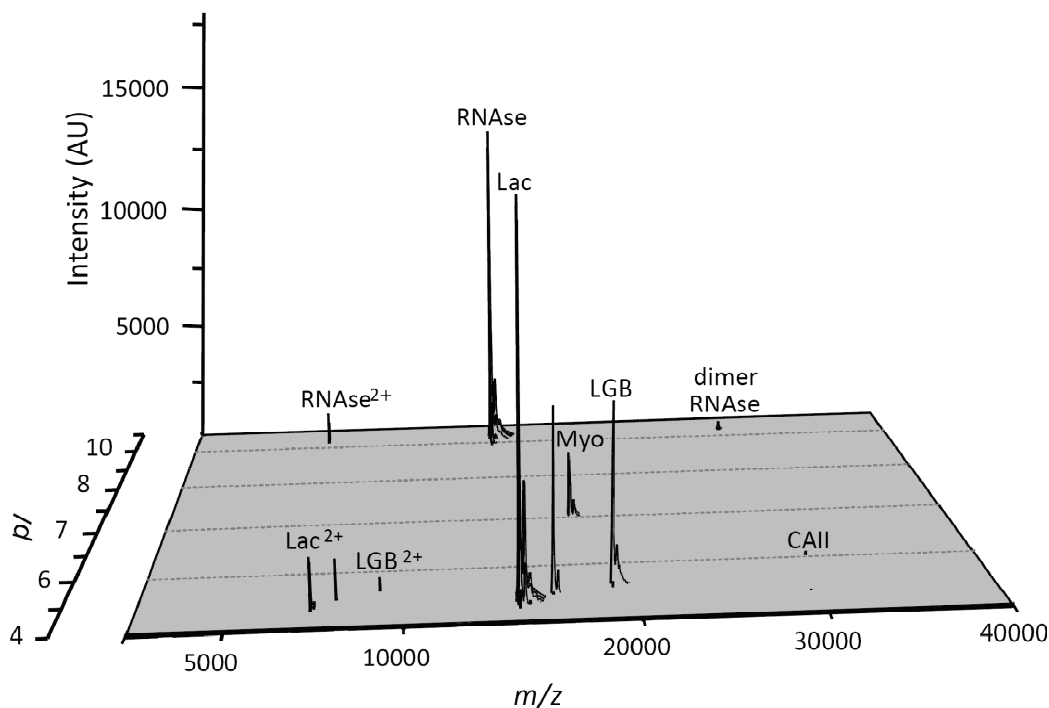

Figure 4.5. Three-dimensional map reconstructed from the mass spectra and UV data of a cIEF-UV-MALDI-TOF-MS run of a mixture of 5 proteins. The m/z values are plotted versus the pI value and the MS signal intensity.

A lower spotting time will then decrease the signal due to the lower amount of protein and not due to the lower amount of additives. Probably, this optimal spotting time is smaller than the LC Packings Probot spotter can handle. For maximum signal intensity in MS, the amount of additives as well as the spotting time should be as low as possible.The optimal conditions were found to be a focusing time of 75 min and a spotting time of 5 s. By combining the data of the cIEF-UV electropherogram and the mass spectra *per* spot, a 3D plot could be constructed for the protein test sample (Fig. 4.5). On the *x*-axis, the *m/z* ratio is plotted (the mass spectra) versus the p*I* on the *Y*-axis. This p*I* is calculated from the migration time in the UV electropherogram. The *z*-axis represents the signal intensity in the MS. Such a 3D plot gives the same information as 2D-GE, *i.e.* p*I* and Mw of the proteins and the formation of dimers, whereas the use of an MS in our cIEF-UV-MS setup can also be used for structure elucidation.Furthermore, cIEF-UV-MS is faster, less labour intensive and offers a higher potential for intact protein analysis.

Applicability

To demonstrate the applicability of this system, a biopharmaceutical formulation is injected into the cIEF-UV-MS system without prior sample clean-up. GlucaGen was incubated with 0.1 M HCl for two weeks at room temperature. This degraded sample was analyzed with MALDI-TOF MS. However, no degradation products could be distinguished. Fig. 4.6a shows the UV and reconstructed MS electropherograms of a non-degraded GlucaGen sample. The monoisotopic peak for glucagon is observed at *m/z* 3482. Fig. 4.6b shows the UV and reconstructed MS electropherograms of the sample degraded by 0.1 M HCl.
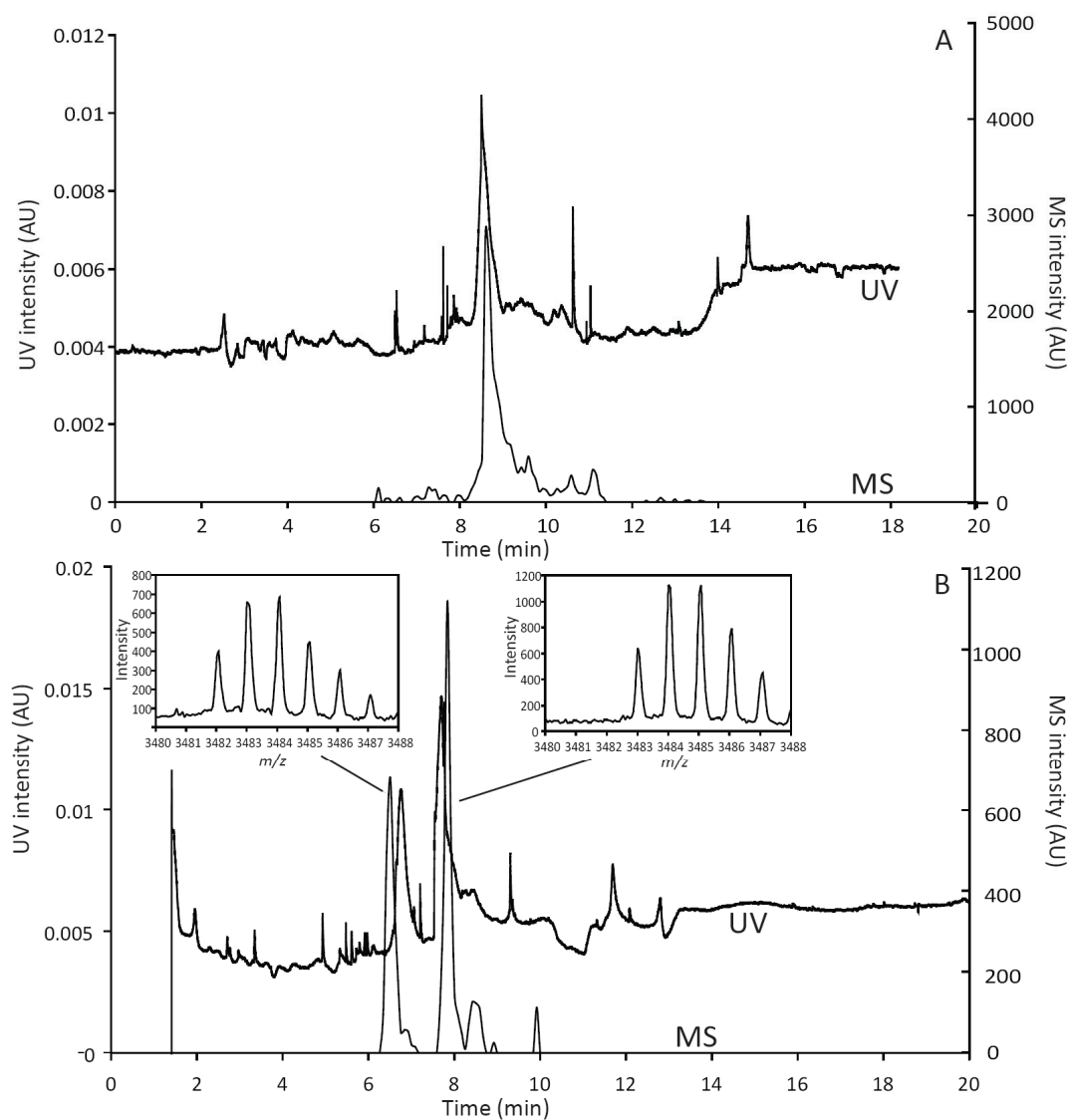
Figure 4.6. Reconstructed MS electropherograms (thin trace) and UV electropherograms (bold trace) of intact glucagon (A) and degraded glucagon (B). The injected sample contains 1mg/mL glucagon in 50 µg/mL 0.3% HEC with 1%w/v Pharmalytes and 0.1% w/v Tween 20. The mass spectra depicted in the graphs are spectra of the corresponding cIEF peaks.

In the UV electropherogram a shift of more than 1 pH unit (from p*I* 6.8 to 5.4) is observed. Furthermore, the mass spectra of the reconstructed electropherogram of degraded glucagon shows an isotope peak shift of +1 *m/z* unit for the degradation peak (*e.g.* from 3482 to 3483 for the monoisotopic peak). The major degradation products of glucagon in acidic solution (1 M HCl or 0.1 M HCl at elevated temperature) are aspartic cleavages at aspartic acid (Asp) residues 9, 15 and 21 and glutaminyl (Gln) deamidation at Gln residues 3, 20 and 24 and at the asparagine (Asn) residue 28 [22-25]. In both the cIEF as well as the MS data, the aspartic cleavage products were not observed. This may be due to the mild degradation conditions and the presence of stabilizing ingredients in the GlucaGen formulation. In contrast, the drop in p*I* of more than 1 pH unit in combination with the difference of 1 mass unit indicates deamidation of one of the Gln residues or the Asn residue. A theoretical pI shift from 6.75 to 5.43 and mass shift of +1 Da is calculated (Expasy).Although in general it is suggested that due to the longer side chain asparaginyl deamidation is more facile [26], it is also reported that this asparaginyl deamidation was not observed for glucagon degradation in acidic solutions [22]. This lower deamidation rate of Asn compared to Gln in an acidic environment was also confirmed by [25]. Therefore it is more probable that one of the Gln residues is deamidated. Although Joshi *et al.* [22] suggest the Gln residue at position 3 is most likely to be deamidated of the three Gln residues, in a more recent paper this group concludes that all three Gln residues have equal deamidation rates [24]. It is, however, still not clear why only one degradation peak is observed in our study.

4.4   Concluding remarks

This paper describes and characterizes the hyphenation of cIEF and MALDI-TOF MS system for the repeatable analysis of proteins. After solving the practical problems encountered when coupling both systems, the effects of focusing and spotting times on repeatability, peak width and signal intensity were studied. Since for shorter migration times, a transitional double peak effect was observed, a longer focusing time than in cIEF-UV systems (60-75 min *versus* 10-15 min) was required. However, migration times and peak widths for the higher focusing times in MS were comparable to UV. A focusing time of 75 min was found to give the best results. A decrease of spotting time led to a lower peak width of the reconstructed MS electropherograms, whereas selectivity did not change, which

affirmed the fact that the resolution increases with decreasing spotting time. The most remarkable and interesting result was the observation of a higher protein signal with decreasing spotting time. Obviously, the effect of the smaller amount of interfering cIEF additives *per* spot has more influence on the signal intensity than the lower amount of proteins *per* spot. This indicates that there is an optimal spotting time where the effect of the additives on protein signal is minimal and the amount of protein is the determining factor for the MS signal intensity. Although a limit of detection of 250 nM or 1 pmol protein was estimated for the conditions described in this paper taking into account the limitations of the spotting device and the signal suppression in MS. Performing cIEF-MALDI-TOF MS is still a compromise between cIEF resolution and MS signal intensity. While the MS data can be used for (semi) identification and peak purity, MALDI-TOF MS data are not suited for quantitative purposes. The combination with cIEF-UV makes quantification possible and contributes to the identification of compounds by supplying information on p*I*.

Applicability of the system has been shown by the degradation of a biopharmaceutical formulation. Deamidation of glucagon would not be observed when using only MS. Therefore it was first separated from its non-degraded component with cIEF. A shift of ca 1 p*I*-unit was observed in cIEF. In combination with the shift of 1 Da, which was now clearly visible in the MS. This confirmed the expected deamidation product. Although it is possible to analyze real biological samples like plasma [11], urine, lysate, *etc.,* it should be taken into account that these matrices are usually too complex to be injected directly onto systems like these without prior sample clean-up. CIEF-MALDI-TOF MS still is a promising technique for the analysis of proteins in biological matrices, and has now also proven its use in stability and degradation studies for biopharmaceutical compounds. As shown here, deamidation products, which only lead to a minimal difference in *m/z*, are clearly observed with cIEF.

Acknowledgements

References

[1]   Hjertén, S., Zhu, M. D., J Chromatogr A 1985, 346, 265-270.

[2]   Tang, Q., Harrata, A. K., Lee, C. S., Anal Chem 1995, 67, 3515-3519

[3]   Foret, F., Muller, O., Thorne, J., Gotzinger, W., Karger, B. L., J Chromatogr A 1995, 716, 157-166.

[4]   Tang, W., Harrata, A. K., Lee, C. S., Anal Chem 1997, 69, 3177-3182.

[5]   Wehr, T., Lc Gc N Am 2004, 22, 998-1000.

[6]   Haselberg, R., de Jong, G. J., Somsen, G. W., J Chromatogr A 2007, 1159, 81-109.

[7]   Silvertand, L. H., Sastre Toraño, J., van Bennekom, W. P., de Jong, G. J., J Chromatogr A 2008.

[8]   Silvertand, L. H., Sastre Toraño, J., de Jong, G. J., van Bennekom, W. P., Electrophoresis 2008, 29, 1985-1996.

[9]   Minarik, M., Foret, F., Karger, B. L., Electrophoresis 2000, 21, 247-254.

[10]   Chartogne, A., Gaspari, M., Jespersen, S., Buscher, B., Verheij, E., Heijden Rv, R., Tjaden, U., van der Greef, J., Rapid Commun Mass Spectrom 2002, 16, 201-207.

[11]   Crowley, T. A., Hayes, M. A., Proteomics 2005, 5, 3798-3804.

[12]   Yu, W., Li, Y., Deng, C., Zhang, X., Electrophoresis 2006, 27, 2100-2110.

[13]   Lechner, M., Seifner, A., Rizzi, A. M., Electrophoresis 2008, 29, 1974-1984.

[14]   Mok, M. L., Hua, L., Phua, J. B., Wee, M. K., Sze, N. S., Analyst 2004, 129, 109-110.

[15]   Fujita, M., Hattori, W., Sano, T., Baba, M., Someya, H., Miyazaki, K., Kamijo, K., Takahashi, K., Kawaura, H., J Chromatogr A 2006, 1111, 200-205.

[16]   Guo, X., Chan-Park, M. B., Yoon, S. F., Chun, J. H., Hua, L., Sze, N. S., Anal Chem 2006, 78, 3249-3256.

[17]   Brinkworth, C. S., Bourne, D. J., J Am Soc Mass Spectrom 2007, 18, 102-105.

[18]   Wehr, T., Handbook of isoelectric focusing and proteomics, Elsevier Inc., San Diego 2005, pp. 181-210.

[19]   Catsimpoolas, N., Campbell, B. E., Griffith, A. L., Biochim Biophys Acta 1974, 351, 196-204.

[20]   Huang, T., Liu, Z., Pawliszyn, J., Anal Bioanal Chem 2005, 382, 783-788.

[21]   Sounart, T. L., Handbook of isoelectric focusing and proteomics
       Elsevier Inc., San Diego 2005, pp. 41-68.

[22]   Joshi, A. B., Rus, E., Kirsch, L. E., Int J Pharm 2000, 203, 115-125.

[23]   Joshi, A. B., Kirsch, L. E., Int J Pharm 2004, 273, 213-219.

[24]   Joshi, A. B., Sawai, M., Kearney, W. R., Kirsch, L. E., J Pharm Sci 2005, 94, 1912-1927.

[25]   Joshi, A. B., Kirsch, L. E., J Pharm Sci 2002, 91, 2331-2345.

[26]   Robinson, A. B., Rudd, C. J., Curr Top Cell Regul 1974, 8, 247-295.

Chapter 5

Development and evaluation of an IEF-iSPR system for biomarker research

L.H.H. Silvertand, M.B.M. Harink, E.C.A. Stigter, R.B.M. Schasfoort, G.J. de Jong, W.P. van Bennekom, submitted.

Abstract

In this paper the proof of principle of hyphenating isoelectric focusing (IEF) with surface plasmon resonance imaging (iSPR) is described for finding protein biomarkers in highly complex matrices. After separation, the proteins in the IEF gel are pressure blotted onto an iSPR sensor surface. It is demonstrated that the polyacrylamide IEF gel allows diffusion of the proteins to the sensor surface and does not have an influence on the binding of proteins to the sensor surface. The maximum binding capacity of the sensor disc is determined to be ca 5 ng per mm^2. This capacity is exceeded for protein gel concentrations in the low µg/mL range after focusing. For synovial fluid (SF) spiked with carbonic anhydrase I, more concentrated SF has a positive effect on focusing properties in IEF increasing the sharpness of the CAI zone. After blotting, anti-CAI antibody is flushed over the immobilized proteins and a 2D iSPR print of the IEF separation for CAI can be constructed. Experiments with the relevant rheumatoid biomarker citrullinated fibrinogen - antibody pair demonstrate the effect of purified antibodies in contrast to whole antiserum and show the applicability for study of auto-immune diseases.

5.1   Introduction

In the search for protein biomarkers in biological fluids nowadays, the bioanalytical chemist has to make a choice in the wide range of available sample pretreatment procedures, separation steps, bioassays and detection methods. Each method has its advantages and disadvantages, forcing the scientist to make a well-considered but difficult choice, since there is no universal approach. However, some strategies seem to be favourable, depending on the sample matrix, the biomarker in question and not in the least on the expertise of the scientist.

One general strategy for an unknown sample may be to start with the removal of matrix components like lipids, sugars and salts from the proteins/peptides, by *e.g.* ultra centrifugation, dialysis, extraction, free flow electrophoresis, etc. The complexity of the remaining protein/peptide sample can be further reduced by fractionation strategies based on protein characteristics like p*I* (isoelectric focusing (IEF), ion-exchange chromatography, off-gel electrophoresis), molecular weight (SDS-PAGE, size exclusion chromatography (SEC)) or hydrophobicity

(RPLC). Each fraction can be separated by a (capillary) liquid chromatographic (LC) or (capillary) electrophoresic (CE) method, with or without digestion and preferably coupled to a mass spectrometer for identification of proteins.

A different strategy can be chosen if an antibody for the biomarker is available. Immunoaffinity methods like surface plasmon resonance (SPR), 2D PAGE-western blot or antibody-based LC (immunoaffinity chromatography or SPE) and CE (immunoaffinity CE) methods are used to isolate proteins. These analytes can, if necessary, be further analyzed after this selective isolation step.

This paper describes the development and the proof of principle of a strategy to find biomarkers for auto-immune diseases and more specifically rheumatoid arthritis (RA). Over the past few years, it has become clear that citrullinated proteins and peptides play an important role in the pathogenesis of RA. These compounds are nowadays used to diagnose RA and can predict the development of RA, since they are present in the earlier stages of the disease [1-8]. The current ELISA-based test methods screen on the presence of antibodies against these citrullinated proteins and peptides [9]. These citrulline-specific antibodies can also be used to find citrullinated antigens in biological fluids like citrullinated fibrinogen (cFib) [10]. Therefore cFib and the anti-citrulline antibody were chosen as antibody-antigen pair for the experiments described in this paper.

A label free immunoaffinity-based method was selected for our study: surface plasmon resonance imaging (iSPR). (i)SPR is an optical biosensor that measures label free and real time protein adsorption and biomolecular interactions by changes in reflectivity of *p*-polarized light at a metal/dielectric surface (thin gold layer/glass). At a certain angle of incidence, the free electrons in the gold (surface plasmons) acquire the maximum resonation (oscillation). Compounds adsorbing or desorbing from this interface change the angle at which this maximum resonance occurs. This angle change, the shift (expressed in mdeg), can be monitored (sensorgram) and is proportional to the mass bound to the surface [11-13]. iSPR uses a CCD camera to image the reflected light and can simultaneously monitor multiple individual interaction spots in real time, so called regions of interest (ROIs). Next to the possibility of real time interaction monitoring, (i)SPR offers the advantage of label-free detection [13, 14], high flexibility and versatility [13], automatization [15, 16] while leaving the compounds intact and available for further analysis like MS [17]. However, (i)SPR also has some disadvantages like the high sensitivity to temperature changes [17]

and low sensitivity (down to 1-10 nM for a 20 kDa protein [18, 19]). However, the major problem in (i)SPR is non-specific binding [13], which makes the direct analysis of low abundant biomarkers in biological fluids like whole blood, synovial fluid, etc. very difficult. To decrease non-specific binding, usually highly diluted matrices are used [20, 21]. However, this reduces sensitivity and the analyte-to-matrix ratio remains the same. Several procedures to reduce the non-specific binding in SPR have been proposed [22-29], but none can completely eliminate the problem.

Here we present yet another approach to circumvent the interference of non-specific binding, *i.e.* isoelectric focusing separation prior to SPR. In isoelectric focusing (IEF), proteins are separated in an immobilized pH gradient gel (IPG) by applying a voltage difference over the ends of the gel. Proteins, peptides and other charged molecules migrate under the influence of the electrical field until their nett charge becomes zero and their *p*I matches the local pH for proteins [30]. Salts will migrate to the electrode compartments. Next to the separation of the proteins, peptides and matrix components, IEF also concentrates the compounds in their zone. This is highly beneficial for the hyphenation with iSPR, since IEF presents a concentrated zone of the compound of interest to the iSPR surface with a reduced local concentration of interfering matrix components, thereby increasing the analyte-to-matrix ratio. Furthermore, instead of immobilizing antibodies on the iSPR surface, the separated and concentrated proteins are immobilized, followed by interaction monitoring with complementary antibodies. This strategy gives higher signals since the antibody is usually the largest compound. Although some papers on (microchip) capillary electrophoresis coupled to SPR has been published, [31-33], the use of gel electrophoresis prior to or after SPR for separation or purification is widely spread, *e.g.* [34-36]. The direct transfer and imaging of proteins from an IEF gel onto an iSPR surface as described in this paper has to our knowledge never been reported.

In this paper, the hyphenation of IEF to iSPR via pressure blotting is described for the purpose of finding biomarkers in a biological fluid. The IEF step separates, desalts and concentrates the possible biomarkers in pH zones. Subsequently, the compounds are transferred to and immobilized on the SPR surface. Finally, after removing of the blotting tool and installing a flow-cell the interaction of these IEF separated and immobilized compounds with specific antibodies is monitored.

Method development and characterization of the system are presented. The proof of principle is demonstrated with synovial fluid spiked with citrullinated fibrinogen.

5.2   Materials and methods

Materials

Immobiline IPG strips (pH 3-10, 7 cm), Immobiline DryStrip Cover fluid (mineral oil) and cellulose electrode strips were obtained form GE Healthcare Life Sciences (Diegem, Belgium). Carbonic anhydrase I (CAI, from human erythrocytes, IEF marker p*I* 6.6), myoglobin (Myo, from equine skeletal muscle, 95-100%), human serum albumin (HSA, 99% by agarose gel electrophoresis), rabbit anti-human serum albumin antibody (anti-HSA, whole antisera), phosphate buffered saline (tablets), ethanolamine hydrochloride ($\geq$ 99.0%), glycine (ACS reagent $\geq$ 99.4%), Coomassie G250, phosphoric acid ($\geq$ 85% wt. in H_2O), sodium hydroxide ($\geq$ 98.0%), ammonium sulphate ($\geq$ 99.0%), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC, $\geq$ 97.0%) and N-hydroxysuccinimide (NHS, 98%) were acquired from Sigma-Aldrich (Steinheim, Germany). Methanol (absolute HPLC Grade) was obtained from Biosolve (Valkenswaard, The Netherlands). Tween 20 (for Analysis) was purchased from Merck Schuchardt OHG (Hohenbrunn, Germany), while acetic acid (100%) was acquired from Merck KGaA (Darmstadt, Germany). Goat polyclonal anti-human carbonic anhydrase I antibody (anti-CAI, raw goat sera) was obtained from DivBioScience (Breda, The Netherlands). Goat polyclonal anti-horse myoglobin antibody (anti-myo, purified) was purchased from Bethyl Labs (Montgomery, TX, US). Human synovial fluid (SF), fibrinogen (Fib), citrullinated fibrinogen (cFib) and anti-human anticitrulline antibodies (anti-cit) were kindly donated by prof. dr. Ger Pruijn (Radboud University, Nijmegen, The Netherlands).

Methods

The immobiline IPG strips 3-10 (7cm) are rehydrated overnight in sample solution (proteins in water or in diluted synovial fluid). Then the duplicate strips are placed in a 3100 Fractionator (kindly donated by dr. Gerard Rozing of Agilent Technologies, Waldbronn, Germany), which is used as an IEF device rather than

for its original purpose *i.e.* off-gel fractionation. Cellulose papers soaked in 10 mM phosphoric acid (anolyte) and 20 mM NaOH (catholyte) are placed between the rehydrated gel and the electrodes at both ends of the gel and the gel is covered with mineral oil. The voltage program in Table 5.1 is applied. An IBIS Easy2Spot G-type disc (with preactivated EDC-NHS dextran) is placed on top of the prism that was positioned in the IBIS iSPR holder. This holder is then properly installed together with an 11 mm IBIS iSPR cuvette (IBIS Technologies Hengelo, The Netherlands).

Table 5.1. Voltage program for the IEF experiments.

Step	Duration (h)	Voltage	Mode	Max I (µA)
1	1	500	Linear	20
2	3	2000	Linear	20
3	4	4000	Linear	20
4	7	6000	Linear	20
5	45	6000	Step	20
6	0.1[*]	50	Step	20

[*] *After 60 hours or 100 kVh, the focusing was assumed to be complete and the voltage was reduced to 50 V to maintain separation.*

The surface of the disc is homogenised with 400 µL of a freshly prepared EDC-NHS solution (200 mM EDC, 50 mM NHS) and after 20 min followed by three rinse steps with 450 µL 10 mM acetate buffer pH 4.0. During the last SPR washing step, the IPG strips are removed from the fractionator and washed 5 times with 10 mM acetate buffer pH 4.0 to dilute the mineral oil.

One of the gel strips is stained with a colloidal Coomassie solution [37]. The other strip is placed gel side up on a glass slide and the required piece of gel, typically 9 mm, is cut out with a surgical knife and transferred to the blotting tool (Fig. 5.1). The gel is attached to this tool with double-sided tape. The blotting tool is then placed in the 11 mm cuvette in a way that enables contact between the gel and the disc. The proteins are allowed to transfer from the gel onto the disc for 8 min under the weight of a blotting tool (192.5 g per cm^2). After these 8 min, the blotting tool, including the gel, is removed from the system and the remaining active sites on the disc are blocked with 1 M ethanolamine pH 8.0 for 10 min which is followed by three washing steps with PBS. The 11 mm cuvette is then replaced by an IBIS flow cell (IBIS Technologies) and this system is flushed five

times with PBST. A baseline signal is constructed with PBST at 2 μL s^{-1}, before running the interaction method which consists of: 10 min of PBST at 2 μL min^{-1} followed by the antibody interaction step (usually 1 to 50 diluted anti-body at 2 μL min^{-1} for 60 min) and a washing step with PBST to remove all the unbound components (at 2 μL min^{-1} for 60 min). A last regeneration step with 1 M glycine HCl adjusted to pH 2.0 removes all the antibodies from the sensor disc and allows the signal to return to baseline (at 2 μL min^{-1} for 10 min). The interaction procedure was repeated three times. Data were acquired with IBIS iSPR Software version 3.0.

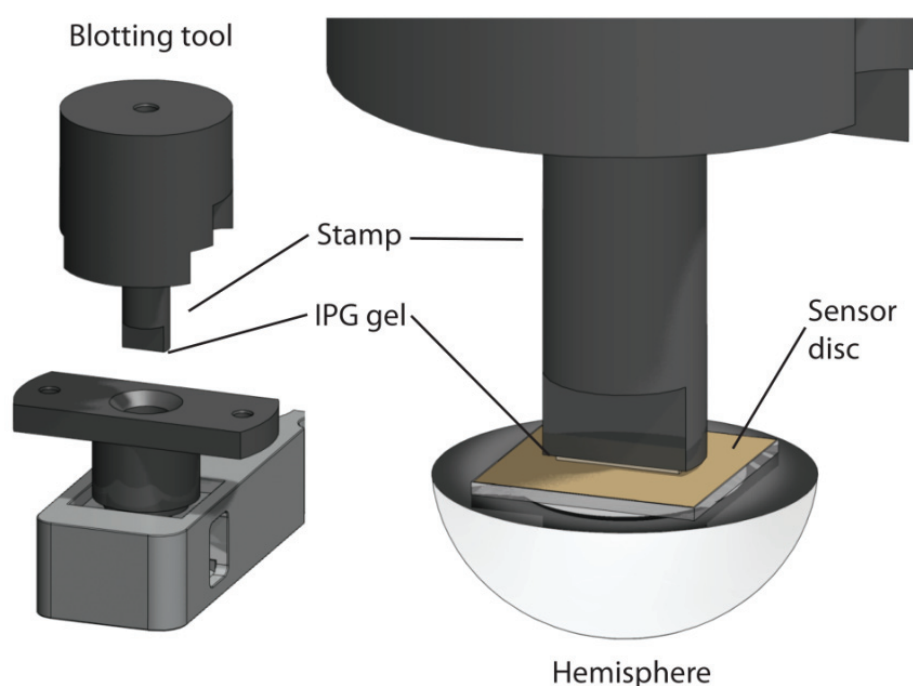*Figure 5.1. The pressure blotting tool.*

5.3   Results and discussion

Development of IEF-iSPR system

Before coupling IEF to iSPR, the two systems have been tuned to be compatible by selecting the appropriate materials and procedures. First, the shortest standard available Immobiline IPG strips were selected (7 cm, pH 3-10) because these give the highest pH window (0.7 pH units per 7 (by 3) mm sensor disc that can be imaged). After soaking the gel in a sample solution (a protein in a diluted matrix), the gel is placed in the fractionator for separation of the compounds.

Second, no carrier ampholytes are added to the system. Free carrier ampholytes were found to interfere with the binding of the proteins to the iSPR sensor surface. Therefore, the applied voltage program is longer than normally used for IEF or off-gel electrophoresis (see Table 5.1). Shorter voltage programs as suggested by GE Healthcare and Agilent lead to double peaks (proteins are not completely focused), which are visible after blotting on the iSPR sensor surface. Third, to minimize the effect of mineral oil on the binding of the proteins to the sensor surface during blotting, the strips are washed in 10 mM acetate buffer pH 4 prior to blotting. An additional advantage of this acetate buffer washing step after the IEF separation, is that the proteins in the gel are protonated, which enhances the electrostatic interaction with the reactive groups on the sensor surface. However, some of the protein in and on the gel might be washed off during this procedure.

One way to blot the gel onto the sensor surface is by pressure blotting. The piece of gel is attached to a home-made blotting tool (Fig. 5.1) using double-sided tape. The tool fits exactly in the IBIS 11 mm cuvette and the weight of the blotting tool presses the gel onto the activated sensor surface. The process from washing the gel to blotting takes less than 20 s, therefore only minimal dispersion takes place during the transfer from the fractionator to the SPR surface. Transfer of the proteins from the gel onto the sensor surface and vice versa reaches a steady state at 8 min. To minimize zone broadening, the gel is therefore allowed to be in contact with the sensor surface for 8 min.

A sensor with a relatively high degree of binding sites for proteins is selected for iSPR, *i.e.* the IBIS Easy2spot G-type disc. This disc consists of a carboxymethyl-ated (CM) dextran gel modified with EDC-NHS. Under low-ionic-strength acidic conditions (10 mM acetate buffer pH 4), unmodified free carboxylic acids in the CM dextran are negatively charged and attract the positively charged proteins, which react with the active esters formed after EDC-NHS activation of the derivatized carboxyl groups [38]. Furthermore, due to the pressure blotting, the liquid in the polyacrylamide gel is squeezed out and will become in close contact with the activated gel leading to an increased transfer and binding of compounds.

Transfer efficiency

The loading capacity of the discs and the effect of the gel on the iSPR disc are determined. The loading capacity is determined by flushing a HSA solution (1 mg mL^{-1}) in acetate buffer over the surface at 2 μL s^{-1} for 1 h. The maximum iSPR angle shift for the ROIs (in these experiments $6.25*10^{-2}$ mm^2) was 294 mdeg. Since a signal of 1 mdeg corresponds to an approximate binding degree of 10.8 pg mm^{-2} [12], the immobilization capacity for the iSPR disc is about 3.2 ng mm^{-2}.

To determine the effect of the gel matrix on the iSPR surface, an IPG strip rehydrated in acetate buffer (10 mM, pH 4) is blotted on the iSPR surface. As can be seen in Fig. 5.2a, the ROIs in the area of the gel exhibit a bulk shift of 500-700 mdeg.
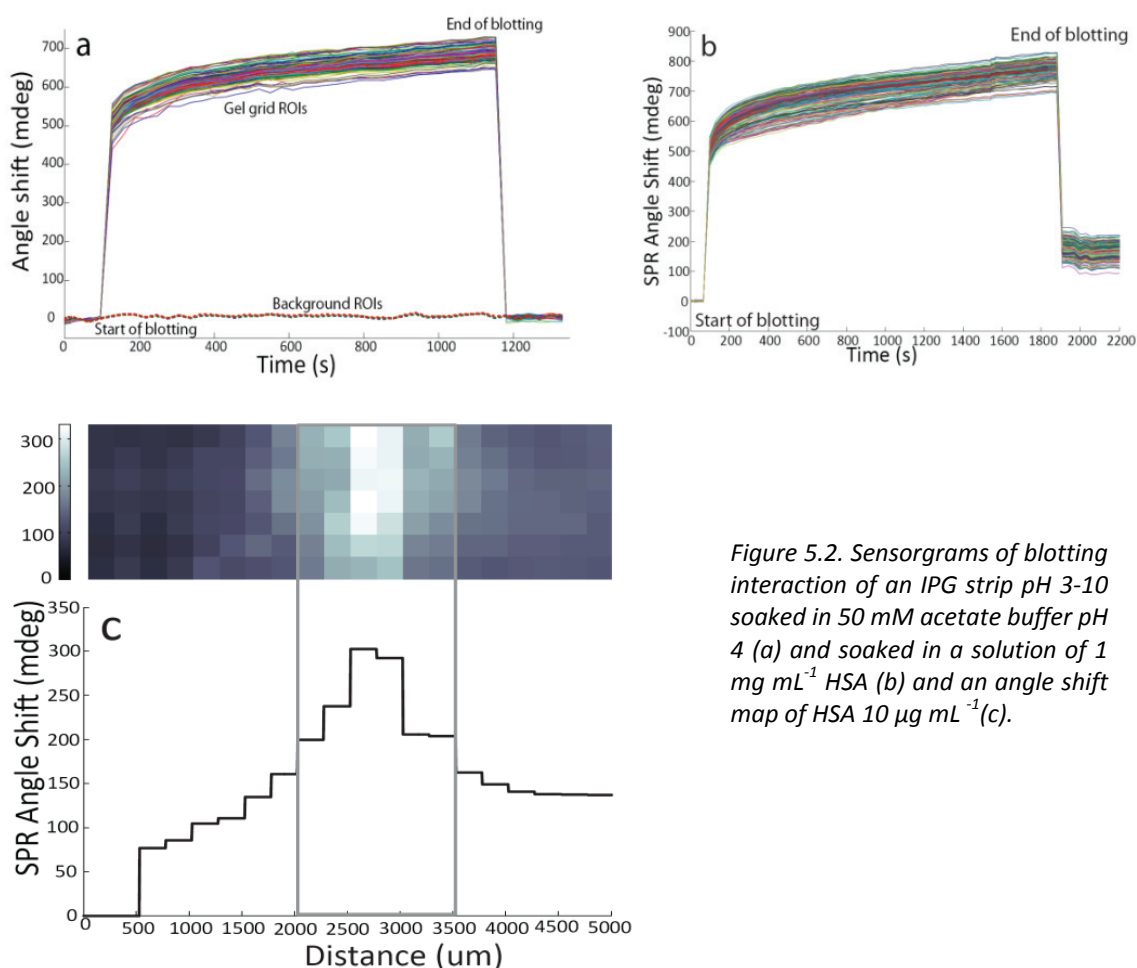

Figure 5.2. Sensorgrams of blotting interaction of an IPG strip pH 3-10 soaked in 50 mM acetate buffer pH 4 (a) and soaked in a solution of 1 mg mL^{-1} HSA (b) and an angle shift map of HSA 10 μg mL^{-1}(c).

This extremely high shift is largely due to the mass of the blotting tool, which compresses the gel and leads to an increasing shift during blotting. After

removing the gel and flushing the disc with PBS, the angle shift returns to 0 mdeg indicating no (non)specific binding of the gel.

The effect of the gel matrix on protein immobilisation is determined by blotting an IPG strip soaked in HSA solution (10 µg mL^{-1}) without prior focussing. The bulk shift is comparable to the blanc strip, *i.e.* 550-800 mdeg (Fig 2b). However, after removal of the gel and flushing the sensor surface with PBS, an average shift of 170 mdeg over the gel grid ROIs remains, indicating a binding of an average of approximately 1.8 ng mm^{-2} HSA of sensor surface. From these experiments it can be concluded that the IPG gel matrix (polyacrylamide) does not cause (non)specific binding, whereas the protein present in the gel (HSA) will diffuse out of the IPG gel and is immobilized on the sensor surface.

These results are also used to calculate the efficiency of the protein transfer. The approximate amount of HSA in the IPG gel is 5.4 ng per mm^2 or 10.8 ng per mm^3 (1250 ng HSA in 231 mm^2 gel or in 116 mm^3), while the observed binding on the disc is 1.8 ng mm^{-2} leading to a transfer efficiency (amount immobilized / amount in IPG gel) of at least 33% for unfocused HSA. However, two notes should be made to these calculations. First, some of the HSA is probably washed off the gel prior to blotting and this amount is not taken into account. Second, a 2D blot on an iSPR surface is made from a 3D IEF gel. In the IPG gel, the proteins are evenly distributed. When blotting, only the proteins near the IPG-gel surface are immobilized on the disc. However, for comparison, it is assumed that all the proteins in the IPG gel are transferred onto the surface and available for immobilization. This assumption makes the estimated transfer efficiencies presented here lower than they are in reality.

Estimating that after focusing approximately 80% of the HSA (1000 ng) is in 2.6 mm^2 (square in Fig. 5.2c), the average concentration in the IPG gel is 382 ng mm^2. The average binding on the disc is calculated to be ca 4.8 ng mm^{-2}. This is slightly lower than the calculated 5.4 ng mm^{-2} for a free HSA solution and is approximately the maximum binding capacity of the sensor disc for HSA. Since this maximum binding capacity (ca 5 ng mm^{-2}), cannot be exceeded and the concentration of HSA in the IPG gel after focusing is about 70 times higher than for an unfocused gel, the transfer efficiency is only 1.3%. Furthermore, 4.8 ng mm^{-2} is an average value for the selected area which includes different local protein concentrations (Fig. 5.2c). From these results it can be concluded that IEF-iSPR is probably well suited for low amounts of proteins.

Resolving power

In order to determine the resolution of the system, two proteins with similar p*I* are chosen: CAI (p*I* 6.6, Mw 29 kDa) and myo (p*I* 6.8 and 7.3, Mw 17 kDa). Directly after blotting the gel onto the surface (Fig. 5.3a), the interaction of the proteins with the sensor surface is hardly visible for myo over the background signal, whereas only a small peak is seen for CAI.
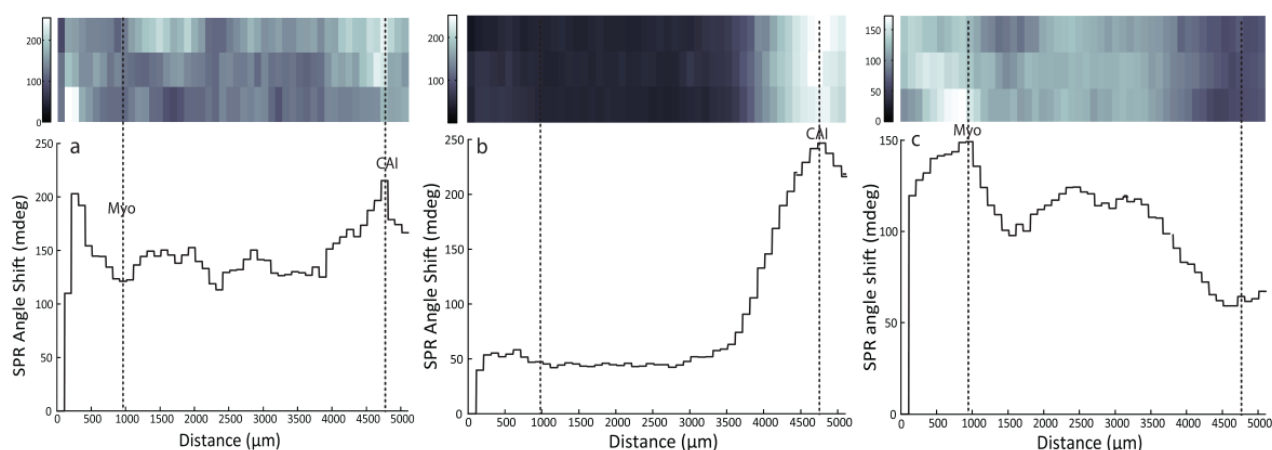

Figure 5.3. SPR angle shift map of isoelectric focused CAI and myo with the sensor surface before (a) and after interaction with anti-CA I antibody (b) and anti-myo antibody (c). In these angle shift map, increasing pH is from right to left.

However, after removing the gel, blocking the surface with ethanolamine and flushing with PBS and anti-CAI antibody (20 µg mL^{-1}, polyclonal), the signal-to-background ratio increases significantly (Fig. 5.3b). The CAI signal is observed as a clear, intense peak at the side of the sensor disc with an angle shift of approximately 250 mdeg. When regenerating the surface with 1 M glycine HCl (pH 2) and flushing with PBS followed by anti-myo antibody (20 µg mL^{-1}, polyclonal), a significant lower interaction is observed on the opposite side of the disc (Fig. 5.3c). Since the interaction steps are repeated three times with the same results, the glycine HCl step does not remove the immobilized protein from the sensor surface and is therefore not the reason for the observed lower interaction for myoglobin. This lower interaction is due to the fact that not the major compound of myo is observed (p*I* 7.3), but the minor component (p*I* 6.8), which is usually present at 5-10% of the total concentration of myoglobin or at ca 1 µg mL^{-1}. Although it is difficult to determine a resolving power from these results, it is clear that these two components with a pI difference of 0.2 can easily be resolved and the resolution is approximately 2.3. The resolving power for a general IEF system is in the range of 0.001-0.01 pH units [30]. For this system the

resolving power is calculated to be 0.09 pH units (for a resolution of 1), which is higher due to the high dpH/dx of the IPG gel (1 pH unit per cm) and can be decreased by using longer IPG strips or zoom gels. In general, the resolving power for an IEF-system is somewhat better than for the IEF-iSPR system due to dispersion of the compounds during the blotting process.

Effect of biological matrix

To determine the effect of the biological matrix on the IEF-iSPR system, two different dilutions of synovial fluid (SF) spiked with a model protein (20 μg mL^{-1} CAI) were separated and blotted (Fig. 5.4) The blanc consisted of the protein in water (without ampholytes). The highest concentration of SF possible is the 1 in 20 dilution. Higher concentrations are too viscous for IEF separation. As can be seen from Figure 5.4, the baseline signal for the two dilutions and the blanc is about 100 mdeg.
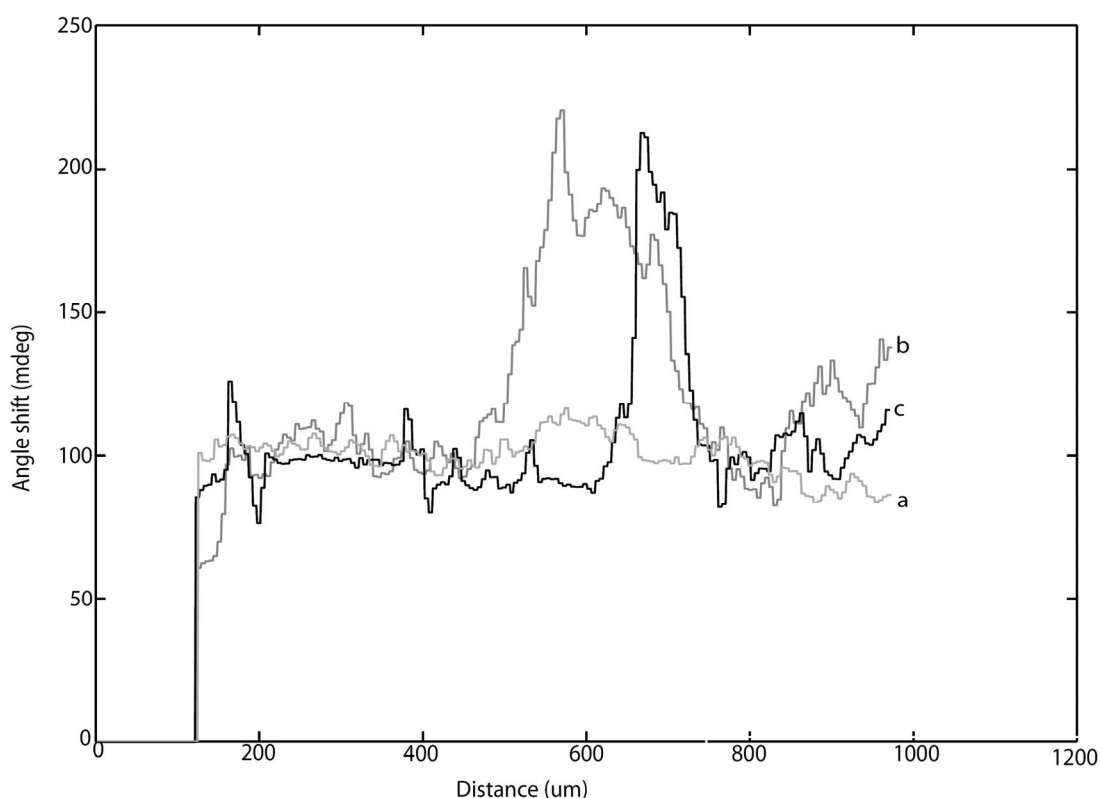

Figure 5 4. Angle shift profiles of 20 μg mL^{-1} CAI in water (a) and different dilutions SF (b: 1 to 100, c: 1 to 20).

This relatively high baseline can be attributed to the non-specific binding of components in the whole antiserum. The baseline is expected to be lower when

purified antibodies are used. However, the observation that all three baselines are equally high irrespective of the concentration of sample matrix also indicates that compounds can be determined in real biological matrices. Fig. 5.4 also shows that a high concentration background matrix in IEF is beneficial for the separation. In the blanc solution, no CAI peak is observed. As for the 1 in 100 dilution, CAI is not completely focused. In the 1 in 20 dilution, CAI is completely focused. This observation could be explained by the absence of ampholytes. During focusing, the proteins and peptides themselves in SF act as ampholytes and migrate to the pH zone where they are electrically neutral [39]. The lower the amount of compounds in a certain zone, the more the water in that zone will migrate to an area that contains compounds, leading to dry spots in the gel.Dry spots interrupt the electrical current and the IEF separation process and therefore no peaks could be observed in the blanc.

Applicability

Now the principle of this IEF-iSPR has been demonstrated, the applicability is shown with a relevant biological antigen-antibody couple, *i.e.* citrullinated fibrinogen (cFib) in diluted SF. Although the concentration (125 μg mL^{-1} cFib in 1 in 100 diluted SF and blanc SF matrix) is far above the relevant concentration of Fib (which should be present in very low amounts in SF), these experiments demonstrate the principle for known RA biomarkers in SF (Fig. 5.5). Contrary to the experiments described previously, a purified polyclonal anti-human anti-cit antibody was used. The background signal is significantly decreased compared to the experiments with whole antiserum. Next to the use of a polyclonal antibody the selection of a different piece of gel can also contribute to a lower background signal. The pH range 6.3-6.9 gel that was used for the CAI in SF experiments contained probably more compounds than the piece of gel used for the cFib experiments, which was also confirmed by Coomassie staining (data not shown).Next to the low matrix signal, the cFib peak is also sharper than e.g. CAI in 1 to 100 diluted SF. This is probabaly due to the size of the protein (340 kDa), as the high amount of amino acids give a very high *d*q/*d*pH (sharp p*I*) value, which may increase the focusing properties of the proteins. Furthermore, a larger antibody will give a higher SPR shift. In combination with a more selective antibody this will increase the sensitivity of the system. On the other hand, large

proteins exhibit a smaller diffusion coefficient, reducing the transfer efficiency slightly.
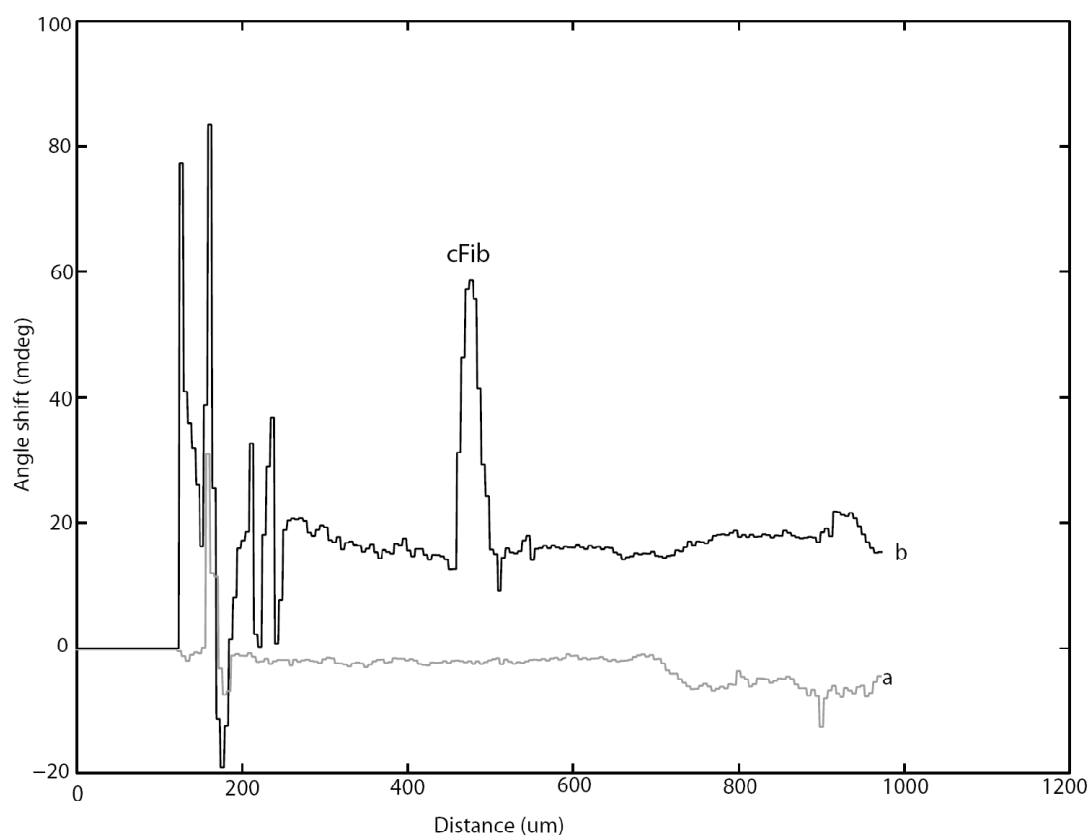

Figure 5.5. Angle shift profiles of blanc (1 to 100 SF (a)) and 125 µg mL^{-1} fibrinogen in 1 to 100 SF (b).

5.4 Concluding remarks

The development and potential of an IEF-iSPR system is demonstrated for the analysis of protein biomarkers in biological fluids. The IEF step is used for sample clean-up and increases the concentration of the compounds of interest. Furthermore, IEF does not modify the proteins like *e.g.* SDS-PAGE, keeping the biological activity intact and available for further interaction studies. The required piece of gel is cut out and pressure blotted via a home-made blotting tool onto the iSPR surface, allowing the proteins to transfer from the gel to the sensor surface. The maximum binding capacity of the sensor disc is in the range of 5 ng per mm^2 and is exceeded when blotting IPG gels with protein concentrations in the low µg/mL range after focusing. For higher concentrations, unbound proteins are washed off the disc and transfer efficiency decreases. It is estimated that proteins concentrations in the low ng/mL range can easily be determined in diluted synovial fluid. Although the dilution factor of SF partially annuls the

concentration of the proteins by IEF, the decrease in matrix-related non-specific binding to the iSPR disc is favorable for the LOD.

By running an antibody solution over the immobilized proteins on the surface, antibody-antigen interaction can be followed by iSPR. By using iSPR instead of SPR, a 2D image of the IEF separation can be constructed, offering information on p*I* and interaction. Although not demonstrated in this paper, kinetic calculations on the purified immobilized proteins in real time can be made [40]. Lower abundant proteins in complex biological matrices can be found due to the separating and concentrating power of IEF in combination with the use of antibodies in the iSPR step. To increase the sensitivity even further, secondary antibodies can be flushed over the antigen-antibody complex, increasing the mass of the complex and thereby the SPR angle shift.

This method is based on the use of antibodies for finding protein biomarkers that bind to these antibodies. Additionally this system is not only applicable to the search of biomarkers for auto-immune diseases, but can also be used for *e.g.* immunogenicity screening in the biopharmaceutical industry where the isoforms of a protein are separated with IEF and the binding kinetics to antibodies for each individual isoform can be determined label free using the iSPR step.

Acknowledgements

References

[1]   Van Venrooij, W. J., Pruijn, G. J., *Arthritis Res* 2000, *2*, 249-251.

[2]   Zendman, A. J., Van Venrooij, W. J., Pruijn, G. J., *Rheumatology (Oxford)* 2006, *45*, 20-25.

[3]   Van Venrooij, W. J., Zendman, A. J., Pruijn, G. J., *Autoimmun Rev* 2006, *6*, 37-41.

[4]   Van Venrooij, W. J., Vossenaar, E. R., Zendman, A. J., *Autoimmun Rev* 2004, *3 Suppl 1*, S17-19.

[5]   Vossenaar, E. R., Van Venrooij, W. J., *Arthritis Res Ther* 2004, *6*, 107-111.

[6]   Nijenhuis, S., Zendman, A. J., Vossenaar, E. R., Pruijn, G. J., Van Venrooij, W. J., *Clin Chim Acta* 2004, *350*, 17-34.

[7]   Tampoia, M., Brescia, V., Fontana, A., Maggiolini, P., Zucano, A., Pansini, N., *Clin Chim Acta* 2005, *357*, 219-225.

[8]   Vannini, A., Cheung, K., Fusconi, M., Stammen-Vogelzangs, J., Drenth, J. P., Dall'Aglio, A. C., Bianchi, F. B., Bakker-Jonges, L. E., Van Venrooij, W. J., Pruijn, G. J., Zendman, A. J., *Ann Rheum Dis* 2007, *66*, 511-516.

[9]   Van Venrooij, W. J., Van Beers, J. J., Pruijn, G. J., *Ann N Y Acad Sci* 2008, *1143*, 268-285.

[10]   Van Venrooij, W. J., Pruijn, G. J., *Arthritis Res Ther* 2008, *10*, 117.

[11]   Smith, E. A., Corn, R. M., *Appl Spectrosc* 2003, *57*, 320A-332A.

[12]   Beusink, J. B., Lokate, A. M., Besselink, G. A., Pruijn, G. J., Schasfoort, R. B., *Biosens Bioelectron* 2008, *23*, 839-844.

[13]   Homola, J., *Anal Bioanal Chem* 2003, *377*, 528-539.

[14]   Mullett, W. M., Lai, E. P., Yeung, J. M., *Methods* 2000, *22*, 77-91.

[15]   Grote, J., Dankbar, N., Gedig, E., Koenig, S., *Anal Chem* 2005, *77*, 1157-1162.

[16]   Natsume, T., Nakayama, H., Jansson, O., Isobe, T., Takio, K., Mikoshiba, K., *Anal Chem* 2000, *72*, 4193-4198.

[17]   Steiner, G., *Anal Bioanal Chem* 2004, *379*, 328-331.

[18]   Adamczyk, M., Johnson, D. D., Mattingly, P. G., Moore, J. A., Pan, Y., *Bioconjug Chem* 1998, *9*, 23-32.

[19]   Wink, T., Van Zuilen, S. J., Bult, A., Van Bennekom, W. P., *Anal Chem* 1998, *70*, 827-832.

[20]   Ladd, J., Lu, H., Taylor, A. D., Goodell, V., Disis, M. L., Jiang, S., *Colloids Surf B Biointerfaces* 2009, *70*, 1-6.

[21]   Ladd, J., Taylor, A. D., Piliarik, M., Homola, J., Jiang, S., *Anal Bioanal Chem* 2009, *393*, 1157-1163.

[22]   Kyo, M., Usui-Aoki, K., Koga, H., *Anal Chem* 2005, *77*, 7115-7121.

[23]   Masson, J. F., Battaglia, T. M., Khairallah, P., Beaudoin, S., Booksh, K. S., *Anal Chem* 2007, *79*, 612-619.

[24]   Battaglia, T. M., Masson, J. F., Sierks, M. R., Beaudoin, S. P., Rogers, J., Foster, K. N., Holloway, G. A., Booksh, K. S., *Anal Chem* 2005, *77*, 7016-7023.

[25]   Phillips, K. S., Han, J. H., Cheng, Q., *Anal Chem* 2007, *79*, 899-907.

[26]   Stigter, E. C., De Jong, G. J., Van Bennekom, W. P., *Biosens Bioelectron* 2005, *21*, 474-482.

[27]   Barrett, D. A., Hartshome, M. S., Hussain, M. A., Shaw, P. N., Davies, M. C., *Anal Chem* 2001, *73*, 5232-5239.

[28]   Reimhult, E., Larsson, C., Kasemo, B., Hook, F., *Anal Chem* 2004, *76*, 7211-7220.

[29]   Wegner, G. J., Wark, A. W., Lee, H. J., Codner, E., Saeki, T., Fang, S., Corn, R. M., *Anal Chem* 2004, *76*, 5677-5684.

[30]   Garfin, D., Ahuja, S., *Handbook of Isoelectric Focusing and Proteomics*, Elsevier Academic Press, Amsterdam 2005, p. 334.

[31]   Whelan, R. J., Zare, R. N., *Anal Chem* 2003, *75*, 1542-1547.

[32]   Krishnamoorthy, G., Carlen, E. T., Kohlheyer, D., Schasfoort, R. B., Van den Berg, A., *Anal Chem* 2009.

[33]   Huh, Y. S., Park, T. J., Yang, K., Lee, E. Z., Hong, Y. K., Lee, S. Y., Kim do, H., Hong, W. H.,

Ultramicroscopy 2008, *108*, 1365-1370.

[34] Catimel, B., Rothacker, J., Catimel, J., Faux, M., Ross, J., Connolly, L., Clippingdale, A., Burgess, A. W., Nice, E., *J Proteome Res* 2005, *4*, 1646-1656.

[35] Borch, J., Roepstorff, P., *Mol Biotechnol* 2006, *33*, 179-190.

[36] Melles, E., Bergman, T., Stahlberg, M., Thirstrup, C., Wahren, J., Jornvall, H., Shafqat, J., *J Biomol Tech* 2005, *16*, 392-397.

[37] Neuhoff, V., Arold, N., Taube, D., Ehrhardt, W., *Electrophoresis* 1988, *9*, 255-262.

[38] Johnsson, B., Löfås, S., Lindquist, G., *Anal Biochem* 1991, *198*, 268-277.

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

[40] Lokate, A. M., Beusink, J. B., Besselink, G. A., Pruijn, G. J., Schasfoort, R. B., *J Am Chem Soc* 2007, *129*, 14013-14018.

Chapter 6

Selective protein removal and desalting using microchip CE

L.H.H. Silvertand, E. Machtejevas, R. Hendriks, K.K. Unger, W.P. van Bennekom, G.J. de Jong, J Chromatogr B 839 (2006) 68.

Abstract

This paper describes the on-line sample pretreatment and analysis of proteins and peptides with a poly(methylmethacrylate) (PMMA) microfluidic device (IonChip^{TM}). This chip consists of two hyphenated electrophoresis channels with integrated conductivity detectors. The first channel can be used for sample preconcentration and sample clean-up, while in the second channel the selected compounds are separated. Isotachophoresis (ITP) combined with zone electrophoresis (CZE) was used to preconcentrate a myoglobin sample by a factor of about 65 before injection into the second dimension and to desalt a mixture of 6 proteins with 100 mM NaCl. However, ITP-CZE could not be used for the removal of two proteins from a protein/peptide sample since the protein zone in the ITP step was too small to remove certain compounds. Therefore we used CZE-CZE for the removal of proteins from a protein/peptide mixture, thereby injecting only the peptides into the second CZE separation channel.

6.1   Introduction

In a time where mankind has revealed the human genome and is finding ways to unravel the human proteome, the challenges, generated in biology, are becoming less easy to solve with standard analytical methods. Faster and more effective separations with preferably integrated sample clean-up procedures are demanded. Combining different areas of expertise, separation sciences and microfluidics technology, provide us with tools to solve the problems mentioned above, at least partly. Although liquid chromatography (LC) has also been configured to chip dimensions just recently, *e.g.* [1,2], the focus seems to be on the more easy implementation of electrophoretic separation methods on a microfluidic device. On-chip analysis offers certain advantages over conventional systems: decrease in analysis time, parallel analysis, possibility of automation, the use of smaller sample volumes, reduced reagent/buffer use, lower production costs, portability, and the main advantage seems to be the possibility to integrate several functions on the chip, thereby bringing the lab to the sample [3,4]. Although these systems work perfectly well for the analysis of small organic and inorganic compounds, the challenge is the analysis of complex biological samples, especially the targeting of proteins in samples like blood, urine or plasma. In the last 10 to 15 years, several papers have been published on the analysis of whole

proteins in different microchip materials, mainly glass [5-8] quartz [7] and polymers including polydimethylsiloxane (PDMS) [4,9-10] and poly(methylmethacrylate) (PMMA) [3,11-14].

When using microchips for analysis, detection is one of the major problems since the channels usually have a low internal diameter. Considering the small sample amounts often used as well, UV detection is not the method of first choice. Therefore more sensitive detection methods are used like fluorescence [3,4,7,8,15-17], mass spectrometry (MS) [6,14] and electrochemical detection methods [10,11-13,18-20].

Another crucial step in the analysis of biological samples when using CE, is the sample pretreatment including *e.g.* removal of salt and other compounds as well as sample preconcentration. Several sample preparation steps have been implemented on chip, *e.g.* microdialysis [21,22], free-flow electrophoresis [23-24], sample stacking [25-27], isotachophoresis [18-20], solid-phase extraction[16,28] and liquid-liquid extraction [15,29].

The PMMA microchip device described in this paper, the IonChip[TM], was originally designed for fast and sensitive analysis of low amounts of small molecules to be analyzed in the ITP [30,31], the CZE [30], the ITP-ITP [31], the ITP-CZE [18-20,30,32] or the CZE-CZE mode [33]. One of the unique features of this IonChip[TM] is the possibility to couple two CE modes in a single analysis. Although several 2D separation systems on chip have been published, combining *e.g.* sample stacking and CZE [27,34,35], ITP and CZE [12,18-20,30-32,36,37], isoelectric focusing (IEF) and CZE [38], open-channel electrochromatography (OCEC) and CZE [17], solid-phase extraction (SPE) and OCEC [39], micellar electrokinetic chromatography (MEKC) and CZE [40], SPE-MEKC [41], or even in-line SPE-CZE-MS [42], only few describe the analysis of intact proteins [12,34,37,38] or protein digests [27,40].

Ölvecka et al. analyzed proteins with the IonChip[TM], but found that depletion of some major proteins from a mixture of proteins was difficult in the ITP-CZE mode [12]. For removal of proteins from a protein/peptide mixture we will therefore focus on CZE-CZE as an alternative for ITP-CZE. Prior to on-line CE separation, different sample pretreatment steps can be carried out on this device, *e.g.* preconcentration, removal of salts and other compounds. Furthermore, the IonChip[TM] has a relatively large sample loop (900 nL) and channel diameter (140 to 200 µm by 200 to 500 µm) which, in combination with the stacking in ITP and

the possibility of removing matrix compounds before injection into the second dimension, increases the sample loadability of the system. This could be of benefit for *e.g.* the search for low concentrations of smaller compounds (drugs/peptides) or proteins in biological matrices or trace constituents in food products [18,32]. Moreover the IonChip^{TM} is a low cost, accessible and user friendly device.

The present paper focuses on the use of the IonChip^{TM} with conductivity detection for sample preparation and subsequent separation, where the ITP-CZE mode is used for preconcentration of myoglobin and for the removal of salt from a protein mixture. Moreover, the CZE-CZE mode is tested for the removal of proteins from a protein/peptide mixture.

6.2   Materials and Methods

Chemicals

Acetonitrile (gradient grade) was obtained from Riedel-de-Haën (Seelze, Germany). Formic acid was purchased from Merck (Darmstadt, Germany). Electrolyte solutions were prepared by the Department of Analytical Chemistry of the Comenius University (Bratislava, Slovak Republic) and were all extra pure. The carrier electrolyte consisted of 20 mM acetic acid without and with 0.1% hydroxyethylcellulose (HEC, pH 3.20). Leading electrolyte: 20 mM ammonium acetate, 2.18 mM acetic acid, 0.05% HEC, pH 5.73. Terminating electrolyte: 10 mM acetic acid, 0.1% HEC, pH 3.36. Angiotensin I, angiotensin II, angiotensin III, neurotensin, bradykinin, human serum albumin (HSA, pI = 4.7; Mw = 66,478 Da), lactoferrin (Lac, from bovine milk, Mw = approx. 90 kDa), ß-lactoglobulin B (Lac B, from bovine milk, pI = 5.3, Mw = 18,276 Da), myoglobin (Myo, from horse skeletal muscle, pI = 7.3 (major component) and 6.8 (minor component), Mw = 17.6 kDa), avidin (Avi, from egg white, pI = 10, Mw = 66 kDa) and cytochrome c (CC, from horse heart, pI = 10.0-10.5; Mw = 12,384 Da) were obtained from Sigma-Aldrich (Steinheim, Germany). All solutions were prepared with water from a MilliQ water purification system (Millipore, Bedford, MA, USA).

ITP-CZE and CZE-CZE

ITP-CZE and CZE-CZE experiments were carried out with the IonChip™, a PMMA microchip device, previously described by Kaniansky et al [36] and

manufactured by Merck KGaA (Darmstadt, Germany). This device consists of (1) the electronic and control unit and (2) the electrolyte and sample handling unit (Fig. 6.1).
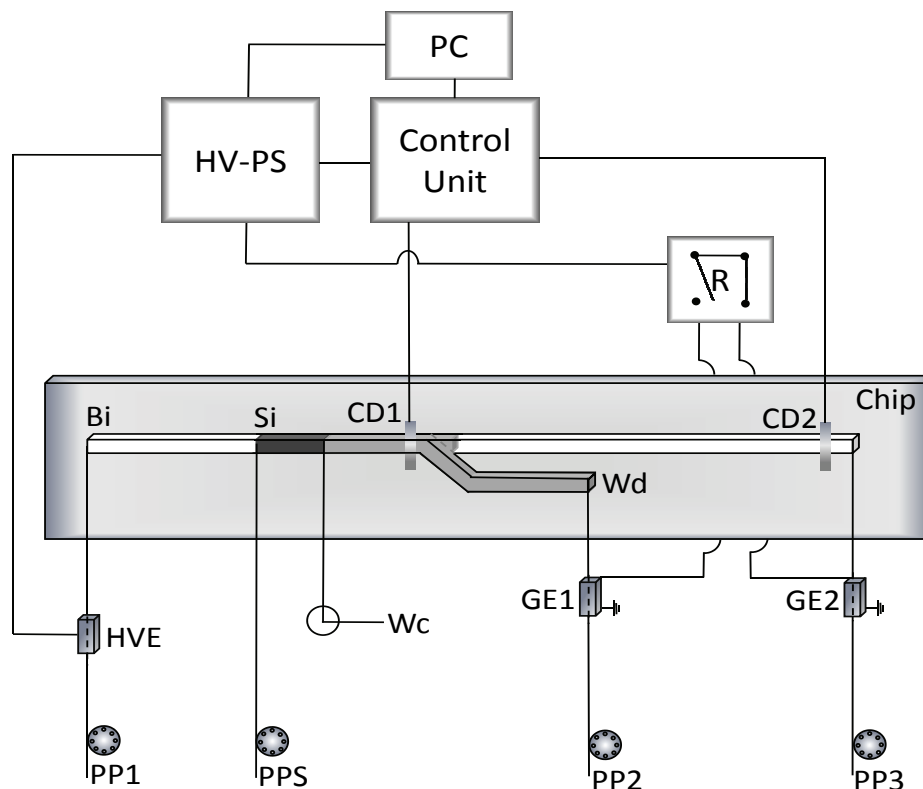

Figure. 6.1. Schematic overview of the IonChip^(TM) column coupling device. A high-voltage power supply (HV-PS; 0-50 µA; 0-7 kV) controls the high-voltage electrode (HVE) located near peristaltic pump 1 (PP1). The chip is filled with electrolyte solution through peristaltic pumps 1, 2 and 3 (PP1, PP2 and PP3), sample is injected from PPS. Excess liquid is directed to a waste channel (Wc). The current from the HV-PS is switched by a relay (R) to either the first ground electrode (GE1) directing compounds to the waste on the chip (Wc), or to the second ground electrode for separation (GE2). Conductivity detectors monitor the sample before (CD1) and after removing compounds (CD2). The whole IonChip^(TM) system is controlled by the control unit and can be operated by a computer (PC).

The latter unit is equipped with three membrane driving electrodes: one high voltage electrode (HVE, 0-7 kV, 1-50 µA) and two ground electrodes (GE1 and GE2). Furthermore, 4 peristaltic pumps (PP1, PP2, PP3 and PPS) are included in the system and are used for filling and flushing the chip. Before an ITP-CZE run, the chip is filled with electrolyte solutions and sample solution through the peristaltic pumps: pump 1 (terminating electrolyte), pump 2 (leading electrolyte), pump 3 (carrier electrolyte) and pump S (sample). When performing a CZE-CZE run, pumps 1, 2 and 3 fill the chip with carrier electrolyte. While flushing the system, excess liquid is directed to a waste channel (Wc). During the electrophoretic runs, the current is kept constant (30 to 20 µA in the ITP-CZE runs

and 20 µA in the CZE-CZE runs). The current is controlled by the polarity of the potential applied between the high voltage electrode and ground electrode 1 (for the first ITP or CZE mode, before and during removal of compounds) or ground electrode 2 (after removal of compounds, separation step).The switching is controlled by a relay (R). Removal of compounds is accomplished by switching the direction of the driving current. First the current is applied between the high voltage electrode and ground electrode 1. When the first conductivity detector (CD1) reaches a predefined threshold value (200 mV in the ITP-CZE runs or 15 mV in the CZE-CZE runs), a preprogrammed time lag (the column switching time or CST) starts counting down, while the driving current is maintained between the high voltage electrode and the first ground electrode, thus directing all the compounds that pass the T-split or bifurcation into the waste channel. After this column switching time, the direction of the current is changed and applied between the high voltage electrode and ground electrode 2, thereby injecting all the compounds that have not passed the bifurcation into the second separation channel. The electronics also controls the peristaltic pumps and interfaces of the device and connects these to a computer. Filling and running of the chip as well as data acquisition and processing is enabled through the use of MicroITP software version 1.0, developed by the Department of Analytical Chemistry of the Comenius University (Bratislava, Slovak Republic). With this software it is also possible to acquire time programmed control of the ITP-CZE and CZE-CZE runs, based on the signal of CD1.

The identities of the compounds in the electropherograms were determined by running each compound separately. Next mixtures were analyzed by adding a single compound to the previous sample, thus eventually injecting all peptides and proteins in a single sample.

6.3   Results and Discussion

Preconcentration and desalting using ITP-CZE

When using the ITP-CZE mode, the IonChip^{TM} is filled with leading, terminating and carrier electrolyte as described in the methods section. The conductivity of the background electrolyte influences the migration times and has an effect on the response of the detectors. In the ITP-CZE as well as in the CZE-CZE experiments we used hydroxyethylcellulose (HEC) in the background electrolytes,

since it suppresses the electroosmotic flow (EOF), it reduces adsorption to the wall and minimizes dispersion [31], especially for certain proteins that are known to adsorb to capillary walls like cytochrome c. Fig. 6.2 shows electropherograms of ITP-CZE runs of cytochrome c without and with using HEC in the background electrolyte.
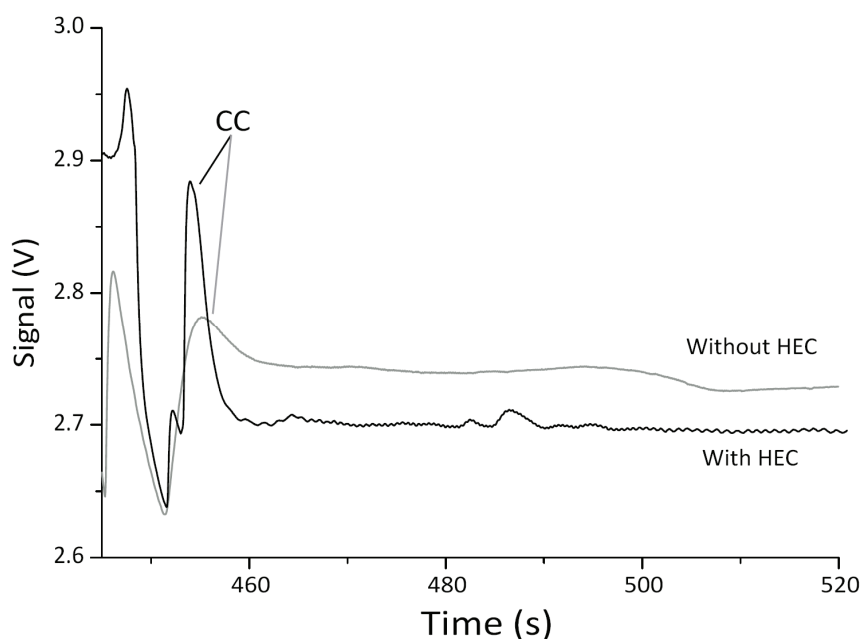

Figure 6.2. ITP-CZE Electropherogram (recorded at CD2) of a sample containing cytochrome c (CC, 100 µg/mL) with (trace a) and without (trace b) HEC in the buffer.

The peak corresponding to cytochrome c is much broader when using a buffer without HEC. ITP-CZE of a mixture of proteins was performed to show that proteins can be analyzed with this system, in a similar way Ölvecka *et al*. did before [12].The possibility of monitoring the ITP step with a conductivity detector in the first channel of this system makes it relatively easy to reproducibly switch fractions from the first to the second channel, because of the staircaselike signal and the sharp zones created in ITP. Fig. 6.3 depicts the separation of the protein mixture by ITP-CZE recorded at the second detector. Most of the proteins are separated and show relatively small peaks, although two of the proteins, myoglobin and HSA, were not separated, probably due to the short channel length in which separation after the ITP step takes place. When performing ITP-CZE on a myoglobin sample of 125 µg/mL, we were able to preconcentrate this protein by a factor of approximately 65 before it was injected into the CZE separation channel.
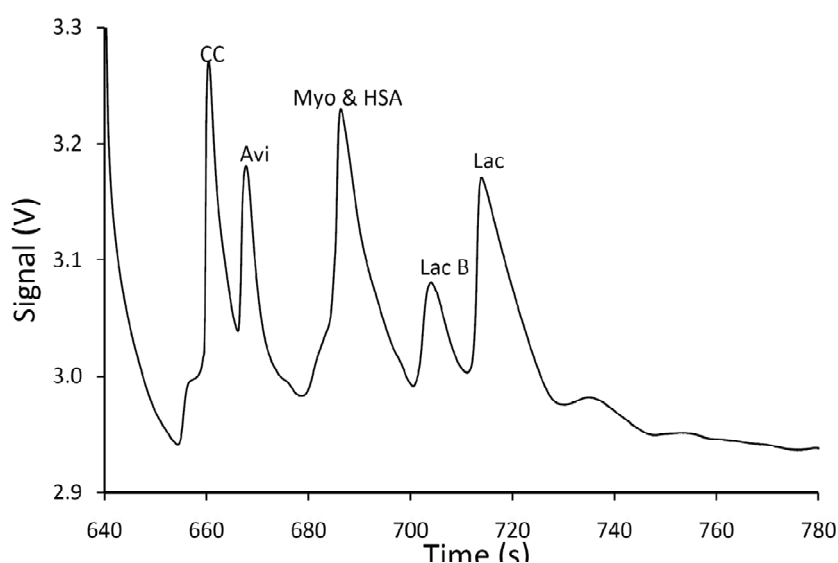
Figure 6.3. ITP-CZE Electropherogram (recorded at CD2) of a sample containing cytochrome c (CC, 100 µg/mL), avidin (Avi, 333 µg/mL), myoglobin (Myo, 100 µg/mL) HSA (100 µg/mL), b-lactoglobulin B (Lac B, 100 µg/mL) and lactoferrin (Lac, 100 µg/mL).

Fig. 6.4a depicts an electropherogram recorded at CD1 during the ITP step. The plug containing myoglobin (3) passes CD1 in 4 s, resulting in a peakvolume of 13.2 nL. Compared to the injection volume of 900 nL, a concentration factor of 65 was obtained before injecting the myoglobin into CZE separation channel. When running the same sample in the CZE-CZE mode, thereby replacing the leading and terminating electrolyte with carrier electrolyte, our peakvolume at CD1 (Fig. 6.4b) was 277 nL before injection into the second separation channel.
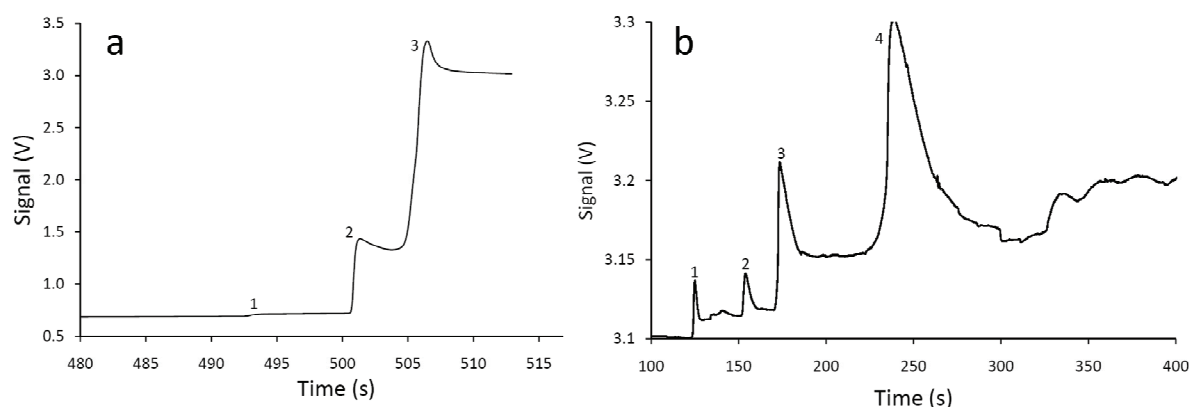
Figure 6.4. ITP-CZE electropherogram recorded at CD1 during th ITP step for a sample containing 125 µg/mL myoglobin (a). The third increase in signal represents the myoglobin passing the detector. CZE-CZE electropherogram recorded at CD1 during the first CZE step for a sample containing 125 µg/mL myoglobin (b).

Compared to the first step in a CZE-CZE system, the plug of myoglobin in the ITP step of ITP-CZE is approximately 20 times smaller, thereby increasing the loadability and the concentration sensitivity of the CZE system when using ITP as the first step, indicating that on-chip preconcentration is also a strong feature of this device.These findings were in line with the findings of Ölvecka *et al.* [12],

where an improvement of the concentration limit of detection of 20-50 was found for different proteins when using ITP-CZE instead of CZE. A main problem in the electrophoretic analysis of biological samples is the presence of high concentrations of salts.
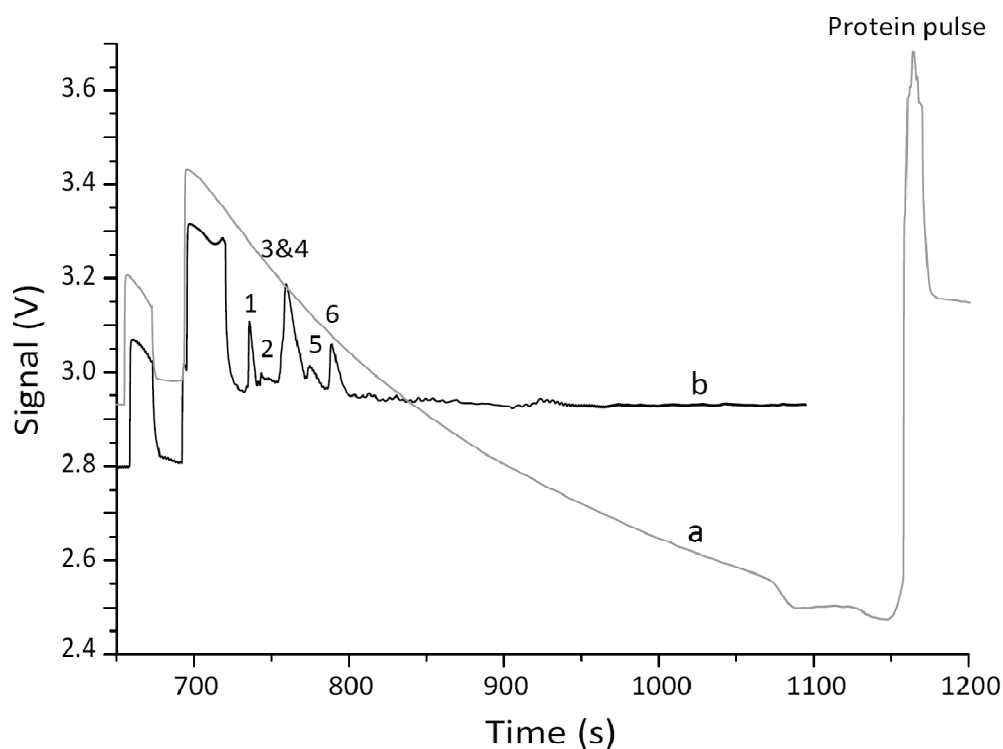

Figure 6.5. ITP-CZE Electropherogram (recorded at CD2) of a sample containing cytochrome c (1; 100 µg/mL), avidin (2; 333 µg/mL), myoglobin (3; 100 µg/ mL) HSA (4; 100 µg/ mL), b-lactoglobulin B (5; 100 µg/ mL) and lactoferrin (6; 100 µg/ mL) in the presence of 100 mM NaCl with a CST of 1s (not desalted, trace a) and 16s (desalted, trace b).

When the IonChip^{TM} is operated in the ITP-CZE mode, it also serves as a fast on-line desalting device with subsequent electrophoretic separation. Since small ions have a higher mobility than large charged molecules, salts can easily be removed from a mixture of proteins by directing the salts to the waste channel. Fig. 6.5 shows a typical ITP-CZE electropherogram recorded at CD2 of a sample containing proteins in the presence of 100 mM NaCl. A large salt plug is present in front of the small protein plug when the salt is not removed (Fig. 6.5a, using a CST = 1s). Furthermore, the proteins are not separated and migrate as one plug. When the salt is removed (Fig. 6.5b) during the ITP step (CST = 16s, directing the salt to Wc), the proteins can be separated in the second channel.

Protein removal using CZE-CZE

When using ITP-CZE for a more complex sample, however, it turns out to be problematic to remove two proteins completely from the sample containing five peptides and two proteins.
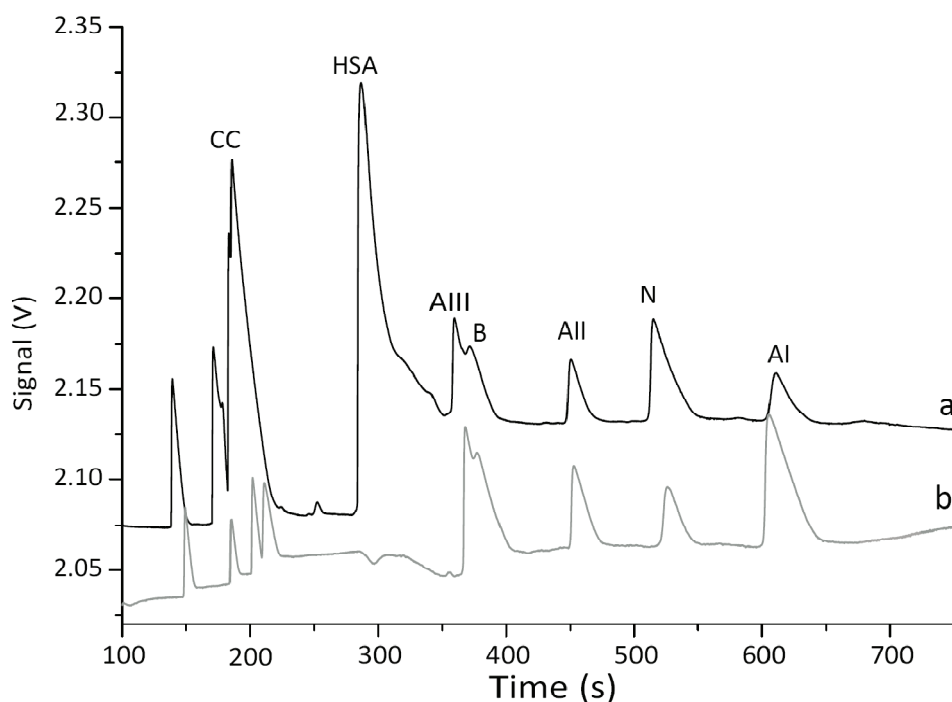

Figure 6.6. Electropherogram of a sample containing 5 peptides (10 µg/mL of each of the following peptides: angiotensin I (AI), angiotensin II (AII), angiotensin III (AIII), bradykinin (B) and neurotensin (N)) and 2 proteins (100 µg/mL CC and 300 µg/mL HSA) recorded at CD2 in the CZE-CZE mode with a CST of 1s (no removal of proteins, black trace a) and 120s (removal of proteins, grey trace b).

Before our findings, Ölvecká *et al*. [12] already came to the same conclusions using a sample of 6 proteins and attributed it to the fact that the protein stack, after the ITP part, is very small.Since one of our goals was to remove certain proteins without affecting the rest of the sample, we have chosen for CZE-CZE. In this mode, however, we lacked the advantage of sample-preconcentration of ITP. Fig. 6.6 shows electropherograms recorded at CD2, with (CST = 120 s) and without (CST = 1 s) the removal of the two proteins from the sample. The two proteins are removed from the sample, while the five peptides are injected into the second CZE separation channel and recorded at CD2. Therefore this method could be useful for sample clean-up and reduction of the complexity of a biological sample, *e.g.* removing high abundant proteins like albumin, before a separation step. Although not specifically designed for protein analysis, the

strong points of this chip, preconcentration and sample clean-up prior to an electrophoretic separation, can also be used for protein and peptide samples.

6.4   Conclusions

Although not designed for protein analysis it is demonstrated that the IonChip^{TM}, is a very powerful device for protein analysis, combining sample pretreatment and separation in a single run. Due to the relatively large sample loop (900 nL) and channel diameter, combined with the isotachophoretic preconcentration, the loadability of the system is relatively high compared to conventional microchip CE. In the ITP step of the ITP-CZE mode, a concentration factor of approximately 65 was obtained for myoglobin prior to injection into the second separation channel. Compared to CZE-CZE, the volume of the plug injected into the second CZE step was approximately 20 times smaller when using ITP-CZE. In the ITP-CZE mode we were also able to remove a high salt concentration present in a protein mixture, thereby increasing the amount/concentration of components to be injected onto the second separation channel. The novelty presented in this paper is the use of CZE-CZE instead of ITP-CZE for the removal of proteins from a protein/peptide mixture, since ITP-CZE could not remove the proteins from this mixture. Although compromising the enrichment factor in the CZE-CZE mode, removal of two proteins from a protein/peptide mixture was accomplished This again demonstrates the sample preparation possibilities of this device.

Acknowledgements

References

[1]   J. Xie, Y. Miao, J. Shih, Y.C. Tai, T.D. Lee, Anal Chem 77 (2005) 6947.

[2]   H. Yin, K. Killeen, R. Brennen, D. Sobek, M. Werlich, T. van de Goor, Anal Chem 77 (2005) 527.

[3]   M. Kato, Y. Gyoten, K. Sakai-Kato, T. Nakajima, T. Toyo'oka, Electrophoresis 26 (2005) 3682.

[4]   D. Wu, Y. Luo, X. Zhou, Z. Dai, B. Lin, Electrophoresis 26 (2005) 211.

[5]   L. Bousse, S. Mouradian, A. Minalla, H. Yee, K. Williams, R. Dubrow, Anal Chem 73 (2001) 1207.

[6]   I.M. Lazar, L. Li, Y. Yang, B.L. Karger, Electrophoresis 24 (2003) 3655.

[7]   C.L. Colyer, S.D. Mangru, D.J. Harrison, J Chromatogr A 781 (1997) 271.

[8]   Y. Liu, R.S. Foote, S.C. Jacobson, R.S. Ramsey, J.M. Ramsey, Anal Chem 72 (2000) 4608.

[9]   D. Xiao, T.V. Le, M.J. Wirth, Anal Chem 76 (2004) 2055.

[10]   Y.H. Dou, N. Bao, J.J. Xu, F. Meng, H.Y. Chen, Electrophoresis 25 (2004) 3024.

[11]   M. Zuborova, Z. Demianova, D. Kaniansky, M. Masar, B. Stanislawski, J Chromatogr A 990 (2003) 179.

[12]   E. Olvecka, D. Kaniansky, B. Pollak, B. Stanislawski, Electrophoresis 25 (2004) 3865.

[13]   M. Galloway, W. Stryjewski, A. Henry, S.M. Ford, S. Llopis, R.L. McCarley, S.A. Soper, Anal Chem 74 (2002) 2407.

[14]   S.H. Chen, W.C. Sung, G.B. Lee, Z.Y. Lin, P.W. Chen, P.C. Liao, Electrophoresis 22 (2001) 3972.

[15]   E.A. Schilling, A.E. Kamholz, P. Yager, Anal Chem 74 (2002) 1798.

[16]   C. Yu, M.H. Davey, F. Svec, J.M. Frechet, Anal Chem 73 (2001) 5088.

17]   N. Gottschlich, S.C. Jacobson, C.T. Culbertson, J.M. Ramsey, Anal Chem 73 (2001) 2669.

[18]   M. Masar, M. Dankova, E. Olvecka, A. Stachurova, D. Kaniansky, B. Stanislawski, J Chromatogr A 1026 (2004) 31.

[19]   R. Bodor, M. Zuborova, E. Olvecka, V. Madajova, M. Masar, D. Kaniansky, B. Stanislawski, J Sep Sci 24 (2001) 802.

[20]   B. Grass, R. Hergenroder, A. Neyer, D. Siepe, J Sep Sci 25 (2002) 135.

[21]   N. Xu, Y. Lin, S.A. Hofstadler, D. Matson, C.J. Call, R.D. Smith, Anal Chem 70 (1998) 3553.

[22]   Y. Jiang, P.C. Wang, L.E. Locascio, C.S. Lee, Anal Chem 73 (2001) 2048.

[23]   D. Raymond, A. Manz, M. Widmer, Anal Chem 66 (1994) 2858.

[24]   H. Lu, S. Gaudet, M.A. Schmidt, K.F. Jensen, Anal Chem 76 (2004) 5705.

[25]   J. Lichtenberg, N.F. de Rooij, E. Verpoorte, Talanta 56 (2002) 233.

[26]   E. Verpoorte, Electrophoresis 23 (2002) 677.

[27]   J. Li, C. Wang, J.F. Kelly, D.J. Harrison, P. Thibault, Electrophoresis 21 (2000) 198.

[28]   S. Ekstrom, J. Malmstrom, L. Wallman, M. Lofgren, J. Nilsson, T. Laurell, G. Marko-Varga, Proteomics 2 (2002) 413.

[29]   D.J. Wilson, L. Konermann, Anal Chem 77 (2005) 6887.

[30]   D. Kaniansky, M. Masar, J. Bielcikova, F. Ivanyi, F. Eisenbeiss, B. Stanislawski, B. Grass, A. Neyer, M. Johnck, Anal Chem 72 (2000) 3596.

[31]   E. Olvecka, M. Masar, D. Kaniansky, M. Johnck, B. Stanislawski, Electrophoresis 22 (2001) 3347.

[32]   M. Masar, M. Dankova, E. Olvecka, A. Stachurova, D. Kaniansky, B. Stanislawski, J Chromatogr A 1084 (2005) 101.

[33]   D. Kaniansky, M. Masar, M. Dankova, R. Bodor, R. Rakocyova, M. Pilna, M. Johnck, B. Stanislawski, S. Kajan, J Chromatogr A 1051 (2004) 33.

[34]   H. Yang, R.L. Chien, J Chromatogr A 924 (2001) 155.

[35]   T. Footz, S. Wunsam, S. Kulak, H.J. Crabtree, D.M. Glerum, C.J. Backhouse, Electrophoresis 22 (2001) 3868.

[36]   D. Kaniansky, M. Masar, M. Dankova, R. Bodor, R. Rakocyova, M. Pilna, M. Johnck, B. Stanislawski, S. Kajan, J Chromatogr A 1051 (2004) 33.

[37]   A. Wainright, S.J. Williams, G. Ciambrone, Q. Xue, J. Wei, D. Harris, J Chromatogr A 979 (2002) 69.

[38]   A.E. Herr, J.I. Molho, K.A. Drouvalakis, J.C. Mikkelsen, P.J. Utz, J.G. Santiago, T.W. Kenny, Anal

Chem 75 (2003) 1180.

[39]   B.S. Broyles, S.C. Jacobson, J.M. Ramsey, Anal Chem 75 (2003) 2761.

[40]   J.D. Ramsey, S.C. Jacobson, C.T. Culbertson, J.M. Ramsey, Anal Chem 75 (2003) 3758.

[41]   J.D. Ramsey, G.E. Collins, Anal Chem 77 (2005) 6664.

[42]   A.P. Dahlin, S.K. Bergstrom, P.E. Andren, K.E. Markides, J. Bergquist, Anal Chem 77 (2005) 5356.

Chapter 7

Conclusions and recommendations

7.1   Conclusions

In this Ph.D. thesis, some sample pretreatment steps were evaluated for the goal of proteomics on a chip. Furthermore, (c)IEF is studied as separation technique and coupled to MS and iSPR. This section gives an overview of the general conclusions of this research in relation to the goals as stated in Chapter 1. Moreover, ideas for improvement of the systems as well as suggestions for future research are presented.

Sample pretreatment

In Chapter 5, the use of IEF for preconcentration and isolation prior to iSPR analysis is described. Carrier ampholytes were not used in the IEF-iSPR system since these competed with proteins for binding spots on the iSPR disc. Proteins and peptides in the synovial fluid also act as carrier ampholytes, thereby eliminating the need for CAs when analyzing biological matrices. IEF was found to separate and concentrate the proteins in their zone, thereby presenting highly concentrated, relatively pure protein zones to the iSPR sensor surface. The separation and preconcentration properties of IEF circumvent the local aspecific binding in iSPR and lower the LOD of the system. These properties make IEF an excellent sample pretreatment step when using low-abundant proteins in highly complex matrices, as is also demonstrated by the popularity of 2D-GE in proteomics, where IEF is the first separation step.

Chapter 6 describes the use of the IonChip^{TM} , which was originally designed for the analysis of small ions in food and beverages, *e.g.* [1]. In this Ph.D. thesis, the IonChip^{TM} is used for salt removal (with ITP) and depletion of proteins from a peptide sample (with CZE) before injection into a separation channel. Due to the large sample loop of 900 nL, a relatively large sample volume can be injected, increasing the loadability of the system. ITP in the first mode removes salt from the sample and concentrates by a factor of *ca.* 65. However, in the ITP mode it is very difficult to deplete proteins from a sample since the protein ITP stack is too small. When the system is run in the CZE-CZE mode, however, the components are in separated, larger zones and it becomes less difficult to remove unwanted analytes from the system, although the concentration factor is lost.

Since the IonChip™ was not developed for protein analysis, it suffers from some minor disadvantages like the length of the second separation channel, software limitations and the use of low budget construction parts. The column coupling principle as presented here is very effective in preconcentration and the removal of salt and other unwanted components and is relatively easy to implement on chip as demonstrated with the IonChip™ and besides CZE other CE techniques like cIEF or CGE can be used as second separation step. This offers the possibility to remove interfering compounds like salt, HSA, lipids, *etc.* from a biological sample and to inject the remaining proteins into cIEF or CGE, increasing the separation efficiency and reproducibility of these techniques.

Separation

The capillary format of IEF is investigated in Chapters 3 and 4, where the main goal was to hyphenate cIEF to MS. Chapter 3 describes the effect of compounds that are usually incorporated in a cIEF system (ampholytes, detergents and viscosity increasing agents) on the separation efficiency, repeatability and correlation of pI to migration time. When looking at the RSD of migration time and peak area in cIEF-UV of proteins with different detergents, zwitterionic and PEO detergent perform equally well and CHAPSO and Tween would be favorable, although other detergents might be more suited for other samples. Low concentrations viscosity increasing agents give lower RSD values of migration time and peak area. All cellulose and dextran components tested (except for high concentration methylcellulose) perform equally well, with HPMC and dextran giving the best results.

When coupled to UV detection, cIEF is an excellent separation and quantitation method for proteins and peptides. cIEF-UV is repeatable and can separate a wide range of proteins (pI 3-10 and Mw 1-160 kDa) in one single run. When selecting the right additives, RSDs of migration times and peak area are low which makes cIEF-UV a good method for quantitative analysis with LODs in the low to medium ng/mL range. Although not demonstrated in this thesis, cIEF of complex biological matrices is still difficult due to the presence of interfering compounds like salts and high abundant proteins like HSA. Sample pretreatment steps as described above are often required to remove these compounds.

Hyphenation

When coupling (c)IEF to a detection method, BGE additives may interfere. This is demonstrated for coupling to MS (Chapters 3 and 4) and to iSPR (Chapter 5). Carrier ampholytes lead to a significant decrease of the protein signal intensity in MS and to competition with proteins for binding spots on the iSPR sensor surface. For detergents and viscosity increasing agents, similar MS results are obtained. Even low amounts of these compounds cause severe reduction of the protein signal. Moreover, the reproducibility of the MS signal is poor, making cIEF-MALDI-TOF MS less suited for quantitative purposes as opposed to the cIEF-UV-MALDI-TOF MS system. To increase the signal in MS several strategies are proposed. First is to keep the amount of these additives as low as possible. This however, may impair cIEF separation. A second option, demonstrated in Chapter 4, is the reduction of the spotting time. Although the absolute amount of proteins per spot will decrease, still an increased signal has been found due to the fact that also the amount of signal suppressing substances is reduced. Obviously the suppressing effect of the additives is larger than the decrease of the amount of proteins. This implies that regular cIEF circumstances can be used when the spotting time is low enough. One should keep in mind that at a certain amount of proteins per spot, this amount will determine the intensity of the MS signal and the effect of the additives is less important.

In general, cIEF-UV can be used for quantitative purposes but hyphenation of cIEF to MS would not be first choice for the quantitative analysis of proteins. However, a combined cIEF-UV-MALDI-TOF MS system can perform quantitative analysis (UV results) and at the same time perform qualitative analysis of the separated peaks (MS data or even MS/MS). This makes it a well-suited system for the analysis of biopharmaceuticals (Chapter 4). Degradation products can be separated and identified at the same time. Next to stability studies, heterogeneity testing, in process analysis and quality control in the biopharmaceutical industry are now the major application fields of this technique.

Although some groups have already performed proteomics studies with cIEF-MS, as reviewed in Chapter 2, real biological samples remain difficult to analyze with this system, due to the sensitivity of cIEF-MS to compounds like salts, *etc*.

IEF hyphenation with iSPR is presented in Chapter 5. The purpose was to isolate and preconcentrate possible biomarker antigens with IEF and immobilize these compounds onto an iSPR sensor surface. With the IEF step minimization of aspecific binding of the biological matrix components was obtained and at the same time the local concentration of proteins presented to the iSPR surface is increased. A blotting tool presses the proteins out of the IPG gel into the dextran gel of the iSPR disc. Next to the proof that this approach is as simple as it is successful, the resolving power of this system was determined to be at least 0.09 pH units (for a 7cm pH 3-10). The resolving power can be increased by selecting strips with a larger dpH/dx ratio (longer strips or zoom gels). Experiments with synovial fluid spiked with CAI show that the principle can be used for biological matrices, leading to distinct peaks for CAI. Unfortunately the background signal is high in these experiments due to aspecific adsorption of the raw anti-CAI antiserum. If purified antibodies were used, this background signal would be lower, as is demonstrated in the citrullinated fibrinogen experiments. A further observation in experiments with synovial fluid is the autofocusing properties of the proteins and peptides. A more concentrated biological matrix causes the proteins to focus in sharper zones, thereby enhancing the IEF separation efficiency. Assuming a limit of detection for an iSPR system is in the low nM range [2, 3], and therefore samples with protein concentrations in the medium pM range should be applied to the IPG strip to obtain reasonable results. However, the transfer efficiency for real samples after IEF is still not known and in Chapter 5 only spiked 1 to 20 diluted synovial fluid has been analyzed. Further investigation and optimization is required for various biological matrices.

Although this IEF-iSPR system has originally been designed for the search of biomarkers, interesting application fields are kinetic proteins models, interaction studies (of proteins with drugs, DNA, proteins, antibodies, micelles, cells, *etc*.). Immunogenicity testing of biopharmaceuticals is another interesting application field: IEF first separates all the different isomers and in iSPR the affinity of the isomers to different (auto) antibodies is determined.

General conclusion

Although spiked synovial fluid samples were analyzed, real biological samples, as intended in the project goals, were still not studied. When looking for low abundant biomarkers in complex matrices like synovial fluid, blood or tissue, it is recommended to start with a sample pretreatment step that combines preconcentration and clean-up. ITP has a concentrating factor of ca 65 (for myoglobin when using the IonChip™), is able to remove salts and other interfering components and can be easily coupled to the preferred separation strategy (cIEF) via *e.g.* the column coupled strategy of the IonChip™. Since cIEF-UV is able to analyze proteins in the medium ng/mL range (or nM), an increase with a factor of ca 65 (ITP) will result in detection limits in the low nM or high pM range, at which many proteins are present in biological samples. Depending on the information needed, this system can be coupled to either MS or iSPR. Both methods should only be used for qualitative purposes, since reproducibility in cIEF-MALDI-TOF MS and IEF-iSPR are poor. However, identification of the proteins can be performed with MS, while with iSPR bioaffinity interactions and kinetic models can be studied. A combination of iSPR and MS was recently reviewed by Visser and Heck [4] and offers additional information on the proteins: The iSPR is an affinity enrichment step and besides isolation of part of the proteome, also provides real time binding data. For structural analysis, the iSPR sensor disc with multiple interaction spots can be inserted in a MALDI source, where the interaction spots can be analyzed individually with a TOF MS.

7.2   Recommendations

cIEF-UV-MS system

The cIEF-UV-MALDI-TOF MS setup as described in this paper is powerful, but some suggestions for improvement are presented.

To reduce the influence of BGE additives, these compounds can be removed from the sample before injection into the MS in several ways: CA free cIEF [5], free flow electrophoresis [6], microdialysis [7, 8], binding of proteins onto the MALDI target plate and washing away the other components [9] etc. All of these approaches have their pro's and con's (mainly zone-broadening). But, with new developments in cIEF, also a new suggestion to couple cIEF to MS can be

presented: Dynamic cIEF (dIEF, [10]) as shown in Fig. 2.3, uses additional electrodes to perform focusing within isoelectric focusing. By placing two extra electrodes in a valve in a separation channel, proteins zones of interest can be selected by varying the voltage applied over the two electrodes in the valve. With this approach it is possible to isolate very sharp zones from a cIEF separation, thus a high amount of protein against a low amount of additives. Subsequently, these selected compounds can be injected into a second CE separation or digestion channel by switching the valve, where further separation or digestion can be accomplished. Direct injection into the MS is also possible. Advantages are the high purity of the compounds before MS analysis and the possibility to perform a second separation. But the disadvantage is that a valve can only transfer fractions into the second channel or the MS (or any other detection method). When the compound of interest is known, this method will be very effective. When a biological sample should be screened for possible biomarkers, all fractions need to be sampled and long analysis times are required.

IEF-iSPR system

The IEF-iSPR system shown in this thesis is only a proof of principle and needs further optimization.

Adjustments to the IEF-iSPR system. The resolution of the IEF system can be tuned by selecting appropriate IPG strip. By using non-linear IPG strips or longer linear strips, the resolution is increased. The disadvantage of this is that fewer proteins can be brought onto the sensor surface. Other blotting methods like electroblotting can increase the transfer efficiency. The proteins are protonated by washing the gel in an acidic solution like the acetate buffer that is used in Chapter 5. By grounding the gold layer, it should be possible to electrophoretically transfer the proteins from the gel towards the sensor surface. This will lead to the transfer of all proteins from the IPG gel to the sensor surface. However, the maximum binding capacity of the sensor surface is not affected and the unbound proteins are washed off the sensor disc by the PBS flushing step. This makes electroblotting especially interesting for low protein concentrations (lower than maximum binding capacity), where all proteins that are transferred will be immobilized. Another improvement to the system is the increase of iSPR sensitivity using secondary antibodies or liposomes, etc. Since SPR is in fact a very

sensitive mass detector, increasing the mass of bound antibody will enhance the signal in iSPR.

Suggestions for alternative techniques

One of the major disadvantages of the IEF-iSPR system is that it is the labor intensive and time consuming. Therefore an alternative, faster strategy is suggested: cIEF-iSPR. The same setup as used for the cIEF-UV-MALDI-TOF experiments can be used, but now the MALDI target plate is replaced by an iSPR disc. The same problems as in the MS experiments may play a role in the coupling of cIEF to iSPR: the additives prevent the proteins from binding to the surface by either competing for binding sites (ampholytes), blocking binding sites (viscosity increasing agents) or modifying proteins or antibodies (detergents). Preliminary experiments not presented in this thesis show that the cIEF system may be made compatible with iSPR by selecting the appropriate conditions: dextran with 0.01% Tween as separation medium for cIEF results in a good separation and may be compatible with the dextran modified sensor surface. Furthermore, the sheath liquid should not interfere with the binding of proteins to the sensor disc. This can be accomplished by reversing the cIEF (inlet is alkaline, outlet is acidic, reversed polarity) separation and using acetate buffer as sheath liquid and anolyte. After performing iSPR, the sensor disc can be attached to a MALDI target plate and further analyzed by MALDI-TOF MS.

A different alternative for the IEF step is the original OGE setup as presented in [11], where a piece of immobilized pH gradient gel is placed on top of a channel. The biological sample is either loaded onto the IPG gel or in the channel. By applying a voltage over the gel, only analytes with a pI matching the pH of the gel are present in the channel, the other components migrate through the gel to their isoelectric pH. This approach reduces the need for ampholytes and viscosity increasing agents. When the compound of interest is already known, it is just a matter of selecting the correct pH interval and the protein can be purified, transferred (hydrodynamically) and immobilized onto the sensor surface.

IonChip

Although the column-coupling principle of ITP-CZE and CZE-CZE has been demonstrated to be successful, the construction of the IonChip™ suffers from a number of limitations that can be solved. First, increasing the length of the

second separation channel improves the separation efficiency. Second, decreasing the diameter of all channels allows for more efficient heat transfer and therefore less zone broadening but also reduces the loadability of the system. Third, placing a UV detector instead of CD at the end of the separation channel will make the IonChip more suited for protein analysis: UV does not detect compounds like salts and other small, non-UV absorbing compounds that interfere with protein detection.

The coupling of the IonChip^{TM} to an ESI-iontrap MS has been attempted (data not presented in this thesis), but was not successful due to limitations of the IonChip^{TM}. However, suggestions to improve the design of this hyphenation are presented here as well. First, integration of an MS sprayer on the chip at the end of the second separation channel (see also Section 6.1) will circumvent the zone broadening that occurs in the corners and different materials of the channels after the second CD detector. Furthermore, by using more powerful peristaltic or syringe pumps, the sample can be hydrodynamically mobilized to the MS.

References

[1]   Bodor, R., Kaniansky, D., Masar, M., Silleova, K., Stanislawski, B., *Electrophoresis* 2002, *23*, 3630-3637.

[2]   Adamczyk, M., Johnson, D. D., Mattingly, P. G., Moore, J. A., Pan, Y., *Bioconjug Chem* 1998, *9*, 23-32.

[3]   Wink, T., Van Zuilen, S. J., Bult, A., Van Bennekom, W. P., *Anal Chem* 1998, *70*, 827-832.

[4]   Visser, N. F., Heck, A. J., *Expert Rev Proteomics* 2008, *5*, 425-433.

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

[6]   Chartogne, A., Tjaden, U. R., Van der Greef, J., *Rapid Commun Mass Sp* 2000, *14*, 1269-1274.

[7]   Lamoree, M. H., van der Hoeven, R. A. M., Tjaden, U. R., van der Greef, J., *J Mass Spectrom* 1998, *33*, 453-460.

[8]   Huh, Y. S., Park, T. J., Yang, K., Lee, E. Z., Hong, Y. K., Lee, S. Y., Kim do, H., Hong, W. H., *Ultramicroscopy* 2008, *108*, 1365-1370.

[9]   Yu, W. J., Li, Y., Deng, C. H., Zhang, X. M., *Electrophoresis* 2006, *27*, 2100-2110.

[10]   Montgomery, R., Jia, X. G., Tolley, L., *Anal Chem* 2006, *78*, 6511-6518.

[11]   Ros, A., Faupel, M., Mees, H., Oostrum, J., Ferrigno, R., Reymond, F., Michel, P., Rossier, J. S., Girault, H. H., *Proteomics* 2002, *2*, 151-156.

Samenvatting

Analytisch-chemische en biochemische technieken zijn tegenwoordig onmisbaar in de zoektocht naar specifieke lichaamseigen signaaleiwitten die in bepaalde concentraties voorkomen in lichaamsvloeistoffen (bloed, urine, speeksel, synoviaalvocht, *etc*.) of weefsels in de ontwikkeling van of tijdens een ziekteproces. De vraag naar kosteffectieve methoden die snel resultaat opleveren is groot. Op capillaire electroforese (CE) gebaseerde technieken leveren snel resultaat, zijn eenvoudig te koppelen met andere scheidings- en detectiemethoden, hebben een hoge scheidingsefficiëntie en zijn daarnaast relatief goedkoop in onderhoud en milieuvriendelijk door het gebruik van kleine hoeveelheden, op water gebaseerde buffers.

Naast het gebruik van capillaire zone electroforese (CZE) en isotachoforese (ITP), staat in dit onderzoek het gebruik van isoelectrisch focuseren (IEF) centraal zowel in een gel als in een capillair. In IEF worden componenten gescheiden op basis van hun isoelectrisch punt (p*I*: de pH waarbij de netto lading van de component nul is), wat IEF een uitermate geschikte scheidingstechniek maakt voor eiwitten en peptiden. IEF biedt de mogelijkheid tot het analyseren van componenten met een brede pI range (3 ≤ p*I* ≤ 11) en een gelijktijdige lokale concentrering van de eiwitten of peptides gedurende deze scheiding. Nadelen van IEF zijn echter het gebruik van drager-amfolieten die UV detectie bij 214 nm bemoeilijken en daarmee de detectielimiet verhogen. Daarnaast speelt ook de kleine optische weglengte van de detector parten. Door het gebruik van detectietechnieken als massaspectrometrie (MS, Hoofdstukken 3 en 4) en surface plasmon resonantie imaging (iSPR, Hoofdstuk 5) is gepoogd de mogelijkheden van cIEF te vergroten.

Een tweede probleem van IEF is de gevoeligheid van de scheiding voor zouten. Bij analyses van biologische monsters als bloed, urine of synoviaalvocht, is een monstervoorbewerkingsstap daarom onmisbaar om ongewenste componenten als zout, albumine, etc. te verwijderen. Daar de detectielimiet in cIEF-UV relatief hoog is, is preconcentrering tijdens deze stap een bijkomend voordeel. Een aantal monstervoorbewerkingsstappen is geëvalueerd in Hoofdstuk 5 (IEF als monstervoorbewerking voor iSPR) en in Hoofdstuk 6 (ITP en CZE voor het verwijderen van zouten en eiwitten voorafgaande aan een CE scheiding).

Het doel van dit proefschrift is drieledig:

- Het onderzoeken van verschillende monstervoorbewerkingstechnieken om een biologisch monster te ontdoen van bij de scheiding storende componenten als zouten en albumine.

- De evaluatie van cIEF voor de scheiding van eiwitten.

- De koppeling van (c)IEF aan detectiemethoden als MS en SPR om een gevoeligere detectie en/of extra informatie over structuur en biologische affiniteit te verkrijgen.

In **Hoofdstuk 1** wordt het doel van het onderzoek en het project "Proteomics on a chip for monitoring auto-immune diseases" beschreven. Verder worden er de gebruikte analysetechnieken kort gepresenteerd.

Hoofdstuk 2 geeft een overzicht van de ontwikkelingen op het gebied van capillaire IEF over de periode 2003-2007. Technologische aspecten, methodiek en nieuwe cIEF technieken worden beschreven. Daarnaast komen onderwerpen als detectie, multidimensionale en microchip systemen alsmede recente toepassingen van cIEF aan de orde.

Hoofdstuk 3 beschrijft de effecten van additieven (drager-amfolieten, detergentia en viscositeitsverhogers) die gebruikt worden in cIEF, op validatieparameters als herhaalbaarheid (migratietijd en piekoppervlakte) en lineariteit (p/ t.o.v. migratietijd). Verder wordt de invloed van deze componenten op het signaal in MS onderzocht. Drager-amfolieten worden toegevoegd om een stabiele pH gradient in het capillair te creëren, nodig voor een goede cIEF scheiding. Het optimale amfolietmengsel blijkt afhankelijk van de gekozen detectiegolflengte in cIEF-UV. Bij 280 nm geven Pharmalytes het vlakste en meest stabiele achtergrondsignaal, terwijl HR ampholyte beter bij 214 nm kan worden gebruikt. De MS resultaten zijn eenduidiger: alle amfolietmengsels zorgen voor een significante en vergelijkbare daling van het MS signaal, zelfs bij lage concentraties. Detergentia worden in cIEF gebruikt om de oplosbaarheid te verhogen en de interactie van eiwitten met de capillairwand te verminderen. De polyethyleenoxide en zwitterionische detergentia (met name CHAPSO en Tween 20) geven een goede herhaalbaarheid voor zowel de migratietijd (RSD < 4,5%) als het piekoppervlak (RSD voornamelijk < 10%). De MS resultaten geven een minder positief beeld: vrijwel alle detergentia zorgen voor een sterke daling van het

signaal in de MS, hoewel de op glucoside gebaseerde detergentia een relatief geringer effect hebben. Viscositeit-verhogende componenten als cellulose derivaten en dextran dienen als dynamische coating en minimaliseren de interactie tussen de eiwitten en de wand en de invloed van de electro-osmotische stroming (EOF). Alle geteste cellulose derivaten en dextran hebben een positief effect op de herhaalbaarheid van de migratietijd (voor lage concentraties) en het piekoppervlak (RSD < 7,5% uitgezonderd hoge concentraties van methylcellulose en hydroxyethylcellulose). Echter ook bij het toevoegen van viscositeitsverhogers treedt een significante signaalonder-drukking in de MS op.

De herhaalbaarheid in cIEF kan sterk verbeterd worden door het toevoegen van de juiste componenten. Er dient wel rekening te worden gehouden met het feit dat al deze componenten een sterk negatief effect kunnen hebben op het eiwitsignaal in de MS. Een compromis dient derhalve te worden gevonden tussen cIEF reproduceerbaarheid enerzijds en behoud van voldoende MS signaal anderzijds.

In **Hoofdstuk 4** wordt de koppeling van cIEF-UV met MALDI-TOF MS gerealiseerd via een spotter. Resultaten uit Hoofdstuk 3 vormen de basis voor de selectie van de cIEF methode: 1% Pharmalyte, 0,3% HEC en 0,1% Tween 20. Hoewel de gekozen methode niet optimaal blijkt voor MALDI-TOF MS geeft deze wel een goede scheiding. Na focuseren worden de gescheiden componenten hydrodynamisch gemobiliseerd richting MALDI plaat waarop ze druppelsgewijs gedeponeerd (spotten) worden, gebruik makend van een sheath liquid koppeling. Deze sheath liquid dient niet alleen als kathodische oplossing (basisch) maar ook als elektrische aarde en zorgt voor een gesloten stroomcircuit. De koppelingsproblemen worden voornamelijk veroorzaakt door de overdracht van de vloeistof vanuit de naald op de plaat en kunnen worden opgelost door de juiste sheath liquid te kiezen (200 mM NH_4OH in 50% MeOH en 0,01% Tween 20). Na optimalisering van het systeem wordt het effect van verschillende focustijden op de scheiding geëvalueerd, waarbij focustijden tussen de 60 en 75 min als optimaal worden gevonden. Naast focustijden is ook het effect van de spottijd bestudeerd. Geheel volgens verwachting leidt een kortere spottijd tot een hoger eiwitsignaal in MS, ondanks de geringere hoeveelheid eiwit per spot. Bij kortere spottijden heeft een kleinere hoeveelheid additieven (amfolieten, detergentia en viscositeitverhogers) een groter effect op het MS signaal dan de lagere

hoeveelheid eiwit die aanwezig is. Hieruit kan geconcludeerd worden dat reguliere cIEF omstandigheden gebruikt kunnen worden zolang de spottijd maar kort genoeg is. Er moet wel rekening mee worden gehouden dat bij een bepaalde spottijd de hoeveelheid componenten (inclusief eiwitten) zo laag is dat de hoeveelheid eiwit weer bepalend is voor het MS signaal. De detectielimiet van het systeem zoals hier beschreven zal rond de 250 nM of 1 pmol liggen. De toepasbaarheid van dit cIEF-UV-MALDI-TOF MS systeem wordt gedemonstreerd aan de hand van een gedegradeerd eiwitgeneesmiddel: glucagon. Glucagon en gedeamideerd glucagon worden van elkaar gescheiden in cIEF waarna massabepaling in MS volgt.

Hoofdstuk 5 beschrijft de koppeling van gel IEF met SPR imaging (iSPR) via een zelfgemaakte blotting tool voor het zoeken naar biomarkers in biologische monsters, in dit geval in synoviaal vocht (SF). IEF wordt naast het scheiden van eiwitten in SF ook gebruikt voor het concentreren van deze componenten in een zone in de gel. Hierdoor wordt de eiwit/achtergrond verhouding in de gel verhoogd, wat leidt tot minder niet-specifieke binding op het SPR oppervlak. De eiwitten uit de gel worden met lichte druk overgebracht vanuit de IEF-gel naar het iSPR oppervlak. De polyacrylamide gel heeft geen interactie met het iSPR oppervlak en alleen eiwitten in de gel worden overgebracht. De maximale bindingscapaciteit van de iSPR schijf is ca 5 ng/mm^2 en wordt aloverschreden bij eiwitconcentraties van 1-100 µg/mL. Wanneer verschillende verdunningen van SF gespiked worden met koolzuuranhydrase II (CAII), lijkt een geconcentreerde verdunning van SF een positieve invloed te hebben op de focusserende eigenschappen: de eiwitten en peptiden in SF gedragen zich als amfolieten en zorgen ervoor dat koolzuuranhydrase in een scherpe band focusseert. Na blotten, wordt een anti-koolzuuranhydrase antilichaam over de geïmmobiliseerde eiwitten op de SPR schijf geleid en wordt er een 2-dimensionale reconstructie van de IEF scheiding gemaakt. Vervolgens is synoviaal vocht gespiked met een relevante rheuma biomarker: gecitrullineerd fibrinogeen (cFib). cFib wordt in IEF als nauwe band gefocuseerd. Na blotten en spoelen met anti-citrulline antilichaam is een scherpe interactie piek waarneembaar in iSPR.

Hoofdstuk 6 beschrijft het gebruik van de IonChip^{TM} met als doel het verwijderen van zout uit een eiwitmengsel en eiwitten uit een eiwit/peptide mengsel vóór een CZE scheiding. Dit systeem werd oorspronkelijk ontwikkeld voor de analyse van kleine organische en anorganische componenten in voedingsmiddelen als

water, wijn en andere dranken. De IonChip^{TM} bestaat uit 2 aan elkaar gekoppelde scheidingskanalen, waarbij het eerste kanaal wordt gebruikt voor monstervoorbewerking en het tweede kanaal voor de scheiding van de overgebleven componenten. Door het relatief grote injectievolume is de laadbaarheid groter dan bij een conventioneel CE systeem, wat leidt tot lagere detectielimieten. Het gebruik van ITP als monstervoorbewerkingsstap, leidt tot zowel een zeer effectieve verwijdering van zout uit een monster als tot een circa 65-voudige concentrering van de overgebleven eiwitten vóór injectie in het (CZE) scheidingskanaal. Helaas is het lastig om de ITP stap te gebruiken voor de verwijdering van eiwitten uit een peptidemonster aangezien de eiwit-peptide zone erg smal is. Vandaar dat CZE als monstervoorbewerkingsstap is geselecteerd voor deze doeleinden. Vóór de verwijdering van eiwitten worden alle componenten al gescheiden, waarna depletie van eiwitten wordt uitgevoerd en de overgebleven peptiden in het tweede scheidingskanaal worden geïnjecteerd.

Hoofdstuk 7 tenslotte, geeft een evaluatie van de onderzochte monster-voorbewerkingsstrategiëen, van cIEF als scheidingstechniek en van de koppeling van op IEF gebaseerde technieken met MS en SPR. Tevens worden er aanbevelingen gedaan voor vervolgonderzoek.

.

Abbreviations

2D PAGE	2 dimensional polyacrylamide gel electrophoresis
2D-GE	2 Dimensional gel electrophoresis
ACN	Acetonitrile
AGP	α-1-Acid glycoprotein
AI	Angiotensin I
AII	Angiotensin II
AIII	Angiotensin III
Avi	Avidin
B	Bradykinin
BGE	Background electrolyte
C	Concentration
CA	Carrier ampholyte
CAF-IEF	Carrier ampholyte free isoelectric focusing
CAI	Carbonic anhydrase I
CAII	Carbonic anhydrase II
CC	Cytochrome c
CCD	Charged-coupled device
CCK	Cholecystokinin flanking peptide
CD	Conductivity detection
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
cFib	Citrullinated fibrinogen
CGE	Capillary gel electrophoresis
cIEF	Capillary isoelectric focusing
CITP	Capillary isotachophoresis
CM dextran	Carboxymethyl dextran
cNGSE	capillary non-gel sieving electrophoresis
CST	Column switching time
CZE	Capillary zone electrophoresis
DDM	N-dodecyl-ß-D-maltoside
dIEF	Dynamic isoelectric focusing
DNA	Desoxyribunucleic acid
EDC	N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide
ELISA	Enzyme-linked immunosorbent assay
EOF	Electroosmotic flow
EPG	Electropherogram
ESI	Electrospray ionization
FFIEF	Free flow isoelectric focusing
Fib	Fibrinogen

FTICR	Fourier transform ion cyclotron resonance
FWHM	Full width half maximum
GE	Ground electrode
gIEF	Gel isoelectric focusing
GO	Glucose oxidase
HDMS	Hexa(dimethylsiloxane)
HEC	Hydroxyethyl cellulose
HFF-FFF	Hollow fiber flow field-flow fractionation
HPC	Hydroxypropylcellulose
HPMC	Hydroxypropylmethylcellulose
HR	High resolution
HRP	Horseradish peroxidase
HSA	Human serum albumin
HVE	High voltage electrode
I	Current
IACE	Immunoaffinity CE
EF	Isoelectric focusing
IPG	Immobilized pH gradient
iSPR	Surface plasmon resonance imaging
ITP	Isotachophoresis
IXC	Ion exchange chromatography
Lac	Alfa-lactalbumin
Lac B	ß-Lactoglobulin B
Lact	Lactoferrin
LC	Liquid chromatography
LE	Leading electrolyte
LGA	ß-lactoglobulin A
LGB	ß-lactoglobulin B
LIF	Laser induced fluorescence
MALDI	Matrix-assisted laser desorption ionisation
MC	Methylcellulose
MEKC	Micellar electrokinetic chromatography
MeOH	Methanol
MHEC	Methylhydroxyethylcellulose
M-IPG	Monolithic immobilized pH gradient
MS	Mass spectrometry
Myo	Myoglobin
N	Neurotensin
NHS	*N*-Hydroxysuccinimide

OCEC	Open channel electrochromatography
OGE	Off-gel electrophoresis
OLED	Organic light emiting diode
PAA	Polyacrylamide
PB-PEG	Pyrenebutanoate poly(ethylene glycol)
PDMS	Poly(dimethylsiloxane)
PEG	Poly (ethylene glycol)
PEHA	Pentaethylenehexamine
PEO	Polyethyleneoxide
PGDE	PH gradient driven electrophoresis
PMMA	Poly(methylmethacrylate)
PMT	Photon multiplier tube
PP	Peristaltic pump
PVA	Polyvinylalcohol
PVP	Poly(vinylpyrrolidone)
R	Relay
RA	Rheumatoid arthritis
RIA	Radio-immunoassay
RNAse	Ribonuclease
ROI	Region of interest
RPLC	Reversed phase liquid chromatography
SDS	Sodium dodecylsulphate
SDS-PAGE	Sodium dodecylsulphate – polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SF	Synovial fluid
SPE	Solid phase extraction
SPME	Solid phase microextraction
SPR	Surface plasmon resonance
TE	Terminating electrolyte
TFA	Trifluoroacetic acid
ToF	Time of flight
UV	Ultra violet
Wc	Waste channel
WCI	Whole column imaging

Curriculum Vitae

Linda Henriette Hermina Silvertand kwam op 21 juni 1979 ter wereld in Heerlen, Nederland. Na het afronden van de basisschool Op Gen Hei, waar de eerste bouwstenen voor dit proefschrift werden gelegd, werd het VWO diploma met lof behaald in 1997 aan het Eijkhagen college te Landgraaf. In datzelfde jaar werd gestart met de studie Farmaceutische Wetenschappen aan de Universiteit Utrecht, die in 2003 werd afgerond met het behalen van het apothekersdiploma. Tijdens het bijvakonderzoek in het vierde jaar in de groep van prof.dr. Maes en prof.dr. Beijnen werd haar belangstelling gewekt voor bioanalytisch onderzoek, waar ze een HPLC-UV methode opzette voor de analyse van melfalan in bloed, tumorweefsel en leverperfusaat. Dit onderzoek, in combinatie met de ziekenhuisstage in het Slotervaart Ziekenhuis tijdens het zesde jaar van de opleiding, leidde tot een aanstelling van 6 maanden als apotheker onderzoeker in het Slotervaart Ziekehuis. Daar werkte Linda aan een HPLC-MS/MS methode voor de bepaling van cyclofosfamide en fludarabine in EDTA en heparine bloed. In maart 2004 volgde een aanstelling als aio aan de Universiteit Utrecht binnen de disciplinegroep Biomedische Analyse op een project gefinancierd door STW en NWO. Dit project was een samenwerkingsverband tussen vier universitaire groepen en verschillende bedrijven onder de naam 'Proteomics on a chip for monitoring auto-immune diseases', met als doel het vinden van nieuwe marker eiwitten voor het vaststellen van rheumatoide artritis. De disciplinegroep Biomedische Analyse was hierbij verantwoordelijk voor de scheiding van de componenten in de aangeleverde biologische matrices. Tijdens dit onderzoek heeft Linda met verscheidene analytische scheidingsmethoden gewerkt en deze aan elkaar gekoppeld. Na afloop van dit promotieonderzoek is Linda begonnen als Application Chemist bij Prince Technologies B.V. in Emmen, alwaar zij nu mede verantwoordelijk is voor de ontwikkeling van de verschillende CE producten.

Publications

Papers

1] R.W. Sparidans, L.H.H. Silvertand, F. Dost, J. Rothbarth, G.J. Mulder, J.H. Schellens, J.H. Beijnen. Simple high-performance liquid chromatographic assay for melphalan in perfusate, rat liver and tumour tissue. Biomed Chromatogr 2003;17 (7):458-64.

[2] L.H.H. Silvertand, F. Vazvaei, P. Weigl, H. Rosing, M.J. Hillebrand, M.J. van Maanen, J.H. Beijnen. Simultaneous quantification of fludarabine and cyclophosphamide in human plasma by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry. Rapid Commun Mass Spectrom 2005;19 (24):3673-80.

[3] L.H.H. Silvertand, E. Machtejevas, R. Hendriks, K.K. Unger, W.P. van Bennekom, G.J. de Jong. Selective protein removal and desalting using microchip CE. J Chromatogr B 2006;839 (1-2):68-73.

[4] L.H.H .Silvertand, J. Sastre Toraño, G.J. de Jong, W.P. van Bennekom. Improved repeatability and matrix-assisted desorption/ionization - time of flight mass spectrometry compatibility in capillary isoelectric focusing. Electrophoresis 2008;29 (10):1985-96.

[5] L.H.H. Silvertand, J. Sastre Toraño, W.P. van Bennekom, G.J. de Jong. Recent developments in capillary isoelectric focusing. J Chromatogr A 2008;1204 (2):157-70.

[6] L.H.H. Silvertand, J. Sastre Toraño, G.J. de Jong, W.P. van Bennekom. Development and characterization of cIEF-MALDI-TOF MS for protein analysis. Electrophoresis 2009;30 (10):1825.

[7] L.H.H. Silvertand, M.B. Harink, R.B.M. Schasfoort, E.C.A. Stigter, G.J. de Jong, W.P. van Bennekom. Development RB and evaluation of an IEF-iSPR system for biomarker research. Submitted to Biosensors and Bioelectronics 2009.

Oral and poster presentations

Silvertand, L. H. H.; Smits-Huijbrechts, M.; Van Bennekom, W. P.; De Jong, G. J., Separation of proteins in complex biological samples by capillary electrophoresis. Analytische chemie dagen 2004, Lunteren, The Netherlands.

Silvertand, L. H. H.; Machtejevas, E.; Hendriks, R.; Unger, K. K.; Van Bennekom, W. P.; De Jong, G. J., Protein depletion and desalting with microchip CE using conductivity and MS detection. MSB 2005, Utrecht, The Netherlands.

Silvertand, L. H. H.; Machtejevas, E.; Hendriks, R.; Unger, K. K.; Van Bennekom, W. P.; De Jong, G. J., Protein depletion and desalting with microchip CE using conductivity and MS detection. Analytische chemie dagen 2005, Lunteren, The Netherlands.

Silvertand, L. H. H.; Machtejevas, E.; Hendriks, R.; Unger, K. K.; Van Bennekom, W. P.; De Jong, G. J., Protein depletion and desalting using microchip CE. MSB 2006, Utrecht, (The Netherlands).

Silvertand, L. H. H.; Sastre Toraño, J.; De Jong, G. J.; Van Bennekom, W. P., Fractionation of proteins using Off-Gel electrophoresis. Analytische chemie dagen 2006, Lunteren, The Netherlands.

Silvertand, L. H. H.; Sastre Toraño, J.; Van Bennekom, W. P.; De Jong, G. J., Reproducible analysis of protein mixtures by capillary IEF. Analytische chemie dagen 2006, Lunteren, The Netherlands.

Silvertand, L. H. H.; Harink, M. B. M.; Sastre Toraño, J.; Schasfoort, R. B. M.; De Jong, G. J.; Van Bennekom, W. P., Coupling of IEF and cIEF with iSPR. Analytische chemie dagen 2007, Lunteren, The Netherlands.

Silvertand, L. H. H.; Sastre Toraño, J.; De Jong, G. J.; Van Bennekom, W. P., Optimization and characterization of cIEF-MALDI-ToF-MS. HPLC 2007, Gent, Belgium.

Silvertand, L. H. H.; Sastre Toraño, J.; De Jong, G. J.; Van Bennekom, W. P., Optimization of repeatability and MS compatibility of capillary IEF. HPLC 2007, Gent, (Belgium).

Silvertand, L. H. H.; Sastre Toraño, J.; De Jong, G. J.; Van Bennekom, W. P., Coupling (c)IEF with iSPR. ISCCE 2007, Albuquerque, NM, (US).

Silvertand, L. H. H.; Sastre Toraño, J.; De Jong, G. J.; Van Bennekom, W. P., Optimization and characterization of cIEF-MALDI-ToF-MS. ISCCE 2007, Albuquerque, NM, US.

Silvertand, L. H. H.; Sastre Toraño, J.; De Jong, G. J.; Van Bennekom, W. P., cIEF–MALDI-TOF-MS for the analysis of (therapeutic) proteins. FABIAN 2008, Groningen, The Netherlands.

Silvertand, L. H. H.; Sastre Toraño, J.; Van Bennekom, W. P.; De Jong, G. J., CIEF-MALDI-ToF MS for protein analysis. KNCV dag 2007, Ede, The Netherlands.

Acknowledgements

Toen ik $5^1/_2$ jaar geleden bij Ad de Jong aanklopte met de vraag of hij nog aio's zocht, had ik niet kunnen vermoeden dat het op deze manier uit zou pakken. Over het algemeen is deze tijd een van de leukste en vooral leerzaamste van mijn leven geweest, maar zoals bij elke promovendus waren er wel de nodige tegenslagen en frustraties. Vooral de afronding van het proefschrift, de laatste loodjes, vielen wat tegen. Maar… het is af! Dit proefschrift is echter niet alleen mijn bloed, zweet en tranen, maar het resultaat van de inzet van een aantal mensen die ik dan bij deze ook wil bedanken.

Ten eerste mijn promotor Ad de Jong. Ad, ik wil je bedanken voor de kans die je me gegeven hebt om als promovendus in je groep aan de slag te mogen gaan. Je kennis en inzicht heeft een groot stempel gedrukt op dit proefschrift. We waren het niet altijd met mekaar eens en dat leidde vaak tot goede wetenschappelijke discussies. Ik kan je frustratie over mijn "farmaceutentrekjes" (hoezo te lang en te uitgebreid?) echter goedbegrijpen. Er ligt nu wel een boekje waar we trots op kunnen zijn.

Dan mijn co-promoter en mentor Wout van Bennekom. Wout, jij hebt me zóveel meer geleerd dan alleen op wetenschappelijk gebied. Onder andere de vorming als persoon in deze tijd heb ik vooral aan jou te danken. Je hebt me altijd vrij gelaten in mijn manier van denken en experimenteren. Dit heeft geleid tot een aantal brainstormsessies en leuke ideeën, waarvan de meeste helaas niet zijn uitgevoerd of al door anderen bedacht waren. Ook je expertise op andere gebieden dan CE heeft geleid tot nieuwe projectjes en leuke contacten met andere groepen, waarvan ik nu nog profijt heb. Onze bijbabbelsessies en verhaaltjes (van vroeger) waren altijd een welkome afwisseling op de wetenschappelijke discussies. Ik ben ontzettend dankbaar dat jij mijn co-promoter bent geweest. Ik hoop dat jij (samen met Fenna) nog lang (in beste gezondheid) van je welverdiende rust mag genieten de komende jaren!

Bij deze zou ik graag prof. dr. Wätzig, prof. dr. Jiskoot, prof. dr. Verpoorte, prof. dr. Heck en dr. ir. Schasfoort willen bedanken voor het lezen en beoordelen van het proefschrift. Ook zou ik graag de Stichting Technologische Wetenschappen (met name Lia Kemper) en de STW-gebruikerscommissie (René Heideman en Henk Leeuwis (LioniX), Gerard Engbers (IBIS Technologies), Martin Salden (Eurodiagnostica), Jos Joore (Pepscan systems) en Jos Raats (Modiquest) willen bedanken voor de wetenschappelijke en financiële bijdragen aan het IOP-STW project. Niet te vergeten nogmaals Richard Schasfoort, de projectleider en de

andere universitaire deelnemers: Universiteit Twente (Bianca Beusink, Dietrich Kohlheyer, Stefan Schlautman en uiteraard Björn Harink), Universiteit Nijmegen (Ger Pruijn en Angelique Segbers-Lokate). De vakgroep Biomoleculaire Massaspectrometrie wil ik even apart noemen aangezien ik daar vaak te vinden was. Naast nogmaals Albert Heck wil ik met name Natasja Carol-Visser, Mirjam Damen en Arjan Barendregt bedanken voor de ondersteuning bij het gebruik van de MALDI (en iSPR), de wetenschappelijke en niet-wetenschappelijke babbeltjes, maar vooral voor het feit dat ik altijd bij hen aan kon kloppen als er weer eens een plaat vastzat in de MALDI (nogmaals… sorry!) of als ik de resultaten niet helemaal kon interpreteren.

The Mainz and Merck people. Klaus Unger, Egidijus Machtejevas, Romas Skudas and Tom Hennessey. You guys made my stay in Mainz a lot of fun. And although some didn't really believe in the Ionchip (especially coupled to an MS), we managed to extract an paper out of it (unbelievable!).

Dan de groep waar het allemaal om draaide, de reden waarom de afgelopen $5\frac{1}{2}$ jaar zo leuk waren: Farmaceutische Analyse (inclusief adoptie aio's en studenten). Naast de serieuze, werkgerelateerde conversaties is er erg veel afgelachen in de koffiekamer, op het lab en tijdens de vele etentjes en borreltjes. Naast nuttige contacten heb ik er ook een aantal goede vrienden aan over gehouden! Dus… Govert, Paul, Joop, Lidija (die kan toveren), Marica, Gerard, Edwin, Roelof, Johan, Frits, Rawi, Stefan, Dragana, Remco, Maurice, Michiel, Yvon en last but zeker not least Javier, Andrea, Rob, Bregje en Thierry…. THANX!!!!!

Neëve 't werrek zint d'r twiëe luu die 30 joar gelejje begoos zint aan de zjwoar opgaaf mich groët te trekke (pfff, alle achtung). Die twiëe zint och de reeä woerum ich hej noe sjton. Ze hont ummer i mich geglofd, kenne mich va binne en va boete en hont mich vaak teage mich zelf en teage angere besjermt, och noe noch. Mam, pap, Ich waardeer gans errug wat ier vuer os hot gedoa en geloate in al die joare, zoedat vuer kenne ziëe wëe vuer noe zint. Danke wa.

Dan mijn paranimfen. Twee mensen die zo dicht bij me staan en ook nog eens weten waar dit boekje over gaat. Ik ben erg blij dat uitgerekend jullie dit wilden doen. Lejo en Javier. Lejo, partynimf en broer heeft over de afgelopen jaren gezorgd voor veel -broodnodige- afleiding (sporten, concertjes, feestjes, vakanties). En Javier, jij hebt een enorme bijdrage geleverd aan dit boekje. De

praktische én mentale ondersteuning waardeer ik zeer. Zonder jou had dit proefschrift er echt heel anders uit gezien.

Dank jullie wel!

Linda

Notes

Notes